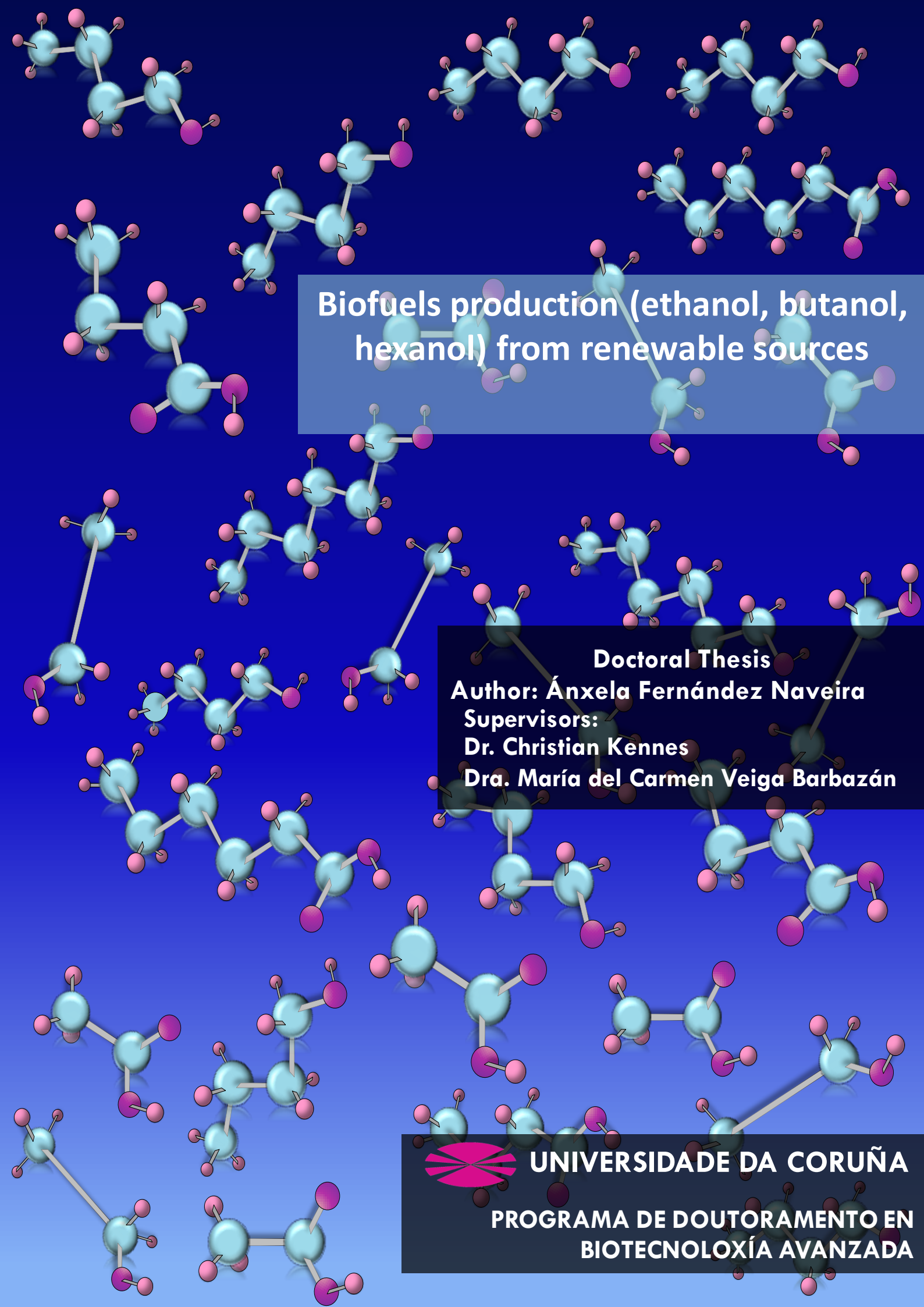

Biofuels production (ethanol, butanol, hexanol) from renewable sources

Doctoral Thesis

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PROGRAMA DE DOUTORAMENTO EN BIOTECNOLOXÍA AVANZADA

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Producción de biocombustibles (etanol, butanol, hexanol) a partir de fuentes renovables

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INFORMAN

Que el trabajo titulado "Biofuels production (ethanol, butanol, hexanol) from renewable sources" ha sido realizado por Ánxela Fernández Naveira en el Departamento de Química y que, como Directores del mismo, autorizan su presentación para optar al grado de Doctor.

Y para que así conste, expiden y firman la presente en:

A Coruña,a de de 2018.

Christian Kennes María del Carmen Veiga Barbazán

A mi familia

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ABSTRACT

The search for new energy sources or fuels has increased in recent years. Besides, the increasing demand and greater shortage of conventional fuels, creates the need to replace them by renewable energy sources. Some possible renewable energy sources are (higher) alcohols such as ethanol, butanol, and hexanol.

The biological production of ethanol and higher alcohols can be a good and inexpensive way for the obtention of new renewable energy sources. The anaerobic fermentation of waste materials or waste gases is an energy-inexpensive and feedstock-flexible technology for the production of those bioalcohols. The use of those raw materials in biorefineries has two major advantages : it is an efficient method for the elimination of pollutants and compounds with a greenhouse effect (CO, CO_2) and it allows to produce solvents with a high commercial value.

The main objective of this dissertation is the obtention of higher alcohols using different carbon sources (glucose, carbon monoxide and syngas) with the anaerobic bacterium *Clostridium carboxidivorans* as biocatalyst. The experimental results are reported in the different chapters of this dissertation, aiming at optimizing the fermentation conditions and operating parameters of the bioconversion of these different carbon sources into ethanol and higher alcohols.

RESUMEN

La busqueda de nuevas fuentes de energía o combustibles ha incrementado recientemente. Además el incremento de la demanda y de la gran escasez de combustibles convencionales, crea la necesidad de reemplazarlos por fuentes de energía renovables. Algunas de estas posibles fuentes de energía renovables son los alcoholes como etanol, butanol y hexanol

La producción biológica de etanol y alcoholes de cadena larga puede ser una buena y barata forma de obtener nuevas fuentes de energía renovable. La fermentación de materiales y gases residuales es una energía barata y una tecnología de materias primas flexibles para la producción de esos bioalcoholes. El uso de esos materiales residuales en biorefinerías tiene dos ventajas principales: es un método eficiente para la eliminación de contaminantes y gases de efecto invernadero (CO, CO_2) y permite producir solventes de elevado valor comercial.

El principal objetivo de esta tesis es la obtención de alcoholes de cadena larga empleando diferentes fuentes de carbono (glucosa, monóxido de carbono y gas de síntesis) empleando a *Clostridium carboxidivorans* como biocatalizador. Los resultados están recogidos en los distintos capítulos de esta tesis doctoral, con el objetivo de optimizar las condiciones de fermentación y los parámetros operativos de bioconversión de estas fuentes de carbono en alcoholes.

RESUMO

A busqueda de novas fontes de enerxía ou combustibles alternativos incrementou recentemente. Ademais o incremento da demanda e da gran escaseza de combustibles convencionais, crea a necesidade de reemplazarlos por fontes de enerxía renovables. Algunhas destas posibles fontes de enerxía renovables son os alcohois como etanol, butanol e hexanol.

A produción biolóxica de etanol e alcohois de cadea longa pode ser unha boa e barata forma de obter novas fontes de enerxía renovables. A fermentación de materiais e gases residuais é unha enerxía barata e unha tecnoloxía de materias primas flexibles para a produción deses bioalcohois. O uso de todos eses materiais residuais en biorefinerías ten dúas vantaxes principais: é un método eficiente para a eliminación de contaminantes e gases de efecto invernadoiro (CO, CO_2) e isto permite producir liquides con alto valor comercial.

O principal obxectivo desta tese é a obtención de alcohois de cadea longa empregando diferentes fontes de carbono (glucosa, monóxido de carbono e gas de síntese) empregando a *Clostridium carboxidivorans* como biocatalizador. Os resultados están recollidos nos distintos capítulos desta tese doctoral, co obxectivo de optimizar as condicións de fermentación e os parámetros operativos da bioconversión destas fontes de carbono en alcohois.

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1 INTRODUCTION

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Worldwide energy consumption is expected to double by 2030. One of the main reasons for this increase is the rapid growth of the transportation sector reaching about 3 % per year. Because of such rapid growth, the limited availability of fossil fuels will not be able to cope with this energy demand. Therefore, in recent years, the environmental impact of fossil fuels and issues related to climate change have forced the search for new alternative fuels that can be obtained from renewable sources or from pollutants and that can be produced in a sustainable way. In addition to the environmental impact generated from the use of fossil fuels (e.g. greenhouse effect), there is also another set of problems including economic (e.g. the instability of oil prices) as well as political issues requiring the development of new techniques for the production of alternative fuels (Gowen and Fong 2011; Li and Liu 2015; Liu et al. 2016). The European Union, through its Renewable Energy Directive RED 2009/28/EC, sets a target for the year 2020, stating that the use of renewable energy should reach 20 % with a 10 % share of renewable energy in the transportation sector (Van Groenestijn et al. 2013). Biorefineries have recently emerged as a potential solution to such problem.

Nowadays numerous studies are focusing on the development and optimization of technologies for obtaining biofuels (biologically sourced fuels) such as ethanol, butanol, biogas and biodiesel as environmentally-friendly energy sources in substitution of fossil fuels. Ethanol and longer chain alcohols such as butanol are suitable substitutes of fossil fuels such as gasoline. They can also be used as chemicals and solvents. Mixtures of alcohols such as butanol and ethanol can potentially be produced from wastes and renewable sources in bioreactors, which is an advantage compared to alcohols obtained from non renewable fossil sources. The most extensively studied bioprocess is based on the fermentation of carbohydrates to obtain ethanol and butanol (i.e. fermentation of sugars or starch feedstocks), whereas biogas is produced through the anaerobic fermentation of organic matter, and biodiesel results from a transesterification reaction between an alcohol and vegetable oils (e.g. soybean, sunflower, coconut oils) in most cases, although animal fats or even algae have also been used (Kennes and Veiga 2013). In the case of bioalcohols, conventional techniques developed for obtaining biofuels do often lead to food–fuel competition. Therefore, another alternative has more recently been developed based on the use of lignocellulosic feedstocks in order to overcome such a drawback. Energy crops and agricultural wastes are the most common renewable and

inexpensive sources of lignocellulosic materials (Gowen and Fong 2011). The efficiency and cost-effectiveness of this alternative still needs to be further improved.

The use of lignocellulosic materials for sugar fermentation is a complex process, as they contain carbohydrates in the form of long chain polysaccharides which are difficult to metabolize directly by microorganisms. Therefore, pretreatment of the feedstock is necessary in order to hydrolyze those polysaccharides into fermentable sugars which can then be used readily and metabolized by several microbial species. The hydrolytic route presents some other drawbacks. Indeed, lignocellulosic biomass is composed of cellulose, hemicellulose and lignin mainly. Cellulose and hemicelluloses yield fermentable sugars while lignin does not (Kennes et al. 2016). The lignin fraction which may, in some cases, represent up to more than 30 % of the feedstock can thus not be used in the fermentation process. On the other hand, those feedstocks can be converted into syngas which can also be fermented into ethanol as well as higher alcohols such as butanol or hexanol by some anaerobic acetogenic bacteria. In such a case, contrary to the hydrolytic fermentation process, the whole feedstock, i.e. cellulose, hemicelluloses and lignin can be gasified. Lignin is thus not lost. Besides, a wider range of starting materials are suitable for gasification than for hydrolysis as many carbon-containing materials can be gasified. Syngas is mainly composed of a mixture of CO, CO_2 and H_2 at variable concentrations depending on the feedstock and gasification conditions. Several industrial waste gases do also contain some or several of those gases and can therefore be used as feedstock as well, with an obvious associated environmental benefit (Abubackar et al. 2011).

The human interest in the use of microbial fermentation to produce ethanol began by the year BC 10 000 (Patrick 1952). On the other hand, the commercial bacterial production of butanol from carbohydrates dates back to about 100 years ago (Dürre 1998), while studies on the potential biological production of hexanol is still more recent. Today research is still being undertaken on improving the biological production of alcohols. Many of those studies deal with ethanol production and its commercial applications. However, ethanol presents some limitations as an individual fuel or mixed with fossil fuels such as gasoline. It has a rather low caloric content. Besides, it is hygroscopic and has a low density, which limits its use with current infrastructures (Ahmed and Lewis 2007; Henstra et al. 2007; Shaw et al. 2008).

On the other hand, butanol has the advantage of being less hygroscopic and it has a higher energy content than ethanol (Mayank et al. 2013; Wallner et al. 2009), besides being less volatile, less corrosive, absorb less water, and being less explosive which makes it safer to use than ethanol (Lee et al. 2008; Schiel-Bengelsdorf et al. 2013). All those advantages result in

growing interest in that alcohol. It is considered a chemical of great industrial importance and could replace gasoline as a fuel (Dürre 2007; Lee et al. 2008), as there is no need for any adjustment in vehicles or engines run on butanol rather than fossil fuels. Moreover, blending of butanol and gasoline is possible at basically any concentrations (Schiel-Bengelsdorf et al. 2013).

In recent years, hexanol has also been established as an alcohol with a high industrial interest. Recent studies aim at maximizing the production of that 6 carbons alcohol which can be considered a suitable alternative fuel as well (Diender et al. 2016). Hexanol, similarly to butanol, is a higher carbon number alcohol, thus characterized by a higher energy content than ethanol. Several assays have recently been done with hexanol to check its potential for use as an aviation fuel (Chuck and Donelly 2014; Vigneswaran and Thirumalini 2015), although the viscosity of pure alcohols such as hexanol and butanol may be too great to be compatible with aviation kerosene (Chuck and Donelly 2014). Hexanol–diesel blends have also been tested as well as gasoline–alcohols blends using multiple alcohols (i.e. ethanol, butanol, and hexanol) in combination (Masum et al. 2014). Besides, hexanol has several other industrial applications. It is not toxic at low concentrations and is also used in the pharmaceutical and cosmetic/perfumes industry, textile industry, in detergents, in pesticides, and as a finishing agent in the leather industry, among others.

1.1 Production of alcohols from carbohydrates

Butanol and some other alcohols can be obtained by the anaerobic fermentation of carbohydrates (ABE fermentation), briefly revised hereafter, as well as from the anaerobic bioconversion of syngas/waste gases (i.e. CO, CO_2, and H_2) (HBE fermentation), as will be described in more detail in later sections.

1.1.1 Clostridial alcohol producing strains

Despite numerous scientific advancements in the area of biofuels production, it is necessary to identify the best-performing microorganisms to maximize their production and minimize costs, and thus to be able to better compete with fossil fuels. High productivity of the desired product along with low production costs will be the desired characteristics in a model organism for a cost-competitive process (Gowen and Fong 2011). *Clostridium* spp. have been identified as suitable bacteria for the production of higher alcohols such as butanol.

The use of clostridial strains in butanol production through conventional ABE (acetone-butanol-ethanol) fermentation is a well known process (Jones and Woods 1986), which started being studied around the First World War with carbohydrates as substrates. Acetone, butanol and of

them having commercial uses either as biofuels or as platform chemicals. After the Second World War, the relevance of fermentation as a major production process of those chemicals decreased significantly and it was largely replaced by petrochemical production as a result of the strong development of the petrochemical industry. However, interest in this type of bioconversion has again grown dramatically in recent years for economic reasons and because of environmental problems related to the use of fossil fuels.

Various species of the genus *Clostridium* have been studied for their ability to naturally produce butanol from carbohydrates or similar carbon sources. This includes *Clostridium acetobutylicum*, *Clostridium aurantibutyricum*, *Clostridium beijerinckii*, *Clostridium butyricum*, *Clostridium cadavaris*, *Clostridium carboxidivorans*, *Clostridium chauvoei*, *Clostridium felsineum*, *Clostridium pasteurianum*, *Clostridium puniceum*, *Clostridium roseum*, *Clostridium saccharobutylicum*, *Clostridium saccharoperbutylacetonicum*, *Clostridium septicum*, *Clostridium sporogenes*, and *Clostridium tetanomorphum* (Dürre 2011). The most common and best known solventogenic clostridial strains for commercial butanol fermentation are *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* (Jones and Keis 1995). *Clostridium acetobutylicum* is the most extensively studied species. On the other hand, there are some other microorganisms, belonging to other genera, which are also able to produce butanol, such as *Butyribacterium* (e.g. *Butyribacterium methylotrophicum*) and *Thermoanaerobacterium* (e.g. *Thermoanaerobacterium thermosaccharolyticum* W16).

1.1.2 Fermentation of sugars

Clostridia are anaerobic bacteria, which have been shown for years to be able to metabolize and completely ferment various carbohydrates, including glucose, fructose, mannose, sucrose, xylose, and lactose, among others (Tracy et al. 2012). Others, such as trehalose and rhamnose have more recently also been found to be fermented into biofuels by bacteria such as *Clostridium butyricum* TM9A (Junghare et al. 2012; Rafieenia and Chaganti 2015). Different carbohydrates are fermented through different pathways by clostridia. In the case of hexoses, their metabolism follows the Embden-Meyerhof-Parnas (EMP) pathway (Figure 1.1 A) whereas the metabolism of pentoses takes place through the pentose phosphate (PP) pathway (Figure 1.1 B) (Shinto et al. 2008; Raganati et al. 2015). Several additional substrates, besides carbohydrates, can be metabolized into butanol as well. For example, glycerol and also polysaccharides such as carboxymethylcellulose were found to be fermented by some clostridia, such as the new isolate *C. acetobutylicum* YM1 (Al-Shorgani et al. 2016).

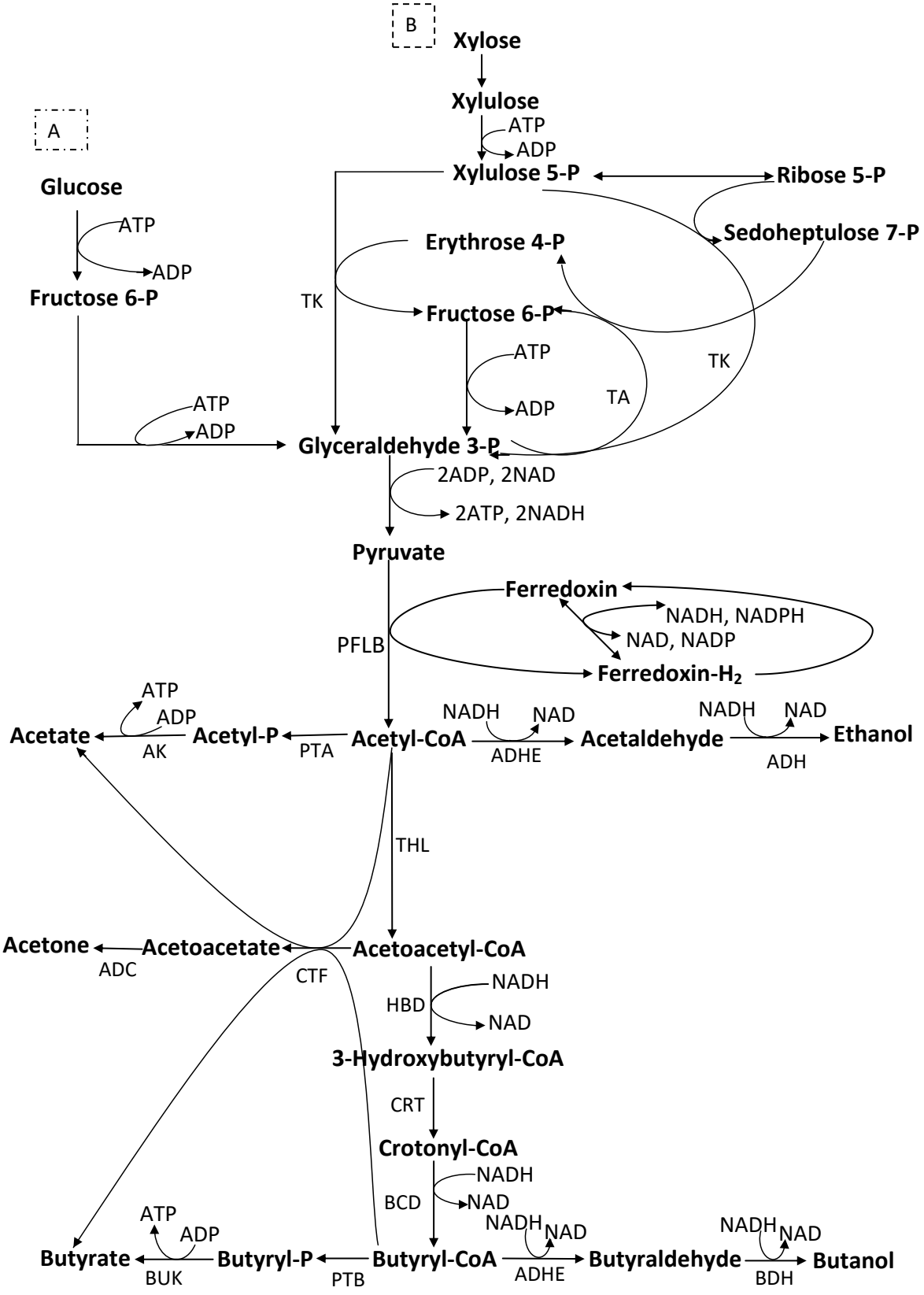

Figure 1.1 (A) Metabolic EMP pathway in *C. acetobutylicum* ATCC 824T with glucose (hexose sugar) as carbon source. (B) Metabolic PP pathway *of C. acetobutylicum* with xylose (pentose sugar) as carbon source

Recently, numerous studies have focused on the effects of sugars fromagricultural feedstocks on the fermentation process (Ezeji et al. 2007; Ezeji and Blascheck 2008; Raganati et al. 2012). Typical sugar composition of the most commonly used feedstocks includes glucose, arabinose, mannose, xylose, fructose, sucrose and lactose, mainly. In this area, scientists have tried to identify the potentially best carbohydrate to use in butanol fermentation. *Clostridium acetobutylicum* was found to prefere glucose, for which the conversion rates were the highest, followed, in decreasing order, by mannose and fructose, arabinose, xylose, and finally lactose (Ezeji et al. 2007; Ezeji and Blascheck 2008; Raganati et al. 2012; Raganati et al. 2015). When comparing monosaccharides, such as glucose, with disaccharides, such as sucrose and lactose, the first difference with glucose is the transport system into the cells. Indeed, sucrose and lactose move across the cell membrane using the carbohydrate phosphotransferase system (PTS). That system is slower than the one used for glucose transport (Raganati et al. 2015). On the other hand, sucrose and lactose are hydrolysed into simple sugars, i.e. fructose-6-P and glucose-6-P, in the case of sucrose; glucose-6-P and galactose-6-P, in the case of lactose. The products obtained from sucrose and lactose will be metabolized through different pathways. Indeed, fructose-6-P and glucose-6-P obtained from sucrose can be converted via the Embden–Meyerhof–Parnas pathway (Tangney et al. 1998). Conversely, in products obtained from lactose, glucose is phosphorylated and incorporated into the glycolytic pathway, while galactose-6-P is metabolized via the tagatose 6-P pathway and does subsequently enter glycolysis.35 These differences in terms of metabolism result in different consumption rates between sucrose and lactose, as a result of the bottleneck in the metabolism of galactose-6-P, as the metabolism of the latter is much more complex than the metabolism of fructose-6-P and glucose-6-P formed in the sucrose metabolism.

The hydrolysis of lignocellulosic feedstocks yields a mixture of carbohydrates including hexoses such as glucose and pentoses such as xylose, which will result in carbon catabolic repression (CCR) mainly in Gram-positive bacteria like *C. acetobutylicum*. CCR consists in the preferential use of glucose over other carbohydrates, e.g. xylose, which will be metabolized only once the former is exhausted. This leads to an inefficient use of the different carbon sources present in the fermentation broth. It is necessary to deal with this CCR issue for an optimal ABE fermentation and for the efficient use of all the carbon sources present in the medium in the case of complex lignocellulosic feedstocks. Working with strains non-affected by CCR or recombinant bacteria would be a suitable solution (Bruder et al. 2015).

The above described characteristics show that the nature of the sugars present in the raw material is a key parameter that will affect the efficiency of the fermentation process and the production of metabolites.

The fermentation metabolism of *C. acetobutylicum* and other similar bacteria is divided into two phases: acidogenesis and solventogenesis. In the acidogenic phase, cells grow exponentially while producing butyric and acetic acids mainly with a typical molar ratio of 2:1. In addition, CO_2, H_2, and ethanol are produced to some extent as well. In the solventogenic phase, the cells are predominantly in stationary phase, and take up the acids produced during acidogenesis, which are then converted into acetone, butanol and ethanol, with a typical molar butanol:acetone:ethanol ratio of 6:3:1. Such a ratio is typically found in *C. acetobutylicum* but may be different in other species. Besides, all three compounds do not necessarily appear in all clostridia (e.g. absence of acetone). Acetoin and lactate may also be produced but in minor amounts and only under specific conditions. It has been reported that the shift from acid production (acidogenesis) to the production of solvents (solventogenesis) is due to a change in gene expression (Dürre et al. 1987). Besides, such a shift has been claimed to be highly dependent on the pH value. An initially slightly high pH (i.e. 5.75) is optimal for the acidogenic phase. Acetic and butyric acids will mainly be formed during that phase, resulting in a pH decrease. It is considered that the solventogenic phase will generally start as soon as a critical pH value has been reached, leading to reassimilation of the acids with concomitant production of solvents, i.e. acetone, butanol and ethanol. Numerous studies have reported that a low pH is necessary for the solventogenic phase and the production of solvents (Jones and Woods 1986; Grethlein et al. 1991; Lee et al. 2008; Phillips et al. 2015; Millat et al. 2013). It is worth taking into account that the bacteria need to have produced sufficient acids before reaching a pH below 4.5 and switch to the solventogenic phase. If only low amounts of acids are available, then the solventogenic stage would be too short and produce only reduced amounts of solvents. This could be avoided by using a medium with a high buffering capacity which will lead to a longer acidogenic phase with better carbohydrate utilization and increased growth which does generally take place simultaneously to the production of acids (Bryant and Blascheck 1988). It has been hypothesized that solvent production might be a response to stress conditions, such as a low pH. When the pH reaches a value of 4.5, undissociated acetic and butyric acids are able to cross the cytoplasmic membrane by diffusion (Mohammadi et al. 2011). When the value of the internal pH is about 5, undissociated acids start to dissociate inside the cell, releasing protons. As a result collapse of the proton gradient over the cytoplasmic membrane takes place and cell death will occur. In order to avoid such phenomenon, the cells will start converting the acids to

solvents, which have a neutral charge. Nevertheless, it should be reminded that there is a point where the concentration of solvents can become toxic to the cells; thus producing solvents is often concomitant to cell sporulation, in order to ensure cell survival in the long-term (Dürre and Bahl 1996; Dürre 1998).

1.2 Production of (higher) alcohols from co-rich gases

Syngas, a mixture of primarily CO, CO_2, and H_2, is an inexpensive and flexible substrate and can be used by acetogens in fermentation processes to produce renewable fuels and chemicals. Acetogens are anaerobic microorganisms able to grow on C1 compounds such as CO, CO_2+H_2 or formate and produce acetate mainly, via the Wood-Ljungdahl pathway with acetyl-CoA as main intermediate (Müller 2003). Besides, occasionally, other organic acids, such as butyrate, as well as alcohols can also be produced by a limited number of strains. The process is flexible because it can be generated from a wide variety of organic materials through gasification. Gasification is a thermal process that converts most of the lignocellulosic materials and other carbonaceous feedstocks into syngas at elevated temperatures (Maschio et al. 1994). This gas mixture (i.e., syngas) is not only obtained through gasification of biomass or waste, it is also found in some industrial gaseous effluents, among others in steel producing processes. Syngas conversion into liquid fuels with biological catalysts is a more effective and efficient process compared with the use of chemical catalysts. When comparing two different case studies, in terms of ethanol production, the efficiency of carbon conversion to fuel was claimed to reach 40.7 % in the thermochemical route and 51.6 % in the biological route (Griffin and Schultz 2012). Besides, the energy in the feedstock converted to final product (LHV %, low heating value basis) was reported to reach 45 % and 57 %, respectively, in the thermochemical and the bioconversion routes. Finally, fuel yields (gal/dry US ton) of 83.8 and 117.6 were found in the thermochemical and biological processes, respectively. One of the reasons for this higher efficiency is the lower energy requirement and infrastructure set-up costs (Henstra et al. 2011).

Indeed, bioconversion takes place at much lower temperature and pressure (30–37 ^{o}C and near atmospheric pressure) than catalytic chemical reactions (200–350^{o} C and 10–200 atm). Besides, a wide range of CO:CO_2:H_2 ratios can be used in bioprocesses, while such flexibility in terms of ratios is highly restricted in chemical processes (H_2/CO ratios of 1–4 in chemical catalysis; that need most often to be close to 2); also chemical processes are more sensitive to impurities (sulfur compounds, chlorine...) than the biological conversion of syngas. On the other hand, the use of syngas rather than dissolved sugars as feedstock has the advantage of allowing uncoupling of the hydraulic retention time from the substrate supply in suspended growth

bioreactors, as the substrate is fed through the gas phase while nutrients are supplied as liquid phase (Henstra et al. 2011). Besides syngas, CO-rich industrial waste gases are also suitable for bioconversion to ethanol and higher alcohols. This is the case of waste gases from numerous steel industries.

Similarly to sugars, syngas and the aforementioned industrial waste gases can be fermented by clostridia and a few other acetogenic bacteria (Drake et al. 2008). In a few strains, this may yield ethanol and, occasionally, higher alcohols. Contrary to the first generation biorefinery processes which are based on the use of sugar containing food crops as feedstock and lead to food-fuel competition (Abubackar et al. 2011; Kennes et al. 2016), the present alternative uses lignocellulosic biomass or wastes mainly or even waste gases and does not generate such food-fuel dilemma. Besides, this gas fermentation technology can reduce the emissions of gaseous pollutants and greenhouse gases such as carbon dioxide and it gives some commercial use to industrial pollutants and agricultural wastes.

Many microorganisms can metabolize syngas as carbon and energy source (Oelgeschläger and Rother 2008), but only a feware able to convert it into (bio)fuels. Carbon monoxide can be metabolized by acetogens as single substrate and can be used both as carbon and energy source. On the other hand, CO_2 can be used as carbon source but needs the presence of hydrogen as energy source. Acetogenicbacteria follow the Wood-Ljungdahl (WL) pathway to produce biofuels from CO, CO_2, and H_2, or syngas/waste gas (Figure 1.2). The WL route is divided into three phases: (i) synthesis of acetyl-CoA by reducing CO or CO_2+H_2; (ii) conservation of energy; and (iii) CO_2 assimilation into cellular carbon (Drake et al. 2008).

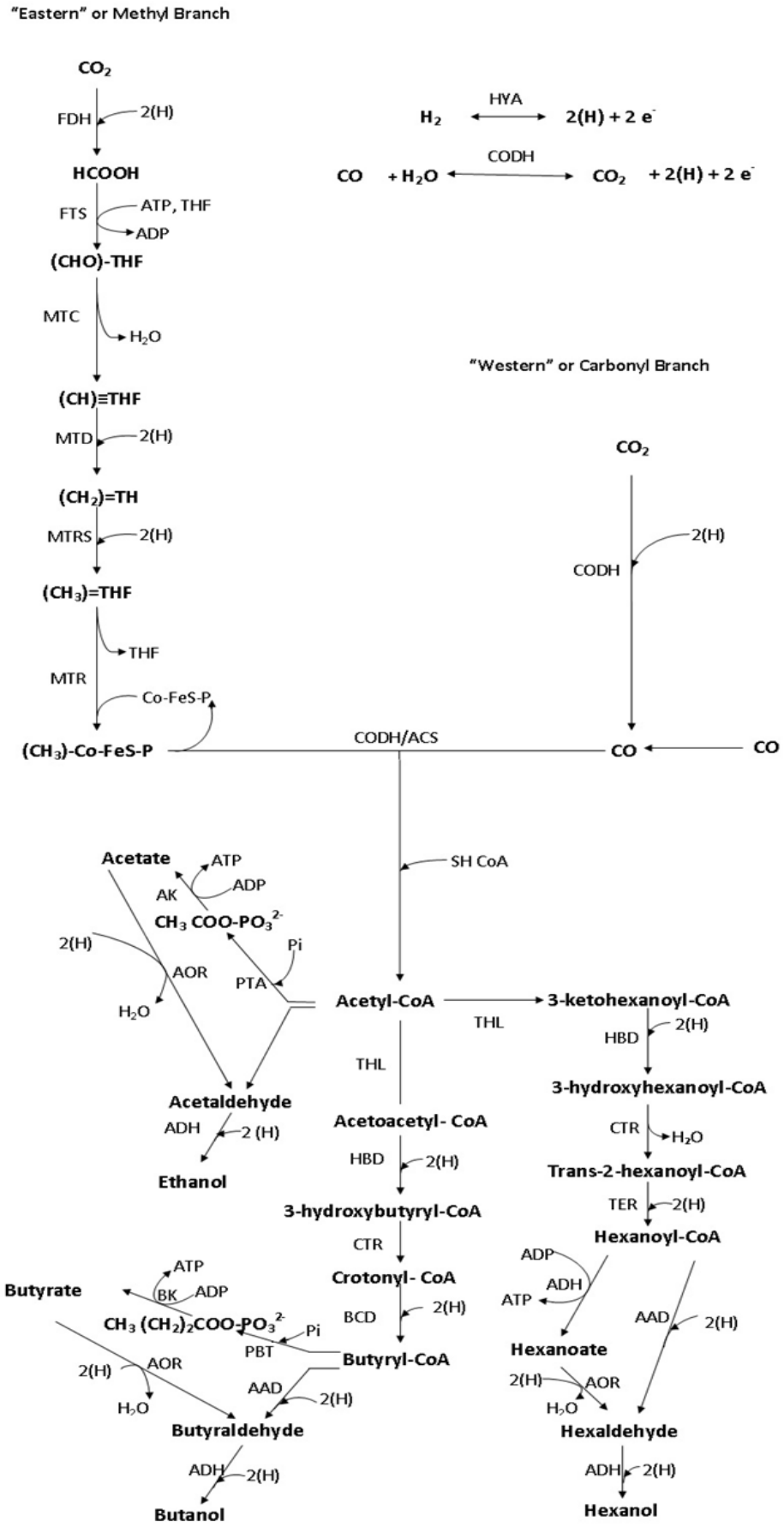

Figure 1.2 Wood-Ljungdahl pathway of acetogens, for H-B-E fermentation (Daniell et al. 2012).

The different steps of the WL pathway leading to the production of different end metabolites are shown in Figure 1.2. The figure shows how the pathway is composed of two branches known as Eastern and Western branches. In the Eastern branch, formate is obtained first through the reduction of CO_2. In the Western branch, the figure makes clear how CO can be taken directly or, otherwise, how CO_2 can be transformed into CO. The Wood-Ljungdahl pathway will eventually lead to the production of acetyl-CoA. Then, the latter is used by different enzymes to generate several end products. On one hand, acetyl-CoA could be converted to both acetate and ethanol. On the other hand, in the case of butanol production, acetyl-CoA is first enzymatically transformed into butyryl-CoA, which could also directly be converted to butyric acid and butanol as end products. Acids, i.e. acetate or butyrate, formed from acetyl-CoA or butyryl-CoA, can also further be converted to the corresponding alcohols. In case of acetate, the acid yields acetaldehyde in a reaction catalyzed by ferredoxin: aldehydeoxydoreductase (AOR). Then, ethanol is produced through the reduction of acetaldehyde. Similar enzymes are used for the conversion of butyric acid into butyraldehyde first and subsequently into butanol. The pathway for the production of higher alcohols such as hexanol leads to the conversion of acetyl-CoA to hexanoyl-CoA which could directly be converted to hexanoate or, otherwise, to hexanol via hexaldehyde as intermediate metabolite (Figure 1.2). Hexanoate and hexanol production in clostridial species is believed to be catalyzed by thiolase enzymes (Figure 1.2), for example 2-keto thiolase. In some cases, butyryl-CoA is elongated with one molecule of acetyl-CoA, by a thiolase enzyme, which results in the production of 3-oxo-hexanoyl-CoA, which is then converted into hexanoyl-CoA. That reaction is believed to be carried out by the same enzymes that convert acetoacetyl-CoA into butyryl-CoA (Tracy et al. 2012).

Finally, as previously explained, hexanol can also be produced by some acetogenic bacteria grown on gaseous C1 substrates (CO, CO_2, and H_2), depending on the bacterial species used in the fermentation process. The presence of hexanoic acid has also been detected during the gas fermentation process and can subsequently be converted to hexanol, similarly as for ethanol and butanol which can be obtained simultaneously, from the fermentation of C1 gases and, to a larger extent, from the corresponding C2 and C4 organic acids (i.e. acetic and butyric acids).

The complex fermentation process can be summarized through the equations shown hereafter.

Potential reactions for ethanol and acetic acid production from CO, CO_2, and H_2 would be (Vega et al. 1989):

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \qquad \Delta G^o = -217.4 \text{ kJ/mol} \qquad \text{Eq. 1.1}$$

$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O$	$\Delta G^o = -97.0$ kJ/mol	Eq. 1.2
$2CO + 4H_2 \rightarrow C_2H_5OH + H_2O$	$\Delta G^o = -137.1$ kJ/mol	Eq. 1.3
$3CO + 3H_2 \rightarrow C_2H_5OH + CO_2$	$\Delta G^o = -157.$ kJ/mol	Eq. 1.4
$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2$	$\Delta G^o = -154.6$ kJ/mol	Eq. 1.5
$4H2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$	$\Delta G^o = -74.3$ kJ/mol	Eq. 1.6
$2CO + 2H_2 \rightarrow CH_3COOH$	$\Delta G^o = -114.5$ kJ/mol	Eq. 1.7

The net reactions for butanol and butyric acid production from CO, CO_2, and H_2 are:

$12CO + 5H_2O \rightarrow C_4H_9OH + 8CO_2$	$\Delta G^o = -486.4$ kJ/mol	Eq. 1.8
$12H_2 + 4CO_2 \rightarrow C_4H_9OH + 7H_2O$	$\Delta G^o = -245.6$ kJ/mol	Eq. 1.9
$6CO + 6H_2 \rightarrow C_4H_9OH + 2CO_2 + H_2O$	$\Delta G^o = -373.0$ kJ/mol	Eq. 1.10
$4CO + 8H_2 \rightarrow C_4H_9OH + 3H_2O$	$\Delta G^o = -334.0$ kJ/mol	Eq. 1.11
$10CO + 4H_2O \rightarrow CH_3 (CH_2)_2 COOH + 6CO_2$	$\Delta G^o = -420.8$ kJ/mol	Eq. 1.12
$10H_2 + 4CO_2 \rightarrow CH_3 (CH_2)_2 COOH + 6H_2O$	$\Delta G^o = -220.2$ kJ/mol	Eq. 1.13
$6CO + 4H_2 \rightarrow CH_3 (CH_2)_2 COOH + 2CO_2$	$\Delta G^o = -317.0$ kJ/mol	Eq. 1.14

Equations for hexanol and hexanoic acid (i.e. caproic acid) production from CO, CO_2, and H_2 are:

$18CO + 7H_2O \rightarrow C_6H_{13}OH + 12CO_2$	$\Delta G^o = -753.0$ kJ/mol	Eq. 1.15
$18H_2 + 6CO_2 \rightarrow C_6H_{13}OH + 11H_2O$	$\Delta G^o = -395.0$ kJ/mol	Eq. 1.16
$6CO + 12H_2 \rightarrow C_6H_{13}OH + 5H_2O$	$\Delta G^o = -514.0$ kJ/mol	Eq. 1.17
$16CO + 6H_2O \rightarrow CH_3 (CH_2)_4 COOH + 10CO_2$	$\Delta G^o = -656.0$ kJ/mol	Eq. 1.18
$16H_2 + 6CO_2 \rightarrow CH_3 (CH_2)_4 COOH + 10H_2O$	$\Delta G^o = -341.0$ kJ/mol	Eq. 1.19
$10CO + 10H_2 \rightarrow CH_3 (CH_2)_4 COOH + 4CO_2$	$\Delta G^o = -540.0$ kJ/mol	Eq. 1.20

A similar two stage fermentation pattern is observed in the production of alcohols from C1 gases (CO, CO_2, and H_2), i.e. the HBE fermentation, as in the more conventional ABE fermentation

using carbohydrate substrates. First the gaseous substrates are converted into acids, during the acidogenic phase, at pH values optimal for growth, usually close to 6.0. Both acetic and butyric acids are formed but, as mentioned earlier, also some hexanoic acid has been detected. Based on the few results published so far, acetic acid appears first and is produced at somewhat higher concentrations than butyric acid in *C. carboxidivorans*. Afterwards, hexanoic acid does also appear, though at lower concentration than the other two acids (Shen et al. 2017). This is different from the more conventional ABE fermentation in which higher concentrations of butyric acid are generally produced compared with acetic acid (Kittithanesuan and Phisalaphong 2015; Wang et al. 2015). The production of acids from pure CO or $CO+CO_2+H_2$ is concomitant with biomass growth and results in acidification of the medium. As a result, the pH decrease inhibits any further biomass growth and stimulates solventogenesis. During the solventogenic phase, alcohols are generated. In a system with no automatic pH regulation, pH is then often found to increase again. In the ABE fermentation, *C. acetobutylicum* metabolizes carbohydrates to produce acetone, butanol and ethanol as solvents. Conversely, in the fermentation of CO-rich gases by *C. carboxidivorans* only ethanol and higher alcohols are produced but no acetone has ever been detected. Ethanol, butanol, and also some hexanol appear as end metabolites. The genome of *C. carboxidivorans* strain P7 was recently characterized and shown to lack genes for acetone production (Bruant et al. 2010). It is worth mentioning that, although acetone is produced in the ABE fermentation in *C. acetobutylicum* and several other studied species, some clostridial strains (e.g. *C. pasteurianum*) appear not to have the ability to produce acetone from carbohydrates in such a process.55 As explained above, butanol is the major solvent in ABE fermentation from sugars, while ethanol has so far always been reported to be the dominant alcohol in the fermentation of C1 gases. This is related to the higher amounts of butyric acid appearing during the acidogenic stage in the ABE fermentation with *C. acetobutylicum* and other clostridia, while more acetic acid is generally detected in the fermentation of C1 gases by *C. carboxidivorans*.

In most of the few studies published so far, usually in batch assays or in continuous gas-fed bioreactors without any continuous addition of nutrients in the form of a liquid phase, final butanol concentrations hardly reached 1 g/L or less, when pH was allowed to fluctuate freely.

In acetogenic bacteria, energy conservation is highly limited. One ATP is produced in the last step of the conversion of acetyl-CoA to acetate catalyzed by the acetate kinase enzyme (Figure 1.2). The same would take place in butyrate and hexanoate production. However, additional ATP can also be formed by the Rnf complex involved in pumping H^+ or Na^+ anions across the cytoplasmic membrane which results in an ion gradient and in concomitant ATP synthesis by the

ATPase protein complexes (Dürre 1998). The Rnf complex has been identified as a proton-translocating ferredoxin: NAD^+ oxidoreductase in *C. ljungdahlii* (Tremblay et al. 2012). Examples of other bacteria that have been found to possess such Rnf complex are *A. woodii* and *C. autoethanogenum* (Müller et al. 2008; Krack et al. 2016). In some cases, the proton gradient in autotrophic acetogens can also be created by an energy converting hydrogenase, or Ech. Only limited research has been published on energy conservation in *C. carboxidivorans*, but some recent reports suggest that in that organism ATP could also be produced by a membrane gradient via the Rnf complex found on its genome.

1.2.1 Anaerobic bacteria converting C1 gases to (higher) alcohols

So far, only very few bacteria have been isolated and proven to produce butanol or higher alcohols from CO, CO_2, and/or H_2 (Table 1-1 Characteristics of different acetogens converting CO-rich gases into alcohols).

Table 1-1 Characteristics of different acetogens converting CO-rich gases into alcohols

	C. ljungdahlii	*C. autoethanogenum*	*C. carboxidivorans P7*	*C. drakei*	*C. ragsdalei P11*	*B. methylotrophicum*	*Alkalibaculum bacchi*
Size (µM)	0.6 x 2 - 3	0.5 x 3.2	0.5 x 3	0.6 x 3 - 4	0.7 - 0.8 x 4 - 5	0.8 - 1.0 x 2.7 -3.2	0.5-0.8 x 1.5–2.2
Temperature range (° C)	30 - 40	20 - 44	24 - 42	18 - 42	18 - 37	10 – 50	15 - 40
Temperature optimum (°C)	37	37	37 - 40	30 - 37	37	37	37
pH range	4.0 - 7.0	4.5 - 6.5	4.4 - 7.6	4.6 - 7.8	4.0 - 8.5	6.0 – 9.0	6.5 - 10.5
pH optimum	6.0	5.8 - 6.0	5.0 - 7.0	5.5 - 7.5	6.3	7.5	8.0 - 8.5
Principal substrate	Syngas (CO, CO_2, H_2 and Ar) and sugars such as glucose, fructose…	Syngas (CO, CO_2, H_2 and N_2) and sugars such as Xylose	Syngas (CO, CO_2, N_2 and H_2) and sugars such as glucose	Syngas (H_2–CO_2, and CO–CO_2) and sugars such as cellobiose, glucose, xylose…	Syngas (CO, CO_2, H_2)	CO, H_2-CO_2, glucose, methanol	H_2 : CO_2, CO : CO_2, glucose, fructose, mannose, turanose, ribose, trimethylamine, pyruvate, methanol, ethanol, n-propanol and n-butanol
Principal products	acetic acid, butanediol and ethanol	Acetic acid, Butanediol, ethanol	Acetic acid, Butyric acid, Ethanol and Butanol	acetic acid, butiric acid, ethanol and butanol	acetic acid, butanediol and ethanol	acetic acid, butiric acid, lactate, ethanol and n-butanol	acetic acid, ethanol,
Reference	Tanner et al. 1993; Mohammadi et al. 2014	Abrini et al. 1994	Liou et al. 2005	Liou et al. 2005	Huhnke et al. 2008	Zeikus et al. 1980; Heiskanen et al. 2007	Allen et al. 2010

Their main characteristics of each bacteria strain are briefly summarized hereafter. In the case of *C. carboxidivorans* (the bacterial strain used in all the experimental section of this thesis) studied further.

Clostridium drakei (ATCC BAA-623T; DSM 12750T)

This species was renamed in recognition of the contributions that Harold L. Drake has made to our understanding of the microbiology of acetogens. This bacterium is an obligate anaerobe, growing autotrophically on H_2/CO_2 or CO, to produce acetic and butyric acids, ethanol, and butanol as end-products of its metabolism (Gößner et al. 2008).

Clostridium ragsdalei (ATCC BAA-622, DSM 15248)

This acetogen ferments syngas ($CO:CO_2:H_2$) into acetic acid and ethanol. One report describes the detection of small amounts of butanol, up to 0.47 g/L, in a bioreactor inoculated with *Clostridium* strain P11, which is expected to belong to the species *C. ragsdalei*, based on other publications and data bases (Kundiyana et al. 2010). However, production of higher alcohols from syngas by *C. ragsdalei* has otherwise never been confirmed nor reported elsewhere. One study has shown that that organism is able to reduce organic acids such as propionic acid, butyric acid, pentanoic acid, and hexanoic acid into the corresponding primary alcohol (propanol, butanol, pentanol, and hexanol) (Isom et al. 2015).

Butyribacterium methylotrophicum

This organism is a catabolically spore-forming anaerobe that ferments multicarbon substrates (e.g. glucose, lactate, and pyruvate) or single-carbon substrates (CO, H_2/CO_2) to produce varying amounts of acetic and butyric acids, ethanol and butanol (Kerby and Zeikus 1987; Worden et al. 1989; Grethlein et al. 1991).

Clostridium carboxidivorans (ATCC BAA-624; DSM-15243)

This organism was isolated from an agricultural settling lagoon in Oklahoma (USA) (Liou et al. 2005). The 16S rRNA gene sequence analysis showed that that species is closely related to *C. scatologenes* ATCC 25775 T and *C. drakei*, but DNA reassociation analysis showed that these three bacteria are different species. *Clostridium carboxidivorans* is an acetogenic anaerobic bacterium, which can grow autotrophicaly with syngas (CO or H_2/CO_2) and chemoorganotrophically with a great variety of sugars such as glucose, xylose, fructose, cellobiose and arabinose. Moreover, it is one of the few bacteria known to produce solvents from syngas (Köpke et al. 2011), including long-chain alcohols (e.g. butanol and hexanol) besides ethanol (Table 1-2) (Phillips et al. 2015; Ramió-Pujol et al. 2015).

Table 1-2 Wild type acetogenic bacteria producing long chain fatty acids and alcohols (C4, C6) from CO, CO_2/H_2, or mixtures of all three gases.

Bacteria	Butyrate	Hexanoate	Butanol	Hexanol	Reference
Acetonema longum	+	NR/ND	NR/ND	NR/ND	Kane and Breznak 1991
Butyribacterium methylotrophicum(*)	+	NR/ND	+	NR/ND	Shen et al. 1999
Clostridium carboxidivorans	+	+	+	+	Liou et al. 2005; Philips et al. 2015; Ramió-Pujol et al. 2015
Clostridium difficile	+	NR/ND	NR/ND	NR/ND	Köpke et al. 2013
Clostridium drakei	+	NR/ND	NR/ND	NR/ND	Gößner et al. 2008
Clostridium scatologenes	+	NR/ND	NR/ND	NR/ND	Küsel et al. 2000
Eubacterium limosum	+	NR/ND	+	NR/ND	Jeong et al. 2015

There are numerous studies on the metabolic and genomic characteristics of that organism. Results indicate that *C. carboxidivorans* follows a Wood-Ljungdahl related pathway for the production of acetic, butyric, and hexanoic acids, as well as ethanol, butanol, and hexanol, metabolizing CO or syngas, used as carbon and energy sources (Figure 1.3) (Liou et al. 2005; Bruant et al. 2010; Ukpong et al. 2012).

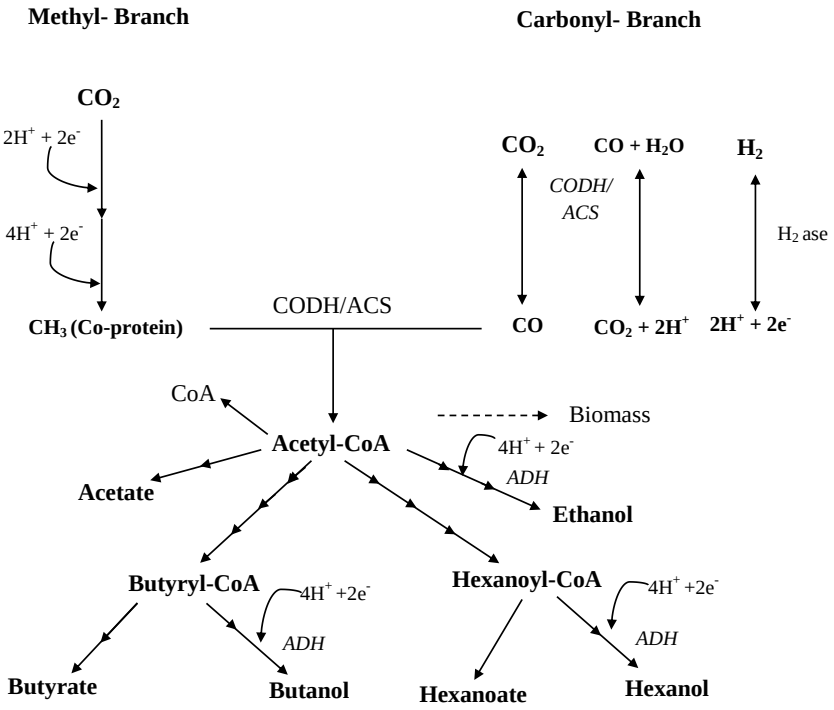

Figure 1.3 Wood–Ljungdahl pathway of *Clostridium carboxidivorans* (Liou et al. 2005; Bruant et al.2010; Ukpong et al. 2012).

In the case of CO and CO_2 the organism used those compounds as carbon source whereas H_2 is used as source of electrons by means of the enzyme "hydrogenase" (Krasna 1979). Under conditions of inhibition of the hydrogenase enzyme, the bacteria will need another source of electrons, which can then be obtained from CO. As described more in detail below, this is the case in presence of compounds such as NO, which can appear as minor compound in syngas, and has been shown to inhibit the hydrogenase enzyme. However, this provokes also a limitation in the use of CO for the formation of desired metabolites and does consequently result in a less efficient fermentation process (Ahmed et al. 2006).

So far, in terms of solvents, the highest end product concentration has always been found for ethanol followed by butanol and finally hexanol. Those alcohols are produced in that same chronological order during carbon monoxide or syngas fermentation, with short chain alcohols appearing first while longer chain ones appear later on. *C. carboxidivorans* has the typical "biphasic fermentation pattern" of many acetogens producing alcohols, and usually the gas fermentation process takes place in two stages; initially carboxylic acids are produced from the gaseous substrates followed by the subsequent conversion of those acids and remaining gases into alcohols. Besides, exponential biomass growth and acidogenesis (with production of acids) are two related processes and take place simultaneously. The solventogenic phase in clostridia

has been considered to start when the conditions are not favourable anymore for growth, i.e. low pH, low ATP levels, accumulation of high concentrations of organic acids, sporulation, low level of availability of reducing energy (Meyer and Papoutsakis 1989; Dürre et al. 1995; Guedon et al. 1999; Dürre and Hollergschwandner 2004). When alcohols are the desired end products, it is necessary to identify the optimum medium composition and conditions for an efficient conversion of accumulated organic acids with the concomitant production of solvents. The culture conditions and another important parameters for the alcohols production with this bacteria strain are described in the Chapter 3.

Besides the main carbon and energy sources, several nutrients and trace compounds may be needed as well. In case of *C. carboxidivorans*, a recent study was published in which the effect of different media compositions were analyzed for their effect on growth and butanol production. Removing copper (Cu) from the culture medium and increasing the molybdate (Mo) concentration allowed to improve the production of butanol (Phillips et al. 2015). It was concluded that Mo can be considered to be analogous to tungsten (W), which is related with the enzyme AOR (*aldehyde:ferredoxin oxidoreductase*), an enzyme involved in the conversion of acids to alcohols. Similarly, the presence of W had previously been proven to stimulate the conversion of carbon monoxide and acetic acid into ethanol in *C. autoethanogenum* (Abubackar et al. 2015). Micronutrients, trace metals or vitamins play a key role in the activity of specific enzymes and in favouring a given metabolic route. Other parameters, described in the next section, such as temperature and pH, will also affect growth, the metabolic behavior and the bioconversion process.

1.3 Parameters affecting gas fermentation to higher alcohols

For the commercial production of (bio)butanol and other alcohols from C1 gases in an efficientway, a key issue consists in identifying optimal conditions for the fermentative process. Therefore, several parameters need to be considered, as described bellow.

1.3.1 pH

The importance of pH to promote the shift from acidogenic to solventogenic phase has been explained previously. A low pH has been considered to be more favourable for the solventogenic stage, i.e. ethanol, butanol, and hexanol production in the present case whereas such a low pH will have a negative effect on cell growth (Lee et al. 2008; Millat et al. 2013). This is a major drawback limiting the optimal conversion of syngas to alcohols, as a lower pH will inhibit

bacterial growth and eventually lead to cell death. It may then also limit the overall specific productivity of butanol and other alcohols in the process.

In the acidogenic phase, it is necessary to know and take into account the optimum growth pH of the microorganism, which is strain specific. A pH value below optimum has a negative impact on growth rates and cell viability and may even result in biomass decay. *Clostridium* strains have an optimum pH for growth ranging between 5.5 and 7.5 depending on the species. The optimum pH of butanol- or higher alcohols-producing species appears in Table 1-1.

1.3.2 Temperature

Similarly to pH, bacteria exhibit their growth and metabolic activity over a given limited range of temperatures characterized by defined optimal values. Temperature does not only affect microbial growth and substrate bioconversion rates in syngas fermentation; it does also affect the solubility of gaseous substrates in liquid medium. For example, CO and H_2 are very poorly soluble in aqueous phase and higher temperatures have a negative impact on such solubility (Table 1-3).

Table 1-3. Solubilities of CO, H_2 and CO_2 in aqueous phase, at different temperatures and constant pressure of 1 atm, expressed in g/L water

Temperature ° C	CO	CO_2	H_2
15	0.031	2.01	0.0017
25	0.028	1.50	0.0016
30	0.026	1.32	0.0015
37	0.023	1.13	0.0014
45	0.019	0.86	0.0013
60	0.015	0.59	0.0012

To the best of our knowledge, all clostridia isolated so far and producing (bio)fuels from C1 gases are mesophilic organisms. Mesophiles typically grow in a temperature range between about 15 and 40° C, with optima between 30 and 37° C in most cases, depending on the species. In the case of carboxydotrophs, the optimum temperature for incubation is often close to 35–37° C. The most common temperature ranges of the few anaerobic bacteria typically used in the production of butanol or higher alcohols from C1 gases are given in Table 1-1.

It has been suggested that temperature may affect 'acid crash'. 'Acid crash' results from the accumulation of high concentrations of undissociated acids produced during acidogenesis, mainly in batch fermentations, leading to the inhibition of solventogenesis and limiting the

production of alcohols. It was observed experimentally that a lower incubation temperature during the conventional ABE fermentation, may help avoiding 'acid crash', as a result of the lower rates of production of acids at such lower temperatures (Maddox et al. 2000).

In a recent study, the syngas-fermenting acetogenic bacterium *C. carboxidivorans* P7 was also incubated at a sub-optimal temperature of 25° C, leading to a lower metabolic activity, slow growth and longer lag phase. It also led to enhanced ethanol and butanol production, reaching concentrations of 32.1 and 14.5 mmol/L, respectively (Ramió-Pujol et al. 2015). Some hexanol and caproic acid were also produced in that experiment, at concentrations of 8.21 and 9.02 mmol/L, respectively. The concentrations of alcohols were found to be significantly higher at these lower temperatures than when incubating at 37° C. On the other hand, other authors did also study the effect of temperature on *C. carboxidivorans* (Zhang et al. 2013). They found that a high temperature (37° C) improved the reassimilation of acids into alcohols compared with a lower temperature (25° C), but that a lower temperature improves the elongation of the carbon chain. Therefore, a low temperature is favourable for obtaining long chain acids and alcohols, improving the production of alcohols such as butanol and hexanol. In the case of butanol and hexanol, the highest concentrations of alcohols were found to reach 0.57 g butanol/L, and 0.48 g hexanol/L in a study performed at 25° C, while at a higher temperature of 37° C butanol and hexanol were not detected (Ramió-Pujol et al. 2015).

1.3.3 Pressure

Another important factor in syngas fermentation is the gas pressure, as solubility inwater for gases such as CO, H_2 or CO_2 increases basically linearly with pressure at moderate pressures as used in bioconversion processes. An increase in pressuremay also improve mass-transfer. Consequently, higher pressures result in a better supply of the carbon/energy source in the culture media and increases the availability of carbon/energy sources to the cells, leading to higher growth and higher concentrations of fermentation products. Abubackar et al. (2011) compared the effect of different nutrients and different initial pressures on ethanol fermentation using *C. autoethanogenum* as biocatalyst. The authors concluded that increasing the initial pressure led to higher final ethanol concentrations, as higher pressures will positively affect gas solubility and the efficiency of bioconversion (Abubackar et al. 2011).

1.3.4 Effect of medium composition

Recently, aspects such as culture medium composition have been optimized, aimed at maximizing the conversion of gaseous substrates and acids into alcohols.

The carbon/energy source is probably one of the most important factors affecting the nature of end metabolites obtained in the fermentation process. It has been known for decades that clostridia are able to use different sugars as carbon source. More recently, studies have also focused on the use of CO, CO_2 +H_2, syngas, or waste gases as carbon and/or energy sources for the production of (bio)fuels. However, so far, only quite few acetogens have proven to be able to produce butanol or higher alcohols from such gases. In case of ethanol production from CO, CO_2, and H_2, some studies have described the effect on bioconversion of other components of the culture medium than the carbon and energy sources. However, hardly any information is available in the case of butanol or higher alcohols production. Compounds that have been demonstrated to improve the microbial productivity of alcohols are metal co-factors. Nickel is one of those. Some authors studied the effect of that metal on ethanol production (Ragsdale 2009; Simpson et al. 2011). It was concluded that nickel improves CO uptake and alcohol (e.g. ethanol) production in gas fermentation, due to the importance of that trace metal for the enzymes CO dehydrogenase and acetyl-CoA synthase. The effect of various trace metals on ethanol production was also studied in *C. ragsdalei* (Saxena and Tanner 2011). It was shown that removing Cu^{2+} and increasing the concentrations of Ni^{2+}, Zn^{2+}, SeO_4^{2-}, and WO_4^{2-} increased ethanol production, due to the increase of the activity of specific metalloenzymes in the WL pathway. Abubackar et al. (2016) found that the presence of tungsten significantly improved alcohol production and increased the ethanol/acetate ratio in *C. autoethanogenum* grown on CO, while the presence of selenium did either not improve ethanol production or even reduce it under the experimental conditions used in their study (Abubackar et al. 2015; Abubackar et al. 2016). Metalloenzymes and the presence of trace metals play thus a key role in ethanol production and are expected to have a similar influence in the bioconversion of gaseous C1 substrates to higher alcohols such as butanol and hexanol.

In a recent report, the effect of different trace compounds was described (e.g. W, Ni, Cu, Mo, and Zn) for batch butanol and hexanol production in *C. carboxidivorans* (Phillips et al. 2015). Media in which copper was removed while increasing the molybdate concentration (×10) resulted in improved butanol and hexanol production. With the standard non-modified medium, maximum butanol and hexanol concentrations of 0.83 g/L and 0.24 g/L, respectively, were obtained. Conversely, with the modified medium described above, butanol and hexanol concentrations reached 1.09 g/L and 0.94 g/L, respectively, resulting in 26 % and 70 % increase in butanol and hexanol production under such conditions (Phillips et al. 2015). Molybdate (Mo) is an analog of tungsten (W) and binds in the active sites of some enzymes, such as the AOR

involved in the conversion of acids (e.g. butyric acid) into alcohols (e.g. butanol), as shown in Figure 1.2.

Other compounds have been studied for their influence on solventogenesis such as the nature and concentration of nitrogen sources or the presence of vitamins. No study has been reported for butanol and hexanol, but a related behavior can be foreseen as for ethanol. Generally, richer media with high nitrogen concentrations or the presence of yeast extract will stimulate acidogenesis, result in the production of acids and improve biomass growth, while nutrient limiting conditions are more suitable for the solventogenic phase. Although most researchers add vitamins to their culture media, it was observed that *C. autoethanogenum* can efficiently produce ethanol without addition of any vitamins (Abubackar et al. 2016).

A low redox potential is also required to produce butanol, ethanol, and higher alcohols, and, as a general rule, a reducing agent such as cysteine-HCl needs to be added into the medium, in order to increase the production of alcohols (Mitchel 1998). Reducing agents are involved in the conversion of NAD(P) to NAD(P)H, which favours the production of alcohols.

1.3.5 Inhibitory compounds

Although biomass-derived syngas is composed of CO, CO_2, and H_2 as major constituents, it does usually also contain a range of additional compounds such as methane, ethylene, ethane, and acetylene, among others (Ahmed et al. 2006; Haryanto et al. 2009), which may affect the fermentation process as they are potential inhibitors of bacterial growth and/or of their metabolic activity, eventually leading to poor bioconversion yields.

Even trace amounts of additional constituents such as acetylene or NO (nitrogen oxide) are known to inhibit the activity of some enzymes such as the hydrogenase enzyme (Xu and Lewis 2012), which is involved in the generation of electrons from the reaction with H_2. A NO mole fraction above 0.015 % was found to exert a strong inhibitory effect on hydrogenase (Ahmed and Lewis 2007). The result of such inhibition is that electrons for ethanol, butanol, or hexanol formation must be obtained from CO rather than H_2, thus reducing the available carbon amount for product formation. The inhibition of this enzyme forces the cell to obtain electrons from CO using CODH enzymes. Another problem associated with the presence of NO in syngas fermentation is initial growth inhibition, besides reduction of the carbon conversion efficiency of the process. Similarly, when using syngas or waste gases containing CO, CO_2 and H_2 as substrate mixture, high concentrations of CO have been proven to inhibit the activity of hydrogenases in acetogens and thus inhibit hydrogen consumption. This may result in CO consumption while H_2

and CO_2 would then remain unused (Bertsch and Müller 2015). So far most bioconversion studies on alcohol production and HBE fermentation have been performed in suspended-growth stirred tank bioreactors. The low solubility of carbon monoxide in aqueous phase might explain why hydrogen consumption has been found to be still possible in such cases, minimizing the inhibition of hydrogenases.

Sulphur compounds may also appear in syngas. In *C. ljungdahlii*, H_2S concentrations as high as 5.2 % (v/v) were found to hardly affect bacterial growth (Klasson et al. 1993). The presence of sulphur compounds may even contribute to further reduce the redox potential of the anaerobic culture medium. On the other hand, sulphur compounds can easily poison chemical catalysts, when they are used for the conversion of syngas into industrial products, which is a clear advantage of the biological process.

Ammonia (NH_3) is another impurity to be taken into account in syngas. Its presence can lead to non-negligible accumulation of ammonium ions (NH^{4+}) in media used for bioconversion. Ammonium ions have been shown to be able to inhibit hydrogenase activity and cell growth of *C. ragsdalei* (Xu and Lewis 2012).

Tars in syngas obtained from biomass were assumed to be the likely cause of cell dormancy in *C. carboxydivorans* and product redistribution, leading to increased alcohol production (in terms of ethanol) and decreased acetic acid production (Ahmed and Lewis 2007). *Clostridium carboxydivorans* appeared to be able to adapt to tars after prolonged exposure. If needed a gas cleaning unit, e.g. filtering system, may be used in order to reduce or avoid the effect of tars present in the gaseous substrate. A recent study using *C. ljungdahlii* showed that concentrations of 0.01 g/L of $CaCO_3$ increased cell growth and the production ethanol. However, higher concentrations (0.02 g/L) will rather decrease growth and solvent production (Xie et al. 2015).

Other compounds and factors may affect the bioconversion process. The salt concentration may have inhibitory effects that may need to be taken into account. Sodium chloride may accumulate in some fermentation processes, for example when adjusting the bioreactor´s pH through the addition of either hydrogen chloride or sodium hydroxide (Bailón et al. 2007). Although this has scarcely been studied, some reports described the effect of salt accumulation in bioreactors and batch experiments in the case of ABE-producing strains. Maddox *et al.* found that concentrations of 30 g/L sodium chloride or higher completely inhibit growth of *C. acetobutylicum* with lactose as a carbon source (Maddox et al. 1995). A concentration of 15 g/L of that compound resulted in 50 % growth inhibition. At lower concentrations, growth took place but the fermentation pattern shifted from solventogenic to acidogenic.

1.3.6 Solvent toxicity

Solvent (i.e. alcohols from the HBE fermentation) toxicity has been identified as a critical problem in the fermentation. Under normal conditions, the clostridial cellular activity decreases in the presence of 20 g/L or more solvents in the conventional ABE production process (Woods 1995). This is one of the most important factors to take into account in butanol fermentation as anaerobic bacteria fermenting carbohydrates or other similar soluble substrates have been found to rarely tolerate more than 2 % butanol (Liu and Qureishi 2009). Butanol has shown to be a fermentation product more toxic than acetone or ethanol in ABE fermentation (Moreira et al. 1981), and it was observed that the cells can tolerate higher concentrations of ethanol and acetone than butanol during growth. Although as much as 40 g/L acetone and ethanol were required to reduce growth on hexoses by 50 %, butanol concentrationsofonly 7–13 g/L were already high enough to exert a similar inhibitory effect (Jones and Woods 1986).

Some of the suggested reasons for the high toxicity of butanol are summarized hereafter. In a study on the mechanism of butanol toxicity in *C. acetobutylicum* in ABE fermentation it was found that 8–12 g/L butanol caused 50 % inhibition of cell growth and the sugar uptake rate by negatively affecting the ATPase activity (Moreira et al. 1981). This is because butanol is a lipophilic solvent. It can alter the fatty acids and phospholipids composition of the cell membrane, which will lead to an increase in membrane fluidity. This will alter some of the membrane functions, such as membrane ATPase activity, transport functions, and substrate uptake (Bowles and Ellefson 1985).

Several studies have focused on trying to improve solvent (e.g. butanol) tolerance in clostridial strains. Two major alternatives have been put forward: (i) using mutagenesis and genetic manipulation; and (ii) maintaining a low concentration of solvents such as butanol in the fermentation broth. At least one recent report has shown that in the fermentation of CO-rich gases, mutant strains were obtained that were able to grow in the presence of ethanol concentrations of around up to 50 g/L (Koepke et al. 2012). For maintaining low solvent concentrations in the fermentation broth, different methods are available to separate end metabolites in order to avoid reaching concentrations that may be inhibitory to the bacteria and that would affect the fermentation process (Zheng et al. 2009). These techniques have been applied in ABE fermentation and, although the end metabolites in CO, syngas and waste gas fermentation are slightly different, similar separation processes would be suitable. A detailed description of these methods is beyond the scope of this review, but common removal techniques are adsorption, liquid–liquid extraction, pervaporation and gas stripping, besides

some other somewhat less studied alternatives (Kumar and Gayen 2011). Adsorption is commonly set-up as an external unit, but otherwise most of the techniques can either be integrated in the fermentation process itself or they can be used offline. Studies reported in the literature concern mainly ABE fermentation, but they could similarly be applied to the HBE process.

In adsorption the solvents are transferred to a solid material, e.g. zeolites or activated carbon. It is a rather easy to use technique, requires generally little energy compared with other alternatives, does not damage bacterial cells and is effective in separating solvents such as butanol from the medium (Agueda et al. 2013; Xue et al. 2016). Liquid–liquid extraction consists in using a solvent with a high extraction efficiency for the metabolites to be separated from the fermentation broth. At the same time, for *in situ* separation, one should make sure that the solvent chosen for extraction does not exhibit any inhibitory effect on the bacteria. Oleyl alcohol is quite popular for the extraction of butanol in ABE fermentation or for other similar products. Ionic liquids, as non-volatile extractants, have more recently been used as well. In gas stripping, an inert gas or gas generated from the fermentation process itself allow to strip solvent-metabolites from the medium. This technique is simple and does not damage the cells. However, a potential drawback is that it can result in foam formation, above all when working with small bubbles, which can negatively affect the stability and performance of the fermentation process. Low selectivity is another potential drawback to be taken into account (Li et al. 2016). Pervaporation is a membrane-separation process. It has a relatively low energy consumption and does not damage the cells. A possible problem is membrane fouling (Groot et al. 1992; Abdehagh et al. 2014; Huang et al. 2014).

Some studies calculated the energy needed for 1-butanol recovery with these different techniques. For adsorption a value of 1.3 MJ/kg-butanol was reported, but it may go up to (Raganati et al. 2012); using pervaporation, energy requirements between 2 and 145 MJ/kg-butanol were reported; values between 7 and 14 kg/butanol are typical for liquid–liquid extraction and finally values in the range of 14–31MJ/kg-butanol were reported for gas stripping (Li et al. 2016; Outram et al., 2016).

1.3.7 Mass transfer limitation in syngas fermentation

The efficiency of bioconversion of syngas and related waste gases to butanol and other alcohols is limited by the low water solubility of their gaseous components, i.e. CO, CO_2, H_2, and gas–liquid mass transfer is therefore a rate-limiting step in the fermentation process (Worden et al. 1991; Klasson et al. 1993; Worden et al., 1997; Bredwell et al. 1999). Generally, gas–liquid mass

transfer limits the conversion rates in bioprocesses that use poorly soluble gases as key components, i.e. carbon and/or energy sources (e.g. CO or H_2 in homoacetogens).

Solubility data of syngas/waste gas components are given in Table 1-3. As can be observed the solubilities of CO and H_2 are quite low. In order to compare with another gas such as oxygen, which is the main electron acceptor in aerobic fermentation processes and whose solubility is about 5mg/L at room temperature, the solubilities of CO and H_2 appear to be only about 60 % and 3 % of oxygen solubility, respectively, on a mass basis. Conversely, the solubility of CO_2 is more than fifty times higher than that of carbon monoxide (Table 1-3).

There are several steps in the diffusion process where mass transfer limitations are inevitable in suspended growth bioreactors: (i) the transport of the gaseous substrate into the gas–liquid interface; (ii) its transport through the nutritive liquid phase to reach the microbial cell surface; and finally (iii) gas diffusion into the microbial cell (Seedorf et al. 2008). Depending on the bioreactor configuration, parameters such as the composition and properties of the liquid, interfacial adsorption, bubble size, mixing intensity, and other factors may influence the magnitude of the mass transfer resistances (Klasson et al. 1992; Seedorf et al. 2008; Munasinghe and Khanal 2010a). The mass transfer coefficient (K_La) helps understand the rate of mass transfer. K_La for a slightly soluble gaseous substrate can be determined using the following equation (Klasson et al. 1992):

$$\frac{1}{V_L}\frac{dN_s^g}{dt} = \frac{K_L a}{H}\left(p_s^g - p_s^L\right)$$

where N_S^g (mol) is the moles of substrate transferred from the gas phase, V_L is the liquid working volume of the reactor, p_S^g (atm) is the partial pressure of the volatile substrate in gas phase and p_S^{gL} (atm) is the partial pressure of the volatile substrate in a gas phase that would be in equilibrium with the actual concentration of that substrate in the liquid phase, H (L atm mol^{-1}) is the Henrýs law constant, and $K_L a$ (s^{-1}) is the overall mass transfer coefficient.

It is clear from the above equation that the efficiency of the fermentation process, in terms of mass transfer and gaseous substrate supply to the biocatalyst, will improve when increasing the gas mass transfer coefficients or at higher concentration (i.e. pressure) gradients. High gas–liquid mass transfer conditions are strongly desired in commercial syngas or waste gas fermentation (Abubackar et al. 2011). Working with pressurized bioreactors would be a suitable means to improve gas supply to the bacteria. However, this would also increase the solubility of carbon monoxide in the liquid phase in suspended-growth bioreactors and, as suggested above, may eventually lead to inhibitory effects such as the inhibition of cell growth and of the activity

of hydrogenases, with the concurrent accumulation of H_2 and CO_2 whenever produced or originally present in the syngas or waste gas mixture. Nevertheless, Chang et al. (2001) reported that high cell density cultures are less affected by the potential inhibitory effects of high carbon monoxide pressures compared with fermentations at low biomass densities (Chang et al. 2001). Such high cell concentrations could be reached, in continuous suspended-growth bioreactors, through the use of cell recycling modules. Another characteristic of pressurized bioreactors is that they would result in higher operating costs.

Various substances such as surfactants, alcohols, salts, catalyst and small particles can be added to increase the gas–liquid mass transfer rates (Zhu et al. 2009). Besides, the addition of nanoparticles to batch bottle systems or stirred tank bioreactors for carbon monoxide or syngas fermentation have shown to lead to increased mass transfer. The addition of functionalized nanoparticles yielded better results than non-functionalized ones. The dissolved concentrations of CO, CO_2, and H_2 were found to increase by 273 %, 200 %, and 156 %, in the presence of methyl functionalized silica nanoparticles at a concentration of 0.3 % by weight. Similarly, the cell concentration of *C. ljungdahlii*, producing ethanol, increased between 29 % and 166 % (Kim et al. 2014). However, such approach might not be realistic nor cost-effective at present in full-scale reactors.

Bioreactors suitable for HBE fermentation are reviewed in the next section. The k_La values in such reactors may vary depending on several parameters, such as gas and liquid flow rates, agitation speed in CSTR or gas bubble size in suspended-growth bioreactors. A few authors recently compared mass transfer in different bioreactor configurations, in the case of both packed-bed and suspended-growth systems mainly with pure carbon monoxide. Gas-lift bioreactors have been considered to represent a suitable configuration because of their simple design and low energy requirements (e.g. no agitation) combined with the highest mass transfer coefficient compared with other bioreactors including column diffusers, hollow fiber membrane bioreactors (HFMB) and biotrickling filters (Munasinghe and Khanal 2010b). Other authors compared innovative attached-growth bioreactors, namely an HFMB and a monolith bioreactor, with more conventional systems for gas fermentation with *C. carboxidivorans* (Shen et al. 2014a). High k_La values were found for the HFMB compared with suspended-growth bioreactors, such as the CSTR and the bubble column bioreactor. Next, the monolith bioreactor also showed high k_La values, though somewhat lower than the HFMB. Such mass transfer coefficients increased at higher gas or liquid flow rates both in the HFMB and the monolith bioreactor. Biotrickling filters are also characterized by relatively high k_La coefficients for which values exceeding 100 h^{-1} have sometimes been reported (Bredwell et al. 199; Munasinghe and

Khanal 2010b), while such values are generally lower for other systems, except the HFMB or the CSTR when applying very high agitation speeds, which would, however, not be suitable for full-scale application.

1.3.8 Kinetics

The production of solvents by acetogens through the WL pathway in the presence of CO and/or CO_2, generates less ATP than through glycolysis. The bacterial species does also play a role, and it was observed that bacteria following the glycolytic pathway may exhibit different growth rates depending on the species and carbohydrate used as substrate. For example, lower specific growth rates were found in *C. tyrobutyricum* grown on glucose than on xylose, which was assumed to be due to the higher amount energy required for transportation of xylose across the cell membrane resulting in less ATP available for growth (Zhu et al. 2002). Generally the formation of fatty acids (e.g. acetic acid, butyric acid) in acetogenic bacteria leads to more net ATP production from C1 gases than the formation of alcohols such as ethanol, butanol, or higher alcohols. Moreover, butanol production with CO as electron donor has a more positive energy balance than in the presence of H_2+CO_2 (Bertsch and Müller 2015). However, one major drawback from an environmental point of view is that CO_2, which is a greenhouse gas, is released as end-product together with butanol when using CO as electron donor, while CO_2 is not produced and is even consumed when using H_2+CO_2 as substrates (see stoichiometric equations above). In presence of syngas or waste gases containing a mixture of CO+CO_2+H_2, complete CO_2 removal might also be possible. This is also related to the amount H_2 available, as hydrogen is required to metabolize carbon dioxide.

Although only limited information has been published on biomass growth rates and yields of clostridia on C1 gases, as a general rule biomass grows better on carbohydrates than on CO-related compounds. Table 1-4 compares kinetic parameters of different *Clostridium* spp. grown either on sugars or C1 gases. Major parameters such as pH and other culture conditions are also given in Table 1-3, whenever reported, as they affect biomass growth and bioconversion rates. Other factors such as the presence of micronutrients or trace metals can also have some influence and details of the exact media compositions can be found in the original publications. Reported data suggest that growth rates on carbohydrates are higher than on CO or CO_2, except for xylose which does also exhibit weak growth rates. Besides, biomass yields and build-up are also low on C1 gases and the concentration of bacterial cells accumulating in bioreactor studies is therefore a limiting factor in the bioconversion of CO-rich gases to alcohols. Alternatives such

as the use of immobilized biomass or cell recycling will improve the amount active biocatalyst in the bioreactor and thus increase bioproduction rates.

Table 1-4 Specific growth rates of wild type *Clostridium* spp. grown on CO or carbohydrates.

Microorganism	Carbon source	Culture conditions	Specific growth rate (μ) (h^{-1})	References
C. carboxidivorans	CO/CO_2, H_2/CO_2	Batch pH= N.R. (*) T= N.R.(*)	0.16, 0.12	Liou et al. 2005
C. acetobutylicum	Glucose	Batch pH=6.0 T=N.R.(*)	0.48	Srivastava et al. 1990
	Lactose	Batch pH=5.0 T=35° C	0.23 - 0.28	Napoli et al. 2012
C. tyrobutyricum	Glucose	Fed-Batch pH=6.0 T=37° C	0.214 ± 0.044	Liu et al. 2006
	Xylose	Fed-Batch pH= 6.0 T= 37° C	0.116 ± 0.009	Liu et al. 2006

N.R.: Not Reported; (*)Presumably under optimal conditions of pH (6.2) and T (38° C)

1.4 Bioreactor configuration

The reactor design is an important factor in syngas fermentation. Most studies on syngas fermentation and their large-scale application have been performed in suspended-growth bioreactors but, besides such systems, other bioreactors can be used as well, several of which have been applied at full-scale for handling gases and waste gases, including other types of stirred tank suspended-growth bioreactors, biofilters, biotrickling filters or trickle bed bioreactors, bioscrubbers, gas-lift bioreactors, bubble column bioreactors, moving bed biofilm reactors, and membrane bioreactors (Kennes and Veiga 2001; Kennes et al. 2009; Abubackar et al. 2011). The most widely studied bioreactor for the conversion of C1 gases to alcohols, at lab-scale, is the stirred tank fermentor. Some of the keys to design an efficient reactor are high mass transfer rates, high bioconversion rates, low operation and maintenance costs, and easy scale-up. So far, only very few results have been reported in the literature on bioreactors for the production of butanol and higher alcohols from C1 gases.

1.4.1 Continuous stirred tank reactors (CSTR)

The CSTR is a typical suspended-growth fermentation unit (Figure 1.4). The fermentation broth contains freely growing bacteria, with continuous supply of the gaseous substrate using gas

diffusers. The agitation mechanism allows break-up of large bubbles into smaller ones, improving the gas–liquid mass transfer. A similar effect can be reached through the use of a microbubble sparging system (Bredwell and Worden 1998). Increasing the impeller speed is a way to increase the mass transfer of sparingly soluble gases, as it will improve mixing and reduce the bubble size. However, a relatively high input of energy per unit volume is required to increase the bubble break-up. Consequently, stirred tank fermentors with high agitation speeds would not be economically viable for large-scale production processes due to excessive operational costs (Abubackar et al. 2011). The gas–liquid mass transfer rate in stirred tank bioreactors will not only increase with the agitation speed, but its value will also depend on the gas retention time, i.e. the gas flow rate.

In the CSTR, the gaseous substrate is continuously fed through the bottom part of the reactor and flows upwards through the fermentation broth. Recent studies have proven that good bioconversion yields and ethanol productivities can be reached from either CO or gas mixtures (CO, CO_2, H_2) in semi-continuous stirred tank bioreactors, in which part of the aqueous medium is occasionally removed after a given period of time and replaced by fresh medium (Abubackar et al. 2016) . Periodic pH shifts in such process allow switching, in a cyclic way, from the acidogenic to the solventogenic stages, with progressive accumulation of increasing concentrations of alcohols and near complete consumption of accumulated acids.

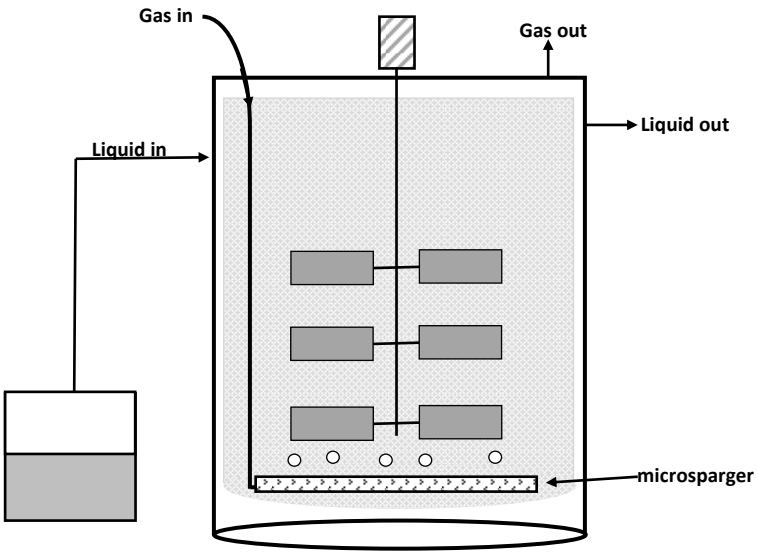

Figure 1.4 Continuous stirred tank reactors (CSTR)

1.4.2 Bubble column (BC) and gas-lift (GL) bioreactors

BC and GL bioreactors are similar in that gas injection through the bottom of the reactors allows for liquid mixing without the need for any mechanical or other power consuming agitation system. These bioreactors differ from each other by the fact that the GL bioreactor contains either an internal draft tube or an external loop for liquid circulation, while the BC reactor does not (Figure 1.5). The most commonly known airlift bioreactor and the gas-lift bioreactor are the same, except that air is fed in the first case while any gas can be introduced in gas-lift bioreactors. BC and GL reactors are economical alternatives to the CSTR (Bielefeldt et al. 2001; Ritcher et al. 2013), as they do not use mechanical agitation but still allow for good mixing, due to the presence of rising gas bubbles, and low shear rate. This is interesting as good mixing can be achieved while avoiding high shear rates that might inhibit or damage microbial cells. Mixing of the gaseous substrate is achieved through gas sparging. Some other advantages of these bioreactors are their low maintenance and operational costs, whereas back mixing and coalescence of gaseous substrates are the main drawbacks. The BC bioreactor has been used as a second stage of a two-stage syngas fermentation, where the first stage was a 1 L CSTR with sustained growth of *Clostridium ljungdahlii*, while the second stage was a 4 L BC reactor used to maximize the production of alcohols (Ritcher et al. 2013).

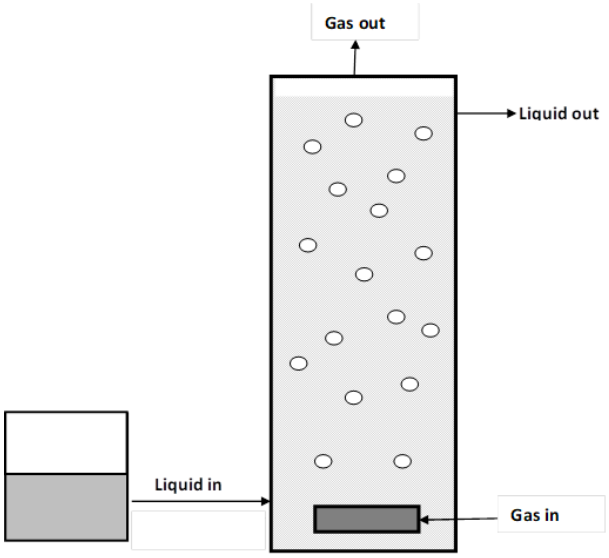

Figure 1.5 Bubble column reactor (BC)

In the GL bioreactor (Figure 1.6), the gas is injected into the riser and allows for fluid circulation. The liquid travels upwards through the riser zone and recirculates down to the bottom of the reactor, together with residual gas bubbles, through the downcomer zone. The use of GL bioreactors for the treatment of waste gases, mainly polluted air, has only been reported in a limited number of studies (Bielefeldt et al. 2001), although it is seen as an attractive alternative for the anaerobic bioconversion of syngas and CO-rich waste gases.

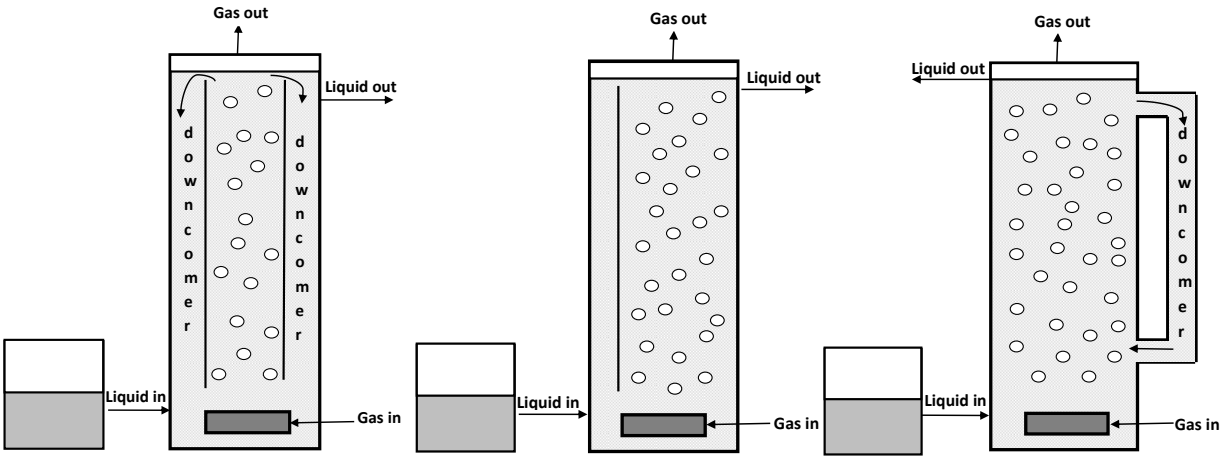

Figure 1.6 Gas-lift reactor (GL): (a) concentric loop, (b) split cylinder, (c) external loop.

1.4.3 Biotrickling filters (BTF)

Other bioreactor configurations such as the biotrickling filter (BTF) or trickle bed reactor (TBR) have widely been used in the full-scale treatment of polluted air from industrial waste gases, or those generated at waste water treatment plants, amongothers, in which the pollutants are present in the gas phase at concentrations usually not exceeding a few g/m^3 (Kennes and Veiga 2001; Kennes and Veiga 2013). They are also used with anaerobic gases, among others for biogas upgrading and for the removal of hydrogen sulphide from that biofuel (López et al. 2012; Fernández et al. 2014). Their efficiency has only scarcely been evaluated for the bioconversion of carbon monoxide and syngas, mainly at lab-scale, and hardly any information is available so far on such applications.

The BTF is a packed-bed bioreactor in which the cells are naturally growing on a solid support material (Figure 1.7). They accumulate on the solid surface in the form of a biofilm. This allows increasing the amount of biocatalyst, through its immobilization on the packing material (Kennes et al. 2009). The gaseous substrate is supplied continuously to the bioreactor while a nutritive aqueous phase is trickling over the solid support and recycled through the system with a pump, without any mechanical agitation, which reduces energy requirements compared with

the CSTR (Figure 1.4). The liquid phase enters through the top of the bioreactor while the gas phase can be fed either concurrently or counter currently. However, countercurrent flows allow for a more efficient driving force distribution along the bioreactor. Part of that aqueous medium can be withdrawn and renewed whenever appropriate. In packed-bed bioreactors such as the biofilter and the biotrickling filter, the amount of liquid medium present in the system is small compared with suspended-growth bioreactors (Kennes and Veiga 2013). This has been observed to reduce the resistance to mass transfer as there is only a thin liquid layer between the substrate in gas phase and the biofilm where the biocatalyst will metabolize the volatile compound (Jin et al. 2009). Conversely, in suspended-growth bioreactors, the substrate needs to diffuse from the gas bubbles to the liquid phase and to the biocatalyst growing in suspension. A semi-continuous trickle bed reactor was recently used with *C. ragsdalei* as a biocatalyst in order to estimate the possibility to improve syngas bioconversion (Devarapalli et al. 2016). A large amount of biomass could build up in the packed-bed bioreactor in the form of a biofilm, allowing the bacteria to take up more H_2 and avoiding CO inhibition. Consequently, 5.7 g/L ethanol was formed and a higher production of acetic acid, reaching 12.3 g/L, was observed.

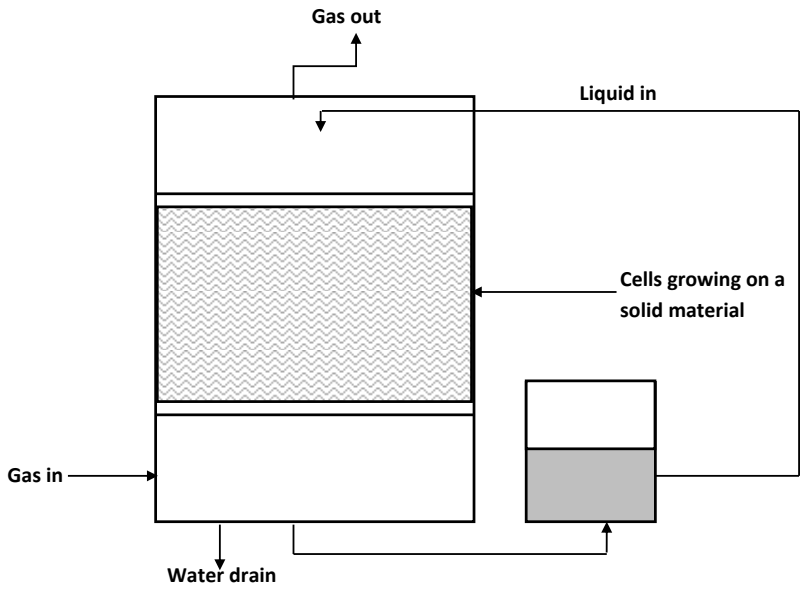

Figure 1.7 Biotrickling filter (BTF)

1.4.4 Hollow fiber membrane bioreactors (HFMB)

In the HFMB, hollow fiber membranes are introduced inside the reactor either in a tubular form or in the form of flat sheets (Kennes et al. 2009). Syngas is generally fed through the membrane lumen. It then diffuses through that membrane and is subsequently metabolized by bacteria forming a biofilm on the outer surface of the membrane, i.e. on the shell side (Figure 1.8). Only

very few research studies have been performed and reported on syngas fermentation in HFMB, none of them dealt with the production of alcohols such as butanol (Munasinghe and Khanal 2012; Zhang et al. 2013; Zhao et al. 2013). Shen et al. (2014) used a continuous HFMB with *C. carboxidivorans*, and compared its behaviour with a CSTR during continuous syngas fermentation for ethanol production. They obtained good resultswith the HFMB in terms of alcohol production, confirming the suitability and advantage of such bioreactor configuration compared with the CSTR. Recent studies have also been focused on optimizing mass transfer through the use of an HFMB, as mass transfer is a major limiting factor in syngas fermentation. In that sense, Yasin et al. (2014) developed a new HFMB configuration with a high mass transfer, able to support an efficient microbial fermentation. The authors reached a high driving force inside the bioreactor with low substrate pressures while increasing the headspace inside the system.

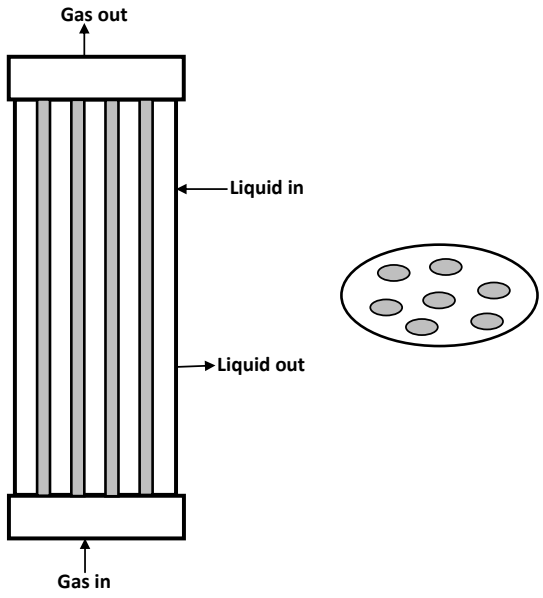

Figure 1.8 Hollow fiber membrane bioreactor (HFMB)

1.4.5 Moving bed biofilm reactors (MBBR)

The MBBR is considered to be more recent than the other bioreactors described above. This reactor was initially designed for application in municipal wastewater treatment (Barwal and Chaudhary 2014). It contains a tank with the culture broth and a gas injection system at the bottom of that tank for gas diffusion (Figure 1.9). Gas injection improves gas diffusion and turbulence. Large amounts of microorganisms grow as a biofilm. Some of the advantages of the systeminclude its ability to provide a high surface area per volume for biofilm development, its

rather simple operation, and the fact that it requires less space than, for instance, traditional wastewater treatment systems (Hickey 2009).

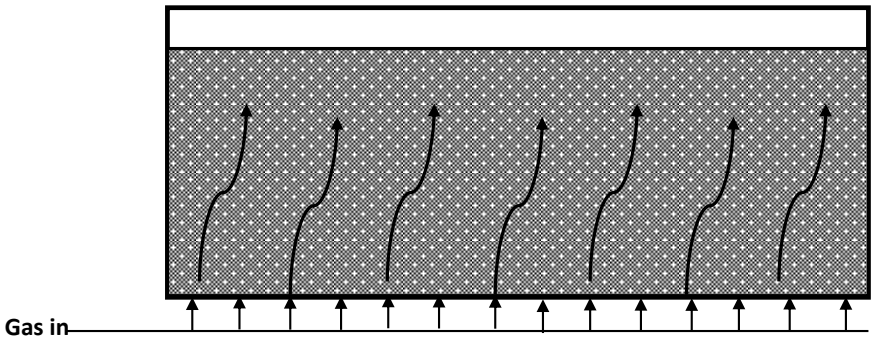

Figure 1.9 Moving bed biofilm reactor (MBBR)

1.4.6 Monolith bioreactors

The monolith bioreactor is a reactor with a structured packing inside. The packing is formed of regular channels that allow for a more homogeneous flow distribution than when working with random packing materials such as used in biotrickling filters (Figure 1.10). This may affect the efficiency and results in a better control of the pressure drop along the reactor height. The monolith bioreactor was first tested somewhat more than a decade ago for biological waste gas treatment and the removal of air pollutants. Good results were obtained for the removal of different volatile organic compounds from air, at concentrations of a few g/m3, typical of many industrial waste gases (Jin et al. 2006; Jin et al. 2008). One recent study described the bioconversion of syngas by *C. carboxidivorans* in amonolith bioreactor for alcohol production. Under the applied operating conditions, that reactor was reported to perform better, in terms of syngas utilization efficiency and alcohol/acid ratio, than a BC bioreactor (Shen et al. 2014b). The k_La values appeared to be similar or higher in the monolith bioreactor than in the BC reactor, depending on the operating conditions.

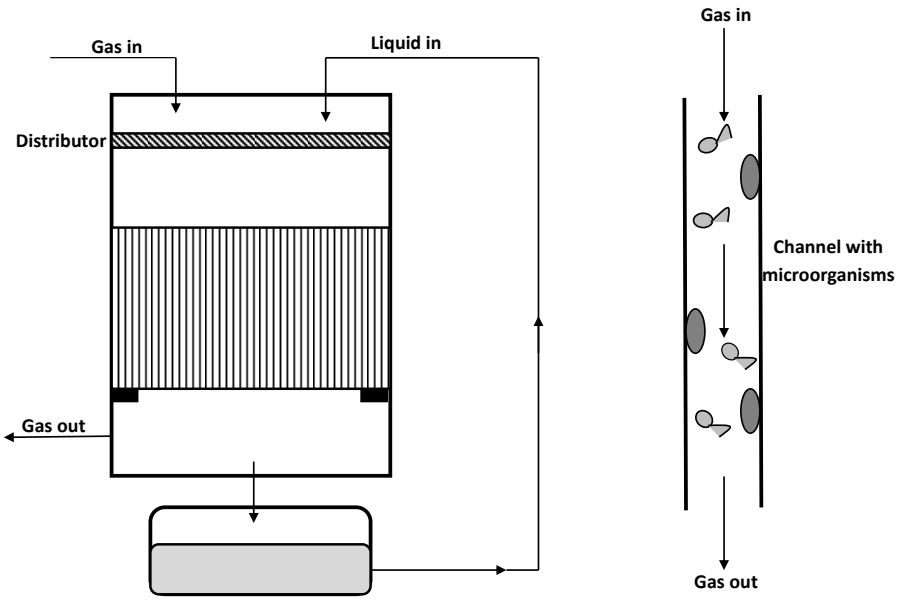

Figure 1.10 Monolith bioreactor

1.5 Present and future industrial perspectives

The production of ethanol and higher alcohols, such as butanol or hexanol, by acetogenic bacteria from C1 gases is not a favourable process from an energetic point of view (Latif et al. 2014). However, although it was originally considered that reaching concentrations approaching one gram per liter in wild type bacteria would be impossible or, at least, challenging, recent data confirm that the production of several g/L of butanol and hexanol mixtures, besides ethanol is feasible through this hexanol–butanol–ethanol (HBE) fermentation process. Optimization of the bioreactor operating conditions would allow to further improve such values. The use of metabolically engineered strains is another possible alternative for the improvement of yields and of the end concentrations of metabolites. Some research has been performed in that respect for butanol production with recombinant strains grown on carbon monoxide (Köpke and Liew 2011). However, improvements are necessary and higher butanol concentrations would still need to be reached from C1 gases with such engineered clostridia. Other bacterial strains are able to produce ethanol as single alcohol from syngas/waste gas, sometimes together with butanediol, but with no accumulation of either butanol or hexanol (Table 1-2). This is the case of *C. autoethanogenum*, *C. ljungdahlii*, and *C. ragsdalei*, among others (Abubackar et al. 2011). Recent studies undertaken at pre-commercial stage confirmed that such a process may be cost-effective (van Groenestijn et al. 2013). Some demonstration plants have recently allowed to produce ethanol, either from syngas or from waste gases from steel producing industries, with such acetogenic bacteria, reaching promising results. In terms of public safety, it is worth

mentioning that, with the exception of only four or five species, most clostridia are non pathogenic at all and do not cause any diseases in humans. Some clostridia can even be used for therapeutic purposes (Kubiak and Minton 2015). Concerning the environmental benefit, it is worth to remind that this HBE fermentation process consumes carbon dioxide, a greenhouse gas, but does also allow to remove carbon monoxide. Although carbon monoxide as such has only a very weak greenhouse effect, it contributes to tropospheric ozone generation, the formation of carbon dioxide, and reacts with hydroxyl radicals in the atmosphere. Those OH radicals would otherwise be involved in reducing the concentration of greenhouse gases such as methane.

The gas fermentation process has attracted interest of some industries and, as indicated above, some demonstration plants have been build recently. The technology has reached pre-commercial stage for the production of ethanol, but not yet for other routes such as the HBE fermentation, and an exhaustive overview of the industrial landscape, among others for the HBE process, would thus be behind the scope of this review. Information on the industrial lansdscape, mainly for ethanol production, can be found in other recent literature (van Groenestijn et al. 2013; Latif et al. 2014). One of the major companies developing this technology is, among others, LanzaTech which produces ethanol using waste gases from industry or using syngas obtained through the gasification of biomass or wastes. In 2013, that company started pre-commercial operation of a plant in China. Similarly, Coskata, in the US, was using a large variety of biomass sources to obtain syngas and ferment it into fuels and chemicals. Finally, INEOS Bio focuses largely on ethanol production through sugar fermentation, but has also evaluated possible commercialization of the biomass gasification process and its subsequent fermentation.

1.6 Syngas fermentation vs other biological and non-biological alternatives

Biomass, agro-industrial waste or other related feedstocks can be used to obtain either carbohydrates or syngas as potential fermentable substrates, which can both be metabolized by clostridia to produce ethanol and higher alcohols such a butanol. Expensive pretreatments are needed in order to extract carbohydrates from cellulose and hemicellulose, two major polymers of lignocellulosic feedstocks. However, lignin which is the third polymer found in such feedstocks, does not yield any carbohydrates and is thus useless for this fermentation process. Conversely, all three major polymers of lignocellulosic materials can be gasified to yield syngas, resulting in a better use of the complete feedstock in the gas fermentation process (Liew et al. 2016).

When comparing the biological and the non-biological syngas conversion routes, the former does also present some technical and economical advantages compared to the latter. The biological conversion, through the Wood–Ljungdahl pathway, takes place at near room temperature and atmospheric pressure, or if needed with slight overpressure. Conversely, the chemical Fischer Tropsch (FT) process for the production of chemicals is more complex and requires higher temperatures (150–350° C) and elevated pressures (e.g., 30 bar). Besides, for the FT process, a specific H_2: CO ratio close to 2:1 is needed (de Klerk et al. 2013), while syngas composition does generally not reach such ratio. A pretreatment consisting in a water–gas shift reaction is then required in order to adjust the gas ratio, with the concomitant increased process costs (Liew et al. 2016). On the other side, *C. carboxidivorans* and some other clostridia can metabolize different gas compositions to produce ethanol or higher alcohols, including pure CO, mixtures of CO_2/ H_2, or mixtures of all three gases, among others. Syngas fermentation is thus simpler and less restrictive. Finally, although the possible inhibitory effect of some trace compounds on bioconversion processes has been mentioned above, the FT process is much more sensitive to some chemicals such as sulphur compounds and has a lower tolerance to their presence than the Wood–Ljungdahl process (Michael et al. 2011; Mohammadi et al. 2011).

However, some potential drawback needs also to be discussed. The most important one is the low aqueous solubility of the volatile compounds of the syngas mixture, when working with bioreactors in which the bioconversion takes place in liquid phase. This results in a poor gas–liquid mass transfer and in limiting rates of supply of the gaseous substrates to the microbial cells, which limits the alcohols production yields. Mass transfer of the volatile substrates can be improved when using microbubble spargers. Using pressurized bioreactors would be another alternative to improve the gas solubility and mass transfer, although this will also increase operating costs. Packed-bed bioreactors, such as biofilters or biotrickling filters, with a reduced amount of water and a small water layer between the gas phase and the biofilm (Kennes et al. 2009), have also been suggested to improve the microbial use of substrates such as carbon monoxide in gas-phase bioreactors (Jin et al. 2009).

1.7 Microbial culture: *Clostridium carboxidivorans*

Clostridium carboxidivorans P7 (= ATCC BAA-624 = DSM 15243) used in this study was originally isolated from a settling lagoon in Oklahoma, USA (Liou et al. 2005). It is an obligate anaerobic carboxydotroph, Gram positive and mesophilic. Its cells are mobile with rod shape having a size of 0.5 × 3 μm along. That strain can present sporulated forms, which could be located in the terminal or subterminal with the appearance of a protuberance (Liou et al. 2005; Fernández-

Naveira et al. 2017a). Figure 1.11 show a SEM picture where could be appreciate the shape of *Clostridium carboxidivorans*.

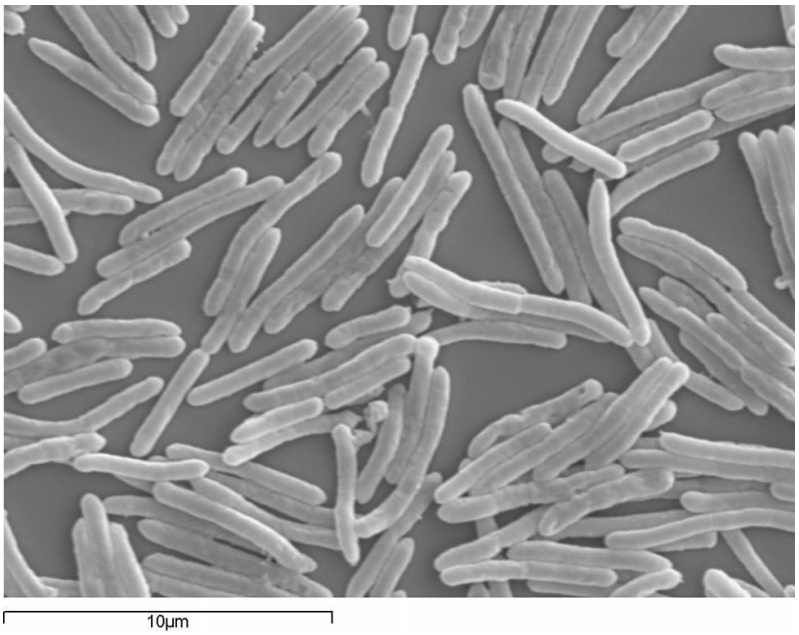

Figure 1.11 SEM picture of *Clostridium carboxidivorans* grown on carbon monoxide

Clostridium carboxidivorans P7 is able to use different carbon sources for growing; in the chemoorganotrophically growth, *C. carboxidivorans* is able to use differents sugars such as glucose, xylose, fructose, cellobiose and arabinose; in the autotrophicaly growth that bacteria can use syngas or CO as a carbon source. The final products of the fementation of all those carbon sources are acids (mainly acetic acid,butyric acid, and hexanoic acid) and alcohols (ethanol, butanol and hexanol) (Liou et al. 2005; Liu et al. 2014; Phillips et al. 2015). However in the case of sugars fermentation was recent detected the production of other acids: lactic acid, propionic acid and formic acid (Fernández-Naveira et al. 2017b).

The principal morphological, metabolic and growth characteristics of *C. carboxidivorans* are summarized in Table 1-5.

Table 1-5 Main characteristics of *Clostridium carboxidivorans*

	C. carboxidivorans P7
Morphology	Rod shape
Size (µm)	0.5x3
Temperature range (° C)	24-42
Temperature optimum (° C)	37-40
pH range	4.4-7.6
pH optimum	5.0-7.0
Reference	Liou et al. 2005

1.8 References

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2 OBJETIVES

The main objective of the present study is to develop an innovative technology based on the use of biomass or other lignocellulosic materials as a raw material for the production of ethanol, butanol and hexanol. This alternative allows to use the complete raw material (including lignin) to obtain a mixture of fermentable gaseous substrates, in its totality, metabolized by the same microbial culture.

This innovative technology is based on the gasification of the raw material instead of its hydrolysis (or chemical / enzymatic treatment). Gasification of biomass and waste convert them into synthesis gas, that is to say mainly CO, as well as variable concentrations of CO_2 and H_2. Some waste gases from different industries could also be used by the anaerobic bacteria to produce these metabolites.

Therefore, this doctoral thesis consists in:

- The optimization of the conditions for ethanol, butanol and hexanol production.
- The optimization of the production of these biofuels in gas-phase bioreactors.

3 MATERIAL AND METHODS

3.1 Microbial culture

In all the experiments, Clostridium carboxidivorans was used as a biocatalyst using different carbon sources, for the production of higher alcohols.

C. carboxidivorans P7 DSM 15243 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The bacteria was inoculated at a firt time in anaerobic conditions on modified basal medium at pH 5.75 using glucose as a carbone source. After the growing, the bacteria was maintained in anaerobic mediums with CO as a carbon source. The principal components of the medium are summarized in the Table 3-1:

Table 3-1 Media composition for *C carboxidivorans*

Chemical	Concentration per L of distilled water
Yeast stract	1 g
Mineral solution	25 mL
Trace metals solution	10 mL
Vitamins solution	10 mL
Resazurine	1 mL
Cystein-HCl	0.90 g

The composition of the mineral stock solution are summarized in the Table 3-2

Table 3-2 Mineral solution composition

Chemical	Concentration (g/L)
Sodium chloride	80
Amonium chloride	100
Potasium chloride	10
Potasium monophosphate	10
Magnesium sulphate	20
Calcium chloride	4

The composition of the trace metals stock solution are summarized in the Table 3-3

Table 3-3 Trace metals solution composition

Chemical	Concentration (g/L)
Nitriloacetic acid	2
Manganesium sulphate	1
Ferrus ammonium sulphate	0.20
Zinc sulphate	0.20
Cupric chloride	0.02
Niquel chloride	0.02
Sodium molybdate	0.02
Sodium selenate	0.02
Sodium tunsgtate	0.02

The composition of the vitamins stock solution are summarized in the Table 3-4

Table 3-4 Vitamins stock solution composition

Chemical	Concentration (g/L)
Pyridoxine	2
Thyamine	1
Riboflabin	0.20
Calcium pantothenate	0.20
Thiotic acid	0.02
Paraminobenzoic acid	0.02
Nicotinic acid	0.02
Vitamine B12	0.02
d- biotin	0.02
Folic acid	0.02
2-mercatoethanesulfonic acid	0.02

The media used foreach experimental studies are described in their corresponding chapter.

3.2 Bioconversion studies

3.2.1 Bottle batch experiments

Serum vials bottles of 200 mL, with 100 mL of total anaerobic basal medium (all prepared under the same condition and methods) for the experiments described in the Chapter 7 and 4.

For each experiment all the medium was prepared together to avoid different on the medium composition. 100 mL of medium was transferred into each bottle. With the aim of remove the oxygen of the medium, all the bottles was boiling for a few minutes. After boiling, the bottles were flushing with N_2 for 15 minutes. When the bottles were cooled (30-35° C) then Cysteine – HCl was added, and the pH of the medium was adjusted by adding either 2M HCl or 2M NaOH. After pH adjustment, all bottles were sealed with viton rubber stoppers and aluminium crimps. The bottles were autoclaved at 120° C for 15 minutes.

In the moment of inoculation, two 3-way stop cocks with a needle were inserted through the stopper on each bottle for sampling purpose (one for liquid sampling and the another one for gases samplings). Then the bottles were inoculated with 10 % of bacteria on exponential phase, which was grown with CO as a carbon source during 72 h. After inoculation, vitamins from a stock solution were added, and finally all bottles were pressurized to 1.2 bar with 100 % CO. Finally all the bottles were maintained at 30° C inside an orbital incubator at 150 rpm. The experimental setup and the method used for media preparation as well as sampling details are described on the Chapter 1 and 3 (Abubackar et al. 2011; Abubackar et al. 2015; Fernández-Naveira et al. 2016a; Fernández-Naveira et al. 2016b).

3.2.2 Continuous gas-fed bioreactors experiments

All the bioreactor experiments were carried out in 2 L BIOFLO 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA) with 1.2 L of total medium. Differences carbon sources were used in the different experiments (CO, syngas and glucose). In the case of the experiments with gaseous carbon sources, the gas was feeding continuously at a rate of 10 using a mass flow controller (Aalborg GFC 17, Müllheim, Germany).

The medium inside the bioreactor was autoclaved at 120° C during 20 minutes. After autoclaving, to ensure anaerobic condition the bioreactor was flushed with N2 during 2 hours and when that was cooling cysteine-HCl (0.90 g/L) was added.

Once the bioreactor reached the anaerobic conditions, a 10 % of biomass in exponential phase was added into the bioreactor. The temperature was maintained in all experiment at 33° C. The pH was automatically maintained through addition of either a 2 M NaOH solution or a 2 M HCl solution, using peristaltic pump.

The pH values and conditions were different in the different experiments. The complete conditions and methods of each experiment are related in each respective chapter.

3.3 Analytical equipment and measurement protocols

3.3.1 Biomass measurements

Biomass was estimated daily or twice in a day depending on the moment of the experiment. The quantification of biomass was madding by the measurement of the absorbance of the liquid

phase at a λ 600 nm using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain).

Previously a calibration of the biomass concentration (g/L) was made. That way, the measured absorbance allowed to estimate the biomass concentration (mg/L) by the comparison with this previously generated calibration curve.

3.3.2 Water soluble products

1 mL of liquid sample of reactor or bottle batch experiments was daily (or twice per day) retire for the measurement of sugars, acids and alcohols, using an HPLC (HP1100, Agilent Co., USA) equipped with a supelcogel C-610 column having a UV detector at a wavelength of 210 nm and a refractive index detector (RID). 0.1 % ortho-phosphoric acid solution fed at a flow rate of 0.5 ml/min was using as a mobile phase and a constant 30° C was set in the column.

The principal metabolites analyzed (depending on the experiment) were: glucose, formic acid, acetic acid, propionic acid, isobutiric acid, butyric acid, hexanoic acid, ethanol, butanol and hexanol

3.3.3 CO, CO_2 measuments

1mL in reactor experiment or 0.2 mL in batch experiment of the head space was retired daily for CO and CO_2 measurements.

In the case of CO measurement, an HP 6890 gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) was used. The GC was fitted with a 15 m HP-PLOT Molecular Sieve 5A column (ID: 0.53 mm, film thickness: 50 μm). The oven temperature was initially kept constant at 50° C, for 5 min, and then raised by 20° C min^{-1} for 2 min, to reach a final temperature of 90° C. The temperature of the injection port and the detector were maintained constant at 150° C. Helium was used as the carrier gas.

For CO_2 measurement, an HP 5890 gas chromatograph, equipped with a thermal conductivity detector (TCD) was used. The GC column used was Porapak Q 80/100 (inox) column (2 m × 1/8”). Helium was used as the carrier gas at a flow rate of 15 mL/min. The injection, oven and detection temperatures were maintained at 90, 30 and 100° C, respectively.

A calibration curves was made for each gas using peaks obtained from known concentrations of gas.

3.3.4 Redox potential

The redox potential was monitored continuously using a Ag/AgCl electrode (M300, Mettler Toledo, Inc. USA).

3.3.5 16S rRNA analysis

In the case of the Chapter 6, where glucose was used as a carbon source, to be ensure any contamination happened in the experiment a 16S rDNA gene sequencing was made

Liquid samples of the reactor was taken, in the moment of inoculation and at the end of the each bioreactor in order to confirm the stability and purity of the inoculated strain in each experiment.

The complete protocol was described in the section of material and methods on the Chapter 6.

3.4 References

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4 EFFICIENT BUTANOL-ETHANOL (B-E) PRODUCTION FROM CARBON MONOXIDE FERMENTATION BY *CLOSTRIDIUM CARBOXIDIVORANS*

EFFICIENT BUTANOL-ETHANOL (B-E) PRODUCTION FROM CARBON MONOXIDE FERMENTATION BY CLOSTRIDIUM CARBOXIDIVORANS

Abstract The fermentation of waste gases rich in carbon monoxide using acetogens is an efficient way to obtain valuable biofuels like ethanol and butanol. Different experiments were carried out with the bacterial species *Clostridium carboxidivorans* as biocatalyst. In batch assays with no pH regulation, after complete substrate exhaustion, acetic acid, butyric acid, and ethanol were detected while only negligible butanol production was observed. On the other side, in bioreactors, with continuous carbon monoxide supply and pH regulation, both C2 and C4 fatty acids were initially formed as well as ethanol and butanol at concentrations never reported before for this type of anaerobic bioconversion of gaseous C1 compounds, showing that the operating conditions significantly affect the metabolic fermentation profile and butanol accumulation. Maximum ethanol and butanol concentrations in the bioreactors were obtained at pH 5.75, reaching values of 5.55 and 2.66 g/L, respectively. The alcohols were produce d both from CO fermentation as well as from the bioconversion of previously accumulated acetic and butyric acids, resulting in low residual concentrations of such acids at the end of the bioreactor experiments. CO consumption was often around 50 % and reached up to more than 80 %. Maximum specific rates of ethanol and butanol production were reached at pH 4.75, with values of 0.16 g/h*g of biomass and 0.07 g/h*g of biomass, respectively, demonstrating that a low pH was more favorable to solventogenesis in this process, although it negatively affects biomass growth which does also play a role in the final alcohol titer.

Keywords *Clostridium carboxidivorans* .Butanol . Ethanol . Syngas . Waste Gas

With minor editorial changes to fulfil formatting requirements, this chapter is substantially as it appears in: Appl Microbiol Biotechnol. **Published online: 25 January 2016**. DOI: 10.1007/s00253-015-7238-1.

4.1 Introduction

In recent years, the low availability of fossil fuels and their environmental impact have forced to look for new alternative fuels obtainable, in a cost-effective way, from renewable sources or from pollutants. In addition to the environmental impact and increasing scarcity of conventional fuels, other aspects, e.g., economic and political, have also led to an ever increasing interest in techniques for the production of such alternative fuels (Gowen and Fong 2011; Abdehagh et al. 2014). Most studies have focused on the production of new energy sources and biofuels (biologically sourced fuels) such as (bio)ethanol, biogas, (bio)hydrogen, and biodiesel (Kennes and Veiga 2013). Additionally, (bio)butanol is also a suitable alternative fuel more similar to gasoline than (bio)ethanol and with interesting characteristics. Butanol exhibits several advantages, e.g., it is less hygroscopic and has a higher caloric content than ethanol (Wallner et al. 2009). It is considered a chemical of great industrial importance and has a high potential to replace gasoline (Dürre 2007; Lee et al. 2008), as there is no need for any adjustment of vehicles and engines using butanol. Besides, blending of butanol and gasoline is possible at any concentrations, and blends have also been reported to be possible in case of diesel (Jin et al. 2011).

Alcohols such as ethanol and butanol can be obtained through fermentation of sugars from sugarcane, corn, or starch feedstocks, among others, which is the conventional and common commercial process nowadays for ethanol, known as a first-generation process. However, this technique for obtaining biofuels leads to food-fuel competition (Kennes et al. 2016). This disadvantage can be avoided by using lignocellulosic feedstocks from agricultural wastes or energy crops, which are inexpensive and renewable starting materials for biofuels production, and do not adversely affect food supplies. After some pretreatments and hydrolytic steps, simple sugars can be obtained from those polymeric feedstocks which can then be fermented into ethanol and/or butanol, in the so-called second-generation process (Kennes et al. 2016). However, there are still numerous scientific and technical challenges involved in the utilization of lignocellulosic materials for biofuel production (Gowen and Fong 2011), and there is a need for further research in order to improve costcompetitiveness of such alternative compared to the more conventional first-generation process.

The conventional second-generation process for the bioconversion of lignocellulosic feedstocks into biofuels is still a complex process (Balat and Balat 2009). As an alternative, gasification of biomass in order to obtain carbon monoxide-rich syngas represents another viable option. Syngas as well as most of its individual dominant components (like CO) can be introduced into a

fermentor inoculated with anaerobic bacteria, under specific process conditions to produce biofuels (Abubackar et al. 2011; Bengelsdorf et al. 2013; Mohammadi et al. 2011). Not only syngas but also industrial waste gases rich in carbon monoxide have recently been shown to be suitable substrates for their bioconversion into biofuels in bioreactors (Kennes and Veiga 2013). Both suspended-growth as well as attached-growth bioreactors can efficiently be used for gas-phase biodegradation or bioconversion of such volatile substrates (Kennes and Veiga 2001, 2013). Initially, research on the fermentation of CO-rich gases focused only on ethanol production, which can be either an independent fuel as mentioned above or act as a substitute for gasoline supplemented with MTBE to reduce emissions of CO and NOx (Ahmed and Lewis 2007; Henstra et al. 2007; Shaw et al. 2008). However, its hygroscopic nature and low caloric content limits the use of ethanol with current infrastructures; therefore, very recent research has also focused on butanol production through the fermentation of such gaseous substrates as an alternative alcohol-biofuel.

Fermentation of CO-rich gases, i.e., syngas or waste gases, has been shown to be an attractive and likely cost-effective alternative able to compete with the conventional second-generation process based on the fermentation of carbohydrates (Kennes et al. 2016). This is above all true whenever using waste gases as substrates. Therefore, this process has recently attracted interest from some companies and some demonstration and pre-commercial projects are now being set up for ethanol production (Abubackar et al. 2011; Kennes and Veiga 2013). However, several challenges remain to be addressed in order to further improve the efficiency and cost-effectiveness of this technology. One of those challenges is related to the low water solubility of carbon monoxide and other volatile compounds (e.g., H_2, CO_2), which limits the mass transfer rate of the substrate to the liquid phase in suspended-growth bioreactors or to the biofilm in attached-growth bioreactors, limiting at the same time the production yields of (bio)fuels or platform chemicals of interest. Some previous and on-going studies are focusing on minimizing such drawback. Among others, the use of membrane systems as well as attached-growth bioreactors seems to allow a more efficient mass transfer of poorly soluble compounds (Jin et al. 2009; Shen et al. 2014) as well as microbubble spargers in suspended-growth bioreactors (Bredwell and Worden 1998). Another drawback to be taken into account and already previously observed in conventional acetone-butanol-ethanol (ABE) fermentation from carbohydrates is solvent toxicity. This is an important factor to take into account in butanol fermentation as acetogenic bacterial cells rarely tolerate more than 2 % butanol (Liu and Qureshi 2009). However, although new strategies can still be developed, experience has already been gained from the conventional ABE fermentation aimed at reducing such inhibitory problems. These

strategies may include the use of continuous in situ removal of produced solvents from the fermentation broth, among others (Schugerl 2000). It is also worth mentioning that setting up bioreactors under anaerobic conditions with CO-related gases as substrates may be somewhat more challenging than the conventional fermentation of carbohydrates. However, such harsher conditions will also reduce potential microbial contamination of the bioreactor. Finally, optimizing the fermentation and bioreactor operating conditions is another aspect that will allow improving the yield and selectivity of the biochemical reactions and which is addressed in this paper. Research on such aspects will further improve the efficiency and cost effectiveness of this process appearing as a promising alternative.

In the present study, the conversion of CO into butanol and ethanol was carried out by the bacterium *Clostridium carboxidivorans*, which was grown first in batch bottles with no pH regulation and, afterwards, in continuous gasfed bioreactors using a defined medium under controlled conditions and continuously fed CO gas. The objectives were to develop and optimize culture conditions for a relatively high production of alcohols through anaerobic CO fermentation and to compare the growth and fermentation products between the batch bottle assays and the bioreactors with continuous CO supply. Bioreactor operating conditions were optimized.

4.2 Material and methods

4.2.1 Microorganism and culture media

C. carboxidivorans P7 DSM 15243 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was maintained anaerobically on modified basal medium (Liou et al. 2005; Tanner 2007) at pH 5.75 with CO (100 %) as the sole gaseous substrate. This medium was composed of (per liter distilled water) the following compounds: yeast extract, 1 g; mineral solution (a source of sodium, ammonium, potassium, phosphate, magnesium, sulfate and calcium), 25 mL; trace metal solution, 10 mL; vitamin solution, 10 mL; resazurin, 1 mL; and cysteine-HCl, 0.60 g.

The mineral stock solution contained (per liter distilled water) 80 g sodium chloride, 100 g ammoniumchloride, 10 g potassium chloride, 10 g potassium monophosphate, 20 g magnesium sulfate, and 4 g calcium chloride.

The vitamin stock solution contained (per liter distilled water): 10 mg pyridoxine, 5 mg each of thiamine, riboflavin, calcium pantothenate, thioctic acid, paraamino benzoic acid, nicotinic acid, and vitamin B12, and 2 mg each of D-biotin, folic acid, and 2-mercaptoethanesulfonic acid.

The trace metal stock solution contained (per liter distilled water): 2 g nitrilotriacetic acid, 1 g manganese sulfate, 0.80 g ferrous ammonium sulfate, 0.20 g cobalt chloride, 0.20 g zinc sulfate, and 20 mg each of cupric chloride, nickel chloride, sodium molybdate, sodium selenate, and sodium tungstate.

4.2.2 Bottle batch experiments

For batch experiments, 10 % seed culture in the early exponential growth phase, grown with CO as sole carbon source, was aseptically inoculated into 200-mL serum vials containing 100 mL medium at pH = 5.75. In order to remove oxygen, all the media in the bottles were boiled and later flushed with N_2 while cooling down the medium. When the temperature of the medium reached 40° C, 0.06 g cysteine-HCl was added as a reducing agent, and the pH was adjusted to 5.75 with 2 M NaOH while continuing flushing with N_2. The bottles were then sealed with Viton stoppers and capped with aluminium crimps and were then autoclaved for 20 min at 121° C. The bottles were maintained under anaerobic conditions. They were pressurized with 100 % CO to reach a total headspace pressure of 1.2 bar and were agitated at 150 rpm on an orbital shaker, inside an incubation chamber at 33° C. Every 24 h, a headspace sample of 0.2 mL and 2 mL liquid sample were taken for CO measurements and to measure the optical density (OD_λ = 600 nm), which is directly related to the biomass concentration. Besides, 1 mL of those 2 mL was centrifuged at 7000 rpm for 3 min in order to measure the concentration of soluble products in the supernatant, using the same methods as described in Fermentation products for the analyses of fermentation products in the continuous bioreactors. All experiments were carried out in triplicate.

4.2.3 Continuous gas-fed bioreactor experiments

Two bioreactor experiments were carried out in 2 L BIOFLO 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA) using the same medium as in the batch bottle experiments. Both experiments were done with 1.2 L optimized medium and CO (100 %) as the sole gaseous substrate, fed continuously at a rate of 10 mL/min using a mass flow controller (Aalborg GFC 17, Müllheim, Germany) and a microsparger used for sparging CO. The bioreactor with the medium was autoclaved, and when the temperature was below 40° C, cysteine-HCl (0.60 g/L) was added, together with nitrogen feeding to ensure anaerobic conditions. The temperature of the

bioreactor was maintained at 33° C by means of a water jacket. Four baffles were symmetrically arranged to avoid vortex formation of the liquid medium and to improve mixing. A constant agitation speed of 250 rpm was maintained throughout the experiments. Ten percent seed culture in the early exponential growth phase, which was grown for 72 h with CO as sole carbon source, was used as the inoculums and was aseptically transferred to the bioreactor. The pH of the medium was automatically maintained at a constant value of either 5.75 or 4.75, through the addition of either a 2-M NaOH solution or a 2-M HCl solution, fed by means of a peristaltic pump. The redox potential was continuously monitored in each experimental run.

When the bioreactor reached its maximum production of acids, the pH in one of the reactors (experiment 1) was maintained at pH 5.75 while it was changed to pH 4.75 in the other reactor (experiment 2). Later, when most of the acids were consumed, part of the medium (around 600 mL) was replaced with the same amount of fresh medium in both bioreactors and the pH was maintained at 5.75 again. During the partial medium replacement procedure, the CO gas flow rate was maintained through the bioreactor and was even slightly increased in order to ensure maintenance of anaerobic conditions inside the bioreactor. Then, when the production of acids reached its maximum value, the pH of the bioreactor in experiment 1 was changed to 4.75.

4.2.4 Growth measurement

One milliliter liquid sample was daily withdrawn from the reactor, in order to measure the optical density (OD_λ = 600 nm), using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The measured absorbance allowed to estimate the biomass concentration (mg/L) by comparing it with a previously generated calibration curve.

4.2.5 Gas-phase CO and CO_2 concentrations

Gas samples of 1 mL were taken from the outlet sampling ports of the bioreactors to monitor the CO and CO_2 concentrations.

Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD). The GC was fitted with a 15-m HP-PLOT Molecular Sieve 5A column (ID, 0.53 mm; film thickness, 50 µm). The oven temperature was initially kept constant at 50° C, for 5 min, and then raised by 20° C/min for 2 min, to reach a final temperature of 90° C. The temperature of the injection port and the detector was maintained constant at 150° C. Helium was used as the carrier gas.

Similarly, CO_2 was analyzed on an HP 5890 gas chromatograph, equipped with a TCD. The injection, oven, and detection temperatures were maintained at 90, 25, and 100° C, respectively.

4.2.6 Fermentation products

The water-soluble products, acetic acid, butyric acid, ethanol, and butanol, were analyzed for each of the two bioreactors from liquid subsamples (1 mL) every 24 h using an HPLC (HP1100, Agilent Co., USA) equipped wi t h a 5 µm × 4 mm × 250 mm Hypersil ODS column and a UV detector at a wavelength of 284 nm. The mobile phase was a 0.1 % ortho-phosphoric acid solution fed at a flow rate of 0.5 mL/min. The column temperature was set at 30° C. The samples were centrifuged (7000 g, 3 min) using a centrifuge (ELMI Skyline ltd CM 70M07) before analyzing the concentration of water-soluble products by HPLC.

4.2.7 Redox potential

The redox potential was constantly monitored in each bioreactor using an Ag/AgCl reference electrode connected to a transmitter (M300, Mettler Toledo, Inc., Bedford, MA, USA) and maintained inside the bioreactor.

4.3 Results

4.3.1 Bottle batch experiments

In the batch experiments, *C. carboxidivorans* started growing immediately after inoculation, without any lag phase (Figure 4.1). A maximum biomass concentration of 0.13 g/L was reached after 30 h (Figure 4.1), while a maximum acetic acid concentration of 0.890 g/L was found after 45 h and a maximum concentration of butyric acid of 0.48 g/L had accumulated at the end of the experiment (Figure 4.2).

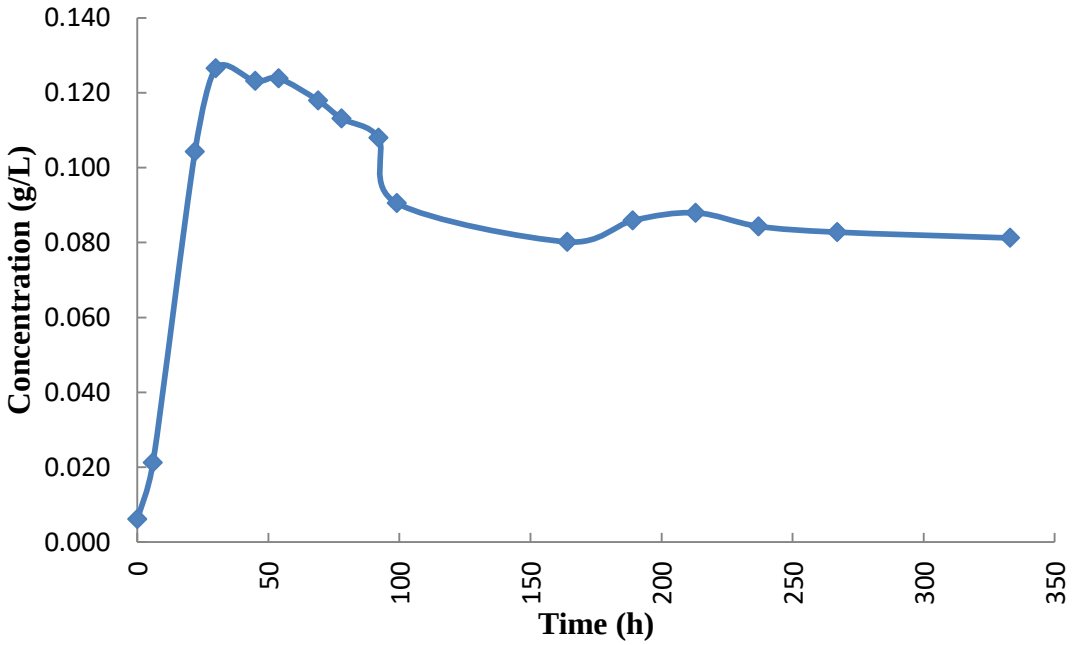

Figure 4.1 Batch experiment: a measured growth expressed in g/L over time, with data represented as mean values ± standard deviations.

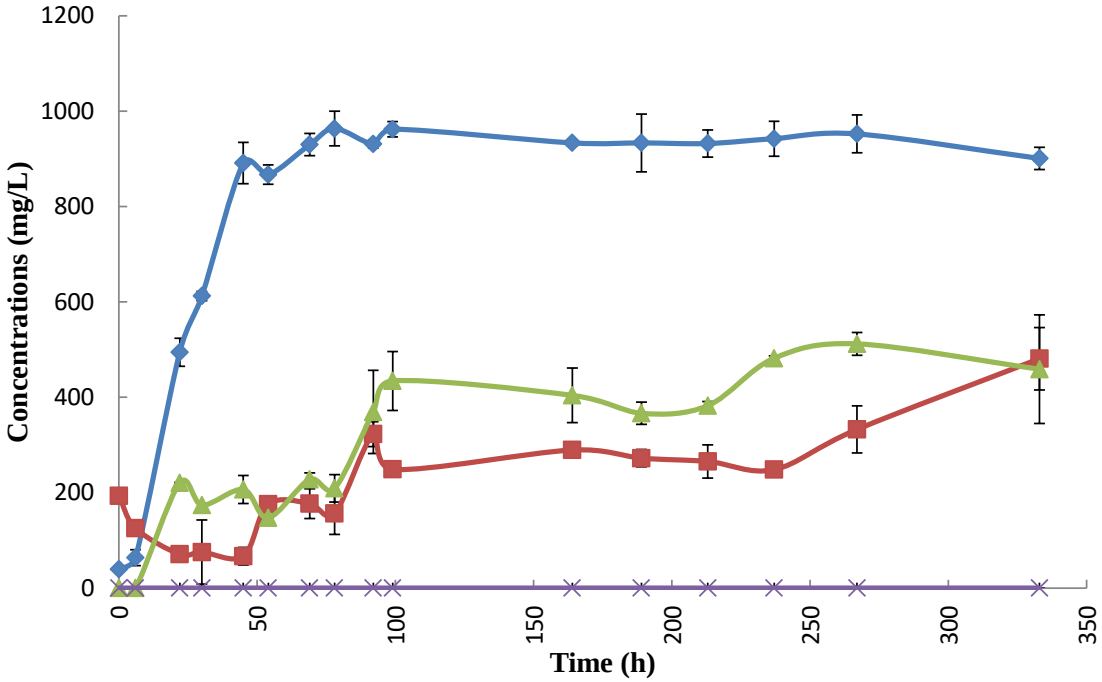

Figure 4.2 Batch experiment production of metabolites, acetic acid (blue diamonds), butyric acid (red squares), ethanol (green triangles), and butanol (purple cross marks), expressed in mg/L over time, with data represented as mean values ± standard deviations.

On other hand, a maximum ethanol concentration of 0.48 g/L was reached, after 267 h. Its production did not seem to take place at the expense of any acetic acid consumption as that

acid was basically not consumed during ethanol accumulation, although clostridia are known to be able to convert acids into alcohols in processes such as the ABE fermentation from carbohydrates (Jones and Woods 1986; Ndaba et al. 2015) as well as in some other fermentation processes such as CO bioconversion to acetic acid followed by the production of ethanol from the accumulated fatty acid (Abubackar et al. 2015). The alcohol was thus formed here directly from the conversion of CO. Although *C. carboxidivorans* is known to be able to produce butanol, that alcohol was generally not found or produced at low trace levels in these batch bottle assays.

The initial pH of the medium was 5.75 in this experiment. However, when the acetogenic phase started, acids were formed leading to medium acidification, as there was not any pH regulation. Therefore, the pH dropped gradually during the batch assays and reached a minimum value around 4.30 at the end of the experiment. Also, anaerobes are very sensitive to changes in redox potential. The reading oxidoreduction potential (ORP) values are directly linked to the pH of the medium, and a lower pH of the medium will result in less negative values of the redox potential.

4.3.2 Continuous gas-fed bioreactor experiments

Experiment 1

In this continuous CO-fed bioreactor experiment, *C. carboxidivorans* started growing immediately after inoculation, without any lag phase, similarly as in the batch assays. The growth and fermentation products followed a pattern common to acetogenic clostridia (Figure 4.3 and Figure 4.4). After 96 h, the biomass reached its maximum value of 0.52 g/L (Figure 4.3) whereas the maximum concentrations of acetic acid and butyric acid, reached after 144 h, were 5.30 and 1.43 g/L, respectively (Figure 4.4).

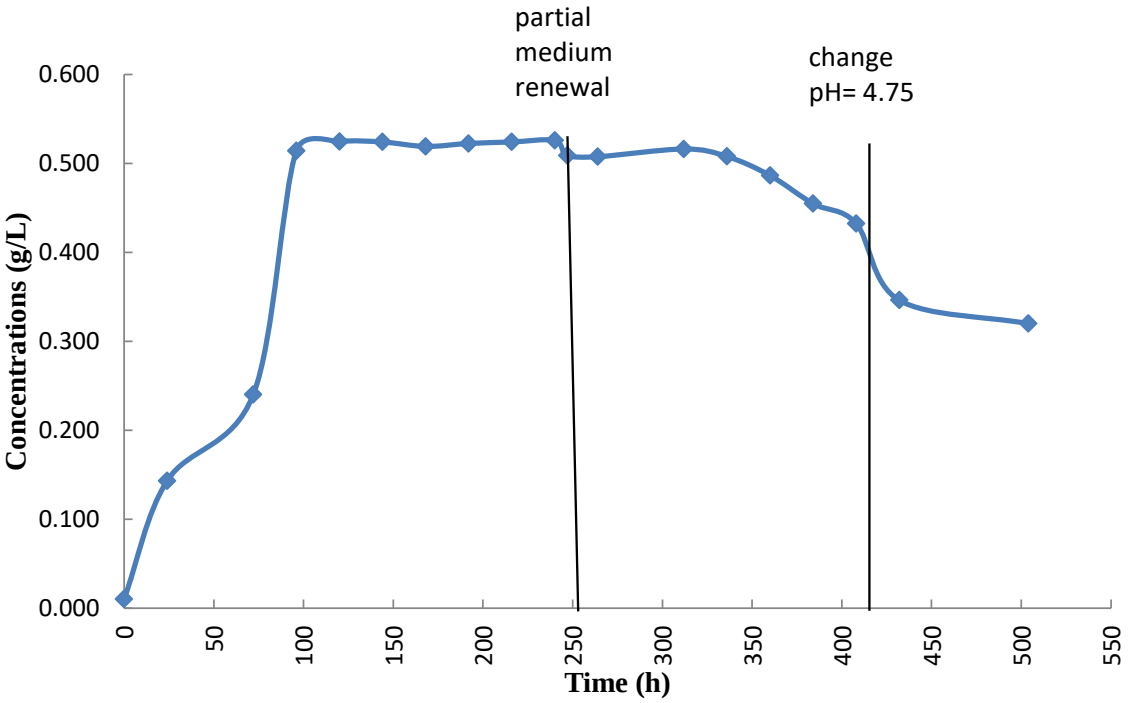

Figure 4.3 Continuous gas-fed bioreactor experiment 1: measured growth expressed in g/L over time.

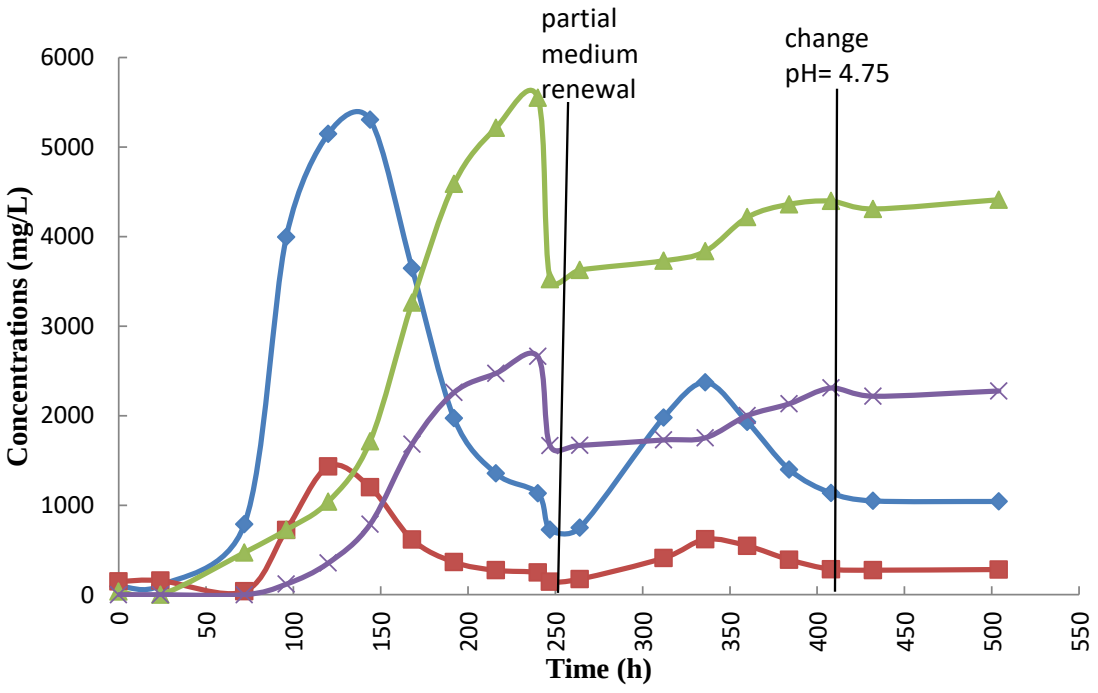

Figure 4.4 Continuous gas-fed bioreactor experiment 1: production of metabolites, acetic acid (blue diamonds), butyric acid (red squares), ethanol (green triangles), and butanol (purple cross marks), expressed in mg/L over time.

The production of alcohols did also start quite soon after inoculation but initially at a quite slower rate than observed for the acids. Alcohols continued accumulating after the fatty acids had reached their highest concentrations. After 240 h, the production of alcohols leveled off, because ethanol and butanol had accumulated up to potentially inhibitory levels. By then, ethanol and butanol had reached quite high maximum concentrations of 5.55 and 2.66 g/L, respectively (Figure 4.4).

A different behavior was observed than in the batch experiments, as ethanol production appeared to increase at the expense of acetic acid consumption, and both the decrease in acid concentration and increase in alcohol concentration occurred simultaneously. Similarly, butanol production appeared to take place at the expense of butyric acid conversion. Contrary to what was observed in the batch assay, in the present experiment with pH regulation and continuous CO supply, butyric acid was almost completely consumed (83 %) and 78 % of acetic acid was also converted. Besides, a rather high final concentration of butanol was reached (2.66 g/L), never reported before in the literature for this type of CO fermentation by clostridia.

After 247 h, part of the bioreactor medium (600 mL) was replaced by fresh medium in order to alleviate the otential inhibitory effect of the high concentrations of alcohols and to check if this partial medium renewal might promote a new acids and alcohols production cycle. The biomass was recycled in the bioreactor; thus, its concentration remained constant at 0.51 g/L. The concentrations of alcohols decreased as a result of the dilution effect due to medium replacement, reaching an ethanol concentration of 3.50 g/L and a butanol concentration of 1.70 g/L (Figure 4.4).

While the amount of biomass remained constant until 360 h, the concentrations of acids started to increase again. The maximum concentrations of acetic acid and butyric acid were reached after 336 h with values of 2.40 and 0.617 g/L, respectively (Figure 4.4).Despite maintaining the pH at 5.75, the formation of some alcohols started immediately, although at much lower rates than for the acids. The pH was later changed to 4.75 after 408 h in order to check if this could further improve the production of alcohols, as a lower pH is expected to be favorable to solventogenesis.

However, at that lower pH, the biomass concentration decreased while there was not any production of acids and alcohols nor any consumption of acids. Finally, the experiment was stopped after 504 h. At that moment, the biomass concentration had decreased to 0.32 g/L, and acetic acid and butyric acid concentrations were 1.04 and 0.28 g/L, respectively. The concentrations of alcohols were 4.41 g/L for ethanol and 2.3 g/L for butanol at the end of the

experiment, which is significantly higher than in any previously reported study (Figure 4.4). Total net ethanol production in this experiment was 7.52 g corresponding to 6.66 g (in 1.2 L reactor medium) before partial medium replacement and 0.86 g after its replacement, and total net butanol production was 3.91 g corresponding to 3.19 g before partial medium replacement and 0.72 g after its replacement. This type of CO fermentation yields reproducible profiles.

During the experimental production phase, the specific rate of ethanol production was 0.12 g/h*g of biomass between 144 and 192 h, while the specific rate of butanol production was 0.06 g/h*g of biomass during that same period of time (Table 4-1). Other production and consumption rates are summarized in Table 4-1 as well. Carbon monoxide consumption was also monitored during the experiment and is shown in Figure 4.5.

Table 4-1 Comparison of the different production and consumption rates between experiments 1 and 2 (The rates are expressed in g/h*g-biomass).

	Acetic acid consumption rate	Butyric acid consumption rate	Ethanol production rate	Butanol production rate
Experiment 1 (pH 5.75)	0.13	0.027	0.12	0.06
Experiment 2 (pH4.75)	0.15	0.039	0.16	0.07

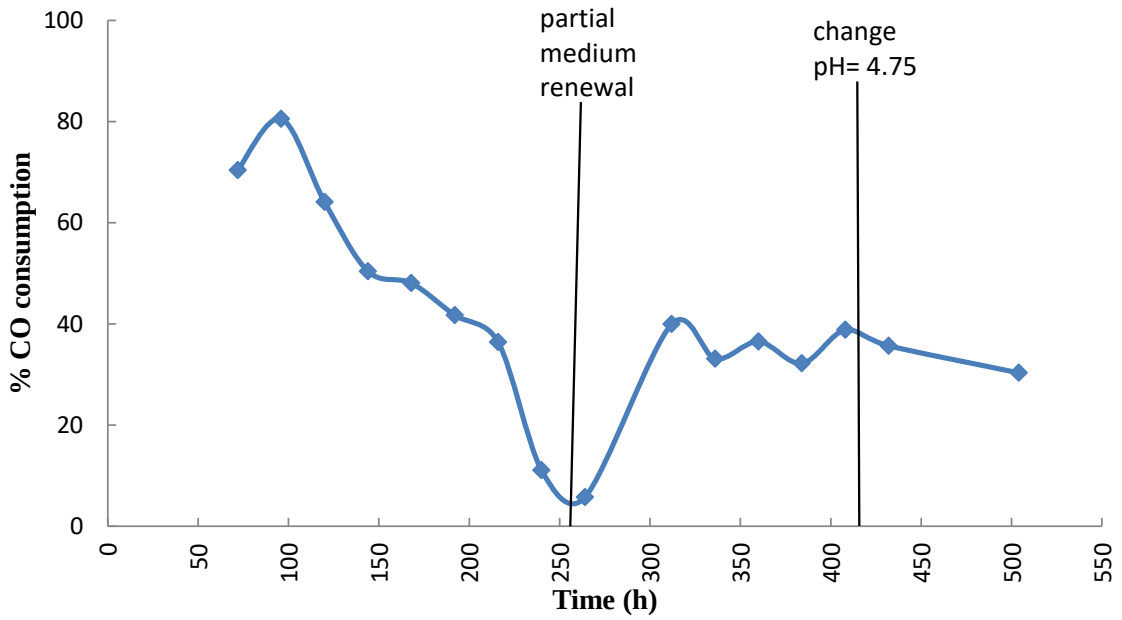

Figure 4.5 Continuous gas-fed bioreactor: percentage CO consumption over time.

A constant carbon monoxide loading rate was maintained throughout the experiment. Most of the time, the average CO consumption was close to 50 %, although higher percentages were observed in the early stages of the experiment, reaching the highest value of 81 % CO removal on the fourth day. It is worth mentioning that, as a general rule, the highest percentage substrate conversions are observed at high pH (5.75) and do then often exceed 50 % (up to 81 %), while conversion decreased when lowering the pH (4.75), which is also concomitant with some biomass decay. CO consumption also dropped within the first few hours after medium replacement.

During the first operation days, and for almost 1 week, the only carbon source for the production of metabolites and biomass is carbon monoxide. As explained above, C2 and C4 acids produced during the first stages become, later on, additional substrates and are then converted to the corresponding alcohols. During the first stage of the experiment, although part of the gaseous substrate is also used for biomass growth, if production of metabolites from CO is only considered during the first experimental stage, then the following reactions would take place:

$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2$ Eq. 4.1

$10CO + 4H_2O \rightarrow CH_3(CH_2)2COOH + 6CO_2$ Eq. 4.2

$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2$ Eq. 4.3

$12CO + 5H_2O \rightarrow C_4H_9OH + 8CO_2$ Eq. 4.4

This would result in CO_2 to CO ratios (g/g) of 0.79 for reaction (Eq. 4.1) (acetic acid production), 0.94 for reaction (Eq. 4.2) (butyric acid production), and 1.04 for reactions (Eq. 4.3) and (Eq. 4.4) (ethanol and butanol production, respectively). CO_2 to CO ratios could be measured experimentally and could be estimated to reach around 0.77, during the first week of operation, with around 10 % fluctuation as this is a dynamic system. This is in agreement with the above equations and theoretical ratios and shows a good fit between the experimental and theoretical substrate to product mass balance calculations.

The redox potential was constantly monitored during each experimental run. Except for the reducing agent added initially to the medium, its value was later on allowed to fluctuate naturally. In experiment 1, before replacing part of the medium, the redox potential (ORP) value was −180 ± 20 mV, while after partial medium replacement, it was −100 ± 10 mV. After the pH change to 4.75, ORP was −62.1 ± 20 mV, and finally at the end of the experiment, it became positive and reached +42.9 mV, which explains the complete inhibition of the anaerobic strain. Inhibition after pH modification could have been due to the fast decrease in pH from 5.75 to 4.75 resulting in an acid shock. As will be explained below, in experiment 2, pH was decreased gradually and no inhibition was observed, allowing to maintain active cells and a negative ORP.

Experiment 2

Similarly as in experiment 1, *C. carboxidivorans* started to grow immediately after seeding the reactor, without any lag phase. A pattern common for acetogenic clostridia for growth and fermentation products was here also observed (Figure 4.6 and Figure 4.7). After 48 h, the biomass reached its maximum value of 0.33 g/L (Figure 4.6) whereas the maximum acetic acid and butyric acid concentrations were found after 3–4 days and were 4.10 and 1.44 g/L, respectively (Figure 4.7). As already observed in experiment 1, and as expected, growth and accumulation of acids were concomitant. It is worth observing that the maximum suspended biomass concentration in the liquid phase was somewhat lower in this experiment compared to experiment 1 because part of the bacteria remained sticked to the glass wall of the bioreactor, slightly above the upper liquid level.

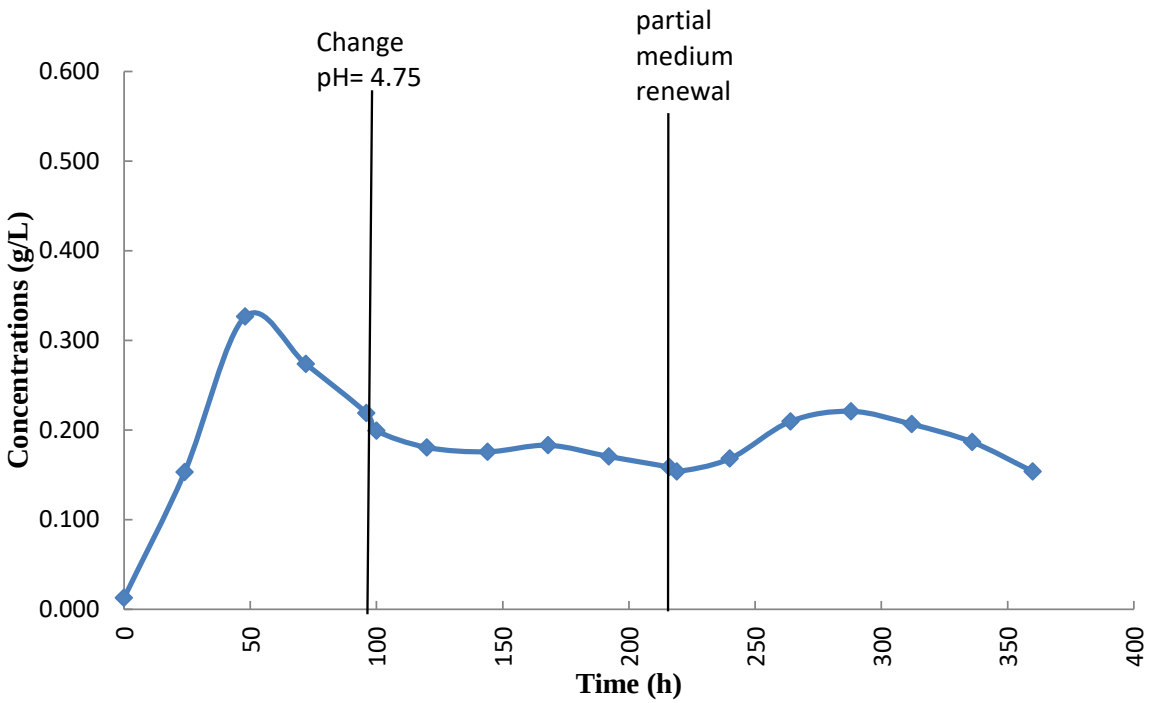

Figure 4.6 Continuous gas-fed bioreactor experiment 2: measured growth expressed in g/L over time.

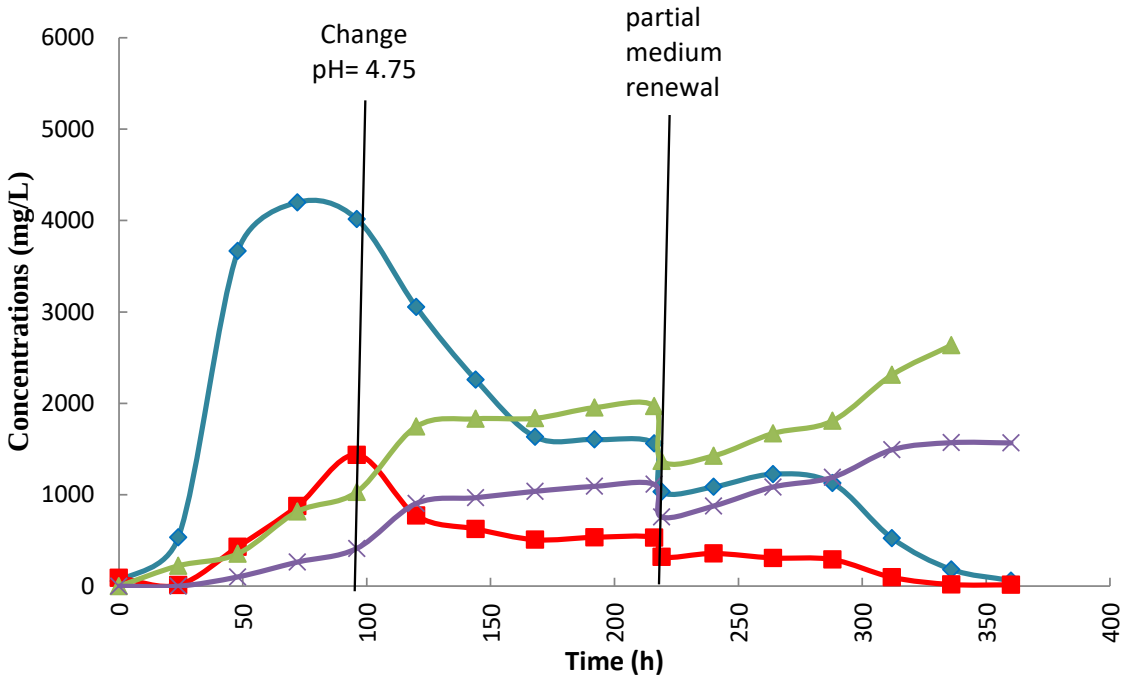

Figure 4.7 Continuous gas-fed bioreactor experiment 2: production of metabolites, acetic acid (blue diamonds), butyric acid (red squares), ethanol (green triangles), and butanol (purple cross marks), expressed in mg/L over time.

After 72 h, once acetic acid accumulation leveled off, the pH of the medium was gradually and slowly decreased to 4.75 over a period of 48 h, in order to avoid any acid shock and inhibition. The pH value was decreased in order to check if this would stimulate solventogenesis. The rate of accumulation of alcohols increased, and their maximum production was reached after 216 h, with ethanol and butanol concentrations of 2.00 and 1.10 g/L, respectively Figure 4.7. This increase was at the expense of acid consumption, and acetic acid and butyric acid concentrations had dropped down to 1.56 and 0.53 g/L respectively, after 216 h (Figure 4.7).

When the concentrations of alcohols stabilized, part of the medium of the bioreactor was replaced by fresh medium, similarly as in experiment 1, and the pH was increased to 5.75 again. As a result of the dilution effect, the concentrations of metabolites decreased to 1.03 g/L for acetic acid, 0.32 g/L for butyric acid, 1.4 g/L for ethanol, and 0.76 g/L for butanol. After partial medium replacement, the remaining concentrations of acids were consumed and converted into alcohols. Finally after 360 h, the biomass started decreasing down to 0.15 g/L and the experiment was stopped. By then, the final concentrations of acids and alcohols were 0.06 g/L acetic acid, 0.01 g/L butyric acid, 2.90 g/L ethanol, and 1.60 g/L butanol, showing a basically complete consumption of both acids and their conversion to alcohols (Figure 4.7).

Total net ethanol production in this experiment was 4.21 g corresponding to 2.40 g (in 1.2 L reactor medium) before partial medium replacement and 1.81 g after its replacement, and total net butanol production was 2.29 g corresponding to 1.32 g before partial medium replacement and 0.97 g after its replacement. These concentrations are somewhat lower than in experiment 1, most probably because of the somewhat lower suspended biomass concentration in this assay.

The specific rate of ethanol production was 0.16 g/h*g-of biomass between 72 and 120 h, while the specific rate of butanol production was 0.07 g/h*g-of biomass during that same period of time, which was thus slightly higher than in experiment 1 (Table 4-1). Other rates are summarized in Table 4-1.

Similarly as in experiment 1, a constant inlet carbon monoxide concentration was maintained during the study and CO consumption was monitored throughout experiment 2. Here again, on an average, close to 50 % of the gaseous carbon source was metabolized by the bacteria (Figure 4.8) with higher values during the first part of the study and when using a high pH, as also observed in experiment 1. The highest CO consumption reached 73 %, at pH 5.75, on the second day of operation.

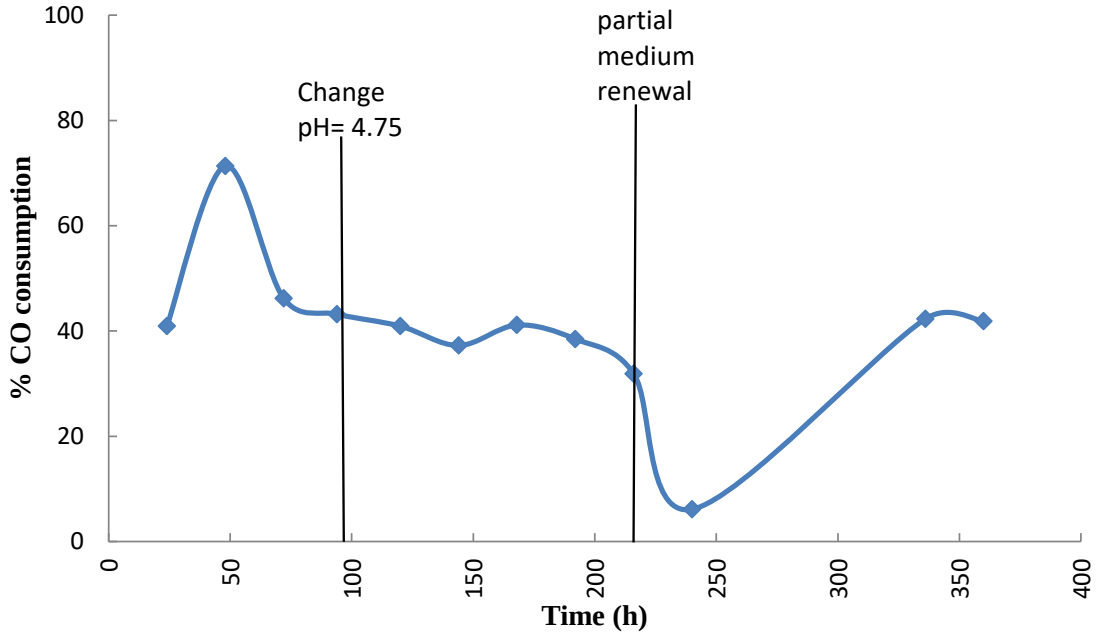

Figure 4.8 Continuous gas-fed bioreactor 2: percentage CO consumption over time.

In terms of redox potential, in experiment 2, when the pH was 5.75, the redox potential (ORP) value was −110 ± 10 mV, while after the pH change to 4.75, it was −80 ± 10 mV. Finally after medium replacement and pH increase again to 5.75, the ORP was −90 ± 10mV. The gradual pH decrease, from 5.75 to 4.75, in this experiment allowed to avoid inhibition of the bacterial activity, and a negative redox potential could be maintained throughout this assay, contrary to what was observed at the end of experiment 1.

4.4 Discusion

In the batch bottle experiments, the maximum biomass concentration was reached after 48 h and the biomass concentration (g/L) was about half the value reached in experiment 2. That difference can be explained by the fact that in the batch assays, there was not any continuous feed of CO, resulting in carbon source limitation for the bacteria. Conversely, in experiments 1 and 2 in bioreactors, CO feeding was continuous, resulting in a higher availability of C source for the biomass. Also, in the batch bottle experiments, there was no continuous pH control. Therefore, the production of acetic acid and butyric acid during growth led to a natural and significant decrease of the pH of the medium. This ended up inhibiting bacterial growth and metabolite production. The initial pH of the medium was 5.75, whereas the final pH value was in the range of 3.80–4.00 for all the batch assays in bottles. *C. carboxidivorans* has an optimum pH

value of 4.4–7.6 (Abubackar et al. 2011; Liou et al. 2005). As there was not any pH control in the batch experiments, its value reached a minimum which was below the optimum range. The lower pH value and lower concentration of C source explain the different growth behaviours between the batch experiments and the bioreactors with continuous CO supply.

In the three experiments, two different growth patterns were observed. First a fast exponential growth rate was observed, between 48 and 96 h in the bioreactors and between 0 and 36 h in the batch assays, concomitant with the acidogenic phase. Due to acid production in the Wood-Ljungdahl pathway, more ATP is produced during acidogenesis than during the production of alcohols (White 2007), which explains that growth and acid production from CO take place simultaneously.

In the case of the production of alcohols, in the batch assays, ethanol accumulation was observed but there was basically no butanol accumulation, whereas in the bioreactors (experiment 1 and 2), there was both significant ethanol and butanol production and the fermentation products followed a pattern common to acetogenic clostridia. It can be assumed that the pH had reached a value lower than the optimum range, which could have inhibited the bacterial metabolism before any significant butanol production could take place in the batch assays.

In both bioreactor experiments, acids were produced first, i.e., acetic acid and butyric acid, followed by ethanol and butanol production, with CO consumption around 50 % and reaching up to somewhat more than 80 % during the early acidogenic stage. Thus, the regulation of the pH value throughout the experiments can be considered to represent an important factor largely affecting both biomass growth and the production of metabolites.

In case of other clostridial strains able to produce only ethanol as alcohol (but no butanol), it was also observed that using different pH values is an effective strategy to promote alcohol production in multi-stage syngas fermentation, in which the acidogenesis and solventogenesis phases are separated in two reactors (Klasson et al. 1992). Here, in both bioreactor experiments, we tried to compare the effect of a pH change after the acidogenic phase. That way, in experiment 1, pH was maintained constant, whereas in experiment 2, the pH was changed to 4.75 to promote the solventogenic phase. No clear separate acidogenic and solventogenic phases were observed in *C. carboxidivorans* during experiment 2, whereas in experiment 1 (without pH change), more pronounced separate acidogenic and solventogenic phases were found. In their study with a different organism and for ethanol production, Klasson et al. (1992) performed a two-stage syngas fermentation experiment, with two reactors in series, using

Clostridium ljungdahlii, with the first reactor at pH 5.0 and the second one at pH 4.0~4.5 to promote ethanol production in that second reactor at the expense of acetate. By using two different pH, 30 times more ethanol production was obtained than in a single continuous gas fed bioreactor.

The rates of alcohol production were lower in experiment 1 than in experiment 2 at a lower pH. In case of ethanol, its production rate was 0.12 g/h*g of biomass in experiment 1, while it was 0.16 g/h*g of biomass in experiment 2. On the other hand, the rate of butanol production was 0.06 g/h*g of biomass in experiment 1 while it was 0.07 g/h*g of biomass in experiment 2. The same relationship was observed between the acid consumption rates, which were lower in experiment 1 than in experiment 2. The acetic acid conversion rates were 0.13 g/h*g of biomass and 0.15 g/h*g of biomass in experiments 1 and 2, respectively. On the other hand, butyric acid conversion rates were 0.03 g/h*g of biomass and 0.04 g/h*g of biomass in experiments 1 and 2, respectively. So, these results show higher acid to alcohol conversion rates at pH 4.75 than at pH 5.75 (Table 4-1).

So far, only few studies have focused on butanol production from CO-rich gases in clostridia. In all few previous reports using *C. carboxidivorans*, butanol concentrations did generally range between a few milligrams per liter and hardly 1 g/L, while butanol concentrations up to 2.66 g/L were reached in the present study together with ethanol concentrations of 5.55 g/L. Bruant et al. (2010), performing a similar batch experiment as ours in our bottles assays, accumulated a minor, near negligible, amount of butanol (below 0.05 mmol) of a few milligrams. Phillips et al. (2015) checked different media in batch experiments and their maximum reported concentrations for ethanol and butanol were 3.25 and 1.09 g/ L, respectively. In another study, Ukpong et al. (2012) checked the bioconversion of CO-rich gases by *C. carboxidivorans* in a gas-fed bioreactor reaching maximum ethanol and butanol accumulation of 2.8 and 0.52 g/L, respectively. In all those studies, the amounts of butanol and ethanol obtained with the same bacterial species were significantly lower than the values obtained in the present work. Overall, comparing the batch assays and the different bioreactor studies, it clearly appears that relatively high butanol-ethanol (BE) accumulation can be reached when optimizing the experimental conditions. It is worth mentioning that, contrary to the ABE fermentation from carbohydrates in clostridia, here, no acetone is produced at all in CO or syngas fermentation in such acetogenic bacteria, which is interesting as butanol is the main desired end-product as a biofuel and attempts do generally need to be made in order to reduce acetone accumulation in the conventional ABE fermentation (Han et al. 2011).

In a nutshell, it can be concluded that (a) a high pH was favorable to CO conversion to fatty acids; (b) reducing the pH value stimulated the production of alcohols but had a profound negative effect on biomass production; (c) acetic acid and butyric acid are produced first and can then be converted to the alcohols (ethanol, butanol), with complete conversion at higher rates under acidic conditions; (d) contrary to the ABE fermentation, no acetone was formed here from the conversion of C1 gases; and (e) the experimental conditions in this study allowed to produce significantly more butanol and ethanol (B-E) than in any other study reported in the literature on the conversion of CO-rich gases.

4.5 Acknowledgments

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5 EFFECT OF THE pH CONTROL ON ANAEROBIC H-B-E FERMENTATION OF SYNGAS IN BIOREACTORS

EFFECT OF THE pH CONTROL ON ANAEROBIC H-B-E FERMENTATION OF SYNGAS IN BIOREACTORS

Abstract

BACKGROUND: Syngas and some waste gases are composed mainly of carbon monoxide, carbon dioxide and hydrogen, which can be used by some acetogenic bacteria to produce ethanol, butanol or hexanol, and represents an attractive alternative to the conventional ABE (acetone–butanol–ethanol) fermentation. Experiments were carried out in bioreactors under different conditions (pH 5.75 and 4.75) with continuous supply of a mixture of $CO/CO_2/H_2/N_2$ converted by *Clostridium carboxidivorans* into hexanol, butanol and ethanol (H-B-E fermentation).

RESULTS: Applying different pH control strategies will affect the syngas fermentation pattern, among others in terms of bioconversion rates as well as final concentrations of acids and alcohols. The highest concentrations of alcohols were obtained at pH 5.75, i.e. 2.7 g/L ethanol, 1.9 g/L butanol and 0.85 g/L hexanol, whereas the maximum production rates were observed at pH 4.75, reaching 0.048 g-ethanol/h*g of-biomass, 0.037 g-butanol/h*g of-biomass, and 0.026 g-hexanol/h*g-of biomass. However, a low pH negatively affects growth and acids production in the first metabolic step, with lower growth and acids production at pH 4.75 than 5.75. Growth rates reached 0.0057 h^{-1} and 0.072 h^{-1}, respectively, at pH 4.75 and 5.75.

CONCLUSIONS: Maintaining initially a higher pH of 5.75 allows accumulating higher concentrations of acids than when natural acidification takes place. Such higher concentrations of acids allow the production of higher amounts of alcohols as end metabolites, showing the importance of pH on bioconversion and biomass growth.

Keywords: acetogens; butanol; ethanol; hexanol; waste gas; *Clostridium carboxidivorans*

With minor editorial changes to fulfil formatting requirements, this chapter is substantially as it appears in: J Chem Technol Biotechnol **Published online: 9 February 2017**. DOI 10.1002/jctb.5232.

5.1 Introduction

Biorefineries are emerging as environmentally-friendly alternatives to conventional refineries for the commercial production of fuels and platform chemicals. They are based on the use of renewable feedstocks, such as biomass, or even waste or pollutants, which can be fermented into added-value end metabolites. Ethanol and higher alcohols, such as butanol, are examples of metabolites of commercial interest. (Bio)ethanol is mainly used as a fuel. Higher alcohols such as butanol and hexanol may also be suitable fuels (Diender et al. 2016), but can be used as platform chemicals as well. Ethanol is an oxygenated, water-free, high octane alcohol that can replace gasoline or it can be mixed with it at different ratios (Abubackar et al. 2011). Butanol is more similar to gasoline than ethanol, besides being less hygroscopic, less corrosive and having a higher caloric content than ethyl alcohol (Fernández-Naveira et al. 2017; Groenestijn et al. 2013; Wallner et al. 2009). It can be blended with gasoline at higher concentrations than ethanol without the need for modifying existing engines (Dürre 2007; Lee et al. 2008). Hexanol has also been tested as a fuel, among others in the form of diesel–hexanol blends and also in aviation fuel, although its high viscosity limits its potential use as jet fuel (Chuck and Donnelly 2014; Fernández-Naveira et al. 2017; Vigneswaran and Thirumallini 2015). In terms of bioprocesses, the best known alternative for their production is the ABE fermentation, allowing production of butanol, together with other solvents such as ethanol and acetone, through the bioconversion of carbohydrates by clostridia or other anaerobic bacteria (Fernández-Naveira et al. 2017).

Bioalcohols have traditionally been produced in biorefineries through the so-called first generation process in which fermentable sugars are obtained from food supplies, which results in food–fuel competition and represents a poorly sustainable alternative (Kennes et al. 2016). The second generation process avoids that problem as it uses lignocellulosic materials from agricultural waste or energy crops as feedstock. It is more sustainable but less cost-effective, among others because of the complex pretreatments required to obtain simple sugars fermentable into alcohols (Balat and Balat 2009). On the other hand, the fermentation of C1 gases has recently been shown to be a promising alternative as it can also generate higher alcohols such as butanol and, to a lower extent, hexanol (Fernández-Naveira et al. 2017). C1 gases, mainly CO and CO_2, can be obtained from the gasification of wastes, coal, biomass and other feedstocks (Abubackar et al. 2016; Heidenreich and Foscolo 2015; Kennes et al. 2016; Vassilev et al. 2015).

Syngas is a complex mixture of gases, composed mainly of CO, CO_2, and H_2 (Hernández et al. 2013), which can be fermented by clostridia and other acetogens, mostly into acids and/or

alcohols. Interestingly, those same gaseous substrates are found in some waste gases, among others in gaseous effluents from steel industries. In such case, their bioconversion would allow the simultaneous removal of air pollutants as well as a greenhouse gas such as carbon dioxide, combined with their conversion into valuable products. This is a promising technology able to compete with second generation carbohydrates fermentation (Kennes et al. 2016), although it still faces some challenges such as the low solubility of CO and H_2 mainly (Fernández-Naveira et al. 2017), or the presence of other minor compounds in the case of syngas, that could be toxic to the producing strains (Ahmed et al. 2006; Haryanto et al. 2009).

In the present study, the anaerobic bioconversion of syngas or waste gas into ethanol and higher alcohols (butanol, hexanol) was carried out by the acetogenic bacterium *Clostridium carboxidivorans* in a continuous gas-fed bioreactor containing a defined aqueous culture broth and continuously supplied a mixture of CO, CO_2, H_2, N_2. The objective of this research was to develop and optimize the operating conditions in order to reach an efficient production of alcohols. The production of metabolites was compared under two different bioreactor operating conditions, either with pH regulation or without pH regulation. In an attempt to increase the production of acids and the subsequent accumulation of alcohols, pH conditions were adjusted throughout the fermentation runs in order to optimize the production of acids and alcohols and increase their concentrations and the overall efficiency of the bioconversion process.

5.2 Material and methods

5.2.1 Microorganism and culture media

Clostridium carboxidivorans P7 DSM 15243 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was maintained anaerobically on modified basal medium at pH 5.75 and with a mixture of the following gases as volatile substrates: $CO:CO_2:H_2:N_2$ (20:20:10:50) (Liou et al. 2005; Tanner 2007). The basal medium had the following composition (per liter distilled water): 1 g yeast extract; 25 mL mineral solution; 10mL trace metal solution; 10 mL of vitamins stock solution; 1mL resazurin; 0.60 g cysteine-HCl.

The mineral stock solution was composed of (per liter distilled water): 80 g sodium chloride, 100 g ammonium chloride, 10 g potassium chloride, 10 g potassium monophosphate, 20 g magnesium sulfate, and 4 g calcium chloride.

The trace metal solution was obtained from a stock solution, whose composition is as follows (per L distilled water): 2 g nitrilotriacetic acid, 1 g manganese sulfate, 0.80 g ferrous ammonium sulfate, 0.20 g cobalt chloride, 0.20 g zinc sulfate, and 20 mg each of cupric chloride, nickel chloride, sodium molybdate, sodium selenate, and sodium tungstate.

The vitamin stock solution was composed of (per L distilled water): 10mg pyridoxine, 5mg each of thiamine, riboflavin, calcium pantothenate, thioctic acid, paraamino benzoic acid, nicotinic acid, and vitamin B12, and 2mg each of D-biotin, folic acid, and 2-mercaptoethanesulfonic acid.

5.2.2 Continuous gas-fed bioreactor experiments

Two bioreactor experiments were carried out in 2 L BIOFLO 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA). The two experiments were carried out with 1.2 L of the same medium composition and the mixture of gases, described above, as gaseous substrate. The syngas mixture was continuously fed at a flow rate of 10 mL/min using a mass flow controller (Aalborg GFC 17, Müllheim, Germany) and the gas was sparged by a microsparger. The medium inside the bioreactor was autoclaved, and afterwards it was flushed with N_2 to ensure anaerobic conditions, while the bioreactor medium was cooled by means of a water jacket. When the temperature reached 40° C the vitamins solution and cysteine-HCl were added. The temperature of the medium was maintained at 33° C throughout the experimental process with a constant agitation speed of 250 rpm. Inside the bioreactor, four baffles avoided vortex formation and allowed improved liquid mixing.

When the conditions were completely anaerobic, N_2 flushing was stopped and constant syngas feeding was started. Then, 10 % seed culture in the early exponential growth phase (which was previously grown for 72 h with CO as sole carbon source) was inoculated in the bioreactor. The redox potential and the pH value were continuously monitored. The pH was automatically maintained constant through the addition of either 2 mol/L NaOH or 2mol/L HCl solutions, by means of a peristaltic pump.

In the first experiment, there was no pH regulation, except during the first day of operation, when the pH was maintained at 5.75, allowing for good biomass growth. Later on, as a result of the production of acids the pH dropped naturally to a value of 4.75, which was then maintained constant in order to avoid any possible inhibitory effect. Afterwards, when the consumption of organic acids stabilized, the pH was slowly and gradually increased to 5.75 with the aim of starting a new production cycle. The new cycle did not start, contrary to what was expected, and part of the medium was then replaced with the same volume of fresh medium. Conditions of

natural pH shift were again maintained in the bioreactor after medium replacement. In the second experiment, the pH was maintained constant at 5.75 throughout the study.

5.2.3 Growth measurement

A 1 mL liquid sample was withdrawn daily from the bioreactors, and was used to measure the optical density (OD $_{\lambda}$=600nm) on a UV-visible spectrophotometer (Hitachi, Model U-200, Pacis and Giralt, Madrid, Spain) in order to estimate the biomass concentration (mg/L) using a previously generated calibration curve.

5.2.4 Gas-phase CO and CO_2 concentrations

Gas samples of 1mL were taken from the outlet sampling ports of the bioreactors to monitor the CO and CO_2 concentrations. Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD). The GC was fitted with a 15m HP-PLOT Molecular Sieve 5A column (ID, 0.53 mm; film thickness, 50 µ m). The oven temperature was initially kept constant at 50° C, for 5 min, and then raised at 20° C min^{-1} for 2 min, to reach a final temperature of 90° C. The temperature of the injection port and the detector were maintained constant at 150° C. Helium was used as the carrier gas. Similarly, CO_2 was analyzed on an HP 5890 gas chromatograph, equipped with a TCD. The injection, oven, and detection temperatures were maintained at 90, 25, and 100° C, respectively.

5.2.5 Fermentation products

Bioconversion products were detected with an HPLC (HP1100, Agilent Co., USA) equipped with a 5 µm x 4mm x 250mm Hypersil ODS column and a UV detector at a wavelength of 284 nm. The mobile phase was a 0.1 % ortho-phosphoric acid solution fed at a flow rate of 0.5mL/min. The column temperature was set at 30° C. 1mL liquid samples were centrifuged (7000x g, 3min) using a centrifuge (ELMI Skyline ltd CM 70 M07) before analyzing the concentration of water-soluble products (acetic acid, butyric acid, hexanoic acid, ethanol, butanol and hexanol) by HPLC, at least once every 24 h.

5.2.6 Redox potential

The redox potential was constantly monitored in each bioreactor using an Ag/AgCl reference electrode connected to a transmitter (M300, Mettler Toledo, Inc., Bedford, MA, USA) and maintained inside the bioreactor.

5.3 Results and discussion

5.3.1 Continuous gas-fed bioreactor with natural acidification

Biomass growth and production of metabolites

In order to allow for a fast start-up and initial biomass growth, in this experiment the pH was maintained constant at a value of 5.75 during the first day. Afterwards, it was allowed to fluctuate freely. *Clostridium carboxidivorans* started growing immediately after inoculation, without any lag phase. The maximum biomass concentration was found after 236 h, reaching a value of 0.42 g/L (Figure 5.1). However, as can be observed in Figure 5.1, there was a clear difference between biomass growth at pH 4.75 compared with its growth during the first few hours at pH 5.75. At the highest pH, a biomass concentration of 0.18 g/L was already reached in only 27 h, while growth was much slower at the lower pH. The growth rates were calculated and compared under both conditions. At pH 5.75 (first 27 h), the bacterial growth rate was 0.072 h^{-1}, while its value dropped by more than a factor of ten, to 0.0057 h^{-1}, in the next stage at pH 4.75. This agrees with data reported on the optimal pH range for *Clostridium carboxidivorans* grown on syngas (Abubackar et al. 2012; Fernández-Naveira et al. 2017; Liou et al. 2005). The growth rate found here at pH 5.75 is also close to the maximum value reported recently for that same strain grown under optimal conditions in batch assays on carbon monoxide (μ max =0.086 h^{-1}) (Fernández-Naveira et al. 2016a). A pH value of 4.75 is very close to growth inhibitory conditions (unpublished data), which explains the observed low biomass build-up in this case. Growth is concomitant with acidogenesis in clostridia metabolizing C1 gases. Therefore, production of acids started soon after seeding the bioreactor. Acetic acid appeared immediately after inoculation, followed by butyric acid production a few hours later; whereas hexanoic acid was detected for the first time after 68 h. All three acids reached their maximum concentrations rather simultaneously, after 92 h, with the accumulation of 3.45 g/L acetic acid, 0.72 g/L butyric acid, and 0.18 g/L hexanoic acid Figure 5.2. As a result of the fast production of organic acids, a pH value of 4.75 was reached only 48 h after reactor start-up, and only a few hours after allowing the pH to drop freely. Although sustained production of acids would result in further acidification, the pH was then maintained constant, as our own experience suggested that a low pH (<4.75) might inhibit the bacteria. Some authors concluded that for some bacteria, a pH of at least 4.7–4.8 is necessary to maximize alcohol production and avoid inhibition of bacterial growth (Ganigué et al. 2016).

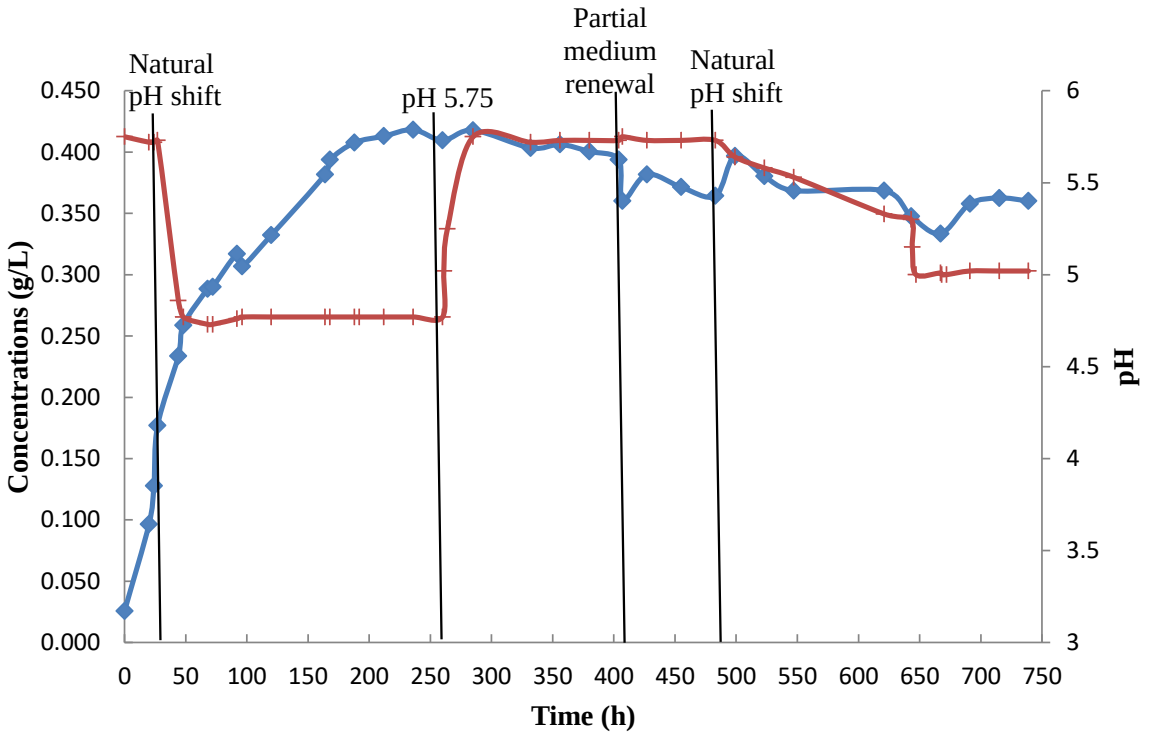

Figure 5.1 Continuous gas-fed bioreactor experiment with natural acidification: measured growth expressed in g/L over time (blue diamonds) and pH values (red plus).

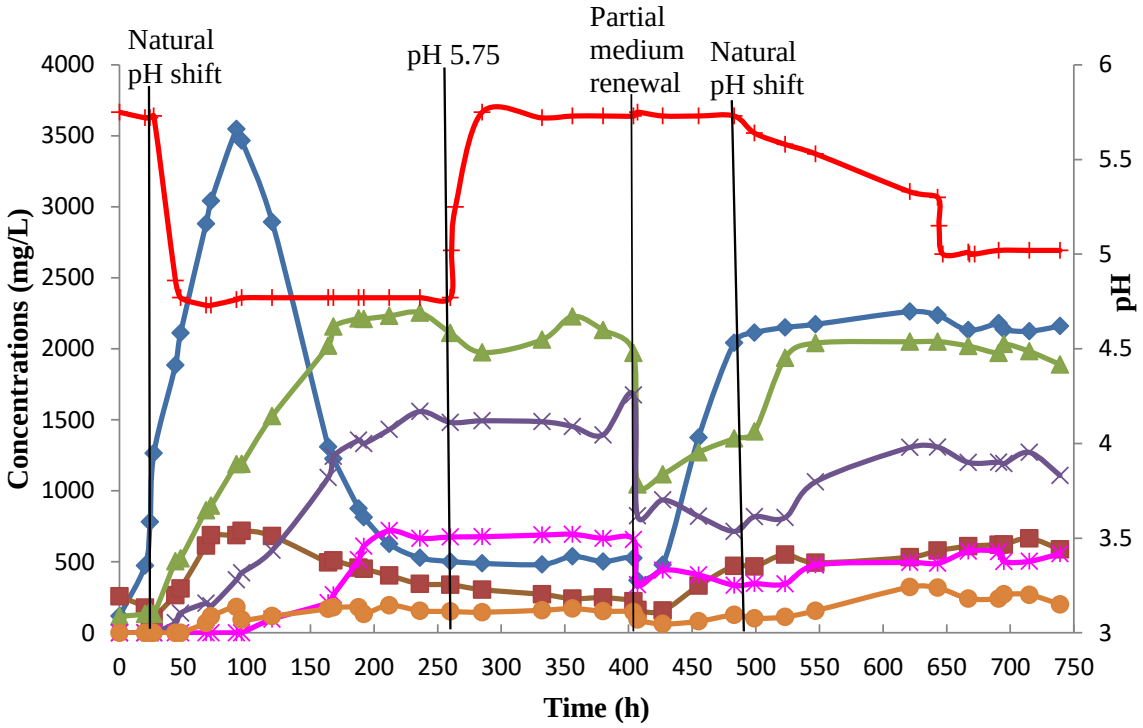

Figure 5.2 Continuous gas-fed bioreactor experiment with natural acidification: production of metabolites: acetic acid (blue diamonds), butyric acid (red squares), hexanoic acid (orange circles), ethanol (green triangles), butanol (purple crossmarks), hexanol (pink star) expressed in mg/L over time, and pH values (red plus)

Medium acidification is generally considered to stimulate solventogenesis and the conversion of organic acids into alcohols (Fernández-Naveira et al. 2017; Grethlein et al. 1991; Jones and Woods 1986; Kennes et al. 2016; Lee et al. 2008; Millat et al. 2011; Phillips et al. 2015). After 100 h, at pH 4.75, both acetic and butyric acid concentrations started gradually decreasing. However, ethanol and butanol appeared after 44 h, when the concentration of acids was still increasing exponentially, suggesting that, at that time, the alcohols could already be produced from the gaseous substrates rather than from the acids and/or that both acid production (from the gaseous substrates) and conversion (to alcohols) was taking place. There was no clear drop in the concentration of hexanoic acid; but significant hexanol production started after 120 h. According to the Wood–Ljungdahl pathway summarized in Figure 5.3 and based on the results shown in Figure 5.1, higher alcohols such as hexanol could potentially be produced from gas fermentation and the bioconversion of acetyl-CoA to hexanoyl-CoA and, subsequently, to the corresponding alcohol. Maximum accumulation of alcohols was observed after 212 h, with 2.25 g/L ethanol, 1.43 g/L butanol and 0.72 g/L hexanol (Figure 5.2). Again, this suggests that some hexanol could presumably directly be produced from the gaseous substrates through acetyl-CoA, besides the potential conversion of the corresponding acid to the alcohol. The results can be compared with a previous study (Fernández-Naveira et al. 2016b) in which the pH was initially maintained constant (pH 5.75) for a longer period than in this assay (with natural acidification here) and was only decreased later on, artificially, to pH 4.75. In the present case, with natural acidification, pH drops sooner; this results in somewhat lower maximum concentrations of acids (due to the low pH). Conversely, the production of alcohols starts earlier and higher maximum, final, concentrations of alcohols are obtained.

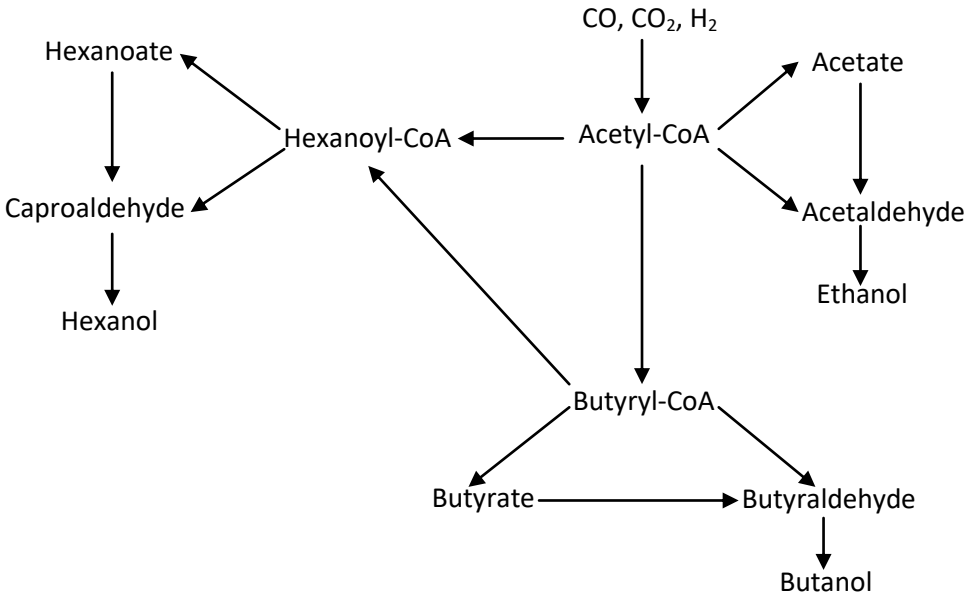

Figure 5.3 Wood–Ljungdahl pathway in H-B-E fermentation

After 260 h, once alcohol production had stabilised, the pH was increased gradually and slowly to 5.75 over a period of 25 h. The goal was to check if increasing the pH would again stimulate the production of acids and help restart a cycle of acids production followed by their consumption and conversion to alcohols, as was recently shown to be feasible in another strain, *C. autoethanogenum*, grown on carbon monoxide and producing ethanol (Abubackar et al. 2016a; Abubackar et al. 2016b). However, 4 days later all concentrations remained basically unchanged Figure 5.2, suggesting some possible inhibitory effect impeding initiation of a new cycle. The accumulation of alcohols at relatively high concentrations has been reported to have toxic effects on clostridia grown on carbon monoxide (Fernández-Naveira et al. 2016a). However, at total alcohol concentrations not exceeding about 4 g/L, as in this experiment, inhibition by end metabolites should be minimal. It is unclear if the presence or absence of any specific compound could have hindered the start of a new cycle. In order to clarify this, after 407 h, part of the fermentation broth was replaced. Immediately after removing part of the old medium and introducing fresh culture broth, the bioconversion process started again and organic acids were produced. The biomass concentration decreased somewhat, down to a value of 0.36 g/L, because of the dilution effect due to medium replacement (Figure 5.1). The amount of acids and alcohols present in the medium decreased too, again as a result of the dilution effect, and their concentrations after partial medium renewal were 0.37 g/L acetic acid, 0.16 g/L butyric acid, 0.09 g/L hexanoic acid, 1.04 g/L ethanol, 0.82 g/L butanol and 0.34 g/L hexanol (Figure 5.2).

The pH of the medium was not artificially maintained constant after partial medium replacement, so that acidification and natural pH shift could take place anew. After 499 h, the biomass concentration increased again and reached a value of 0.40 g/L, which is near to the maximum value of the previous cycle (Figure 5.1). Although acids started accumulating immediately after medium replacement, their production was not high enough to allow the pH to drop down to 4.75 again. Therefore, a minimum pH value of 5.00 was reached after 647 h, but none of the acids nor the alcohols were consumed anymore. At the end of that new cycle, the maximum concentrations of acids were 2.20 g/L acetic acid, 0.61 g/L butyric acid and 0.32 g/L hexanoic acid. They remained roughly constant until the end of the experiment (Figure 5.2). After 499 h, when the pH started dropping, alcohols accumulated at a higher rate, whereas the production of acids leveled off. The following concentrations of alcohols were then detected at the end of the experiment: 2.03 g/L ethanol, 1.20 g/L butanol and 0.57 g/L hexanol.

The total net amount of alcohols generated during the process was calculated, reaching 3.88 g ethanol (2.70 g in the first cycle and 1.18 g in the second cycle), 2.18 g butanol (1.72 g in the first cycle and 0.46 g in the second cycle) and 1.14 g hexanol (0.86 g in the first cycle and 0.28 g in the second cycle) in the 1.2 L bioreactor. The experimental production rates of alcohols were calculated as well (pH 4.75). In the case of ethanol, the production rate was 0.048 g/h*g of biomass between 27 h and 168 h. The butanol production rate was 0.037 g/h*g of biomass between 72 h and 168 h; and the hexanol production rate was 0.026 g/h*g of biomass between 168 h and 212 h.

The redox potential was monitored throughout the experimental process. In the first part of the experiment, after inoculation, when the pH was around 5.75, the redox potential was −100±5 mV, whereas when the bacteria started to produce acids, at a pH around 4.75, the redox potential changed to −68±2 mV. After partial medium replacement (pH 5.75 again) the redox potential was −85±4 mV. Finally, at the end of the experiment (pH 5.00) the redox potential reached −40±20 mV.

Consumption of gaseous C1-substrates

Consumption of the gaseous C1-substrates is hardly ever reported in studies on syngas or waste gas bioconversion to alcohols, while this is an important aspect, among others in the case of waste gas treatment as emission to the atmosphere of pollutants such as CO_2, a potent greenhouse gas, should be avoided. Similarly CO has clear indirect effects on climate change. CO consumption was monitored throughout the experiment (Figure 5.4).The maximum CO

bioconversion was reached 44 h after inoculation, with a value of 69 %, and in the second stage (i.e. after partial medium renewal) the maximum consumption was 56.5 % after 455 h. During the rest of the experiment CO conversion remained around 30–40 %. It is interesting to observe that, after partial renewal of the fermentation broth, a jump in the levels of CO consumption was detected with sudden improvement of CO assimilation. This could be due to a possible presence of some inhibitory compound or to limitation of a specific essential nutrient in the fermented medium before introducing fresh medium. However, it is also worth noting that the period after partial medium replacement corresponds to an acidogenic stage with production of acids mainly, and that the experimental data suggest that acidogenesis would be related to higher consumption of the gaseous carbon substrates.

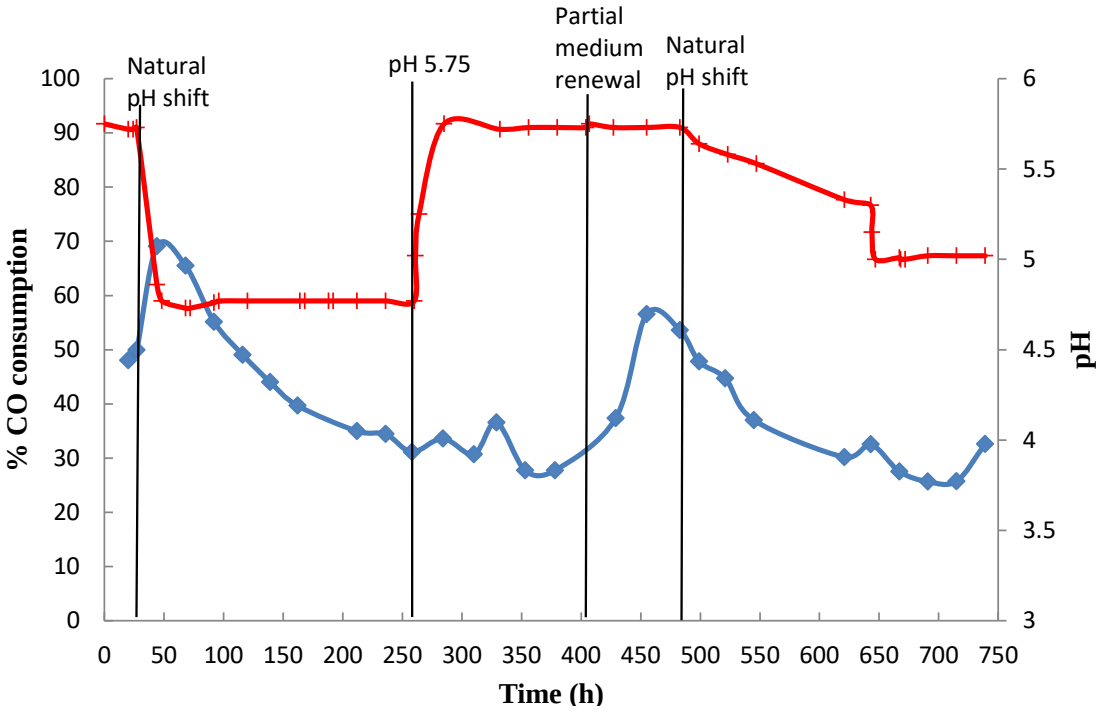

Figure 5.4 Continuous gas-fed bioreactor experiment with natural acidification: percentage of CO consumption over time (blue diamonds), and pH values (red plus).

On the other hand, CO_2 was monitored too (Figure 5.5). Its fate is somewhat more difficult to elucidate, as carbon dioxide is not only a substrate but also a metabolite. It can be both consumed and produced, even simultaneously. However, reasonable explanations of the observed trends can be hypothesized. During the first few hours after bioreactor start-up, as well as just after partial medium replacement, higher CO_2 concentrations were detected at the outlet than at the inlet of the system, with net carbon dioxide production and 18–21 % higher concentrations detected at the outlet port compared with the inlet one (Figure 5.5). During the rest of the experiment net CO_2 removal was observed. A short peak of maximum CO_2 production

appeared at t=50 h; it then started to drop to reach a minimum outlet carbon dioxide concentration after 160 h of bioreactor operation. The maximum consumption reached 40 % of the amount of CO_2 present in the feed. During the remaining experimental period, until partial medium renewal, the average carbon dioxide bioconversion remained, on average, close to 15 % of CO_2 consumption refered to the inlet concentration (negative values on Figure 5.5).

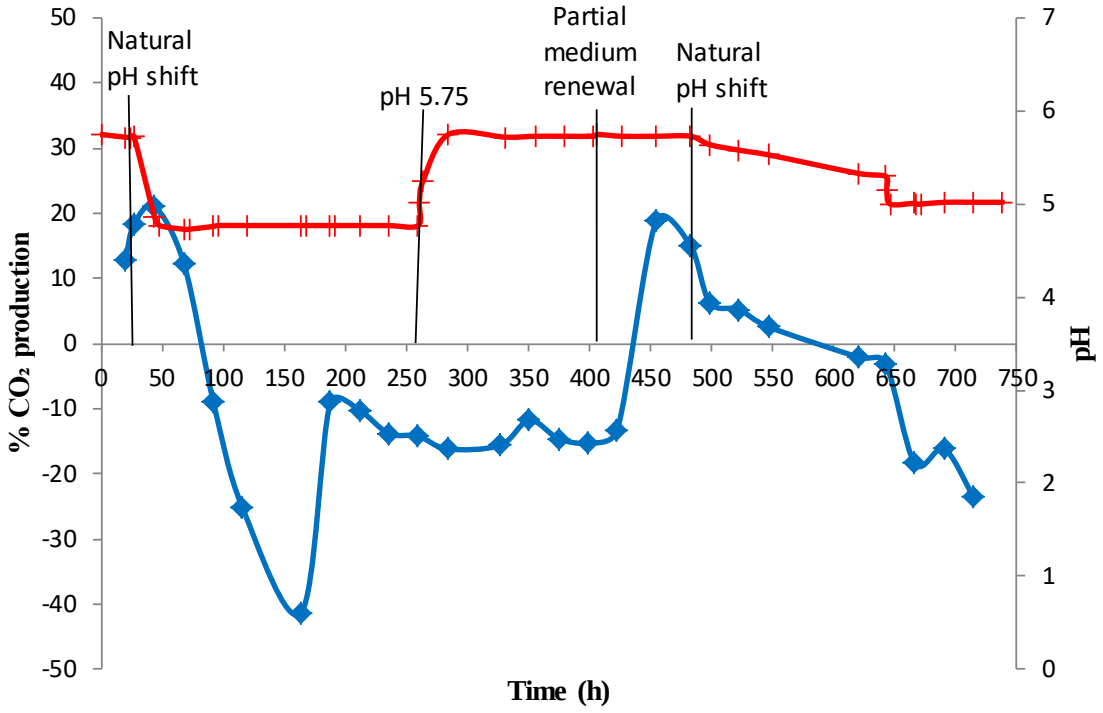

Figure 5.5 Continuous gas-fed bioreactor experiment with natural acidification: percentage of CO2 production over time (blue diamonds), and pH values (red plus).

Maximum CO bioconversion and maximum CO_2 removal (minimum CO_2 concentrations at the outlet of the fermentor) were detected simultaneously. The peaks of net carbon dioxide production observed occasionally, just after start-up (t=50 h) and after medium replacement (t=450 h) correspond to the exponential accumulation of organic acids (mainly acetic, as well as some butyric, acids). It is thus also simultaneous with biomass growth. This sounds logical, as the production of acids from substrates such as carbon monoxide, present in syngas or waste gases, leads to the simultaneous accumulation of carbon dioxide as suggested from the Wood–Ljungdahl pathway and as shown in Eq. 5.1 for acetic acid and Eq. 5.2 for butyric acid (Fernández-Naveira et al. 2017):

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad \text{Eq. 5.1}$$

$$10CO + 4H_2O \rightarrow CH_3(CH_2)2COOH + 6CO_2 \quad \text{Eq. 5.2}$$

Once the production of acids stops, no more carbon dioxide is generated, resulting in net consumption of that gaseous C1 substrate to produce ethanol, butanol or eventually hexanol as shown in Eq. 5.3, Eq. 5.4 and Eq. 5.5 (Fernández-Naveira et al. 2017):

$$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3\ H_2O \quad \text{Eq. 5.3}$$

$$12\ H_2 + 4\ CO_2 \rightarrow C_4H_9OH + 7\ H_2O \quad \text{Eq. 5.4}$$

$$18H_2 + 6CO_2 \rightarrow C_6H_{13}OH + 11H_2O \quad \text{Eq. 5.5}$$

Moreover, typical reactions of bioconversion of organic acids to alcohols do not produce any carbon dioxide either, as can be seen in Eq. 5.6, Eq. 5.7 and Eq. 5.8, for ethanol, butanol and hexanol, respectively(Fernández-Naveira et al. 2017):

$$CH_3COOH + 2H_2 \rightarrow C_2H_5OH + H_2O \quad \text{Eq. 5.6}$$

$$CH_3\ (CH_2)_2\ COOH + 2H_2 \rightarrow C_4H_9OH + H_2O \quad \text{Eq. 5.7}$$

$$CH_3\ (CH_2)_4\ COOH + 2H_2 \rightarrow C_6H_{13}OH + H_2O \quad \text{Eq. 5.8}$$

5.3.2 Continuous gas-fed bioreactor at constant pH

Biomass growth and production of metabolites

This experiment was started in a similar way to the previous one, except that a high pH value was maintained constant throughout the study in order to check how this would affect the production of acids and if their concentration might reach higher values, without any inhibition, eventually resulting in increased accumulation of alcohols. Again, the bacteria started growing immediately after inoculation, without any lag phase. The maximum biomass concentration was observed 91 h after inoculation (Figure 5.6). Growth data appearing in Figure 5.6 confirm the trend already observed in the previous experiment in Figure 5.1, with high growth rates (0.056 h^{-1}) and fast biomass production at high pH (5.75) (Figure 5.1 and Figure 5.6) compared with slower kinetics at pH 4.75 (Figure 5.1). In the previous experiment, acidification and omission of pH regulation resulted in slow growth with maximum biomass concentration reached after 236 h, whereas the constant higher pH of 5.75 in this new experiment allowed the bacteria to grow faster, reaching a maximum bacterial concentration after 91 h.

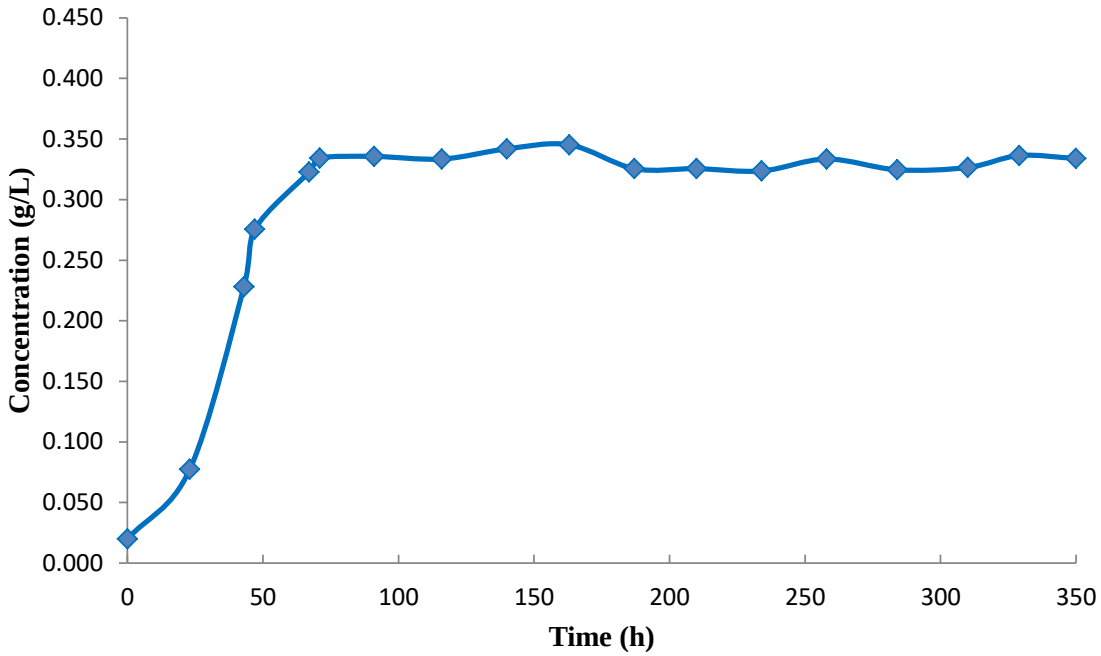

Figure 5.6 Continuous gas-fed bioreactor experiment at constant pH: measured growth expressed in g/L over time.

The first acid produced in this experiment, under pH regulated conditions, was acetic acid followed by butyric acid and finally hexanoic acid, similarly to the previous experiment with natural acidification. The highest concentrations of acetic and butyric acids were found, in both cases, after 116 h, although acetic acid started being produced earlier than butyric acid. Those maximum values were 6.20 g/L for acetic acid and 1.40 g/L for butyric acid, whereas the maximum hexanoic acid concentration was 0.40 g/L and was reached later, after 258 h (Figure 5.7). Hexanoic acid production started only on the fourth day, after 100 h. Maintaining a constant high pH value of 5.75 in this experiment allowed higher total concentrations of acids than in the experiment with the pH naturally dropping down to a value of 4.75. This was also observed in another experiment in which CO was used as substrate rather than syngas (Fernández-Naveira et al. 2016b). The maximum amount of organic acids accumulating in the case of natural acidification was about half the amount obtained at a constant pH of 5.75 as it corresponded to 3.45 g/L acetic acid, 0.72 g/L butyric acid and 0.18 g/L hexanoic acid Figure 5.2) at low pH (4.75) compared with 6.20 g/L acetic acid, 1.40 g/L butyric acid and 0.40 g/L hexanoic acid at this higher pH (5.75).

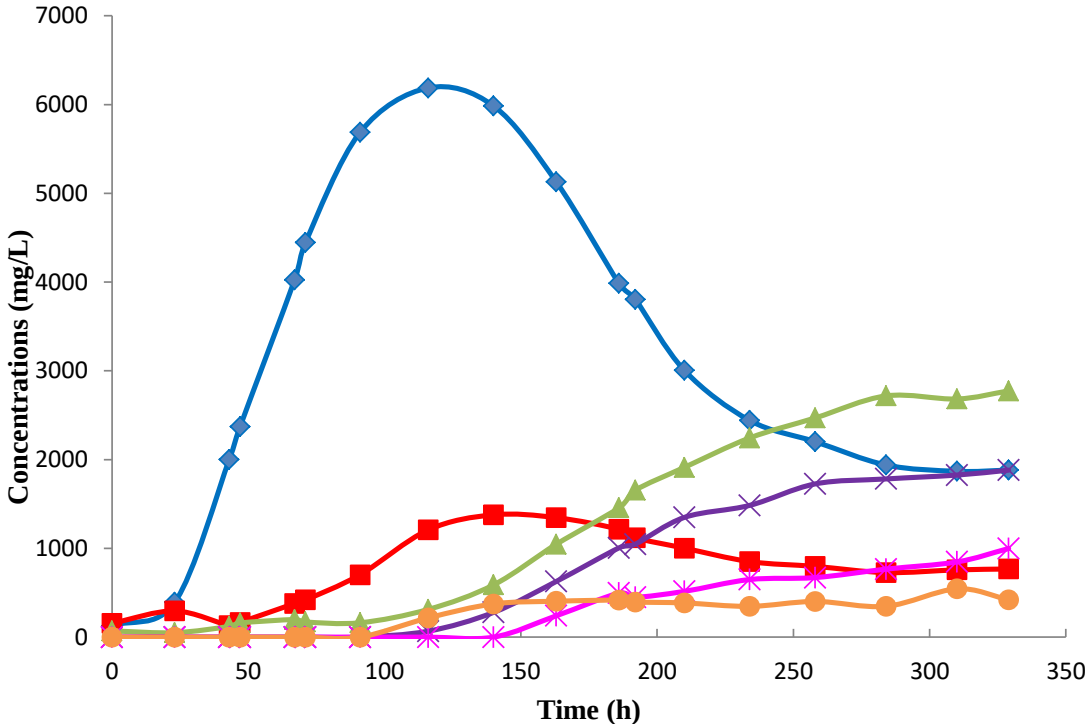

Figure 5.7 Continuous gas-fed bioreactor experiment at constant pH: production of metabolites: acetic acid (blue diamonds), butyric acid (red squares), hexanoic acid (orange circles), ethanol (green triangles), butanol (purple crossmarks), hexanol (pink star) expressed in mg/L over time.

In the previous experiment, the production of alcohols took place as soon as acidification started, almost immediately after inoculation. This means that, at such low pH, there is coexistence of acidogenesis and solventogenesis in *C. carboxidivorans* and it indicates that, under such experimental conditions, both bioconversion processes can take place simultaneously. Conversely, in this new experiment at higher pH, the exponential production of ethanol appeared later, several hours after inoculation (around t=96 h), while butanol and hexanol production started after 116 and 163 h, respectively. The maximum alcohol concentrations in this assay at constant pH were 2.70 g/L ethanol, 1.90 g/L butanol and 0.85 g/L hexanol, which were reached after 310 h (Figure 5.7). It is agreed that the gas (i.e. substrate) composition may have an effect on the bioconversion pattern. In the present study with syngas (CO, CO_2, H_2) a somewhat lower conversion of acids to alcohols was observed compared with results for pure CO as substrate reported recently (Fernández-Naveira et al. 2016b). In both experiments described in the present paper, the pH remained constant during the solventogenic phase. Indeed, a low pH value of 4.75 was quickly reached in the first experiment, through natural acidification, and it was then maintained constant during solvent production in order to avoid any inhibitory effect. Similarly, in this new experiment the pH remained constant during solventogenesis but at a higher value of 5.75. In the latter experiment (pH 5.75), as already

indicated above, the maximum concentrations of ethanol, butanol and hexanol were 2.7 g/L, 1.9 g/L and 0.85 g/L, respectively (Figure 5.7), which were higher values than in the experiment at pH 4.75, where the maximum concentrations of ethanol, butanol and hexanol were 2.25 g/L, 1.43 g/L, and 0.72 g/L, respectively (Figure 5.2). The higher concentrations of solvents at higher pH can thus be assumed to be related to the higher production of acids at constant high pH. When the production rates of each alcohol are analyzed, different patterns are observed in the two experiments, as summarized in Table 5-1. In all cases, the production rates of alcohols were higher in the first experiment than in the second one. This can be attributed to the fact that a lower pH simulates a more effective solventogenic phase and, consequently, the production rates of alcohols are higher (Lee et al. 2008; Millat et al. 2011). A similar pattern was observed in a previous study in which pure CO was fermented, rather than syngas, by the same microbial strain and at two different pH values (Fernández-Naveira et al. 2016b). In that experiment ethanol and butanol production rates were higher at pH 4.75 than at pH 5.75.

Table 5-1 Comparison of the different production rates of alcohols in the experiment at high pH (5.75) and the experiment with natural medium acidification (4.75). The rates are expressed in g/h*g-biomass.

	Ethanol production rate	Butanol production rate	Hexanol production rate
Experiment 1 (pH 4.75)	0.048	0.037	0.026
Experiment 2 (pH 5.75)	0.044	0.035	0.014

In the present experiment, at constant high pH, the following total net productions of alcohols were found: ethanol, 3.24 g; butanol, 2.28 g; and hexanol, 1.02 g; in a 1.2 L bioreactor. The production rates, at pH 5.75, were calculated for each alcohol. The ethanol production rate reached 0.044 g/h*g of biomass between 116 h and 284 h; the butanol production rate reached 0.035 g/h*g of biomass over that same period; and the hexanol production rate was 0.014 g/h*g of biomass between 163 h and 330 h. Besides, the total net production of butanol and ethanol can also be compared between the CO fermentation study reported previously (Fernández-Naveira et al. 2016b) and this syngas fermentation at pH 4.75, corresponding to the first experiment described in the previous section. In the CO fermentation process, the total net production of ethanol and butanol was 4.21 g and 2.29 g, respectively (Fernández-Naveira et al. 2016b), whereas 3.88 g ethanol and 2.18 g butanol were obtained at the end of this syngas

fermentation process. Thus both values are quite similar. It is worth mentioning that in our previous recent study on CO fermentation (Fernández-Naveira et al. 2016b), the presence of hexanol was not reported, as it had not been measured at that time, although subsequent analysis revealed that hexanol was actually also produced, in similar amounts to that in syngas fermentation. The redox potential was also measured throughout the study. When the pH was maintained at 5.75 the redox potential remained stable at −85±3mV.

Consumption of gaseous C1-substrates

Concerning CO consumption in this second experiment, at constant pH, the results can be seen in Figure 5.8. When the pH was maintained at 5.75, such higher pH seemed to favour higher levels of CO assimilation. The highest percentage CO consumption was observed during the first stages of the experiment reaching a value of 76 % after 91 h, which is higher than the maximum value found in the previous experiment with acidification of the culture medium. During the rest of the process, mainly during the solventogenic stage, CO consumption gradually decayed, reaching a minimum value of 38 % at the end of the experiment. On average, CO consumption levels were also somewhat higher in this experiment at constant high pH (5.75) compared with the previous experiment with culture medium acidification down to pH 4.75.

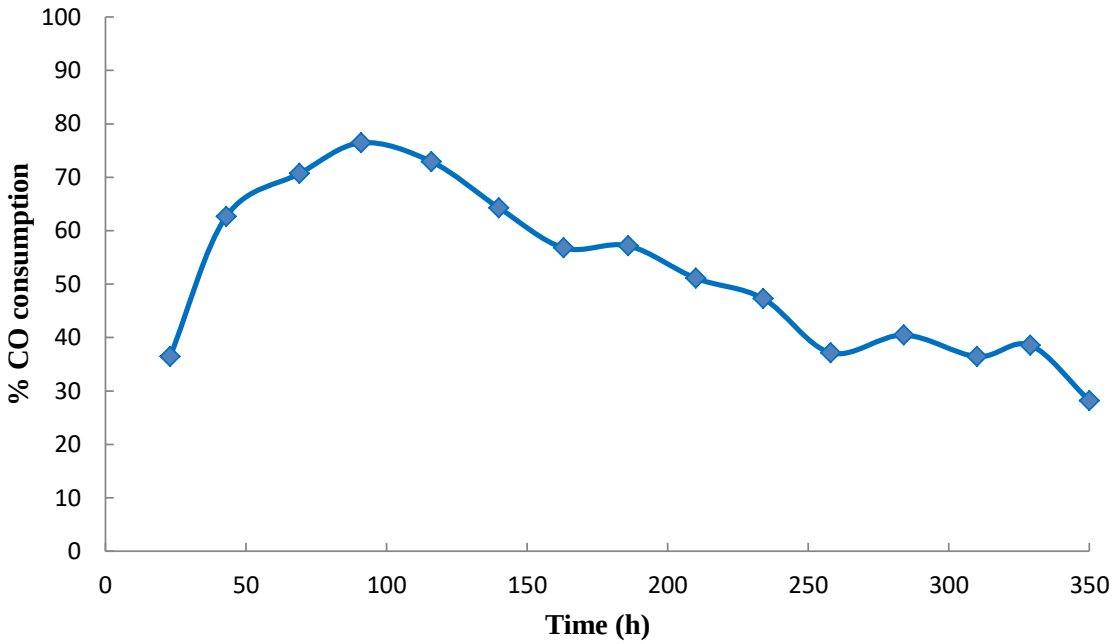

Figure 5.8 Continuous gas-fed bioreactor experiment at constant pH: percentage of CO consumption over time.

A similar behaviour is observed in this experiment to that in the previous one, leading to similar explanations and confirming the conclusions hypothesized in the first fermentation run. High carbon monoxide consumption levels are observed initially, after start-up. This leads to fast biomass growth as well as exponential production of organic acids, mainly acetic acid. As explained earlier and as shown in Eq. 5.1 and Eq. 5.2, the accumulation of acids leads to the simultaneous net production of carbon dioxide, reaching its highest peak during the early stages of the fermentation process (Figure 5.9).

This is also related to the Wood–Ljungdahl (WL) pathway in which CO can be converted into CO_2. Both C1 gases can later on also be metabolized through the WL pathway to generate acetyl-CoA which is an intermediate metabolite in the subsequent bioconversion to organic acids and/or alcohols (Figure 5.3). After 100 h, the concentration of carbon dioxide decays and, at the same time, organic acids are metabolized and converted to alcohols (Eq. 5.6 to Eq. 5.8), with carbon dioxide consumption. Once the fermentation of organic acids stops, net CO_2 consumption is observed (Figure 5.9).

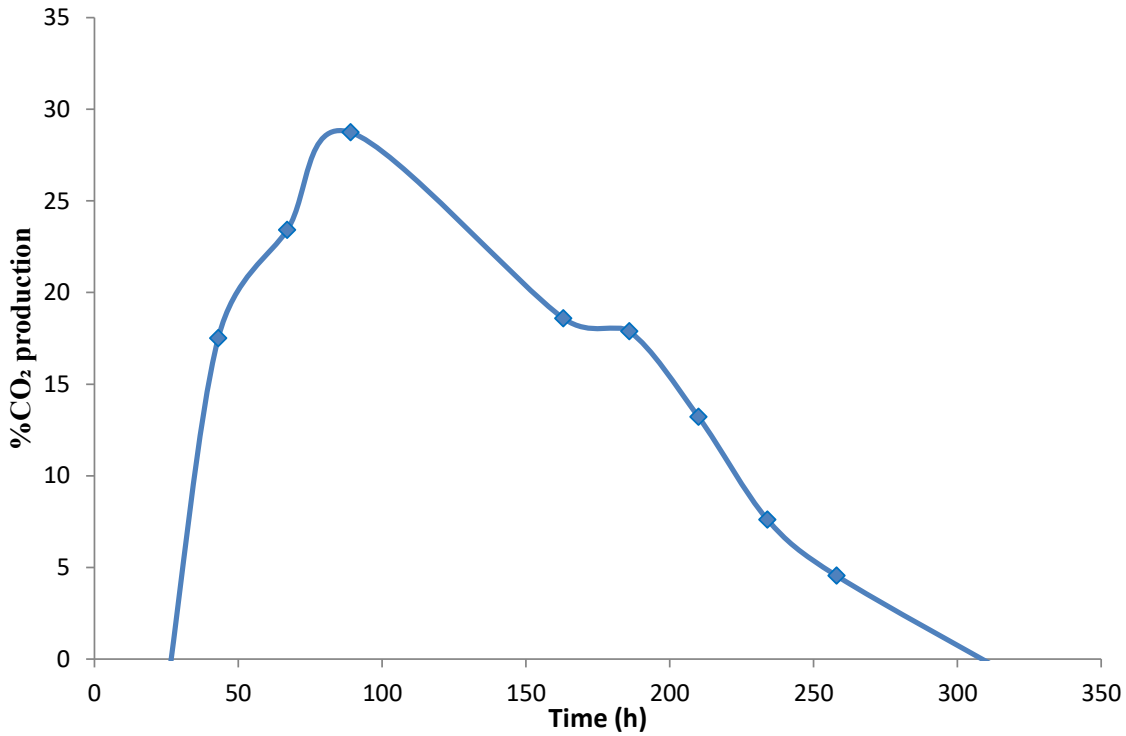

Figure 5.9 Continuous gas-fed bioreactor experiment at constant pH: percentage of CO_2 production over time.

5.4 Conclusions

It can be concluded that: (a) a high pH (5.75) valuewas more favourable to syngas/waste gas bioconversion to organic acids than a low pH (4.75), thus reaching a higher transient accumulation of such acids; (b) faster production rates of alcohols were observed at lower pH (favourable to solventogenesis), while a higher total amount of alcohols accumulated at higher pH as a result of the presence of higher transient concentrations of organic acids; (c) organic acids and alcohols are produced in the following order in all cases: C2 > C4 > C6; (d) although it stimulates solventogenesis, a lower pH has a negative effect on bacterial growth, reaching near inhibition below pH 4.75; and (e) H–B–E fermentation allows net removal of volatile pollutants having a greenhouse effect, such as CO_2 or CO, while producing ethanol and higher alcohols.

5.5 Acknowledgements

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6 GLUCOSE BIOCONVERSION PROFILE IN THE SYNGAS-METABOLIZING SPECIES *CLOSTRIDIUM CARBOXIDIVORANS*

GLUCOSE BIOCONVERSION PROFILE IN THE SYNGAS-METABOLIZING SPECIES CLOSTRIDIUM CARBOXIDIVORANS

Abstract Some clostridia produce alcohols (ethanol, butanol, hexanol) from gases (CO, CO_2, H_2) and others from carbohydrates (e.g., glucose). *C. carboxidivorans* can metabolize both gases as well as glucose. However, its bioconversion profile on glucose had not been reported. It was observed that *C. carboxidivorans* does not follow a typical solventogenic stage when grown on glucose. Indeed, at pH 6.20, it produced first a broad range of acids (acetic, butyric, hexanoic, formic, and lactic acids), several of which are generally not found, under similar conditions, during gas fermentation. Medium acidification did not allow the conversion of fatty acids into solvents. Production of some alcohols from glucose was observed in *C. carboxidivorans* but at high pH rather than under acidic conditions, and the total concentration of those solvents was low. At high pH, formic acid was produced first and later converted to acetic acid, but organic acids were not metabolized at low pH.

Keywords Acidogenesis, Butanol, Ethanol, Hexanol, *Clostridium carboxidivorans*

With minor editorial changes to fulfil formatting requirements, this chapter is substantially as it appears in: Bioresource Technology. **Published online:.2 August 2017.** DOI: 10.1016/j.biortech.2017.07.174.

6.1 Introduction

Many fuels and platform chemicals are, nowadays, still largely being obtained from crude oil as starting material in conventional refineries. Because of the instability of oil prices, the environmental impact and the increasing scarcity of crude oil, scientists and industries are being forced to look for new alternative feedstocks to produce metabolites of interest in a more sustainable way in biorefineries (Abdehagh et al. 2014; Gowen and Fong 2011; Liu et al. 2016). Some examples of such metabolites are acetone, methanol, ethanol, butanol and hexanol, to cite just few. The latter compounds can be produced from renewable materials such as lignocellulosic biomass, but also from municipal or agricultural wastes. They can be used to obtain either simple fermentable sugars or, otherwise, syngas, which can be fermented by clostridia into biofuels and other bioproducts.

Lignocellulosic materials are composed of cellulose (35–50 %), hemicellulose (20–35 %), and lignin (15–20 %) (Anwar et al. 2014). Cellulose is mainly a glucose polymer, while hemicellulose is largely composed of hexoses and pentoses, e.g. glucose, xylose, arabinose, mannose, galactose (Karimi et al. 2006; Saha 2003). Lignin does unfortunately not yield any sugars. Pretreatments are necessary in order to extract carbohydrates from the cellulose and hemicellulose polymers; those carbohydrates can then be fermented by different microorganisms (Hendriks and Zeeman 2009). The most studied organism is *Clostridium acetobutylicum*. There are two major different types of pretreatments of lignocellulosic materials, enzymatic hydrolysis and chemical hydrolysis. The first one is largely used due to the low production of by products which could inhibit the fermentation process compared to chemical hydrolysis (Jönsson et al. 2013; Qureshi and Manderson 1995; Rabinovich et al. 2002). Several studies have focussed on ABE fermentation, which is the bioconversion of carbohydrates by clostridia in order to obtain a mixture of acetone, butanol and ethanol (Jones and Woods 1986). All three metabolites are not necessarily found, depending on the strain.

On the other side, the above mentioned feedstocks can be gasified. This will yield a volatile product called synthetic gas or syngas (Kennes et al. 2016). In case of lignocellulosic materials, all the polymeric structure, i.e. cellulose, hemicellulose, but also lignin, can be gasified and converted to syngas, resulting in a better, full, use of the starting material. This gas mixture can then also be fermented by clostridia. In case of species such as *C. autoethanogenum* or *C. ljungdahlii*, the main products which are formed are acetic acid first and then ethanol (Abubackar et al. 2011; van Groenestijn et al. 2013). In other species, such as *Clostridium carboxidivorans*, a mixture of organic acids appears initially (acetic, butyric, hexanoic acids),

followed by the accumulation of the corresponding C2, C4, and C6 alcohols (ethanol, butanol, hexanol), in the novel HBE fermentation process (Fernández-Naveira et al. 2017a,b). It is a two-steps process characterized by exponential bacterial growth first and the production of different organic acids as major products. When the conditions become favourable, then the second step takes place, in which the acids are converted into ethanol and higher alcohols by the same bacteria (Fernández-Naveira et al., 2017b; Phillips et al., 2015). Alternatively, the accumulation of fatty acids rather than solvents could be stimulated, with further bioconversion of such acids into other products by different microorganisms in a two-stage process (Lagoa-Costa et al. 2017).

Since *C. carboxidivorans* is able to ferment gases and produce acids and then alcohols (HBE fermentation); in the present study, the main goal was to check if that species would also be able to use sugars, such as glucose, as carbon source while following a similar bioconversion profile as in the ABE fermentation, or otherwise what would be its metabolic profile with sugars compared to the HBE and the ABE fermentation processes in clostridia.

The experiments were carried out in three bioreactors in order to elucidate the metabolic profile of glucose fermentation by *C. carboxidivorans*. Besides, another major objective of this research was to study the effect of different pH and operating conditions in order to try to improve the production of alcohols or solvents. Three bioreactors were run with different operational conditions, the first one with constant pH, the second one without pH regulation and the last one with a change of pH at the end of the acidogenic stage; the idea being to find how the operational conditions affect sugar fermentation in *C. carboxidivorans* and to check to what extent the production of solvents from carbohydrates is possible and if it can be optimized.

6.2 Material and methods

6.2.1 Microorganism and culture media

C. carboxidivorans DSM 15243 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was maintained anaerobically on modified basal medium (Liou et al., 2005; Tanner, 2007) at pH 5.75 with glucose as the sole carbon source. The basal medium was composed of (per liter distilled water): 30 g glucose; 1 g yeast extract; 25 mL mineral solution; 10 mL trace metal solution; 10 mL of vitamins stock solution; 1 mL resazurin; 0.60 g cysteine-HCl.

The composition of the mineral stock solution was (per liter) 80 g sodium chloride, 100 g ammonium chloride, 10 g potassium chloride, 10 g potassium monophosphate, 20 g magnesium sulphate, and 4 g calcium chloride.

The composition of vitamin stock solution was (per liter) 10 mg pyridoxine, 5 mg each of thiamine, riboflavin, calcium pantothenate, thioctic acid, paraamino benzoic acid, nicotinic acid, and vitamin B12, and 2 mg each of d-biotin, folic acid, and 2-mercaptoethanesulphonic acid.

The composition of trace metal stock solution was (per liter) 2 g nitrilotriacetic acid, 1 g manganese sulphate, 0.80 g ferrous ammonium sulphate, 0.20 g cobalt chloride, 0.20 g zinc sulphate, and 20 mg each of cupric chloride, nickel chloride, sodium molybdate, sodium selenate, and sodium tungstate.

6.2.2 Continuous bioreactor experiments

Three bioreactor experiments were carried out in 2 L BIOFLO 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA). The final working volume in the three experiments was 1.2 L; using in all cases the same medium composition and glucose concentrations.

The medium with all the compounds, except glucose, vitamins and cysteine-HCl, was introduced in the bioreactor and autoclaved at 120° C for 20 min. Once autoclaving finalized, the bioreactor was kept under an extraction hood where it was flushed with N_2 in order to ensure anaerobic conditions. The vitamins solution, glucose stock solution and cysteine-HCl were added when the bioreactor reached a temperature below 40° C. Once the bioreactor medium was completely anaerobic, N_2 flushing was stopped. Then, 10 % seed culture, in the early exponential growth phase (through previously growing the strain for 72 h with glucose) was inoculated in the bioreactor. The experimental conditions of the three bioreactors were the same, with the temperature of the medium maintained at 33° C and a constant agitation speed of 250 rpm. Inside the bioreactor, four baffles avoided vortex formation and allowed thus to improve liquid mixing. The pH was automatically maintained constant through the addition of either 2 M NaOH or 2 M HCl solutions, by means of a peristaltic pump.

In the first experiment, the pH was maintained constant at 6.20 throughout the study. In the second experiment, the pH was maintained constant at 6.20 during the first 52 h, but afterwards automatic pH regulation was stopped. That way natural acidification took place, as a result of the production of acids, reaching a pH value of 5.20, which was then maintained constant. In the third experiment, the pH was maintained constant at 6.20 during the first hours, and when the

maximum concentration of acids was reached, the pH regulation was changed to 5.20 adding HCl 2 M in a gradual way.

6.2.3 Growth measurement

The optical density (OD_{λ}=600nm) was measured daily on a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain) removing 1 mL liquid samples from the bioreactors. The biomass concentration (g/L) was estimated using a previously generated calibration curve.

6.2.4 Fermentation products

At least every 24 h, 1 mL liquid sample was taken from each bioreactor and then centrifuged (7000g, 3 min) using a bench-centrifuge (ELMI Skyline ltd CM 70M07). The samples were then filtered through a filter with a pore size of 0.22 µm. They were then analyzed on an HPLC (HP1100, Agilent Co., USA) in order to determinate the concentrations of acids and alcohols present in liquid phase in the bioreactors. The HPLC was equipped with a 5 µm × 4 mm× 250 mm Hypersil ODS column and a UV detector at a wavelength of 284 nm. The mobile phase was a 0.1 % ortho-phosphoric acid solution fed at a flow rate of 0.5 mL/min

6.2.5 Redox potential

The redox potential was constantly monitored in each bioreactor using an Ag/AgCl reference electrode connected to a transmitter (M300, Mettler Toledo, Inc., Bedford, MA, USA) and maintained inside the bioreactor.

6.2.6 16S rDNA analysis of bioreactor cells

Samples from the different bioreactors were taken during the experiments as well as at the end of each assay and were analyzed using 16S rDNA gene sequencing in order to confirm the stability and purity of the inoculated strain in each experiment. The DNA extraction procedure was performed as per the manufacturer's protocol using E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek). After the DNA extraction, the quality of the DNA samples was analyzed using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE). A sequence of 1500 bp was amplified to obtain the 16S rDNA using a PCR. To obtain the complete sequence, a genetic analyzer 313XL Apply Biosystem was used using the Sanger method, in order to obtain different fragments of the complete sequence. Later, these sequence fragments were used in the program CLUSTAL W, in order to determinate the query sequence of the 16S rDNA of each sample. The query sequence

allows to identify the microbial strain with help of the BLAST (Basic Local Alignment Search Tool) program, using the database 16S ribosomal RNA sequences (Bacteria and Archaea).

6.3 Results and discussion

6.3.1 Glucose bioreactor at constant pH

A common characteristic of some Clostridium spp. is their ability to produce both organic acids and alcohols or other solvents (e.g. acetone), either through the ABE fermentation with carbohydrates as carbon sources or through the HBE fermentation with gaseous substrates. Those are two-step bioconversion processes in which organic acids are produced first (acidogenesis), at near-neutral or slightly acidic pH, together with biomass growth; followed by a second step consisting in the production of solvents (mainly alcohols, acetone) (solventogenesis) after acidification of the medium as a result of the production and subsequent bioconversion of acids into those solvents. Organisms such as *Clostridium acetobutylicum* convert sugars into volatile fatty acids and produce later acetone, butanol and ethanol (ABE fermentation). In a similar way, *C. carboxidivorans* is a bacterial species able to perform the HBE fermentation using substrates such as CO (Fernández-Naveira et al. 2016) or syngas (CO, CO_2, H_2) (Fernández-Naveira et al. 2017c; Phillips et al. 2015) following the above described two-step bioconversion process. Previous recent studies (Fernández-Naveira et al. 2017c) have shown that both acidogenesis and solventogenesis can take place at slightly acidic pH (around pH 6.00), before any stronger acidification. Experiments were performed here under different pH conditions, nin order to check the bioconversion characteristics of *C. carboxidivorans* and its potential to produce acids and alcohols from glucose, in a similar way as in the ABE fermentation in other clostridia. A first experiment was performed at a constant, slightly acidic, pH of 6.20 and the results were compared to the ABE and HBE fermentation profiles.

In this first experiment at constant pH, after bioreactor inoculation, *C. carboxidivorans* started growing and entered the exponential growth phase quite soon (Figure 6.1). A maximum biomass concentration of 0.50 g/L was reached 71 h after inoculation, with its bacterial growth rate reaching 0.080 h^{-1} in the log phase. This value is very similar to the ones found previously when growing that strain either on CO (Fernández-Naveira et al. 2016) or on syngas (Fernández-Naveira et al. 2017c) at a similar pH (pH 5.75).

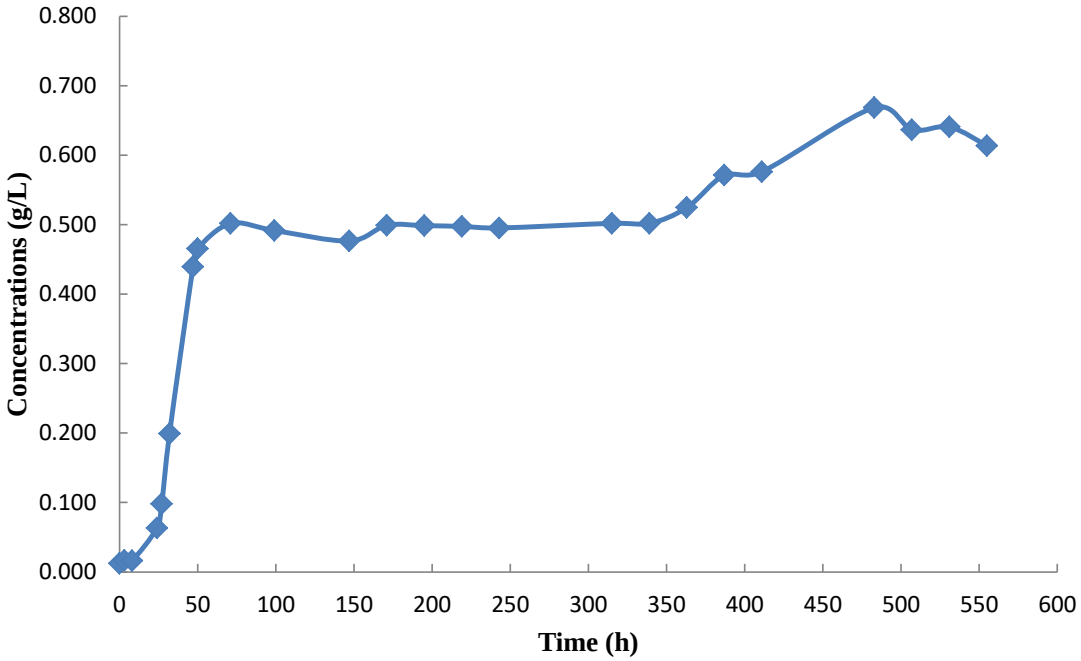

Figure 6.1 Glucose bioreactor at constant pH: measured growth expressed in g/L over time

Glucose bioconversion profile

The exponential consumption of glucose started about 24 h after inoculation and ended approximately 80 h after starting the experiment, concomitant with the log-growth; followed by a lower residual substrate consumption up to about 100 h. At the end of the assay the total net glucose consumption was 26.73 g/L, which corresponds to an overall substrate assimilation of 81 %. However, most of it was consumed during the exponential growth phase, corresponding to 78 % of that overall consumption (Figure 6.2).

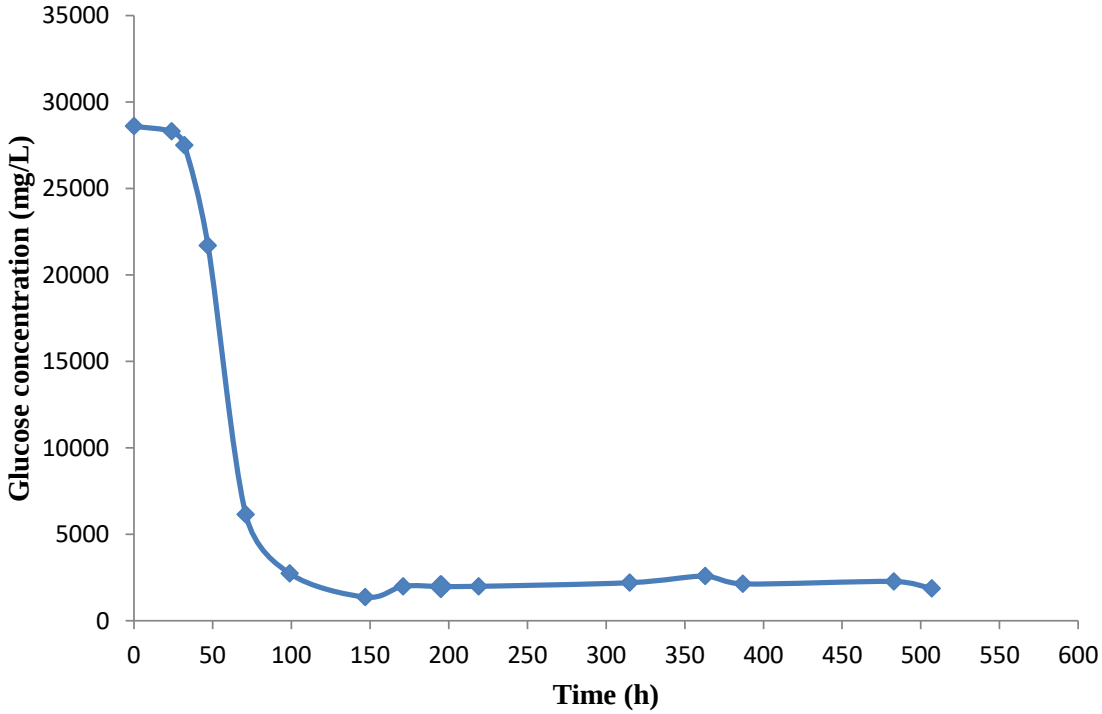

Figure 6.2 Glucose bioreactor at constant pH: glucose consumption over time with concentrations expressed in g/L.

Contrary to the HBE fermentation profile of *C. carboxidivorans* in which a limited number of organic acids (acetic, butyric and hexanoic acids, mainly) and alcohols (ethanol, butanol, hexanol) are produced as sole metabolites, in the present case with glucose as carbon source, a wider range of acids was found in the culture medium, at non-negligible concentrations. Similarly, in the ABE fermentation, with species such as *C. acetobutylicum*, acetic acid and butyric acid are the only major acids produced (Millat and Winzer 2017). In glucose bioconversion by *C. carboxidivorans*, the production of acids started simultaneously to sugar consumption. Acetic acid and formic acid were the first metabolites appearing in the fermentation broth, which is different from the bioconversion of CO or syngas by that organism, in which acetic acid is also the first metabolite to be detected but no formic acid is found. Butyric acid and lactic acid appeared a few hours later and their concentrations increased quite fast. Almost at the same time, isobutyric acid and propionic acid were found as well but in minor concentrations. Hexanoic acid started being produced still later, 71 h after bioreactor start-up (Figure 6.3). This is another difference with the HBE gas-fermentation in *C. carboxidivorans*, in which hexanoic acid is the third most abundant acid after acetic and butyric acids with basically no other acids detected. The presence of formic acid in the culture medium has never been reported in the gas fermentation process, but it is the second most abundant acid in this glucose

fermentation. Lactic acid and formic acid reached their maximum concentrations of 0.97 g/L and 2.92 g/L, respectively, after 99 h already. After 180 h, the maximum concentrations of isobutyric acid and propionic acid were found, with values of 0.38 g/L and 0.17 g/L, respectively. Interestingly, the accumulation of some acids, mainly acetic acid went on for a quite long period of time still after glucose consumption had stopped (after approximately 80–100 h, as explained earlier). The maximum concentration of acetic acid was reached after 363 h, with a value of 7.20 g/L, which is almost twice the concentration reached at the end of glucose consumption, suggesting that acetic acid could be produced from another compound than glucose. The concentration of formic acid started decreasing once glucose consumption leveled off, while the acetic acid concentration kept increasing. Overall, 2.60 g/L of the produced formic acid was consumed, leaving only 0.34 g/L in the medium by the end of the experiment. Therefore, a relationship between formic acid elimination and further acetic acid accumulation can reasonably be hypothesized, as will be discussed in next experiments. Maximum butyric acid and hexanoic acid concentrations of 1.85 g/L and 0.54 g/L, respectively, were reached after 483 h (Figure 6.3). That way the main acids present in the fermentation broth at the end of the experiment were: acetic acid > butyric acid > lactic acid > hexanoic acid > isobutyric acid > formic acid > propionic acid.

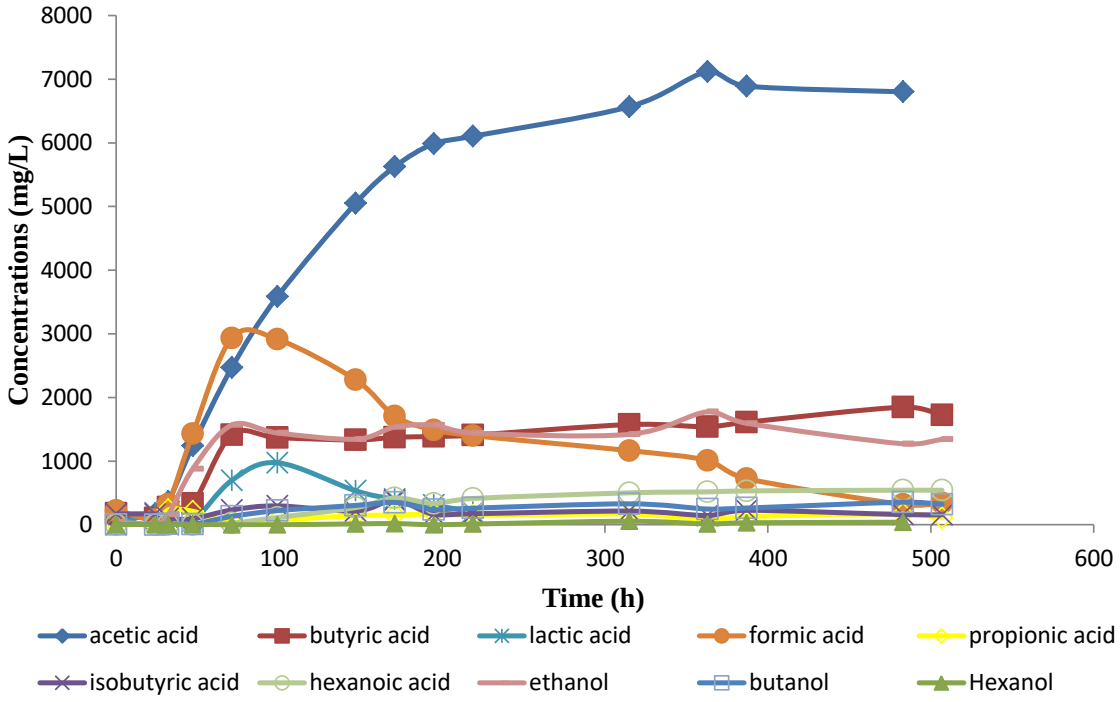

Figure 6.3 Glucose bioreactor at constant pH:product formation over time (acetic acid, butyric acid, isobutyric acid, propionic acid, lactic acid, formic acid, hexanoic acid, ethanol, butanol and hexanol).

In terms of solvents, despite working at a relatively high pH (6.20), ethanol started being produced soon, 24–32 h after start-up. Butanol was detected somewhat later but after 47 h already, when the concentration of acids was still increasing. A similar behavior was reported in the HBE fermentation with *C. carboxidivorans* and CO or syngas as substrates, at pH 5.75 (Fernández-Naveira et al. 2016, 2017c). With all three substrates (CO, syngas, glucose), ethanol appears soon during the acidification stage, and butanol is always detected around the 44–50 h after inoculation with the three different carbon sources. The production of hexanol started later, after 150 h. The maximum concentrations of alcohols were 1.78 g/L ethanol, 0.33 g/L butanol and 0.06 g/L hexanol (Figure 6.3), but contrary to the ABE fermentation with glucose or the HBE fermentation with gases, here most of the alcohols appeared during the consumption of the original substrate (glucose) (first 3–4 days) and no clear correlation could be found between alcohol production and any possible consumption of organic fatty acids. Although simultaneous production and consumption of acetic acid could have taken place, its net concentration did never decrease, suggesting that alcohols such as ethanol and butanol could have been produced directly from glucose rather than from the organic acids. No acetone was found with *C. carboxidivorans*, although this is a common metabolite in ABE fermentation with other clostridia. It was recently shown that organism lacks genes for acetone production (Bruant et al. 2010; Fernández-Naveira et al. 2017b).

The maximum ethanol production rate was much higher than for other alcohols, reaching 0.072 g/h*g of biomass, between 32 h and 71 h. The butanol production rate was 0.008 g/h*g of biomass, between 47 h and 147 h; and the hexanol production rate was only 0.001 g/h*g of biomass, between 219 and 315 h.

The RedOx potential was monitored. On starting-up the bioreactor and during the first hours, organic acids were largely produced and automatic addition of sodium hydroxide was required in order to maintain a constant pH of 6.20. The RedOx potential was then −145.0 ± 5 mV. Interestingly, when the bioreactor started to consume HCl, the RedOx decreased to values of −320.0 ± 5 mV.

6.3.2 Glucose bioreactor with natural acidification

High amounts of acids were produced in the previous experiment at a high, constant, pH of 6.20. In *C. carboxidivorans*, alcohols have been found to be produced (solventogenesis) both at high (e.g., 6 or above) as well as lower pH (e.g., 5 or below), during HBE fermentation (Fernández-Naveira et al. 2017c). However, in ABE fermentation with glucose as substrate, solventogenesis

has frequently been reported to be stimulated during medium acidification, after the production of acids (Schiel-Bengelsdorf et al. 2013; Fernández-Naveira et al. 2017b). Therefore, because of the absence of significant amounts of solvents in the first experiment at a constant pH of 6.20, compared to the HBE fermentation, a new assay was set-up with natural acidification, in order to check if this would change the metabolic profile of *C. carboxidivorans* and if it could stimulate solventogenesis.

The pH value was initially maintained constant at 6.20 for the first two days to ensure good bacterial growth. After 52 h, natural acidification was allowed to take place in order to check its possible stimulating effect on solventogenesis. Similarly as in the previous experiment and since the bioreactor start-up conditions were the same, *C. carboxidivorans* started growing without any lag phase, reaching a maximum biomass concentration of 0.55 g/L 46 h after inoculation (Figure 6.4). The bacterial growth rate was 0.087 h^{-1} measured during the exponential growth phase, up to 30 h. This value is similar as in the previous assay; with a slight difference which can be explained by the limited number of data points available for calculations. pH was allowed to drop naturally just after the maximum amount biomass was reached; that way the final maximum biomass concentration and the biomass growth rate were not affected by the possible inhibitory effect of the pH decrease.

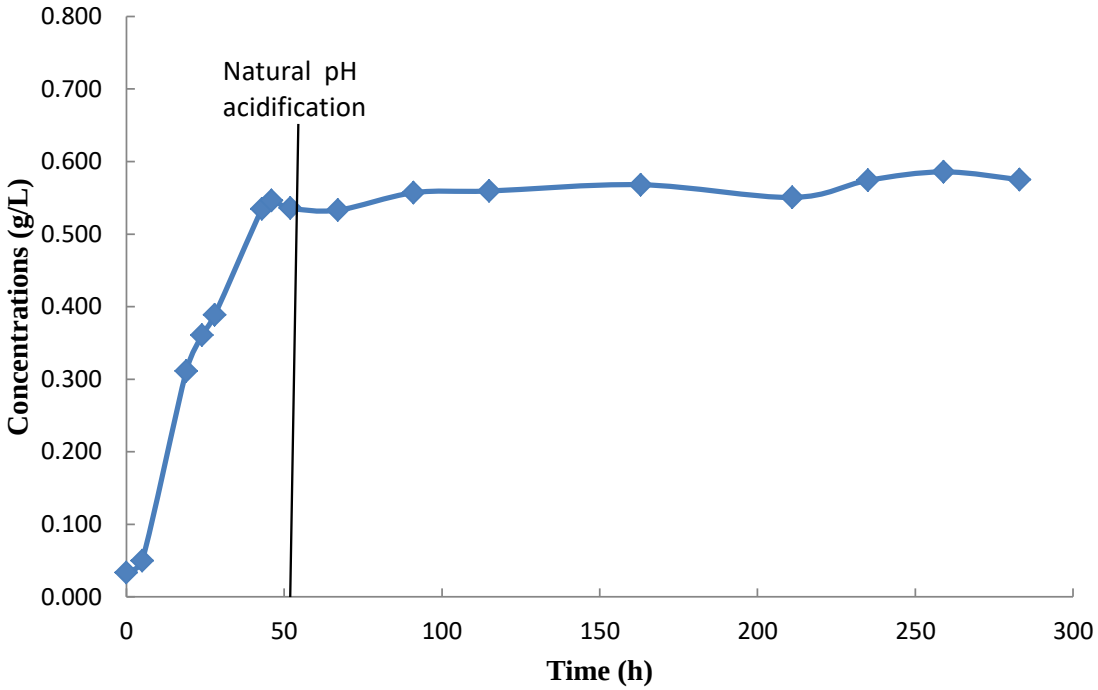

Figure 6.4 Glucose bioreactor with natural acidification: measured growth expressed in g/L over time.

Glucose bioconversion profile

Exponential consumption of glucose started soon after inoculation, but its consumption rate decreased dramatically once natural acidification took place, after 52 h. No more substrate was used at all 67 h after inoculation, at low pH. The total net glucose consumption reached 20.46 g/L by the end of the experiment which corresponds to a total sugar consumption of 82 %, but with the major part of it (72 % of the total consumption) being assimilated during the first 67 h (Figure 6.5). The near instantaneous inhibition of glucose assimilation once pH dropped to 5.20, suggests that it cannot be metabolized at such a low pH. The first acids detected in this experiment were acetic and formic acids, followed by butyric acid, lactic acid, isobutyric acid and propionic acid and finally hexanoic acid (Figure 6.6), similarly as in the previous experiment. This is because the acids appeared during the first 43 h, before natural acidification, when the conditions are the same as in the previous experiment. 52 h after inoculation the pH was allowed to drop down to 5.20 in a natural way as a result of the bacterial production of acids. Only 15 h were necessary to see the pH decrease to 5.20.

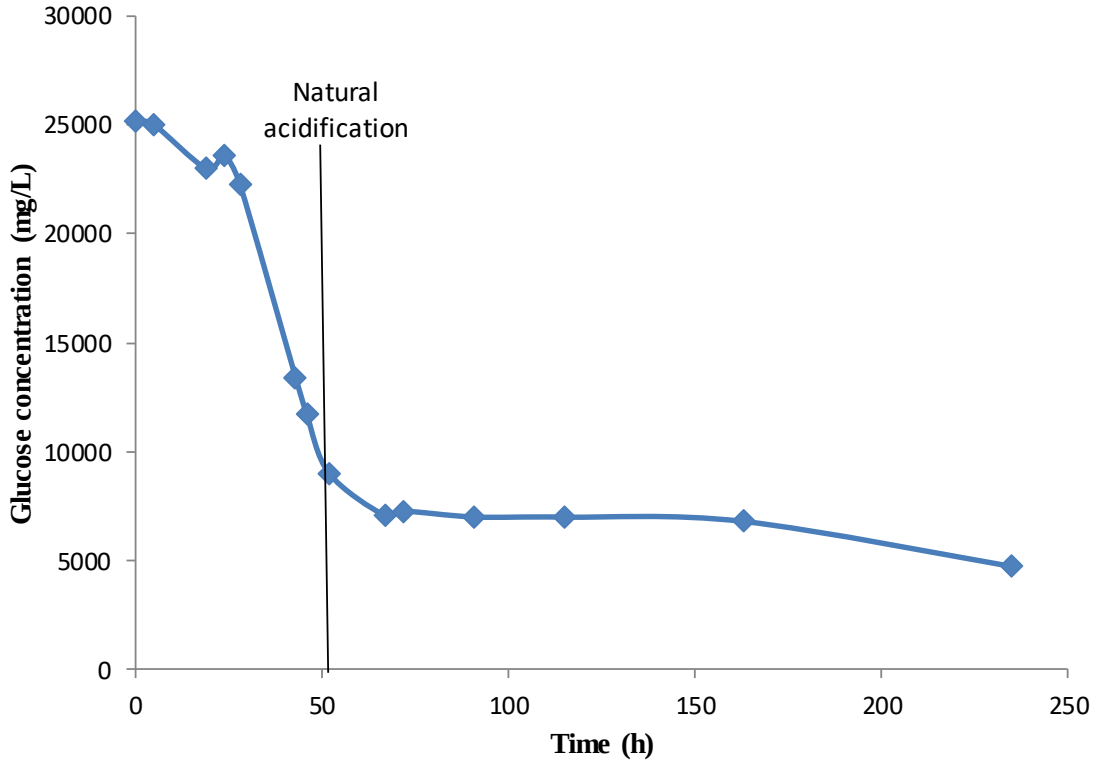

Figure 6.5 Glucose bioreactor with natural acidification: glucose consumption over time with concentrations expressed in g/L.

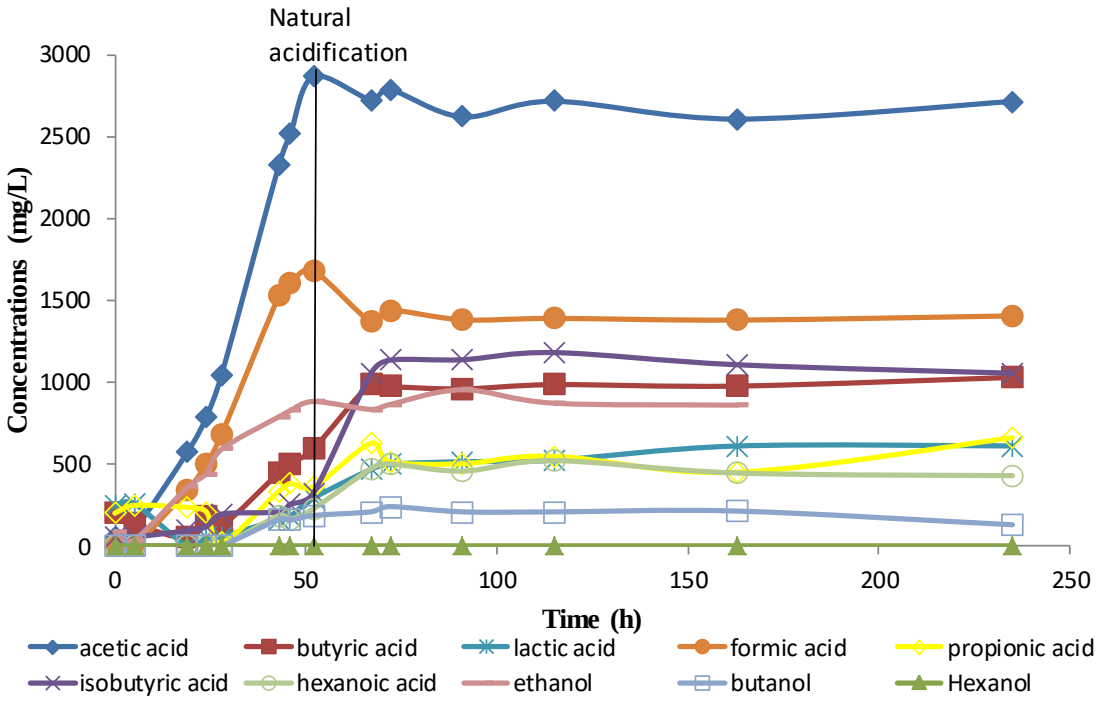

Figure 6.6 Glucose bioreactor with natural acidification: product formation over time (acetic acid, butyric acid, isobutyric acid, propionic acid, lactic acid, formic acid, hexanoic acid, ethanol, butanol and hexanol).

In this case, the maximum concentrations of acetic and formic acids were found after 52 h, reaching respectively 2.86 g/L and 1.68 g/L. After 67 h, the production of butyric acid, isobutyric acid and propionic acid stopped, with maximum concentrations of 1.00 g/L, 1.05 g/L and 0.63 g/L, respectively. Finally, hexanoic acid and lactic acid reached their maximum concentrations after 72 h and 163 h, respectively, with values of 0.52 g/L and 0.61 g/L (Figure 6.6). Suitable pH values for *C. carboxidivorans* have been reported to range from 4.4 to 7.6, with optimal conditions between 5.0 and 7.0 (Liou et al. 2005); however, in this experiment, the production of any metabolites did significantly slowdown after natural acidification down to pH 5.20. Although acidification could have been expected to stimulate solventogenesis, the opposite occurred. There was no consumption of acids at all, at pH 5.20, and no production of solvents. Even formic acid consumption was inhibited at such low pH, while it had almost completely been used up at high pH, in the previous assay. That way the main acids present in the fermentation broth at the end of this experiment were: acetic acid > formic acid > butyric acid =isobutyric acid > propionic acid > lactic acid > hexanoic acid. The proportions of acids are then also different from the previous assay. Since formic acid was not metabolized, its final concentration was higher than that of butyric acid at the end of the experiment. In the previous assay, the maximum concentration of formic acid, after 99 h, was also higher than for butyric

acid, but it was then used up at constant high pH. Therefore, at the end of the experiment there was only a low, residual, concentration of formic acid when maintaining a high pH of 6.20, while it remained present at its maximum concentration when acidification takes place.

Concerning the concentrations of solvents, ethanol was the first alcohol appearing in the fermentation broth. The presence of some ethanol was already detected soon after bioreactor inoculation and its concentration continued immediately increasing, meaning that this solvent appeared during the high pH period while it was not produced anymore after natural acidification. The maximum ethanol concentration was reached after 52 h with a value of 0.88 g/L. There was some delay before butanol was produced and it first appeared 43 h after inoculation. It was mainly produced at high pH (close to 90 % of its final concentration), although its maximum concentration was reached after 72 h, slightly after the pH decreased, with a value of 0.24 g/L. No hexanol was found in this experiment. Thus, the solvents, ethanol and butanol, appeared before any pH drop, when acids were still produced by the bacteria, following a similar pattern as in the previous experiment. This is different from the HBE fermentation with *C. carboxidivorans* (Fernández-Naveira et al. 2016, 2017a,b), as the strain grown on CO or syngas is still active at pH 5.20 and even below pH 5, and it produces solvents at somewhat increased rates at such low pH values. Solventogenesis is stimulated under acidic conditions in HBE fermentation with gaseous substrates while it is not, in the present study, when the strain is grown on glucose.

The alcohols production rates were calculated at pH 6.20. The ethanol and butanol production rates were 0.039 g-ethanol/h*g of biomass (between 5 h and 52 h) and 0.007 g-butanol/h*g of biomass (between 28 h and 52 h). The rates were not calculated at low pH as no significant production of alcohols was observed. In this case, the production rate of ethanol was lower than in the previous experiment. The reason was the inhibition caused by the pH drop on the production of alcohols. However, the production rate for butanol was similar in both experiments.

In assays using gases as substrates, CO or syngas, the highest production rates of alcohols were observed at low pH, during the solventogenic stage, whereas, in the present experiment, with glucose as a carbon source, the production of alcohols at low pH was not significant; that way the highest production rate was obtained at higher pH.

The RedOx potential was also monitored. When the pH was maintained at 6.20 (first 52 h), the RedOx potential was –150 ± 10 mV, as in the first experiment. However, when the pH dropped

to 5.20, the RedOx potential was higher, with a value of –90 ± 10 mV. The RedOx potential is known to be affected by the pH value and to become less negative at lower pH.

6.3.3 Glucose bioreactor with artificial pH change

It is known and accepted, from ABE and HBE fermentations, that maintaining a high pH, optimal for acidogenesis, for a longer period of time, will increase the production of acids during that first stage; which will, afterwards, stimulate solventogenesis and increase the amount of solvents produced during the second stage, as a result of the higher concentrations and availability of fermentable organic acids. In order to check such possible behavior in the case of glucose bioconversion by *C. carboxidivorans*, in this experiment, a high pH was maintained constant for several days in order to produce higher amounts of organic acids, before artificially decreasing the pH and check if this would more efficiently stimulate solventogenesis and result in the accumulation of higher amounts of alcohols. Therefore, the pH was maintained constant until the maximum concentrations of acids were reached (211 h). Thereafter, the pH was decreased to 5.20, through the addition of HCl 2 M. *C. carboxidivorans* started growing immediately after inoculation, without any lag phase (Figure 6.7), and a maximum biomass concentration of 0.432 g/L was reached within 40 h, with a bacterial growth rate of 0.081 h^{-1} during those first 40 h of exponential phase. The pH was modified artificially after the maximum biomass concentration had been reached; that way possible growth inhibition at low pH was avoided.

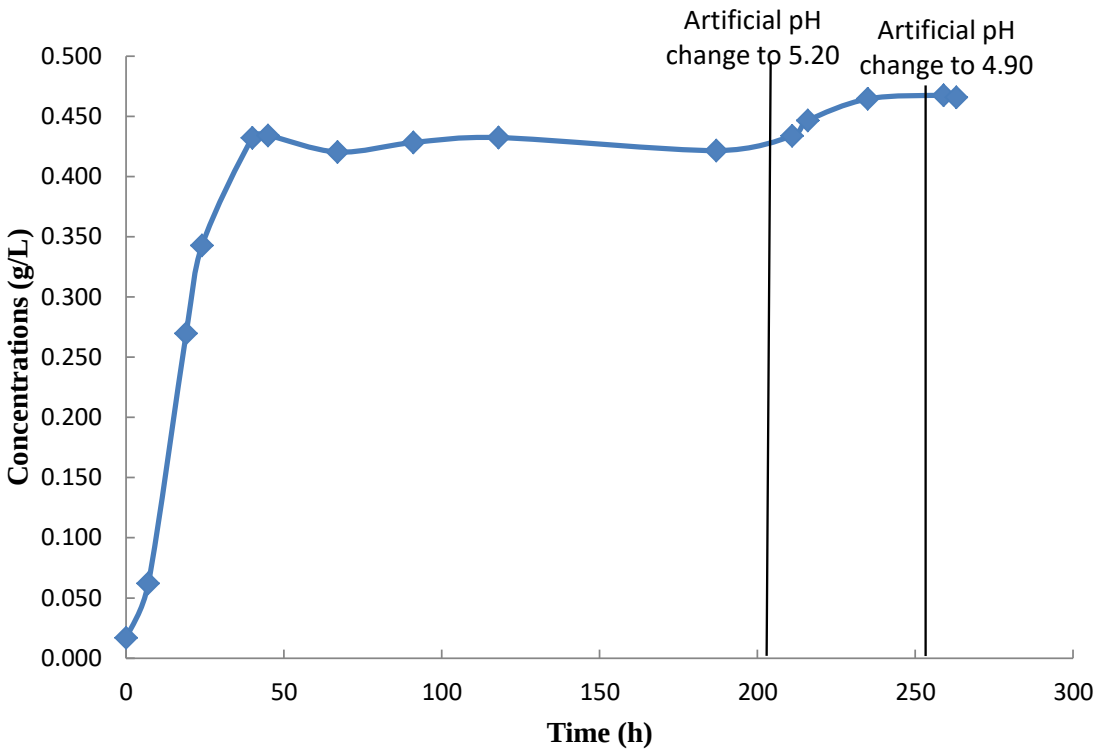

Figure 6.7 Glucose bioreactor with artificial pH change: measured growth expressed in g/L over time.

Glucose bioconversion profile

C. carboxidivorans started to consume glucose soon after inoculation and its consumption stabilized 120 h after bioreactor start-up. The total net glucose consumption was 26 g/L at the end of the experiment, which represents 95 % of the initial concentration. No more glucose was metabolized after the artificial pH change (211 h) (Figure 6.8).

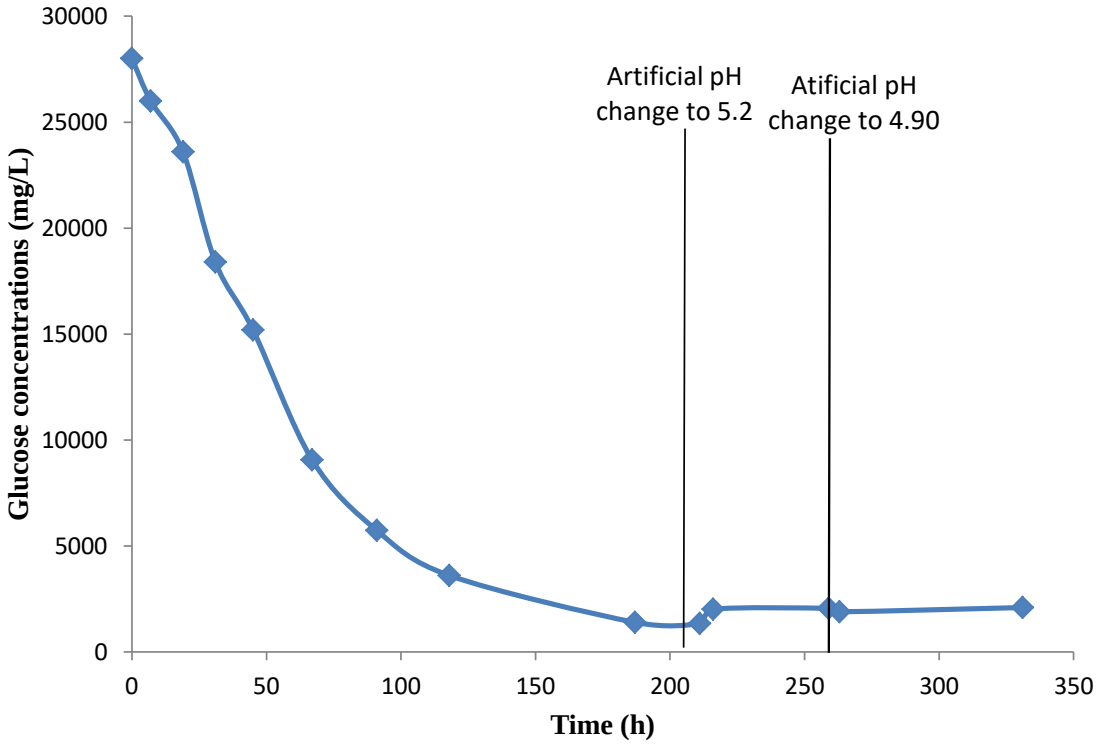

Figure 6.8 Glucose bioreactor with artificial pH change: glucose consumption over time with concentrations expressed in g/L.

As in all cases, acetic acid and formic acid were produced first, as soon as the bioreactor was inoculated. A few hours later, butyric acid, lactic acid, isobutyric acid and propionic acid appeared. Hexanoic acid was the last acid to be detected (Figure 6.9) similarly as in the previous two experiments. All those acids reached their maximum concentrations before the pH was decreased. The first acids reaching their maximum concentrations were lactic acid, formic acid and isobutyric acid, with values of 1.40 g/L, 1.62 g/L and 0.77 g/L, respectively, 118 h after inoculation. The next ones were propionic acid, acetic acid, butyric acid, and hexanoic acid, with maximum values of 0.42 g/L for propionic acid, 6.73 g/L for acetic acid, 2.20 g/L for butyric acid and 1.25 g/ L for hexanoic acid (Figure 6.9). Except for formic acid, for all other organic acids their concentrations kept gradually increasing until the pH change. The behavior was reproducible under similar conditions and comparable trends are observed in Figure 6.9 and Figure 6.3 when the pH was maintained constant at a high value of 6.20.

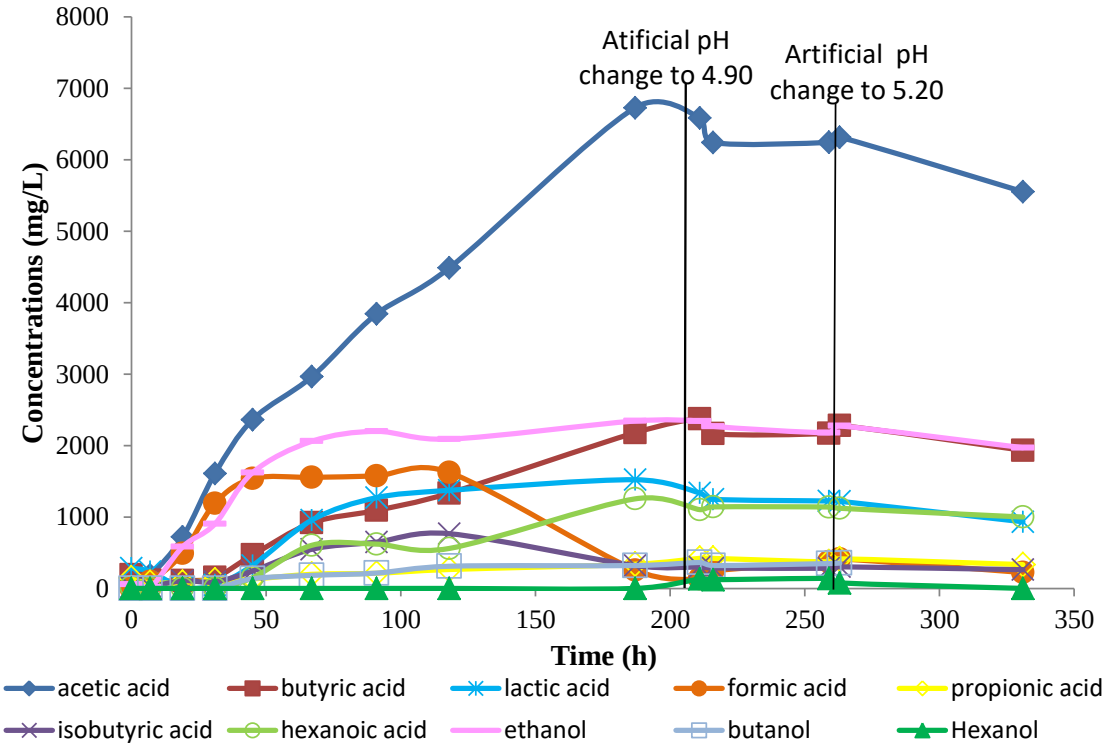

Figure 6.9 Glucose bioreactor with artificial pH change:product formation over time (acetic acid, butyric acid, isobutyric acid, propionic acid, lactic acid, formic acid, hexanoic acid, ethanol, butanol and hexanol).

Before the pH change, 1.40 g/L formic acid was consumed, but not anymore after the pH change towards the end of the experiment. There was some, but only very slight, consumption of the other acids after lowering the pH: 0.50 g/L isobutyric acid, 0.41 g/L lactic acid, 0.72 g/L acetic acid, 0.32 g/L butyric acid and 0.20 g/L hexanoic acid. The most abundant acid was acetic acid and the concentrations of all different acids followed the following pattern from the highest to the lowest concentrations: acetic acid > butyric acid > hexanoic acid > lactic acid > isobutyric acid > propionic acid > formic acid. This is the same pattern as observed in the experiment at constant pH (first experiment above).

Concerning solvents, those were produced, in this case again, at high pH during glucose assimilation, but not anymore after acidification, contrary to the typical successive acidogenic-solventogenic steps in ABE and HBE fermentations (Fernández-Naveira et al. 2017b). Ethanol was produced as soon as the experiment started, and butanol appeared for the first time 45 h after inoculation. This is similar as in the first two experiments, at high pH (6.20), with production of alcohols when the concentrations of acids were still increasing. Hexanol production started after 211 h, just a few hours before the pH was changed. Maximum concentrations of ethanol, butanol and hexanol were reached just before the pH change from

6.20 to 5.20 (211 h after inoculation) with values of 2.34 g/L, 0.36 g/L and 0.13 g/L, respectively (Figure 6.9). 50 h after the pH change to 5.20, a second artificial pH change was decided, down to 4.90, to see if a lower pH would better stimulate the production of alcohols. However, higher concentrations of alcohols were not reached with any of both pH changes. Thus, most of the production took place at high pH, contrary to the common solventogenic stages in ABE and HBE fermentation. This is then also different from the trend observed with gaseous substrates metabolized by *C. carboxidivorans*, where higher productions rates were observed at low pH (4.75) than at high pH (5.75) for all three alcohols. The alcohols production rates were also calculated, but only at pH 6.20, as no more alcohols were produced after the pH drop. The ethanol production rate was 0.085 g/h*g of biomass between 7 h and 67 h. The butanol production rate was 0.009 g/h*g of biomass between 31 h and 118 h; and the hexanol production rate was 0.005 g/h*g of biomass between 187 and 211 h.

Although the production of fatty acids is ubiquitous in clostridia, their further conversion to solvents is much less widespread. Still*, C. carboxidivorans* is known to possess the required enzymes to metabolize gaseous substrates (CO, syngas) and convert acids obtained from gas assimilation into alcohols. Under any of the three experimental conditions described above, the production of solvents was limited and occurred at high, rather than low, pH. Acid crash is a phenomenon that can explain the absence of a clear and efficient solventogenic stage and the lack of conversion of acids into alcohols in clostridia. However, the concentration of fatty acids accumulating in the present experiments are similar or lower than found under similar conditions (e.g. pH) in gas fermentation with the same strain. Thus, a possible inhibition of solventogenesis by fatty acids, such as acetic and butyric acids, is not expected. Other potential inhibitory condition is the accumulation of formic acid (Qi et al. 2016). That compound is observed to accumulate transiently in this glucose fermentation, but it was not found in gas fermentation processes (Fernández-Naveira et al. 2017b). Some studies have reported that formic acid might be more inhibitory than the above mentioned fatty acids and trigger acid crash in *C. acetobutylicum* (Wang et al. 2011). On the other side, some authors did also suggest that formic acid could have a stimulating effect on species such as *C. acetobutylicum* or *C. beijerinckii*, but only when present at low concentrations (Cho et al. 2012).

Formic acid is produced during the early stages of the fermentation process in *C. carboxidivorans*, concomitant to the consumption of glucose. In the experiment at constant pH 6.2, after glucose consumption, formic acid starts disappearing while the acetic acid concentration keeps increasing. Once glucose is roughly exhausted, it sounds reasonable to assume that the increase in concentration of the C2-fatty acid results from the conversion of

formic acid. This agrees with data reported in a recent study suggesting that *C. carboxidivorans* would convert formic acid into acetic acid, to different extents, depending on conditions such as the pH of the medium, such bioconversion would mainly occur at higher pH (e.g. pH 6.0, but not pH 5.0) (Ramió-Pujol et al. 2014). Although some authors reported that formic acid does not support growth (Liou et al. 2005), others hypothesized that some growth could simultaneously take place at high pH (e.g., 6.0), but not a low pH (Ramió-Pujol et al. 2014), which might explain formic acid consumption and a concomitant slight increase in biomass concentration observed here in the late stage of the experiment at constant pH 6.20, as shown in Figure 6.1 and Figure 6.3, while formic acid consumption and additional growth were not observed in the experiment with natural acidification down to pH 5.2 (Figure 6.4 and Figure 6.6). Such two-phase growth on mixtures of organic acids and gaseous substrates such as CO has also been reported in other anaerobic bacteria (Haddad et al. 2013). There is no evidence from any of the experiments that formic acid might significantly have inhibited solventogenesis. From Figure 6.9, it appears that, even several days after complete elimination of that acid, no additional relevant increase in concentrations of alcohols (ethanol, butanol, hexanol) was found. *C. carboxidivorans* should posses all the required enzymatic machinery to produce alcohols and does indeed produce some of them, mainly ethanol (up to 2 g/L, depending on culture conditions), but mainly at the beginning of the experiments during glucose consumption, usually at high pH (6.20), rather than through any possible conversion of acids into alcohols during a specific solventogenic stage at lower pH.

6.3.4 16S rDNA analysis

The experiments were carried out under sterile operating conditions. However, glucose is a suitable carbon source for many microorganisms. Therefore, the 16S rDNA sequence was analyzed at the end of each experiment, showing a query sequence with a similarity of 99 % to *C. carboxidivorans* in all three experiments. This analysis confirms the purity and stability of the inoculated culture

6.4 Conclusions

It is known that, when grown on $CO/CO_2/H_2$, *C. carboxidivorans* produces first a mixture of acetic, butyric and hexanoic acids, resulting in medium acidification and the concomitant conversion of such acids into hexanol, butanol, ethanol (HBE fermentation). It can also grow on glucose, but has not a typical ABE fermentation pattern, which would consist in the production

of acids followed by the production of solvents after acidification similarly as in HBE fermentation. Instead, organic acids and low amounts of alcohols are found at high pH, although this is not followed by solventogenesis after the pH drops down to lower values.

6.5 Acknowledgements

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7 CARBON MONOXIDE BIOCONVERSION TO BUTANOL-ETHANOL BY *CLOSTRIDIUM CARBOXIDIVORANS*: KINETICS AND TOXICITY OF ALCOHOLS

CARBON MONOXIDE BIOCONVERSION TO BUTANOL-ETHANOL BY CLOSTRIDIUM CARBOXIDIVORANS: KINETICS AND TOXICITY OF ALCOHOLS

Abstract Butanol production from carbon monoxide-rich waste gases or syngas is an attractive novel alternative to the conventional acetone-butanol-ethanol (ABE) fermentation. Solvent toxicity is a key factor reported in ABE fermentation with carbohydrates as substrates. However, in the gas-fermentation process, kinetic aspects and the inhibition effect of solvents have not thoroughly been studied.

Therefore, different batch bottle experiments were carried out with the bacterial species *Clostridium carboxidivorans* using CO as carbon source for butanol-ethanol fermentation.

A maximum specific growth rate of 0.086 ± 0.004 h^{-1} and a biomass yield of 0.011 g biomass/g CO were found, which is significantly lower than in other clostridia grown on sugars.

Besides, three assays were carried out to check the inhibitory effect of butanol, ethanol, and their mixtures. Butanol had a higher inhibitory effect on the cells than ethanol and showe d a lower IC_{50}, reduced growth rate, and slower CO consumption with increasing alcohol concentrations. A concentration of 14–14.50 g/L butanol caused 50 % growth inhibition in *C. carboxidivorans*, and 20 g/L butanol resulted in complete inhibition, with a growth rate of 0 h^{-1}. Conversely, 35 g/L ethanol decreased by 50 % the final biomass concentration respect to the control and yielded the lowest growth rate of 0.024 h^{-1}. The inhibitory effect of mixtures of both alcohols was also checked adding similar, near identical, concentrations of each one. Growth decreased by 50 % in the presence of a total concentration of alcohols of 16.22 g/L, consisting of similar amounts of each alcohol. Occasional differences in initially added concentrations of alcohols were minimal. The lowest growth rate (0.014 h^{-1}) was observed at the highest concentration assayed (25 g/L).

Keywords: *Clostridium carboxidivorans*. Butanol. Ethanol. Inhibitory effect. Batch experiment. IC_{50}.

With minor editorial changes to fulfill formatting requirements, this chapter is substanti ally as it appears in: Appl Microbiol Biotechnol. **Published online: 27 February 2016**. DOI: 10.1007/s00253-016-7389-8.

7.1 Introduction

Nowadays, the instability of the oil price as well as recent concerns about the increasing scarcity of fossil fuels and their negative environmental impact have led to a growing interest in biofuels such as ethanol and butanol (Gowen and Fong 2011; Abdehagh et al. 2014). Butanol has recently been recognized as a highly promising biofuel (Bellido et al. 2014) and has several advantages. It is less hygroscopic and less corrosive and has a higher caloric content than ethanol (Qureshi et al. 2001; Wallner et al. 2009). For those reasons, in recent years, butanol has been considered a chemical of great industrial importance with a high potential to replace gasoline (Dürre 2007; Lee et al. 2008).

Ethanol and butanol can be obtained through fermentation of different sugars available in food crops, which is the conventional and most common commercial technology nowadays, known as first generation process. However, this process leads to food-fuel competition. Recently, in order to avoid such drawback, a new alternative has been developed using lignocellulosic feedstocks from agricultural waste and energy crops, which are inexpensive and renewable starting materials for biofuels production and do not adversely affect food supplies (Gowen and Fong 2011). Extracting simple, carbohydrates from the polymeric lignocellulosic structure is a complex process (Balat and Balat 2009) requiring physical, chemical, or enzymatic pretreatments in order to hydrolyze the biomass into fermentable sugars in the so-called second generation process. Carbohydrates can be converted to butanol by clostridial strains, together with other side products, i.e., ethanol and acetone, through the acetone-butanol-ethanol (ABE) fermentation. Based on advances in biotechnology and process engineering, new fermentation processes are being developed, using renewable carbon sources, for a more efficient production of butanol (Dürre 2007; Lee et al. 2008; Papoutsakis 2008).

Besides butanol production from carbohydrates, a novel production route has been suggested, consisting of converting biomass or any other carbonaceous feedstocks into CO-rich gases, such as syngas. The gaseous substrate can then be fermented into ethanol and/or butanol by some bacterial species, mainly clostridia. Interestingly, this alternative route can also use CO-rich waste gases as substrates (Abubackar et al. 2011; Kennes and Veiga 2013). Under optimized conditions, a mixture of butanol and ethanol (B-E) is obtained as end-products (Fernández-Naveira et al. 2016). Only very few bacteria have been isolated so far and shown to produce butanol from carbon monoxide. *Clostridium carboxidivorans* is one such bacterium able to grow on synthesis gas, by using CO, CO_2, and H_2 to produce the liquid biofuels ethanol and butanol using a variation of the classical Wood-Ljungdahl pathway (Ukpong et al. 2012).

In the more extensively studied conventional ABE fermentation from carbohydrates, solvent toxicity is a critical problem. Under normal conditions, the clostridial cellular activity decreases significantly in the presence of 20 g/L or more solvents (Woods 1995). This is one of the most important factors to be considered in butanol fermentation as bacterial cells rarely tolerate concentrations exceeding 2 % butanol (Liu and Qureshi 2009). This should thus be taken into account in order to get a more efficient production process. Although studies have been performed on the inhibitory effect of butanol on cell growth of specific bacteria with sugars as a carbon source in the conventional ABE fermentation (Moreira et al. 1981), no data are yet available in the literature on the kinetics and the inhibition effect of solvents (butanol, ethanol) in the more recently developed clostridial butanol-ethanol (B-E) fermentation from CO-rich gases as carbon source.

In order to increase butanol production from gaseous substrates, it is necessary to know its inhibitory effect as well as the inhibitory effect of other end-product solvents (i.e. ethanol) on the B-E fermentation process. Therefore, this work focussed on evaluating the inhibitory effect of end-products of the B-E fermentation on the growth kinetics and bioconversion of CO to valuable metabolites, by *C. carboxidivorans* in batch bottle experiments.

7.2 Material and methods

7.2.1 Microorganism and culture media

C. carboxidivorans P7 DSM 15243 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was maintained anaerobically on modified basal medium (Liou et al. 2005; Tanner 2007) at pH 5.75 with CO (100 %) as the sole gaseous substrate.

The medium composition (per liter distilled water) was as follows: Yeast extract, 1 g; mineral solution 25 mL; trace metal solution, 10 mL; resazurin, 1 mL; cysteine-HCl, 0.60 g.

The mineral stock solution contained (per liter distilled water): 80 g sodium chloride, 100 g ammonium chloride, 10 g potassium chloride, 10 g potassium monophosphate, 20 g magnesium sulfate, and 4 g calcium chloride.

The trace metal stock solution contained (per liter distilled water) the following: 2 g nitrilotriacetic acid, 1 g manganese sulfate, 0.80 g ferrous ammonium sulfate, 0.20 g cobalt

chloride, 0.20 g zinc sulfate, and 20 mg each of cupric chloride, nickel chloride, sodium molybdate, sodium selenate, and sodium tungstate.

7.2.2 Bottle batch experiments

Batch experiments were carried out in order to check the inhibitory effect of ethanol, butanol, and mixtures of both ethanol and butanol.

All media used for the batch experiments were prepared with the same methods and under the same conditions. For the experiments, 10 % seed culture in the early exponential growth phase, grown with CO as sole carbon source, was aseptically inoculated into 200 mL serum vials containing 100 mL medium at pH = 5.0 ± 0.1. The bottles were maintained under anaerobic conditions. They were pressurized to 1.2 bar with 100 % CO and were agitated at 150 rpm inside an orbital incubator at 30° C. The experimental setup and the method used for media preparation as well as sampling details are described elsewhere (Abubackar et al. 2011; Abubackar et al. 2015; Fernández-Naveira et al. 2016). All the batch experiments were carried out in duplicate, reaching statistically highly reproducible results, and some were even repeated in order to confirm data whenever needed.

Three separate experiments were carried out in order to analyze the inhibitory effect of each compound separately. Butanol was checked at the following concentrations: control (0 g/L), 1, 5, 10, 15, and 20 g/L. The effect of ethanol, which appeared to be somewhat less inhibitory, was checked at the following concentrations: control (0 g/L), 1, 5, 20, 25, and 35 g/L. Similarly, the combined inhibitory effect of both alcohols together was analyzed, using the following total final concentrations: control (0 g/L), 2, 6, 15, and 25 g/L, using identical concentrations of each alcohol. There was only a slight difference in the initial concentrations at the highe st values assayed of 15 and 25 g/L, but always below 10 %.

7.2.3 Growth measurement

One milliliter liquid sample was withdrawn daily in order to avoid affecting too much the total liquid volume. Two daily samples were occasionally taken, mainly during the exponen tial growth phase, in order to have more data points allowing to calculate the exponential growth rate. The optical density (OD_λ = 600 nm) was measured for each sample, in order to estimate the biomass concentration, using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa and Giralt, Madrid, Spain). The measured absorbance allowed to estimate the biomass concentration (mg/L) by comparing it with a previously generated calibration curve.

Growth rates, (μ), expressed in h^{-1}, were calculated using the following formula:

$$\mu = \frac{[ln(N_t) - ln(N_0)]}{(t - t_0)} \quad \text{Eq. 7.1}$$

Where N_t is the cell density (g/L) at time t (expressed in hours) and N_0 is the cell density at time 0 (t_0).

Such maximum growth rate was estimated during the exponential growth phase based on the best slope fit to the experimental data and making sure reproducible results were obtained. Whenever needed, an additional experiment was performed to confirm reproducibility.

One of the most common parameters used in toxicity assays is the IC_{50} (Leboulanger et al. 2001), i.e., the concentration of the tested substance that decreases the growth by 50 %. IC_{50} values were calculated using non-linear regression analysis (four parameters sigmoidal) of transformed alcohol concentration as natural logarithm data versus percentage of growth inhibition. The regression analysis was performed using the regression Wizard software (Sigma-Plot 12.5, SPSS Inc.).

Cell yield coefficient (YX/S) is defined as the amount of cell mass produced per amount of substrate consumed (CO). It was estimated through the following equation:

$$Y_{x/s} = \frac{\mathrm{N} - \mathrm{N_0}}{\mathrm{S} - \mathrm{S_0}} \quad \text{Eq. 7.2}$$

Where N is the cell density at the end of the exponential growth phase (g/L), N_0 is the cell density at time 0, S is the final substrate concentration at the end of the exponential growth phase, and S_0 is the substrate concentration at time 0.

7.2.4 Gas-phase CO concentrations

Gas samples of 1 mL were taken from the headspace of the bottles to monitor the CO concentrations.

Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD).

The GC was fitted with a 15-m HP-PLOT Molecular Sieve 5A column (ID 0.53 mm, film thickness 50 μm). The oven temperature was initially kept constant at 50° C, for 5 min, and then raised by 20° C·min^{-1} for 2 min, to reach a final temperature of 90° C. The temperature of the injection port and the detector were maintained constant at 150° C. Helium was used as the carrier gas.

7.2.5 Ethanol and butanol concentrations

The concentrations of ethanol and butanol were analyzed for each bottle from liquid samples (1 mL) using an HPLC (HP1100, Agilent Co. , USA) equipped with a 5 µm × 4 mm × 250 mm Hypersil ODS column and a UV detector at a wavelength of 284 nm. The mobile phase was a 0.1 % ortho-phosphoric acid solution fed at a flow rate of 0.5 mL/min. The column temperature was set at 30° C. The samples were centrifuged (7000g, 3 min) using a centrifuge (ELMI Skyline Ltd. CM 70M07) before analyzing the concentration of water-soluble compounds by HPLC.

7.3 Results

7.3.1 Growth parameters of *C. carboxidivorans*

Growth parameters of *C. carboxidivorans* were estimated on pure carbon monoxide in experiments repeated in sextuplicate. The maximum specific growth rate (µ) was found to reach 0.086 ± 0.004 h^{-1}. This value is significantly lower than for clostridial strains grown on sugars in ABE fermentation (Table 7-1). Each experiment corresponds to the duplicate controls of the three alcohols inhibition experiments (i.e., a total of six assays) described in the next section and summarized in (Table 7-2, Table 7-3 and Table 7-4). Detailed experimental data can thus be found in the next sections. Besides, the biomass yield (YX/S) was also estimated based on the amount substrate consumed and generated biomass and appeared to reach 0.011 g biomass/g CO.

Table 7-1 Specific growth rates of *Clostridium carboxidivorans* and *Clostridium acetobutylicum* grown, respectively, on CO or carbohydrates (glucose, lactose)

Carbon source	µ (h^{-1})	microorganism	References
Carbon monoxide	0.086 ± 0.004	*C. carboxidivorans*	This study
Glucose	0.48	*C. acetobutylicum*	Srivastave and Volesky 1990
Lactose	0.23–0.28	*C. acetobutylicum*	Napoleti et al. 2012

7.3.2 Butanol toxicity experiment

In the experiments with added butanol, *C. carboxidivorans* started growing immediately after inoculation (Figure 7.1) at all the concentrations assayed, with the exception of the bottle with

the highest butanol concentration (20 g/L) in which no growth at all was observed. Butanol affected the growth of *C. carboxidivorans* and the negative effect on growth was concentration-dependent (Figure 7.1, Table 7-2). The control reached its maximum biomass concentration (0.135 g/L) after 30 h (Figure 7.1), and the maximum growth rate was reached in the control bottles, in the absence of any added butanol, with a value of 0.084 h^{-1} (Table 7-2). All butanol concentrations assayed provoked a decrease in the growth rate of that strain (Table 7-2). The highest concentration assayed (20 g/L) completely inhibited the bacterial growth, with a growth rate of 0 h^{-1}.

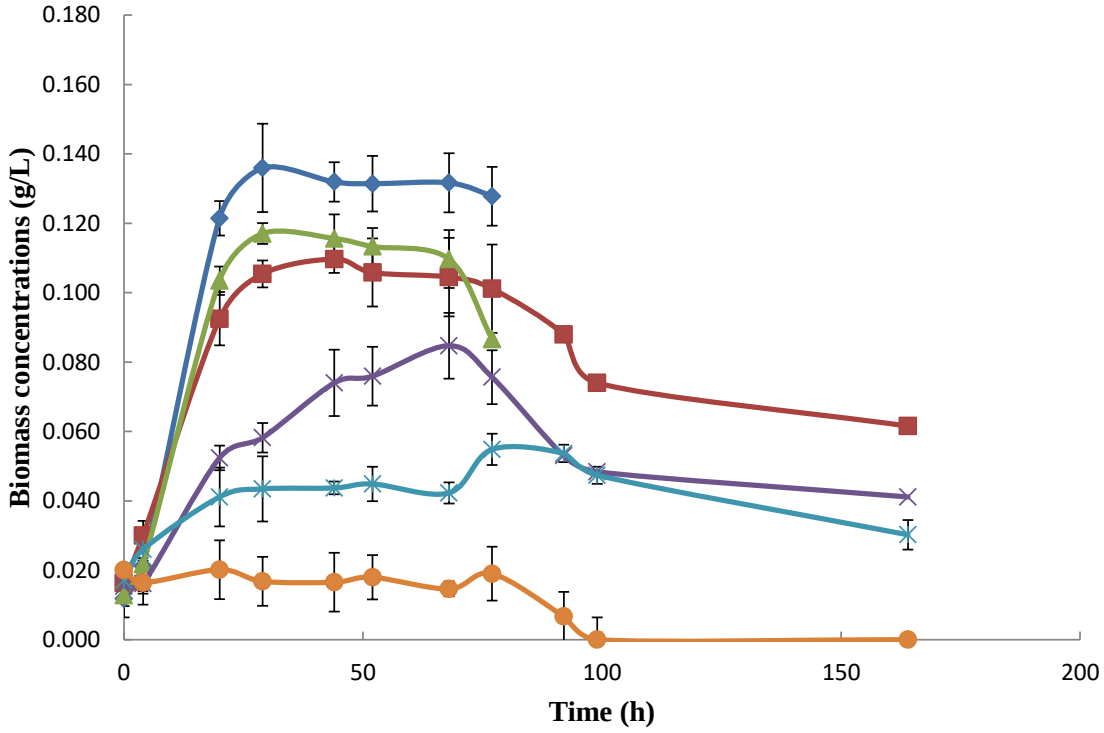

Figure 7.1 Batch experiments with butanol. Measured biomass accumulation over time, expressed in g/L. Data are given as mean values ± standard deviation of the means (control bottles represented as blue diamond, 1 g/L butanol represented as green triangles, 5 g/L butanol represented as red squared, 10 g/L butanol represented as purple X, 15 g/L butanol represented as blue X, and 20 g/L butanol represented as orange circle).

Table 7-2 Batch experiments with butanol. Maximum specific growth rates in the presence of different butanol concentrations, expressed in h^{-1}

Butanol concentration (g/L)	μ (h^{-1})
Control	0.084
1	0.076
5	0.043
10	0.026
15	0.019
20	0.000

The IC_{50} of butanol for growth was 14.50 g/L after 48 h of butanol exposure and 14.20 g/L after 72 h of exposure.

Carbon monoxide consumption was monitored during the experiment and is shown in Figure 7.2. In the control bottles and with 1 g/L of butanol, 50 % CO was already consumed after 24 h and it was totally consumed after 77 h. In the case of butanol concentrations of 5 and 10 g/L, 50 % CO consumption was reached after 28 h of butanol exposure, and total CO consumption was observed after 92 h exposure. Only in the bottles with the highest alcohol concentrations (15 and 20 g/L) was total substrate removal not possible, observing 20 % CO consumption, after 99 h of butanol exposure. This was related to the fact that in those bottles, biomass had scarcely grown and could not consume CO, whereas significant growth was found in the control bottles as well as in the presence of butanol at 1, 5, and 10 g/L, where growth took place at different rates, and reaching different final biomass concentrations. The highest final, total amount biomass was reached in the control bottle. The total maximum biomass concentration gradually decreased at increasing added butanol concentrations

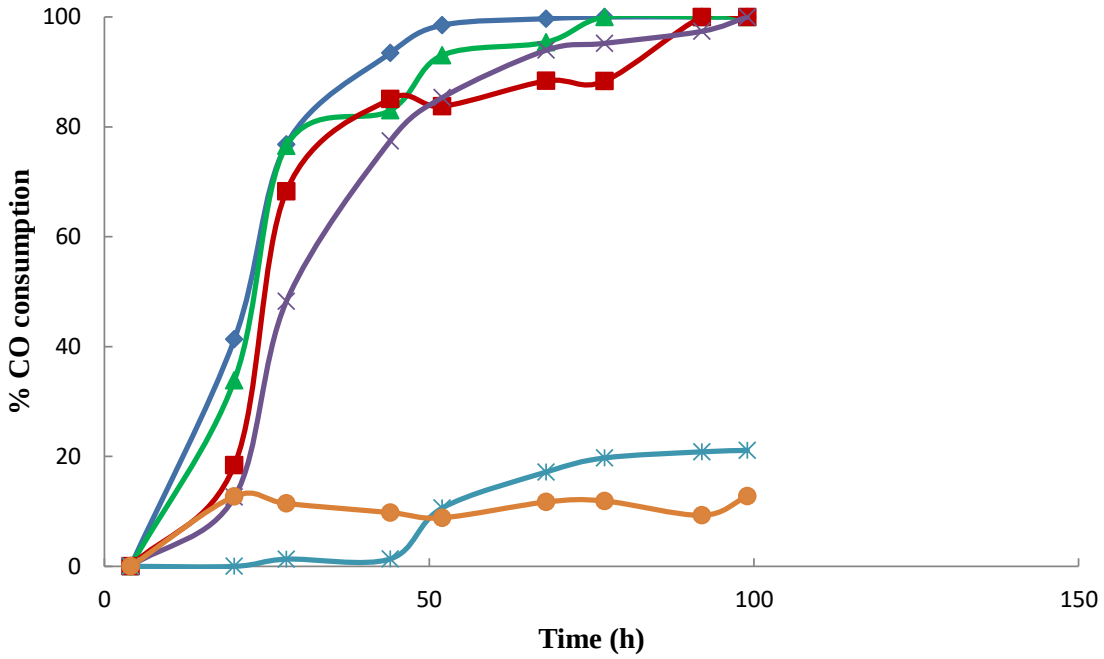

Figure 7.2 Batch experiments with butanol. Percentage CO consumption (control bottles represented as blue diamond, 1 g/L butanol represented as green triangles, 5 g/L butanol represented as red squared, 10 g/L butanol represented as purple X, 15 g/L butanol represented as blue X, and 20 g/L butanol represented as orange circle)

7.3.3 Ethanol toxicity experiment

In case of the ethanol inhibition study, ethanol showed a negative effect on the bacterial growth of *C. carboxidivorans*, and this effect was here also concentration-dependent (Figure 7.3 and Table 7-3) similarly as for butanol. The maximum biomass concentration was observed in the bottles with 1 g/L of ethanol (0.153 g/L) as well as in the control bottles (0.150 g/L), resulting in statistically similar values.

Table 7-3 Batch experiments with ethanol. Maximum specific growth rates in the presence of different ethanol concentrations, expressed in h^{-1}.

Ethanol concentration (g/L)	μ (h^{-1})
Control	0.082
1	0.090
5	0.066
20	0.055
25	0.028
35	0.024

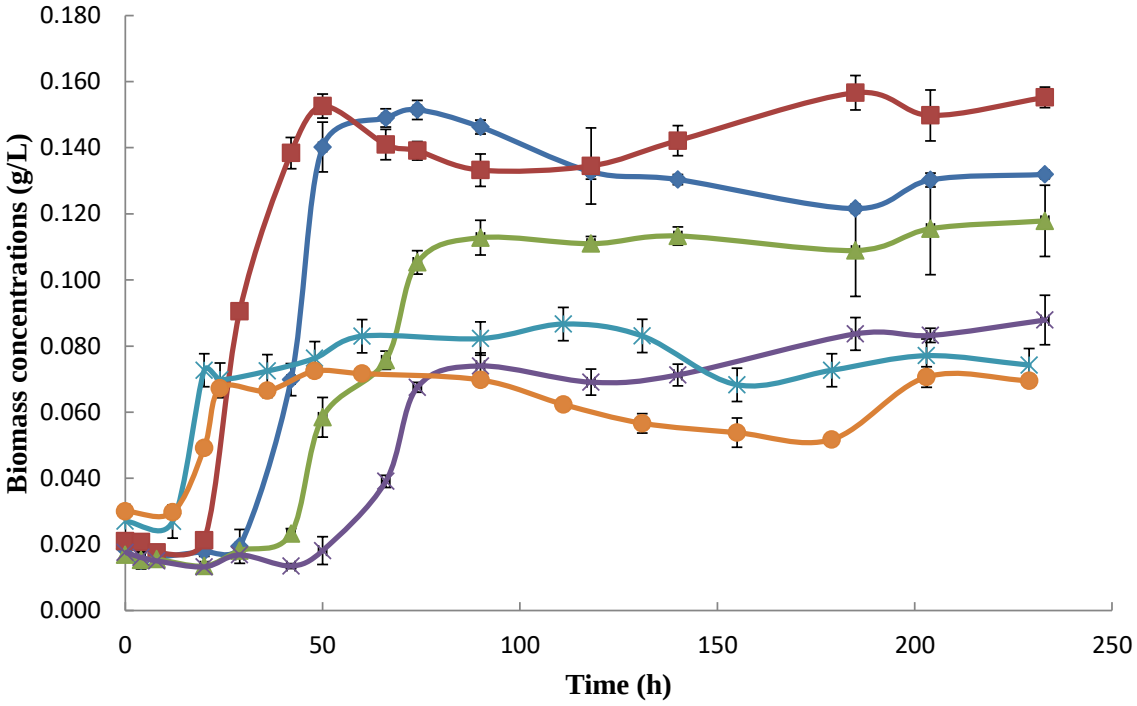

Figure 7.3 Batch experiments with ethanol. Measured biomass accumulation over time, expressed in g/L. Data are given as mean values ± standard deviation of the means (control bottles represented as blue diamond, 1 g/L ethanol represented as red square, 5 g/L ethanol represented as green triangle, 20 g/L ethanol represented as purple X, 25 g/L ethanol represented as blue X, and 35 g/L ethanol represented as orange circle).

It is noteworthy that in bottles with an added ethanol concentration of 1 g/L, in assays performed under exactly the same conditions, and with the same preculture inoculum, a somewhat higher growth rate was found than in the control bottles, with growth rates of 0.090 and 0.082 h^{-1}, respectively. The study was repeated and that effect was observed again in a second experiment, where the growth rate in bottles with 1 g/L added ethanol was slightly higher than the value found in the control bottles. Although the highest ethanol concentration added (35 g/L) was higher than for butanol (20 g/L), growth was detected in all the bottles with ethanol and reached growth rates higher than 0.024 h^{-1} in all cases, even in the most concentrated bottles (25 and 35 g/L) (Table 7-3).

The IC_{50} of ethanol for growth could not be estimated with the statistical software (SigmaPlot) because the most concentrated bottle had only reached 51 % growth inhibition at the end of the experiment, suggesting a much lower inhibitory effect of ethanol compared to butanol. However, a rather accurate estimation of the IC_{50} could be done based on the experimental data obtained, that way the IC_{50} of ethanol for growth appeared to be very close to 35 g/L, where 51 % inhibition was found.

Carbon monoxide consumption is shown in Figure 7.4. Maximum fast CO consumption was observed after a similar time period in all the assays up to an ethanol concentration of 5 g/L. In the bottles with 20 g/L of ethanol, carbon monoxide did not disappear completely, and the maximum consumption was 84 % after 329 h of ethanol exposure. Similarly, in the bottles with the highest alcohol concentrations (25 and 35 g/L), total substrate consumption was not possible either, observing 42 and 39 % CO consumption, respectively, at the end of the experiment after 230 h of ethanol exposure. This was related to the fact that in those bottles, biomass had scarcely grown and could thus not consume CO, whereas in the control bottles and with 1, 5, and 20 g/L added ethanol, growth took place at different rates, and different final biomass concentrations were reached. The final total amount accumulated biomass gradually decreased at increasing ethanol concentrations.

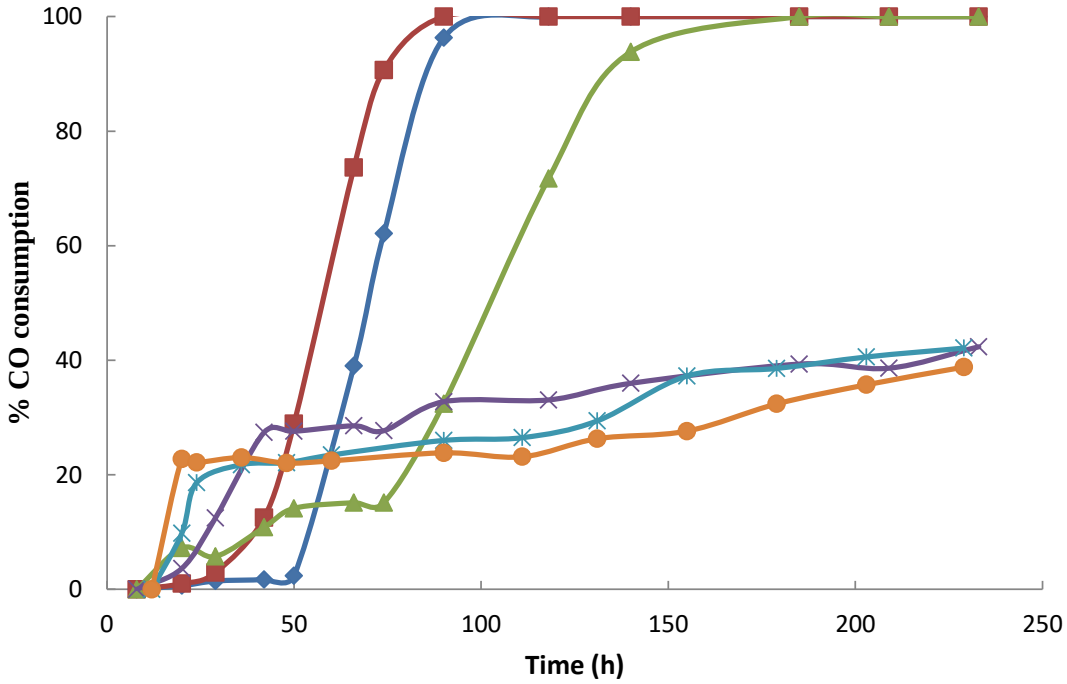

Figure 7.4 Batch experiments with ethanol. Percentage CO consumption (control bottles represented as blue diamond, 1 g/L ethanol represented as red square, 5 g/L ethanol represented as green triangle, 20 g/L ethanol represented as purple X, 25 g/L ethanol represented as blue X, and 35 g/L ethanol represented as orange circle).

7.3.4 Toxicity experiment with mixtures of both alcohols

In this experiment, the bacteria started growing soon after inoculation (Figure 7.5). The negative effect of the mixture of ethanol and butanol on the growth of *C. carboxidivorans* is shown in Figure 7.5 and in Table 7-4. This negative effect of the ethanol-butanol mixture is here also concentration-dependent (Figure 7.5 and Table 7-4). The maximum biomass concentration (0.170 g/L) was reached after 48 h in the control bottle. Growth took place in all the bottles and reached growth rates higher than at least 0.014 h^{-1}. In the bottles with the highest alcohol concentration (25 g/L), growth started initially and reached a maximum biomass concentration of 0.053 g/L after 60 h of alcohols exposure. However, after that time of alcohols exposure, growth stopped and the biomass started slightly decaying.

Table 7-4 Batch experiments with mixtures of alcohols. Maximum specific growth rates in the presence of different total concentrations of butanol and ethanol (1:1), expressed in h^{-1}

Total concentration of alcohols (1:1 mixtures) (g/L)	μ (h^{-1})
Control	0.090
2	0.072
7	0.033
15	0.018
25	0.014

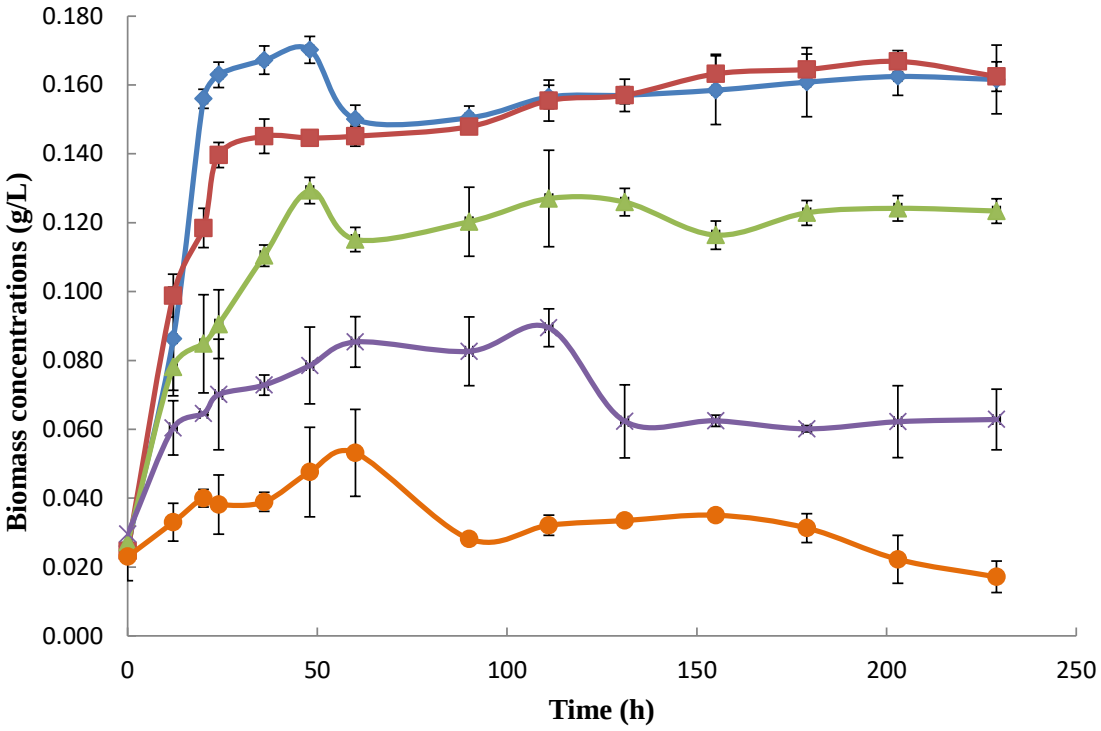

Figure 7.5 Batch experiments with both butanol and ethanol (1:1, w/w). Measured biomass accumulation over time, expressed in g/L. Data are given as mean values ± standard deviation of the means (control bottles represented as blue diamond, 2 g/L represented as red square, 7 g/L represented as green triangle, 15 g/L represented as purple X, 25 g/L represented as orange circle).

The IC_{50} for growth was 16.22 g/L after 111 h of alcohols exposure, which is similar, but slightly higher than in the assays with pure butanol, and can be explained by the lower toxicity of ethanol in the mixture of both alcohols.

Carbon monoxide consumption was monitored during all the experiment and is shown in Figure 7.6. In the control bottles, 50 % CO was consumed after 24 h and it was totally consumed after 60 h. In the case of 2 g/L, complete CO consumption was found after 130 h of alcohols exposure. In the bottles with 7 g/L, the maximum final consumption was 63 % after 60 h of alcohols exposure, and in the bottles with the highest concentrations of alcohols (15 and 25 g/L), only 30 and 22 % CO consumption were respectively observed, after 230 h of alcohols exposure.

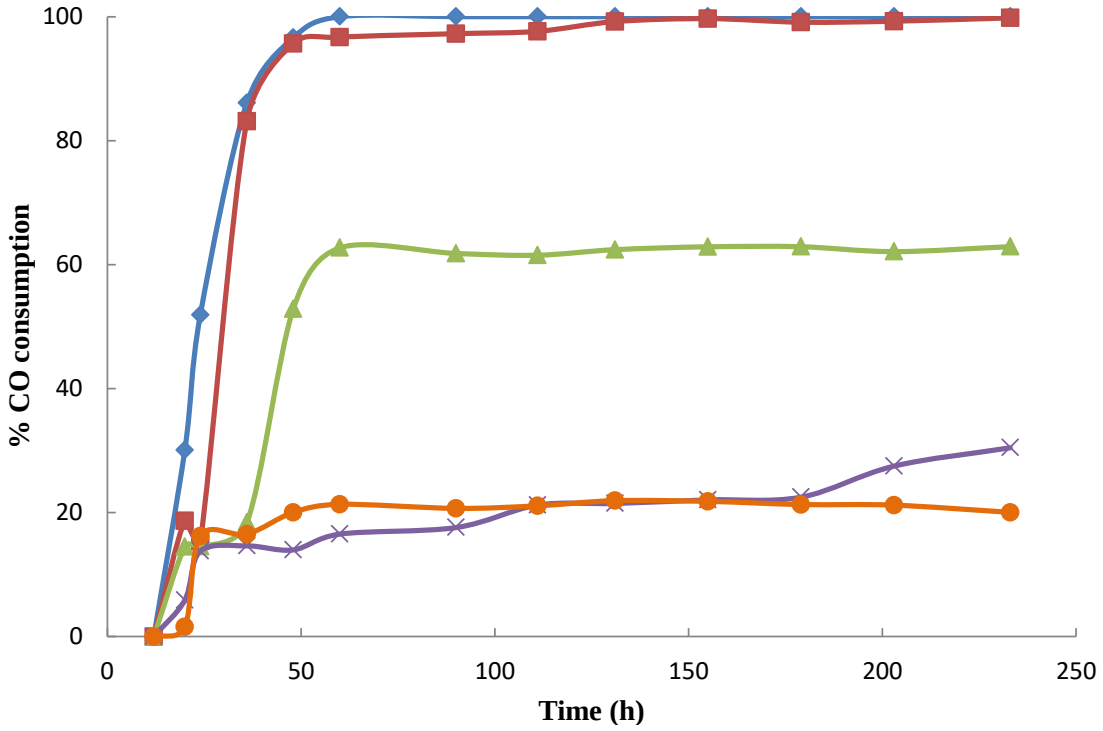

Figure 7.6 Batch experiments with both butanol and ethanol (1:1, w/ w). Percentage CO consumption (control bottles represented as blue diamond, 2 g/L represented as red square, 7 g/L represented as green triangle, 15 g/L represented as purple X, 25 g/L represented as orange circle).

7.3.5 Comparison of inhibitory effects of alcohols

Figure 7.7 compares the inhibitory effect on growth rates of ethanol, butanol, and mixtures of both alcohols. It shows that pure ethanol is the least toxic to bacterial growth, followed by the mixture of alcohols and pure butanol with the highest inhibitory effects. A similar trend can be

found for the inhibitory effects of the different alcohols on substrate consumption as well as IC_{50} data.

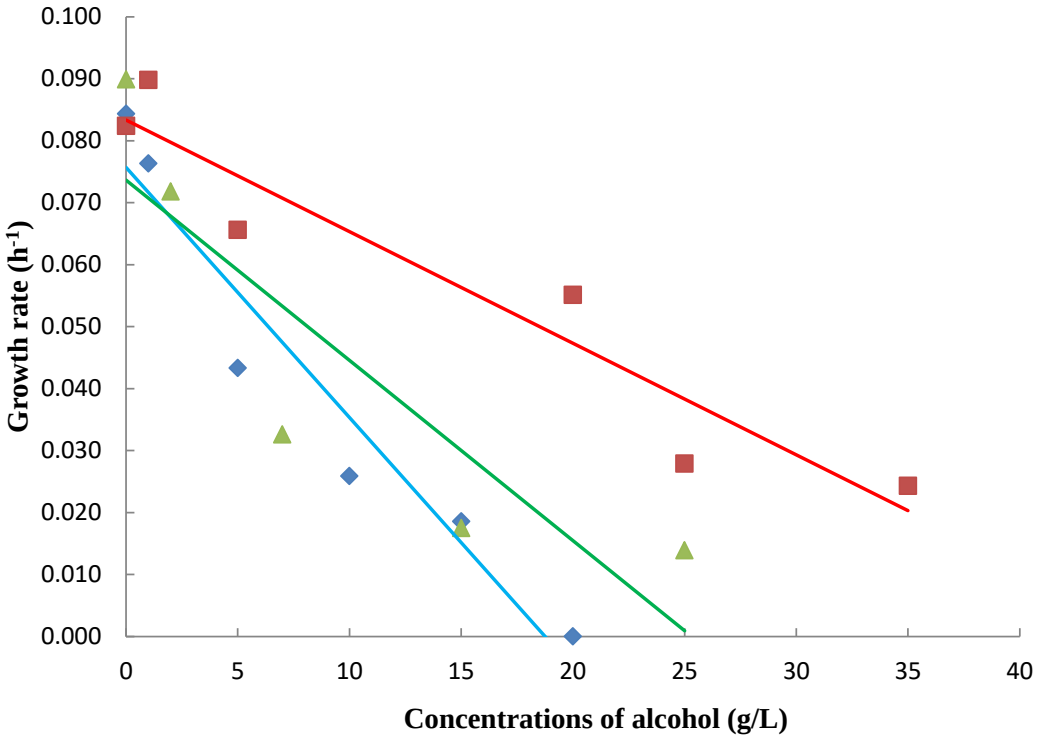

Figure 7.7 Comparison of growth rates in each experiment. Maximum specific growth rates (GR) of each treatment in the three experiments, expressed in h^{-1}. Butanol experiment represented as blue diamond, ethanol experiment represented as red square, mixture of alcohols experiment represented as green triangle. The lines represent the general trend of variation of the growth rates as a function of the concentration of alcohols (butanol experiment represented as blue line, ethanol experiment represented as red line, mixture of alcohols experiment represented as green line).

7.4 Discussion

Bioalcohols such as butanol can be produced through the conversion of lignocellulosic feedstocks into carbohydrates which are then fermented into biofuels. Alternatively, another recent approach consists in using CO-rich gases (i.e., syngas, waste gases) which can also be fermented to butanol and ethanol. Both the carbohydrate and the carbon monoxide routes use clostridia or acetogens in general as biocatalysts and present each their own advantages and drawbacks (Kennes et al. 2016). The lower biomass yield and slower bacterial growth rate is a typical drawback of the syngas approach, studied in this paper, reaching specific growth rates

close to hardly 0.086 h^{-1}, to be compared to values of 0.23–0.48 h^{-1} when clostridia are grown on sugars (Table 7-1). Thus, growth rates appear to be about 4–5 times higher on carbohydrates than on carbon monoxide. Similarly, our data show that the biomass yield on CO is 0.011 g biomass/g CO, while it reaches 0.36–0.53 g biomass/g carbohydrate when growing clostridia on sugars such as lactose (Napoli et al. 2012). Specific strategies are thus needed to overcome the low biomass production on such gaseous substrates in continuous bioreactors for B-E fermentation, such as cell recycling.

Another aspect to be taken into account, common to both the carbohydrate and the carbon monoxide (waste gas, syngas) approach, is the potential toxicity of end-metabolites, i.e., butanol and ethanol, on growth and substrate conversion itself. Toxicity studies have been performed and reported in the literature for clostridial strains converting sugars to solvents (ABE fermentation), but, to the best of our knowledge, no previous report is available on the effect of alcohols on clostridia fermenting CO-rich gases; although, in a recent patent application, it has been shown that recombinant strains of CO-fermenting clostridia might tolerate ethanol concentrations of up to around 50 g/L (Koepke et al. 2012).

The IC_{50} value of butanol obtained for *C. carboxidivorans* grown on CO in the present work (14.50 g/L), and reported here for the first time, is rather close to the values available from studies with other Clostridium species grown on carbohydrates. Similar results in terms of butanol inhibition were obtained by Moreira et al. (1981) using sugars as carbon source in *C. acetobutylicum*. They found that 0.10–0.15 M (7.41–11.12 g/L) butanol caused 50 % inhibition of cell growth and sugar uptake rate by negatively affecting the ATPase activity. A comparable effect was reported by Jones and Woods (1986), who found that 7–13 g/L butanol caused 50 % inhibition of cell growth.

The mechanism of butanol toxicity seems to be related to its hydrophobic nature, as this alcohol is a lipophilic solvent which can disrupt the phospholipid and fatty acid composition of the cell membrane causing an increase in membrane fluidity (Bowles and Ellefson 1985). This increase in the membrane fluidity would cause destabilization of the membrane and disruption of membrane functions such as transport processes, substrate (glucose) uptake, and membrane ATPase activity (Bowles and Ellefson 1985). On the other hand, Gottwald and Gottschalk (1985) also found that butanol can inhibit the ability of *C. acetobutylicum* to maintain its internal pH and abolishes the membrane pH gradient. Butanol toxicity has also been related to the autolytic degradation of the solventogenic cells in *C. acetobutylicum* P262 (Van der Westhuizen et al.

1982), and it was suggested that concentrations of butanol near the inhibitory concentration were involved in the release of autolysin during the solventogenic phase (Barber et al. 1979).

The effect of ethanol was also studied in the present work and it was shown that concentrations of 35 g/L could reduce the growth of *C. carboxidivorans* by 50 % after 200 h of ethanol exposure. That value is similar to the values obtained in the fermentation of sugars, for which it was suggested that the addition of acetone and ethanol up to 40 g/L reduced growth by 50 % (Jones and Woods 1986), and total growth inhibition appeared at concentrations of about 50–60 g/L of ethanol (Leung and Wang 1981; Costa and Moreira 1983).

In the butanol toxicity experiment with *C. carboxidivorans*, the IC_{50} (14.50 g/L) was much lower the value of IC_{50} observed in the ethanol toxicity experiment (35 g/L). A similar trend was observed for the growth rate. In the assay on ethanol toxicity, in the bottle with 35 g/L of ethanol (the most concentrated one), a growth rate of 0.024 h^{-1} was reached, which is a value close to the one obtained in the assays with 10 g/L of butanol. These results show that ethanol has a quite weaker inhibitory effect on *C. carboxidivorans* than butanol, as more ethanol is required to observe the same toxic effect as with butanol.

In both experiments with individual alcohols, the % CO consumed was monitored. This parameter shows that the bottles with ethanol reached higher percentages of CO consumption than the bottles with butanol. The assays with the highest butanol concentrations (20 g/L) did not even reach 20 % CO consumption, whereas in the bottles with the same concentration of ethanol, as much as 83 % CO was consumed. Besides, the bottles with the highest amount ethanol (35 g/L) still reached as much as 40 % CO consumption. There was basically no growth at all of *C. carboxidivorans* in the presence of a concentration of 20 g/L butanol; that way, this concentration would thus be near the concentration of full inhibition. As described above, the effect of alcohols on the level of CO consumption could be related to the fact that butanol disrupts the membrane fluidity, so the uptake of CO is a function of the characteristics of the membrane, which can get damaged as a result of butanol toxicity (Bowles and Ellefson 1985). Also, butanol at high concentrations could be involved in the release of autolysin, with an effect on the autolytic degradation of the cell (Van der Westhuizen et al. 1982; Barber et al. 1979).

Ethanol added at low concentration (up to 1 g/L) appeared not to cause any negative effect on the cells, and a somewhat faster biomass accumulation than in control bottles was even observed. This might be related to the fact that ethanol may favor the uptake of cholesterol or saturated fatty acids into membranes (Goldstein 1986). Goldstein (1986) suggested that the

bacteria he studied could be able to uptake a higher quantity of molecules such as fatty acids or other compounds, which could favor their growth.

In the last experiment, the effect of similar amounts of both alcohols in mixtures was analyzed. In that experiment, the IC_{50} was found to reach a value of 16.22 g/L of the ethanol and butanol mixture. This value is higher than the value obtained in the butanol toxicity assay, and it can most probably be related to the lower inhibitory effect of ethanol in the mixture. The growth rates in that experiment were higher than in the butanol experiment, at all the concentrations assayed. However, compared with the ethanol toxicity experiment, the growth rate was lower in the mixture than in the ethanol experiment. That difference is related to the strong toxic effect of butanol in the mixture in comparison with ethanol.

Also, differences were observed regarding the % CO consumed. A rather similar effect in terms of CO consumption was observed between the butanol experiment and the assay with mixtures of both alcohols, again as a result of the more significant inhibitory effect of butanol compared to ethanol. The bottles with the highest concentrations of alcohols only consumed 20–30 % CO in both cases, whereas in the bottles with ethanol only, the % CO consumption was always higher and exceeded at least 40 % in all cases.

It can be concluded that: (a) The alcohol with lowest inhibitory effect has the highest IC_{50}, meaning that butanol had the highest toxic effect on CO fermentation by *C. carboxidivorans*, followed by the 1:1 mixture of alcohols and finally ethanol; (b) small quantities of ethanol (around up to 1 g/L) have no toxic effect and seem even to exhibit a slightly positive effect on biomass growth and accumulation compared to the control cultures; (c) the alcohols produce a negative effect on the growth rate, on biomass accumulation as well as on the CO consumption rate in all the experiments (except at low ethanol concentrations).

7.5 Acknowledgments

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8 EFFECT OF SALINITY ON C1-GAS FERMENTATION BY *CLOSTRIDIUM CARBOXIDIVORANS* PRODUCING ACIDS AND ALCOHOLS

EFFECT OF SALINITY ON C1-GAS FERMENTATION BY CLOSTRIDIUM CARBOXIDIVORANS PRODUCING ACIDS AND ALCOHOLS

Abstract *Clostridium carboxidivorans* can produce acids and/or alcohols through syngas (C1-gas) fermentation. In the syngas fermentation process, the production of acids takes place at higher pH, during the acidogenic stage, while the solventogenic stage, with the conversion of accumulated acids into alcohols, should be more favourable at a lower pH of the fermentative broth. The pH drop, when switching from the acidogenic stage to the solventogenic stage, can either be natural – and result from the bacterial production of acids - or artificial, or even a combination of both. For a natural acidification process, a strong acid such as hydrogen chloride was added to a syngas fermenting bioreactor in this study. Cycles of high and low pH were applied in order to switch from acidogenic to solventogenic stages. pH increases were possible through the addition of sodium hydroxide. This pH adjustment procedure leads to the accumulation of salts and increased salinity of the medium, estimated in terms of conductivity. The possible inhibitory effect exherted by changes in salinity in the syngas bioreactor was estimated in batch, bottles, assays. Different batch bottle assays were carried out with different salinities (media with different concentrations of sodium chloride) using *C. carboxidivorans* and pure CO as a carbon source. At NaCl concentrations below 9 g/L (conductivity of 26.4 mS/cm), maximum growth rates around 0.055 h^{-1} were obtained, whereas increasing the concentration of sodium chloride had a negative effect on the bacterial growth. Also the maximum biomass accumulation and the CO consumption rate decreased at increased salinities. In the case of the most concentrated bottles, above 15 g/L NaCl (conductivities above 37.9 mS/cm) no relevant growth was observed, getting complete inhibition and growth rates around 0 h^{-1}. Also, the IC_{50}, or concentration yielding 50 % growth inhibition, was estimated with a value around 11 g/L sodium chloride.

Keywords: *Clostridium carboxidivorans*; carbon dioxide; ethanol; butanol; HBE fermentation; syngas

With minor editorial changes to fulfil formatting requirements, this chapter is waiting to be submitted

8.1 Introduction

Biorefineries based on the production of biofuels and platform chemicals from renewable sources, biomass, or wastes are being optimized nowadays to gradually replace the more conventional oil refineries and chemical industries or to coexist with those, as they are considered to be more sustainable and environmentally-friendly. A key challenge in biofuels production is to develop a low cost and effective process, to make such production competitive and viable (Branduardi and Porro, 2016). Environmental sustainability in the biofuel industry can be evaluated through life cycle assessment or using other similar tools (Rosen 2018). Biorefineries are mainly classified into first and second generation processes. In first generation biorefineries, sugars from agricultural crops, such as corn or sugarcane, are metabolized by microorganisms in order to obtain biofuels (e.g., bioalcohols) or bioproducts. The raw material used in this process has to deal with a food-fuel competition which is one of its major drawbacks (Kennes et al. 2016). Therefore, research on first generation processes is generally not funded anymore, among others in the European Union. Feedstocks used in second generation biorefineries are agricultural wastes such as lignocellulosic materials; that way the problem of food-fuel competition is avoided. However, that raw material is composed of cellulose, hemicelluloses and lignin, and rather complex pre-treatments may often be necessary to obtain simple sugars which can then be metabolized by microorganisms. The use of enzymatic, chemical, thermal, or other pretreatments convert cellulose and hemicelluloses into simple sugars, but lignin does not yield any simple sugars (Kennes et al. 2016); that way an important percentage, reaching sometimes up to 30 %, of the lignocellulosic material cannot be used for the sugar fermentation process. The conversion of lignocellulosic biomass or other similar renewable resources into syngas by means of a thermochemical pre-treatment, allows to use the complete feedstock, i.e. cellulose, hemicelluloses but also lignin; to obtain a fermentable gas mixture. That syngas can be metabolized by anaerobic bacteria as carbon and energy source to produce a range of fuels, e.g. bioalcohols, and chemicals (Abubackar et al. 2011; Bengelsdorf et al. 2013; Mohammadi et al. 2011). Some industrial emissions are also rich in C1 gases and sometimes hydrogen, similar as syngas obtained from biomass, and can then also be used by anaerobic bacteria as substrates and fermented into alcohols or other commercial products (Kennes and Veiga 2013). Syngas fermentation by either mixed cultures (Yang 2018; Chakraborty et al. 2019) or pure cultures (Abubackar et al. 2011) has gained increased interest recently, focusing, among others on its potential for biofuels production (e.g., ethanol, methane). Such gas fermentation process needs then to be optimized, trying to determine the best bioreactor operating conditions and avoiding any possible inhibitory issue.

Different parameters may affect the efficiency of a fermentation process, either in a positive or a negative way, and may also affect the nature and concentration of end metabolites. Optimal culture medium composition is one such key parameter. For example, in syngas-metabolizing solventogenic acetogens, the pH of the culture broth as well as the addition or omission of specific trace metals allows to shift the metabolism towards the production of either acids or alcohols (Abubackar et al. 2016; Fernández-Naveira et al. 2017a). Similarly, the salinity of a fermentation medium can play a role in the activity of microorganisms and should be considered when setting-up bioprocesses. The salinity of a medium may vary, among others during pH adjustment, as the addition of chemicals such as sodium hydroxide or hydrogen chloride will result in the accumulation of salts. In first and second generation production of biofuels and other platform chemicals through sugar fermentation, pH adjustment during pretratments or fermentation steps may lead to the accumulation of salts or other potentially inhibitory compounds (Casey et al. 2013; Palmqvist et al. 2000). Similarly as in first and second generation processes, pH is a key parameter strongly affecting the metabolism of solventogenic clostridia and acetogens in general, fermenting syngas, while pH adjustment relies on the addition of acids or bases that will ultimately affect salinity. Studies on the effect of high salt concentrations have been done in pathogenic, medical and food-related clostridia mainly. For example the addition of salt (i.e., NaCl) is known to be a common way to prevent the growth of different microorganisms such as *Clostridium* spp in food products (Lund, 1993). It has also been described that the addition of salt in levels of 8.2-10.5 (W/W) allows to prevent spore outgrowth (Khanipour et al. 2014). To the best of our knowledge, no study has been reported on the influence of salinity or conductivity of the medium on the activity of syngas-metabolizing strains useful in environmental applications.

In this study, anaerobic syngas fermentation was performed in a stirred tank bioreactor and pH was adjusted after a specific processing time in order to stimulate solventogenesis, i.e. the accumulation of alcohols. Salinity was checked in terms of conductivity during the fermentation process and during pH adjustment. Besides, batch assays were set-up in order to check the inhibitory effect of the salt concentration and conductivity on the *C. carboxidivorans* strain used to inoculate the syngas-fed bioreactor. Conductivity data found in the fermentor were compared to inhibitory values identified from batch assays.

8.2 Material and methods

8.2.1 Microorganism and culture media

Clostridium carboxidivorans (P7 DSM 15243) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), and was maintained anaerobically on modified basal medium (Liou et al. 2005; Tanner, 2007) with carbon monoxide (100 %) as a carbon source, and with an initial pH of 5.75.

The general composition of the medium used was (per liter distilled water): Yeast extract, 1 g; mineral solution 25mL; trace metal solution, 10 mL; resazurin, 1 mL; cysteine-HCl, 0.60 g.

The trace metal stock solution was composed of (per liter distilled water): 2 g nitrilotriacetic acid, 1 g manganese sulfate, 0.80 g ferrous ammonium sulfate, 0.20 g cobalt chloride, 0.20 g zinc sulfate, and 20 mg each of cupric chloride, nickel chloride, sodium molybdate, sodium selenate, and sodium tungstate.

The composition of the mineral stock solution was (per liter distilled water): 100 g ammonium chloride, 10 g potassium chloride, 10 g potassium monophosphate, 20 g magnesium sulfate, and 4 g calcium chloride. In the batch assays, different concentrations of NaCl were added to each bottle in order to check its effect on the strain. The NaCl concentration commonly recommended by culture collections for that strain is 2 g/L.

8.2.2 Bottle batch experiments

Different concentrations of sodium chloride were added in serum vials, per duplicate, in order to check the effect of sodium chloride on bacterial growth and activity. Sodium chloride was added at the following final concentrations: 0.2 g/L, 3 g/L, 9 g/L, 10 g/L, 11 g/L, 12 g/L, 15 g/L, 18 g/L and 21 g/L. The medium was introduced in 250 mL anaerobic serum vials with 100 mL working volume.

The pH was adjusted to 5.75, and the medium was boiled and flushed with nitrogen to ensure anaerobic conditions. After that, the bottles were sealed with rubber stoppers and aluminum caps and autoclaved for 20 min at 120° C.

For seeding the medium, 10 % biomass in exponential growth phase was inoculated in each bottle. They were then pressurized to 1.2 bar with 100 % CO and were agitated at 150 rpm inside an orbital incubator at 37° C.

8.2.3 Bioreactor experiment

The continuous gas-fed bioreactor experiment was carried out in a 2 L BIOFLO 110 bioreactor (New Brunswick Scientific, Edison, NJ, USA) with a working value of 1.2 L. A gas mixture of $CO:CO_2:H_2:N_2$ (20:20:10:50) was used as a carbon and energy source and was fed at a flow rate of 10 mL/min using a mass flow controller (Aalborg GFC 17, Müllheim, Germany) during all the experiment.

The original basal medium was prepared as described above, with the addition of 2 g/L NaCl, and was then autoclaved. After sterilization, the medium was flushed with N_2 and cooled down to 33° C. A water jacket allowed to maintain the temperature constant throughout the experiment. During the cooling down process, once the temperature had reached about 35° C, cysteine-HCl and vitamins were added. Once the bioreactor had reached anaerobic conditions, the N_2 feed was interrupted and it was then replaced by a syngas mixture fed to the reactor at a flowrate of 10 mL/min, using a microsparger. The agitation speed was maintained constant at 250 rpm. To avoid the formation of vortex in the culture medium, four baffles were placed inside the bioreactor. The pH and redox potential were constantly controlled. The pH was adjusted to the desired value through the addition of either 1 M NaOH or 1 M HCl solutions. Finally, 10% of the bacterial culture, in exponential growth phase (which was grown with CO as a carbon source during 72h), was inoculated in the bioreactor.

Initially, during the first experimental period, pH was maintained constant at a value of 5.75, then, when the maximum concentration of acids was reached, the pH was lowered to 4.8 through addition of HCl in a gradual way. 170 hours after the pH drop, it value was increased up to 5.75 again, adding NaOH gradually. 50 hours later, the medium of the reactor was partially replaced. When the maximum concentration of acids was reached again, HCl was added again to decrease the pH to a value of 4.8.

8.2.4 Growth measurement

For the batch assays in bottles, samples were taken at least twice a day during the exponential growth phase and once a day after that, withdrawing 1 mL of liquid sample from each bottle. The optical density (OD_λ = 600 nm) was measured on a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain) in order to estimate the biomass concentration. A biomass calibration curve had previously been plotted, representing biomass concentration *vs* optical density, with the aim to estimate the biomass concentration (g/L) based on the absorbance readings.

The growth rates (μ), expressed in $hour^{-1}$, for each bottle, were calculated during the exponential growth phase using the following equation:

$$\mu = \frac{[ln(N_t) - ln(N_0)]}{(t - t_0)} \quad \text{Eq. 8.1}$$

where N_t is the cell density (g/L) at time t (expressed in hours) and N_0 is the cell density at time 0 (t_0).

Another parameter estimated in that assay was the IC_{50} (concentration of the tested substance that decreases the growth rate by 50 %). It is one of the most commonly used parameters in toxicity assays (Leboulanger et al. 2001). The IC_{50} for each concentration of sodium chloride were estimated using a non-linear regression analysis (four parameters sigmoidal) of the concentration of sodium chloride as logarithm versus the percentage of growth inhibition. The percentage of inhibition is calculated considering that the control bottles correspond to 0 % inhibition. All the calculations were made using the regression Wizard software (Sigma-Plot 12.5, SPSS Inc.).

8.2.5 Gas-phase CO concentrations

Similarly as for biomass sampling, samples were taken once or twice a day in bottles assays, depending on the growth phase. 1 mL gas sample was removed from the headspace of each bottle to estimate CO consumption.

The CO concentration in each bottle was measured using an HP 6890 gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD) using Helium as a carrier gas. The GC was fitted with a 15 m HP-PLOT Molecular Sieve 5A column (ID: 0.53 mm, film thickness: 50 μm). The oven temperature was maintained constant at 50° C, while the temperature of the injection port and the detector were maintained constant at 150° C.

8.2.6 Fermentation products

In case of the bioreactor studies, 1 mL liquid samples were taken daily to check the nature and concentration of fermentation products on an HPLC (HP1100, Agilent Co., USA) equipped with a supelcogel C-610 column and a UV detector at a wavelength of 210 nm. A 0.1 % ortho-phosphoric acid solution was used as a mobile phase at a flow rate of 0.5 mL/min. The column temperature was set at 30° C. The samples were centrifuged (7000 g, 3 min) using a benchtop centrifuge (ELMI Skyline ltd CM 70M07) and filtered using a 0.22 μm filter, before analyzing them on the HPLC.

8.2.7 Conductivity measurement

In the bioreactor studies, conductivity was checked at the beginning of the experiment, at the end and after pH changes, when adding NaOH or HCl. For batch bottles experiments, the conductivity was only checked after inoculation checked, because those assays did not have any pH control and conductivity remained constant. For the measurements, 1 mL liquid sample was removed from the medium and conductivity was measured with a conductimeter (EUTECH INSTRUMENTS αlpha CON560).

8.3 Results and discusion

8.3.1 Continuous gas-fed bioreactor

The bioreactor was set-up with continuous syngas feed and its performance as well as salinity (conductivity) data were monitored as described hereafter.

Bioreactor performance

Acidogenesis, with the production of organic acids, started immediately after bioreactor inoculation. The first acid to be produced by *C. carboxidivorans* was acetic acid, which was the only acid to be detected in large amounts during the first three days of operation (Figure 8.1).

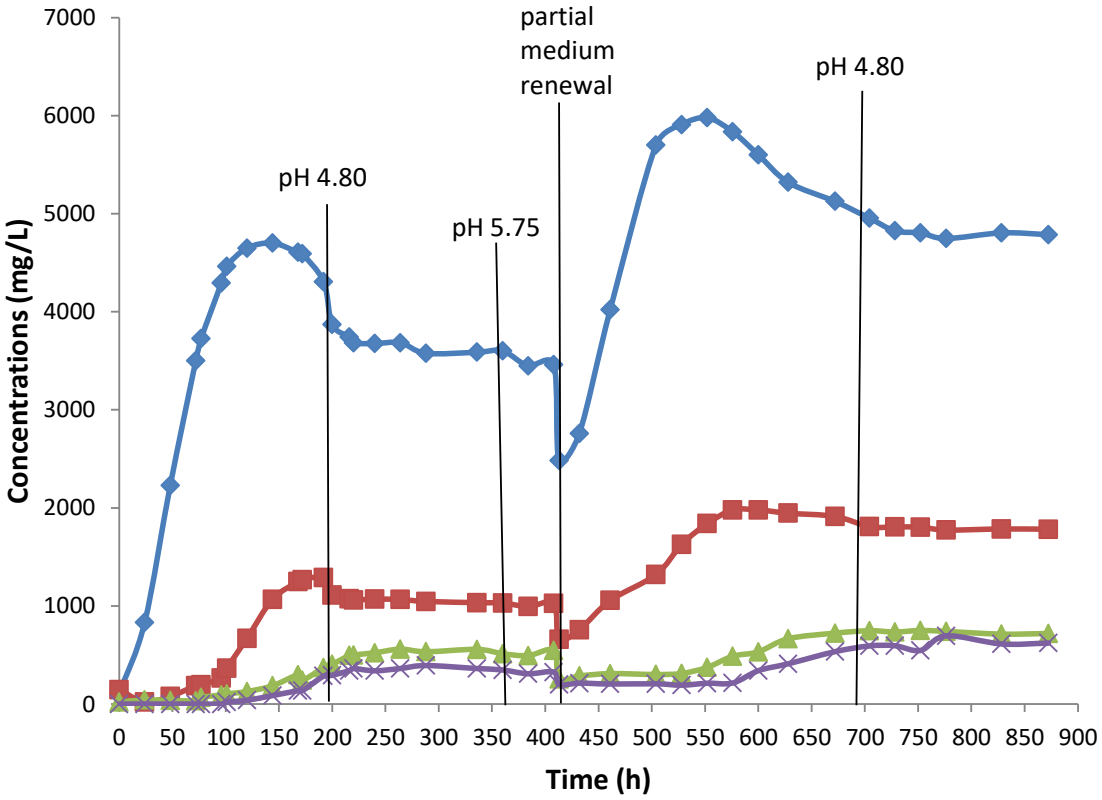

Figure 8.1 Fermentation products expressed in mg/L over the time in continuous gas-fed bioreactor. (Acetic acid represented as ♦, butyric acid represented as ■, ethanol represented as ▲ and butanol represented as X).

Later, 72 hours after inoculation, butyric acid started to appear, when acetic acid had already reached a high concentration of 3.50 g/L. The maximum concentrations of these acids were 4.70 g/L for acetic acid, 144 hours after inoculation, and 1.30 g/L for butyric acid, 192 hours after inoculation. These values agree with data reported recently under very similar conditions, except for a somewhat higher concentration of acetic acid found in that previous work, which could be due to the slightly lower maximum concentration of biomass obtained in the present experiment compared to the previously reported study (Fernández Naveira et al. 2017b). Growth of *C. carboxidivorans* was simultaneous to the production of acetic acid, following a common pattern, as also observed in other related bioreactor studies (Fernández-Naveira et al. 2016a; 2017b). A maximum biomass concentration of 0.29 g/L was reached after 72h (Figure 8.2), slightly before the concentration of acetic acid stopped increasing (Figure 8.1). The growth rate was calculated in the exponential phase, between 0 and 48 hours, reaching a value of 0.063 h^{-1}. These values of maximum biomass concentration and growth rate are similar to values reported in other studies at the same pH and with similar experimental conditions (Fernández-Naveira et al. 2017b). During that first experimental stage, at pH 5.75, bacterial growth and the

production of acids were concomitant to a fast CO consumption, starting soon after inoculation. During the first 120 h, a maximum CO consumption, between 90-93 %, was observed (Figure 8.3). After 120 h, the CO consumption started to decrease around 83 % at that pH value of 5.75. Concerning the production of alcohols, some ethanol was first detected 72 h after inoculation whereas butanol was not observed until t = 101 h (Figure 8.1). The maximum concentrations of ethanol and butanol, before the artificial pH drop (t = 200 h), were 0.40 and 0.29 g/L, respectively.

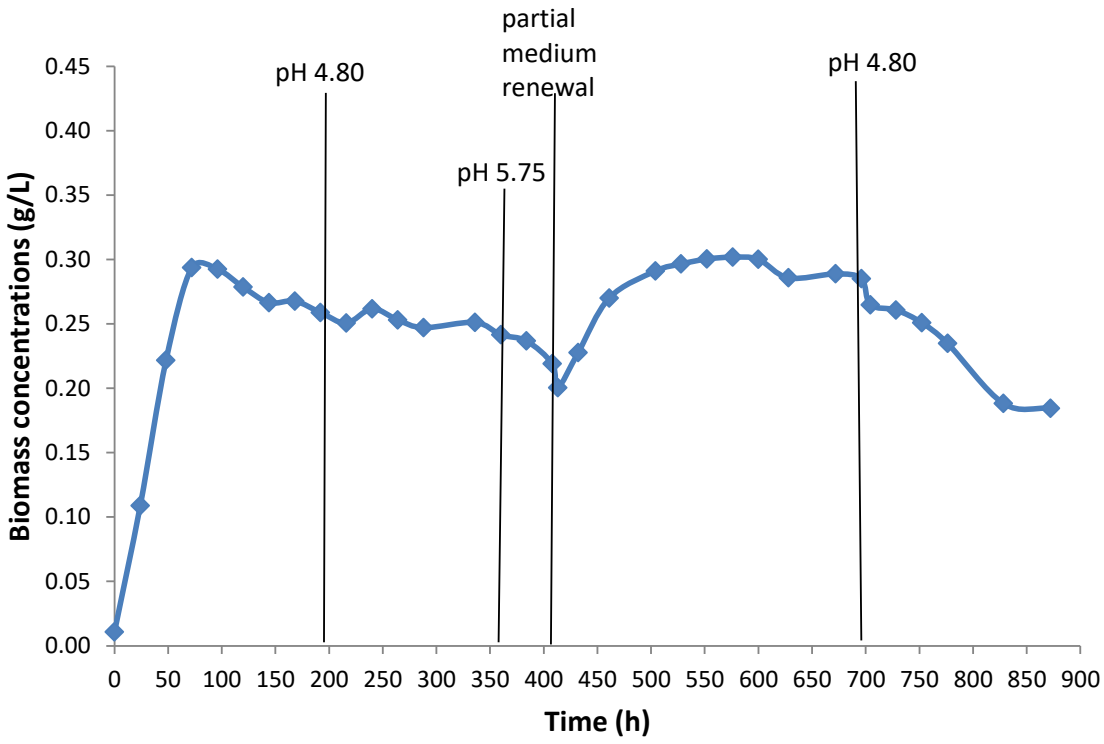

Figure 8.2 Biomass measurement in g/L over time in gas-fed bioreactor.

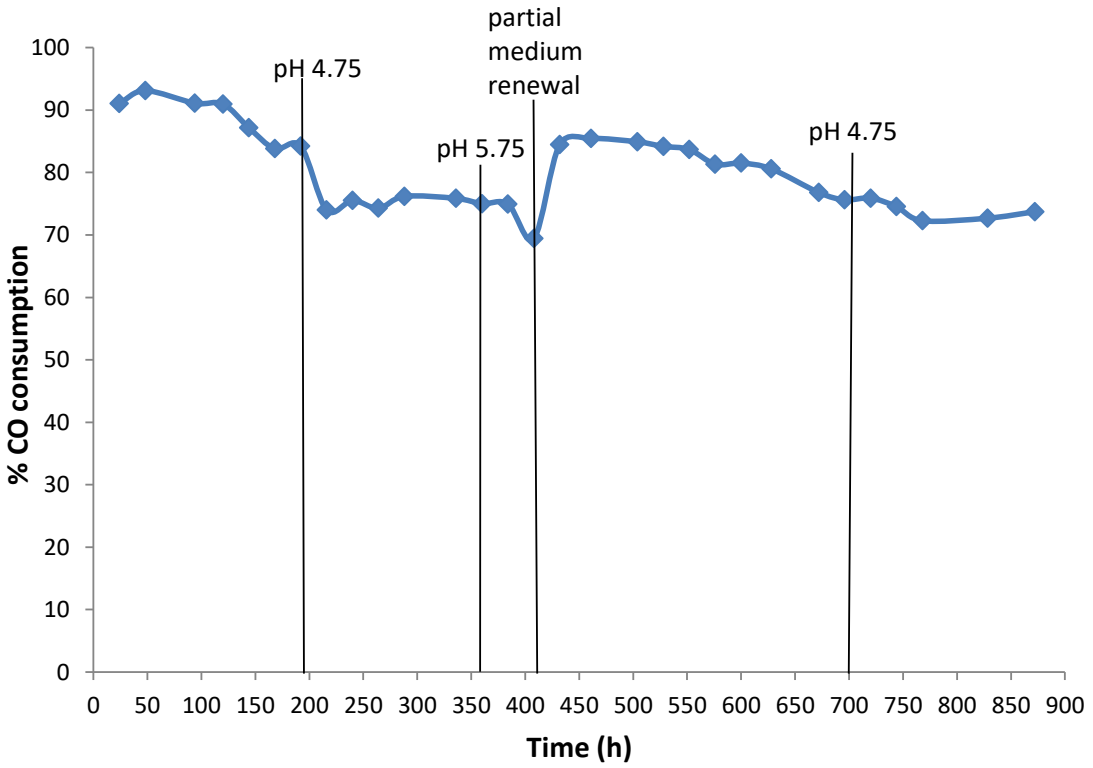

Figure 8.3 Percentage of CO consumption over time.

In order to stimulate the solventogenic phase, artificial acidification down to pH 4.80 was applied at t = 200 h. After the pH change, both acetic and butyric acids started to be consumed; although the acetic acid concentration had also already been found to slightly decrease before lowering the pH value. In Figure 8.1, consumption of 0.70 g/L acetic acid and 0.30 g/L butyric acid was observed during this period. After the pH drop, hexanoic acid was detected in the medium. However, for technical reasons the concentration of C6 compounds could only be measured later, after the experiment had finalized. Since the exact concentrations detected (corresponding to a few hundreds of mg/L) were not fully reliable, the data have not been plotted in Figure 8.1, as this does also not affect the main goal and conclusions of the study. As a general rule, it can be stated that the production of such compound was similar as in other similar studies. During the low pH conditions, CO consumption started decreasing down to 74-76 % (Figure 8.3). Although biomass appeared initially to decay somewhat at pH 4.80, the biomass concentration remained then roughly constant around 0.260 g/L during this experimental stage (Figure 8.2). After the artificial pH drop, only 0.16 g/L ethanol and 0.10 g/L butanol were produced and hardly any acids were consumed, although a low pH is expected to be more favourable to solventogenesis than higher pH values. Therefore, it was decided to check if

changes in the salt concentration and conductivity, due to the artificial pH drop reached by adding HCl to the medium, could be a possible reason for the low alcohol productivity, slight biomass decay and reduced gas consumption. This was checked in batch assays described in the next section.

Because of the lower bioreactor performance, it was decided to increase the pH again to 5.75. However, no significant improvement in acids consumption or production was observed (Figure 8.1), while the biomass concentration kept slowly decreasing ((Figure 8.2). Therefore, a partial medium replacement was decided and part of the bioreactor´s fermented medium was replaced by fresh medium, to check if any inhibitory medium component may have accumulated, including salts, or if some key compound may have become limiting. After the partial medium renewal, biomass started immediately to grow again, reaching a maximum value of 0.300 g/L, at t = 528 h (Figure 8.2), which is similar to the original highest steady-state cell concentration reached in the first stages of the experiment. It can also be observed, in Figure 8.1, how the partial medium renewal exerted a positive effect on the production of acids, with a fast increase of their concentrations up to maximum values of 6.00 g/L for acetic acid (t = 552 h) and 1.98 g/L for butyric acid (t = 600 h). As a consequence of the bacterial growth and acids production, CO consumption recovered as well, reaching a maximum value of 85 %. Then, later on, the CO consumption started to decrease again, around t = 600 h, when the maximum concentration of acids was reached. With this partial medium renewal, the production of alcohols was also stimulated, reaching maximum concentrations of ethanol and butanol for that second period of 0.75 g/L and 0.60 (respectively), at t = 700 h (Figure 8.1).

Subsequently, in the last experimental period, after t = 700 h, HCl was gradually added in order to decrease the pH value to 4.80 again, aiming at stimulating solventogenesis. A slow conversion of acids had already taken place at pH 5.75, while the pH decrease seemed to inhibit any further alcohols production. As observed in Figure 1 no more alcohols were produced after the pH was modified. A dramatic decrease of the biomass concentration (Figure 8.2) and slowdown of gas consumption (Figure 8.3) were also found and the experiment was then stopped.

The total net production of alcohols was analysed, reaching the following values: 1.05 g/L of ethanol, where 0.56 g/L was produced between t = 0 h and t = 264 h, and 0.49 g/L between t = 413 h and t = 704 h, in both cases such production took mainly place at pH 5.75. There was no production of acids either at low pH although this was expected as a low pH might stimulate the production of alcohols through acids consumption, but would not favor the production of such acids. In the case of butanol, an overall production of 0.89 g/L was observed, with 0.39 g/L

produced during the first pH 5.75 period, between t = 101 h and t = 288 h, and 0.50 g/L during the second pH 5.75 period, between t = 576 h and t = 776 h, in a similar way as for ethanol. That way, a total amount of 1.26 g ethanol and 1.07 g butanol were accumulated in the 1.2L bioreactor.

In another similar experiment with a natural pH drop (Fernández-Naveira et al. 2017b), the low pH did indeed stimulate the solventogenic stage. However, the artificial acidification applied here, through the addition of hydrogen chloride, seemed to have a negative effect on the process. This negative effect could be related with a higher sensitivity of *C. carboxidivorans* to these artificial pH changes and/or an increased salinity provoked by the artificial pH decrease, not expected when allowing a natural acidification process.

Salinity effect and conductivity measurements

Several hypotheses were considered in order to try to clarify the problem with the solventogenic stage. Inhibition could have been provoked by the salinity due to the addition of NaOH and HCl required for the artificial pH decrease. On the other side, *C. carboxidivorans* might be sensitive to the pH changes and the way they were applied in the present study, and the artificial pH decrease down to pH 4.80 might not be a fully suitable strategy to stimulate solventogenis in this case. Finally, a combination of artificial acidification, low pH and salinity, and may be other conditions, might altogether have affected the bacterial solventogenic activity. In order to estimate the possible effect of pH, a full set of experiments was performed at somewhat higher solventogenic pH values (e.g., pH 5.00), applying natural acidification strategies as reported elsewhere (Fernández-Naveira et al. submitted publication), while the effect of salinity, estimated in terms of conductivity, was evaluated in this study. The data and main conclusions are detailed below.

At different stages of the fermentation process described above, samples were taken in order to check the conductivity of the culture medium and estimate a possible relationship between salinity and inhibitory conditions. The following values were obtained: 13.85 mS/cm at t = 0 h, on starting the experiment; 14.59 mS/cm after the first pH drop, at t = 240 h; 17.33 mS/cm after the pH increase and before the partial medium replacement, at t = 384 h; and 15.30 mS/cm after the partial medium replacement (t = 432 h); and 16.21 mS/cm at the end of the experiment.

Since fluctuations of conductivity were observed during the different experimental periods, batch bottle inhibition experiments were set-up using media with different salt additions and conductivities, as described hereafter.

8.3.2 Batch bottle experiments

Batch assays were performed in presence of different salt concentrations and thus medium conductivities to evaluate their effect of the growth and activity of *C. carboxidivorans*.

Growth parameters of C. carboxidivorans

Biomass concentration

Three different growth patterns can be described, depending on the sodium chloride concentration, i.e. conductivity (Figure 8.4). In media with 0.20 g/L and 3 g/L, *Clostridium carboxidivorans* started growing soon upon inoculation of the bottles, without any significant lag phase, and reached similar maximum biomass concentrations of 0.140 g/L after 46 hours in 0.2 g/L bottles, and 0.144 g/L after 85 hours in 3 g/L bottles. Those maximum biomass concentrations agree with other data available in recent literature describing batch assays with that same strain (Fernández-Naveira et al. 2016a; 2016b). The growth pattern observed in the bottles with 9 g/L sodium chloride can still be combined with the other assays at the two concentrations described above, only that in this case *C. carboxidivorans* showed a slightly longer lag phase of 22h, reaching a maximum biomass value of 0.161 g/L after 61 hours, which is close to the values obtained at the two previous concentrations assayed. The second pattern observed is for concentrations ranging between 10 and 12 g/L sodium chloride, in which inhibitory effects started already be observed, and *C. carboxidivorans* exhibited a lag phase of about 100 h after inoculation, or even 122 h at the highest concentration of 12 g/L. Although the 10 g/L assay still reached a maximum biomass accumulation of 0.108 g/L, after 166 hours, those values were lower in the other two cases. At 11g/L sodium chloride, the biomass concentration was only 0.072 g/L after 190 hours; and that parameter reached 0.060 g/L, after 240 hours, at a salt concentration of 12 g/L. The third pattern corresponds to bottles with still higher concentrations of sodium chloride, of 15, 18 and 21 g/L. In those bottles, complete inhibition was found and the highest amount biomass in those experiments did not exceed 0.003 g/L. That way, as can be seen in Figure 8.4, increasing the concentration of sodium chloride in the fermentative broth, results in a negative effect on the growth pattern of *C. carboxidivorans* when surpassing a given threshold salt level.

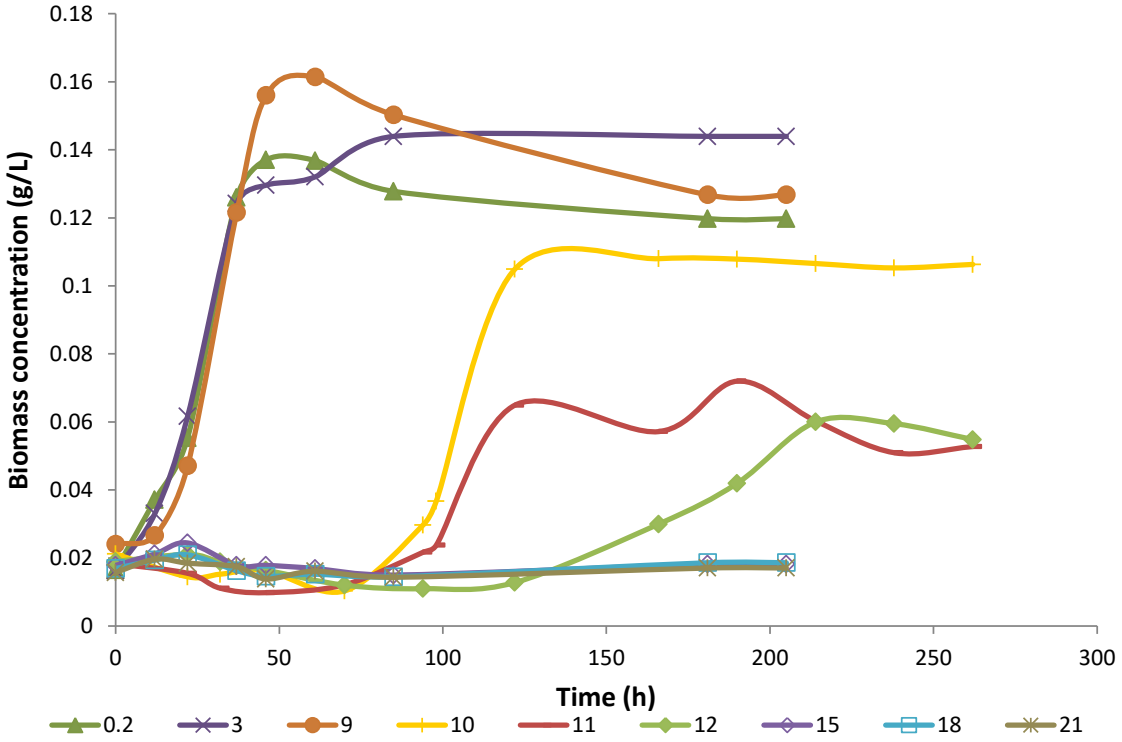

Figure 8.4 Measured biomass concentration expressed as g/L over time. (0.2 g/L of sodium chloride represented as ▲, 3 g/L of sodium chloride represented as X, 9 g/L of sodium chloride represented as ●, 10 g/L of sodium chloride represented as +, 11 g/L of sodium chloride represented as -, 12 g/L of sodium chloride represented as ♦, 15 g/L of sodium chloride represented as ◊, 18 g/L of sodium chloride represented as □ and 21 g/L of sodium chloride represented as ∗.

Specific growth rates

The specific growth rates were estimated for each concentration of sodium chloride used in this study (Table 8-1). The specific growth rates in bottles in which no inhibition took place agree with values obtained with that same species under similar conditions in other batch studies described in recent literature (Fernández-Naveira et al. 2016b). A negative effect on the growth rate of *C. carboxidivorans* could be observed at increasing sodium chloride concentrations above a threshold value. Up to nearly 9 g/L NaCl, no clear inhibition was detectable, although inhibition seems then to start at such value of around 9 g/L. The slight variations in growth rates over that concentration range of 0-9 g/L could be related with the sampling time or with the state of the inoculum or even with the experimental accuracy, but not significant differences were observed. However, at higher NaCl concentrations, both the growth rates and maximum biomass

concentrations decreased, even reaching total inhibition of *C. carboxidivorans* when the amount NaCl reached 15 to 21 g/L (Table 8-1).

Table 8-1 Maximum specific growth rates for each sodium chloride concentration.

Sodium chloride concentration (g/L)	Specific growth rate (μ) (h^{-1})
0.2	0.055
3	0.055
9	0.052
10	0.044
11	0.032
12	0.021
15	0
18	0
21	0

Hardly any research has been reported on the effect of salinity on clostridia and acetogens used in environmental applications, while studies can be found for pathogenic clostridia or strains typically found in food research. Although the salt concentration in the bioreactor was below the inhibitory values identified for *C. carboxidivorans* in batch assays, that strain seems to be more sensitive to salinity than previously described clostridia in food research. A strain of C. botulinum 62A was found to tolerate NaCl concentrations up to 3 % without any inhibition (Montville 1983). Similarily, the parent strain of C. botulinum ATCC 3502 with a growth rate of 0.31 h^{-1} in presence of 1 % NaCl, was still able to grow in presence of 4 % NaCl, but with a quite lower growth rate of 0.10 h^{-1} (Derman et al. 2015). For other bacteria such as C. tyrobutyricum different strains found in dairy products tolerated at least 3 % salt in milk, but complete inhibition was often already observed at 3.5% (Ruusunen et al. 2012). In cooked ham and beef, among three C. perfringens strains, all could develop at NaCl concentrations up to 2 % but complete inhibition was generally found above 3 % salt (Zaika. 2003). Many of those bacteria do generally still grow in presence of about 2-4 % NaCl, depending on the strain, while *C. carboxidivorans* appears to be already completely inhibited at such concentrations.

IC_{50}

An important parameter in toxicity assays is the IC_{50}. To check how restrictive the salinity of the fermentation broth could be on the activity of *C. carboxidivorans*, the IC_{50} values were estimated 181 and 202 hours after inoculation. The values obtained were 10.79 g/L and 11.02 g/L, respectively. Those values correspond to the concentration at which inhibitory effects started being detected in the experiment, as shown in Figure 8.3, where no inhibition was found

between 0.2 and 9 g/L, while longer lag phases and reduced maximum biomass concentrations started being observed at concentrations exceeding 9 g/L.

Conductivity measurement

When gas fermentation experiments are carried out to obtain alcohols with *Clostridium carboxidivorans,* non negligible amounts of NaOH and HCl are consumed during the acidogenic and solventogenic stages in order to adjust pH. The addition of those two chemicals would change the salinity and conductivity of the fermentation broth. As a result, this could have a negative effect on the bioconversion process. For each sodium chloride concentration assayed, the corresponding conductivity was measured (Table 8-2), in order to estimate the conductivity range that would cause a negative effect on *C. carboxidivorans*, and eventually complete inhibition.

Table 8-2 Conductivity measurement for each concentration of sodium chloride.

Sodium chloride concentration (g/L)	Conductivity (mS/cm)
0.2	10
3	17.1
9	26.4
10	27.1
11	29.9
12	32.8
15	37.9
18	43.6
21	48

With these values, and the data obtained in the IC_{50} analysis (10.79 and 11 g/L of NaCl), the value of conductivity which would cause 50 % inhibition on the growth of *C. carboxidivorans* was around 29.9 mS/cm.

The medium used in the continuous gas-fed bioreactor experiment described here, and in similar experiments with *C. carboxidivorans* and syngas as a carbon source described in recent literature (Fernández-Naveira et al. 2016a, 2017b) corresponds to the compositions commonly added in the standard culture medium (2 g/L NaCl). This corresponds to an initial conductivity around 14 mS/cm. At the end of the bioreactor experiment, after consumption of NaOH and HCl, the conductivity of the fermentative broth was around 16- 18.7 mS/cm. These values observed in the bioreactors are far from the IC_{50}. Therefore, the conductivities reached in the

fermentation broth due to the addition of NaOH and HCl should not represent a problem for the bioconversion process and no inhibition should be expected.

CO consumption

Similarly as observed for the biomass concentration, three patterns were also observed for the percentage of CO consumption (Figure 8.5). Similar fast CO consumptions were typical at NaCl concentrations between 0.2 and nearly 9 g/L (Figure 8.5). In all those bottles, except at 9 g/L, CO started to be consumed soon after inoculation, reaching 100 % consumption quite fast and exhausting the carbon source 61 hours after starting the experiment. In the case of bottles with 9 g/L sodium chloride, the consumption of CO started a few hours later (12 hours) corresponding also to the moment when growth started. A second pattern can be observed for concentrations between 10 and 12 g/L sodium chloride. In those bottles, CO consumption was slower and the substrate did not get fully exhausted. The maximum CO consumptions were 79 % after 166 h, 53.5 % after 214 h, and 56 % after 204 h for the bottles with 10, 11 and 12 g/L, respectively (Figure 8.5). The lower CO consumption in those bottles is also related with the lower biomass concentration obtained in comparison with the first set of assays at lower NaCl concentrations. For the last group of bottles, corresponding to 15, 18 and 21 g/L sodium chloride, still lower CO consumptions were observed with maximum values of 24 %, 27.6 % and 35 % after 204 hours for each concentration. In those bottles no significant growth was detected, and the biomass concentration remained low tough constant during all the experimental process. That way some consumption of CO was observed which could be logical as it could have been used for biomass maintenance or to satisfy other metabolic aspects. Some minimal loss during the sampling can also not completely be excluded.

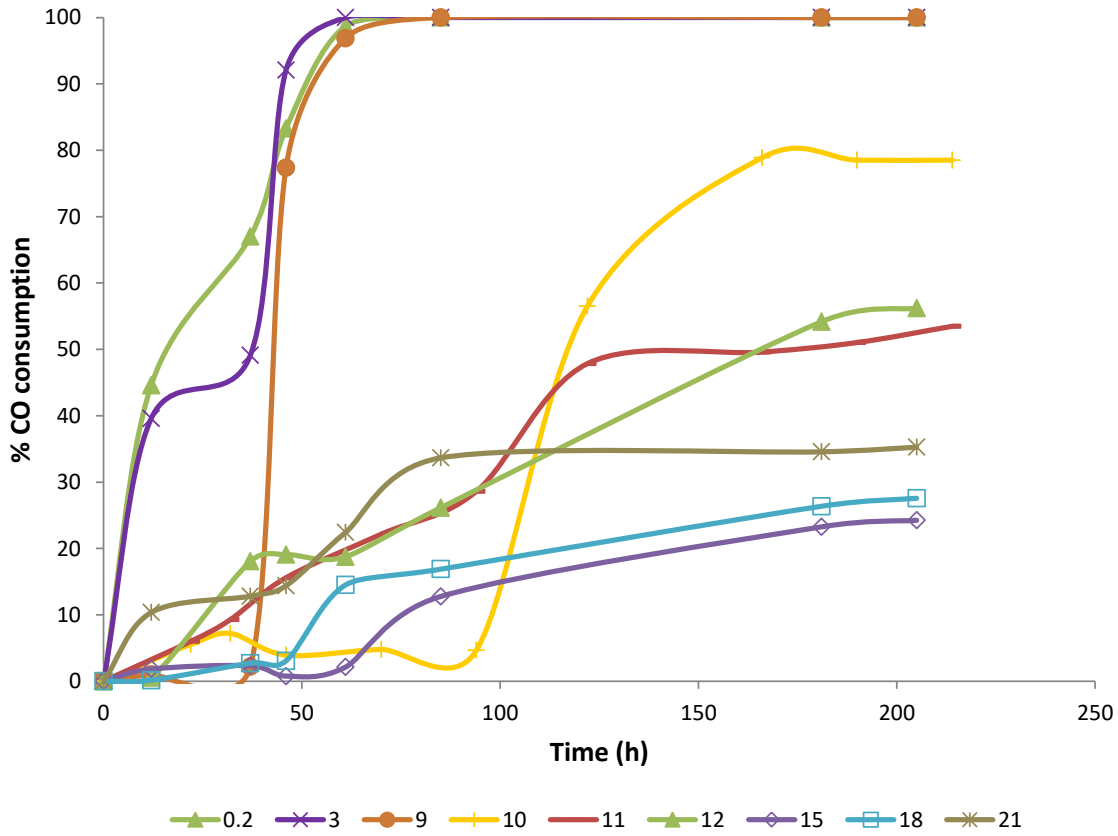

Figure 8.5 Percentage of carbon monoxide consumption over time. (0.2 g/L of sodium chloride represented as ▲, 3 g/L of sodium chloride represented as X, 9 g/L of sodium chloride represented as ●, 10 g/L of sodium chloride represented as +, 11 g/L of sodium chloride represented as -, 12 g/L of sodium chloride represented as ♦, 15 g/L of sodium chloride represented as ◊, 18 g/L of sodium chloride represented as □ and 21 g/L of sodium chloride represented as ∗.

8.4 Conclusions

In syngas bioconversion in bioreactors with pH regulation, an increase in salinity/conductivity is observed during the experiment, related to the pH adjustment procedure and the addition of sodium hydroxide or hydrogen chloride. The salinity (conductivity) of the medium (e.g., NaCl concentration) can affect the internal functions of the cell, causing reduction of the performance of the process at high salt concentrations. However, the salt concentrations found in this fermentation process are below possible inhibitory values. Therefore, it should not significantly affect the long term efficiency of the bioconversion. The results show that concentrations below 9 g/L do not affect the strain´s activity while concentrations of 15 g/L or higher do completely

inhibit *C. carboxidivorans*, with intermediate inhibitory effects at salt concentrations between 9 and 15 g/L. It has been shown that, in solventogenic clostridia, shifting the pH from a high to a low value, may allow to completely consume acids, produced from the gas fermentation, and convert those into alcohols, provided other conditions are optimal (e.g., medium composition, presence of key trace metals) (Abubackar et al. 2015; Fernández-Naveira et al. submitted publication). Since conductivity did not seem to affect the stability of the process and the low conversion of acids in syngas fermentation, the pH value could have played a role. Indeed, the optimal pH range for that strain has been suggested to be 5-7 (Liou et al. 2005, Fernández-Naveira et al. 2017c) and a pH around 4.80, - although not reported to be inhibitory -., could be close to a lower threshold value that could have avoided complete conversion of acids to alcohols, as observed in this paper. Conversely, in another recent study it was found that limiting the pH drop to a minimum value of 5.0 (instead of 4.80) does allow the complete conversion of acids (e.g., acetic acid) into alcohols (e.g, ethanol) in *C. carboxidivorans* (Fernández-Naveira et al. submitted). Besides, a natural acidification process, without any addition of acid or base, may be less stressful for the bacteria, than artificial acidification, and thus still allow a better conversion of acids into alcohols. However, it remains unclear why partial medium replacement significantly improved the production process, and thus aspects such as the salinity or a low pH do not completely allow to explain the limited conversion of acids into alcohols and the highly positive effect of partial medium replacement.

8.5 Acknowledgements

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9 SELECTIVE ANAEROBIC FERMENTATION OF SYNGAS INTO EITHER ORGANIC ACIDS OR ETHANOL AND HIGHER ALCOHOLS

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Abstract. *Clostridium carboxidivorans* is one of the few bacteria able to produce (bio)alcohols, e.g. ethanol, butanol and hexanol, from C1 gases (e.g., CO, CO_2) and syngas, following the Wood-Ljungdhal pathway. In that pathway, gases are first converted into fatty acids, i.e., acetic, butyric and hexanoic acids (acidogenesis), to be subsequently converted into alcohols (solventogenesis). This research focused on identifying conditions that allow to selectively produce either fatty acids or alcohols, which is industrially relevant as it represents a means to select for the desired metabolites. The conversion of gases into acids and the subsequent conversion of the latter into alcohols are catalysed by metalloenzymes whose activity would be stimulated by the presence of specific trace metals, e.g. tungsten (W), selenium (Se). Therefore, different bioreactor experiments were set-up either with or without addition of W or Se and at different pH values. The results showed that combining the presence of those trace metals with a low pH (5.00) allows to accumulate high amounts of alcohols as major end products (8.04 g/L total alcohols; 3.03 g/L total acids) produced from the conversion of fatty acids obtained from C1 gas fermentation. Instead, maintaining a higher pH (6.20), in the absence of those trace metals, allows for the selective production of organic acids (9.55 g/L) while a much lower total concentration of alcohols was found (0.68 g/L). Omitting W, but not Se, at such higher pH (6.20), led to a still higher total concentration of acids (11.30 g/L) and lower conversion to alcohols (2.02 g/L).

Keywords: Butanol; ethanol; hexanol; selenium; tungsten; waste gas

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9.1 Introduction

Many energy sources are still produced from nonrenewable sources in mostly non environmentally-friendly oil refineries. There is a need to look for more sustainable production processes based on a circular economy concept, reducing pollution and considering the advantages of reusing contaminants in a cost-effective way (Andersen 2007). Biomass and other similar feedstocks as well as pollutants are good candidates to produce biofuels at industrial scale (Martínez et al. 2017). Bioethanol is one of those biofuels suitable to replace transportation fuels such as gasoline (Kennes and Veiga 2013). Bioethanol production based on the fermentation of carbohydrates obtained from lignocellulosic materials has been quite extensively reported (Kennes et al. 2016). Another, more recent and innovative alternative, consists in gasifying those feedstocks in order to obtain syngas that can also be converted into ethanol, with pure anaerobic bacteria (Mohammadi et al. 2012; Abubackar et al., 2015) or with mixed cultures (Chakraborty et al. 2019). That process can also use some waste gases, e.g. from steel industries, as they have similar gas compositions, and all contain mainly CO, CO_2 and H_2. Some recent cost analyses have proven that such technology is cost-effective, industrially feasible and competitive compared to carbohydrate fermentation (Roy et al. 2015; He et al. 2011). Gas fermentation to ethanol has now reached precommercial scale (Phillips et al. 2017). Besides, the technology allows to mitigate greenhouse gas emissions, while yielding a biofuel, which is a significant aspect as the sustainability of a biofuel production process includes energy and greenhouse gas saving, and a low environmental impact (Rathore et al. 2016).

It has been recently shown that, besides ethanol, other metabolites, such as biopolymers, can be obtained as well from anaerobic waste gas or syngas fermentation (Drzyzga et al. 2015; Lagoa et al. 2017); but in order to broaden the range of compounds that can be obtained through anaerobic C1-gas fermentation, research gaps related to the production of energy sources (e.g., ethanol, higher alcohols) need to be addressed. C1-gas fermentation for the accumulation of higher alcohols, besides ethanol, which is the focus of this paper, is a novel process with only a limited number of reports on optimization (Fernández-Naveira et al. 2017a). Higher alcohols are interesting as novel energy sources. Butanol is a C4 alcohol that can be used as a fuel, besides having other industrial applications (Dong et al. 2012). It has several advantages compared to ethanol and is more similar to gasoline (Ramey et al. 2007). It is less hygroscopic than ethanol, less volatile, less corrosive and has a higher energy content (Lagoa et al. 2017). It can be mixed at high ratios and concentrations with gasoline. On the other hand, hexanol is a longer chain, C6, alcohol being considered as a suitable substitute of refinery diesel. It can also be blended with

fossil fuels and other alcohols (Atmanli 2016; Yilmaz et al. 2017). Besides, studies have been done on the compatibility of hexanol with Jet aviation fuel (Chuck et al. 2014), on oxidation kinetics and on engine emissions in presence of hexanol (Babu and Anand, 2017; Togbé et al. 2010). Additionally, it has been reported that hexanol can be used as a starting material for obtaining other liquid transportation fuels (Luggren et al. 2016). Ethanol and higher alcohols are thus interesting biofuels, but some research gaps related to the optimization of their production still need to be further addressed, among others for the novel HBE (hexanol-butanol-ethanol) gas fermentation process (Fernández-Naveira et al. 2017a).

Clostridium carboxidivorans is able to metabolize syngas for the production of ethanol and higher alcohols, via the Wood-Ljungdahl (WL) pathway (Müller 2003; Fernández-Naveira et al. 2017b). Generally, solvent producing clostridia using that pathway for C1-gas fermentation, will first generate fatty acids, which can later be converted to solvents further down in the WL pathway. If the desired end products are ethanol and higher fuel-alcohols, optimizing conditions that allow to minimize the accumulation of acids while maximizing the production of alcohols, as addressed in this paper, will provide a new body of knowledge that will improve the efficiency of the process and its industrial application. In that sense, some trace metals are used as cofactors by metalloenzymes of the WL pathway. Bioreactor studies on their involvement if HBE fermentation have not yet been reported. In the WL pathway, acetogenic bacteria convert gas-substrates into the common intermediate acetyl-CoA, for their growth and the production of organic acids and alcohols. From basic biochemical studies, it appears that tungsten (W) and selenium (Se) can be considered as two key trace metals involved either in the assimilation of C1 gases or in the conversion of acids, produced from such gases, into alcohols. Se plays a role in formate dehydrogenase (FDH), an enzyme catalyzing the reduction of C1 gases, mainly CO_2, in bacteria such as *C. ljungdahlii* (Çakar et al. 2018) and *C. carboxidivorans* (Alissandratos et al. 2013). Some authors have mentioned the involvement of W, and sometimes molybdate, in the activity of formate dehydrogenases (e.g., in *C. thermoaceticum*) (Andreesen et al. 1973; Saxena and Tanner 2011). Depending on the strain, either tungsten or molybdate would thus be involved in the activity of FDH. On the other side, W is considered to be a key trace metal for the activity of aldehyde:ferredoxin oxidoreductase (AFOR), an enzyme catalyzing the conversion of acids into an aldehyde. Later on alcohol dehydrogenase (ADH) will catalyze the conversion of the aldehyde into alcohols in several clostridia metabolizing CO, CO_2 or syngas (Abubackar et al. 2015; Fernández-Naveira et al. 2017a). The combined effect of W and Se is therefore expected to affect the assimilation of C1 gases and their overall conversion into acids and later alcohols in HBE fermentation, while omitting W only would mostly affect the last step of the pathway in

which acids are converted into alcohols. The selection of optimized bioreactor operating conditions and the presence of specific trace metals would presumably allow to maximize the production of fuel-alcohols.

In this study, four experiments were carried out with syngas as substrate mixture and *C. carboxidivorans* as a biocatalyst, using different trace element compositions, i.e. absence or presence of tungsten or selenium, in order to study how it affects the bioconversion profile in the HBE fermentation, i.e. the conversion of C1 gases to acids and, later, ethanol and higher alcohols (butanol, hexanol). Objectives of this research were to evaluate the combined effect of tungsten and selenium as well as the individual effect of those trace metals on bacterial growth and on the production of acids and alcohols, and that way maximize the production of novel biofuels (e..g, higher alcohol). Four bioreactors were operated under different conditions. First, a reactor was set-up using the complete medium composition (i.e., presence of all trace metals) and allowing natural acidification (no pH control) as a result of the initial accumulation of acids. Then, two additional bioreactors were used with a medium in which tungsten and selenium were omitted and applying different operating conditions in each system, i.e. in one reactor the pH was maintained constant throughout the experiment and in the other one there was no pH control (i.e., with acidification). Finally, a bioreactor without tungsten, at constant pH, was operated in order to check the effect of that specific trace metal.

9.2 Material and methods

9.2.1 Microorganism and culture media

C. carboxidivorans P7 DSM 15243 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was maintained under anaerobic conditions on modified basal medium at pH 6.2 and with a mixture of the following gases as substrate: $CO:CO_2:H_2:N_2$ (30:10:20:40) (Liou et al. 2005; Tanner 2007). The medium used in the experiments had the following composition (per liter distilled water): 1 g yeast extract; 25 mL mineral solution; 10 mL trace metal solution; 10 mL vitamins stock solution; 1 mL resazurin; 0.60 g cysteine-HCl.

The mineral stock solution was prepared with the following composition (per liter distilled water): 80 g sodium chloride, 100 g ammonium chloride, 10 g potassium chloride, 10 g potassium monophosphate, 20 g magnesium sulfate, and 4 g calcium chloride.

Three trace metal solutions were prepared in order to carry out the different experiments. The composition of the complete trace metal solution was (per liter distilled water): 2 g nitrilotriacetic acid, 1 g manganese sulfate, 0.80 g ferrous ammonium sulfate, 0.20 g cobalt chloride, 0.20 g zinc sulfate, and 20 mg each of cupric chloride, nickel chloride, sodium molybdate, sodium selenate, and sodium tungstate. The second solution had the same trace metals composition but omitting sodium tungstate, and in the third stock solution the addition of sodium selenate and sodium tungstate was omitted.

The vitamin stock solution had the following composition (per liter distilled water): 10 mg pyridoxine, 5 mg each of thiamine, riboflavin, calcium pantothenate, thioctic acid, para-amino benzoic acid, nicotinic acid, and vitamin B12, and 2 mg each of D-biotin, folic acid, and 2-mercaptoethanesulfonic acid.

9.2.2 Continuous gas-fed bioreactor experiments

Four experiments were carried out in 2L BIOFLO 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA) with a working volume of 1.2 L. In one of them the complete basal medium described above was used. In other two, tungsten and selenium were omitted in the trace elements solution. In the fourth one only tungsten was omitted. The four bioreactors were all fed the same syngas mixture, which was continuously supplied at a flow rate of 10 mL/min using a mass flow controller (Aalborg GFC 17, Müllheim, Germany). A microsparger was used for optimal diffusion of the gas. Each bioreactor was filled with 1.2 L of the desired medium and was autoclaved for 20 minutes at 121° C. After autoclaving, N_2 was flushed through the medium in order to remove oxygen and, at the same time, the medium was cooled down using a water jacket. When the temperature reached 40° C, cysteine-HCl and vitamins were added from a stock solution.

When the medium reached anaerobic conditions, N_2 flushing was replaced by syngas feeding. The operating conditions of the bioreactors were, in all cases, a constant temperature of 33° C and a constant agitation speed of 250 rpm during all the experiment. Besides, four baffles were placed inside those bioreactors in order to improve medium mixing and to avoid vortex formation.

A 10 % seed culture, in early exponential growth phase, was used to inoculate the bioreactors. It was grown for 72 h with CO as a carbon source and with the same medium composition as for each bioreactor, meaning that the inoculum for the first bioreactor (with the two trace metals) was grown in a complete culture medium while, for the two bioreactors without selenium and

tungsten the inocula were grown without these two trace metals to avoid their presence in the bioreactor and finally for the bioreactor without tungsten the inoculum was grown without W.

Regarding the pH, the latter was maintained at a given value, whenever required, using peristaltic pumps for the addition of either 2M NaOH or 2M HCl solutions. In the case of the control bioreactor with both W and Se and one of the two reactors without tungsten and selenium, the pH was regulated and maintained constant at 6.2 during the first hours of the experiment until reaching an acetic acid concentration of about 2.0 g/L. When such concentration was reached, automatic pH regulation was switched on, and the pH could drop down naturally, as a result of the production of acids, to a value of 5.2. In the other bioreactor without selenium and tungsten and in the bioreactor without tungsten, the pH was maintained at a constant value of 6.2 during all the experiment. Tungsten and Selenium concentrations of samples taken from the bioreactors were determined and confirmed using an HR-ICP-MS (Element Thermo Finnigan).

9.2.3 Growth measurement

1 mL of liquid sample was daily removed from the bioreactors for measurement of the biomass concentration. The concentration was measured on a UV-Visible spectrophotometer at a wavelength (λ) of 600 nm (Hitachi, Model U-200, Pacis & Giralt, Madrid, Spain). A biomass calibration was done previously to be able to estimate the biomass concentration (g/L) from the optical density reading (OD λ=600nm) obtained with the spectrophotometer.

9.2.4 Gas-phase CO and CO_2 concentrations

In order to quantify the concentrations of CO and CO_2, 1 mL of gaseous sample was daily removed from the outlet port of the bioreactor.

An HP 6890 gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD) was used for measured the gas-phase CO concentrations. The GC was fitted with a 15-m HP-PLOT Molecular Sieve 5A column (ID, 0.53 mm; film thickness, 50 µm), the oven temperature was maintained constant at 50° C and in the injection port and in the detector the temperature was maintained constant at 150° C (Fernández-Naveira et al. 2016). Helium was used as the carrier gas. Similarly, an HP 5890 gas chromatograph, equipped with a TCD was used for the CO_2 analyses. The injection, oven, and detection temperatures were kepted at 90, 25, and 100° C, respectively.

9.2.5 Fermentation products

1 mL liquid samples were taken from the reactor at least every 24 h. The concentrations of acids, ethanol and higher alcohols were measured using an HPLC (HP1100, Agilent Co., USA) equipped with a supelcogel C-610 column and a UV detector at a wavelength of 210 nm. A 0.1 % ortho-phosphoric acid solution fed at a flow rate of 0.5 mL/min was used as mobile phase. The column temperature was set at 30° C. The samples were centrifuged (7000 g, 3 min) using a benchtop centrifuge (ELMI Skyline ltd CM 70M07) before analyzing the concentration of water-soluble products by HPLC.

9.3 Results

9.3.1 Continuous gas-fed bioreactor with W and Se and natural acidification from pH 6.20 to 5.00

Biomass and solvent production

The experiment was carried out with the culture medium containing both W and Se in the trace elements solution and at an initial pH of 6.20. The pH was maintained at such value for 48 hours in order to allow acidogenesis to take place, as a higher pH has been proven to stimulate bacterial growth and acids production (Fernández-Naviera et al. 2017c). Once the biomass concentration reached a value of 0.29 g/L and the amount accumulated acetic acid was 1.91 g/L, automatic pH regulation was stopped in order to allow natural acidification until reaching a final pH value of 5.00, expected to stimulate solventogenesis with conversion of the organic acids into alcohols. That pH value was then maintained constant throughout the remaining experimental period. Previous studies have indeed shown that lowering the pH down to values around 5 would generally effectively allow metabolisation of acids in clostridia with simultaneous accumulation of alcohols, which is desirable when fuel-ethanol and other higher alcohols want to be obtained (Abubackar et al. 2015; Fernández-Naviera et al. 2017c). However, lower pH values might become inhibitory, depending on the strain and specific culture conditions.

A short lag phase of 19 h was observed after inoculation (Figure 9.1); then *Clostridium carboxidivorans* reached its highest biomass concentration of 0.38 g/L within 72 h after seeding the reactor, with a maximum growth rate of 0.074 h^{-1} calculated in the exponential phase, just before natural acidification started. Figure 9.1 shows how biomass growth slowed down

simultaneously to the natural acidification process and ultimately leveled off; that way between t= 48 h (when natural acidification was started) and t= 72 h (when the maximum biomass concentration was reached) the amount biomass only increased by 0.09 g/L. This was expected, as it has been reported that a pH decrease would generally inhibit biomass growth and acid production in the HBE (Hexanol-Butanol-Ethanol) fermentation process (Fernández-Naveira et al. 2017a). The same effect of natural acidification on biomass growth was also recently found in another study using the same strain (Fernández-Naviera et al. 2017c).

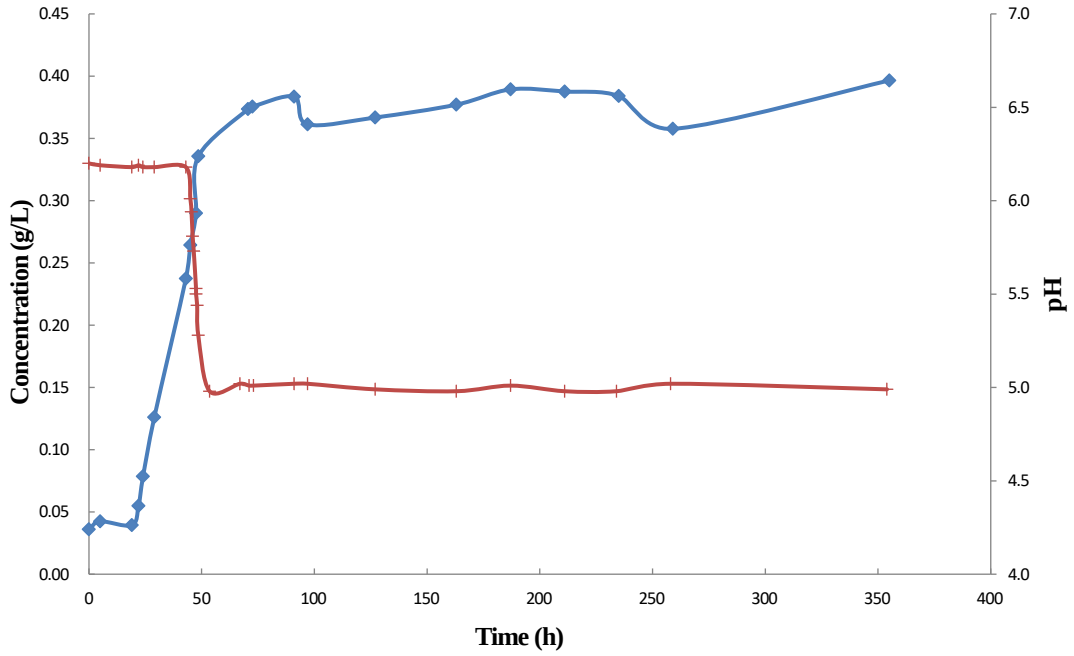

Figure 9.1 Control experiment with trace metals and natural acidification from pH 6.20 to 5.00. Measured growth expressed in g/L over time (blue diamonds) and pH values (red plus)

The production of acids was observed as soon as biomass started to increase (Figure 9.2), with acetic acid being the first acid to be detected, around t= 19 h. That acid reached a maximum concentration of 2.52 g/L, 54 h after inoculation. Later on, butyric acid appeared, at t= 48 h, reaching a maximum value of 0.51 g/L about 67h after seeding the reactor. C2, C4 and then C6 fatty acids would generally appear in that chronological order, with increasing number of carbon atoms. Hexanoic acid would be the last acid to be produced, after acetic and butyric acids; but that acid was not detected in this experiment, which could be attributable to the fast pH decrease in the early stages of the experiment, prematurely finalizing the acidogenic stage. For that same reason, the fast acidification down to pH 5.00 led to lower concentrations of all other acids (acetic acid, butyric acid) as well, due to the negative effect of such low pH conditions on the acidogenic stage. In some other recently published report hexanoic acid was detected 50-60

h after inoculation when maintaining a higher pH (Fernández-Naviera et al. 2017c). Similarily, hexanoic acid was detected when natural acidification was allowed but only down to a less acidic pH of 5.60 (unpublished data) (compared to pH 5.00 in the present study). In both those previous cases hexanoic acid was found in the medium, confirming the negative effect on the accumulation of longer chain fatty acids (C4 and C6, mainly) of early strong acidification or acidification down to low pH values. Provided the final pH is not too low, natural acidification of the medium will have a positive effect on the solventogenic stage, not only in gas fermentation but also in the fermentation of carbohydrates by acetogenic bacteria; and the conversion of acids into alcohols would be stimulated as a result of acidification (Kennes et al. 2016; Millat et al. 2013), while slowing down or ending the production of more acids. This explains the consumption of acids and the conversion of those acids into alcohols when pH 5.00 had been reached. Nevertheless, the production of ethanol started already at a higher pH value, at the same time as acetic acid was detected, which does also largely agree with data reported in other studies with *Clostridium carboxidivorans* (Fernández-Naviera et al. 2016; Fernández-Naviera et al. 2017c). Thereby, alcohols could presumably be produced from the assimilation of acids as well as from the gaseous substrates to some extent. The appearance of ethanol during the acidogenic stage is assumed to be related to the presence of trace metals (W, Se) in the medium, potentially involved in the activity of specific enzymes. Other experiments, shown hereafter, support such hypothesis, as the absence of one or both trace metals in the culture medium largely impedes ethanol production during the acidogenic stage. Besides, acetic acid production stopped at low pH (5.00), while the concentration of ethanol kept increasing after such acidification. In Figure 9.2, it can be observed that the acids were basically completely consumed and reached values near 0 g/L soon after the pH had dropped down to 5.00.

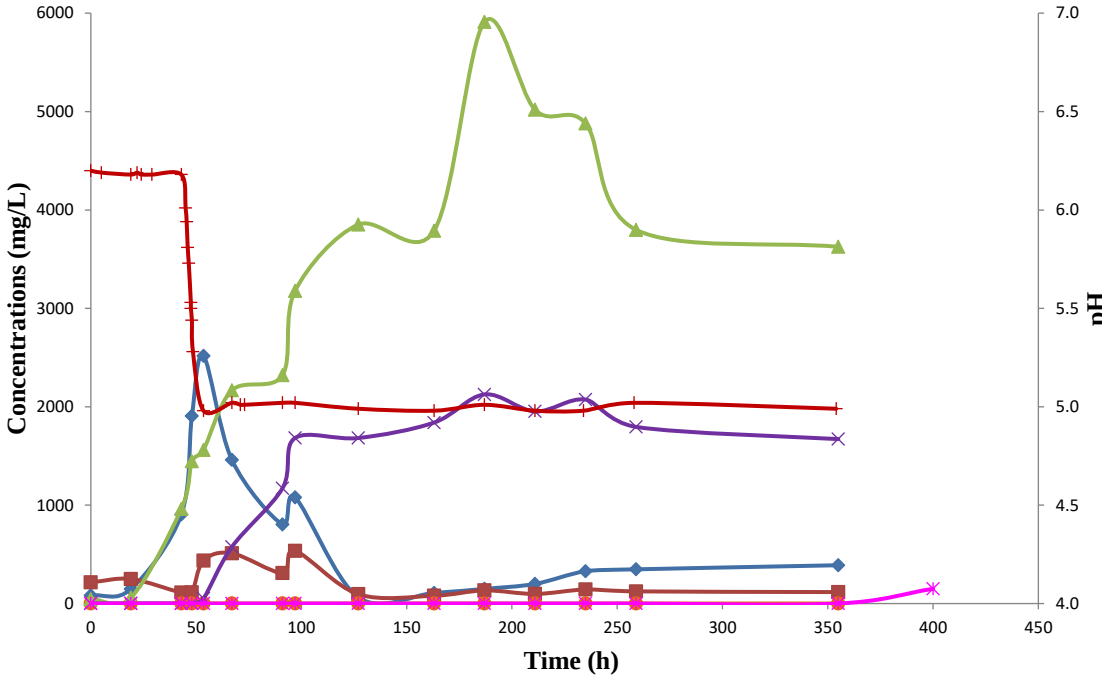

Figure 9.2 Control experiment with trace metals and natural acidification from pH 6.20 to 5.00. production of metabolites: acetic acid (blue diamonds), butyric acid (red squares), hexanoic acid (orange circles), ethanol (green triangles), butanol (purple crossmarks), hexanol (pink star) expressed in mg/L over time, and pH values (red plus).

The maximum accumulation of alcohols was observed 187 h after inoculation, corresponding to 5.91 g/L for ethanol and 2.13 g/L for butanol. No hexanol was detected throughout the experiment, which would largely be due to the absence of hexanoic acid production, as mentioned above (Figure 9.2). The alcohol production rates, calculated in this experiment, were 0.096 g ethanol/h*g of biomass and 0.043 g butanol/h*g of biomass.

The results reached under these experimental conditions are relevant from an industrial viewpoint as they allow to selectively produce ethanol and higher alcohols such as butanol or even hexanol, with only minor amounts of by-products, e.g. fatty acids. Obtaining a fermented medium highly enriched in the desired end products (i.e. alcohols) simplifies downstream processing of any industrial process and reduces the corresponding costs. The presence of various fermentation by-products at non-negligible concentrations would otherwise significantly increase production costs (Ranjan et al. 2012), while their accumulation is largely avoided under the operating conditions evaluated in this study. The production of ethanol as single alcohol and major end product through C1-gas fermentation has been studied in clostridia and has recently been considered for cost-effective precommercial application confirming the industrial relevance of this gas fermentation technology (Daniell et al. 2012). Besides, as reported

recently, in biobased industries, it is more economical and environmentally-friendly to produce alcohols from cheap and abundant gases (pollutants) such as CO and CO_2, as suggested here, rather than from carbohydrates derived from biomass or food/feed (Dürre 2016).

CO and CO_2 evolution

CO consumption was measured during all the experiment (Figure 9.3). A maximum carbon monoxide consumption of 89 % was found 67 h after inoculation, when the highest biomass concentration was reached. This is understandable as biomass growth is known to take place during acidogenesis, simultaneous to a high consumption of the gaseous substrate (Fernández-Naveira et al. 2017a). Once the maximum biomass concentration was reached, the CO consumption decreased progressively, as a consequence of the cessation of bacterial growth at the onset of the solventogenic stage.

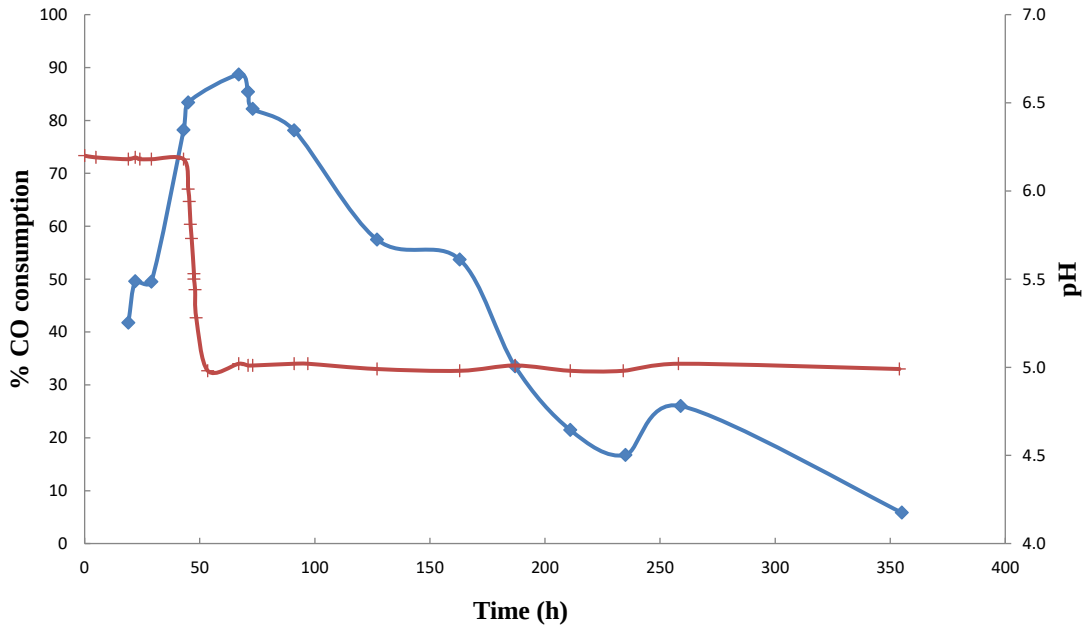

Figure 9.3 Control experiment with trace metals and natural acidification from pH 6.20 to 5.00. Percentage of CO consumption over time (blue diamonds), and pH values (red plus)

As soon as the fermentor was inoculated, *C. carboxidivorans* started producing CO_2 (Figure 9.4), as a consequence of CO consumption, known to be converted to acids and CO_2 in the HBE fermentation process (Fernández-Naveira et al. 2017a). Afterwards, when natural acidification took place, CO_2 started to be consumed resulting in a decrease of biomass growth and the beginning of the production of alcohols (Figure 9.4). In HBE fermentation, carbon dioxide would thus initially be produced while it will subsequently be consumed in a later stage, depending

also on the availability of compounds such as hydrogen, present in syngas and required in carbon dioxide consumption.

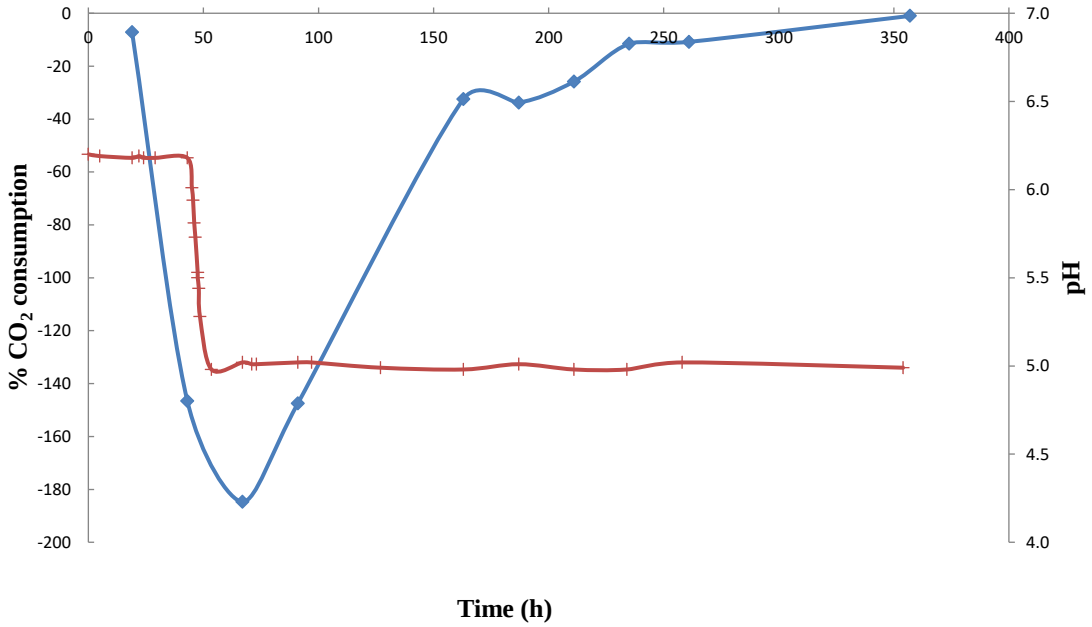

Figure 9.4. Control experiment with trace metals and natural acidification from pH 6.20 to 5.00. Percentage of CO_2 consumption over time (blue diamonds),and pH values (red plus).

In order to confirm that the selective production of alcohols, with negligible accumulation of fatty acids as by-products, is the result of the presence of trace metals and an optimal pH, additional experiments were performed, under different trace metal and pH conditions, as described below.

9.3.2 Continuous gas-fed bioreactor without W and Se and natural acidification from pH 6.20 to 5.00

Biomass and solvent production

In order to avoid and elucidate the possible effect of pH besides the effect of trace metals (W, Se), this experiment was performed under the same conditions as the first one, but without the trace metals. Similarly as in the first experiment, a short (19 h) lag phase was observed after inoculating *C. carboxidivorans* (Figure 9.5). Afterwards, the strain started growing exponentially until natural acidification was allowed, 48 h after inoculation. The maximum biomass concentration was 0.38 g/L, reached 90 h after start-up of the bioreactor (Figure 9.5). The growth rate was estimated during the exponential phase (between 22 and 46 hours after inoculation) and was found to reach a value of 0.068 h^{-1}. The maximum amount biomass was

similar as in the first experiment, but the growth rate was somewhat lower in this modified medium, without W and Se, compared to the control experiment. As expected, (natural) acidification exhibited the same negative effect on biomass growth as observed in the first experiment; growth does first slowdown to eventually end up stopping at pH 5.00.

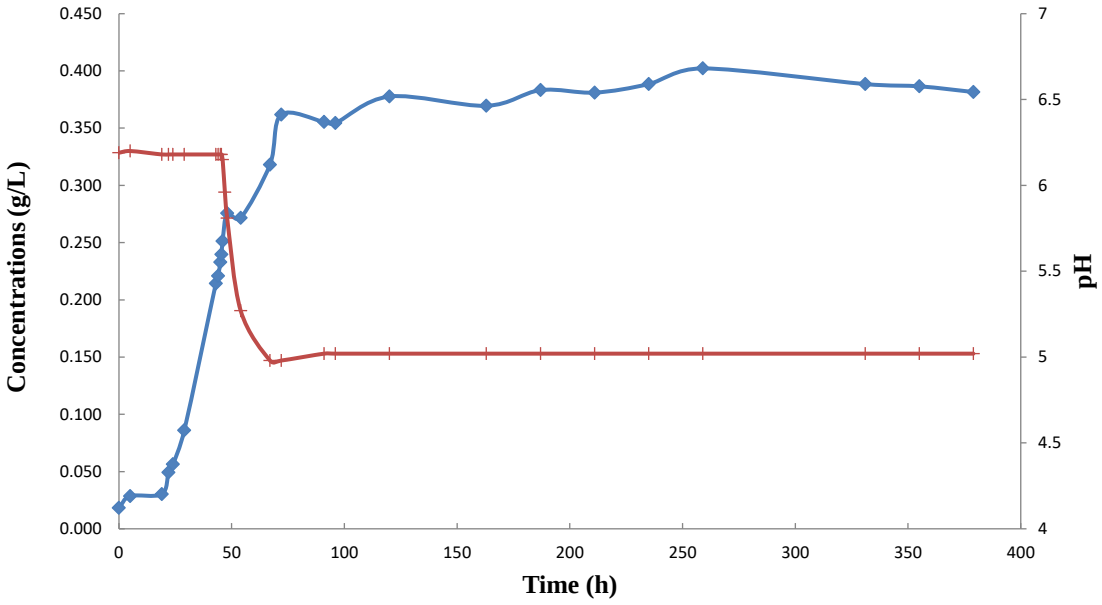

Figure 9.5 Continuous bioreactor without W and Se and natural acidification from pH 6.20 to 5.00. Measured growth expressed in g/L over time (blue diamonds) and pH values (red plus).

A typical acids production pattern was found in this assay. Both here as well as in the other experiment described above, acetic acid was the first acid to be produced, simultaneously with bacterial growth. A maximum acetic acid concentration of 4.11 g/L was detected, 91 hours after inoculation, when the highest biomass concentration was reached (Figure 9.6). Butyric acid and hexanoic acid appeared around the end of the acidification stage, with maximum values of 0.72 g/L and 0.21 g/L, respectively, 91 h after inoculation (Figure 9.6). W and Se are expected to stimulate the activity of enzymes used in anaerobic gas fermentation or used in the conversion of acids into alcohols. Selenium has, among others, been found to affect the activity of formate dehydrogenase in the gas fermenting species *C. ljungdahlii* (Çakar et al. 2018), while tungsten has been suggested to stimulate the conversion of acetic acid into acetaldehyde in *C.*

autoethanogenum due to its presence in aldehyde:ferredoxin oxidoreductase (AFOR), the step before of the conversion of that acetaldehyde into ethanol via the alcohol dehydrogenase (ADH) (Abubackar et al. 2015). The absence or presence of such trace metals did not notably affect the production of organic fatty acids; but mainly their conversion into alcohols. That way, the absence of those trace metals might justify the higher maximum concentrations of all three acids in this experiment (Figure 9.6) compared to the previous experiment with W and Se (Figure 9.2). For that same reason, hardly any ethanol is produced here during the acidogenic stage in absence of those trace metals, contrary to what was observed in their presence in the previous assay with the trace metals.

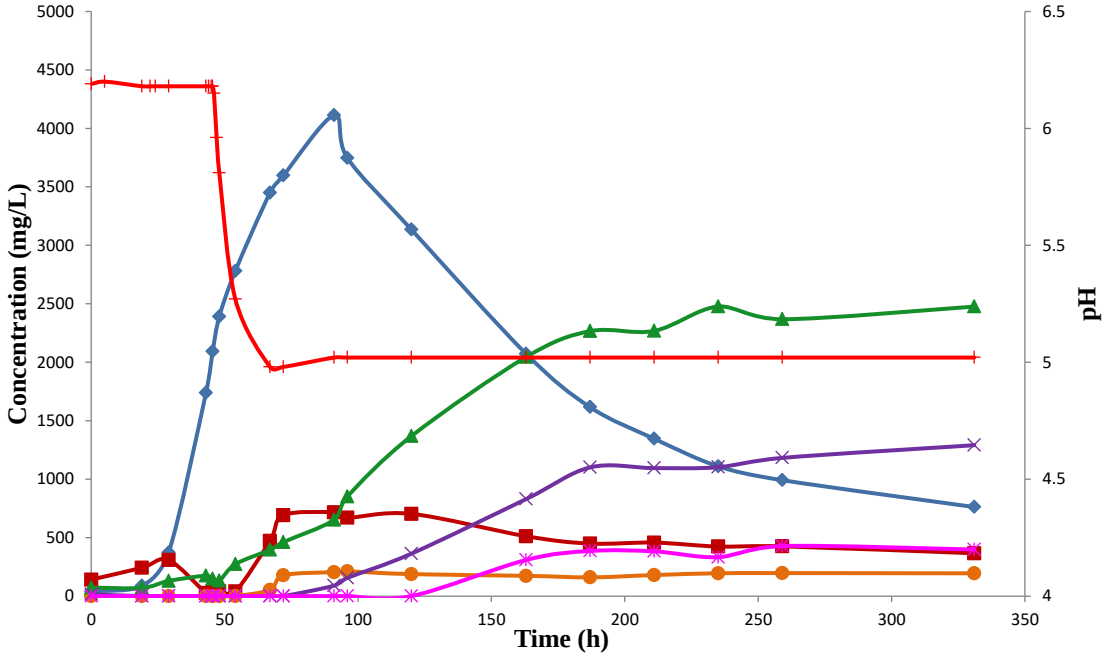

Figure 9.6 Continuous bioreactor without W and Se and natural acidification from pH 6.20 to 5.00. Production of metabolites: acetic acid (blue diamonds), butyric acid (red squares), hexanoic acid (orange circles), ethanol (green triangles), butanol (purple crossmarks), hexanol (pink star) expressed in mg/L over time, and pH values (red plus).

Although some ethanol appeared a few hours after acetic acid was first detected (28h), most of the alcohol was produced during the solventogenic stage, reaching its maximum value of 2.48 g/L 235 h after inoculation (Figure 9.6). The first traces of butanol were found 91 hours after the experimental start-up; and it then reached a maximum concentration of 0.83 g/L, 163 h after inoculation. At that time, between t= 120 h and t= 163 h, hexanol just appeared, reaching its

maximum value of 0.39 g/L, 187 h after starting the experiment (Figure 9.6). Similarly as for the acids, shorter chain alcohols are produced first, following a chronological order of production with increasing carbon numbers, from C2 to C4 and finally C6.

The alcohol production rates were found to be 0.069 g ethanol/h*g of biomass, 0.029 mg butanol/h*g of biomass and 0.020 g hexanol/h*g of biomass. Compared with the first experiment, which was operated under the same conditions (natural acidification down to pH 5.00), except for the absence of W and Se, here lower maximum concentrations of alcohols were reached together with lower production rates, which can be considered to be due to the lack of relevant trace metals in the culture medium. However, hexanol was produced and detected in this experiment as well as hexanoic acid that can be converted to the corresponding C6-alcohol, resulting therefore in the presence of hexanol. The production of alcohols is thus clearly stimulated by the presence of specific trace metals, while omitting such trace metals would allow to selectively accumulate organic acids in the HBE fermentation process. This confirms the original hypothesis on the key role of specific trace metals on the production of alcohols in this HBE fermentation process using syngas as substrate mixture.

CO and CO_2 evolution

CO consumption and growth of *C. carboxidivorans* started simultaneously, soon after inoculation. The maximum CO consumption was reached 68 h after inoculation with a value of 76 % (Figure 9.7). Afterwards, it quickly dropped when medium acidification was allowed, down to minimum values around 15-19 %. Although CO consumption was slightly lower than in the previous experiment, the pattern was otherwise very similar, meaning that both CO assimilation and biomass growth decreased when natural acidification took place. Besides, CO concentrations were measured once day; thus a somewhat higher maximum could have been reached, though not detected.

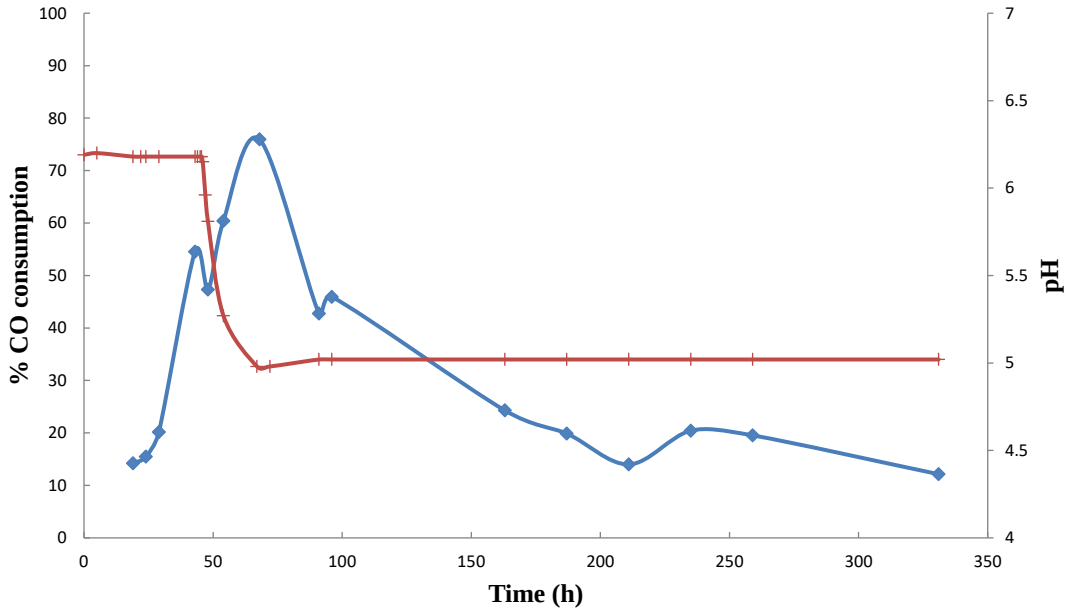

Figure 9.7 Continuous bioreactor without W and Se and natural acidification from pH 6.20 to 5.00. Percentage of CO consumption over time (blue diamonds), and pH values (red plus).

CO_2 consumption and production are shown in Figure 9.8. CO_2 was produced at the same time as CO was being consumed, reaching its maximum production 67 h after inoculation, exactly when CO consumption reached its maximum value. When CO consumption started deceasing, CO_2 production started dropping as well. At the end of the experiment a lower CO_2 production was detected, due to the decrease of CO consumption. As explained above and based on stoichiometric bioconversion equations published elsewhere (Fernández-Naveira et al. 2017a), it can be considered that the amount hydrogen present in the syngas mixture used in this study might not have been high enough to remove all the amount carbon dioxide originally present in the syngas mixture together with the amount produced during the early stages of the process. This is true both in presence or absence of W and Se, and was observed in this experiment as well as in the experiment with a complete trace metals solution.

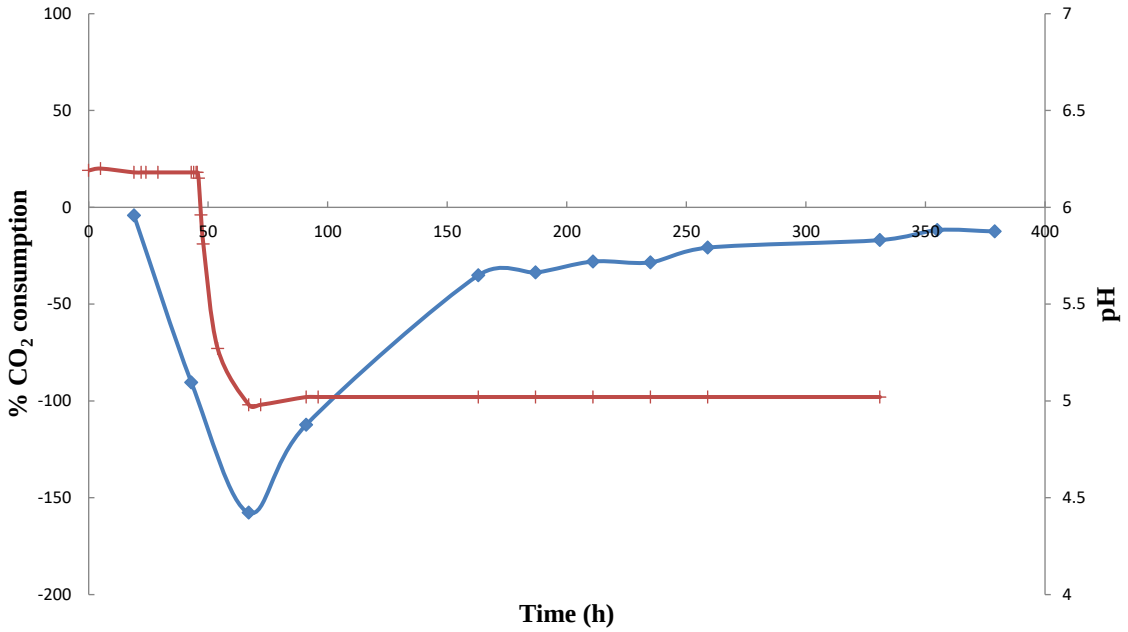

Figure 9.8 Continuous bioreactor without W and Se and natural acidification from pH 6.20 to 5.00. Percentage of CO_2 consumption over time (blue diamonds) and pH values (red plus).

9.3.3 Continuous gas-fed bioreactor without W and Se at constant pH 6.20

Biomass and solvent production

The absence of trace metals such as W or Se together with bioreactor operation at a constant high pH is expected to favour the production of acids while significantly reducing the presence of alcohols, since both a high pH and the absence of such trace metals would be favourable to acidogenesis and unfavourable to solventogenesis. Such hypothesis was indeed confirmed in this experiment. Growth of *C. carboxidivorans* started soon after its inoculation, with a maximum biomass concentration of 0.33 g/L reached 95 h after starting the bioreactor operation (Figure 9.9). A growth rate of 0.076 h^{-1} was calculated during the exponential phase. In this assay the pH was maintained constant at a value of 6.20 throughout all the experimental period. By maintaining the pH at a relatively high value of 6.20, without any natural acidification, a somewhat higher growth rate was found, due to the fact that a higher pH has been observed to promote bacterial growth (Millat et al. 2013; Lee et al. 2008).

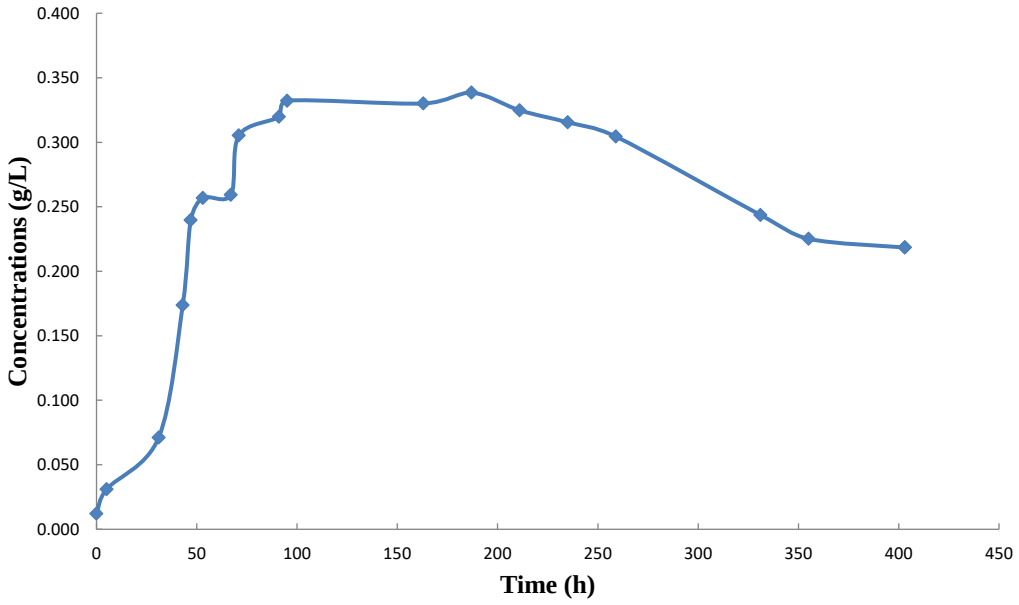

Figure 9.9 Continuous bioreactor without W and Se and constant pH of 6.20. Measured growth expressed in g/L over time.

As observed in the other experiments described above, acetic acid production and bacterial growth appeared simultaneously, but a significantly higher maximum acetic acid concentration, of 7.95 g/L, was found compared to the previous assays, 163 h after inoculation (Figure 9.10). The presence of butyric acid and hexanoic acid was observed 47 h and 91 h after inoculation, reaching maximum values of 1.23 g/L and 0.32 g/L, respectively, 211 h after start-up of the experiment (Figure 9.10). As hypothesized initially, the maximum concentrations of fatty acids were higher in this experiment due to the higher pH value, maintained constant during all the experiment and considered to limit the conversion of acids into alcohols (Phillips et al. 2015). Only ethanol and butanol were produced and at relatively low concentrations, and no hexanol was detected throughout the experiment due to the negative effect of a high pH on the solventogenic stage. Besides, as expected and as observed in the previous experiment, the absence of W and Se, played a key role in the low production of alcohols. The high pH value and the absence of those trace metals exerted cumulative negative effects. That way, alcohols production was lower and delayed in time; ethanol and butanol appeared, respectively, 48 h and 163 h after inoculation. Maximum ethanol and butanol concentrations of hardly 0.61 g/L and 0.069 g/L were reached, 187 h and 259 h after inoculation, respectively. Those concentrations are lower than the concentrations obtained (2.72 g/L ethanol, 1.92 g/L butanol and 0.85 g/L

hexanol) in experiments with syngas, at a higher constant pH of 5.75 and with W and Se added to the medium (Fernández-Naveira et al. 2017c).

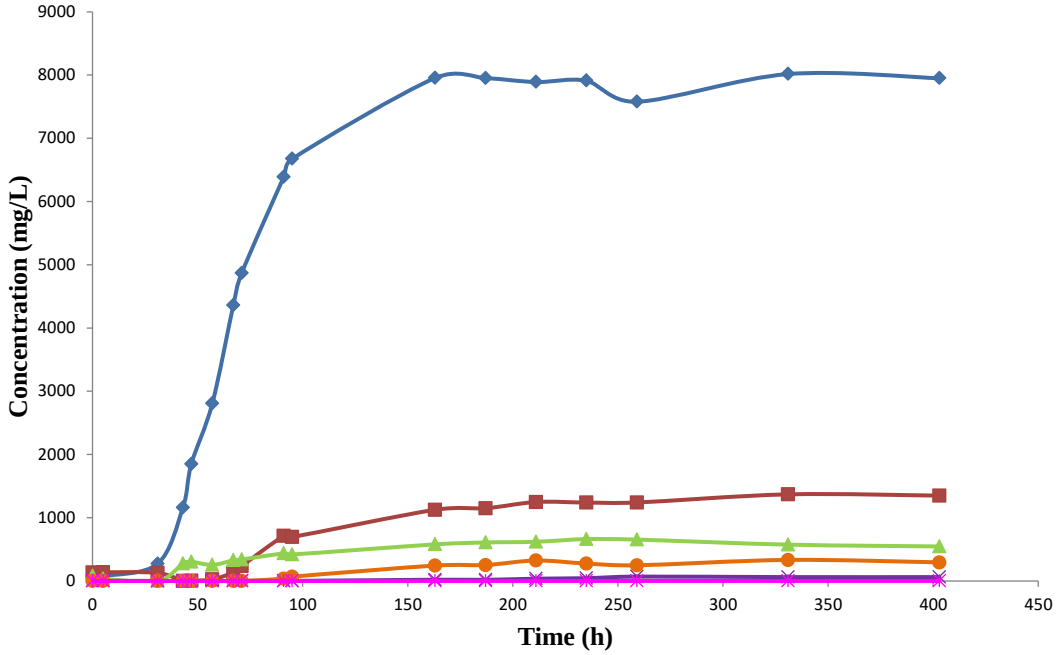

Figure 9.10 Continuous bioreactor without W and Se and constant pH of 6.20. Production of metabolites: acetic acid (blue diamonds), butyric acid (red squares), hexanoic acid (orange circles), ethanol (green triangles), butanol (purple crossmarks), hexanol (pink star) expressed in mg/L over time.

Ethanol and butanol production rates were estimated, with values of 0.006 g ethanol/h*g of biomass and 0.002 g butanol/h*g of biomass. Compared with the previous experiments, those are the lowest alcohol production rates obtained. Those rates are also lower than those obtained in other experiments at higher pH, in presence of W and Se and with syngas as gaseous substrate mixture (Fernández-Naveira et al. 2017c). The absence of W and Se, but also the pH value, do thus clearly affect the metabolic profile and do directly affect the level of production of alcohols in HBE fermentation. Contrary to the first assay, in which the operating conditions allowed for selective production of alcohols, the conditions applied in this experiment resulted in the selective production of organic acids, in both cases with minor presence of by-products. At industrial scale production of either organic acids or alcohols would thus be possible.

CO and CO_2 evolution

Again, CO consumption and cell growth started at the same time. A maximum CO consumption of 67 % was reached 67 h after inoculation. Then, the consumption started gradually to decrease down to values of 20-30 % in the last phase of the experiment (Figure 9.11).

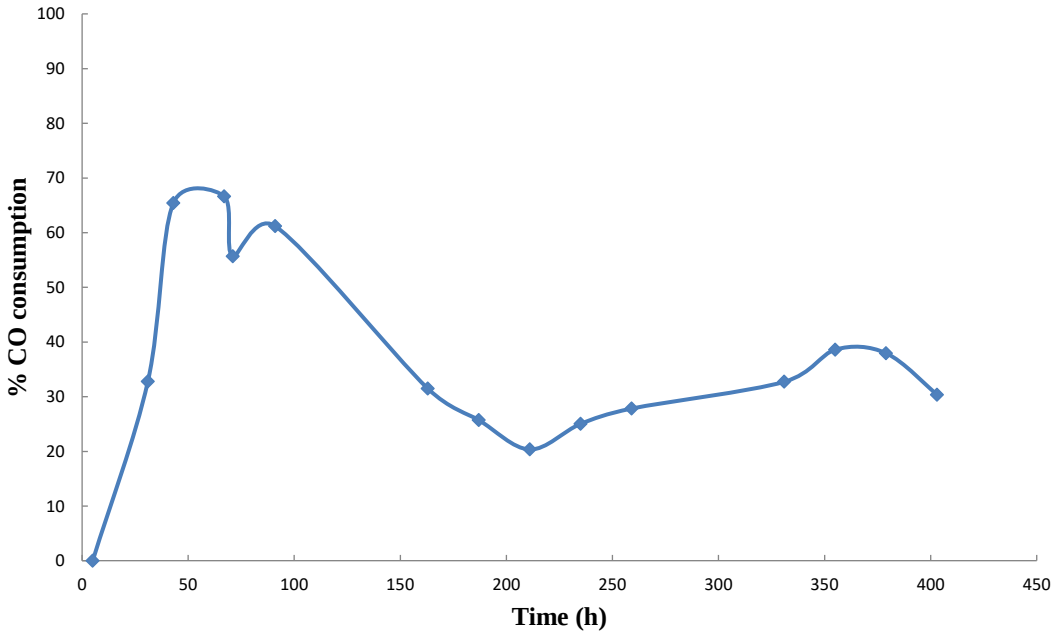

Figure 9.11 Continuous bioreactor without W and Se and constant pH of 6.20. Percentage of CO consumption over time

As in the previous experiments, CO_2 was produced in the first part of the study, during the acidogenic stage. When CO consumption deceased, the production of CO_2 dropped and it started being consumed, 150 h after inoculation. Some net CO_2 consumption was observed (Figure 9.12).

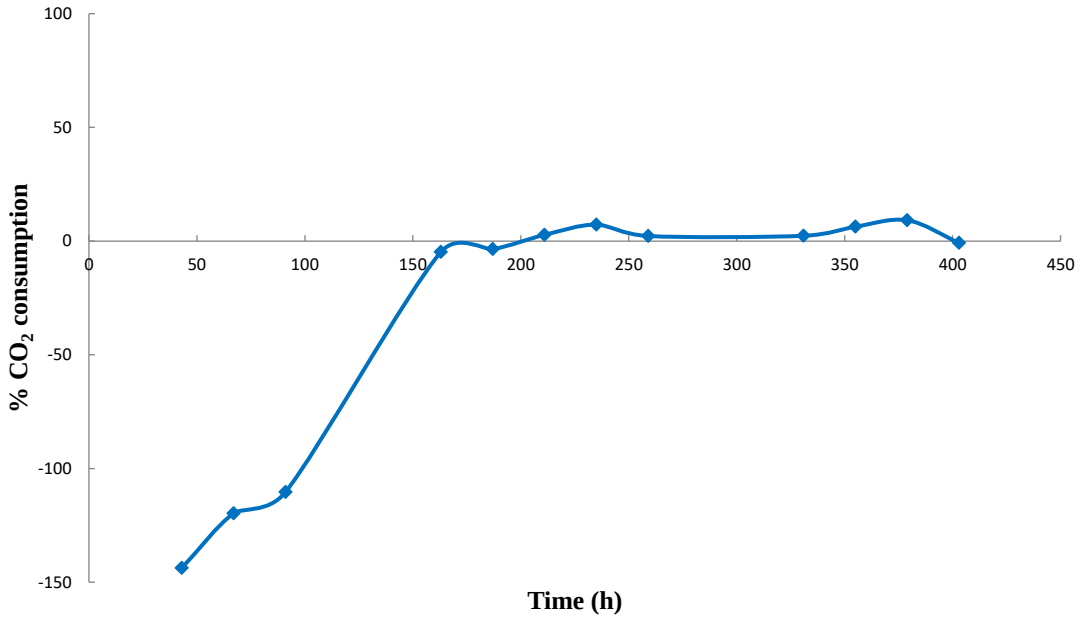

Figure 9.12 Continuous bioreactor without W and Se and constant pH of 6.20. percentage of CO_2 consumption over time

9.3.4 Continuous gas-fed bioreactor without W at constant pH 6.20

Biomass and solvent production

Although W and Se together were considered to be potentially able to affect the overall pathway from the assimilation of C1 gases all the way down to the production of alcohols, a reasonable assumption was raised suggesting that W might have played the most relevant role both in the higher production of acids from C1 gases as well as in the production of alcohols. In order to elucidate to effect of W as individual trace metal compared to Se, an additional experiment was undertaken without W but in the presence of Se. At such pH, growth of *C. carboxidivorans* was observed immediately after inoculation without any lag phase (Figure 9.13). The maximum biomass concentration was reached 48 h after inoculation with a value of 0.42 g/L (Figure 9.13). A growth rate of 0.100 h^{-1} was calculated during the exponential phase. The maximum biomass concentration obtained and growth rate were somewhat higher than in the previous experiment. This could be due to the absence of tungsten in the fermentation broth, as tungsten is a trace metal necessary to metalloenzymes of the WL pathway for the production of alcohols. The production of alcohols is a process very expensive energetically for the bacteria; as

a consequence, it could be assumed that the absence or reduction of production of alcohols allows the bacteria to be more efficient in their growth and production of acids.

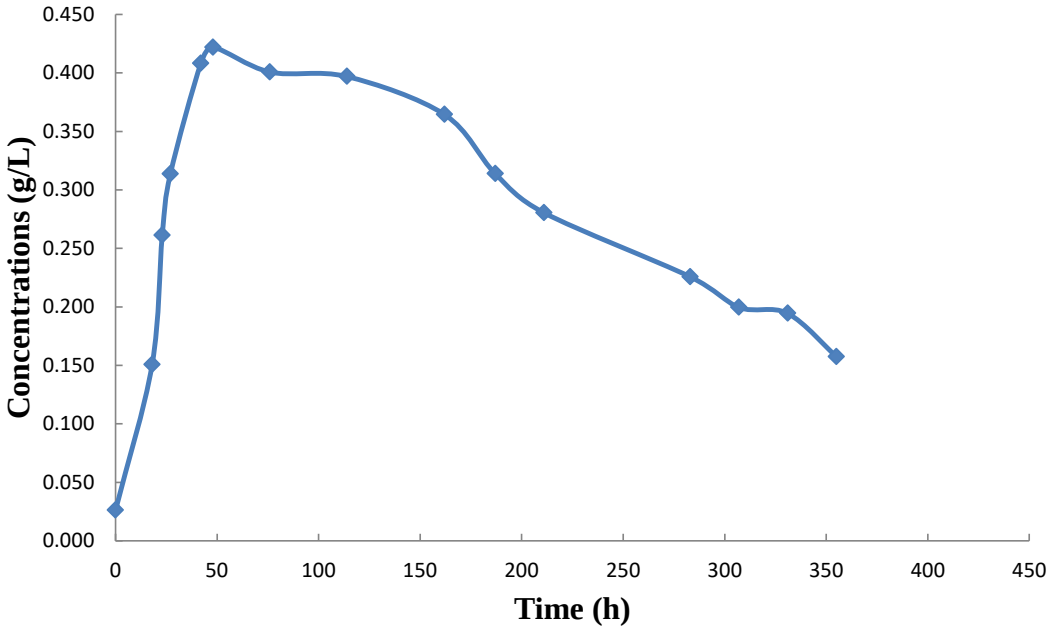

Figure 9.13 Continuous bioreactor without W and constant pH of 6.20. Measured growth expressed in g/L over time

The same pattern of acids production was observed as in the previous experiments, where acetic acid was the first acid to appear, followed by butyric acid and finally hexanoic acid (Figure 9.14). Likewise, acetic acid was produced simultaneously to the bacterial growth, reaching a maximum value of 10.05 g/L 162 h after inoculation (Figure 9.14). *Clostridium carboxidivorans* started to produce butyric acid 50 h after inoculation, while hexanoic acid was observed 211 h after inoculation. Besides, the maximum concentration of those acids was 1.12 g/L for butyric acid and 0.13 g/L for hexanoic acid, around the end of the experiment, after 331 h, when it remained basically constant (Figure 9.14). As was previously hypothesized, the absence of tungsten results in a clear increase in the production of acids, mainly in the case of acetic acid, compared to other data published recently for the same conditions and similar pH values (Fernández-Naveira et al. 2017c).

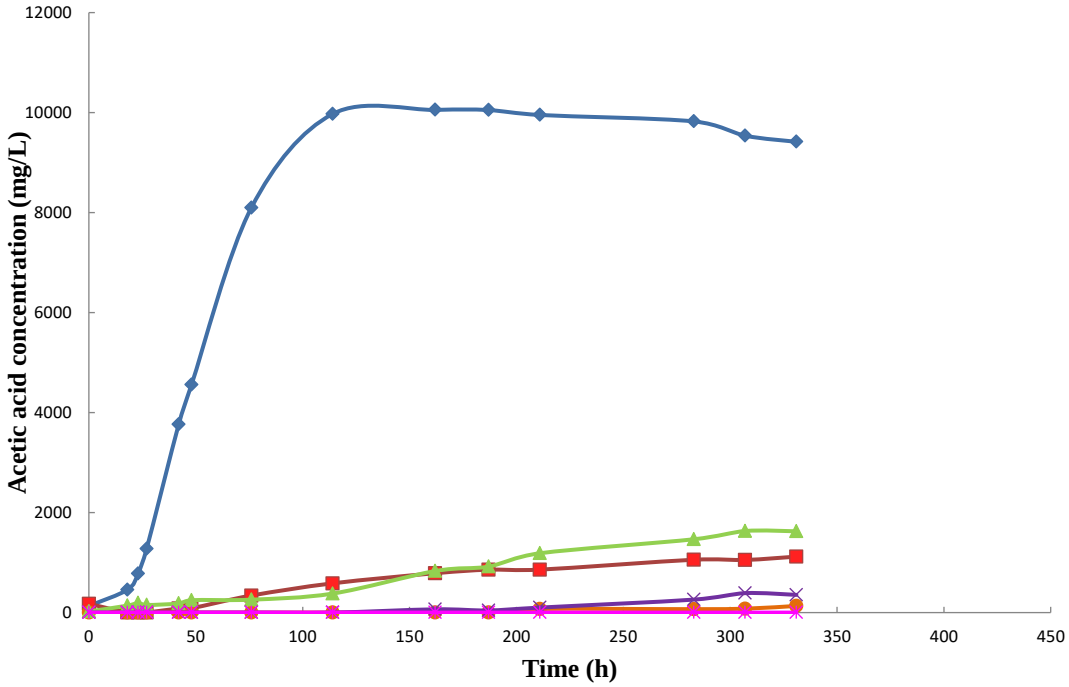

Figure 9.14 Continuous bioreactor without W and constant pH of 6.20. Production of metabolites: acetic acid (blue diamonds), butyric acid (red squares), hexanoic acid (orange circles), ethanol (green triangles), butanol (purple crossmarks), hexanol (pink star) expressed in mg/L over time.

Concerning the alcohols, ethanol was produced as soon as *Clostridium* started to grow, whereas butanol was not observed until 162 h after inoculation (Figure 9.14). Their maximum concentrations were reached simultaneously, 307 h after inoculation, with values of 1.63 g/L and 0.39 g/L, respectively. However, hexanol was not produced at all in this experiment (Figure 9.14). Comparing this experiment with the previous ones and with recent literature data using similar pH conditions (Fernández-Naveira et al. 2016; Fernández-Naveira et al. 2017c), the absence of tungsten appears to lead to lower concentrations of all alcohols in HBE fermentation. However the absence of the two trace metals together (previous experiment) has a negative effect on the maximum biomass concentration and growth rate when these values are compared with this experiment omitting tungsten only. Also, lower concentrations of acids were obtained in the previous experiments in comparison with the present one (Table 9-1), this could be related with the lower biomass concentration obtained during the third experiment in comparison with this last experiment under same pH conditions. In this way, it can be suggested that the lack of both trace metals had more negative effect on the production of alcohols than

the absence of tungsten only, however the major negative effect seems to be produced by the absence of tungsten (Table 9-1). A similar effect was observed by Abubackar et al. (2015) using *C. autoethanogenum* grown in a medium without selenium or tungsten in the medium, for the production of ethanol. *C. autoethanogenum* does not perform the full HBE fermentation process, but still produces acetic acid and ethanol through gas fermentation (Abubackar et al. 2015).

Table 9-1 Effect of trace metals and pH on the selective production of acids and alcohols during the HBE fermentation process.

	Selenium	Tungsten	pH conditions	acids concentrations (g/L)	alcohols concentrations (g/L)
Experiment 1	+	+	Natural acidification (6.2->5.0)	Acetic acid: 2.52 Butyric acid: 0.51 Hexanoic acid: 0 Total: 3.03	Ethanol: 5.91 Butanol: 2.13 Hexanol: 0 Total: 8.04
Experiment 2	-	-	Natural acidification (6.2->5.0)	Acetic acid: 4.11 Butyric acid: 0.72 Hexanoic acid: 0.21 Total: 5.04	Ethanol: 2.48 Butanol: 0.83 Hexanol: 0.39 Total: 3.70
Experiment 3	-	-	Constant pH 6.2	Acetic acid: 7.95 Butyric acid: 1.28 Hexanoic acid: 0.32 Total: 9.55	Ethanol: 0.61 Butanol: 0.07 Hexanol: 0 Total: 0.68
Experiment 4	+	-	Constant pH 6.2	Acetic acid: 10.05 Butyric acid: 1.12 Hexanoic acid: 0.13 Total: 11.30	Ethanol: 1.63 Butanol: 0.39 Hexanol: 0 Total: 2.02

The ethanol and butanol production rates were estimated, reaching 0.021 g ethanol/h*g of biomass and 0.005 g butanol/h*g of biomass, which are, as expected, lower values than obtained in the previous experiments, described above. The data summarized in Table 9-1 clearly allow to see the effect of trace metals and/or pH on the selective production of acids and alcohols.

CO and CO_2 evolution

Carbon monoxide consumption was concomitant with the bacterial growth. Figure 9.15 shows a maximum CO consumption of 84 %, 23 h after inoculation. When the bacterial growth ceased, CO consumption started to decrease as well (50 h after inoculation). Maximum CO consumption was reached somewhat later in the previous experiments. Also, the maximum consumption was

slightly higher in the previous experiment (89 %) with respect to this one (84 %). Although such difference is low, this could be related to the fact that the bacteria can theoretically also produce some amount of alcohols directly from CO based on the WP pathway, which would also be affected by the presence of trace metals, such as W, in the last steps of the pathway. Nevertheless, for energetic reasons, most of the alcohols produced should go through the production of acids first. As mentioned above, *Clostridium carboxidivorans* produces CO_2 during the acidogenic step, as a consequence of CO consumption. This can also be seen in Figure 9.16, where a maximum consumption of -121 % was observed, 43 hours after inoculation, coinciding with the maximum biomass growth and CO consumption. Subsequently, CO_2 was consumed, until it stabilized and reached an approximate value of -20 % (Figure 9.16). When comparing with the previous experiment, it was observed that the production of CO_2 was higher in that experiment, also related to the higher CO consumption.

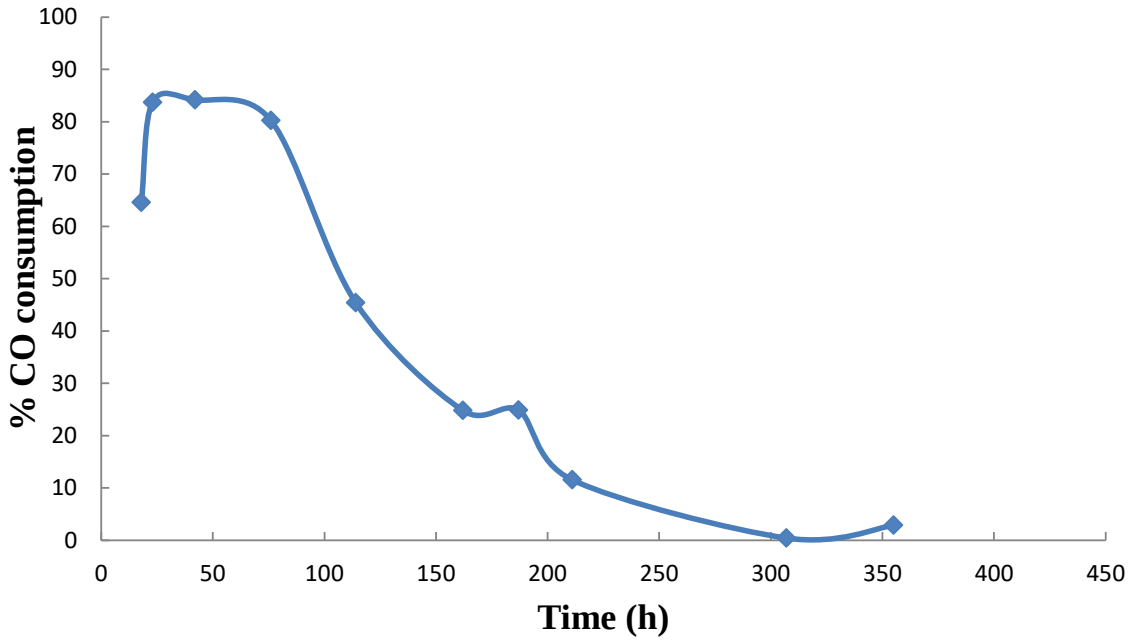

Figure 9.15 Continuous bioreactor without W and constant pH of 6.20. Percentage of CO consumption over time

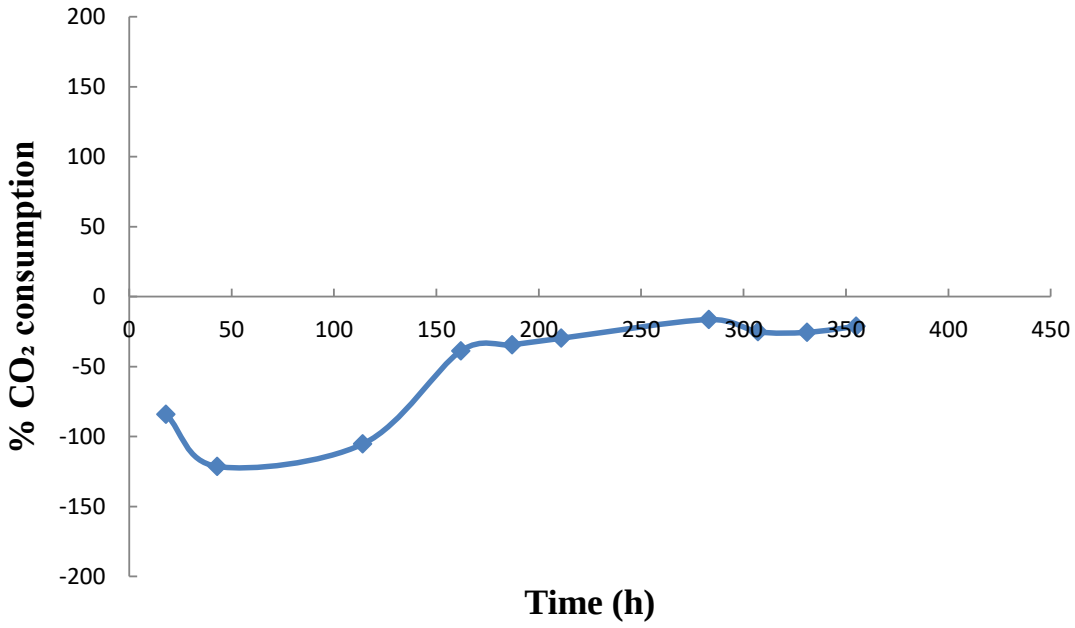

Figure 9.16 Continuous bioreactor without W and constant pH of 6.20. Percentage of CO_2 consumption over time

9.4 Conclusions

It can be concluded that, in syngas HBE fermentation, experimental conditions can be selected in order to direct the bioconversion pattern towards the production of specific metabolites. Tungsten and selenium are components of some metalloenzymes of the Wood-Ljungdhal pathway involved in C1 gas fermentation and in their bioconversion into fatty acids and later into alcohols. In terms of fatty acids and alcohols production, the accumulation of fatty acids was the highest in the absence of tungsten and at constant high pH, followed by conditions without the studied trace metals at constant high pH, then conditions without those trace metals and natural acidification (i.e., low pH), and finally the medium with all trace metals and natural acidification (i.e., low pH). As general rule, it can then be concluded that W plays a major role in HBE fermentation and in solventogenesis in *C. carboxidivorans*, as its presence is required to stimulate the conversion of acids into alcohols, reaching a higher conversion rate at low pH (e.g., around pH 5.00) than at high pH (e.g, around pH 6.20), favourable for the accumulation of alcohols rather than fatty acids. The effect of selenium seemed to be less significant compared to tungsten. Instead a higher pH and the absence of specific trace metals such as W will

stimulate the accumulation of fatty acids rather than alcohols in that organism. The identification of optimal conditions, in terms of trace metals supply, for the selective production of specific metabolites, either organics acids or alcohols, will result in a more efficient and cost-effective industrial production process.

9.5 Acknowledgements

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10 PRODUCTION OF HIGHER ALCOHOLS USING A TWO STAGE BIOREACTOR AND SYNGAS AS CARBON/ENERGY SOURCE

PRODUCTION OF HIGHER ALCOHOLS USING A TWO STAGE BIOREACTOR AND SYNGAS AS CARBON/ENERGY SOURCE

Abstract Obtaining biofuels from biomass is a novel process with several advantages: it helps the development of high commercial value fuels and avoids environmental problems. Acidogenic bacteria use the Wood-Ljungdhal pathway to produce alcohols using different gases as carbon/energy source (for example, CO, CO_2 or syngas). This conversion is carried out in two stages: acidogenesis where the bacterial growth and the production of acids are carried out and solventogenesis where the production of alcohols takes place. In this study, a continuous bioreactor experiment was carried out in two stages, with the objective of separating acidogenesis and solventogenesis in two different bioreactors, that way in one bioreactor (without tungsten, at constant pH) acidogenesis will be stimulated, while in the second one (natural acidification) solventogenesis will take place. The results showed a good separation of acidogenesis from solventogenesis in the two fermenters, obtaining a continuous production of mainly acids in bioreactor 1 and mainly alcohols in bioreactor 2.

Keywords: *Clostridium carboxidivorans*, continuous two stages system, HBE fermentation, syngas.

10.1 Introduction

Higher alcohols are a good option to use as fuels instead of oil (Diender et al. 2016). These compounds are environmentally-friendly and can be obtained in biorefineries using renewable feedstocks, such as biomass, agricultural wastes, waste gases, and other pollutants. That way the production of alcohols from those kinds of feedstocks is a good alternative due to the obtention of metabolites of important commercial value and also because of the use of part of the wastes produced in industrial processes and in other human activities (Li et al. 2018).

HBE (Hexanol-Butanol-Ethanol) fermentation is a novel process allowing to obtain higher alcohols from waste gases. *C. carboxidivorans* is one of the few microorganisms able to carry out that process by means of the Wood-Ljungdahl pathway. This pathway is divided into two branches: the Eastern and the Western branches (Fernández-Naveira et al. 2017a). In the first branch, CO_2 is reduced to obtain formate whereas in the second one CO is taken directly or through the CO_2 transformation. Using this pathway, *C. carboxidivorans* is able to produce acids from gases (CO_2 and CO) with acetyl-CoA as an intermediate metabolite and later these acids could be converted into higher alcohols (Fernández-Naveira et al. 2017a). The Wood-Ljungdahl pathway is a complex process involving numerous enzymes. Each enzyme is responsible of different parts on the pathway, that way their correct activity is necessary for acids and/or alcohols production.

Low productivity rates of alcohols make the production of ethanol and higher alcohols by acetogenic bacteria a costly process (Doll et al. 2018); for that reason an optimization of the parameters and operational system are necessary. There are several parameters and inhibitors of the process to be taken into account: ***pH***, an adequate pH is not only necessary for the specific bacterial growth (Fernández-Naveira et al. 2017a), it is also necessary for the shift between acidogenic and solventogenic stages (Lee et al. 2008; Jones and Woods 1986). ***Temperature***, it is related with the optimal bacterial growth, with the solubility of gaseous substrates and also with the "acid crash" which could inhibit the solventogenic stage (Ramió-Pujol et al. 2015). ***Media composition***, the energy/carbon source is one of the most important parameters in syngas fermentation (Fernández-Naveira et al. 2017a), including the possible effect of some trace metals such as selenium, copper, tungsten… (Fernández-Naveira et al. submitted; Phillips et al. 2015; Saxena and Tanner 2011; Abubackar et al. 2015; Abubackar et al. 2016); the effect of the addition of some reducing compounds to the medium, such as cysteine-HCl (Abubackar et al. 2016), or others such as yeast extract (Mitchell et al., 1998). ***Gas pressure***, to increase the mass-transfer of the carbon source into the liquid media. ***Inhibitory compounds***,

such as methane, ethylene, ethane, acetylene, nitrogen oxide, sulphur compounds that can be present in syngas or waste gases (Ahmed et al., 2006; Ahmed and Lewis 2007; Haryanto et al., 2009; Xu and Lewis 2012). ***Solvent toxicity***, higher alcohols can inhibit the bacterial activity at high concentrations (Woods 1995; Jones and Woods 1986; Liu and Qureshi 2009; Fernández-Naveira et al., 2016a). ***Mass transfer limitation***, the low mass transfer and solubility of the gases used in the fermentation proccess into the aqueous medium is a key aspect of the process, since the transfer of the gas to the medium is related to the efficiency of assimilation by the biocatalyst and the production of acids and alcohols (Klasson et al. 1993; Bredwell et al. 1999). ***Bioreactor configurations,*** is related with a few parameters mentioned before, such as the agitation, the mass transfer limitation, the bubbler system (Fernández-Naveira et al. 2017a).

Taking into account the need for high, cost-effective, productivities and that optimal conditions are different to promote either the production of acids or the production of alcohols, in the last years the development of continuous fermentation systems has become a priority in order to improve the accumulation of alcohols based on the separation of the two bioconversion processes (acidogenesis/solventogenesis) in two different bioreactors. With such bioreactors configuration, in one bioreactor acidogenesis can be promoted in a continuous way, while in the other bioreactor the medium and operating condition are optimized for alcohol production.

In this study the use of a two stage continuous system is promoted using *C. carboxidivorans* as a biocatalyst and syngas as a carbon/energy source, with the principal aim of developing a continuous production of ethanol and higher alcohols by means of the HBE fermentation.

10.2 Material and methods

10.2.1 Microorganism and culture media

Clostridium carboxidivorans P7 DSM 15243, was the strain used in the following experiments. It was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was maintained under anaerobic conditions on modified basal medium at pH 6.0 with carbon monoxide as a carbon source (Liou et al. 2005; Tanner 2007). The medium used in the different bioreactors was prepared using the following composition (per liter distilled water): 1 g yeast extract; 25 mL mineral solution; 10 mL trace metal solution; 10 mL vitamins stock solution; 1 mL resazurin; 0.60 g cysteine-HCl.

The mineral stock solution used in the basal medium, was previously prepared with the following composition (per liter distilled water): 80 g sodium chloride, 100 g ammonium chloride, 10 g potassium chloride, 10 g potassium monophosphate, 20 g magnesium sulfate, and 4 g calcium chloride.

Each one of the two stage bioreactors used in that assay contained different trace metals in the medium, that way, it was necessary to prepare two specific trace metal solutions. One of the bioreactors (bioreactor 2) had the complete trace metal solution, with the following composition (per liter distilled water): 2 g nitrilotriacetic acid, 1 g manganese sulfate, 0.80 g ferrous ammonium sulfate, 0.20 g cobalt chloride, 0.20 g zinc sulfate, and 20 mg each of cupric chloride, nickel chloride, sodium molybdate, sodium selenate, and sodium tungstate. The second trace metal solution used for bioreactor 1, had the same composition but omitting the addition of sodium selenate and sodium tungstate.

Finally a vitamin stock solution was prepared, with the following composition (per liter distilled water): 10 mg pyridoxine, 5 mg each of thiamine, riboflavin, calcium pantothenate, thioctic acid, para-amino benzoic acid, nicotinic acid, and vitamin B12, and 2 mg each of D-biotin, folic acid, and 2-mercaptoethanesulfonic acid.

10.2.2 Two stage bioreactors

The continuous two stage bioreactors, consisted in the use of two bioreactors of 2 L (New Brunswick Scientific, Edison, NJ, USA) with a working volume of 1.2 L each, interconnected among them, where acidogenesis (production of acids) will mainly take place in bioreactor 1 (F1), while solventeogénesis (production of alcohols) will take place in bioreactor 2 (F2).

To achieve this separation of the two phases of the fermentation process in 2 separate bioreactors, the methodology used is split into the two steps described below:

The first step of the experimental study consisted of the individual assembly of F1 and F2 operated discontinuously with a 3-day lapse of time between start-up of F1 and, later, F2. The discontinuous assembly of each of these fermenters followed the methodology previously described in other studies (Fernández-Naveira et al. 2016b; Fernández-Naveira et al., 2017b).

In the case of bioreactor 1 (F1), the composition of the medium consisted of a complete medium but without the addition of Se and W in the trace metals solution in order to improve the production of acids and limit the production of alcohols, as was described in the previous section. A gas mixture was used as carbon/energy source with the following composition $CO:CO_2:H_2:N_2$ (30:10:20:40) which was supplied with a constant flow rate of 10 mL/min using a

mass flow controller (Aalborg GFC 17, Mülheim, Germany) throughout the experiment. In this case, the pH remained constant with a value of 6.00 throughout the experiment.

In bioreactor 2 (F2), a complete medium with all the required trace metals was used, as described in the previous section. CO was used as the sole carbon/energy source at a constant flow rate of 10 mL/min using a mass flow controller (Aalborg GFC 17, Mülheim, Germany). This gas feeding, unlike bioreactor 1, was only supplied during the 1st phase (discontinuous operation) since during the 2nd phase this gaseous feeding was removed, because during the solventogenesis there is no CO consumption. In this case, the initial pH was the same as in F1 (6.00) and a natural acidification was performed down to a value of 5.00 when the concentration of acetic acid reached 2.50 – 3.00 g/L.

The second step of the experiment consisted in connecting F1 and F2, to carry out a continuous fermentation process. This step was carried out once F2 had consumed practically all the acids, more or less coinciding with the maximum concentration of acids reached in F1. In this way, with the assembly of the continuous system, it is intended that F1 would continue to produce acids, while F2 would receive the acids from F1 and convert them into alcohols.

The connection process between F1 and F2 in the continuous experiment consisted of:

- A fresh medium feed with the same initial composition as F1 (without Se and without W), which was supplied to F1 with a flow rate of 293 mL/day.

- A connection between F1 and F2, in such a way that F2 receives biomass and acids from F1 with the same flow rate as in the previous case, 293 mL/day.

- A feeding of a metal solution of Se and W, which is supplied to F2 in order to avoid the dilution of these metals in this bioreactor, due to the addition of the F1 medium.

- Recirculation of biomass in F2: consisting in the use of a cell filter (Cellflo polyethersulfone with 500 cm^2 of surface area and 0.20 µm of pore size (Spectrum laboratories, Inc., USA)) which filters the content of F2 to a flow rate of 293 mL/days, the bacterial content returns to the bioreactor, while the remaining medium is eliminated.

In all cases oxygen-resistant tygon tubes were used.

10.2.3 Growth measurement

1 mL of liquid sample was withdrawn daily from both bioreactors to measure the biomass concentration (g/L) using a UV-Visible spectrophotometer at a wavelength (λ) of 600 nm (Hitachi, Model U-200, Pacis & Giralt, Madrid, Spain). The optical density value obtained (OD

λ=600nm) was used to estimate the biomass concentration (g/L), using a calibration curve obtained previously.

The growth rates (μ), expressed in $hour^{-1}$, were estimated during the exponential bacterial growth using the following equation:

$$\mu = \frac{[ln(N_t) - ln(N_0)]}{(t - t_0)} \qquad \text{Eq. 10.1}$$

where N_t is the cell density (g/L) at time t (expressed in hours) and N_0 is the cell density at time 0 (t_0).

10.2.4 Gas-phase CO and CO_2 concentrations

1 mL of gaseouse sample from the outlet of the bioreactor was removed in order to quantify the concentrations of CO and CO_2 using an HP 6890 gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD). The GC was fitted with a 15-m HP-PLOT Molecular Sieve 5A column (ID, 0.53 mm; film thickness, 50 μm), using helium as a carried gas, a temperature of 50° C was maintained constant as the oven temperature and 150° C was maintained constant in the injection port and in the detector (Fernández-Naveira et al. 2017b). The measurement of CO_2 was made using an HP 5890 gas chromatograph, equipped with a TCD and with helium as a carrier gas. The injection temperature used was kepted at 90° C, the oven temperature was kepted at 25° C, and detection temperature was kepted at 100° C.

10.2.5 Fermentation products

1 mL of liquid samples were removed from the bioreactors at least every 24 h. When the maximum acids or alcohols production was reached, more than one sample per day was analyzed. The concentrations of the different metabolites were measured using an HPLC (HP1100, Agilent Co., USA) equipped with a supelcogel C-610 column and a UV detector at a wavelength of 210 nm and as a mobile phase, a 0.1 % ortho-phosphoric acid solution at a flow rate of 0.5 mL/min was used. The column temperature was kept at 30° C.

All the samples were previously centrifuged at 7000 g during 3 min using a benchtop centrifuge (ELMI Skyline ltd CM 70M07) and filtered through a filter with a pore size of 0.20 μm before analyzing by HPLC.

10.3 Results and discussion

10.3.1 Growth measurement

In both bioreactors (F1 and F2), *Clostridium carboxidivorans* started to grow immediately after inoculation, without any lag phase, as can be observed in Figure 10.1 and Figure 10.2. In the case of bioreactor 1, the maximum of biomass was reached a few hours sooner than in bioreactor 2, with the following values: 0.47 g/L 64 hours after inoculation and 0.55 g/L 72 hours after inoculation, respectively (Figure 10.1 and Figure 10.2).

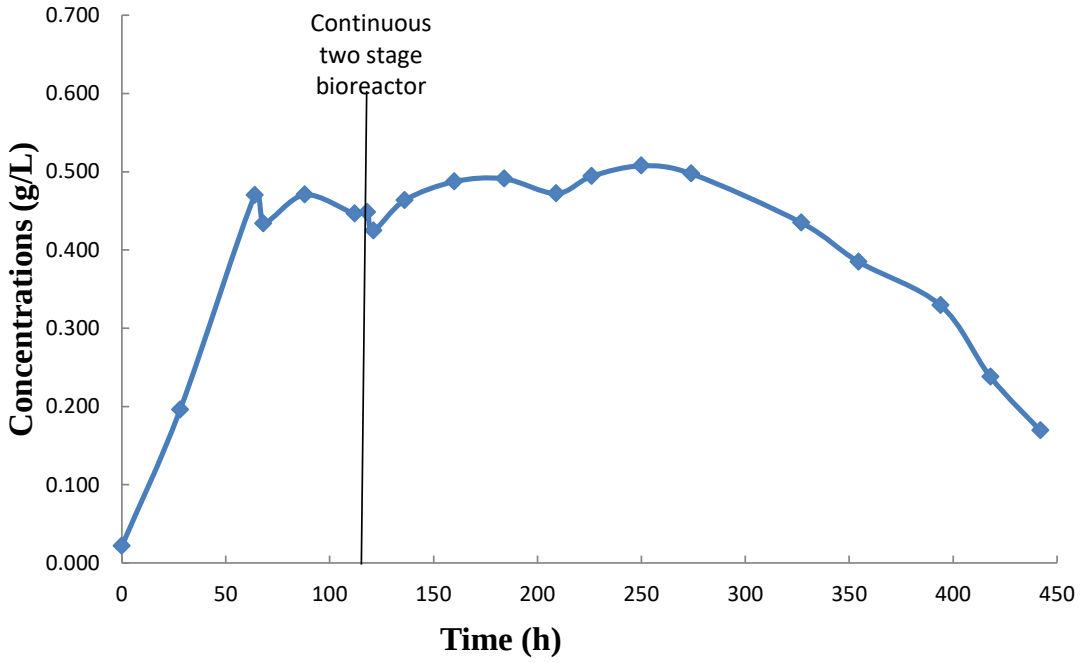

Figure 10.1 Bioreactor 1: Growth of *Clostridium carboxidivorans* expressed in g/L over time.

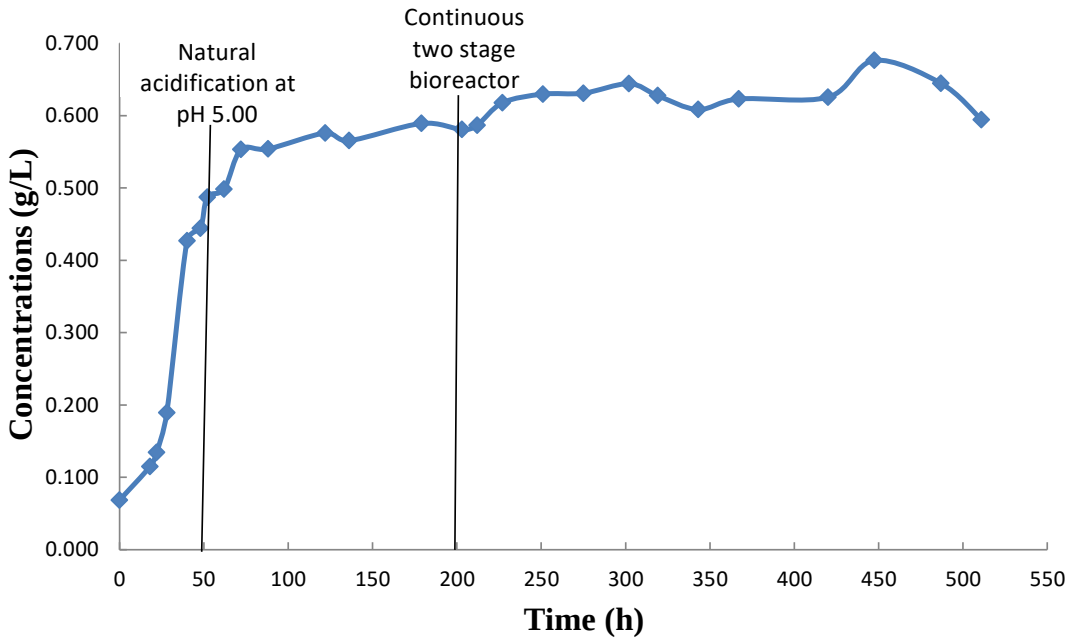

Figure 10.2 Bioreactor 2: Growth of *Clostridium carboxidivorans* expressed in g/L over time.

The growth ratee were calculated during the exponential phase of growth, in the case of bioreactor 1 a value of 0.079 h^{-1} was obtained between 0 and 28 hours whereas in bioreactor 2 a lower value of 0.068 h^{-1} was obtained between 28 and 40 hours. Comparing these results, it was observed that bioreactor 1 has a higher growth rate than bioreactor 2, as was also seen in another experiment (Fernández-Naveira et al. 2017b). This is because the lack of tungsten inhibits the production of alcohols, and this way the bacteria are more efficient in terms of growth and production of acids.

Figure 10.1 shows that the continuous assembly was done, approximately, 100 hours after inoculation. The biomass growth after the continuous assembly remained constant until 250 hours, but then from that moment the biomass started to decrease. In the case of bioreactor 2, 206 hours after inoculation, the two stage continuous system was assembled and as shown in Figure 10.2 the biomass remained constant from that moment and throughout the entire experiment.

10.3.2 Metabolites production

As had been seen in previous experiments, using similar conditions, the first acid to appear in both bioreactors was acetic acid, which started to be produced immediately after inoculation. As expected, the concentration of this acid was higher in bioreactor 1 (without selenium and

without tungsten) than in bioreactor 2, reaching maximum values of 9.28 g/L after 112h and 4.07 g/L 62 h after inoculation, respectively (Figure 10.3, Figure 10.4).

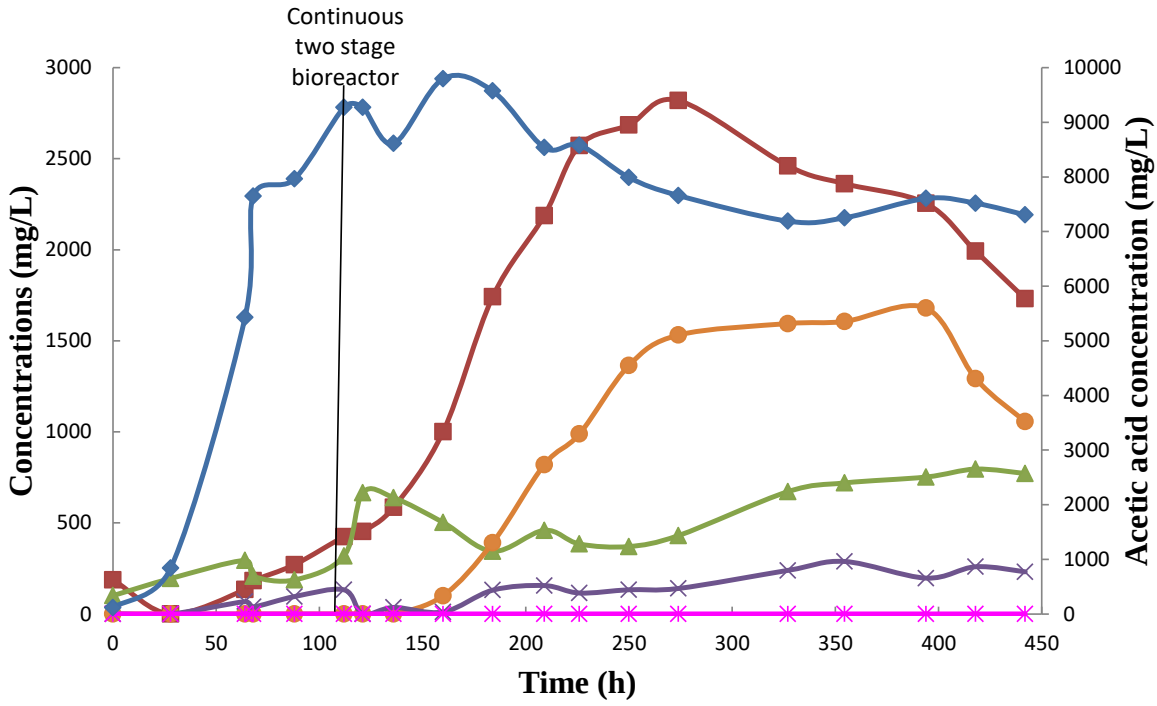

Figure 10.3 Bioreactor 1: Production of metabolites: acetic acid (blue diamonds), butyric acid (red squares), hexanoic acid (orange circles), ethanol (green triangles), butanol (purple crossmarks), hexanol (pink star) expressed in mg/L over time.

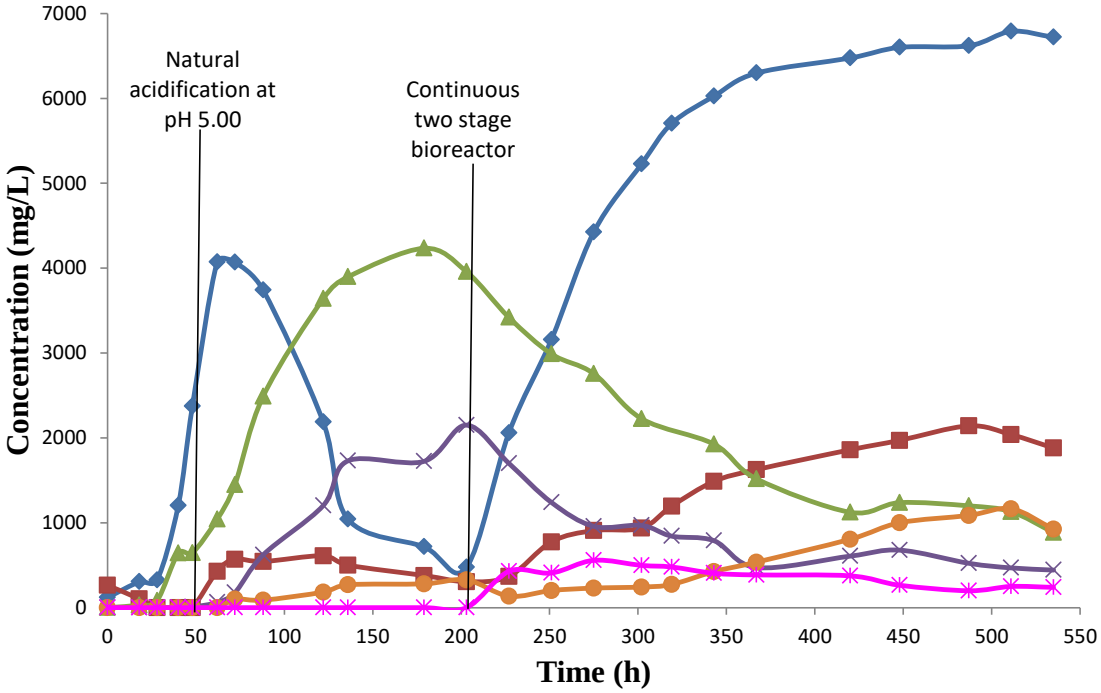

Figure 10.4 Bioreactor 2: Production of metabolites: acetic acid (blue diamonds), butyric acid (red squares), hexanoic acid (orange circles), ethanol (green triangles), butanol (purple crossmarks), hexanol (pink star) expressed in mg/L over time.

However, after the assembly of the continuous system in two stages, the concentration of acetic acid continued to increase, in the case of bioreactor 1, reaching a maximum value of 9.800 g/L 160 h after inoculation (Figure 10.3), while in the case of bioreactor 2 this increase in concentration of acetic acid corresponds to the feeding of medium received from bioreactor 1 (Figure 10.4).

After this, in bioreactor 1 it is observed that the concentration of acetic acid started to decrease (Figure 10.3), which may be due to the dilution effect generated by the feeding of 293 mL daily of fresh medium that was added to the bioreactor. To test the effect of this dilution, the theoretical and experimental production of acetic acid were represented from the moment of the assembly of the continuous system (Figure 10.5). It can be observed how there is still some acetic acid production, obtaining experimental values greater than the theoretical ones, which in the initial moments of the continuous system is up to 3.09 g/L as shown in Figure 10.6. The daily acetic acid productivity is around 1.500 g/L per day, as shown in Figure 10.6. One of the reasons why the concentration seems to decrease in Figure 10.3, could be that a feed of 293 mL/day is too high and consequently the dilution exerted in the bioreactor is higher than the production of acids.

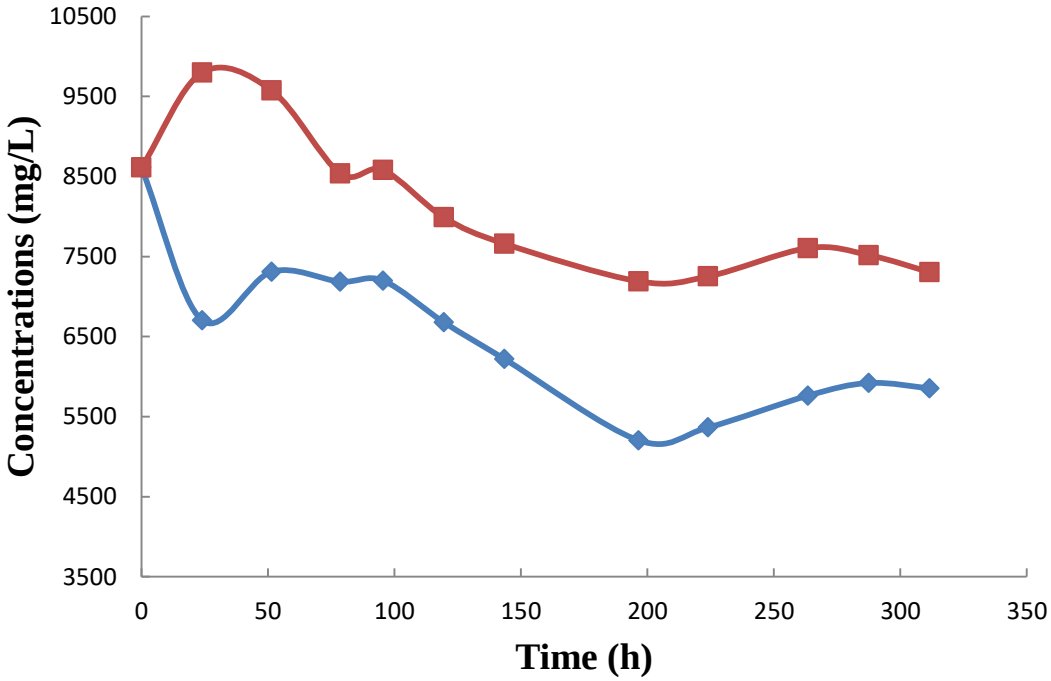

Figure 10.5 Bioreactor 1: Comparison between the theoretical (blue diamonds) and experimental productivity (red squares) for acetic acid expressed in mg/L over time.

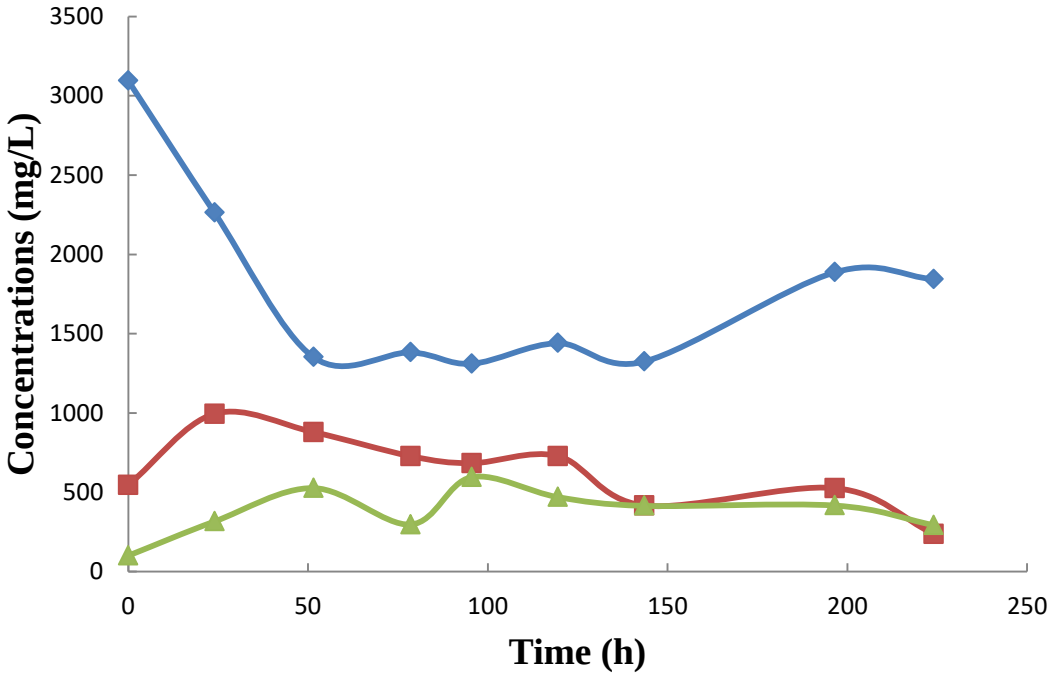

Figure 10.6 Bioreactor 1: Daily productivity of the three acids expressed in mg/L over time

In the case of butyric acid, it started to be produced 64 hours after inoculation in bioreactor 1, as shown in Figure 10.3. At the time of assembly of the continuous system, the concentration of

butyric acid observed was 0.42 g/L (Figure 10.3). However, the maximum concentration was observed 274 hours after inoculation, with a value of 2.82 g/L. In the case of bioreactor 2, butyric acid started to be produced 72 h after inoculation and reached a maximum value of 0.61 g/L at 122 hours (Figure 10.4). As in the previous case, the production of that acid is higher in bioreactor 1 than in bioreactor 2 due to the lack of trace metals, that impedes the conversion of these acids into alcohols, thus allowing a higher accumulation of the acids.

As in the previous case, in bioreactor 2 an increase in the concentration of butyric acid is observed after the assembly of the continuous system, which is due to the feeding of acid-rich medium of bioreactor F1 that receives bioreactor F2 (Figure 10.4). While in bioreactor 1, Figure 10.3 shows a decrease in the concentration of butyric acid due to the dilution effect caused by the feeding of fresh medium. As in the case of acetic acid, the theoretical and experimental production was checked after the assembly of the continuous system (Figure 10.7),and it was observed that there is still production of butyric acid, obtaining a daily productivity value of 0.70 g/L (Figure 10.6).

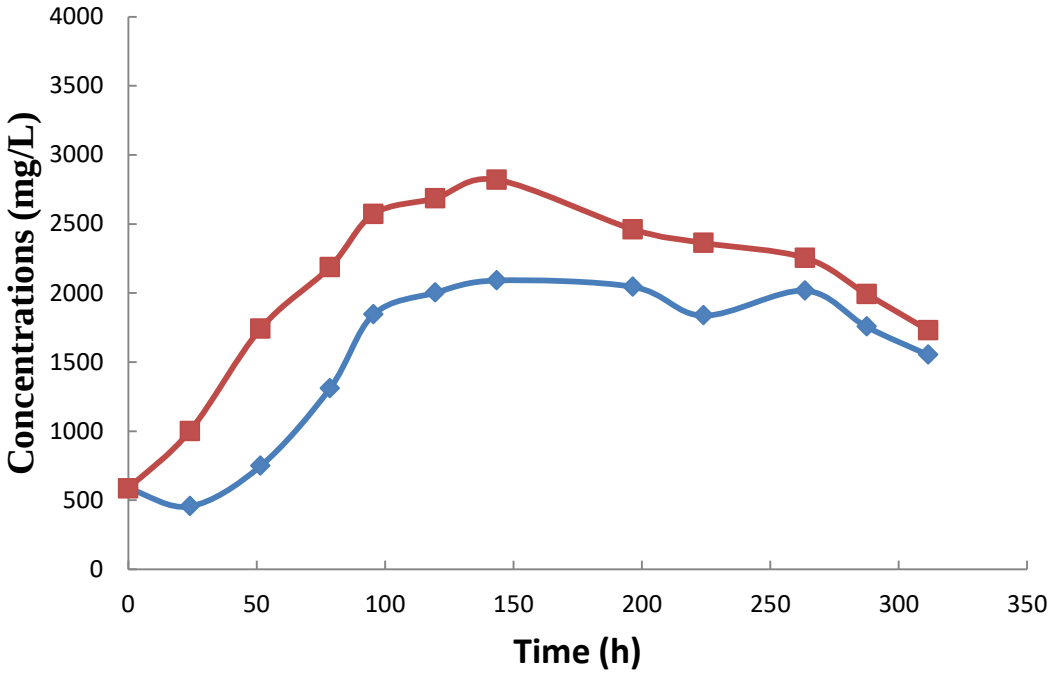

Figure 10.7 Bioreactor 1: Comparison between the theoretical (blue diamonds) and experimental productivity (red squares) for butytic acid expressed in mg/L over time.

The last acid to be produced was hexanoic acid, which in the case of bioreactor 1, is not observed until the assembly of the continuous system, specifically 160 hours after inoculation, as shown in Figure 10.3, reaching a maximum value of 1.68 g/L 394 hours after the inoculation;

while in bioreactor 2, hexanoic acid started to be detected after 72 h, reaching a maximum value of 0.33 g/L at 203 hours, just before the assembly of the continuous system (Figure 10.4). In the same way as in the previous cases, the theoretical productivity values of hexanoic acid for bioreactor 1 were estimated (Figure 10.8) and they were compared to the experimental ones obtained, to determine if there is a dilution effect, although in this case this is not observed in Figure 10.3. In this way, the daily productivity of hexanoic acid was observed in Figure 10.6, and had a constant value of approximately 0.30 g/L daily. In the case of bioreactor 2, the increase observed after the continuous assembly, as in the previous cases, was given by the acid-rich feed of F1 to such bioreactor.

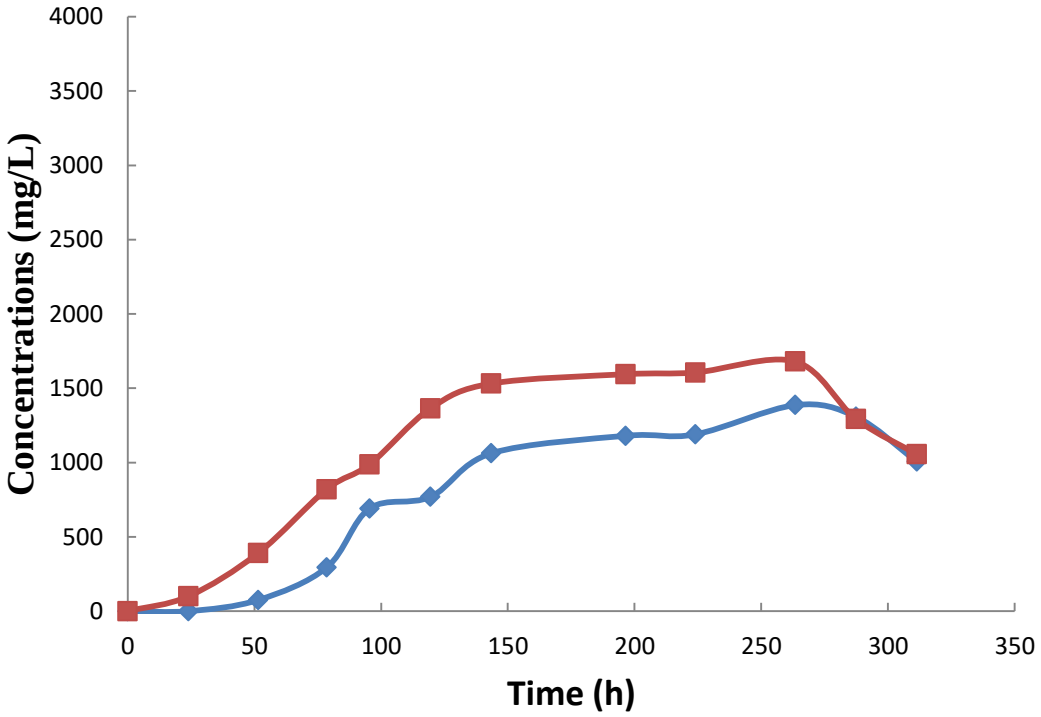

Figure 10.8 Bioreactor 1: Comparison between the theoretical (blue diamonds) and experimental productivity (red squares) for hexanoic acid expressed in mg/L over time.

As for the production of alcohols, it was observed in bioreactor 1 (Figure 10.3) that there was production of alcohols throughout the experiment. Ethanol appeared immediately after inoculation, while butanol did not appear until 68 hours after inoculation. Ethanol reached a maximum value of 0.80 g/L 418 hours after inoculation, while butanol reached a maximum value of 0.29 g/L at 355 hours. However, no hexanol was detected during all the experimental process (Figure 10.3).

The production of alcohols in this bioreactor is low because the lack of tungsten inhibits its production; however, the production of acids is favored by the absence of tungsten (Fernández-Naveira et al. submitted).

On the other hand, in bioreactor 2 it was observed that there was production of alcohols before the assembly of the continuous system (Figure 10.4). Ethanol appeared 18 hours after inoculation and butanol 52 hours after inoculation; in the case of hexanol, its appearance was just after the assembly of the continuous system (Figure 10.4). At 179 hours, just before the assembly of the continuous system, ethanol and butanol reached values of 4.23 g/L and 1.73 g/L, respectively; while hexanol reached its maximum concentration after the assembly at 275 hours with a value of 0.58 g/L as shown in Figure 10.4.

In Figure 10.4 it was observed that the concentration of alcohols after the assembly of the continuous system seems to decrease. That decrease was due to the dilution effect generated by the feeding of medium of bioreactor 1, but it was verified through calculations of the theoretical and experimental productivity that, despite this decrease in concentration observed in Figure 10.4, there is still a net production. This fact can be observed for ethanol in Figure 10.9, obtaining a daily productivity of approximately 0.18 g/L; in the case of butanol (Figure 10.10) a productivity of 0.06 g/L daily was obtained; and finally for hexanol, a productivity of 0.09 g/L daily was observed (Figure 10.11).

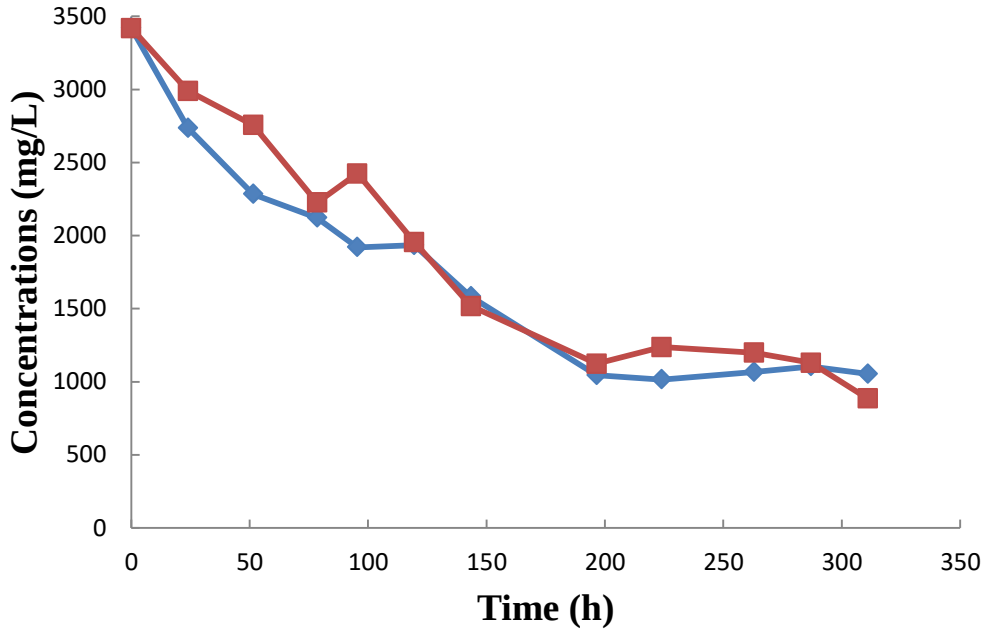

Figure 10.9 Bioreactor 2: Comparison between the theoretical (blue diamonds) and experimental productivity (red squares) for ethanol expressed in mg/L over time.

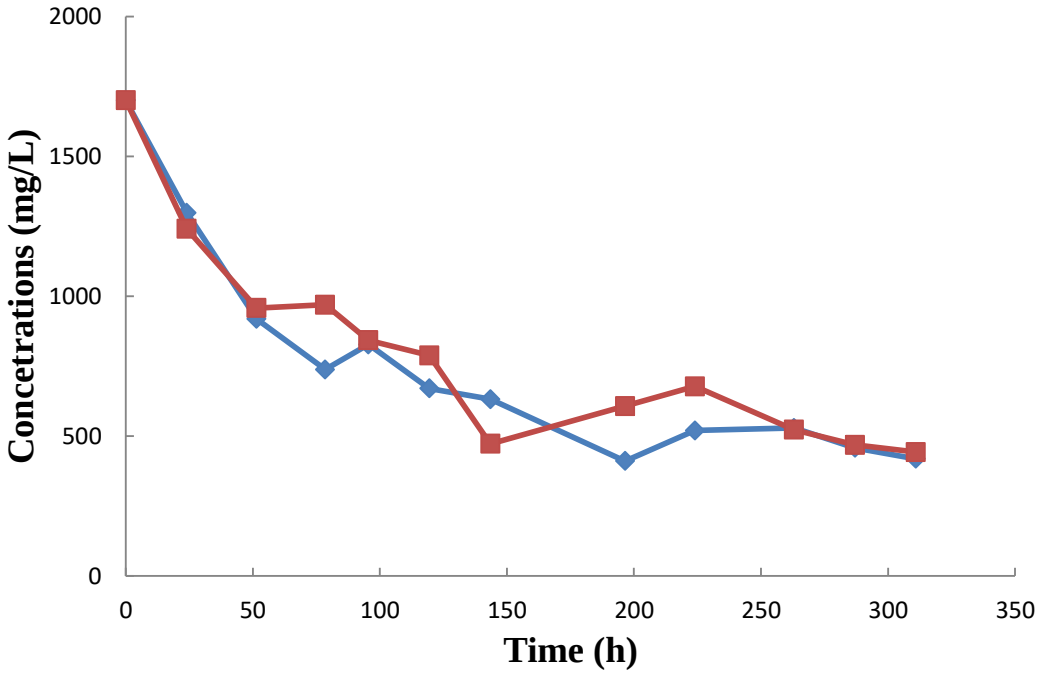

Figure 10.10 Bioreactor 2: Comparison between the theoretical (blue diamonds) and experimental productivity (red squares) for butanol expressed in mg/L over time.

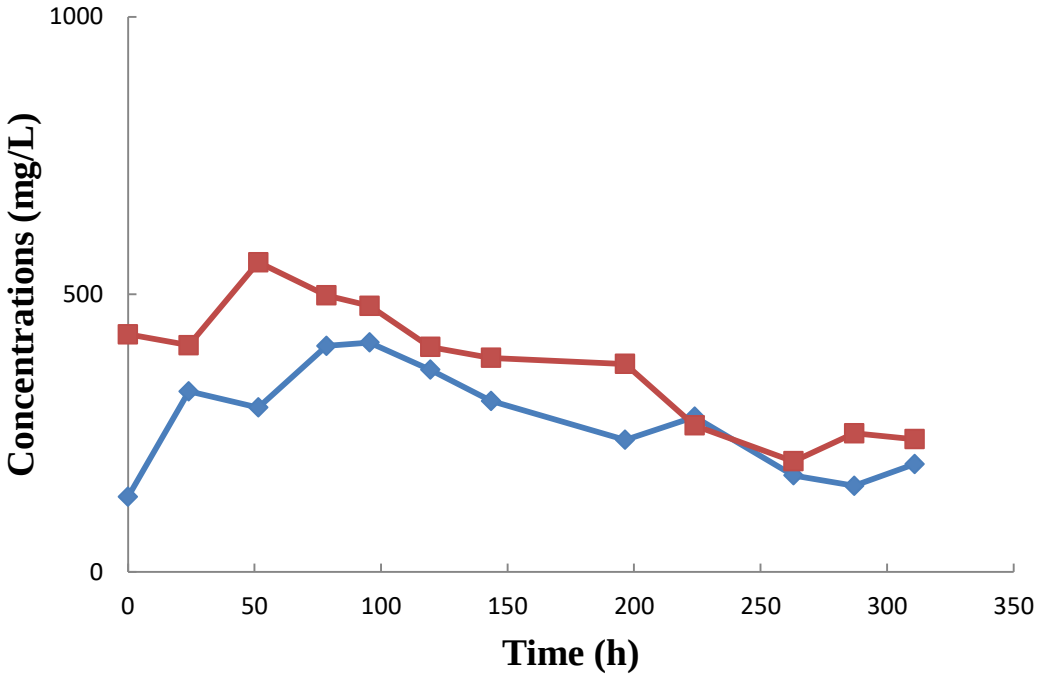

Figure 10.11 Bioreactor 2: Comparison between the theoretical (blue diamonds) and experimental productivity (red squares) for hexanol expressed in mg/L over time.

Likewise, the production rates of the three alcohols were calculated. In the case of ethanol and butanol, they were estimated before the assembly of the continuous system, and for hexanol it was estimated after the assembly of the continuous system. The ethanol production rate was calculated between 62 and 88 hours, obtaining a value of 0.105 g/h g biomass; the butanol production rate was calculated between 122 and 136 hours with a value of 0.066 g/h g biomass and finally the hexanol production rate was calculated between 203 and 227 hours with a value of 0.031 g / h g of biomass.

Comparing this with the data obtained from the bibliography at a similar pH (4.75), using CO as a carbon/energy source, the value of the production rates are similar in the case of butanol (0.070 g/h g biomass) and in the case of ethanol it is slightly lower (0.160 g/h g biomass) (Fernández-Naveira et al., 2016).

10.3.3 CO and CO_2 consumptions

In the case of bioreactor 1, the CO consumption of *Clostridium carboxidivorans* was measured. As shown in Figure 10.12, before the continuous assembly, a maximum consumption of 69 % was reached 88 hours after the inoculation; after that, the CO consumption started to decrease. Later, coinciding with the assembly of the continuous system, the CO consumption increased

again until reaching a maximum value of 95 % at 136 hours. And once the bacterium stopped growing, it was observed that the consumption of CO started to decrease again, until it stabilized at a value of 65 % (Figure 10.12).

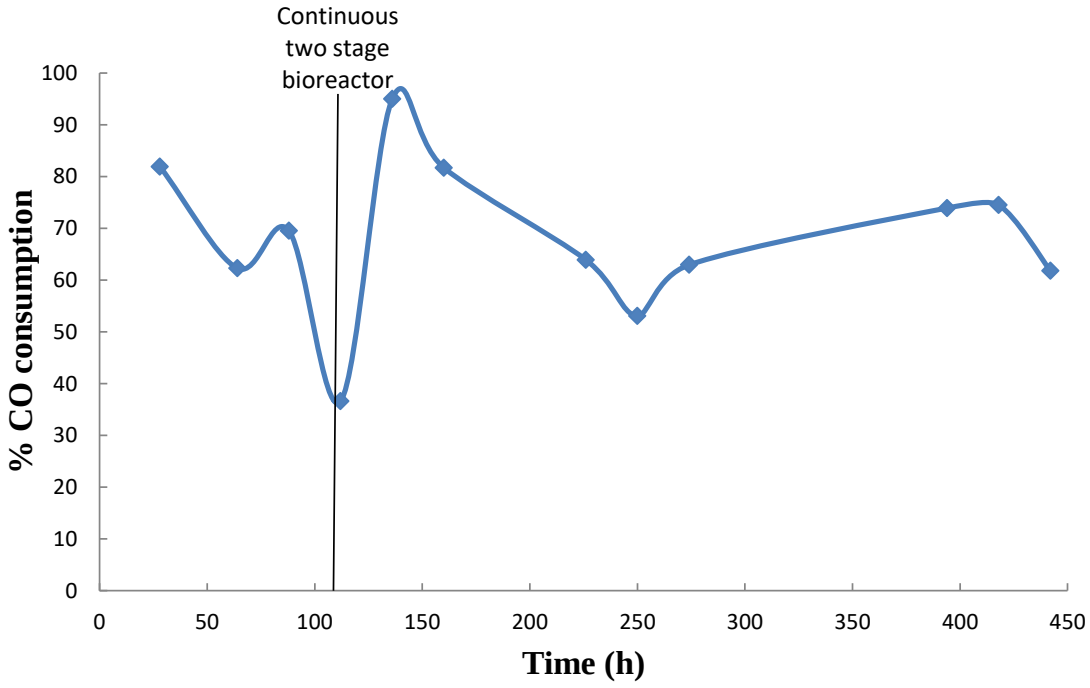

Figure 10.12 Bioreactor 1: Percentage of CO consumption over time.

Clostridium carboxidivorans produces CO_2 as a consequence of the CO consumption during acidogenesis, whereas, in solventogenesis, it consumes this CO_2 to produce alcohols. This was observed in Figure 10.13. In the first hours of the experiment, coinciding with the maximum CO consumption, a (negative) CO_2 consumption of -148 % was observed. After 88 hours, CO_2 started to be consumed, however, after the assembly of the continuous system, the CO_2 consumption decreased again reaching a value of -188 % at 136 hours. After this, the CO_2 started to be consumed and between 250 and 400 hours a consumption of -65 % of CO_2 was observed (Figure 10.13).

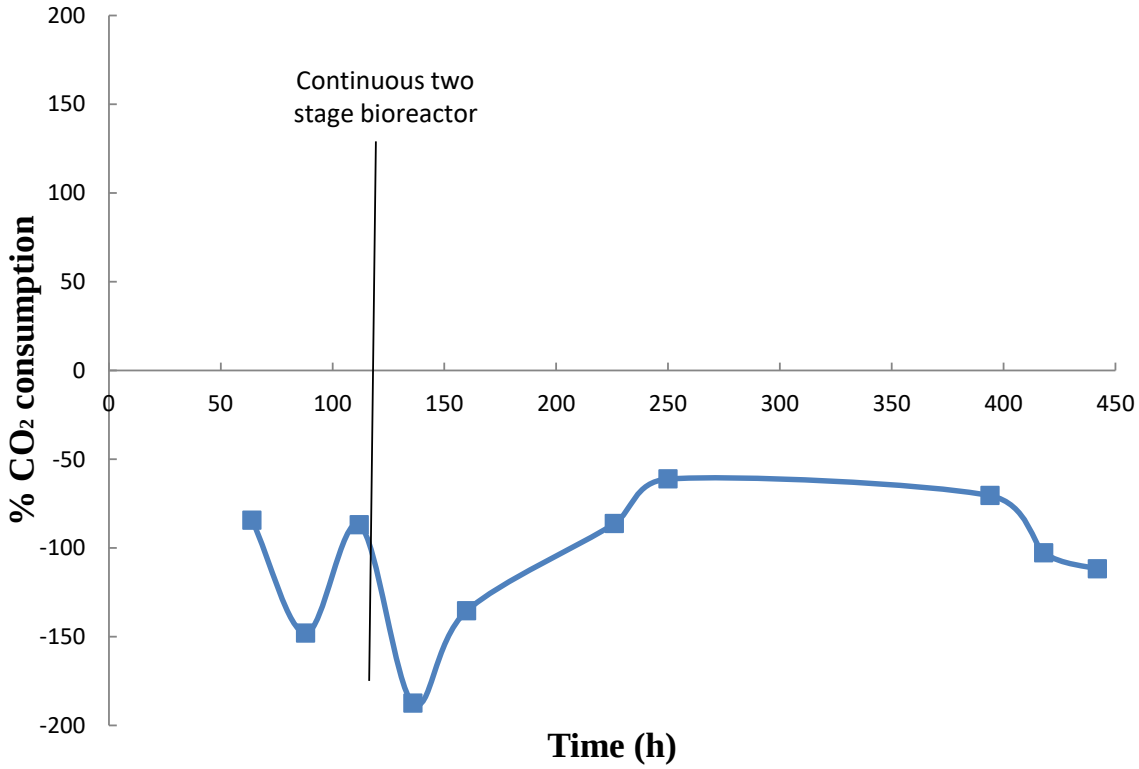

Figure 10.13 Bioreactor 1: Percentage of CO_2 consumption over the time.

In the case of bioreactor 2, in Figure 10.14 it is observed that the maximum consumption of CO was reached 72 hours after inoculation, with a value of 89.50 %, which coincides with the maximum growth of the biomass. Once the bacterium stops growing, a decrease in CO consumption was observed until it stabilizes, reaching an approximate value of 20 %. When the continuous system was assembled, the CO feed was stopped, since the goal is to stimulate the solventogenesis process where CO_2 is consumed up to now.

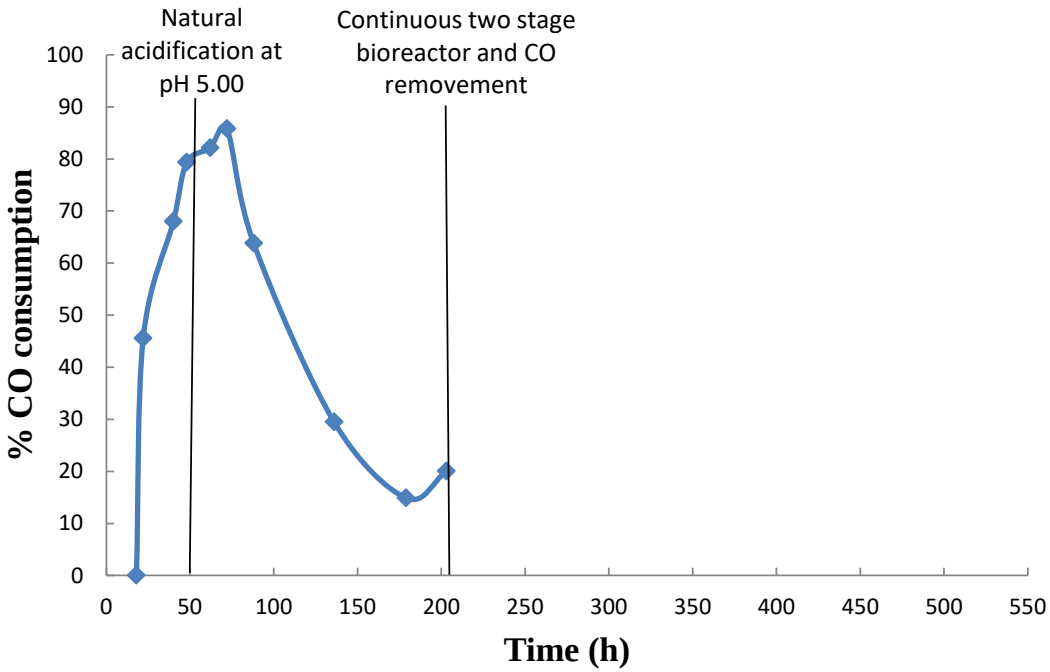

Figure 10.14 Bioreactor 2: Percentage of CO consumption over the time.

In this case, bioreactor 2 measured the CO_2 productivity in g/m^3 as shown in Figure 10.15, where it is observed that it reached a maximum value of 1256 g/m^3 at 72 hours, coinciding with the maximum CO consumption: Due to natural acidification, it was observed how CO_2 production decreases until practically reaching zero. After continuous assembly, there was no CO_2 production, since the CO supply has been switched off.

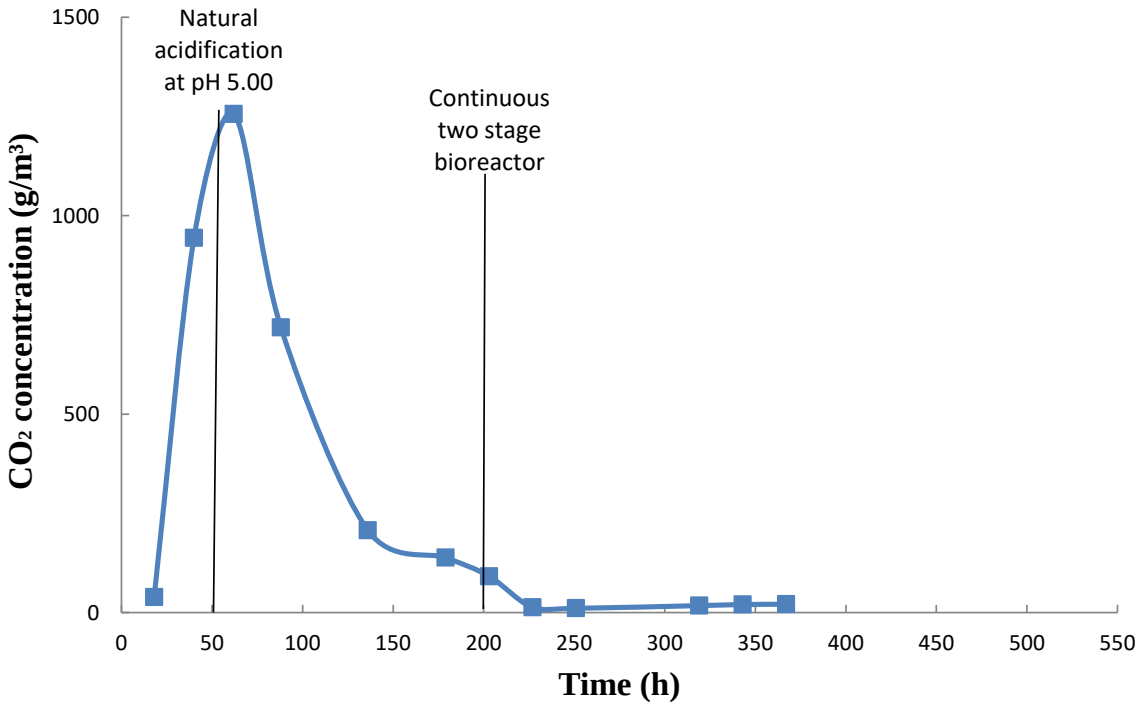

Figure 10.15 Bioreactor 2: Percentage of CO_2 consumption over the time.

10.4 Conclusions

Using a continuous system in two stages, it is possible to separate the acidogenesis and the solventogenesis; obtaining in this way a continuous production of acids in bioreactor 1 (without tungsten and selenium), while the production of alcohols will be continuously carried out in bioreactor 2.

The continuous system of bioreactors in two stages is a novel process for HBE fermentation. It is still necessary to optimize the parameters to be used (composition of the medium, feeding rates, pH, etc.) to maximize the production of metabolites of interest.

10.5 Acknowledgements

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11 GENERAL DISCUSSION AND CONCLUSIONS

Hexanol-Butanol-Ethanol fermentation (HBE) is a novel process, where the obtention of higher alcohols is possible using syngas as a carbon source and *C. carboxidivorans* as a biocatalyst. There are many studies focused on the production of acetic acid and ethanol; however there are only few focused on the production of higher alcohols such as butanol and hexanol. Till now, the main bacterium reported to be able to produce hexanol was *C. carboxidivorans*. In syngas fermentation, the best case described in the literature reached a hexanol concentration of 1.06 g L^{-1} (Fernández-Naveira et al. 2017).

Typical molar H:B:E ratios found in literature data, using syngas mixutres ($CO:CO_2:H_2:N_2$ (2:2:1:5)) for the fermentation, were 1:2.5:6, or using another syngas mixture ($CO:H_2:N_2:CO_2$) (32:32:28:8)) the H:B:E ratio found was (1:1.64:3.6) (Ramió-Pujol et al. 2015).

The main aim of this thesis was the optimization of the fermentation process and conditions to improve the production of higher alcohols. Also, better understanding the W-L pathway for the fermentation process as well as the role of metalloenzymes involved in the metabolic process were other important objectives on this thesis. Therefore, various studies were carried out under different fermentation conditions: different pH values during the different fermentation steps and the method of varying the pH, different carbon sources (CO, syngas, glucose), solvent inhibition, sodium chloride (salinity) effect and the effect of trace metals.

A summary of this thesis and the main conclusions obtained from the different experiments, are detailed in this section.

1. In order to check the effect of the pH on CO fermentation, in Chapter 4, two bioreactor experiments were carried out using different pH values during the solventogenic stage (5.75 and 4.75). Also, results of the fermentation process in batch bottles and bioreactors were compared. This comparison allowed to observe that non continuous supply of CO and a non control of pH in the batch bottles, create unfavourable conditions for *C. carboxidivorans* obtaining lower biomass concentrations (0.13 g/L) and final fermentation products (0.89 g/L HAc, 0.48 g/L HBu, 0.48 g/L EtOH y 0 g/L BuOH) comparied with the bioreactors. On the other hand, in the assays in bioreactors with a continuous supply of CO, different pH values during the solventogenic stage were compared. Also, during the experiment half of the medium was replaced for new medium to improve the conditions in the bioreactor and that way restart the fermentation process. In these experiments, it can be observed that during the solventogenic stage, the control of pH is a key aspect for the alcohols production. In the

case of bioreactor 1 (pH constant at 5.75) there is higher acids and alcohols accumulation than in bioreactor 2 (lower pH of 4.75 during the solventogenic step): 7.52 g EtOH and 3.91 g BuOH in bioreactor 1 and 4.21 g EtOH and 2.29 g BuOH in bioreactor 2. However, when the production rates of alcohols and acids consumption rates were analysed, a lower pH vale during the solvenogenic stage has a positive effect on these rates: 0.13 g-acetic/h*g-biomass, 0.03 g-butyric/h*g-biomass and 0.15 g-acetic/h*g-biomass, 0.04 g-butyric/h*g-biomasa, for bioreactors 1 and 2 respectively; 0.12 g-ethanol/h*g-biomass and 0.06 g-butanol/h*g-biomass, in bioreactor 1 and 0.16 g-ethanol/h*g-biomass y 0.07 g-butanol/h*g-biomass in bioreactor 2.

2. The effect of using syngas as a carbon source on the H:B:E fermentation was checked in chapter 5; for this two bioreactor experiments using syngas were carried out. Also, a new strategy of pH change was developed (natural acidification). As happened in the bioreactors with CO, a pH drop had a negative effect on the biomass. This can be observed by comparing the growth rates obtained in experiment 1 at high pH and low pH, reaching 0.072 h^{-1} and 0.0057 h^{-1}, respectively. Also, a negative effect on the acid accumulation was observed at a low pH (4.75). However, a positive effect on the solventogenic stage was observed when a low pH is promoted in the bioreactor; that positive effect cannot be observed in the alcohols accumulation, due to the lower accumulation of acids, but can be observed in the alcohols production rates which were higher at a low pH (4.75) than at higher pH (5.75) (0.048 vs 0.044 g-etanol/h*g-biomasa, 0.037-0.035 g-butanol/h*g-biomasa and 0.026-0.014 g-hexanol/h*g-biomasa).
   When comparing the total accumulation of alcohols using either syngas or CO (chapter before), a higher alcohol accumulation was observed when CO is used as a carbon source (4.21 g de EtOH y 2.29 g de BuOH at a pH of 4.75 and 7.52 g EtOH y 3.91 g de BuOH at a higher pH of 5.75).

3. Glucose and other sugars are used in ABE fermentation as carbon source. In bacteria performing HBE fermentation, experiments with sugar as a carbon source were never reported previously. That way, in chapter 6, three bioreactor experiments were carried out to check the effect of glucose as a carbon source with different pHs changes (constant pH at 6.2, natural acidification to pH 5.00 and artificial pH change to 5.20) on the H:B:E fermentation. The consumption of glucose was observed at the same time as the bacterial growth. In the other experiments with CO or syngas as a carbon source, the

consumption of the C1 gas was also concomitant with the bacterial growth. The main difference between the use of gases or glucose as a carbon source is related with the variation of acids observed in the fermentation broth. In the case of glucose more diiferent acids were observed than with syngas (lactic acid, formic acid, propionic acid). The pH effect observed in these experiments with glucose maintain the same tendency as the results observed in the experiments with CO or syngas (higher pH during the acidogenic stage have as a consequence a higher acid accumulation). Contrary with other experiments using CO or syngas as carbon source, formic acid started to be consumed at a higher pH, and in this time more acetic acid was observed, that fact never was reported with gaseous carbon sources. That way, *C. carboxidivorans* could use the produced formic acid as a precursor for acetic acid production and also for the bacterial growth. Contrary to what was observed in experiments with gas as a carbon source, the lower pH didn't seem to stimulate the solventogenic stage, so the conversion of acids into alcohols didn't happen in those three experiments (higher pH or lower pH); that way lower concentrations of alcohols were observed when glucose was used as a carbon source. This could be related with the presence of formic acid in the fermentation broth which was found to have an inhibitory effect of the solventogenic stage in some bacteria (*C. acetobutylicum)*. However, higher production rates of alcohols were observed (0.085 g-ethanol/h*g-biomass, 0.009 g-butanol/h*g-biomass, 0.005 g-hexanol/h*g-biomass in the experiment 3 and 0.039 g-ethanol/h*g-biomass, 0.007 g-butanol/h*g-biomass, 0.000 g-hexanol/h*g-biomass in the experiment 2). The higher production rates could be related with the fact that glucose is a carbon source easier to be consumed than gases; that way *C. carboxidivorans* could be able to produce alcohols directly from sugars in an easier way than with C1 gases.

4. In Chapter 7, three batch experiments, using CO as a cabon source and a pH of 5.75, were carried out to check the toxicity effect of the two alcohols produced by *C. carboxidivorans* in major concentrations (ethanol, butanol and the mixture of both). In this study the IC_{50} for the bacteria and each solvent were stimated: 14.50 g/L for butanol, 35.00 g/L for ethanol and 16.22 g/L for the ethanol and butanol mixture.

5. The optimization of the conditions of the fermentation broth is one important aspect to maximize alcohol production. In the metabolic shift between acidogenesis and solventogenesis a change in the pH of the medium is necessary. Several strategies can

be used to change the pH of the fermentation broth, such as natural acidification and artificial pH drop. This pH change must remain within the optimal pH range of each bacteria; however for the same pH values, a variation in the effiency between natural acidification and artificial pH drop could be observed. A natural pH drop seems to have less stress on the bacteria than the artificial pH change. When an artificial pH change is done, HCl is added into the fermentation broth, as a consequence an increase of the salinity/conductivity is provocked in the medium, which could be the cause of the lower efficiency of this process than in the natural acidification. In chapter 8, a bioreactor with two artificial pH changes and a batch experiment at different conductivities were carried out.

In the bioreactor, the artificial pH change was made after reaching the maximum acids concentration. No alcohols production was observed at a low pH; that way a partial medium replacement was done to restart all the process. However, the same result after the artificial pH drop was observed. Conductivity was measured in the different stages of the bioreactor, reaching 13.85 mS/cm at the beginning, 14.59 mS/cm after the artificial pH drop, 17.33 mS/cm after the pH increase; 15.30 mS/cm after the partial medium replacement and 16.21 mS/cm at the end of the experiment.

In the batch experiments, different concentrations were assayed to test the possible negative effect of conductivity on *C. carboxidivorans*. In this experiment, it could be observed that concentrations lower than 9 g/L of NaCl (conductivities lower than 26.4 mS/cm) didn´t have any negative effect on *C. carboxidivorans*. Concentrations between 9-12 g/L of NaCl (conductivities between 26.4 and 32.8 mS/cm) start to have a negative effect on the bacterial strain, with a IC_{50} around 11 g/L (29.9 mS/cm). Finally, concentrations up to 15 g/L (37.9 mS/cm) have as a consequence a total inhibition.

When the conductivities data observed in the reactor are compared with the data obtained in the batch experiments, the conductivity values of the reactor are lower than the values which start to have a negative effect on *C. carboxidivorans*.

6. In the WL pathway there are many enzymes with an important role for the production of alcohols. Some of those enzymes, known as metalloenzymes, need the presence of some trace metals for their activity. An example is formate dehydrogenase, which needs selenium to catalyse the reduction of C1 gases. Also, the aldehyde:ferredoxin oxidoreductase needs tungsten to catalyse the conversion of acids into aldehyde. In Chapter 9, four bioreactors were set up to check the effect of eliminating tungsten and

selenium from the fermentative broth, and using different pH strategies. In that experiment a higher growth rate could be observed as a consequence of the lack of tungsten (0.100 h^{-1} in comparison with 0.074 h^{-1} in the control experiment), this fact could be related with the fact that alcohols production is an expensive energetic process, so the absence of this trace metal could have a positive effect on the bacterial growth. Also, the lack of the two metals and the use of a higher pH have as a consequence the lower accumulation of alcohols (0.676 g/L) in comparison with the rest of experiments and a higher acid concentration (9.56 g/L) compared with the control. To understand the effect of selenium or tungsten separately, a bioreactor experiment with only selenium but without tungsten was carried out. In that reactor a higher acids concentration was reached (11.30 g/L) and lower alcohol concentration (2.02 g/L) compared with the control, but a little higher compared with the bioreactor without both trace metals. That way, the lack of those metals has a negative effect on the alcohol production and as a consecuence a positive effect on the acids accumulation. Also, tungsten has more effect on the process than selenium.

7. In the last chapter (chapter 10) of this doctoral thesis, a new and innovative continuous system was carried out: the two stage bioreactors. This is a novel technique for anaerobic gas fermentation with the main objective to stimulate the accumulation of higher alcohols and get a continuous production of these. This chapter describes a first set-up and preliminary study where, with data obtained from the optimization done in other chapters, optimum conditions are established in each of the bioreactors separately to promote, on the one hand, the production of acids in bioreactor 1 (F1) in which the optimal conditions were: a high pH and the elimination of selenium and tungsten from the medium; and the production of alcohols in bioreactor 2 (F2), where solventogenesis was stimulated by using a complete medium with acidification at low pH. For the management of the continuous system, F1 receives fresh medium without Se and without W, while F2 receives medium rich in F1-acids for their transformation into alcohols. Likewise, F2 had a filtering system with biomass recirculation to eliminate excess waste without eliminating the biomass. In this first set-up it was possible to observe how in F1 a large quantity of acids was produced (in the discontinuous system (first days) and in the continuous one), reaching a daily production of 1.50 g/L of acetic acid, 0.70 g/L of butyric acid and 0.30 g/L of hexanoic acid. In the case of fermentor 2, the daily productivity of alcohols was 0.18 g/L for ethanol, 0.06 g/L for butanol and in

the case of hexanol 0.09 g/L daily. Other parameters need to be optimized, such as the flow of feeds to try to increase the daily productivities of these alcohols, as well as the feeding of different gaseous sources ect.

Final conclusions:

1. During the acidogenic step, a high pH was necessary to improve the gas (CO) conversion into fatty acids.
2. A low pH of 4.75 is necessary during the solventogenic step to improve the alcohols production; however, a low pH has a negative effect on the biomass production.
3. Higher accumulation of alcohols was observed using a higher pH due to the higher acids accumulation; however, the alcohols production rates were higher when a lower pH was used.
4. Acids and alcohols are produced following the next order: C2>C4>C6.
5. H:B:E fermentation is an option with two major advantages: the removal of volatile contaminants (with greenhouse effect) such as CO, and CO_2, and on the other hand the production of ethanol and higher alcohols which are solvents with a high commercial value.
6. When CO is used as a carbon source in the fermentation process, higher production rates and higher alcohol and ethanol final concentrations are obtained than in syngas fermentation, due to the higher purity and concentration of CO (absence of N_2) compared to syngas.
7. Glucose fermentation by *C. carboxidivorans* had as a consequence the obtention of a higher variety of acids than gaseous fermentation.
8. The solventogenic stage using glucose as a carbon source in the fermentation by *C. carboxidivorans*, seems not to be improved by a pH drop.
9. At higher pH of 6.2, at the end of glucose fermentation, *C. carboxidivorans* is able to use the formic acid (produced previously) as a precursor for actic acid production and growth.
10. Butanol has a higher toxic effect on *C. carboxidivorans* than a mixture of ethanol:butanol (1:1) or ethanol.
11. Ethanol has the lowest toxic effect on *C. carboxidivorans*.
12. A low initial concentration of ethanol seems to cause a slight improvement on the bacterial growth.
13. High salinity (conductivity) inhibits growth and activity of *C. carboxidivorans.*

14. Concentrations lower than 9 g/L of NaCl did´nt provoke inhibition, and partial inhibition occurs between 9-12 g/L of NaCl; IC_{50} (50 % growth rate inhibition) for *C. carboxidivorans* was around 11 g/L of NaCl.
15. Concentrations up to 15 g/L of NaCl cause a total inhibition, with no growth of *C. carboxidivorans.*
16. The presence of tungsten and selenium have a positive effect on the alcohols production.
17. The lack of tungsten has more negative effect on the alcohol production than the lack of selenium.
18. Lack of tungsten and selenium has as a consequence a higher accumulation of acids in the fermentation broth than in control conditions.
19. The development of a two stage continuous system for the continuous accumulation of alcohols is possible.
20. More studies and optimization are necessary for the complete development of the two stage continuous system.

11.1 References

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12 RESUMEN EN CASTELLANO

En los últimos años la escasez de los combustibles fósiles junto con una serie de problemas económicos (inestabilidad del precio) políticos y medioambientales (la emisión de gases de efecto invernadero a consecuencia de su uso), todo esto sumado a que cada vez hay más demanda del uso de los mismos, el cual se espera que se duplique en el año 2030 debido al incremento del 3 % anual del sector de transporte, han forzado a que en los últimos años se busquen fuentes de energía alternativas y renovables.

Además, la Unión Europea, a través de su Directiva de Energías Renovables RED 2009/28 / CE, establece como objetivo que para el año 2020, que el uso de energía renovable debe alcanzar el 20 %, donde un 10 % de participación debe corresponder al sector del transporte.

El uso de biocombustibles como son los bioalcoholes, etanol, butanol y hexanol, es una opción más ecológica y que permite disminuir el uso de dichos combustibles fósiles. De hecho en los últimos años se ha visto que el butanol tiene un futuro muy prometedor como biocombustible debido a todas las ventajas que tiene (se puede mezclar directamente con la gasolina en cualquier proporción, usarse directamente en la infraestructura actual sin necesidad de ninguna modificación, menos higroscópico, con mayor contenido energético y más seguro que el etanol).

La metodología convencional y más conocida para la obtención de bioalcoholes es mediante la fermentación de azúcares, los alcoholes obtenidos a través de este proceso son conocidos como biocombustibles de primera generación. El problema de esta metodología es el uso como materia prima de distintos cultivos (por ejemplo la caña de azúcar) los cuales son empleados como fuentes de alimentación humana y animal. Esto genera un dilema, debido a que cultivos que tienen aplicación alimentaria se desvían para la producción de biocombustibles.

Es por ello, por lo que se ha desarrollado una segunda via de obtención de estos bioalcoholes, la denominada biocombustibles de segunda generación, en la cual se fermenta biomasa lignocelulosica para la obtención de los bioalcoholes. Esta biomasa lignoclulósica está compuesta principalmente por lignina, celulosa y hemicelulosa, en distintas proporciones. Para que sea posible el uso de este material lignocelulosico por los microorganismos, es necesario realizar un pretratamiento, que puede ser de dos tipos: la hidrólisis enzimática y tratamientos termoquímicos o gasificación. En el primer caso, la celulosa y hemicelulosa son fácilmente degradables en azúcares (pentosas y hexosas) mediante el uso de diferentes enzimas, mientras que la lignina presente en el material lignocelulósico (hasta un 30 %) es muy difícil de degradar, lo que supone un problema en este proceso; la dificultad en la degradación de la lignina, sumado a que solo las hexosas son fermentables por los microorganismos (mientras que las pentosas no) junto con el encarecimiento que supone el empleo de dichas enzimas hidrolíticas,

supone una desventaja global en la obtención de los bioalcoholes con este proceso. El segundo pretratamiento consiste en una vía alternativa, en la que mediante tratamientos termoquímicos se obtiene una mezcla gaseosa, la cual es conocida como "gas de síntesis".

Este gas de síntesis, compuesto principalmente por monóxido de carbono, dióxido de carbono e hidrogeno, puede ser fermentado por las bacterias anaerobias para obtener ácidos y como resultado final alcoholes (etanol, butanol y hexanol). A diferencia del empleo de este gas por una vía química (Fischer-Tropsch), el proceso biológico de obtención de alcoholes es un proceso más económico y sencillo, algunas de las ventajas que supone son: La baja temperatura y presión necesaria en el proceso biológico, mayor rendimiento y uniformidad del producto y por tanto una mayor facilidad para su posterior purificación…

El proceso de fermentación de gas de síntesis por bacterias acetogénicas, además permite la fermentación no solo del gas de síntesis obtenido tras la gasificación de material lignocelulósico, sino que también permite la fermentación de gases industriales residuales tales como los de la industria del refinado de petróleo, industria del acero… Esto supone una doble ventaja, por un lado el empleo de esos gases residuales elimina la emisión de gases tóxicos y de efecto invernadero a la atmósfera y por otro lado, la obtención de metabolitos de elevado interés industrial.

En esta tesis se ha empleado la cepa *Clostridium carboxidivorans* (DSM 15243), para realizar distintos estudios para maximizar la obtención de alcoholes de cadena larga (etanol, butanol y hexanol). Diferentes fuentes de carbono fueron empleadas en los distintos experimentos las cuales son descritas en los distintos capítulos, en estos ensayos se obtuvieron diferentes ácidos (ácido acético, ácido butírico, ácido hexanoico entre otros) y como resultado final alcoholes de cadena larga (etanol, butanol y hexanol). Como se describe en el **capítulo 1**, estos alcoholes suponen una serie de ventajas unos frente a otros y pueden ser empleados como biocombustibles. Así mismo en este capítulo se recoge información sobre distintos microorganismos y procesos para la obtención de ácidos y alcoholes, en donde se puede observar como *C. carboxidivorans* es la única hasta el momento capaz de sintetizar etanol, butanol y hexanol.

Este ha sido uno de los motivos de la elección de esta bacteria para realizar esta tesis doctoral, la innovación que supone la obtención de estos tres alcoholes en un único proceso y con una única bacteria. Como se dijo con anterioridad, el objetivo principal de esta tesis es optimizar las condiciones de fermentación para así maximizar la obtención de estos alcoholes de cadena larga. Para ello se han ensayado distintas fuentes de carbono, distintos pH, distintas condiciones

de medio de cultivo, distintas estrategias y parámetros los cuales irán recogidos en distintos capítulos. Para poder realizar estos estudios es necesario comprender las condiciones óptimas de crecimiento de la bacteria empleada (pH, temperatura ect) y su proceso de fermentación; toda esta información, se encuentra recogida y esquematizada en el **capítulo 1**.

Los diferentes protocolos empleados para los distintos estudios, se encuentran descritos de una forma general, en el **capítulo 3**; posteriormente en cada uno de los capítulos siguientes se encuentran cada una de las metodologías empleadas, descritas con más detalle para cada uno de los casos específicos.

En el **capítulo 4**, se realizaron experimentos en batch y en fermentadores empleando CO como fuente de carbono, con el objetivo de ver como el control de pH y los distintos cambios de pH afectan a la producción y crecimiento de la bacteria. En los ensayos en batch, se pudieron observar como la falta de un suministro continuo de fuente de carbono (CO) junto a la falta de control de pH durante el proceso, hacen que la biomasa (0.13 g/L) y concentración de ácidos y alcoholes máxima (0.89 g/L HAc, 0.48 g/L HBu, 0.48 g/L EtOH y 0 g/L BuOH) obtenida sea menor que en los ensayos en reactores. En los dos ensayos en reactores, con un suministro continuo de CO, se compara el efecto que tienen dos valores distintos de pH durante la solventogénesis. Así mismo, en ambos fermentadores se realiza un reciclaje parcial del medio, de forma aséptica y anaerobia, para estimular de nuevo la producción de ácidos y el crecimiento (acidogénesis). En estos experimentos, se pudo observar como el pH durante la solventogénesis tiene un efecto determinante para la producción de alcoholes. En el caso del fermentador 1, en el cual se mantiene el pH constante a 5.75 durante todo el proceso, se obtiene una acumulación de ácidos y de alcoholes más alta que en el fermentador 2, donde tras las 72H se realiza una bajada de pH a 4.75, siendo la acumulación neta al final del experimento de alcoholes la siguiente: 7.52 g EtOH y 3.91 g BuOH en el fermentador 1 y 4.21 g EtOH y 2.29 g BuOH en el fermentador 2. No obstante cuando se analizan las tasas de consumo de ácidos y de producción de alcoholes, se puede observar como el pH más bajo durante la solventogénesis estimula el consumo de ácidos y por tanto la producción de alcoholes, obteniendo los siguiente valores: Tasa de consumo de ácidos, para el fermentador 1 0.13 g-ácetico/h*g-biomasa, 0.03 g-butírico/h*g-biomasa, 0.15 g-ácetico/h*g-biomasa, 0.04 g-butírico/h*g-biomasa, para el fermentador 2; Tasa de producción de alcoholes, 0.12 g-etanol/h*g-biomasa y 0.06 g-butanol/h*g-biomasa, en el fermentador 1 y 0.16 g-etanol/h*g-biomasa y 0.07 g-butanol/h*g-biomasa.

Para comprobar el efecto que tenía la mezcla de gas de síntesis sobre nuestra bacteria, se decidió realizar una serie de experimentos con gas de síntesis y dos estrategias de pH diferentes,

los cuales se encuentran recogidos en el **capítulo 5.** Se realizaron 2 experimentos, uno a pH constante, y el segunto con el mismo pH incial que el anterior pero con acidificación natural a pH 4.75.

En estos experimentos, se puede ver como una bajada de pH tiene un efecto negativo sobre la biomasa, lo cual se puede observar (en el experimento 1 de este capítulo) cuando comparamos la tasa de crecimiento a pH 5.75 y 4.75 (0.072 h^{-1} y 0.0057 h^{-1} respectivamente); este efecto negativo también se puede observar en la acumulación de ácidos, ya que se obtiene el doble de concentración final de ácidos en el experimento 2 (a pH constante 5.75) que en el experimento 1 (con acidificación natural). Este mayor crecimiento y mayor acumulación de ácidos, viene como consecuencia de un mayor consumo de CO durante la acidogénesis a pH 5.75 que a 4.75 (76 % frente a un 69 %). No obstante, los pH bajos tienen un efecto positivo en la solventogénesis del proceso, aunque la cantidad final de alcoholes sea mayor a pH 5.75 (2.70 g/L EtOH, 1.90 g/L BuOH y 0.85 g/L HeOH) comparado con pH 4.75 (2.25 g/L EtOH, 1.43 g/L BuOH y 0.72 g/L HeOH); este echo es debido a la mayor acumulación de ácidos a pH elevado, no obstante, la tasa de producción es más alta a pH 4.75 que a 5.75 (0.048 frente 0.044 g-etanol/h*g-biomasa, 0.037-0.035 g-butanol/h*g-biomasa,y 0.026-0.014 g-hexanol/h*g-biomasa). Como conclusiones generale de este capítulo, se puede destacar:

El efecto positivo de un pH alto (5.75) sobre la acidogénesis, es decir sobre el consumo de CO, el crecimiento bacteriano, la producción de ácidos y en consecuencia la acumulación final de alcoholes, frente a un pH bajo (4.75).

El efecto positivo de un pH bajo (4.75) sobre la solventogénesis, es decir sobre la tasa de producción de alcoholes.

Así mismo se puede observar como bajo las mismas condiciones cuando se compara los experimentos con CO (**capítulo anterior**) con los de este capítulo, se obtiene una acumulación neta mayor cuando trabajamos solo con CO (4.21 g de EtOH y 2.29 g de BuOH en el caso de fermentadores con pH bajo y 7.52 g EtOH y 3.91 g de BuOH en el fermentador con pH alto).

En el siguiente capítulo (**capítulo 6**) se analiza el efecto de otra fuente de carbono distinta sobre el crecimiento y producción de *C. carboxidivorans*. Como en los capítulos anteriores, también se comparan distintas estrategias de pH durante el proceso; 3 experimentos fueron llevados a cabo: el primero a pH constante 6.20, el segundo con el mismo pH inicial y una posterior acidificación natural a pH 5.20 y el tercero de ellos con el mismo pH inicial que los anteriores y una bajada gradual de pH a 5.20 empleando HCl 1 M una vez se alcanzó el máximo de biomasa.

La biomasa máxima alcanzada y las tasas de crecimiento, fueron similares entre estos tres experimentos aunque ligeramente más altas que con las otras fuentes de carbono empleadas en los capítulos anteriores. En los tres casos, el consumo de glucosa es paralelo al crecimiento bacteriano y solo ocurre a pH elevado, cuando se baja el pH (bien de forma natural o artificialmente) el sustrato deja de ser consumido. Se obtuvieron valores de consumo del 81 % en el experimento 1, 72 % en el experimento 2 y 95 % en el experimento 3. Una de las principales diferencias del uso de glucosa como fuente de carbono con las fuentes de carbono gaseosas empleadas en los otros capítulos, es que en este caso aparece una mayor variación de ácidos que en los casos anteriores. Con fuentes de carbono gaseosas, se obtenía ácido acético, ácido butírico y ácido hexanoico; no obstante con glucosa se obtiene una mayor variedad: ácido acético, ácido butírico, ácido hexanoico, ácido láctico, ácido fórmico, ácido propiónico y ácido isobutírico. Como cabría esperar, la concentración de ácidos es más elevada en el experimento 1 y 3 que en el 2 ya que se emplea un pH alto durante toda la acidogénesis. Además, se puede observar un echo nunca hasta ahora expuesto en ninguna publicación, esto es que a pH elevado el ácido fórmico comienza a ser consumido una vez que el consumo de glucosa cesa por *C. carboxidivorans,* en este mismo momento comienza a consumirse y en consecuencia vuelve a producirse más ácido acético. En este capítulo, se hipotetiza por tanto el posible empleo de ácido fórmico para el crecimiento bacteriano y así mismo como precursor de ácido ácetico. En cuanto a la producción de alcoholes, estos son producidos solo a pH elevados y durante el consumo de glucosa, no obstante en ninguno de los 3 casos se observa un consumo de ácidos y en consecuencia una producción de alcoholes a partir de estos. Las concentraciones obtenidas por tanto son menos que en los experimentos llevados a cabo con gas de síntesis o con CO; no obstante, si se observan tasas de producción más altas con glucosa como fuente de carbono que con gases, esto puede deberse a la habilidad de *C. carboxidivorans* de producir directamente alcoholes a partir de glucosa o CO; la glucosa al ser más fácilmente fementable que el CO por la bacteria, permite obtener tasas de conversión de alcoholes más elevadas a pH altos ya que, contrariamente a las fuentes de carbono gaseosas, con glucosa no hay solventogénesis a pH bajo (0.085 g-etanol/h*g-biomasa, 0.009 g-butanol/h*g-biomasa, 0.005 g-hexanol/h*g-biomasa en el experimento 3 frente a 0.039 g-etanol/h*g-biomasa, 0.007 g-butanol/h*g-biomasa, 0.000 g-hexanol/h*g-biomasa en el experimento 2). La presencia de ácido fórmico a elevadas concentraciones, es conocida por algunos autores por causar un efecto inhibitorio de la solventogénesis en *C. acetobutylicum*, lo cual podría ser uno de los motivos de obtener tan bajas concentraciones de alcoholes cuando se comparan estos experimentos con los de los capítulos

anteriores. Otra posibilidad sería que los pH bajos no sean adecuados para la solventogénesis en las fermentaciones de azúcares por *C. carboxidivorans*.

Uno de los primeros estudios que se realizaron, el cual se encuentra descrito en el **capítulo 7** de esta tesis, fue llevado a cabo en botellas con distintas concentraciones de etanol, butanol y una mezcla de ambos alcoholes. El objetivo de este estudio, fue conocer cuáles son las concentraciones límites ante las cuales nuestra bacteria de estudio, *C. carboxidivorans,* es capaz de crecer con normalidad. Con este estudio se pudo ver el potencial efecto inhibitorio que tiene la presencia de butanol sobre *C. carboxidivorans*, donde empleando el programa estadístico Sigma Plot, se determinaron las IC_{50} (concentración inhibitoria del 50 %). Con esto, se pudo observar que para *C. carboxidivorans* 14.50 g/L de butanol son suficientes para causar una inhibición del 50 % del crecimiento, siendo así el butanol mucho más inhibitorio para esta bacteria que la mezcla de ambos alcoholes (IC_{50} 16.24 g/L) o que el etanol solo (IC_{50} 35 g/L).

Uno de los mayores retos a la hora de maximizar la producción de alcoholes con una bacteria dererminada, es la optimización de las condiciones del medio de cultivo. Numerosos estudios se han basado es la adición/supresión de vitaminas, fuentes de nitrógeno como el cloruro amónico, temperatura y sobre todo el pH. *C. carboxidivorans* necesita dos pH distintos durante la fase de acidogénesis y solventogénesis; en fermentadores, realizar el cambio de pH de una forma poco agresiva (siempre dentro de las condiciones óptimas de crecimiento) que no suponga una alteración para el estado metabólico de la bacteria supone un reto. Varias estrategias de cambio de pH se han realizado en esta tesis (cambio artificial, cambio natural…) siendo el cambio natural de pH el que parece tener un menor efecto negativo sobre la bacteria. Con la adición de HCl al medio de forma gradual y progresiva, se consigue realizar el cambio de pH artificial, esta adicción de HCl junto con la NaOH consumido durante la acidogénesis, provoca un incremento en la salinidad/conductividad del medio, siendo una posible causa de la condición desfavorable de esta metodología. Con el objetivo de explicar una posible causa ante este efecto menos favorable del cambio artificial de pH, se propuso un experimento en fermentador, seguido de un batch con distintas conductividades en el **capítulo 8.**

Tras la obtención de la máxima concentración de ácidos en el fermentador (4.700 g/L de ácido acético y 1.300 g/L de butírico), se procedió a una bajada de pH gradual y progresiva mediante la adición de HCl. No obstante tras esta bajada de pH, no se produjo ningún cambio en el fermetador, por lo que se cambio parcialmente el medio del fermentador para restaurar de nuevo el ciclo y la aplicación de una bajada artificial de pH. En el segundo ciclo, tampoco se observa solventogénesis tras el cambio de pH. Durante todo el experimento se realizaron

mediciones de conductividad, observándose los siguientes valores: 13.85 mS/cm al inicio del experimento, 14.59 mS/cm despúes del primer cambio artificial de pH, 17.33 mS/cm después de la subida de pH al valor inicial, 15.30 mS/cm después del cambio parcial de medio y finalmente 16.21 mS/cm al final del experimento.

En los experimentos en batch, distintas concentraciones de NaCl (0.2, 3, 9, 10, 11, 12, 15, 18 y 21 g/L) fueron ensayadas para determinar la conductividad inhibitoria para *C. carboxidivorans*. En estos experimentos, se pudo observar que concentraciones menores de 9 g/L de NaCl (lo cual corresponde a conductividades inferiores a 26.4 mS/cm) no causan ningún efecto sobre la bacteria. Serán las concentraciones entre 9-12 g/L de NaCl (correspondientes a conductividades entre 26.4 y 32.8 mS/cm) las que causen en la bacteria una inhibición al 50 % de su crecimiento, determinando la IC_{50} en torno a los 11 g/L (29.9 mS/cm). Y por último la total inhibición se observó a concentraciones de NaCl superiores a 15 g/L (37.9 mS/cm).

Comparando estos datos de inhibición con los observados en el fermentador, podemos obs ervar como cualquiera de dichos valores están muy por debajo de la IC_{50}, estando dentro de los valores en los cuales no se observó ningún tipo de inhibición en la bacteria en los ensayos en batch. De este modo, se puede descartar que la causa de la poca eficiencia del cambio artificial de pH se vea causado por un incremento en la salinidad del medio de cultivo, quedando como otra posible causa el echo de que un cambio artificial aunque sea gradual, suponga mayor estrés para la bacteria que un cambio natural de pH.

Las metalloenzimas son las enzimas resposables de la convesión de gas a ácido y posteriormente de destos ácidos a alcoholes. La actividad de estas enzimas se caracteriza por estar estimulada por la presencia de ciertas trazas de metales. Según la enzima de la que hablemos, estará estimulada por distintos metales. La FDH es una metaloenzyma involucrada en la reducción de gases C1 a ácidos, la cuál su actividad se ve fomentada por la presencia de selenio y de tungsteno. Así mismo la AFOR, es la responsable de la conversión de ácido a aldehído, un paso intermedio en la formación de alcoholes, la actividad de esta última está estimulada por la presencia de tungsteno. Para estudiar el efecto de estos dos metales, los cuales están involucrados en dos pasos de la ruta W-L, se decidió realizar cuatro experimentos (experimento control con acidificación natural, experimento sin tungsteno y sin selenio con acidificación natural, experimento sin los metales a pH contstante y finalmente un experimento sin tunsgteno pero con selenio a pH constante), los cuales se encuentran recogidos en el **capítulo 9.**

En los cuatro experimentos la biomasa máxima obtenida es similar, no obstante en la tasa de crecimiento es donde se pueden apreciar diferencias; en el caso del experimen to a pH constante

sin tungsteno, se aprecia una tasa de crecimiento de 0.10 h^{-1} la cuál es una de las tasas de crecimiento más altas obtenidas en condiciones similares. Este echo podría justificarse debido a que con la ausencia de tungsteno se inhibe la formación de alcoholes, lo cual es un proceso muy costoso para la bacteria, de este modo la bacteria podría ser energéticamente más eficiente en la producción de ácidos y en su crecimiento. En cuanto a la producción de ácidos y alcoholes, se puede observar como la carencia de estos metales reduce la producción de alcoholes y en consecuencia aumenta la acumulación de ácidos comparado con el control, pudiendo considerarse el tungsteno el metal con mayor efecto en esta inhibición. Así mismo este efecto se intensifica cuando el pH del medio se mantiene alto, ya que cuando se permite una acidificación natural, sigue estando presente una inhibición en la acumulación de alcoholes comparado con el control, pero el pH bajo permite un ligero ascenso en la producción de alcoholes comparado con el pH alto, ya que se estimula la solventogenesis.

En el último capítulo de esta tesis, **capítulo 10**, se desarrolla una técnica de bioreactores continuos en dos etapas. Esta es una técnica novedosa en el campo con la que se permitiría aumentar la concentración de alcoholes de cadena larga además de su producción de forma contínua con una separación espacial en dos bioreactores distintos. En este capítulo, se describe una primera puesta a punto donde con datos conseguidos en las optimizaciones realizadas en otros capítulos, se establecen condiciones óptimas en cada uno de los fermentadores por separado para impulsar, por un lado la producción de ácidos en el fermentador 1 y de alcoholes en el fermentador 2. Las condiciones llevadas a cabo fueron: En el fermentador 1, un pH alto y la eliminación de selenio y tungsteno del medio, y de ese modo se inhibe la solventogénesis estimulando la acidogénesis; En el fermentador 2 por otro lado, se estimula la solventogénesis mediante un medio completo con acidificación a pH bajo. Para el manejo del sistema contínuo, el F1 recibe medio fresco sin Se y sin W, mientras que el F2 recibe medio rico en ácidos del F1 para su transformación en alcoholes. Así mismo el F2 tendrá un sistema de filtrado con recirculación de biomasa para eliminar medio sobrante sin eliminar la biomasa. En esta primera puesta a punto se pudo observar como el F1 produce una gran cantidad de ácidos tanto en el sistema discontínuo (primeros días) como en el contínuo, llegando a unas producciones diarias de 1.50 g/L de ácido acético, 0.70 g/L de ácido butírico y 0.30 g/L de ácido hexanoico. En el caso del fermentador 2, la productividad diaria de alcoholes fue 0.18 g/L para el caso del etanol, 0.06 g/L para el butanol y en el caso del hexanol 0.09 g/L diario. Quedarían pendientes otros parámetros a optimizar, como el flujo de las alimentaciones para intentar aumentar las productividades diarias de estos alcoholes, así como la alimentación de distintas fuentes gaseosas ect.

APENDIX

Review

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H-B-E (hexanol-butanol-ethanol) fermentation for the production of higher alcohols from syngas/waste gas

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Abstract

CO, H_2, and CO_2 are major components of syngas and some industrial CO-rich waste gases (e.g. waste gases from steel industries), besides some additional minor compounds. It was recently shown that those gases can be bioconverted, by acetogenic/solventogenic bacteria, into ethanol and higher alcohols such as butanol, but also hexanol, through the so-called HBE fermentation. That process presents some advantages over existing chemical conversion processes. This paper reviews HBE fermentation from C1-gases after briefly describing the more conventional ABE (acetone-butanol-ethanol) fermentation from carbohydrates by *Clostridium acetobutylicum*, in order to allow for comparison of both processes. Although acetone may appear in carbohydrate fermentation, alcohols are the only major end-metabolites in the HBE process with *Clostridium carboxidivorans*. The few acetogenic bacteria known to metabolize C1-gases and produce butanol or higher alcohols are described. *Clostridium carboxidivorans* has been used in most cases. Bioconversion of the gaseous substrates takes place in two stages, namely acidogenesis (production of acids) followed by solventogenesis (production of alcohols), characterized by different optimal fermentation conditions. Major parameters affecting each bioconversion stage as well as the overall fermentation process are analyzed. Although it has been claimed that acidification is required in ABE fermentation to initiate the solventogenic stage, strong acidification seems to some extent not to be a prerequisite for solventogenesis in the HBE process. Bioreactors potentially suitable for this type of bioconversion process are described as well.

Keywords: butanol; carbon dioxide; carbon monoxide; *Clostridium*; hexanol; Wood–Ljungdahl pathway

ABBREVIATIONS

AAD	alcohol/aldehyde dehydrogenase
ACS	acetyl-CoA synthase
ADC	acetoacetate decarboxylase
ADH	alcohol dehydrogenase
ADHE	acetaldehyde dehydrogenase
AK	acetate kinase
AOR	aldehyde:ferredoxin oxidoreductase
BCD	butyryl-CoA dehydrogenase
BDH	butanol dehydrogenase
BUK	butyrate kinase
CODH	CO dehydrogenase
Co-FeS-P	corrinoid iron-sulphur protein
CRT	crotonase
CTF	CoA-transferase
FDH	formate dehydrogenase
FTS	formyl-THF synthetase
HBD	3-hydroxybutyryl-CoA dehydrogenase
MTC	methenyl-THF cylcohydrolase
MTD	methylene-THF dehydrogenase
MTR	methyltransferase
MTRS	methylene-THF reductase
PTA	phosphotransacetylase
PTB	phosphotransbutyrylase
PFLB	pyruvate-ferredoxin oxidoreductase
TA	transaldolase
TER	Trans enoyl-CoA reductase
THL	thiolase
TK	transketolase

INTRODUCTION

Worldwide energy consumption is expected to double by 2030. One of the main reasons for this increase is the rapid growth of the transportation sector reaching about 3% per year. Because of such rapid growth, the limited availability of fossil fuels will not be able to cope with this energy demand. Therefore, in recent years, the environmental impact of fossil fuels and issues related to climate change have forced the search for new alternative fuels that can be obtained from renewable sources or from pollutants and that can be produced in a sustainable way. In addition to the environmental impact generated from the use of fossil fuels (e.g. greenhouse effect), there is also another set of problems including economic (e.g. the instability of oil prices) as well as political issues requiring the development of new techniques

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for the production of alternative fuels.[1–3] The European Union, through its Renewable Energy Directive RED 2009/28/EC, sets a target for the year 2020, stating that the use of renewable energy should reach 20% with a 10% share of renewable energy in the transportation sector.[4]

Nowadays numerous studies are focusing on the development and optimization of technologies for obtaining biofuels (biologically sourced fuels) such as ethanol, butanol, biogas and biodiesel as environmentally-friendly energy sources in substitution of fossil fuels. Ethanol and butanol can be obtained through fermentation of sugars or starch feedstocks, whereas biogas is produced through the anaerobic fermentation of organic matter, and biodiesel results from a transesterification reaction between an alcohol and vegetable oils (e.g. soybean, sunflower, coconut oils) in most cases, although animal fats or even algae have also been used.[5] In the case of bioalcohols, conventional techniques developed for obtaining biofuels do often lead to food–fuel competition. Therefore, another alternative has more recently been developed based on the use of lignocellulosic feedstocks in order to overcome such a drawback. Energy crops and agricultural wastes are the most common renewable and inexpensive sources of lignocellulosic materials.[1] The efficiency and cost-effectiveness of this alternative still needs to be further improved.

The use of lignocellulosic materials for sugar fermentation is a complex process, as they contain carbohydrates in the form of long chain polysaccharides which are difficult to metabolize directly by microorganisms. Therefore, pretreatment of the feedstock is necessary in order to hydrolyze those polysaccharides into fermentable sugars which can then be used readily and metabolized by several microbial species. The hydrolytic route presents some other drawbacks. Indeed, lignocellulosic biomass is composed of cellulose, hemicellulose and lignin mainly. Cellulose and hemicellulose yield fermentable sugars while lignin does not.[6] The lignin fraction which may, in some cases, represent up to more than 30% of the feedstock can thus not be used in the fermentation process. On the other hand, those feedstocks can be converted into syngas which can also be fermented into ethanol as well as higher alcohols such as butanol or hexanol by some anaerobic acetogenic bacteria. In such a case, contrary to the hydrolytic fermentation process, the whole feedstock, i.e. cellulose, hemicellulose and lignin can be gasified. Lignin is thus not lost. Besides, a wider range of starting materials are suitable for gasification than for hydrolysis as many carbon-containing materials can be gasified. Syngas is mainly composed of a mixture of CO, CO_2 and H_2 at variable concentrations depending on the feedstock and gasification conditions. Several industrial waste gases do also contain some or several of those gases and can therefore be used as feedstock as well, with an obvious associated environmental benefit.[7]

The human interest in the use of microbial fermentation to produce ethanol began by the year BC 10 000.[8] On the other hand, the commercial bacterial production of butanol from carbohydrates dates back to about 100 years ago,[9] while studies on the potential biological production of hexanol is still more recent. Today research is still being undertaken on improving the biological production of alcohols. Many of those studies deal with ethanol production and its commercial applications. However, ethanol presents some limitations as an individual fuel or mixed with fossil fuels such as gasoline. It has a rather low caloric content. Besides, it is hygroscopic and has a low density, which limits its use with current infrastructures.[10–12]

On the other hand, butanol has the advantage of being less hygroscopic and it has a higher energy content than ethanol,[13,14] besides being less volatile, less corrosive, absorb less water, and being less explosive which makes it safer to use than ethanol.[15,16] All those advantages result in growing interest in that alcohol. It is considered a chemical of great industrial importance and could replace gasoline as a fuel,[15,17] as there is no need for any adjustment in vehicles or engines run on butanol rather than fossil fuels. Moreover, blending of butanol and gasoline is possible at basically any concentrations.[16]

In recent years, hexanol has also been established as an alcohol with a high industrial interest. Recent studies aim at maximizing the production of that 6 carbons alcohol which can be considered a suitable alternative fuel as well.[18] Hexanol, similarly to butanol, is a higher carbon number alcohol, thus characterized by a higher energy content than ethanol. Several assays have recently been done with hexanol to check its potential for use as an aviation fuel,[19,20] although the viscosity of pure alcohols such as hexanol and butanol may be too great to be compatible with aviation kerosene.[19] Hexanol–diesel blends have also been tested as well as gasoline–alcohols blends using multiple alcohols (i.e. ethanol, butanol, and hexanol) in combination.[21] Besides, hexanol has several other industrial applications. It is not toxic at low concentrations and is also used in the pharmaceutical and cosmetic/perfumes industry, textile industry, in detergents, in pesticides, and as a finishing agent in the leather industry, among others.

The aim of this paper is to focus on reviewing a novel biological alternative for the production of ethanol and higher alcohols (butanol and hexanol) through the HBE fermentation, explaining the main reactions taking place in this process and describing the main microorganisms involved in the bioconversion of gases to alcohols as well as the most common bioreactors and their modes of operation. The HBE process is an interesting alternative to the more conventional ABE fermentation used, among others, for butanol production.

PRODUCTION OF ALCOHOLS FROM CARBOHYDRATES

Butanol and some other alcohols can be obtained by the anaerobic fermentation of carbohydrates (ABE fermentation), briefly revised hereafter, as well as from the anaerobic bioconversion of syngas/waste gases (i.e. CO, CO_2, and H_2) (HBE fermentation), as will be described in more detail in later sections.

Clostridial alcohol producing strains

Despite numerous scientific advancements in the area of biofuels production, it is necessary to identify the best-performing microorganisms to maximize their production and minimize costs, and thus to be able to better compete with fossil fuels. High productivity of the desired product along with low production costs will be the desired characteristics in a model organism for a cost-competitive process.[1] *Clostridium* spp. have been identified as suitable bacteria for the production of higher alcohols such as butanol.

The use of clostridial strains in butanol production through conventional ABE (acetone-butanol-ethanol) fermentation is a well known process,[22] which started being studied around the First World War with carbohydrates as substrates. Acetone, butanol and ethanol are the end products from the fermentation process, all

of them having commercial uses either as biofuels or as platform chemicals. After the Second World War, the relevance of fermentation as a major production process of those chemicals decreased significantly and it was largely replaced by petrochemical production as a result of the strong development of the petrochemical industry. However, interest in this type of bioconversion has again grown dramatically in recent years for economic reasons and because of environmental problems related to the use of fossil fuels.

Various species of the genus *Clostridium* have been studied for their ability to naturally produce butanol from carbohydrates or similar carbon sources. This includes *Clostridium acetobutylicum*, *Clostridium aurantibutyricum*, *Clostridium beijerinckii*, *Clostridium butyricum*, *Clostridium cadavaris*, *Clostridium carboxidivorans*, *Clostridium chauvoei*, *Clostridium felsineum*, *Clostridium pasteurianum*, *Clostridium puniceum*, *Clostridium roseum*, *Clostridium saccharobutylicum*, *Clostridium saccharoperbutylacetonicum*, *Clostridium septicum*, *Clostridium sporogenes*, and *Clostridium tetanomorphum*.[23] The most common and best known solventogenic clostridial strains for commercial butanol fermentation are *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum*.[24] *Clostridium acetobutylicum* is the most extensively studied species. On the other hand, there are some other microorganisms, belonging to other genera, which are also able to produce butanol, such as *Butyribacterium* (e.g. *Butyribacterium methylotrophicum*) and *Thermoanaerobacterium* (e.g. *Thermoanaerobacterium thermosaccharolyticum* W16).

Fermentation of sugars

Clostridia are anaerobic bacteria, which have been shown for years to be able to metabolize and completely ferment various carbohydrates, including glucose, fructose, mannose, sucrose, xylose, and lactose, among others.[25] Others, such as trehalose and rhamnose have more recently also been found to be fermented into biofuels by bacteria such as *Clostridium butyricum* TM9A.[26,27] Different carbohydrates are fermented through different pathways by clostridia. In the case of hexoses, their metabolism follows the Embden-Meyerhof-Parnas (EMP) pathway (Fig. 1(A)), whereas the metabolism of pentoses takes place through the pentose phosphate (PP) pathway (Fig. 1(B)).[28,29] Several additional substrates, besides carbohydrates, can be metabolized into butanol as well. For example, glycerol and also polysaccharides such as carboxymethylcellulose were found to be fermented by some clostridia, such as the new isolate *C. acetobutylicum* YM1.[30]

Recently, numerous studies have focused on the effects of sugars from agricultural feedstocks on the fermentation process.[31–33] Typical sugar composition of the most commonly used feedstocks includes glucose, arabinose, mannose, xylose, fructose, sucrose and lactose, mainly. In this area, scientists have tried to identify the potentially best carbohydrate to use in butanol fermentation. *Clostridium acetobutylicum* was found to prefere glucose, for which the conversion rates were the highest, followed, in decreasing order, by mannose and fructose, arabinose, xylose, and finally lactose.[28,31–33] When comparing monosaccharides, such as glucose, with disaccharides, such as sucrose and lactose, the first difference with glucose is the transport system into the cells. Indeed, sucrose and lactose move across the cell membrane using the carbohydrate phosphotransferase system (PTS). That system is slower than the one used for glucose transport.[28] On the other hand, sucrose and lactose are hydrolysed into simple sugars, i.e. fructose-6-P and glucose-6-P, in the case of sucrose; glucose-6-P and galactose-6-P, in the case of lactose. The products obtained from sucrose and lactose will be metabolized through different pathways. Indeed, fructose-6-P and glucose-6-P obtained from sucrose can be converted via the Embden–Meyerhof–Parnas pathway.[34] Conversely, in products obtained from lactose, glucose is phosphorylated and incorporated into the glycolytic pathway, while galactose-6-P is metabolized via the tagatose 6-P pathway and does subsequently enter glycolysis.[35] These differences in terms of metabolism result in different consumption rates between sucrose and lactose, as a result of the bottleneck in the metabolism of galactose-6-P, as the metabolism of the latter is much more complex than the metabolism of fructose-6-P and glucose-6-P formed in the sucrose metabolism.

The hydrolysis of lignocellulosic feedstocks yields a mixture of carbohydrates including hexoses such as glucose and pentoses such as xylose, which will result in carbon catabolic repression (CCR) mainly in Gram-positive bacteria like *C. acetobutylicum*. CCR consists in the preferential use of glucose over other carbohydrates, e.g. xylose, which will be metabolized only once the former is exhausted. This leads to an inefficient use of the different carbon sources present in the fermentation broth. It is necessary to deal with this CCR issue for an optimal ABE fermentation and for the efficient use of all the carbon sources present in the medium in the case of complex lignocellulosic feedstocks. Working with strains non-affected by CCR or recombinant bacteria would be a suitable solution.[36]

The above described characteristics show that the nature of the sugars present in the raw material is a key parameter that will affect the efficiency of the fermentation process and the production of metabolites.

The fermentation metabolism of *C. acetobutylicum* and other similar bacteria is divided into two phases: acidogenesis and solventogenesis. In the acidogenic phase, cells grow exponentially while producing butyric and acetic acids mainly with a typical molar ratio of 2:1. In addition, CO_2, H_2, and ethanol are produced to some extent as well. In the solventogenic phase, the cells are predominantly in stationary phase, and take up the acids produced during acidogenesis, which are then converted into acetone, butanol and ethanol, with a typical molar butanol:acetone:ethanol ratio of 6:3:1. Such a ratio is typically found in *C. acetobutylicum* but may be different in other species. Besides, all three compounds do not necessarily appear in all clostridia (e.g. absence of acetone). Acetoin and lactate may also be produced but in minor amounts and only under specific conditions. It has been reported that the shift from acid production (acidogenesis) to the production of solvents (solventogenesis) is due to a change in gene expression.[37] Besides, such a shift has been claimed to be highly dependent on the pH value. An initially slightly high pH (i.e. 5.75) is optimal for the acidogenic phase. Acetic and butyric acids will mainly be formed during that phase, resulting in a pH decrease. It is considered that the solventogenic phase will generally start as soon as a critical pH value has been reached, leading to reassimilation of the acids with concomitant production of solvents, i.e. acetone, butanol and ethanol. Numerous studies have reported that a low pH is necessary for the solventogenic phase and the production of solvents.[15,22,38–40] It is worth taking into account that the bacteria need to have produced sufficient acids before reaching a pH below 4.5 and switch to the solventogenic phase. If only low amounts of acids are available, then the solventogenic stage would be too short and produce only reduced amounts of solvents. This could be avoided by using a medium with a high buffering capacity which

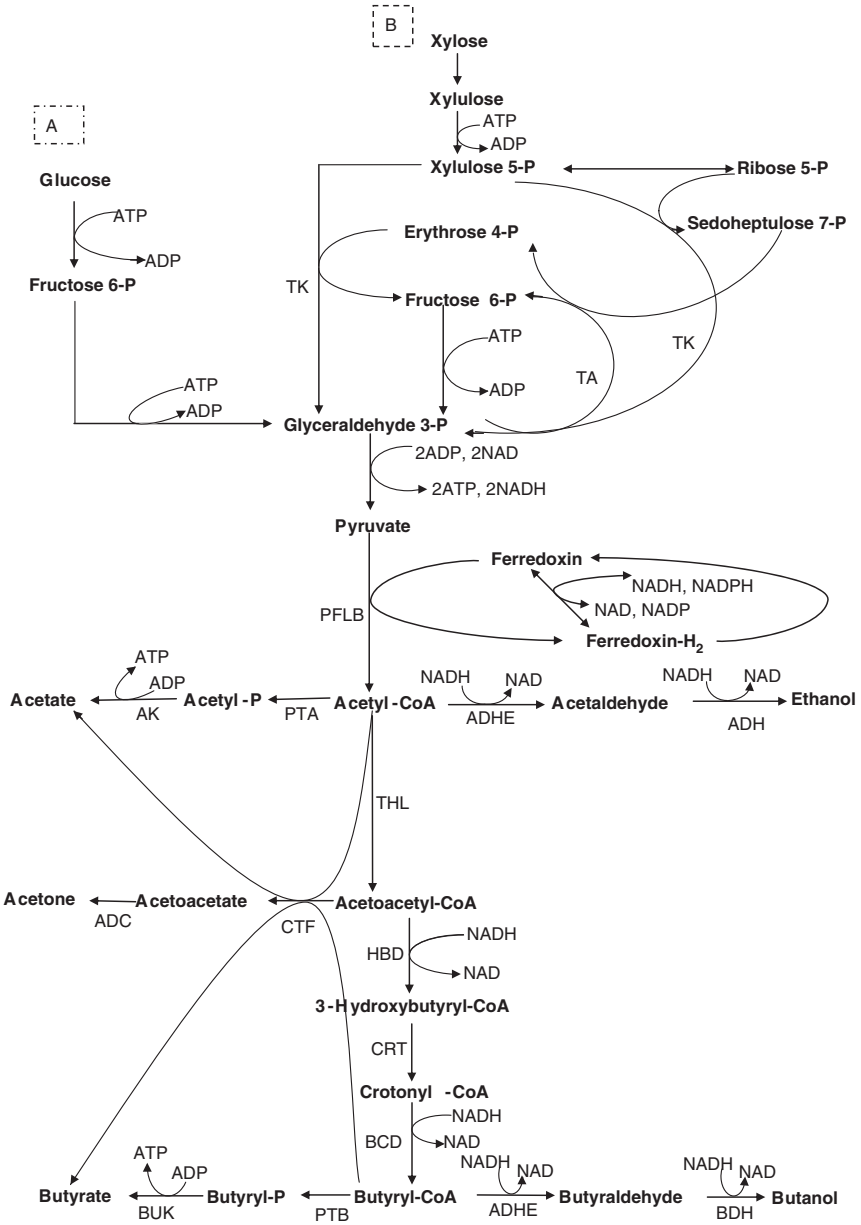

Figure 1. (A) Metabolic EMP pathway in *C. acetobutylicum* ATCC 824^T with glucose (hexose sugar) as carbon source. (B) Metabolic PP pathway of *C. acetobutylicum* with xylose (pentose sugar) as carbon source.

will lead to a longer acidogenic phase with better carbohydrate utilization and increased growth which does generally take place simultaneously to the production of acids.[41] It has been hypothesized that solvent production might be a response to stress conditions, such as a low pH. When the pH reaches a value of 4.5, undissociated acetic and butyric acids are able to cross the cytoplasmic membrane by diffusion.[42] When the value of the internal pH is about 5, undissociated acids start to dissociate inside the cell, releasing protons. As a result collapse of the proton gradient over the cytoplasmic membrane takes place and cell death will occur. In order to avoid such phenomenon, the cells will start converting the acids to solvents, which have a neutral charge. Nevertheless, it should be reminded that there is a point where the concentration of solvents can become toxic to the cells; thus producing solvents is often concomitant to cell sporulation, in order to ensure cell survival in the long-term.[9,43]

PRODUCTION OF (HIGHER) ALCOHOLS FROM CO-RICH GASES

Syngas, a mixture of primarily CO, CO_2, and H_2, is an inexpensive and flexible substrate and can be used by acetogens in fermentation processes to produce renewable fuels and chemicals. Acetogens are anaerobic microorganisms able to grow on C1 compounds such as CO, $CO_2 + H_2$ or formate and produce acetate mainly, via the Wood-Ljungdahl pathway with acetyl-CoA as main intermediate.[44] Besides, occasionally, other organic acids, such as butyrate, as well as alcohols can also be produced by a limited number of strains. The process is flexible because it can be generated from a wide variety of organic materials through gasification. Gasification is a thermal process that converts most of the lignocellulosic materials and other carbonaceous feedstocks into syngas at elevated temperatures.[45] Syngas conversion into liquid

fuels with biological catalysts is a more effective and efficient process compared with the use of chemical catalysts. When comparing two different case studies, in terms of ethanol production, the efficiency of carbon conversion to fuel was claimed to reach 40.7% in the thermochemical route and 51.6% in the biological route.[46] Besides, the energy in the feedstock converted to final product (LHV%, low heating value basis) was reported to reach 45% and 57%, respectively, in the thermochemical and the bioconversion routes. Finally, fuel yields (gal/dry US ton) of 83.8 and 117.6 were found in the thermochemical and biological processes, respectively. One of the reasons for this higher efficiency is the lower energy requirement and infrastructure set-up costs.[11] Indeed, bioconversion takes place at much lower temperature and pressure (30–37 °C and near atmospheric pressure) than catalytic chemical reactions (200–350 °C and 10–200 atm). Besides, a wide range of CO:CO_2:H_2 ratios can be used in bioprocesses, while such flexibility in terms of ratios is highly restricted in chemical processes (H_2/CO ratios of 1–4 in chemical catalysis; that need most often to be close to 2); also chemical processes are more sensitive to impurities (sulfur compounds, chlorine …) than the biological conversion of syngas. On the other hand, the use of syngas rather than dissolved sugars as feedstock has the advantage of allowing uncoupling of the hydraulic retention time from the substrate supply in suspended growth bioreactors, as the substrate is fed through the gas phase while nutrients are supplied as liquid phase.[11] Besides syngas, CO-rich industrial waste gases are also suitable for bioconversion to ethanol and higher alcohols. This is the case of waste gases from numerous steel industries.

Many microorganisms can metabolize syngas as carbon and energy source,[47] but only a few are able to convert it into (bio)fuels. Carbon monoxide can be metabolized by acetogens as single substrate and can be used both as carbon and energy source. On the other hand, CO_2 can be used as carbon source but needs the presence of hydrogen as energy source. Acetogenic bacteria follow the Wood-Ljungdahl (WL) pathway to produce biofuels from CO, CO_2, and H_2, or syngas/waste gas (Fig. 2). The WL route is divided into three phases: (i) synthesis of acetyl-CoA by reducing CO or $CO_2 + H_2$; (ii) conservation of energy; and (iii) CO_2 assimilation into cellular carbon.[48]

The different steps of the WL pathway leading to the production of different end metabolites are shown in Fig. 2. The figure shows how the pathway is composed of two branches known as Eastern and Western branches. In the Eastern branch, formate is obtained first through the reduction of CO_2. In the Western branch, the figure makes clear how CO can be taken directly or, otherwise, how CO_2 can be transformed into CO. The Wood-Ljungdahl pathway will eventually lead to the production of acetyl-CoA. Then, the latter is used by different enzymes to generate several end products. On one hand, acetyl-CoA could be converted to both acetate and ethanol. On the other hand, in the case of butanol production, acetyl-CoA is first enzymatically transformed into butyryl-CoA, which could also directly be converted to butyric acid and butanol as end products. Acids, i.e. acetate or butyrate, formed from acetyl-CoA or butyryl-CoA, can also further be converted to the corresponding alcohols. In case of acetate, the acid yields acetaldehyde in a reaction catalyzed by ferredoxin:aldehydeoxydoreductase (AOR). Then, ethanol is produced through the reduction of acetaldehyde. Similar enzymes are used for the conversion of butyric acid into butyraldehyde first and subsequently into butanol. The pathway for the production of higher alcohols such as hexanol leads to the conversion of acetyl-CoA to

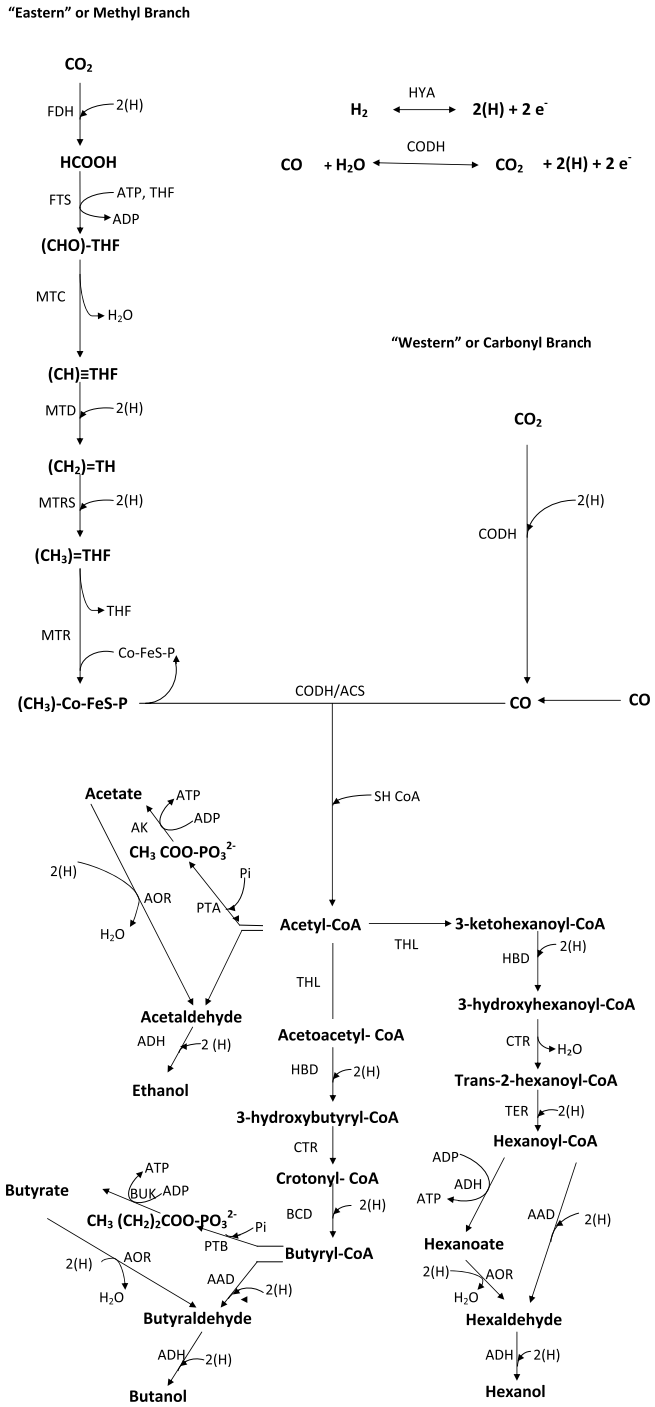

Figure 2. Wood-Ljungdahl pathway of acetogens,[143] for H-B-E fermentation.

hexanoyl-CoA which could directly be converted to hexanoate or, otherwise, to hexanol via hexaldehyde as intermediate metabolite (Fig. 2). Hexanoate and hexanol production in clostridial species is believed to be catalyzed by thiolase enzymes (Fig. 2), for example 2-keto thiolase. In some cases, butyryl-CoA is elongated with one molecule of acetyl-CoA, by a thiolase enzyme, which results in the production of 3-oxo-hexanoyl-CoA, which is then converted into hexanoyl-CoA. That reaction is believed to be carried out by the same enzymes that convert acetoacetyl-CoA into butyryl-CoA.[25]

The complex fermentation process can be summarized through the equations shown hereafter.

Potential reactions for ethanol and acetic acid production from CO, CO_2, and H_2 would be:[49]

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \quad \Delta G^o = -217.4 \text{ kJ mol}^{-1} \quad (1)$$

$$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O \quad \Delta G^o = -97.0 \text{ kJ mol}^{-1} \quad (2)$$

$$2CO + 4H_2 \rightarrow C_2H_5OH + H_2 \quad \Delta G^o = -137.1 \text{ kJ mol}^{-1} \quad (3)$$

$$3CO + 3H_2 \rightarrow C_2H_5OH + CO_2 \quad \Delta G^o = -157.2 \text{ kJ mol}^{-1} \quad (4)$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad \Delta G^o = -154.6 \text{ kJ mol}^{-1} \quad (5)$$

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad \Delta G^o = -74.3 \text{ kJ mol}^{-1} \quad (6)$$

$$2CO + 2H_2 \rightarrow CH_3COOH \quad \Delta G^o = -114.5 \text{ kJ mol}^{-1} \quad (7)$$

The net reactions for butanol and butyric acid production from CO, CO_2, and H_2 are:

$$12CO + 5H_2O \rightarrow C_4H_9OH + 8CO_2 \quad \Delta G^o = -486.4 \text{ kJ mol}^{-1} \quad (8)$$

$$12H_2 + 4CO_2 \rightarrow C_4H_9OH + 7H_2O \quad \Delta G^o = -245.6 \text{ kJ mol}^{-1} \quad (9)$$

$$6CO + 6H_2 \rightarrow C_4H_9OH + 2CO_2 + H_2O \quad \Delta G^o = -373 \text{ kJ mol}^{-1} \quad (10)$$

$$4CO + 8H_2 \rightarrow C_4H_9OH + 3H_2O \quad \Delta G^o = -334 \text{ kJ mol}^{-1} \quad (11)$$

$$10CO + 4H_2O \rightarrow CH_3(CH_2)_2COOH + 6CO_2 \quad \Delta G^o = -420.8 \text{ kJ mol}^{-1} \quad (12)$$

$$10H_2 + 4CO_2 \rightarrow CH_3(CH_2)_2COOH + 6H_2O \quad \Delta G^o = -220.2 \text{ kJ mol}^{-1} \quad (13)$$

$$6CO + 4H_2 \rightarrow CH_3(CH_2)_2COOH + 2CO_2 \quad \Delta G^o = -317 \text{ kJ mol}^{-1} \quad (14)$$

Finally, as previously explained, hexanol can also be produced by some acetogenic bacteria grown on gaseous C1 substrates (CO, CO_2, and H_2), depending on the bacterial species used in the fermentation process. Till now, hexanol production has been reported in *C. carboxidivorans* mainly, reaching concentrations of up to 1.06 g L^{-1} in the best case described so far on syngas fermentation, in a bioreactor with continuous gas feed and batch operation for the aqueous nutrient phase.[50] The presence of hexanoic acid has also been detected during the gas fermentation process and can subsequently be converted to hexanol, similarly as for ethanol and butanol which can be obtained simultaneously, from the fermentation of C1 gases and, to a larger extent, from the corresponding C2 and C4 organic acids (i.e. acetic and butyric acids).

Equations for hexanol and hexanoic acid (i.e. caproic acid) production from CO, CO_2, and H_2 are:

$$18CO + 7H_2O \rightarrow C_6H_{13}OH + 12CO_2 \quad \Delta G^o = -753 \text{ kJ mol}^{-1} \quad (15)$$

$$18H_2 + 6CO_2 \rightarrow C_6H_{13}OH + 11H_2O \quad \Delta G^o = -395 \text{ kJ mol}^{-1} \quad (16)$$

$$6CO + 12H_2 \rightarrow C_6H_{13}OH + 5H_2O \quad \Delta G^o = -514 \text{ kJ mol}^{-1} \quad (17)$$

$$16CO + 6H_2O \rightarrow CH_3(CH_2)_4COOH + 10CO_2 \quad \Delta G^o = -656 \text{ kJ mol}^{-1} \quad (18)$$

$$16H_2 + 6CO_2 \rightarrow CH_3(CH_2)_4COOH + 10H_2O \quad \Delta G^o = -341 \text{kJ mol}^{-1} \quad (19)$$

$$10CO + 10H_2 \rightarrow CH_3(CH_2)_4COOH + 4CO_2 \quad \Delta G^o = -540 \text{ kJ mol}^{-1} \quad (20)$$

A similar two stage fermentation pattern is observed in the production of alcohols from C1 gases (CO, CO_2, and H_2), i.e. the HBE fermentation, as in the more conventional ABE fermentation using carbohydrate substrates. First the gaseous substrates are converted into acids, during the acidogenic phase, at pH values optimal for growth, usually close to 6.0. Both acetic and butyric acids are formed but, as mentioned earlier, also some hexanoic acid has been detected. Based on the few results published so far, acetic acid appears first and is produced at somewhat higher concentrations than butyric acid in *C. carboxidivorans*. Afterwards, hexanoic acid does also appear, though at lower concentration than the other two acids.[50] This is different from the more conventional ABE fermentation in which higher concentrations of butyric acid are generally produced compared with acetic acid.[51,52] The production of acids from pure CO or $CO + CO_2 + H_2$ is concomitant with biomass growth and results in acidification of the medium. As a result, the pH decrease inhibits any further biomass growth and stimulates solventogenesis. During the solventogenic phase, alcohols are generated. In a system with no automatic pH regulation, pH is then often found to increase again. Besides, a higher pH leads to some increase of the redox potential, reaching slightly less negative values.[53] In the ABE fermentation, *C. acetobutylicum* metabolizes carbohydrates to produce acetone, butanol and ethanol as solvents. Conversely, in the fermentation of CO-rich gases by *C. carboxidivorans* only ethanol and higher alcohols are produced but no acetone has ever been detected. Ethanol, butanol, and also some hexanol appear as end metabolites. The genome of *C. carboxidivorans* strain P7 was recently characterized and shown to lack genes for acetone production.[54] It is worth mentioning that, although acetone is produced in the ABE fermentation in *C. acetobutylicum* and several other studied species, some clostridial strains (e.g. *C. pasteurianum*) appear not to have the ability to produce acetone from carbohydrates in such a process.[55] As explained above, butanol is the major solvent in ABE fermentation from sugars, while ethanol has so far always been reported to be the dominant alcohol in the fermentation of C1 gases. This is related to the higher amounts of butyric acid appearing during the acidogenic stage in the ABE fermentation with *C. acetobutylicum* and other clostridia, while more acetic acid is generally detected in the fermentation of C1 gases by *C. carboxidivorans*. Based on the limited available literature data,[50] typical molar hexanol:butanol:ethanol (H:B:E) ratios in that species from the fermentation of $CO:CO_2:H_2:N_2$ mixtures

(2:2:1:5) are 1:2.5:6, to be compared with acetone:butanol:ethanol (A:B:E) molar ratios of 6:3:1 in ABE fermentation in clostridia with monosaccharides as substrates.

In most of the few studies published so far, usually in batch assays or in continuous gas-fed bioreactors without any continuous addition of nutrients in the form of a liquid phase, final butanol concentrations hardly reached 1 g L^{-1} or less, when pH was allowed to fluctuate freely. However, in some recent studies, butanol concentrations as high as 2.66 g L^{-1} were obtained during CO fermentation[53] and 1.98 g L^{-1} from syngas fermentation[50] ($CO:CO_2:H_2:N_2$ mixture, 2:2:1:5, on a molar basis) with the wild type strain of *C. carboxidivorans*. This higher alcohol production was reached when using pH regulation during the fermentation process. Indeed, pH was initially maintained automatically constant at a value of 5.75, suitable for growth and production of acids, resulting in the accumulation of relatively high concentrations of such acids. Subsequently, after growth leveled off and acids had accumulated in the medium, the pH was maintained at a lower value of 4.75, favourable to the further bioconversion of the gaseous substrates, but also the conversion of accumulated acids to the corresponding alcohols.[53] Similarly to the ABE fermentation, a two stage process with two bioreactors in series might allow producing high amounts of alcohols, maintaining the first reactor under optimal growth conditions, with accumulation of acids, and the second reactor at a lower pH for conversion of the acids into alcohols.

Performance data available on CO and syngas fermentation to higher alcohols, under different conditions, are summarized in Table 1.

In acetogenic bacteria, energy conservation is highly limited. One ATP is produced in the last step of the conversion of acetyl-CoA to acetate catalyzed by the acetate kinase enzyme (Fig. 2). The same would take place in butyrate and hexanoate production. However, additional ATP can also be formed by the Rnf complex involved in pumping H^+ or Na^+ anions across the cytoplasmic membrane which results in an ion gradient and in concomitant ATP synthesis by the ATPase protein complexes.[9] The Rnf complex has been identified as a proton-translocating ferredoxin:NAD+ oxidoreductase in *C. ljungdahlii*.[56] Examples of other bacteria that have been found to possess such Rnf complex are *A. woodii* and *C. autoethanogenum*.[57,58] In some cases, the proton gradient in autotrophic acetogens can also be created by an energy converting hydrogenase, or Ech. Only limited research has been published on energy conservation in *C. carboxidivorans*, but some recent reports suggest that in that organism ATP could also be produced by a membrane gradient via the Rnf complex found on its genome.[54,59]

Anaerobic bacteria converting C1 gases to (higher) alcohols

So far, only very few bacteria have been isolated and proven to produce butanol or higher alcohols from CO, CO_2, and/or H_2 (Table 2). Their main characteristics are briefly summarized hereafter.

Clostridium carboxidivorans (ATCC BAA-624; DSM-15243)

This organism was isolated from an agricultural settling lagoon in Oklahoma (USA). The 16S rRNA gene sequence analysis showed that that species is closely related to *C. scatologenes* ATCC 25775 T and *C. drakei*, but DNA reassociation analysis showed that these three bacteria are different species. *Clostridium carboxidivorans* is an acetogenic anaerobic bacterium, which can grow chemoautotrophically on syngas, using CO or H_2/CO_2. Moreover, it is one of the few bacteria known to produce solvents from syngas,[63]

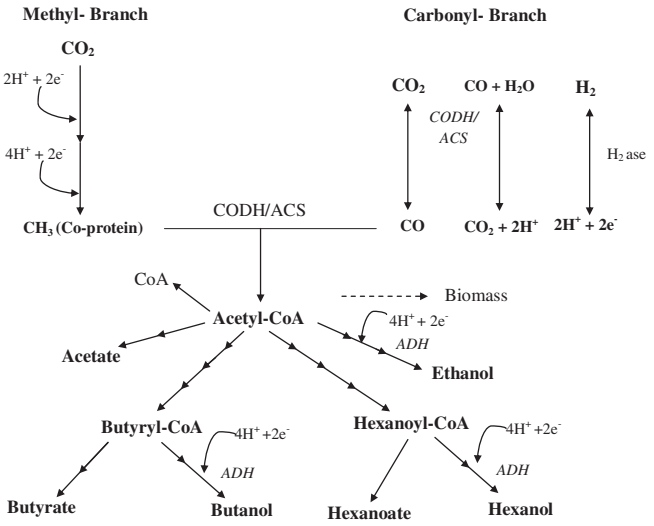

Figure 3. Wood – Ljungdahl pathway of *Clostridium carboxidivorans*[54,64,65].

including long-chain alcohols (e.g. butanol and hexanol) besides ethanol.[39,50,62]

There are numerous studies on the metabolic and genomic characteristics of that organism. Results indicate that *C. carboxidivorans* follows a Wood-Ljungdahl related pathway for the production of acetic, butyric, and hexanoic acids, as well as ethanol, butanol, and hexanol, metabolizing CO or syngas, used as carbon and energy sources (Fig. 3).[54,64,65]

Clostridium drakei (ATCC BAA-623T; DSM 12750T)

This species was renamed in recognition of the contributions that Harold L. Drake has made to our understanding of the microbiology of acetogens. This bacterium is an obligate anaerobe, growing autotrophically on H_2/CO_2 or CO, to produce acetic and butyric acids, ethanol, and butanol as end-products of its metabolism.[66]

Clostridium ragsdalei (ATCC BAA-622, DSM 15248)

This acetogen ferments syngas ($CO:CO_2:H_2$) into acetic acid and ethanol. One report describes the detection of small amounts of butanol, up to 0.47 g L^{-1}, in a bioreactor inoculated with *Clostridium* strain P11, which is expected to belong to the species *C. ragsdalei*, based on other publications and data bases.[67] However, production of higher alcohols from syngas by *C. ragsdalei* has otherwise never been confirmed nor reported elsewhere. One study has shown that that organism is able to reduce organic acids such as propionic acid, butyric acid, pentanoic acid, and hexanoic acid into the corresponding primary alcohol (propanol, butanol, pentanol, and hexanol).[68]

Butyribacterium methylotrophicum

This organism is a catabolically spore-forming anaerobe that ferments multicarbon substrates (e.g. glucose, lactate, and pyruvate) or single-carbon substrates (CO, H_2/CO_2) to produce varying amounts of acetic and butyric acids, ethanol and butanol.[38,69,70]

Parameters affecting gas fermentation to higher alcohols

For the commercial production of (bio)butanol and other alcohols from C1 gases in an efficient way, a key issue consists in identifying

Table 1. Production of higher alcohols using gaseous substrates by acetogenic bacteria

Microorganism	Culture mode	Syngas composition (v/v, %)	pH	Temperature (°C)	Cell density (g L^{-1})	Conversion efficiency	Products concentration	References
C. carboxidivorans								
P7	Bubble column bioreactor	$CO/CO_2/N_2$ (25:15:60)	5.8 - 5.9	37	NR	NR	Ethanol: 0.15; butanol: 0.075; acetic acid: 0.025 Apparent yields (mole C in products per mole CO consumed) Ethanol 1.6 g L^{-1}; acetic acid: 0.3 g L^{-1}; butanol 1.1 g L^{-1} (dropped to 0.6 g L^{-1} reason not determined)	60
	Bubble column bioreactor	$CO/CO_2/N_2$ (25:15:60)	5.2	37	0.35	CO: 60%	0.33 mol ethanol/mol CO; 0.033 mol butanol/mol CO; 0.04 mol acetate/mol CO (based on carbon content)	61
	Continuous gas-fed bioreactor	CO	5.75	33	0.52	CO: 50%	Acetic acid: 1.04 g L^{-1}; butyric acid: 0.28 g L^{-1}; ethanol: 4.41 g L^{-1} and butanol: 2.3 g L^{-1}. Total net ethanol production: 7.52 g (in 1.2 L reactor medium) and total net butanol production: 3.91 g (in 1.2 L reactor medium).	53
	Continuous gas-fed bioreactor	CO	Exponential phase 5.75 and solventogenic phase 4.75	33	0.33	CO: ± 50%	Acetic acid: 0.06 g L^{-1}; butyric acid: 0.01 g L^{-1}; ethanol: 2.90 g L^{-1} and butanol: 1.60 g L^{-1}. Total net ethanol production: 4.21 g (in 1.2 L reactor medium) and total net butanol production: 2.29 g (in 1.2 L reactor medium)	53
	Batch	$CO/H_2/N_2/CO_2$ (32:32:28:8)	6	25	0.288	NR	Ethanol: 1.47 g L^{-1}; butanol: 1.08 g L^{-1}; hexanol: 0.84 g L^{-1}; caproic acid: 1.05 g L^{-1}; acetic acid: 1.65 g L^{-1}.	62

NR: Not reported

optimal conditions for the fermentative process. Therefore, several parameters need to be considered, as described in the following sections.

pH

The importance of pH to promote the shift from acidogenic to solventogenic phase has been explained previously. A low pH has been considered to be more favourable for the solventogenic stage, i.e. ethanol, butanol, and hexanol production in the present case whereas such a low pH will have a negative effect on cell growth.[15,40] This is a major drawback limiting the optimal conversion of syngas to alcohols, as a lower pH will inhibit bacterial growth and eventually lead to cell death. It may then also limit the overall specific productivity of butanol and other alcohols in the process. However, some recent studies showed that in the HBE fermentation efficient conversion of acids into higher alcohols is also possible at higher, slightly acidic, pH (e.g. pH 5.75), theoretically most favorable to acidogenesis.[53]

In the acidogenic phase, it is necessary to know and take into account the optimum growth pH of the microorganism, which is strain specific. A pH value below optimum has a negative impact on growth rates and cell viability and may even result in biomass decay. *Clostridium* strains have an optimum pH for growth ranging between 5.5 and 7.5 depending on the species. For example the optimum pH for *C. ljungdahlii* is 5.8–6.0, while a pH range of 4.4–7.6 has been reported for *C. carboxidivorans*, with an optimum pH between 5.0 and 7.0.[64,71] The optimum

Table 2. Characteristics of different acetogens converting CO-rich gases into alcohols

	C. ljungdahlii	*C. autoethanogenum*	*C. carboxidivorans P7*	*C. drakei*	*C. ragsdalei P11*	*B. methylotrophicum*	*Alkalibaculum bacchi*
Size (μM)	0.6 x 2 - 3	0.5 x 3.2	0.5 x 3	0.6 x 3 - 4	0.7 - 0.8 x 4 - 5	0.8 - 1.0 x 2.7 -3.2	0.5-0.8 x 1.5–2.2
Temperature range (°C)	30 - 40	20 - 44	24 - 42	18 - 42	18 - 37	10 – 50	15 - 40
Temperature optimum (°C)	37	37	37 - 40	30 - 37	37	37	37
pH range	4.0 - 7.0	4.5 - 6.5	4.4 - 7.6	4.6 - 7.8	4.0 - 8.5	6.0 – 9.0	6.5 - 10.5
pH optimum	6.0	5.8 - 6.0	5.0 - 7.0	5.5 - 7.5	6.3	7.5	8.0 - 8.5
Main substrates	Syngas (CO, CO_2, H_2 and Ar) and sugars such as glucose, fructose	Syngas (CO, CO_2, H_2 and N_2) and sugars such as xylose	Syngas (CO, CO_2, N_2 and H_2) and sugars such as glucose	Syngas (H_2–CO_2, and CO–CO_2) and sugars such as cellobiose, glucose, xylose	Syngas (CO, CO_2, H_2)	CO, H_2-CO_2, glucose, methanol	H_2 : CO_2, CO : CO_2, glucose, fructose, mannose, turanose, ribose, trimethylamine, pyruvate, methanol, ethanol, *n*-propanol and *n*-butanol
Main products	Acetic acid, butanediol and ethanol	Acetic acid, butanediol, ethanol	Acetic acid, butyric acid, hexanoic acid, ethanol, butanol and hexanol	Acetic acid, butyric acid, ethanol and butanol	Acetic acid, butanediol and ethanol	Acetic acid, butyric acid, lactate, ethanol and *n*-butanol	Acetic acid, ethanol
References	72,73	74	64	64	75	76,77	78

Table 3. Solubilities of CO, H_2 and CO_2 in aqueous phase, at different temperatures and constant pressure of 1 atm, expressed in g L^{-1}_{water}

Temperature (°C)	CO	CO_2	H_2
15	0.031	2.01	0.0017
25	0.028	1.50	0.0016
30	0.026	1.32	0.0015
37	0.023	1.13	0.0014
45	0.019	0.86	0.0013
60	0.015	0.59	0.0012

pH of butanol- or higher alcohols-producing species appears in Table 2.

Temperature

Similarly to pH, bacteria exhibit their growth and metabolic activity over a given limited range of temperatures characterized by defined optimal values. Temperature does not only affect microbial growth and substrate bioconversion rates in syngas fermentation; it does also affect the solubility of gaseous substrates in liquid medium. For example, CO and H_2 are very poorly soluble in aqueous phase and higher temperatures have a negative impact on such solubility (Table 3).

To the best of our knowledge, all clostridia isolated so far and producing (bio)fuels from C1 gases are mesophilic organisms. Mesophiles typically grow in a temperature range between about 15 and 40 °C, with optima between 30 and 37 °C in most cases, depending on the species. In the case of carboxydotrophs, the optimum temperature for incubation is often close to 35–37 °C. The most common temperature ranges of the few anaerobic bacteria typically used in the production of butanol or higher alcohols from C1 gases are given in Table 2.

It has been suggested that temperature may affect 'acid crash'. 'Acid crash' results from the accumulation of high concentrations of undissociated acids produced during acidogenesis, mainly in batch fermentations, leading to the inhibition of solventogenesis and limiting the production of alcohols. It was observed experimentally that a lower incubation temperature during the conventional ABE fermentation, may help avoiding 'acid crash', as a result of the lower rates of production of acids at such lower temperatures.[79] In a recent study, the syngas-fermenting acetogenic bacterium *C. carboxidivorans* P7 was also incubated at a sub-optimal temperature of 25 °C, leading to a lower metabolic activity, slow growth and longer lag phase. It also led to enhanced ethanol and butanol production, reaching concentrations of 32.1 and 14.5 mmol L^{-1}, respectively.[62] Some hexanol and caproic acid were also produced in that experiment, at concentrations of 8.21 and 9.02 mmol L^{-1}, respectively. The concentrations of alcohols were found to be significantly higher at these lower temperatures than when incubating at 37 °C. On the other hand, other authors did also study the effect of temperature on *C. carboxidivorans*.[80] They found that a high temperature (37 °C) improved the reassimilation of acids into alcohols compared with a lower temperature (25 °C), but that a lower temperature improves the elongation of the carbon chain. Therefore, a low temperature is favourable for obtaining long chain acids and alcohols, improving the production of alcohols such as butanol and hexanol. In the case of butanol and hexanol, the highest concentrations of alcohols were found to reach 0.57 g butanol L^{-1}, and 0.48 g hexanol L^{-1} in a study performed at 25 °C, while at a higher temperature of 37 °C butanol and hexanol were not detected.[62]

Pressure

Another important factor in syngas fermentation is the gas pressure, as solubility in water for gases such as CO, H_2 or CO_2 increases basically linearly with pressure at moderate pressures as used in bioconversion processes. An increase in pressure may also improve mass-transfer. Consequently, higher pressures result in a better supply of the carbon/energy source in the culture media and increases the availability of carbon/energy sources to the cells, leading to higher growth and higher concentrations of fermentation products. Abubackar and co-workers[7] compared the effect of different nutrients and different initial pressures on ethanol fermentation using *C. autoethanogenum* as biocatalyst. The authors concluded that increasing the initial pressure led to higher final ethanol concentrations, as higher pressures will positively affect gas solubility and the efficiency of bioconversion.[7]

Effect of medium composition

Recently, aspects such as culture medium composition have been optimized, aimed at maximizing the conversion of gaseous substrates and acids into alcohols.

The carbon/energy source is probably one of the most important factors affecting the nature of end metabolites obtained in the fermentation process. It has been known for decades that clostridia are able to use different sugars as carbon source. More recently, studies have also focused on the use of CO, $CO_2 + H_2$, syngas, or waste gases as carbon and/or energy sources for the production of (bio)fuels. However, so far, only quite few acetogens have proven to be able to produce butanol or higher alcohols from such gases.

In case of ethanol production from CO, CO_2, and H_2, some studies have described the effect on bioconversion of other components of the culture medium than the carbon and energy sources. However, hardly any information is available in the case of butanol or higher alcohols production. Compounds that have been demonstrated to improve the microbial productivity of alcohols are metal co-factors. Nickel is one of those. Some authors studied the effect of that metal on ethanol production.[81,82] It was concluded that nickel improves CO uptake and alcohol (e.g. ethanol) production in gas fermentation, due to the importance of that trace metal for the enzymes CO dehydrogenase and acetyl-CoA synthase. The effect of various trace metals on ethanol production was also studied in *C. ragsdalei*.[83] It was shown that removing Cu^{2+} and increasing the concentrations of Ni^{2+}, Zn^{2+}, SeO_4^{2-}, and WO_4^{2-} increased ethanol production, due to the increase of the activity of specific metalloenzymes in the WL pathway. Abubackar and co-workers found that the presence of tungsten significantly improved alcohol production and increased the ethanol/acetate ratio in *C. autoethanogenum* grown on CO, while the presence of selenium did either not improve ethanol production or even reduce it under the experimental conditions used in their study.[84,85] Metalloenzymes and the presence of trace metals play thus a key role in ethanol production and are expected to have a similar influence in the bioconversion of gaseous C1 substrates to higher alcohols such as butanol and hexanol.

In a recent report, the effect of different trace compounds was described (e.g. W, Ni, Cu, Mo, and Zn) for batch butanol and hexanol production in *C. carboxidivorans*.[39] Media in which copper was removed while increasing the molybdate concentration(×10) resulted in improved butanol and hexanol production. With the

standard non-modified medium, maximum butanol and hexanol concentrations of 0.83 g L^{-1} and 0.24 g L^{-1}, respectively, were obtained. Conversely, with the modified medium described above, butanol and hexanol concentrations reached 1.09 g L^{-1} and 0.94 g L^{-1}, respectively, resulting in 26% and 70% increase in butanol and hexanol production under such conditions.[39] Molybdate (Mo) is an analog of tungsten (W) and binds in the active sites of some enzymes, such as the AOR involved in the conversion of acids (e.g. butyric acid) into alcohols (e.g. butanol), as shown in Fig. 2.

Other compounds have been studied for their influence on solventogenesis such as the nature and concentration of nitrogen sources or the presence of vitamins. No study has been reported for butanol and hexanol, but a related behavior can be foreseen as for ethanol. Generally, richer media with high nitrogen concentrations or the presence of yeast extract will stimulate acidogenesis, result in the production of acids and improve biomass growth, while nutrient limiting conditions are more suitable for the solventogenic phase. Although most researchers add vitamins to their culture media, it was observed that *C. autoethanogenum* can efficiently produce ethanol without addition of any vitamins.[85]

A low redox potential is also required to produce butanol, ethanol, and higher alcohols, and, as a general rule, a reducing agent such as cysteine-HCl needs to be added into the medium, in order to increase the production of alcohols.[86] Reducing agents are involved in the conversion of NAD(P) to NAD(P)H, which favours the production of alcohols.

Inhibitory compounds

Although biomass-derived syngas is composed of CO, CO_2, and H_2 as major constituents, it does usually also contain a range of additional compounds such as methane, ethylene, ethane, and acetylene, among others,[87,88] which may affect the fermentation process as they are potential inhibitors of bacterial growth and/or of their metabolic activity, eventually leading to poor bioconversion yields.

Even trace amounts of additional constituents such as acetylene or NO (nitrogen oxide) are known to inhibit the activity of some enzymes such as the hydrogenase enzyme,[89] which is involved in the generation of electrons from the reaction with H_2. A NO mole fraction above 0.015% was found to exert a strong inhibitory effect on hydrogenase.[10] The result of such inhibition is that electrons for ethanol, butanol, or hexanol formation must be obtained from CO rather than H_2, thus reducing the available carbon amount for product formation. The inhibition of this enzyme forces the cell to obtain electrons from CO using CODH enzymes. Another problem associated with the presence of NO in syngas fermentation is initial growth inhibition, besides reduction of the carbon conversion efficiency of the process. Similarly, when using syngas or waste gases containing CO, CO_2 and H_2 as substrate mixture, high concentrations of CO have been proven to inhibit the activity of hydrogenases in acetogens and thus inhibit hydrogen consumption. This may result in CO consumption while H_2 and CO_2 would then remain unused.[90] So far most bioconversion studies on alcohol production and HBE fermentation have been performed in suspended-growth stirred tank bioreactors. The low solubility of carbon monoxide in aqueous phase might explain why hydrogen consumption has been found to be still possible in such cases, minimizing the inhibition of hydrogenases.

Sulphur compounds may also appear in syngas. In *C. ljungdahlii*, H_2S concentrations as high as 5.2% (v/v) were found to hardly affect bacterial growth.[91] The presence of sulphur compounds may even contribute to further reduce the redox potential of the anaerobic culture medium. On the other hand, sulphur compounds can easily poison chemical catalysts, when they are used for the conversion of syngas into industrial products, which is a clear advantage of the biological process.

Ammonia (NH_3) is another impurity to be taken into account in syngas. Its presence can lead to non-negligible accumulation of ammonium ions (NH_4^+) in media used for bioconversion. Ammonium ions have been shown to be able to inhibit hydrogenase activity and cell growth of *C. ragsdalei*.[89]

Tars in syngas obtained from biomass were assumed to be the likely cause of cell dormancy in *C. carboxydivorans* and product redistribution, leading to increased alcohol production (in terms of ethanol) and decreased acetic acid production.[10] *Clostridium carboxydivorans* appeared to be able to adapt to tars after prolonged exposure. If needed a gas cleaning unit, e.g. filtering system, may be used in order to reduce or avoid the effect of tars present in the gaseous substrate. A recent study using *C. ljungdahlii* showed that concentrations of 0.01 g L^{-1} of $CaCO_3$ increased cell growth and the production ethanol. However, higher concentrations (0.02 g L^{-1}) will rather decrease growth and solvent production.[92]

Other compounds and factors may affect the bioconversion process. The salt concentration may have inhibitory effects that may need to be taken into account. Sodium chloride may accumulate in some fermentation processes, for example when adjusting the bioreactor´s pH through the addition of either hydrogen chloride or sodium hydroxide.[93] Although this has scarcely been studied, some reports described the effect of salt accumulation in bioreactors and batch experiments in the case of ABE-producing strains. Maddox *et al.* found that concentrations of 30 g L^{-1} sodium chloride or higher completely inhibit growth of *C. acetobutylicum* with lactose as a carbon source.[94] A concentration of 15 g L^{-1} of that compound resulted in 50% growth inhibition. At lower concentrations, growth took place but the fermentation pattern shifted from solventogenic to acidogenic.

Solvent toxicity

Solvent (i.e. alcohols from the HBE fermentation) toxicity has been identified as a critical problem in the fermentation. Under normal conditions, the clostridial cellular activity decreases in the presence of 20 g L^{-1} or more solvents in the conventional ABE production process.[95] This is one of the most important factors to take into account in butanol fermentation as anaerobic bacteria fermenting carbohydrates or other similar soluble substrates have been found to rarely tolerate more than 2% butanol.[96] Butanol has shown to be a fermentation product more toxic than acetone or ethanol in ABE fermentation,[97] and it was observed that the cells can tolerate higher concentrations of ethanol and acetone than butanol during growth. Although as much as 40 g L^{-1} acetone and ethanol were required to reduce growth on hexoses by 50%, butanol concentrations of only 7 – 13 g L^{-1} were already high enough to exert a similar inhibitory effect.[22]

Although no thorough study had been reported until recently, a similar toxic effect was found to take place during the bioconversion of C1 gases by clostridia. Indeed, butanol and ethanol toxicity was studied, using *C. carboxidivorans* as biocatalyst grown on carbon monoxide.[98] The authors found that butanol had a higher toxic effect than ethanol and than 1:1 mixtures (w/w) of both ethanol and butanol. The IC_{50} (i.e. the inhibitory concentration leading to 50% growth inhibition) was observed to correspond to 14.5 g L^{-1} for butanol, while the value of IC_{50} found in the ethanol

toxicity experiments was much higher and reached 35 g L^{-1} (i.e. lower toxic effect). It was concluded that ethanol and butanol are both toxic for *C. carboxidivorans* at high concentrations, but that ethanol had a lower inhibitory effect on *C. carboxidivorans* than butanol. Thus, a higher concentration of ethanol is necessary to exhibit the same toxic effect as butanol, in terms of IC_{50}, growth rate, biomass density and CO consumption. Potential hexanol toxicity has not been evaluated nor reported so far but, as indicated above, hexanol concentrations in HBE fermentation are generally lower than butanol and ethanol concentrations.

Some of the suggested reasons for the high toxicity of butanol are summarized hereafter. In a study on the mechanism of butanol toxicity in *C. acetobutylicum* in ABE fermentation it was found that 8–12 g L^{-1} butanol caused 50% inhibition of cell growth and the sugar uptake rate by negatively affecting the ATPase activity.[97] This is because butanol is a lipophilic solvent. It can alter the fatty acids and phospholipids composition of the cell membrane, which will lead to an increase in membrane fluidity. This will alter some of the membrane functions, such as membrane ATPase activity, transport functions, and substrate uptake.[99]

Several studies have focused on trying to improve solvent (e.g. butanol) tolerance in clostridial strains. Two major alternatives have been put forward: (i) using mutagenesis and genetic manipulation; and (ii) maintaining a low concentration of solvents such as butanol in the fermentation broth. As described elsewhere,[98] at least one recent report has shown that in the fermentation of CO-rich gases, mutant strains were obtained that were able to grow in the presence of ethanol concentrations of around up to 50 g L^{-1}.[100] For maintaining low solvent concentrations in the fermentation broth, different methods are available to separate end metabolites in order to avoid reaching concentrations that may be inhibitory to the bacteria and that would affect the fermentation process.[101] These techniques have been applied in ABE fermentation and, although the end metabolites in CO, syngas and waste gas fermentation are slightly different, similar separation processes would be suitable. A detailed description of these methods is beyond the scope of this review, but common removal techniques are adsorption, liquid–liquid extraction, pervaporation and gas stripping, besides some other somewhat less studied alternatives.[102] Adsorption is commonly set-up as an external unit, but otherwise most of the techniques can either be integrated in the fermentation process itself or they can be used offline. Studies reported in the literature concern mainly ABE fermentation, but they could similarly be applied to the HBE process.

In adsorption the solvents are transferred to a solid material, e.g. zeolites or activated carbon. It is a rather easy to use technique, requires generally little energy compared with other alternatives, does not damage bacterial cells and is effective in separating solvents such as butanol from the medium.[103,104] Liquid–liquid extraction consists in using a solvent with a high extraction efficiency for the metabolites to be separated from the fermentation broth. At the same time, for *in situ* separation, one should make sure that the solvent chosen for extraction does not exhibit any inhibitory effect on the bacteria. Oleyl alcohol is quite popular for the extraction of butanol in ABE fermentation or for other similar products. Ionic liquids, as non-volatile extractants, have more recently been used as well. In gas stripping, an inert gas or gas generated from the fermentation process itself allow to strip solvent-metabolites from the medium. This technique is simple and does not damage the cells. However, a potential drawback is that it can result in foam formation, above all when working with small bubbles, which can negatively affect the stability and performance of the fermentation process. Low selectivity is another potential drawback to be taken into account.[105] Pervaporation is a membrane-separation process. It has a relatively low energy consumption and does not damage the cells. A possible problem is membrane fouling.[106–108]

Some studies calculated the energy needed for 1-butanol recovery with these different techniques. For adsorption a value of 1.3 MJ kg^{-1}-butanol was reported, but it may go up to 33; using pervaporation, energy requirements between 2 and 145 MJ kg^{-1}-butanol were reported; values between 7 and 14 kg/butanol are typical for liquid–liquid extraction and finally values in the range of 14–31 MJ kg^{-1} butanol were reported for gas stripping.[105,109]

Mass transfer limitation in syngas fermentation

The efficiency of bioconversion of syngas and related waste gases to butanol and other alcohols is limited by the low water solubility of their gaseous components, i.e. CO, CO_2, H_2, and gas–liquid mass transfer is therefore a rate-limiting step in the fermentation process.[91,110–112] Generally, gas–liquid mass transfer limits the conversion rates in bioprocesses that use poorly soluble gases as key components, i.e. carbon and/or energy sources (e.g. CO or H_2 in homoacetogens).

Solubility data of syngas/waste gas components are given in Table 3. As can be observed the solubilities of CO and H_2 are quite low. In order to compare with another gas such as oxygen, which is the main electron acceptor in aerobic fermentation processes and whose solubility is about 5 mg L^{-1} at room temperature, the solubilities of CO and H_2 appear to be only about 60% and 3% of oxygen solubility, respectively, on a mass basis. Conversely, the solubility of CO_2 is more than fifty times higher than that of carbon monoxide (Table 3).

There are several steps in the diffusion process where mass transfer limitations are inevitable in suspended growth bioreactors: (i) the transport of the gaseous substrate into the gas–liquid interface; (ii) its transport through the nutritive liquid phase to reach the microbial cell surface; and finally (iii) gas diffusion into the microbial cell.[116] Depending on the bioreactor configuration, parameters such as the composition and properties of the liquid, interfacial adsorption, bubble size, mixing intensity, and other factors may influence the magnitude of the mass transfer resistances.[116–118] The mass transfer coefficient ($K_L a$) helps understand the rate of mass transfer. $K_L a$ for a slightly soluble gaseous substrate can be determined using the following equation: [118]

$$\frac{1}{V_L}\frac{dN_s^g}{dt} = \frac{K_L a}{H}\left(p_s^g - p_s^L\right)$$

where N_s^g (mol) is the moles of substrate transferred from the gas phase, V_L is the liquid working volume of the reactor, p_s^g (atm) is the partial pressure of the volatile substrate in gas phase and p_s^L (atm) is the partial pressure of the volatile substrate in a gas phase that would be in equilibrium with the actual concentration of that substrate in the liquid phase, H (L·atm mol^{-1}) is the Henry´s law constant, and $K_L a$ (s^{-1}) is the overall mass transfer coefficient.

It is clear from the above equation that the efficiency of the fermentation process, in terms of mass transfer and gaseous substrate supply to the biocatalyst, will improve when increasing the gas mass transfer coefficients or at higher concentration (i.e. pressure) gradients. High gas–liquid mass transfer conditions are strongly desired in commercial syngas or waste gas fermentation.[7] Working with pressurized bioreactors would be a suitable means

to improve gas supply to the bacteria. However, this would also increase the solubility of carbon monoxide in the liquid phase in suspended-growth bioreactors and, as suggested above, may eventually lead to inhibitory effects such as the inhibition of cell growth and of the activity of hydrogenases, with the concurrent accumulation of H_2 and CO_2 whenever produced or originally present in the syngas or waste gas mixture. Nevertheless, Chang *et al.* reported that high cell density cultures are less affected by the potential inhibitory effects of high carbon monoxide pressures compared with fermentations at low biomass densities.[119] Such high cell concentrations could be reached, in continuous suspended-growth bioreactors, through the use of cell recycling modules. Another characteristic of pressurized bioreactors is that they would result in higher operating costs.

Various substances such as surfactants, alcohols, salts, catalyst and small particles can be added to increase the gas–liquid mass transfer rates.[120] Besides, the addition of nanoparticles to batch bottle systems or stirred tank bioreactors for carbon monoxide or syngas fermentation have shown to lead to increased mass transfer. The addition of functionalized nanoparticles yielded better results than non-functionalized ones. The dissolved concentrations of CO, CO_2, and H_2were found to increase by 273%, 200%, and 156%, in the presence of methyl functionalized silica nanoparticles at a concentration of 0.3% by weight. Similarly, the cell concentration of *C. ljungdahlii*, producing ethanol, increased between 29% and 166%.[121] However, such approach might not be realistic nor cost-effective at present in full-scale reactors.

Bioreactors suitable for HBE fermentation are reviewed in the next section. The k_La values in such reactors may vary depending on several parameters, such as gas and liquid flow rates, agitation speed in CSTR or gas bubble size in suspended-growth bioreactors. A few authors recently compared mass transfer in different bioreactor configurations, in the case of both packed-bed and suspended-growth systems mainly with pure carbon monoxide. Gas-lift bioreactors have been considered to represent a suitable configuration because of their simple design and low energy requirements (e.g. no agitation) combined with the highest mass transfer coefficient compared with other bioreactors including column diffusers, hollow fiber membrane bioreactors (HFMB) and biotrickling filters.[122] Other authors compared innovative attached-growth bioreactors, namely an HFMB and a monolith bioreactor, with more conventional systems for gas fermentation with *C. carboxidivorans*.[123] High k_La values were found for the HFMB compared with suspended-growth bioreactors, such as the CSTR and the bubble column bioreactor. Next, the monolith bioreactor also showed high k_La values, though somewhat lower than the HFMB. Such mass transfer coefficients increased at higher gas or liquid flow rates both in the HFMB and the monolith bioreactor. Biotrickling filters are also characterized by relatively high k_La coefficients for which values exceeding 100 h^{-1} have sometimes been reported,[110,122] while such values are generally lower for other systems, except the HFMB or the CSTR when applying very high agitation speeds, which would, however, not be suitable for full-scale application.

Kinetics

The production of solvents by acetogens through the WL pathway in the presence of CO and/or CO_2, generates less ATP than through glycolysis. The bacterial species does also play a role, and it was observed that bacteria following the glycolytic pathway may exhibit different growth rates depending on the species and carbohydrate used as substrate. For example, lower specific growth rates were found in *C. tyrobutyricum* grown on glucose than on xylose, which was assumed to be due to the higher amount energy required for transportation of xylose across the cell membrane resulting in less ATP available for growth.[124] Generally the formation of fatty acids (e.g. acetic acid, butyric acid) in acetogenic bacteria leads to more net ATP production from C1 gases than the formation of alcohols such as ethanol, butanol, or higher alcohols. Moreover, butanol production with CO as electron donor has a more positive energy balance than in the presence of $H_2 + CO_2$.[90] However, one major drawback from an environmental point of view is that CO_2, which is a greenhouse gas, is released as end-product together with butanol when using CO as electron donor, while CO_2 is not produced and is even consumed when using $H_2 + CO_2$ as substrates (see stoichiometric equations above). In presence of syngas or waste gases containing a mixture of $CO + CO_2 + H_2$, complete CO_2 removal might also be possible. This is also related to the amount H_2 available, as hydrogen is required to metabolize carbon dioxide.

Although only limited information has been published on biomass growth rates and yields of clostridia on C1 gases, as a general rule biomass grows better on carbohydrates than on CO-related compounds. Table 4 compares kinetic parameters of different *Clostridium* spp. grown either on sugars or C1 gases. Major parameters such as pH and other culture conditions are also given in Table 2, whenever reported, as they affect biomass growth and bioconversion rates. Other factors such as the presence of micronutrients or trace metals can also have some influence and details of the exact media compositions can be found in the original publications. Reported data suggest that growth rates on carbohydrates are higher than on CO or CO_2, except for xylose which does also exhibit weak growth rates. Besides, biomass yields and build-up are also low on C1 gases and the concentration of bacterial cells accumulating in bioreactor studies is therefore a limiting factor in the bioconversion of CO-rich gases to alcohols. Alternatives such as the use of immobilized biomass or cell recycling will improve the amount active biocatalyst in the bioreactor and thus increase bioproduction rates.

Bioreactor configuration

The reactor design is an important factor in syngas fermentation. Most studies on syngas fermentation and their large-scale application have been performed in suspended-growth bioreactors but, besides such systems, other bioreactors can be used as well, several of which have been applied at full-scale for handling gases and waste gases, including other types of stirred tank suspended-growth bioreactors, biofilters, biotrickling filters or trickle bed bioreactors, bioscrubbers, gas-lift bioreactors, bubble column bioreactors, moving bed biofilm reactors, and membrane bioreactors.[7,125,126] The most widely studied bioreactor for the conversion of C1 gases to alcohols, at lab-scale, is the stirred tank fermentor. Some of the keys to design an efficient reactor are high mass transfer rates, high bioconversion rates, low operation and maintenance costs, and easy scale-up. So far, only very few results have been reported in the literature on bioreactors for the production of butanol and higher alcohols from C1 gases.

Continuous stirred tank reactors (CSTR)

The CSTR is a typical suspended-growth fermentation unit (Fig. 4(A)). The fermentation broth contains freely growing bacteria, with continuous supply of the gaseous substrate using gas

Table 4. Specific growth rates of wild type *Clostridium* spp. grown on CO or carbohydrates

Microorganism	Carbon source	Culture conditions	Specific growth rate (μ) (h^{-1})	Reference
C. carboxidivorans	CO	Batch, pH = 5.0, T = 33 °C	0.086 ± 0.004	53
	CO/CO_2, H_2/CO_2	Batch, pH = N.R. (*), T = N.R. (*)	0.16, 0.12	64
C. acetobutylicum	Glucose	Batch, pH = 6.0, T = N.R. (*)	0.48	113
	Lactose	Batch, pH = 5.0, T = 35 °C	0.23 - 0.28	114
Clostridium tyrobutyricum	Glucose	Fed-Batch, pH = 6.0, T = 37 °C	0.214 ± 0.044	115
	Xylose	Fed-Batch, pH = 6.0, T = 37 °C	0.116 ± 0.009	115

N.R.: Not reported; (*)Presumably under optimal conditions of pH (6.2) and T (38 °C)

diffusers. The agitation mechanism allows break-up of large bubbles into smaller ones, improving the gas–liquid mass transfer. A similar effect can be reached through the use of a microbubble sparging system.[127] Increasing the impeller speed is a way to increase the mass transfer of sparingly soluble gases, as it will improve mixing and reduce the bubble size. However, a relatively high input of energy per unit volume is required to increase the bubble break-up. Consequently, stirred tank fermentors with high agitation speeds would not be economically viable for large-scale production processes due to excessive operational costs.[7] The gas–liquid mass transfer rate in stirred tank bioreactors will not only increase with the agitation speed, but its value will also depend on the gas retention time, i.e. the gas flow rate.

In the CSTR, the gaseous substrate is continuously fed through the bottom part of the reactor and flows upwards through the fermentation broth. Recent studies have proven that good bioconversion yields and butanol productivities can be reached from either CO or gas mixtures (CO, CO_2, H_2) in semi-continuous stirred tank bioreactors, in which part of the aqueous medium is occasionally removed after a given period of time and replaced by fresh medium.[53] Periodic pH shifts in such process allow switching, in a cyclic way, from the acidogenic to the solventogenic stages, with progressive accumulation of increasing concentrations of alcohols and near complete consumption of accumulated acids.

Bubble column (BC) and gas-lift (GL) bioreactors

BC and GL bioreactors are similar in that gas injection through the bottom of the reactors allows for liquid mixing without the need for any mechanical or other power consuming agitation system. These bioreactors differ from each other by the fact that the GL bioreactor contains either an internal draft tube or an external loop for liquid circulation, while the BC reactor does not (Fig. 4(B)). The most commonly known airlift bioreactor and the gas-lift bioreactor are the same, except that air is fed in the first case while any gas can be introduced in gas-lift bioreactors. BC and GL reactors are economical alternatives to the CSTR,[128,129] as they do not use mechanical agitation but still allow for good mixing, due to the presence of rising gas bubbles, and low shear rate. This is interesting as good mixing can be achieved while avoiding high shear rates that might inhibit or damage microbial cells. Mixing of the gaseous substrate is achieved through gas sparging. Some other advantages of these bioreactors are their low maintenance and operational costs, whereas back mixing and coalescence of gaseous substrates are the main drawbacks. The BC bioreactor has been used as a second stage of a two-stage syngas fermentation, where the first stage was a 1 L CSTR with sustained growth of *Clostridium ljungdahlii*, while the second stage was a 4 L BC reactor used to maximize the production of alcohols.[128]

In the GL bioreactor (Fig. 4(C), the gas is injected into the riser and allows for fluid circulation. The liquid travels upwards through the riser zone and recirculates down to the bottom of the reactor, together with residual gas bubbles, through the downcomer zone. The use of GL bioreactors for the treatment of waste gases, mainly polluted air, has only been reported in a limited number of studies,[129] although it is seen as an attractive alternative for the anaerobic bioconversion of syngas and CO-rich waste gases.

Biotrickling filters (BTF)

Other bioreactor configurations such as the biotrickling filter (BTF) or trickle bed reactor (TBR) have widely been used in the full-scale treatment of polluted air from industrial waste gases, or those generated at wastewater treatment plants, among others, in which the pollutants are present in the gas phase at concentrations usually not exceeding a few g m^{-3}. [5,125] They are also used with anaerobic gases, among others for biogas upgrading and for the removal of hydrogen sulphide from that biofuel.[130,131] Their efficiency has only scarcely been evaluated for the bioconversion of carbon monoxide and syngas, mainly at lab-scale, and hardly any information is available so far on such applications.

The BTF is a packed-bed bioreactor in which the cells are naturally growing on a solid support material (Fig. 4(D)). They accumulate on the solid surface in the form of a biofilm. This allows increasing the amount of biocatalyst, through its immobilization on the packing material.[126] The gaseous substrate is supplied continuously to the bioreactor while a nutritive aqueous phase is trickling over the solid support and recycled through the system with a pump, without any mechanical agitation, which reduces energy requirements compared with the CSTR (Fig. 4(A)). The liquid phase enters through the top of the bioreactor while the gas phase can be fed either concurrently or counter currently. However, countercurrent flows allow for a more efficient driving force distribution along the bioreactor. Part of that aqueous medium can be withdrawn and renewed whenever appropriate. In packed-bed bioreactors such as the biofilter and the biotrickling filter, the amount of liquid medium present in the system is small compared with suspended-growth bioreactors.[5] This has been observed to reduce the resistance to mass transfer as there is only a thin liquid layer between the substrate in gas phase and the biofilm where the biocatalyst will metabolize the volatile compound.[132] Conversely, in suspended-growth bioreactors, the substrate needs to diffuse from the gas bubbles to the liquid phase and to the biocatalyst growing in suspension. A semi-continuous trickle bed reactor was recently used with *C. ragsdalei* as a biocatalyst in order to estimate the possibility to improve syngas bioconversion.[133] A large amount of biomass could build up in the packed-bed bioreactor

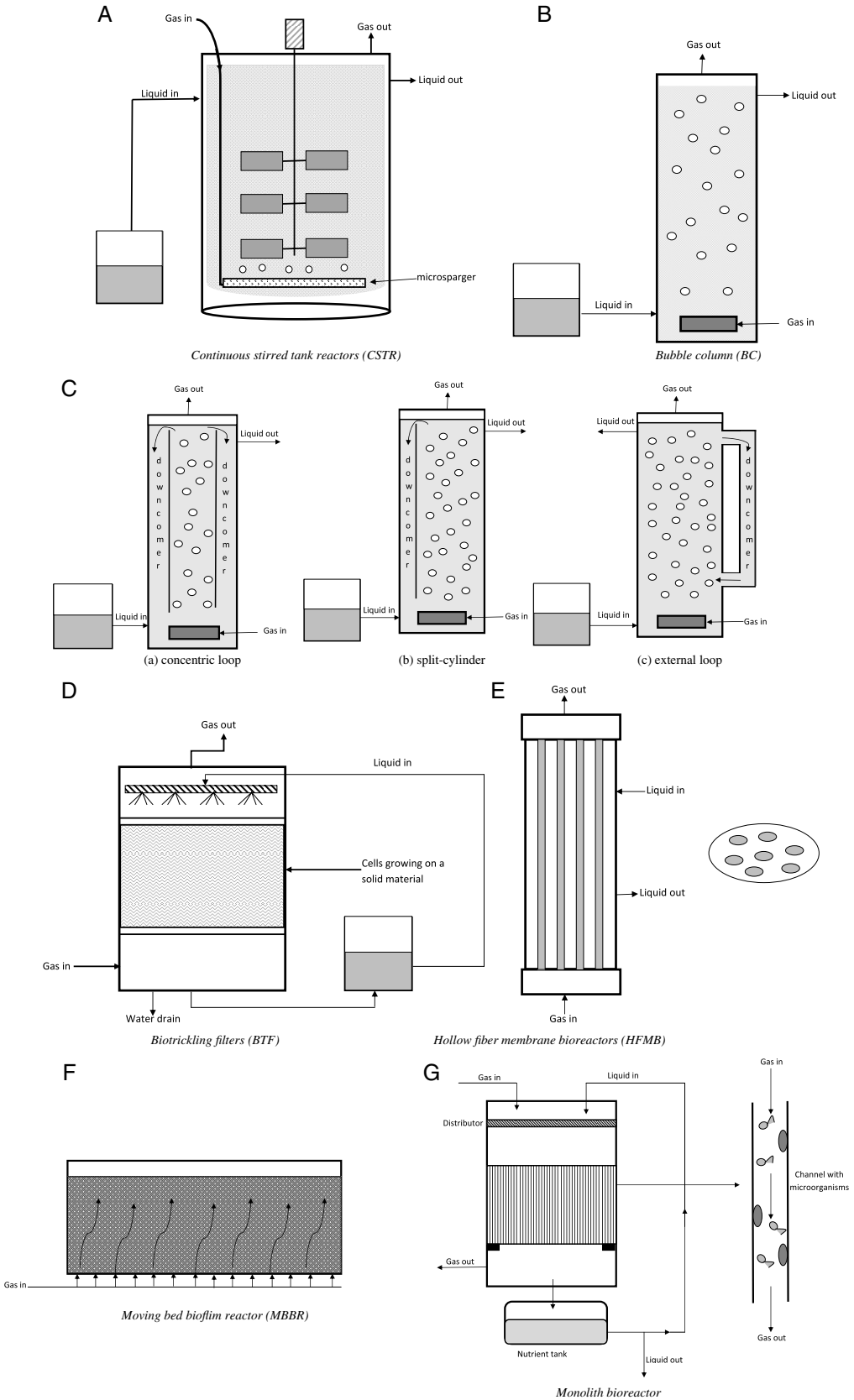

Figure 4. Bioreactors suitable for CO-rich syngas/waste gas fermentation: (A) continuous stirred tank reactor (CSTR); (B) bubble column (BC); (C) gas-lift reactor (GL): (a) concentric loop, (b) split cylinder, (c) external loop; (D) biotrickling filter (BTF); (E) hollow fiber membrane bioreactor (HFMB); (F) moving bed biofilm reactor (MBBR); (G) monolith bioreactor.

in the form of a biofilm, allowing the bacteria to take up more H_2 and avoiding CO inhibition. Consequently, 5.7 g L^{-1} ethanol was formed and a higher production of acetic acid, reaching 12.3 g L^{-1}, was observed.

Hollow fiber membrane bioreactors (HFMB)

In the HFMB, hollow fiber membranes are introduced inside the reactor either in a tubular form or in the form of flat sheets.[126] Syngas is generally fed through the membrane lumen. It then diffuses through that membrane and is subsequently metabolized by bacteria forming a biofilm on the outer surface of the membrane, i.e. on the shell side (Fig. 4(E)). Only very few research studies have been performed and reported on syngas fermentation in HFMB, none of them dealt with the production of alcohols such as butanol.[80,134,135] Shen and co-workers used a continuous HFMB with *C. carboxidivorans*, and compared its behaviour with a CSTR during continuous syngas fermentation for ethanol production.[123] They obtained good results with the HFMB in terms of alcohol production, confirming the suitability and advantage of such bioreactor configuration compared with the CSTR. Recent studies have also been focused on optimizing mass transfer through the use of an HFMB, as mass transfer is a major limiting factor in syngas fermentation. In that sense, Yasin and co-workers developed a new HFMB configuration with a high mass transfer, able to support an efficient microbial fermentation.[136] The authors reached a high driving force inside the bioreactor with low substrate pressures while increasing the headspace inside the system.

Moving bed biofilm reactors (MBBR)

The MBBR is considered to be more recent than the other bioreactors described above. This reactor was initially designed for application in municipal wastewater treatment.[137] It contains a tank with the culture broth and a gas injection system at the bottom of that tank for gas diffusion (Fig. 4(F)). Gas injection improves gas diffusion and turbulence. Large amounts of microorganisms grow as a biofilm. Some of the advantages of the system include its ability to provide a high surface area per volume for biofilm development, its rather simple operation, and the fact that it requires less space than, for instance, traditional wastewater treatment systems.[138]

Monolith bioreactors

The monolith bioreactor is a reactor with a structured packing inside. The packing is formed of regular channels that allow for a more homogeneous flow distribution than when working with random packing materials such as used in biotrickling filters (Fig. 4(G)). This may affect the efficiency and results in a better control of the pressure drop along the reactor height. The monolith bioreactor was first tested somewhat more than a decade ago for biological waste gas treatment and the removal of air pollutants. Good results were obtained for the removal of different volatile organic compounds from air, at concentrations of a few g m^{-3}, typical of many industrial waste gases.[139,140] One recent study described the bioconversion of syngas by *C. carboxidivorans* in a monolith bioreactor for alcohol production. Under the applied operating conditions, that reactor was reported to perform better, in terms of syngas utilization efficiency and alcohol/acid ratio, than a BC bioreactor.[141] The $k_L a$ values appeared to be similar or higher in the monolith bioreactor than in the BC reactor, depending on the operating conditions.

Advantages and challenges of the HBE fermentation process

ABE fermentation as well as the novel HBE process are the two major biological alternatives for the production of ethanol, butanol, and, in this case, higher alcohols such as hexanol. Compared with conventional refineries, bioprocesses and biorefineries present the advantage of using renewable sources, wastes or pollutants as feedstock rather than being based on non renewable oil derived sources. Lignocellulosic biomass or agro-industrial waste are generally used for the ABE fermentation as they yield simple fermentable sugars. Instead, a wider range of carbonaceous materials, – including biomass – is suitable for the HBE process, as long as they can be gasified to produce syngas. Lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin. In most cases, lignin represents 10–25% of the biomass but it may sometimes reach more than 30% of the lignocellulosic material in that biomass.[142] However, simple sugars for the ABE fermentation can only be obtained from cellulose and hemicellulose. Conversely, the whole biomass can be gasified into synthesis gas.[143] Another advantage is that, besides syngas, some industrial waste gases do also contain CO, CO_2, and/or H_2 and have recently been confirmed to be fermentable into end metabolites such as ethanol, among others. This allows simultaneous treatment of industrial waste gases, removing some greenhouse gases such as CO_2, while obtaining valuable end products in a cost-effective way. Recent and on-going research has generated encouraging results. Nevertheless, further studies are still needed in order to improve the production of ethanol or higher alcohols through this HBE process. Some of the challenges to be addressed are the low water solubility of compounds such as CO and H_2, mainly. In that respect, different strategies can be applied such as the use of membrane bioreactors or pressured reactors improving mass transfer rates, although such systems may affect cost-efficiency. On the other side, and similarly to the ABE process, managing to increase the concentration of end products is desirable, but should at the same time avoid inhibitory effects at such higher concentrations. Improving downstream processing and the use of on-line separation of products in continuous bioreactors would be suitable options.

CONCLUSIONS

HBE fermentation is a rather novel process, similar to the more conventional ABE fermentation, involving acetogenic bacteria, mainly clostridia. It represents a suitable promising alternative for the production of ethanol, butanol or higher alcohols. Butanol is a compound which can be used as a fuel as well as an important industrial chemical. Butanol is the chemical with major probability to replace gasoline, but ethanol and hexanol are also interesting alternative fuels. Butanol production from biomass, syngas, and waste gases is a viable process from a technical viewpoint. In terms of cost-effectiveness, in order to reduce the costs of butanol or higher alcohols production, it is necessary to further develop economical and efficient fermentation strategies and optimize productivities. To reach these goals, all factors that may affect the fermentation process and its efficiency need to be taken into account, such as parameters that influence the bioconversion, impurities in the syngas (e.g. NO, acetylene, and compounds that have an antagonistic effect on enzymatic activities), gas–liquid mass transfer that needs to be optimized (improve the diffusion or availability of poorly soluble syngas compounds, e.g. CO and H_2), the type of microorganisms (eventually genetically manipulated), and strategies that alleviate the inhibitory effects of high alcohol concentrations during the fermentation process.

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REVIEW

Production of chemicals from C1 gases (CO, CO_2) by *Clostridium carboxidivorans*

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Abstract Bioprocesses in conventional second generation biorefineries are mainly based on the fermentation of sugars obtained from lignocellulosic biomass or agro-industrial wastes. An alternative to this process consists in gasifying those same feedstocks or even other carbon-containing materials to obtain syngas which can also be fermented by some anaerobic bacteria to produce chemicals or fuels. Carbon monoxide, carbon dioxide and hydrogen, which are the main components of syngas, are also found in some industrial waste gases, among others in steel industries. *Clostridium carboxidivorans* is able to metabolise such gases to produce ethanol and higher alcohols, i.e. butanol and hexanol, following the Wood–Ljungdahl pathway. This does simultaneously allow the removal of volatile pollutants involved in climate change. The bioconversion is a two step process in which organic acids (acetate, butyrate, hexanoate) are produced first, followed by the accumulation of alcohols; although partial overlap in time of acids and alcohols production may sometimes take place as well. Several parameters, among others pH, temperature, or gas-feed flow rates in bioreactors, affect the bioconversion process. Besides, the accumulation of high concentrations of alcohols in the fermentation broth inhibits the growth and metabolic activity of *C. carboxidivorans*.

Keywords Acetogens · Clostridia · Greenhouse gases · Syngas · Waste gas

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Introduction

Most fuels and a wide range of platform chemicals produced in industrialized countries have traditionally been obtained from petroleum in oil refineries. For environmental reasons and because of the near shortage of crude oil reserves, modern societies need to develop new production processes and alternative fuels (Gowen and Fong 2011; Abdehagh et al. 2014). Biorefineries have recently emerged as a potential solution to such problem. Ethanol and longer chain alcohols such as butanol are suitable substitutes of fossil fuels such as gasoline. They can also be used as chemicals and solvents. Mixtures of alcohols such as butanol and ethanol can potentially be produced from wastes and renewable sources in bioreactors, which is an advantage compared to alcohols obtained from non renewable fossil sources. The most extensively studied bioprocess is based on the fermentation of carbohydrates, available from lignocellulosic biomass or similar feedstocks, using anaerobic bacteria, usually clostridia. This is commonly known as the ABE fermentation yielding a mixture of **a**cetone, **b**utanol and **e**thanol. *Clostridium acetobutylicum* has most often been used as biocatalyst for such bioconversion, metabolizing sugars and producing the aforementioned three solvents as end metabolites. Other substrates such as glycerol and other clostridial species have more recently been used as well. Another possible bioconversion process for the production of (bio)alcohols has emerged much more recently. It can use similar feedstocks as for the ABE fermentation, such as biomass, but also municipal solid waste, agro-industrial wastes and a broader range of some other carbon containing materials, which represents a clear advantage (Mohammadi et al. 2011). The feedstock is then gasified in order to obtain syngas on one side, which is a mixture of CO, CO_2 and H_2 mainly and, on the other side,

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some inert solid residue (ash) is formed as well. This is different from the ABE process, in which the starting material does first undergo pretreatments and hydrolytic treatments to extract simple sugars from the polymeric lignocellulosic structure. This gas mixture (i.e., syngas) is not only obtained through gasification of biomass or waste, it is also found in some industrial gaseous effluents, among others in steel producing processes. Similarly to sugars, syngas and the aforementioned industrial waste gases can be fermented by clostridia and a few other acetogenic bacteria (Drake et al. 2008). In a few strains, this may yield ethanol and, occasionally, higher alcohols. Contrary to the first generation biorefinery processes which are based on the use of sugar containing food crops as feedstock and lead to food-fuel competition (Abubackar et al. 2011; Kennes et al. 2016), the present alternative uses lignocellulosic biomass or wastes mainly or even waste gases and does not generate such food-fuel dilemma. Besides, this gas fermentation technology can reduce the emissions of gaseous pollutants and greenhouse gases such as carbon dioxide and it gives some commercial use to industrial pollutants and agricultural wastes. Only very few strains have so far been proven to be able to convert syngas and CO-rich waste gases into ethanol and higher alcohols such as butanol and hexanol. The best known and most studied species is *Clostridium carboxidivorans*, which will be the focus of this review paper.

Clostridium carboxidivorans: major morphological and metabolic characteristics

Clostridium carboxidivorans P7 (= ATCC BAA-624 = DSM 15243) is a Gram positive, mesophilic and obligate anaerobic carboxydotroph, originally isolated from an agricultural settling lagoon in Oklahoma, USA (Liou et al. 2005). Its cells are mobile, with rod shape (0.5 × 3 µm) and can present sporulated forms, which appear like a terminal or subterminal protuberance (Liou et al. 2005) (Fig. 1). Its main morphological, metabolic and growth characteristics are summarized in Table 1 and described more in details below.

Substrates, nutrients and products

Clostridium carboxidivorans P7 is able to grow autotrophicaly with syngas and chemoorganotrophically with a great variety of sugars such as glucose, xylose, fructose, cellobiose and arabinose. It is able to ferment all those carbon sources to produce acids, mainly acetic acid, butyric acid, and hexanoic acid, and alcohols (Liou et al. 2005; Liu et al. 2014; Phillips et al. 2015). Lactic acid, propionic acid and formic acid have also recently been detected in glucose fermentation (Fernández-Naveira et al., non published data). Suitable carbon and energy sources and their main metabolites are listed in Table 2. Recent interest in that species has mainly been focused on its ability to produce alcohols from syngas and waste gases. *C. carboxidivorans* is one of the few bacteria able to grow autotrophically on syngas, using the gaseous CO, CO_2, H_2 compounds as carbon or energy source to produce short chain alcohols such as ethanol as well as longer chain alcohols such as butanol and hexanol (Bruant et al. 2010; Dürre 2016; Fernández-Naveira et al. 2016a; Hemme et al. 2010; Liou et al. 2005; Phillips et al. 2015). That organism uses CO and CO_2 as carbon source whereas H_2 is used as source of electrons by means of the enzyme "hydrogenase" (Krasna 1979). Under conditions of inhibition of the hydrogenase enzyme, the bacteria will need another source of electrons, which can then be obtained from CO. As described more in detail below, this is the case in presence of compounds such as NO, which can appear as minor compound in syngas, and has been shown to inhibit the hydrogenase enzyme. However, this provokes also a limitation in the use of CO for the

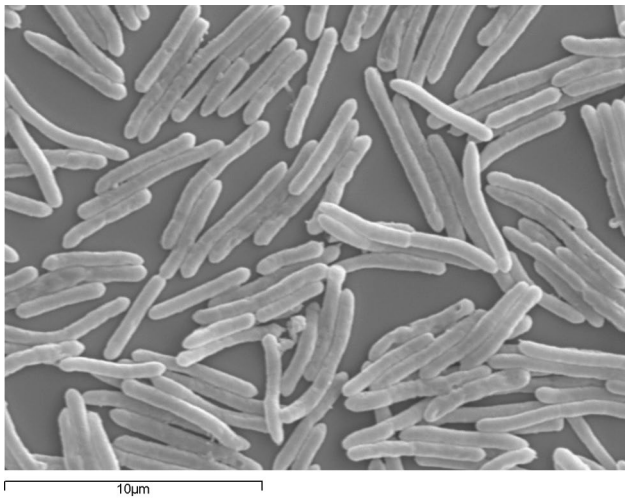


Fig. 1 SEM picture of *Clostridium carboxidivorans* grown on carbon monoxide

Table 1 Main characteristics of *Clostridium carboxidivorans*

	C. carboxidirorans P7
Morphology	Rod shape
Size (µM)	0.5 × 3
Temperature range (°C)	24–42
Temperature optimum (°C)	37–40
pH range	4.4–7.6
pH optimum	5.0–7.0
Reference	Liou et al. (2005)

Table 2 Major substrates and products of the HBE fermentation in *Clostridium carboxidivo rans*

Major substrate	Major products	References
Syngas (CO, CO_2, N_2 and H_2)	Acetic acid, butyric acid, hexanoic acid, ethanol, butanol and hexanol	Fernández-Naveira et al., submitted Phillips et al. (2015) Ramió-Pujol et al. (2015) Ukpong et al. (2012)
CO	Acetic acid, butyric acid, hexanoic acid, ethanol, butanol and hexanol	Fernández-Naveira et al. (2016a), Liou et al. (2005)
Sugars such as glucose	Acetic acid butyric acid, hexanoic acid, ethanol, butanol, hexanol, formic acid, propionic acid and lactic acid	Liou et al. (2005), Fernández-Naveira et al., unpublished data

formation of desired metabolites and does consequently result in a less efficient fermentation process (Ahmed et al. 2006).

So far, in terms of solvents, the highest end product concentration has always been found for ethanol followed by butanol and finally hexanol. Those alcohols are produced in that same chronological order during carbon monoxide or syngas fermentation, with short chain alcohols appearing first while longer chain ones appear later on. *C. carboxidivorans* has the typical "biphasic fermentation pattern" of many acetogens producing alcohols, and usually the gas fermentation process takes place in two stages; initially carboxylic acids are produced from the gaseous substrates followed by the subsequent conversion of those acids and remaining gases into alcohols. Besides, exponential biomass growth and acidogenesis (with production of acids) are two related processes and take place simultaneously. The solventogenic phase in clostridia has been considered to start when the conditions are not favourable anymore for growth, i.e. low pH, low ATP levels, accumulation of high concentrations of organic acids, sporulation, low level of availability of reducing energy (Dürre et al. 1995; Dürre and Hollergschwandner 2004; Guedon et al. 1999; Meyer and Papoutsakis 1989). When alcohols are the desired end products, it is necessary to identify the optimum medium composition and conditions for an efficient conversion of accumulated organic acids with the concomitant production of solvents. The suitable range of conditions depends on the bacterial species, and such conditions are shown in Table 1 for *C. carboxidivorans*.

Besides the main carbon and energy sources, several nutrients and trace compounds may be needed as well. In case of *C. carboxidivorans*, a recent study was published in which the effect of different media compositions were analyzed for their effect on growth and butanol production. Removing copper (Cu) from the culture medium and increasing the molybdate (Mo) concentration allowed to improve the production of butanol (Phillips et al. 2015). It was concluded that Mo can be considered to be analogous to tungsten (W), which is related with the enzyme AOR (*aldehyde:ferredoxin oxidoreductase*), an enzyme involved in the conversion of acids to alcohols. Similarly, the presence of W had previously been proven to stimulate the conversion of carbon monoxide and acetic acid into ethanol in *C. autoethanogenum* (Abubackar et al. 2015). Micronutrients, trace metals or vitamins play a key role in the activity of specific enzymes and in favouring a given metabolic route. Other parameters, described below, such as temperature and pH, will also affect growth, the metabolic behavior and the bioconversion process.

Temperature

The suitable growth temperature of *C. carboxidivorans* ranges between 24 and 42 °C, but its optimum temperature was found to be 37–40 °C (Liou et al. 2005). "Acid crash", which is the accumulation of undissociated acids and can inhibit the solventogenic stage, is a phenomenon that depends on temperature (Maddox et al. 2000). Therefore, it is useful to identify temperature conditions that prevent acid crash and allow an efficient solvent production while maintaning a near optimum temperature for growth. The incubation of *C. carboxidivorans* at suboptimal temperature of 25 °C (which is still within the suitable temperature range for growth) was shown to allow to avoid acid crash (Ramió-Pujol et al. 2015). However, a lag phase and a slower bacterial growth were observed than under optimal temperature conditions, while the concentrations of alcohols were somewhat higher than when incubating the same strain at 37 °C. An overview of the production yields of alcohols obtained at different temperatures and pH is presented in Table 3.

pH

The pH range of *C. carboxidivorans* is between 4.4 and 7.0, but its optimum pH was found to be between 5.0 and 7.0. A few studies have focused on the effect of pH in syngas fermentation. Fernández-Naveira et al. (submitted) studied its effect using the bacterium *C. carboxidivorans* as biocatalyst and a mixture of CO, CO_2, H_2 and

Table 3 Different production yields of *C. carboxidivorans* at different temperatures and different pH using CO or syngas as substrates

Substrates	Fermentation systems		pH	Temperature (°C)	Ethanol production rate (g/L h)	Butanol production rate (g/L h)	Hexanol production rate (g/L h)	References
CO	Bioreactor		4.75 5.75	34	0.032 0.060	0.013 0.031	ND ND	Fernández-Naveira et al. (2016a)
$CO:CO_2:H_2:N_2$	Bioreactor		4.75 5.75	34	14.32 0.014	11.68 0.011	4.54 0.01	Fernández-Naveira et al., submitted
$CO:CO_2:H_2:N_2$	Batch bottles	Growth phase Stationary phase	6	37	0.01 0.0058	ND/NR ND/NR	ND/NR ND/NR	Ramió-Pujol et al. (2015)
	Batch bottles	Growth phase Stationary phase	6	25	8.97 0.15	4.8 0.0082	5.15 0.0092	Ramió-Pujol et al. (2015)
$CO:H_2:CO_2$	Batch bottles	(−Cu/+10 Mo)	NR	37	0.0058	0.0031	0.0026	Phillips et al. (2015)

NR/ND not reported or not detected

N_2 supplied to a continuous gas-fed bioreactor, using two different operating conditions. In a first experiment, a near optimum pH of 5.75 was used and maintained constant during the study. The second experiment was started at pH 5.75, but pH was not regulated in that case and natural acidification took place as a result of the production of organic acids; and once it reached pH 4.75 its value was maintained constant in order to avoid any inhibition at lower pH. The results of that study showed that the highest concentrations of alcohols were observed at pH 5.75, with 2.7 g/L ethanol, 1.9 g/L butanol and 0.85 g/L hexanol; whereas the highest production rates of alcohols were obtained at pH 4.75, reaching 0.048 g ethanol/h g biomass, 0.036 g butanol/h g biomass, and 0.026 g hexanol/h g biomass. Those data, and other related information of production rates, are summarized in Table 3. However, a negative effect on bacterial growth and on the accumulation of acids was observed at lower pH, in the experiment with natural acidification. As a result of the lower accumulation of acids in the first step of that fermentation, lower amounts of alcohols were obtained in the experiment at lower pH compared to the study at a higher, constant, pH of 5.75. Growth rates of 0.0057 and 0.072 h^{-1} were observed at pH 4.75 and pH 5.75, respectively. It was concluded that the pH is a critical factor for growth, the accumulation of acids as well as the efficient production of alcohols. Although it has often been assumed that stress conditions, such as a low pH, are necessary for solventogenesis based on data of the ABE fermentation in *C. acetobutylicum*, strong acidification does not seem to be a prerequisite for solventogenesis in the conversion of organic acids into alcohols in hexanol–butanol–ethanol (HBE) fermentation with *C. carboxidivorans*, as a slightly acidic environment (pH 5.75) allowed the efficient conversion of organic acids into alcohols, compared to lower pH values (e.g., pH 4.75).

Metabolic pathway

Clostridium carboxidivorans uses a variation of the Wood–Ljungdahl pathway for the bioconversion of gaseous substrates to end metabolites, where the eastern branch of its pathway involves the enzymes in charge of the conversion of the C1 substrates (CO, CO_2) to formate, and later on methyl-tetrahydrofolate; and the western branch is composed of the enzymes catalyzing the direct conversion of C1 compounds into acetyl-CoA (Ragsdale and Pierce 2008). Acetyl-CoA is a common intermediate of both branches, and it can either be converted to acetate or to ethanol. Alternatively, acetyl-CoA can also be converted to butyryl-CoA and subsequently into butyrate and/or butanol, or into hexanoyl-CoA and then hexanoate and/or hexanol. Although acetate is a common product of autotrophic acetogens, butyrate and hexanoate are quite more unusual among the acetogenic bacteria isolated so far; the same holds true for butanol and hexanol which are still less common than long chain (C4, C6) fatty acids. Examples of acetogens producing long chain fatty acids (butyric acid, hexanoic acid) and alcohols (butanol, hexanol) from volatile substrates (CO, CO_2, H_2) are listed in Table 4, confirming that the production of alcohols is less common than organic acids in such bacteria. So far, ethanol does generally always appear and has been detected in all acetogenic cultures in which higher alcohols (butanol and/or hexanol) are produced. Acetic acid is present in all cases during the gas fermentation, although its presence may often be transient, as it can further be converted to alcohols, mainly ethanol. The Wood–Ljungdahl pathway does hardly yield any energy. One mole of ATP is generated per mole of acetic acid produced. As explained above, biomass growth and acetic acid production are concomitant. Later on, that organic acid can be converted into acetaldehyde which yields ethanol in turn, but with no generation of ATP and no biomass growth

Table 4 Wild type acetogenic bacteria producing long chain fatty acids and alcohols (C4, C6) from CO, CO_2/H_2, or mixtures of all three gases. All those strains are able to produce acetic acid and all the bacteria producing long chain alcohols do also produce ethanol

Bacteria	Butyrate	Hexanoate	Butanol	Hexanol	References
Acetonema longum	+	NR/ND	NR/ND	NR/ND	Kane and Breznat (1991)
*Butyribacterium methylotrophicum**	+	NR/ND	+	NR/ND	Shen et al. (1999)
Clostridium carboxidivorans	+	+	+	+	Liou et al. (2005) Fernández-Naveira et al., submitted
Clostridium difficile	+	NR/ND	NR/ND	NR/ND	Köpke et al. (2013)
Clostridium drakei	+	NR/ND	NR/ND	NR/ND	Gößner et al. (2008)
Clostridium scatologenes	+	NR/ND	NR/ND	NR/ND	Küsel et al. (2000)
Eubacterium limosum	+	NR/ND	+	NR/ND	Jeong et al. (2015)

NR/ND not reported or not detected

* This O*Butyribacterium methylotrophicum* strain is actually considered to belong to the species *Eubacterium limosum* (Jansen and Hansen 2001)

detected. The Wood–Ljungdahl pathway generating ethanol, butanol and hexanol, besides volatile fatty acids and typical in *C. carboxidivorans*, is shown in Fig. 2.

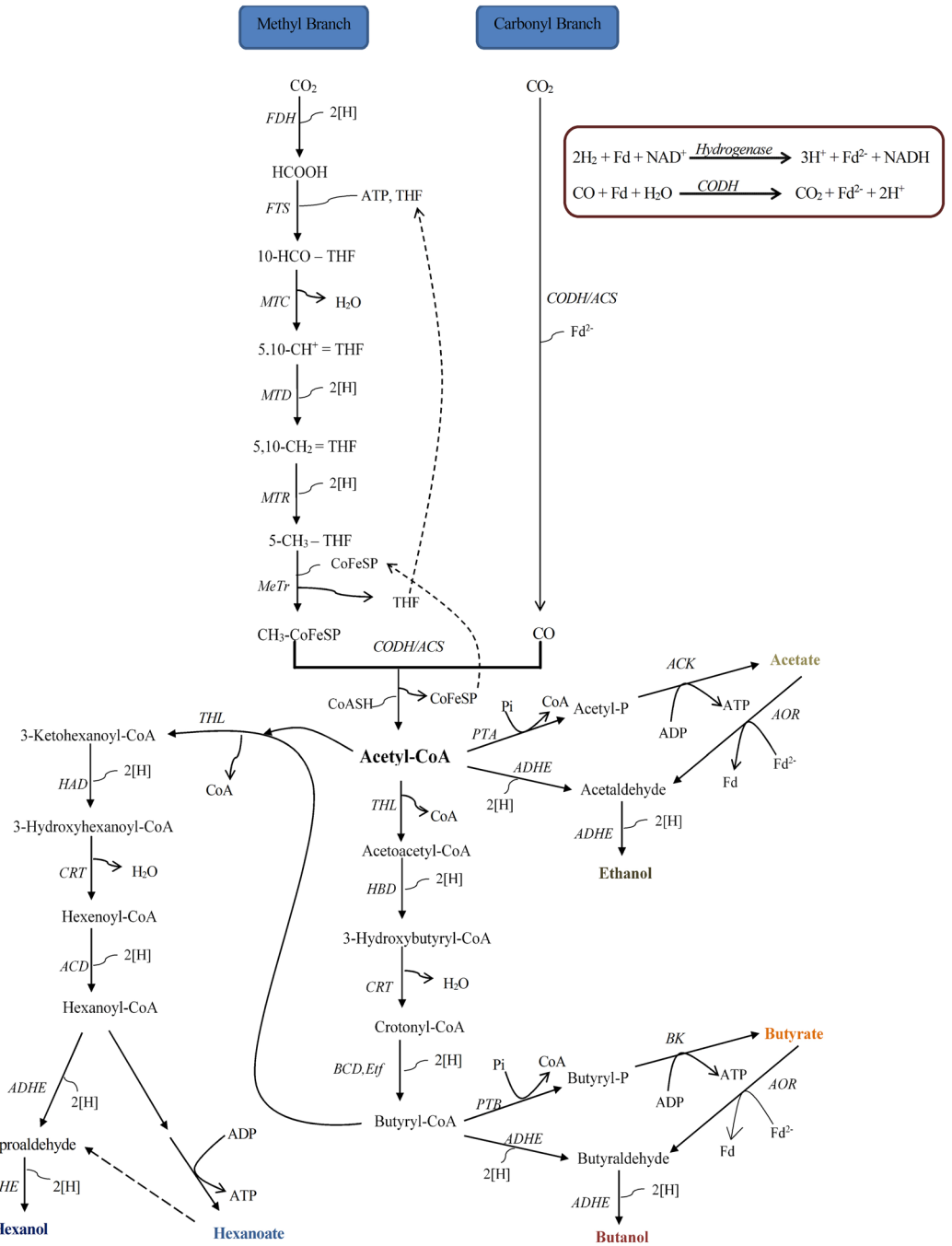
Recent genetic studies have been done on the genomic characterization of novel solventogenic microorganisms such as *C. carboxidivorans* by sequencing the genome and comparing the results with the genome of various other solventogenic bacteria. Bruant et al. (2010) sequenced the entire genomic material of *C. carboxidivorans* and compared that with other major ethanol and butanol producing strains. They found that *C. carboxidivorans* has a complete gene cluster associated with the Wood–Ljungdahl pathway, including the genes involved in CO and CO_2 fixation and conversion to acetyl-CoA, but with the exception of the acetone pathway, as no acetoacetate decarboxylase genes were found in that species. Therefore, the authors concluded that *C. carboxidivorans* is closely related to *C. acetobutylicum* and *C. beijerinckii*, in terms of ABE fermentation pathways for volatile fatty acids, ethanol and butanol, but that it lacks the acetone production pathway. Both *C. carboxidivorans* and *C. acetobutylicum* encode an NADPH-dependent butanol dehydrogenase that allows the conversion of acetyl-CoA into butanol. Other clostridia have been shown to grow on syngas or waste gases (CO, CO_2, H_2), such as *C. autoethanogenum, C. ljungdahlii, C. drakei* but, to the best of our knowledge, none has yet been found to possess such butanol dehydrogenase. The same happens for hexanol, which has so far only been found to be produced in *C. carboxidivorans*. That organism is thus unique in that respect. As shown in Table 5, among the few gas fermenting solventogenic anaerobic bacteria isolated so far, *C. carboxidivorans*, is basically the only species found to be able to produce higher alcohols such as butanol and hexanol.

Solvent inhibition

Alcohols such as ethanol and butanol are known to exert inhibitory effects on strains such as *C. acetobutylicum* during the ABE fermentation. Besides, it is worth reminding that the inhibitory effect may be different depending on the bacterial species and type of alcohol. Therefore, toxicity levels should be evaluated in each specific case. Recently, the toxic effect of different concentrations of ethanol, butanol or their mixtures was estimated in *C. carboxidivorans* grown on carbon monoxide as single carbon source in bottle batch assays (Fernández-Naveira et al. 2016b). No information is available in the literature on hexanol, but that alcohol is generally produced at lower concentrations during HBE fermentation than its C2 and C4 counterparts. The experiments showed that butanol causes a significantly higher inhibitory effect than ethanol in terms of the bacterial growth rate, the final biomass density and the CO consumption rate. That inhibitory effect was quantified by means of the IC_{50} (i.e., the half maximal inhibitory concentration), which reached 14.5 g/L for butanol and 35 g/L for ethanol (Fernández-Naveira et al. 2016b). Mixtures (1:1) of both alcohols have intermediate toxic effects compared to each alcohol individually. The authors concluded that both alcohols have an inhibitory effect on *C. carboxidivorans* at high concentrations. Besides, inhibition is higher in the case of butanol than for ethanol, as a lower IC_{50} value was found for the former than the latter (ethanol). These values are rather similar to those found during ABE fermentation of sugars by *C. acetobutylicum*.

Trace compounds in syngas

Although CO, H_2 and CO_2 are the main components of some industrial waste gases and syngas, they may also



Abbreviations: FDH, Formate dehydrogenase; FTS, 10-formyl-H4folate synthetase; MTC, 5,10-methenyl-H4folate cyclohydrolase; MTD, 5,10-methylene-H4folate dehydrogenase; MTR, 5,10-methylene-H4folate reductase and MeTr, methyltransferase; CODH/ACS, carbon monoxide dehydrogenase /acetyl-CoA synthase; CoFeSP, corrinoid iron sulphur protein, THF, cofactor tetrahydrofolate. **Acetate production:** PTA, phosphotransacetylase; ACK, acetate kinase. **Butyrate production:** BCD, butyryl-CoA dehydrogogenase; BK, butyrate kinase; CRT, crotonase; Etf, electron-transferring flavoprotein; HBD, 3-hydroxybutyryl-CoA dehydrogenase; PTB, phosphotransbutyrylase; THL, thiolase. **Hexanoate production:** ACD, acyl-CoA dehydrogenase; HAD, 3-hydroxyacyl-CoA dehydrogenase. **Butanol/Ethanol /Hexanol production:** ADHE, aldehyde/alcohol dehydrogenase; AOR, aldehyde oxidoreductase. The dotted arrow from hexanoate to caproaldehyde indicates the possible route of hexanoate to hexanol conversion. 2[H], reducing equivalents (NADH or NADPH). Fd, ferredoxin. Fd^{2-}, reduced ferredoxin.

Fig. 2 Wood–Ljungdahl pathway for the production of acetic, butyric and hexanoic acids, as well as ethanol, butanol and hexanol

Table 5 Experiments with differents acetogens; operational parameters, substrates and products of syngas fermentation

	C. ljungdahlii	*C. autoethanogenum*	*C. carboxidivorans* P7	*C. drakei*	*C. ragsdalei* P11	*B. methylotrophicum*	*Alkalibaculum bacchi*
Fermentation system	Two stage fermentation (stage A acidogenesis; stage B solventogenesis)	Continuous syngas bioreactor	Two continuous bioreactors at differents pH: Reactor A (4.75) Reactor B (5.75)	–	Batch bottles	Batch tubes	Fed-batch fermentations
Substrates	60% CO, 35% H_2, and 5% CO	50% N_2, 20% CO, 20% CO_2, and 10% H_2	20% CO, 20% CO_2, 10% H_2 and 50% N_2	(H_2–CO_2, and CO–CO_2)	44% CO, 32% N_2, 22% CO_2, and 2% H_2	70% CO, 30% CO_2	40% CO, 30% CO_2, 30% H_2
Products	Stage A (acidogenesis) 5.04 g/L acetic acid and 0.56 g/L ethanol Stage 2 (solventogenesis) 1.98 g/L acetic acid and 5.67 g/L ethanol Butanediol (ND/NR)	1.40 g/L acetic acid 0.07 g/L ethanol and butanediol NR	Reactor A (pH 4.75): 3.45 g/L acetic acid, 2.25 g/L ethanol, 0.72 g/L butyric acid, 1.43 g/L butanol, 0.18 g/L hexanoic acid and 0.72 g/L hexanol Reactor B (pH 5.75): 6.20 g/L acetic acid, 2.7 g/L ethanol, 1.40 g/L butyric acid, 1.9 g/L butanol, 0.40 g/L hexanoic acid and 0.85 g/L hexanol	Acetic acid (NR), butyric acid (NR), ethanol (NR) and butanol (NR)	1.92 gL/ acetic acid, 1.01 g/L ethanol and 0.18 g/L butanediol	1.3 g/L acetic acid, 0.3 g/L butyric acid and 0.02 g/L ethanol Butanol (NR/ND)	1 g/L acetic acid, 1.7 g/L ethanol
References	Richter et al. (2016)	Cotter et al. (2009)	Fernández-Naveira et al., submitted	Gößner et al. (2008), Liou et al. (2005)	Köpke and Liew (2011)	Heiskanen et al. (2007)	Liu et al. (2012)

NR/ND concentrations not reported or not detected

contain trace amounts of additional compounds, which could have some inhibitory effect on the biocatalyst. The influence of those trace compounds is barely considered in lab-scale research as prepared gas mixtures are generally used, mimicking the composition of only the major compounds of syngas. Other compounds that can be formed during the gasification process include products such as methane, ethylene, ethane, acetylene, NH_3, sulphur compounds and NO, among others (Ahmed et al. 2006; Haryanto et al. 2009).

Although no research has been reported on this with *C. carboxidivorans*, some other alcohol producing species have been studied. Some of those trace compounds have been shown to be potential inhibitors of the fermentation process and bacterial growth. For example acetylene and NO may inhibit the activity of the hydrogenase enzyme, which catalizes the generation of electrons from H_2 (Xu and Lewis 2012). When NO inhibits the hydrogenase enzyme, the cells must generate electrons from CO, using the CODH enzyme. Sulphur compounds and ammonia (NH_3) are other compounds that may appear in syngas. A negative effect on the bacterial growth in presence of sulphur compounds has been reported in the ethanol producing species *C. ljungdahlii* (Klasson et al. 1993). Besides, Xu and Lewis (2012) found that the presence of ammonia can lead to the accumulation of ammonium ions (NH_4^+) in the medium, which was observed to inhibit the hydrogenase activity and bacterial growth of *C. ragsdalei*.

Present and future industrial perspectives

The production of ethanol and higher alcohols, such as butanol or hexanol, by acetogenic bacteria from C1 gases is not a favourable process from an energetic point of view (Latif et al. 2014). However, although it was originally considered that reaching concentrations approaching one gram per liter in wild type bacteria would be impossible or, at least, challenging, recent data confirm that the production of several g/L of butanol and hexanol mixtures, besides ethanol is feasible through this hexanol–butanol–ethanol (HBE) fermentation process. Optimization of the bioreactor operating conditions would allow to further improve such values. The use of metabolically engineered strains is another possible alternative for the improvement of yields and of the end concentrations of metabolites. Some research has been performed in that respect for butanol production with recombinant strains grown on carbon monoxide (Köpke and Liew 2011). However, improvements are necessary and higher butanol concentrations would still need to be reached from C1 gases with such engineered clostridia. Other bacterial strains are able to produce ethanol as single alcohol from syngas/waste gas, sometimes together with butanediol, but with no accumulation of either butanol or hexanol (Table 4). This is the case of *C. autoethanogenum, C. ljungdahlii*, and *C. ragsdalei*, among others (Abubackar et al. 2011). Recent studies undertaken at pre-commercial stage confirmed that such a process may be cost-effective (van Groenestijn et al. 2013). Some demonstration plants have recently allowed to produce ethanol, either from syngas or from waste gases from steel producing industries, with such acetogenic bacteria, reaching promising results. In terms of public safety, it is worth mentioning that, with the exception of only four or five species, most clostridia are non pathogenic at all and do not cause any diseases in humans. Some clostridia can even be used for therapeutic purposes (Kubiak and Minton 2015). Concerning the environmental benefit, it is worth to remind that this HBE fermentation process consumes carbon dioxide, a greenhouse gas, but does also allow to remove carbon monoxide. Although carbon monoxide as such has only a very weak greenhouse effect, it contributes to tropospheric ozone generation, the formation of carbon dioxide, and reacts with hydroxyl radicals in the atmosphere. Those OH radicals would otherwise be involved in reducing the concentration of greenhouse gases such as methane.

The gas fermentation process has attracted interest of some industries and, as indicated above, some demonstration plants have been build recently. The technology has reached pre-commercial stage for the production of ethanol, but not yet for other routes such as the HBE fermentation, and an exhaustive overview of the industrial landscape, among others for the HBE process, would thus be behind the scope of this review. Information on the industrial lansdscape, mainly for ethanol production, can be found in other recent literature (van Groenestijn et al. 2013; Latif et al. 2014). One of the major companies developing this technology is, among others, LanzaTech which produces ethanol using waste gases from industry or using syngas obtained through the gasification of biomass or wastes. In 2013, that company started pre-commercial operation of a plant in China. Similarly, Coskata, in the US, was using a large variety of biomass sources to obtain syngas and ferment it into fuels and chemicals. Finally, INEOS Bio focuses largely on ethanol production through sugar fermentation, but has also evaluated possible commercialization of the biomass gasification process and its subsequent fermentation.

Syngas fermentation vs other biological and non-biological alternatives

Biomass, agro-industrial waste or other related feedstocks can be used to obtain either carbohydrates or syngas as potential fermentable substrates, which can both be metabolized by clostridia to produce ethanol and higher alcohols such a butanol. Expensive pretreatments are

needed in order to extract carbohydrates from cellulose and hemicellulose, two major polymers of lignocellulosic feedstocks. However, lignin which is the third polymer found in such feedstocks, does not yield any carbohydrates and is thus useless for this fermentation process. Conversely, all three major polymers of lignocellulosic materials can be gasified to yield syngas, resulting in a better use of the complete feedstock in the gas fermentation process (Liew et al. 2016).

When comparing the biological and the non-biological syngas conversion routes, the former does also present some technical and economical advantages compared to the latter. The biological conversion, through the Wood–Ljungdahl pathway, takes place at near room temperature and atmospheric pressure, or if needed with slight overpressure. Conversely, the chemical Fischer Tropsch (FT) process for the production of chemicals is more complex and requires higher temperatures (150–350 °C) and elevated pressures (e.g., 30 bar). Besides, for the FT process, a specific H_2:CO ratio close to 2:1 is needed (de Klerk et al. 2013), while syngas composition does generally not reach such ratio. A pretreatment consisting in a water–gas shift reaction is then required in order to adjust the gas ratio, with the concomitant increased process costs (Liew et al. 2016). On the other side, *C. carboxidivorans* and some other clostridia can metabolize different gas compositions to produce ethanol or higher alcohols, including pure CO, mixtures of CO_2/H_2, or mixtures of all three gases, among others. Syngas fermentation is thus simpler and less restrictive. Finally, although the possible inhibitory effect of some trace compounds on bioconversion processes has been mentioned above, the FT process is much more sensitive to some chemicals such as sulphur compounds and has a lower tolerance to their presence than the Wood–Ljungdahl process (Michael et al. 2011; Mohammadi et al. 2011).

However, some potential drawback needs also to be discussed. The most important one is the low aqueous solubility of the volatile compounds of the syngas mixture, when working with bioreactors in which the bioconversion takes place in liquid phase. This results in a poor gas–liquid mass transfer and in limiting rates of supply of the gaseous substrates to the microbial cells, which limits the alcohols production yields. Mass transfer of the volatile substrates can be improved when using microbubble spargers. Using pressurized bioreactors would be another alternative to improve the gas solubility and mass transfer, although this will also increase operating costs. Packed-bed bioreactors, such as biofilters or biotrickling filters, with a reduced amount of water and a small water layer between the gas phase and the biofilm (Kennes et al. 2009), have also been suggested to improve the microbial use of substrates such as carbon monoxide in gas-phase bioreactors (Jin et al. 2009).

Conclusions

Clostridium carboxidivorans is a unique acetogenic bacterium in that it has proven to be basically the only organism isolated so far able to produce a mixture of alcohols, i.e. ethanol, butanol and hexanol, at significant concentrations, from syngas or waste gases which are composed mainly of carbon monoxide, carbon dioxide and hydrogen. From a metabolic point of view, the process does hardly yield any energy; still total concentrations of alcohols of several g/L have already been obtained in stirred tank bioreactors. From a chronological point of view, organic acids (C2, C4, C6, mainly) appear first, followed by the production of the corresponding alcohols. Optimizing the operating conditions, in terms of parameters such as pH, temperature or bioreactor configuration and flow rates, among others, allow to maximize the production of alcohols. The process still needs to be further improved in order to increase yields and productivity, taking into account that the accumulation of high concentrations of alcohols in the fermentation broth will end up inhibiting biomass growth and bioconversion, which may require their removal in-situ from the medium in bioreactors.

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BIOENERGY AND BIOFUELS

Efficient butanol-ethanol (B-E) production from carbon monoxide fermentation by *Clostridium carboxidivorans*

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Abstract The fermentation of waste gases rich in carbon monoxide using acetogens is an efficient way to obtain valuable biofuels like ethanol and butanol. Different experiments were carried out with the bacterial species *Clostridium carboxidivorans* as biocatalyst. In batch assays with no pH regulation, after complete substrate exhaustion, acetic acid, butyric acid, and ethanol were detected while only negligible butanol production was observed. On the other side, in bioreactors, with continuous carbon monoxide supply and pH regulation, both C2 and C4 fatty acids were initially formed as well as ethanol and butanol at concentrations never reported before for this type of anaerobic bioconversion of gaseous C1 compounds, showing that the operating conditions significantly affect the metabolic fermentation profile and butanol accumulation. Maximum ethanol and butanol concentrations in the bioreactors were obtained at pH 5.75, reaching values of 5.55 and 2.66 g/L, respectively. The alcohols were produced both from CO fermentation as well as from the bioconversion of previously accumulated acetic and butyric acids, resulting in low residual concentrations of such acids at the end of the bioreactor experiments. CO consumption was often around 50 % and reached up to more than 80 %. Maximum specific rates of ethanol and butanol production were reached at pH 4.75, with values of 0.16 g/h*g of biomass and 0.07 g/h*g of biomass, respectively, demonstrating that a low pH was more favorable to solventogenesis in this process, although it negatively affects biomass growth which does also play a role in the final alcohol titer.

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Keywords *Clostridium carboxidivorans* · Butanol · Ethanol · Syngas · Waste Gas

Introduction

In recent years, the low availability of fossil fuels and their environmental impact have forced to look for new alternative fuels obtainable, in a cost-effective way, from renewable sources or from pollutants. In addition to the environmental impact and increasing scarcity of conventional fuels, other aspects, e.g., economic and political, have also led to an ever increasing interest in techniques for the production of such alternative fuels (Gowen and Fong 2011; Abdehagh et al. 2014). Most studies have focused on the production of new energy sources and biofuels (biologically sourced fuels) such as (bio)ethanol, biogas, (bio)hydrogen, and biodiesel (Kennes and Veiga 2013). Additionally, (bio)butanol is also a suitable alternative fuel more similar to gasoline than (bio)ethanol and with interesting characteristics. Butanol exhibits several advantages, e.g., it is less hygroscopic and has a higher caloric content than ethanol (Wallner et al. 2009). It is considered a chemical of great industrial importance and has a high potential to replace gasoline (Dürre 2007; Lee et al. 2008), as there is no need for any adjustment of vehicles and engines using butanol. Besides, blending of butanol and gasoline is possible at any concentrations, and blends have also been reported to be possible in case of diesel (Jin et al. 2011).

Alcohols such as ethanol and butanol can be obtained through fermentation of sugars from sugarcane, corn, or starch feedstocks, among others, which is the conventional and common commercial process nowadays for ethanol, known as a first-generation process. However, this

technique for obtaining biofuels leads to food-fuel competition (Kennes et al. 2016). This disadvantage can be avoided by using lignocellulosic feedstocks from agricultural wastes or energy crops, which are inexpensive and renewable starting materials for biofuels production, and do not adversely affect food supplies. After some pretreatments and hydrolytic steps, simple sugars can be obtained from those polymeric feedstocks which can then be fermented into ethanol and/or butanol, in the so-called second-generation process (Kennes et al. 2016). However, there are still numerous scientific and technical challenges involved in the utilization of lignocellulosic materials for biofuel production (Gowen and Fong 2011), and there is a need for further research in order to improve cost-competitiveness of such alternative compared to the more conventional first-generation process.

The conventional second-generation process for the bioconversion of lignocellulosic feedstocks into biofuels is still a complex process (Balat and Balat 2009). As an alternative, gasification of biomass in order to obtain carbon monoxide-rich syngas represents another viable option. Syngas as well as most of its individual dominant components (like CO) can be introduced into a fermentor inoculated with anaerobic bacteria, under specific process conditions to produce biofuels (Abubackar et al. 2011; Bengelsdorf et al. 2013; Mohammadi et al. 2011). Not only syngas but also industrial waste gases rich in carbon monoxide have recently been shown to be suitable substrates for their bioconversion into biofuels in bioreactors (Kennes and Veiga 2013). Both suspended-growth as well as attached-growth bioreactors can efficiently be used for gas-phase biodegradation or bioconversion of such volatile substrates (Kennes and Veiga 2001, 2013). Initially, research on the fermentation of CO-rich gases focused only on ethanol production, which can be either an independent fuel as mentioned above or act as a substitute for gasoline supplemented with MTBE to reduce emissions of CO and NO_x (Ahmed and Lewis 2007; Henstra et al. 2007; Shaw et al. 2008). However, its hygroscopic nature and low caloric content limits the use of ethanol with current infrastructures; therefore, very recent research has also focused on butanol production through the fermentation of such gaseous substrates as an alternative alcohol-biofuel.

Fermentation of CO-rich gases, i.e., syngas or waste gases, has been shown to be an attractive and likely cost-effective alternative able to compete with the conventional second-generation process based on the fermentation of carbohydrates (Kennes et al. 2016). This is above all true whenever using waste gases as substrates. Therefore, this process has recently attracted interest from some companies and some demonstration and pre-commercial projects are now being set up for ethanol production (Abubackar et al. 2011; Kennes and Veiga 2013). However, several challenges remain to be addressed in order to further improve the efficiency and cost-effectiveness of this technology. One of those challenges is related to the low water solubility of carbon monoxide and other volatile compounds (e.g., H_2, CO_2), which limits the mass transfer rate of the substrate to the liquid phase in suspended-growth bioreactors or to the biofilm in attached-growth bioreactors, limiting at the same time the production yields of (bio)fuels or platform chemicals of interest. Some previous and on-going studies are focusing on minimizing such drawback. Among others, the use of membrane systems as well as attached-growth bioreactors seems to allow a more efficient mass transfer of poorly soluble compounds (Jin et al. 2009; Shen et al. 2014) as well as microbubble spargers in suspended-growth bioreactors (Bredwell and Worden 1998). Another drawback to be taken into account and already previously observed in conventional acetone-butanol-ethanol (ABE) fermentation from carbohydrates is solvent toxicity. This is an important factor to take into account in butanol fermentation as acetogenic bacterial cells rarely tolerate more than 2 % butanol (Liu and Qureshi 2009). However, although new strategies can still be developed, experience has already been gained from the conventional ABE fermentation aimed at reducing such inhibitory problems. These strategies may include the use of continuous in situ removal of produced solvents from the fermentation broth, among others (Schugerl 2000). It is also worth mentioning that setting up bioreactors under anaerobic conditions with CO-related gases as substrates may be somewhat more challenging than the conventional fermentation of carbohydrates. However, such harsher conditions will also reduce potential microbial contamination of the bioreactor. Finally, optimizing the fermentation and bioreactor operating conditions is another aspect that will allow improving the yield and selectivity of the biochemical reactions and which is addressed in this paper. Research on such aspects will further improve the efficiency and cost-effectiveness of this process appearing as a promising alternative.

In the present study, the conversion of CO into butanol and ethanol was carried out by the bacterium *Clostridium carboxidivorans*, which was grown first in batch bottles with no pH regulation and, afterwards, in continuous gas-fed bioreactors using a defined medium under controlled conditions and continuously fed CO gas. The objectives were to develop and optimize culture conditions for a relatively high production of alcohols through anaerobic CO fermentation and to compare the growth and fermentation products between the batch bottle assays and the bioreactors with continuous CO supply. Bioreactor operating conditions were optimized.

Material and methods

Microorganism and culture media

C. carboxidivorans P7 DSM 15243 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was maintained anaerobically on modified basal medium (Liou et al. 2005; Tanner 2007) at pH 5.75 with CO (100 %) as the sole gaseous substrate. This medium was composed of (per liter distilled water) the following compounds: yeast extract, 1 g; mineral solution (a source of sodium, ammonium, potassium, phosphate, magnesium, sulfate and calcium), 25 mL; trace metal solution, 10 mL; vitamin solution, 10 mL; resazurin, 1 mL; and cysteine-HCl, 0.60 g.

The mineral stock solution contained (per liter distilled water) 80 g sodium chloride, 100 g ammonium chloride, 10 g potassium chloride, 10 g potassium monophosphate, 20 g magnesium sulfate, and 4 g calcium chloride.

The vitamin stock solution contained (per liter distilled water) 10 mg pyridoxine, 5 mg each of thiamine, riboflavin, calcium pantothenate, thioctic acid, paraamino benzoic acid, nicotinic acid, and vitamin B12, and 2 mg each of D-biotin, folic acid, and 2-mercaptoethanesulfonic acid.

The trace metal stock solution contained (per liter distilled water) 2 g nitrilotriacetic acid, 1 g manganese sulfate, 0.80 g ferrous ammonium sulfate, 0.20 g cobalt chloride, 0.20 g zinc sulfate, and 20 mg each of cupric chloride, nickel chloride, sodium molybdate, sodium selenate, and sodium tungstate.

Bottle batch experiments

For batch experiments, 10 % seed culture in the early exponential growth phase, grown with CO as sole carbon source, was aseptically inoculated into 200-mL serum vials containing 100 mL medium at pH = 5.75. In order to remove oxygen, all the media in the bottles were boiled and later flushed with N_2 while cooling down the medium. When the temperature of the medium reached 40 °C, 0.06 g cysteine-HCl was added as a reducing agent, and the pH was adjusted to 5.75 with 2 M NaOH while continuing flushing with N_2. The bottles were then sealed with Viton stoppers and capped with aluminum crimps and were then autoclaved for 20 min at 121 °C. The bottles were maintained under anaerobic conditions. They were pressurized with 100 % CO to reach a total headspace pressure of 1.2 bar and were agitated at 150 rpm on an orbital shaker, inside an incubation chamber at 33 °C. Every 24 h, a headspace sample of 0.2 mL and 2 mL liquid sample were taken for CO measurements and to measure the optical density ($OD_{\lambda = 600\ nm}$), which is directly related to the biomass concentration. Besides, 1 mL of those 2 mL was centrifuged at 7000 rpm for 3 min in order to measure the concentration of soluble products in the supernatant, using the same methods as described in "Fermentation products" for the analyses of fermentation products in the continuous bioreactors. All experiments were carried out in triplicate.

Continuous gas-fed bioreactor experiments

Two bioreactor experiments were carried out in 2L BIOFLO 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA) using the same medium as in the batch bottle experiments. Both experiments were done with 1.2 L optimized medium and CO (100 %) as the sole gaseous substrate, fed continuously at a rate of 10 mL/min using a mass flow controller (Aalborg GFC 17, Müllheim, Germany) and a microsparger used for sparging CO. The bioreactor with the medium was autoclaved, and when the temperature was below 40 °C, cysteine-HCl (0.60 g/L) was added, together with nitrogen feeding to ensure anaerobic conditions. The temperature of the bioreactor was maintained at 33 °C by means of a water jacket. Four baffles were symmetrically arranged to avoid vortex formation of the liquid medium and to improve mixing. A constant agitation speed of 250 rpm was maintained throughout the experiments. Ten percent seed culture in the early exponential growth phase, which was grown for 72 h with CO as sole carbon source, was used as the inoculum and was aseptically transferred to the bioreactor. The pH of the medium was automatically maintained at a constant value of either 5.75 or 4.75, through the addition of either a 2-M NaOH solution or a 2-M HCl solution, fed by means of a peristaltic pump. The redox potential was continuously monitored in each experimental run.

When the bioreactor reached its maximum production of acids, the pH in one of the reactors (experiment 1) was maintained at pH 5.75 while it was changed to pH 4.75 in the other reactor (experiment 2). Later, when most of the acids were consumed, part of the medium (around 600 mL) was replaced with the same amount of fresh medium in both bioreactors and the pH was maintained at 5.75 again. During the partial medium replacement procedure, the CO gas flow rate was maintained through the bioreactor and was even slightly increased in order to ensure maintenance of anaerobic conditions inside the bioreactor. Then, when the production of acids reached its maximum value, the pH of the bioreactor in experiment 1 was changed to 4.75.

Growth measurement

One milliliter liquid sample was daily withdrawn from the reactor, in order to measure the optical density ($OD_{\lambda = 600\ nm}$), using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The measured absorbance allowed to estimate the biomass concentration (mg/L) by comparing it with a previously generated calibration curve.

Gas-phase CO and CO_2 concentrations

Gas samples of 1 mL were taken from the outlet sampling ports of the bioreactors to monitor the CO and CO_2 concentrations.

Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD). The GC was fitted with a 15-m HP-PLOT Molecular Sieve 5A column (ID, 0.53 mm; film thickness, 50 μm). The oven temperature was initially kept constant at 50 °C, for 5 min, and then raised by 20 °C/min for 2 min, to reach a final temperature of 90 °C. The temperature of the injection port and the detector was maintained constant at 150 °C. Helium was used as the carrier gas.

Similarly, CO_2 was analyzed on an HP 5890 gas chromatograph, equipped with a TCD. The injection, oven, and detection temperatures were maintained at 90, 25, and 100 °C, respectively.

Fermentation products

The water-soluble products, acetic acid, butyric acid, ethanol, and butanol, were analyzed for each of the two bioreactors from liquid subsamples (1 mL) every 24 h using an HPLC (HP1100, Agilent Co., USA) equipped with a 5 μm × 4 mm × 250 mm Hypersil ODS column and a UV detector at a wavelength of 284 nm. The mobile phase was a 0.1 % ortho-phosphoric acid solution fed at a flow rate of 0.5 mL/min. The column temperature was set at 30 °C. The samples were centrifuged (7000*g*, 3 min) using a centrifuge (ELMI Skyline ltd CM 70M07) before analyzing the concentration of water-soluble products by HPLC.

Redox potential

The redox potential was constantly monitored in each bioreactor using an Ag/AgCl reference electrode connected to a transmitter (M300, Mettler Toledo, Inc., Bedford, MA, USA) and maintained inside the bioreactor.

Results

Bottle batch experiments

In the batch experiments, *C. carboxidivorans* started growing immediately after inoculation, without any lag phase (Fig. 1a). A maximum biomass concentration of 0.130 g/L was reached after 30 h (Fig. 1a), while a maximum acetic acid concentration of 0.89 g/L was found after 45 h and a maximum concentration of butyric acid of 0.48 g/L had accumulated at the end of the experiment (Fig. 1b).

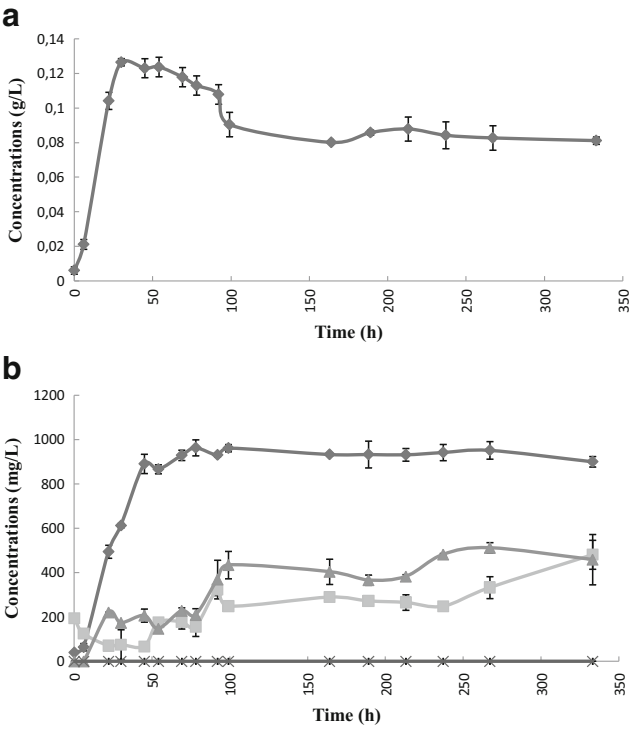

Fig. 1 Batch experiment: **a** measured growth expressed in grams biomass per liter over time, with data represented as mean values ± standard deviations, and **b** production of metabolites, acetic acid (*diamonds*), butyric acid (*squares*), ethanol (*triangles*), and butanol (*cross marks*), expressed in milligrams per liter over time, with data represented as mean values ± standard deviations

On other hand, a maximum ethanol concentration of 0.48 g/L was reached, after 267 h. Its production did not seem to take place at the expense of any acetic acid consumption as that acid was basically not consumed during ethanol accumulation, although clostridia are known to be able to convert acids into alcohols in processes such as the ABE fermentation from carbohydrates (Jones and Woods 1986; Ndaba et al. 2015) as well as in some other fermentation processes such as CO bioconversion to acetic acid followed by the production of ethanol from the accumulated fatty acid (Abubackar et al. 2015). The alcohol was thus formed here directly from the conversion of CO. Although *C. carboxidivorans* is known to be able to produce butanol, that alcohol was generally not found or produced at low trace levels in these batch bottle assays.

The initial pH of the medium was 5.75 in this experiment. However, when the acetogenic phase started, acids were formed leading to medium acidification, as there was not any pH regulation. Therefore, the pH dropped gradually during the batch assays and reached a minimum value around 4.30 at the end of the experiment. Also, anaerobes are very sensitive to changes in redox potential. The reading oxidoreduction potential (ORP) values are directly linked to the pH of the medium, and a lower pH of the medium will result in less negative values of the redox potential.

Continuous gas-fed bioreactor experiments

Experiment 1

In this continuous CO-fed bioreactor experiment, *C. carboxidivorans* started growing immediately after inoculation, without any lag phase, similarly as in the batch assays. The growth and fermentation products followed a pattern common to acetogenic clostridia (Fig. 2a, b). After 96 h, the biomass reached its maximum value of 0.52 g/L (Fig. 2a) whereas the maximum concentrations of acetic acid and butyric acid, reached after 144 h, were 5.30 and 1.43 g/L, respectively (Fig. 2b).

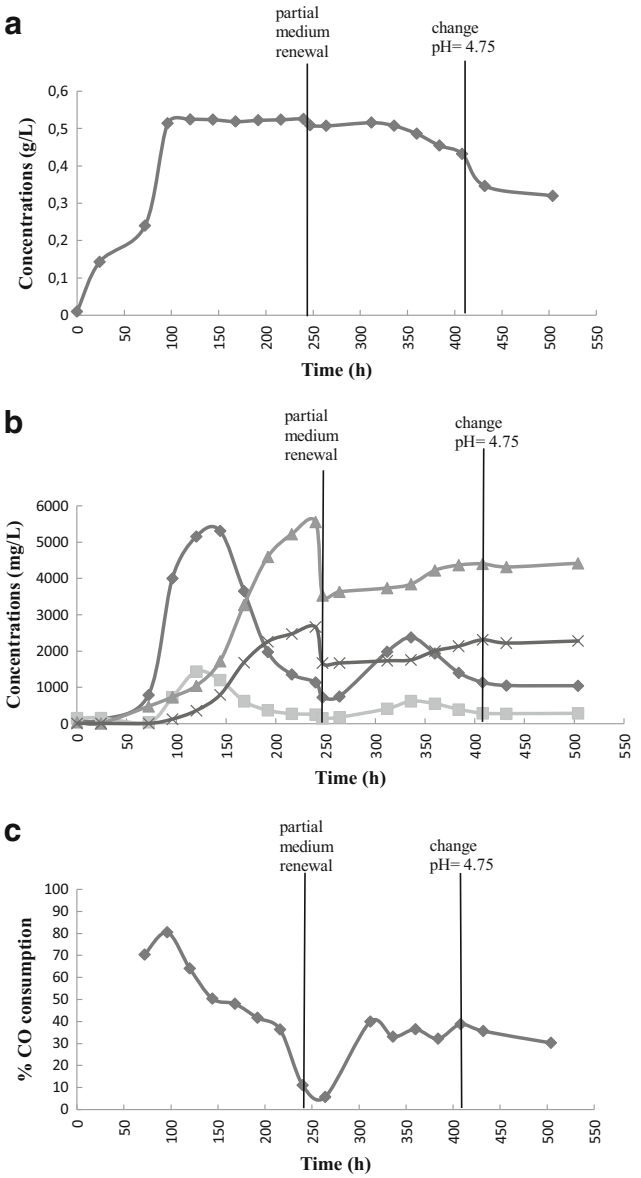

Fig. 2 Continuous gas-fed bioreactor experiment 1: **a** measured growth expressed in grams biomass per liter over time; **b** production of metabolites, acetic acid (*diamonds*), butyric acid (*squares*), ethanol (*triangles*), and butanol (*cross marks*), expressed in milligrams per liter over time; and **c** percentage CO consumption over time

The production of alcohols did also start quite soon after inoculation but initially at a quite slower rate than observed for the acids. Alcohols continued accumulating after the fatty acids had reached their highest concentrations. After 240 h, the production of alcohols leveled off, because ethanol and butanol had accumulated up to potentially inhibitory levels. By then, ethanol and butanol had reached quite high maximum concentrations of 5.55 and 2.66 g/L, respectively (Fig. 2b). A different behavior was observed than in the batch experiments, as ethanol production appeared to increase at the expense of acetic acid consumption, and both the decrease in acid concentration and increase in alcohol concentration occurred simultaneously. Similarly, butanol production appeared to take place at the expense of butyric acid conversion. Contrary to what was observed in the batch assay, in the present experiment with pH regulation and continuous CO supply, butyric acid was almost completely consumed (83 %) and 78 % of acetic acid was also converted. Besides, a rather high final concentration of butanol was reached (2.66 g/L), never reported before in the literature for this type of CO fermentation by clostridia.

After 247 h, part of the bioreactor medium (600 mL) was replaced by fresh medium in order to alleviate the potential inhibitory effect of the high concentrations of alcohols and to check if this partial medium renewal might promote a new acids and alcohols production cycle. The biomass was recycled in the bioreactor; thus, its concentration remained constant at 0.51 g/L. The concentrations of alcohols decreased as a result of the dilution effect due to medium replacement, reaching an ethanol concentration of 3.50 g/L and a butanol concentration of 1.70 g/L (Fig. 2b).

While the amount of biomass remained constant until 360 h, the concentrations of acids started to increase again. The maximum concentrations of acetic acid and butyric acid were reached after 336 h with values of 2.40 and 0.617 g/L, respectively (Fig. 2b). Despite maintaining the pH at 5.75, the formation of some alcohols started immediately, although at much lower rates than for the acids. The pH was later changed to 4.75 after 408 h in order to check if this could further improve the production of alcohols, as a lower pH is expected to be favorable to solventogenesis.

However, at that lower pH, the biomass concentration decreased while there was not any production of acids and alcohols nor any consumption of acids. Finally, the experiment was stopped after 504 h. At that moment, the biomass concentration had decreased to 0.32 g/L, and acetic acid and butyric acid concentrations were 1.04 and 0.28 g/L, respectively. The concentrations of alcohols were 4.41 g/L for ethanol and 2.3 g/L for butanol at the end of the experiment, which is significantly higher than in any previously reported study

(Fig. 2b). Total net ethanol production in this experiment was 7.52 g corresponding to 6.66 g (in 1.2 L reactor medium) before partial medium replacement and 0.86 g after its replacement, and total net butanol production was 3.91 g corresponding to 3.19 g before partial medium replacement and 0.72 g after its replacement. This type of CO fermentation yields reproducible profiles.

During the experimental production phase, the specific rate of ethanol production was 0.12 g/h*g of biomass between 144 and 192 h, while the specific rate of butanol production was 0.06 g/h*g of biomass during that same period of time (Table 1). Other production and consumption rates are summarized in Table 1 as well.

Carbon monoxide consumption was also monitored during the experiment and is shown in Fig. 2c. A constant carbon monoxide loading rate was maintained throughout the experiment. Most of the time, the average CO consumption was close to 50 %, although higher percentages were observed in the early stages of the experiment, reaching the highest value of 81 % CO removal on the fourth day. It is worth mentioning that, as a general rule, the highest percentage substrate conversions are observed at high pH (5.75) and do then often exceed 50 % (up to 81 %), while conversion decreased when lowering the pH (4.75), which is also concomitant with some biomass decay. CO consumption also dropped within the first few hours after medium replacement.

During the first operation days, and for almost 1 week, the only carbon source for the production of metabolites and biomass is carbon monoxide. As explained above, C2 and C4 acids produced during the first stages become, later on, additional substrates and are then converted to the corresponding alcohols. During the first stage of the experiment, although part of the gaseous substrate is also used for biomass growth, if production of metabolites from CO is only considered during the first experimental stage, then the following reactions would take place:

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad (1)$$

$$10CO + 4H_2O \rightarrow CH_3(CH_2)_2COOH + 6CO_2 \quad (2)$$

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \quad (3)$$

$$12CO + 5H_2O \rightarrow C_4H_9OH + 8CO_2 \quad (4)$$

This would result in CO_2 to CO ratios (g/g) of 0.79 for reaction (1) (acetic acid production), 0.94 for reaction (2) (butyric acid production), and 1.04 for reactions (3) and (4) (ethanol and butanol production, respectively). CO_2 to CO ratios could be measured experimentally and could be estimated to reach around 0.77, during the first week of operation, with around 10 % fluctuation as this is a dynamic system. This is in agreement with the above equations and theoretical ratios and shows a good fit between the experimental and theoretical substrate to product mass balance calculations.

The redox potential was constantly monitored during each experimental run. Except for the reducing agent added initially to the medium, its value was later on allowed to fluctuate naturally. In experiment 1, before replacing part of the medium, the redox potential (ORP) value was −180 ± 20 mV, while after partial medium replacement, it was −100 ± 10 mV. After the pH change to 4.75, ORP was −62.1 ± 20 mV, and finally at the end of the experiment, it became positive and reached +42.9 mV, which explains the complete inhibition of the anaerobic strain. Inhibition after pH modification could have been due to the fast decrease in pH from 5.75 to 4.75 resulting in an acid shock. As will be explained below, in experiment 2, pH was decreased gradually and no inhibition was observed, allowing to maintain active cells and a negative ORP.

Experiment 2

Similarly as in experiment 1, *C. carboxidivorans* started to grow immediately after seeding the reactor, without any lag phase. A pattern common for acetogenic clostridia for growth and fermentation products was here also observed (Fig. 3a, b). After 48 h, the biomass reached its maximum value of 0.33 g/L (Fig. 3a) whereas the maximum acetic acid and butyric acid concentrations were found after 3–4 days and were 4.10 and 1.44 g/L, respectively (Fig. 3b). As already observed in experiment 1, and as expected, growth and accumulation of acids were concomitant. It is worth observing that the maximum suspended biomass concentration in the liquid phase was somewhat lower in this experiment

Table 1 Comparison of the different production and consumption rates between experiments 1 and 2

	Acetic acid consumption rate	Butyric acid consumption rate	Ethanol production rate	Butanol production rate
Experiment 1 (pH 5.75)	0.13	0.027	0.12	0.06
Experiment 2 (pH 4.75)	0.15	0.039	0.16	0.07

The rates are expressed in g/h*g of biomass

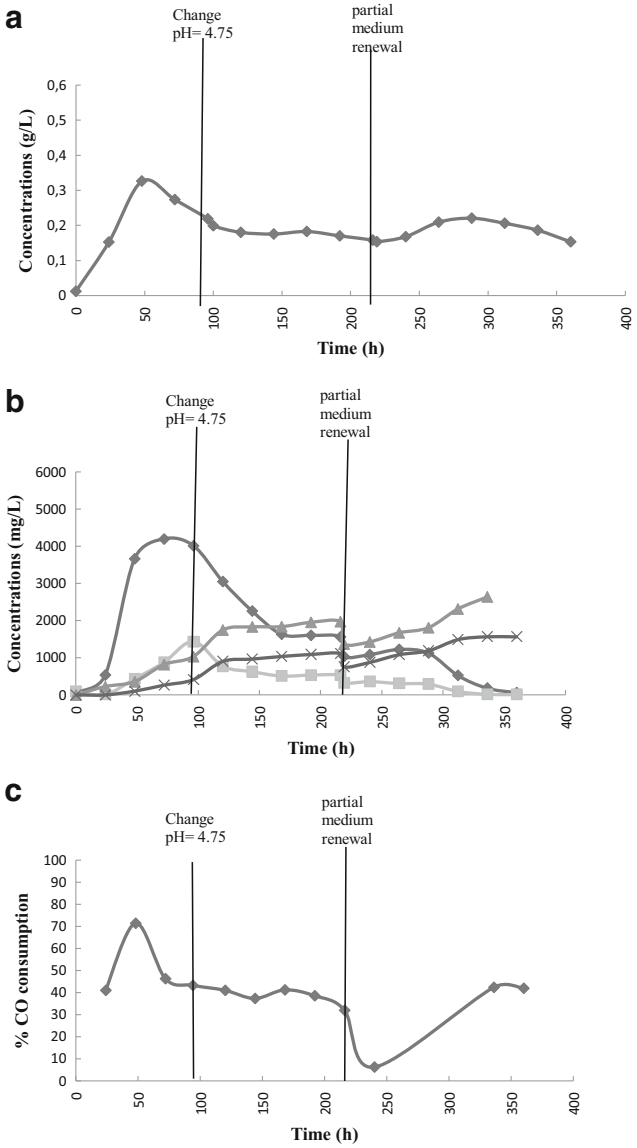

Fig. 3 Continuous gas-fed bioreactor experiment 2: **a** measured growth expressed in grams biomass per liter over time; **b** production of metabolites, acetic acid (*diamonds*), butyric acid (*squares*), ethanol (*triangles*), and butanol (*cross marks*), expressed in milligrams per liter over time; and **c** percentage CO consumption over time

compared to experiment 1 because part of the bacteria remained sticked to the glass wall of the bioreactor, slightly above the upper liquid level.

After 72 h, once acetic acid accumulation leveled off, the pH of the medium was gradually and slowly decreased to 4.75 over a period of 48 h, in order to avoid any acid shock and inhibition. The pH value was decreased in order to check if this would stimulate solventogenesis. The rate of accumulation of alcohols increased, and their maximum production was reached after 216 h, with ethanol and butanol concentrations of 2.00 and 1.10 g/L, respectively (Fig. 3b). This increase was at the expense of acid consumption, and acetic acid and butyric acid concentrations had dropped down to 1.56 and 0.53 g/L respectively, after 216 h (Fig. 3b).

When the concentrations of alcohols stabilized, part of the medium of the bioreactor was replaced by fresh medium, similarly as in experiment 1, and the pH was increased to 5.75 again. As a result of the dilution effect, the concentrations of metabolites decreased to 1.03 g/L for acetic acid, 0.32 g/L for butyric acid, 1.4 g/L for ethanol, and 0.76 g/L for butanol. After partial medium replacement, the remaining concentrations of acids were consumed and converted into alcohols. Finally after 360 h, the biomass started decreasing down to 0.15 g/L and the experiment was stopped. By then, the final concentrations of acids and alcohols were 0.06 g/L acetic acid, 0.01 g/L butyric acid, 2.90 g/L ethanol, and 1.60 g/L butanol, showing a basically complete consumption of both acids and their conversion to alcohols (Fig. 3b).

Total net ethanol production in this experiment was 4.21 g corresponding to 2.40 g (in 1.2 L reactor medium) before partial medium replacement and 1.81 g after its replacement, and total net butanol production was 2.29 g corresponding to 1.32 g before partial medium replacement and 0.97 g after its replacement. These concentrations are somewhat lower than in experiment 1, most probably because of the somewhat lower suspended biomass concentration in this assay.

The specific rate of ethanol production was 0.16 g/h*g of biomass between 72 and 120 h, while the specific rate of butanol production was 0.07 g/h*g of biomass during that same period of time, which was thus slightly higher than in experiment 1 (Table 1). Other rates are summarized in Table 1.

Similarly as in experiment 1, a constant inlet carbon monoxide concentration was maintained during the study and CO consumption was monitored throughout experiment 2. Here again, on an average, close to 50 % of the gaseous carbon source was metabolized by the bacteria (Fig. 3c) with higher values during the first part of the study and when using a high pH, as also observed in experiment 1. The highest CO consumption reached 73 %, at pH 5.75, on the second day of operation.

In terms of redox potential, in experiment 2, when the pH was 5.75, the redox potential (ORP) value was −110 ± 10 mV, while after the pH change to 4.75, it was −80 ± 10 mV. Finally after medium replacement and pH increase again to 5.75, the ORP was −90 ± 10 mV. The gradual pH decrease, from 5.75 to 4.75, in this experiment allowed to avoid inhibition of the bacterial activity, and a negative redox potential could be maintained throughout this assay, contrary to what was observed at the end of experiment 1.

Discussion

In the batch bottle experiments, the maximum biomass concentration was reached after 48 h and the biomass

concentration (g/L) was about half the value reached in experiment 2. That difference can be explained by the fact that in the batch assays, there was not any continuous feed of CO, resulting in carbon source limitation for the bacteria. Conversely, in experiments 1 and 2 in bioreactors, CO feeding was continuous, resulting in a higher availability of C source for the biomass. Also, in the batch bottle experiments, there was no continuous pH control. Therefore, the production of acetic acid and butyric acid during growth led to a natural and significant decrease of the pH of the medium. This ended up inhibiting bacterial growth and metabolite production. The initial pH of the medium was 5.75, whereas the final pH value was in the range of 3.80–4.00 for all the batch assays in bottles. *C. carboxidivorans* has an optimum pH value of 4.4–7.6 (Abubackar et al. 2011; Liou et al. 2005). As there was not any pH control in the batch experiments, its value reached a minimum which was below the optimum range. The lower pH value and lower concentration of C source explain the different growth behaviors between the batch experiments and the bioreactors with continuous CO supply.

In the three experiments, two different growth patterns were observed. First a fast exponential growth rate was observed, between 48 and 96 h in the bioreactors and between 0 and 36 h in the batch assays, concomitant with the acidogenic phase. Due to acid production in the Wood-Ljungdahl pathway, more ATP is produced during acidogenesis than during the production of alcohols (White 2007), which explains that growth and acid production from CO take place simultaneously.

In the case of the production of alcohols, in the batch assays, ethanol accumulation was observed but there was basically no butanol accumulation, whereas in the bioreactors (experiment 1 and 2), there was both significant ethanol and butanol production and the fermentation products followed a pattern common to acetogenic clostridia. It can be assumed that the pH had reached a value lower than the optimum range, which could have inhibited the bacterial metabolism before any significant butanol production could take place in the batch assays.

In both bioreactor experiments, acids were produced first, i.e., acetic acid and butyric acid, followed by ethanol and butanol production, with CO consumption around 50 % and reaching up to somewhat more than 80 % during the early acidogenic stage. Thus, the regulation of the pH value throughout the experiments can be considered to represent an important factor largely affecting both biomass growth and the production of metabolites.

In case of other clostridial strains able to produce only ethanol as alcohol (but no butanol), it was also observed that using different pH values is an effective strategy to promote alcohol production in multi-stage syngas fermentation, in which the acidogenesis and solventogenesis phases are separated in two reactors (Klasson et al. 1992). Here, in both bioreactor experiments, we tried to compare the effect of a pH change after the acidogenic phase. That way, in experiment 1, pH was maintained constant, whereas in experiment 2, the pH was changed to 4.75 to promote the solventogenic phase. No clear separate acidogenic and solventogenic phases were observed in *C. carboxidivorans* during experiment 2, whereas in experiment 1 (without pH change), more pronounced separate acidogenic and solventogenic phases were found. In their study with a different organism and for ethanol production, Klasson et al. (1992) performed a two-stage syngas fermentation experiment, with two reactors in series, using *Clostridium ljungdahlii*, with the first reactor at pH 5.0 and the second one at pH 4.0~4.5 to promote ethanol production in that second reactor at the expense of acetate. By using two different pH, 30 times more ethanol production was obtained than in a single continuous gas fed bioreactor.

The rates of alcohol production were lower in experiment 1 than in experiment 2 at a lower pH. In case of ethanol, its production rate was 0.12 g/h*g of biomass in experiment 1, while it was 0.16 g/h*g of biomass in experiment 2. On the other hand, the rate of butanol production was 0.06 g/h*g of biomass in experiment 1 while it was 0.07 g/h*g of biomass in experiment 2. The same relationship was observed between the acid consumption rates, which were lower in experiment 1 than in experiment 2. The acetic acid conversion rates were 0.13 g/h*g of biomass and 0.15 g/h*g of biomass in experiments 1 and 2, respectively. On the other hand, butyric acid conversion rates were 0.03 g/h*g of biomass and 0.04 mg/h*g of biomass in experiments 1 and 2, respectively. So, these results show higher acid to alcohol conversion rates at pH 4.75 than at pH 5.75 (Table 1).

So far, only few studies have focused on butanol production from CO-rich gases in clostridia. In all few previous reports using *C. carboxidivorans*, butanol concentrations did generally range between a few milligrams per liter and hardly 1 g/L, while butanol concentrations up to 2.66 g/L were reached in the present study together with ethanol concentrations of 5.55 g/L. Bruant et al. (2010), performing a similar batch experiment as ours in our bottles assays, accumulated a minor, near negligible, amount of butanol (below 0.05 mmol) of a few milligrams. Phillips et al. (2015) checked different media in batch experiments and their maximum reported concentrations for ethanol and butanol were 3.25 and 1.09 g/L, respectively. In another study, Ukpong et al. (2012) checked the bioconversion of CO-rich gases by *C. carboxidivorans* in a gas-fed bioreactor reaching maximum ethanol and butanol accumulation of 2.8 and 0.52 g/L, respectively. In all those studies, the amounts of butanol and ethanol obtained with the same bacterial species were significantly lower than the values obtained in the present work. Overall, comparing the batch assays and the different bioreactor studies, it clearly appears that relatively high butanol-ethanol (B-E) accumulation can be reached when optimizing the

experimental conditions. It is worth mentioning that, contrary to the ABE fermentation from carbohydrates in clostridia, here, no acetone is produced at all in CO or syngas fermentation in such acetogenic bacteria, which is interesting as butanol is the main desired end-product as a biofuel and attempts do generally need to be made in order to reduce acetone accumulation in the conventional ABE fermentation (Han et al. 2011).

In a nutshell, it can be concluded that (a) a high pH was favorable to CO conversion to fatty acids; (b) reducing the pH value stimulated the production of alcohols but had a profound negative effect on biomass production; (c) acetic acid and butyric acid are produced first and can then be converted to the alcohols (ethanol, butanol), with complete conversion at higher rates under acidic conditions; (d) contrary to the ABE fermentation, no acetone was formed here from the conversion of C1 gases; and (e) the experimental conditions in this study allowed to produce significantly more butanol and ethanol (B-E) than in any other study reported in the literature on the conversion of CO-rich gases.

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Effect of pH control on the anaerobic H-B-E fermentation of syngas in bioreactors

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Abstract

BACKGROUND: Syngas and some waste gases are composed mainly of carbon monoxide, carbon dioxide and hydrogen, which can be used by some acetogenic bacteria to produce ethanol, butanol or hexanol, and represents an attractive alternative to the conventional ABE (acetone–butanol–ethanol) fermentation. Experiments were carried out in bioreactors under different conditions (pH 5.75 and 4.75) with continuous supply of a mixture of $CO/CO_2/H_2/N_2$ converted by *Clostridium carboxidivorans* into hexanol, butanol and ethanol (H-B-E fermentation).

RESULTS: Applying different pH control strategies will affect the syngas fermentation pattern, among others in terms of bioconversion rates as well as final concentrations of acids and alcohols. The highest concentrations of alcohols were obtained at pH 5.75, i.e. 2.7 g L^{-1} ethanol, 1.9 g L^{-1} butanol and 0.85 g L^{-1} hexanol, whereas the maximum production rates were observed at pH 4.75, reaching 0.048 g-ethanol h^{-1} g^{-1}-biomass, 0.037 g-butanol h^{-1} g^{-1}-biomass, and 0.026 g-hexanol h^{-1} g^{-1}-biomass. However, a low pH negatively affects growth and acids production in the first metabolic step, with lower growth and acids production at pH 4.75 than 5.75. Growth rates reached 0.0057 h^{-1} and 0.072 h^{-1}, respectively, at pH 4.75 and 5.75.

CONCLUSIONS: Maintaining initially a higher pH of 5.75 allows accumulating higher concentrations of acids than when natural acidification takes place. Such higher concentrations of acids allow the production of higher amounts of alcohols as end metabolites, showing the importance of pH on bioconversion and biomass growth.

Keywords: acetogens; butanol; ethanol; hexanol; waste gas; *Clostridium carboxidivorans*

INTRODUCTION

Biorefineries are emerging as environmentally-friendly alternatives to conventional refineries for the commercial production of fuels and platform chemicals. They are based on the use of renewable feedstocks, such as biomass, or even waste or pollutants, which can be fermented into added-value end metabolites. Ethanol and higher alcohols, such as butanol, are examples of metabolites of commercial interest. (Bio)ethanol is mainly used as a fuel. Higher alcohols such as butanol and hexanol may also be suitable fuels,[1] but can be used as platform chemicals as well. Ethanol is an oxygenated, water-free, high octane alcohol that can replace gasoline or it can be mixed with it at different ratios.[2] Butanol is more similar to gasoline than ethanol, besides being less hygroscopic, less corrosive and having a higher caloric content than ethyl alcohol.[3–5] It can be blended with gasoline at higher concentrations than ethanol without the need for modifying existing engines.[6,7] Hexanol has also been tested as a fuel, among others in the form of diesel–hexanol blends and also in aviation fuel, although its high viscosity limits its potential use as jet fuel.[3,8,9] In terms of bioprocesses, the best known alternative for their production is the ABE fermentation, allowing production of butanol, together with other solvents such as ethanol and acetone, through the bioconversion of carbohydrates by clostridia or other anaerobic bacteria.[3]

Bioalcohols have traditionally been produced in biorefineries through the so-called first generation process in which fermentable sugars are obtained from food supplies, which results in food–fuel competition and represents a poorly sustainable alternative.[10] The second generation process avoids that problem as it uses lignocellulosic materials from agricultural waste or energy crops as feedstock. It is more sustainable but less cost-effective, among others because of the complex pretreatments required to obtain simple sugars fermentable into alcohols.[11] On the other hand, the fermentation of C1 gases has recently been shown to be a promising alternative as it can also generate higher alcohols such as butanol and, to a lower extent, hexanol.[3] C1 gases, mainly CO and CO_2, can be obtained from the gasification of wastes, coal, biomass and other feedstocks.[2,10,12,13] Syngas is a complex mixture of gases, composed mainly of CO, CO_2, and H_2,[14] which can be fermented by clostridia and other acetogens, mostly into acids and/or alcohols. Interestingly, those same gaseous substrates are found in some waste gases, among others in gaseous effluents from steel industries. In such case, their bioconversion would allow the simultaneous removal of air pollutants as well as a greenhouse gas such as carbon dioxide, combined with their conversion into valuable products. This is a promising technology able to compete with second generation

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carbohydrates fermentation,[10] although it still faces some challenges such as the low solubility of CO and H_2 mainly,[3] or the presence of other minor compounds in the case of syngas, that could be toxic to the producing strains.[15,16]

In the present study, the anaerobic bioconversion of syngas or waste gas into ethanol and higher alcohols (butanol, hexanol) was carried out by the acetogenic bacterium *Clostridium carboxidivorans* in a continuous gas-fed bioreactor containing a defined aqueous culture broth and continuously supplied a mixture of CO, CO_2, H_2, N_2. The objective of this research was to develop and optimize the operating conditions in order to reach an efficient production of alcohols. The production of metabolites was compared under two different bioreactor operating conditions, either with pH regulation or without pH regulation. In an attempt to increase the production of acids and the subsequent accumulation of alcohols, pH conditions were adjusted throughout the fermentation runs in order to optimize the production of acids and alcohols and increase their concentrations and the overall efficiency of the bioconversion process.

MATERIAL AND METHODS

Microorganism and culture media

Clostridium carboxidivorans P7 DSM 15243 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was maintained anaerobically on modified basal medium at pH 5.75 and with a mixture of the following gases as volatile substrates: $CO:CO_2:H_2:N_2$ (20:20:10:50).[17,18] The basal medium had the following composition (per liter distilled water): 1 g yeast extract; 25 mL mineral solution; 10 mL trace metal solution; 10 mL of vitamins stock solution; 1 mL resazurin; 0.60 g cysteine-HCl.

The mineral stock solution was composed of (per liter distilled water): 80 g sodium chloride, 100 g ammonium chloride, 10 g potassium chloride, 10 g potassium monophosphate, 20 g magnesium sulfate, and 4 g calcium chloride.

The trace metal solution was obtained from a stock solution, whose composition is as follows (per L distilled water): 2 g nitrilotriacetic acid, 1 g manganese sulfate, 0.80 g ferrous ammonium sulfate, 0.20 g cobalt chloride, 0.20 g zinc sulfate, and 20 mg each of cupric chloride, nickel chloride, sodium molybdate, sodium selenate, and sodium tungstate.

The vitamin stock solution was composed of (per L distilled water): 10 mg pyridoxine, 5 mg each of thiamine, riboflavin, calcium pantothenate, thioctic acid, paraamino benzoic acid, nicotinic acid, and vitamin B12, and 2 mg each of D-biotin, folic acid, and 2-mercaptoethanesulfonic acid.

Continuous gas-fed bioreactor experiments

Two bioreactor experiments were carried out in 2 L BIOFLO 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA). The two experiments were carried out with 1.2 L of the same medium composition and the mixture of gases, described above, as gaseous substrate. The syngas mixture was continuously fed at a flow rate of 10 mL min^{-1} using a mass flow controller (Aalborg GFC 17, Müllheim, Germany) and the gas was sparged by a microsparger. The medium inside the bioreactor was autoclaved, and afterwards it was flushed with N_2 to ensure anaerobic conditions, while the bioreactor medium was cooled by means of a water jacket. When the temperature reached 40 °C the vitamins solution and cysteine-HCl were added. The temperature of the medium was maintained at 33 °C throughout the experimental process with a constant agitation speed of 250 rpm. Inside the bioreactor, four baffles avoided vortex formation and allowed improved liquid mixing.

When the conditions were completely anaerobic, N_2 flushing was stopped and constant syngas feeding was started. Then, 10% seed culture in the early exponential growth phase (which was previously grown for 72 h with CO as sole carbon source) was inoculated in the bioreactor. The redox potential and the pH value were continuously monitored. The pH was automatically maintained constant through the addition of either 2 mol L^{-1} NaOH or 2 mol L^{-1} HCl solutions, by means of a peristaltic pump.

In the first experiment, there was no pH regulation, except during the first day of operation, when the pH was maintained at 5.75, allowing for good biomass growth. Later on, as a result of the production of acids the pH dropped naturally to a value of 4.75, which was then maintained constant in order to avoid any possible inhibitory effect. Afterwards, when the consumption of organic acids stabilized, the pH was slowly and gradually increased to 5.75 with the aim of starting a new production cycle. The new cycle did not start, contrary to what was expected, and part of the medium was then replaced with the same volume of fresh medium. Conditions of natural pH shift were again maintained in the bioreactor after medium replacement. In the second experiment, the pH was maintained constant at 5.75 throughout the study.

Growth measurement

A 1 mL liquid sample was withdrawn daily from the bioreactors, and was used to measure the optical density ($OD_{\lambda=600nm}$) on a UV-visible spectrophotometer (Hitachi, Model U-200, Pacis and Giralt, Madrid, Spain) in order to estimate the biomass concentration (mg L^{-1}) using a previously generated calibration curve.

Gas-phase CO and CO_2 concentrations

Gas samples of 1 mL were taken from the outlet sampling ports of the bioreactors to monitor the CO and CO_2 concentrations. Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD). The GC was fitted with a 15 m HP-PLOT Molecular Sieve 5A column (ID, 0.53 mm; film thickness, 50 μm). The oven temperature was initially kept constant at 50 °C, for 5 min, and then raised at 20 °C min^{-1} for 2 min, to reach a final temperature of 90 °C. The temperature of the injection port and the detector were maintained constant at 150 °C. Helium was used as the carrier gas. Similarly, CO_2 was analyzed on an HP 5890 gas chromatograph, equipped with a TCD. The injection, oven, and detection temperatures were maintained at 90, 25, and 100 °C, respectively.

Fermentation products

Bioconversion products were detected with an HPLC (HP1100, Agilent Co., USA) equipped with a 5 μm × 4 mm × 250 mm Hypersil ODS column and a UV detector at a wavelength of 284 nm. The mobile phase was a 0.1% ortho-phosphoric acid solution fed at a flow rate of 0.5 mL min^{-1}. The column temperature was set at 30 °C. 1 mL liquid samples were centrifuged (7000× g, 3 min) using a centrifuge (ELMI Skyline ltd CM 70 M07) before analyzing the concentration of water-soluble products (acetic acid, butyric acid, hexanoic acid, ethanol, butanol and hexanol) by HPLC, at least once every 24 h.

Redox potential

The redox potential was constantly monitored in each bioreactor using an Ag/AgCl reference electrode connected to a transmitter (M300, Mettler Toledo, Inc., Bedford, MA, USA) and maintained inside the bioreactor.

RESULTS AND DISCUSSION

Continuous gas-fed bioreactor with natural acidification

Biomass growth and production of metabolites

In order to allow for a fast start-up and initial biomass growth, in this experiment the pH was maintained constant at a value of 5.75 during the first day. Afterwards, it was allowed to fluctuate freely. *Clostridium carboxidivorans* started growing immediately after inoculation, without any lag phase. The maximum biomass concentration was found after 236 h, reaching a value of 0.42 g L^{-1} (Fig 1(a)). However, as can be observed in Fig. 1(a), there was a clear difference between biomass growth at pH 4.75 compared with its growth during the first few hours at pH 5.75. At the highest pH, a biomass concentration of 0.177 g L^{-1} was already reached in only 27 h, while growth was much slower at the lower pH. The growth rates were calculated and compared under both conditions. At pH 5.75 (first 27 h), the bacterial growth rate was 0.072 h^{-1}, while its value dropped by more than a factor of ten, to 0.0057 h^{-1}, in the next stage at pH 4.75. This agrees with data reported on the optimal pH range for *Clostridium carboxidivorans* grown on syngas.[3,17,19] The growth rate found here at pH 5.75 is also close to the maximum value reported recently for that same strain grown under optimal conditions in batch assays on carbon monoxide ($\mu_{max} = 0.086\ h^{-1}$).[20] A pH value of 4.75 is very close to growth inhibitory conditions (unpublished data), which explains the observed low biomass build-up in this case. Growth is concomitant with acidogenesis in clostridia metabolizing C1 gases. Therefore, production of acids started soon after seeding the bioreactor. Acetic acid appeared immediately after inoculation, followed by butyric acid production a few hours later; whereas hexanoic acid was detected for the first time after 68 h. All three acids reached their maximum concentrations rather simultaneously, after 92 h, with the accumulation of 3.45 g L^{-1} acetic acid, 0.72 g L^{-1} butyric acid, and 0.18 g L^{-1} hexanoic acid (Fig. 1(b)). As a result of the fast production of organic acids, a pH value of 4.75 was reached only 48 h after reactor start-up, and only a few hours after allowing the pH to drop freely. Although sustained production of acids would result in further acidification, the pH was then maintained constant, as our own experience suggested that a low pH (<4.75) might inhibit the bacteria. Some authors concluded that for some bacteria, a pH of at least 4.7–4.8 is necessary to maximize alcohol production and avoid inhibition of bacterial growth.[21]

Medium acidification is generally considered to stimulate solventogenesis and the conversion of organic acids into alcohols.[3,7,10,22–25] After 100 h, at pH 4.75, both acetic and butyric acid concentrations started gradually decreasing. However, ethanol and butanol appeared after 44 h, when the concentration of acids was still increasing exponentially, suggesting that, at that time, the alcohols could already be produced from the gaseous substrates rather than from the acids and/or that both acid production (from the gaseous substrates) and conversion (to alcohols) was taking place. There was no clear drop in the concentration of hexanoic acid; but significant hexanol production started after 120 h. According to the Wood–Ljungdahl pathway summarized in Fig. 2 and based on the results shown in Fig. 1(a), higher alcohols such as hexanol could potentially be produced from gas fermentation and the bioconversion of acetyl-CoA to hexanoyl-CoA and, subsequently, to the corresponding alcohol. Maximum accumulation of alcohols was observed after 212 h, with 2.25 g L^{-1} ethanol, 1.43 g L^{-1} butanol and 0.72 g L^{-1} hexanol (Fig. 1(b)). Again, this suggests that some hexanol could presumably directly be produced from the gaseous substrates through acetyl-CoA, besides the potential conversion of the corresponding acid to the alcohol. The results can be compared with a previous study[26] in which the pH was initially maintained constant (pH 5.75) for a longer period than in this assay (with natural acidification here) and was only decreased later on, artificially, to pH 4.75. In the present case, with natural acidification, pH drops sooner; this results in somewhat lower maximum concentrations of acids (due to the low pH). Conversely, the production of alcohols starts earlier and higher maximum, final, concentrations of alcohols are obtained.

After 260 h, once alcohol production had stabilised, the pH was increased gradually and slowly to 5.75 over a period of 25 h. The goal was to check if increasing the pH would again stimulate the production of acids and help restart a cycle of acids production followed by their consumption and conversion to alcohols, as was recently shown to be feasible in another strain, *C. autoethanogenum*, grown on carbon monoxide and producing ethanol.[27,28] However, 4 days later all concentrations remained basically unchanged (Fig. 1(b)), suggesting some possible inhibitory effect impeding initiation of a new cycle. The accumulation of alcohols at relatively high concentrations has been reported to have toxic effects on clostridia grown on carbon monoxide.[20] However, at total alcohol concentrations not exceeding about 4 g L^{-1}, as in this experiment, inhibition by end metabolites should be minimal. It is unclear if the presence or absence of any specific compound could have hindered the start of a new cycle. In order to clarify this, after 407 h, part of the fermentation broth was replaced. Immediately after removing part of the old medium and introducing fresh culture broth, the bioconversion process started again and organic acids were produced. The biomass concentration decreased somewhat, down to a value of 0.36 g L^{-1}, because of the dilution effect due to medium replacement (Fig. 1(a)). The amount of acids and alcohols present in the medium decreased too, again as a result of the dilution effect, and their concentrations after partial medium renewal were 0.37 g L^{-1} acetic acid, 0.16 g L^{-1} butyric acid, 0.09 g L^{-1} hexanoic acid, 1.04 g L^{-1} ethanol, 0.82 g L^{-1} butanol and 0.34 g L^{-1} hexanol (Fig. 1(b)).

The pH of the medium was not artificially maintained constant after partial medium replacement, so that acidification and natural pH shift could take place anew. After 499 h, the biomass concentration increased again and reached a value of 0.40 g L^{-1}, which is near to the maximum value of the previous cycle (Fig. 1(a)). Although acids started accumulating immediately after medium replacement, their production was not high enough to allow the pH to drop down to 4.75 again. Therefore, a minimum pH value of 5.00 was reached after 647 h, but none of the acids nor the alcohols were consumed anymore. At the end of that new cycle, the maximum concentrations of acids were 2.2 g L^{-1} acetic acid, 0.61 g L^{-1} butyric acid and 0.32 g L^{-1} hexanoic acid. They remained roughly constant until the end of the experiment (Fig. 1(b)). After 499 h, when the pH started dropping, alcohols accumulated at a higher rate, whereas the production of acids leveled off. The following concentrations of alcohols were then detected at the end of the experiment: 2.03 g L^{-1} ethanol, 1.20 g L^{-1} butanol and 0.57 g L^{-1} hexanol.

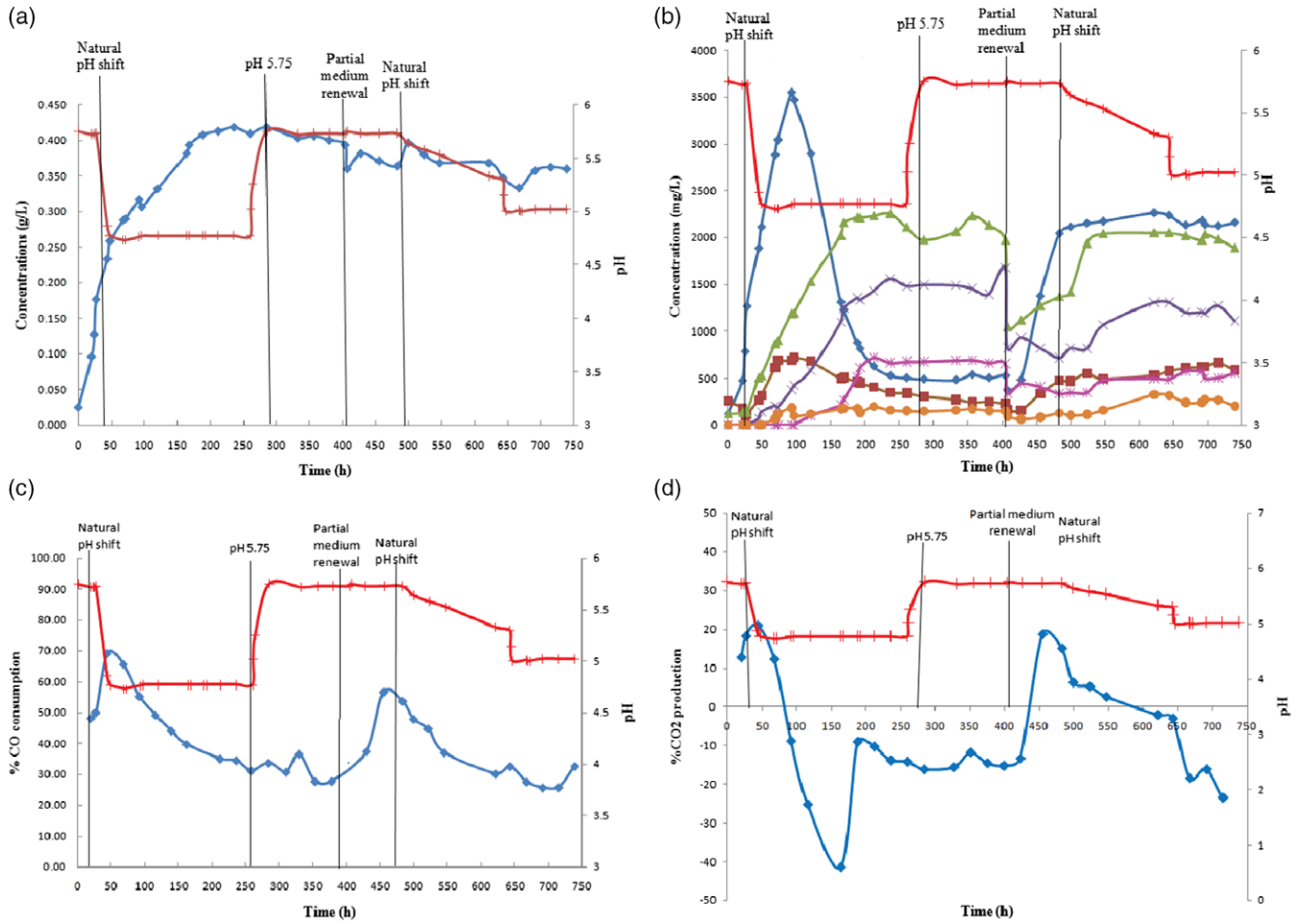

Figure 1. Continuous gas-fed bioreactor experiment with natural acidification: (a) measured growth expressed in g L^{-1} over time (♦) and pH values (+); (b) production of metabolites: acetic acid (♦), butyric acid (■), hexanoic acid (●), ethanol (▲), butanol (X), hexanol (*) expressed in mg L^{-1} over time, and pH values (+); (**c**) percentage of CO consumption over time (♦), and pH values (+); (**d**) percentage of CO_2 production over time (♦),and pH values (+).

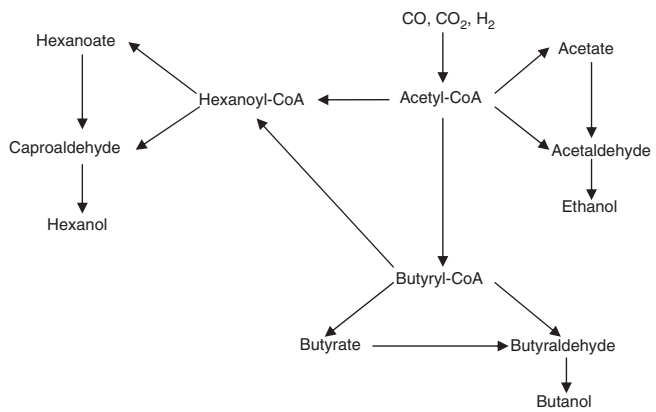

Figure 2. Wood–Ljungdahl pathway in H-B-E fermentation.

The total net amount of alcohols generated during the process was calculated, reaching 3.88 g ethanol (2.7 g in the first cycle and 1.18 g in the second cycle), 2.18 g butanol (1.72 g in the first cycle and 0.46 g in the second cycle) and 1.14 g hexanol (0.86 g in the first cycle and 0.28 g in the second cycle) in the 1.2 L bioreactor. The experimental production rates of alcohols were calculated as well (pH 4.75). In the case of ethanol, the production rate was 0.048 g h^{-1} g^{-1}-biomass between 27 h and 168 h. The butanol production rate was 0.037 g h^{-1} g^{-1}-biomass between 72 h and 168 h; and the hexanol production rate was 0.026 g h^{-1} g^{-1}-biomass between 168 h and 212 h.

The redox potential was monitored throughout the experimental process. In the first part of the experiment, after inoculation, when the pH was around 5.75, the redox potential was -100 ± 5 mV, whereas when the bacteria started to produce acids, at a pH around 4.75, the redox potential changed to -68 ± 2 mV. After partial medium replacement (pH 5.75 again) the redox potential was -85 ± 4 mV. Finally, at the end of the experiment (pH 5.00) the redox potential reached -40 ± 20 mV.

Consumption of gaseous C1-substrates

Consumption of the gaseous C1-substrates is hardly ever reported in studies on syngas or waste gas bioconversion to alcohols, while this is an important aspect, among others in the case of waste gas treatment as emission to the atmosphere of pollutants such as CO_2, a potent greenhouse gas, should be avoided. Similarly CO has clear indirect effects on climate change. CO consumption was monitored throughout the experiment (Fig. 1(c)). The maximum CO bioconversion was reached 44 h after inoculation, with a value of 69%, and in the second stage (i.e. after partial medium renewal) the maximum consumption was 56.5% after 455 h. During the rest

of the experiment CO conversion remained around 30–40%. It is interesting to observe that, after partial renewal of the fermentation broth, a jump in the levels of CO consumption was detected with sudden improvement of CO assimilation. This could be due to a possible presence of some inhibitory compound or to limitation of a specific essential nutrient in the fermented medium before introducing fresh medium. However, it is also worth noting that the period after partial medium replacement corresponds to an acidogenic stage with production of acids mainly, and that the experimental data suggest that acidogenesis would be related to higher consumption of the gaseous carbon substrates.

On the other hand, CO_2 was monitored too (Fig. 1(d)). Its fate is somewhat more difficult to elucidate, as carbon dioxide is not only a substrate but also a metabolite. It can be both consumed and produced, even simultaneously. However, reasonable explanations of the observed trends can be hypothesized. During the first few hours after bioreactor start-up, as well as just after partial medium replacement, higher CO_2 concentrations were detected at the outlet than at the inlet of the system, with net carbon dioxide production and 18–21% higher concentrations detected at the outlet port compared with the inlet one (Fig. 1(d)). During the rest of the experiment net CO_2 removal was observed. A short peak of maximum CO_2 production appeared at t = 50 h; it then started to drop to reach a minimum outlet carbon dioxide concentration after 160 h of bioreactor operation. The maximum consumption reached 40% of the amount of CO_2 present in the feed. During the remaining experimental period, until partial medium renewal, the average carbon dioxide bioconversion remained, on average, close to 15% of CO_2 consumption refered to the inlet concentration (negative values on Fig. 1(d)). Maximum CO bioconversion and maximum CO_2 removal (minimum CO_2 concentrations at the outlet of the fermentor) were detected simultaneously. The peaks of net carbon dioxide production observed occasionally, just after start-up (t ≈ 50 h) and after medium replacement (t ≈ 450 h) correspond to the exponential accumulation of organic acids (mainly acetic, as well as some butyric, acids). It is thus also simultaneous with biomass growth. This sounds logical, as the production of acids from substrates such as carbon monoxide, present in syngas or waste gases, leads to the simultaneous accumulation of carbon dioxide as suggested from the Wood–Ljungdahl pathway and as shown in Equation (1) for acetic acid and Equation (2) for butyric acid:[3]

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad (1)$$

$$10CO + 4H_2O \rightarrow CH_3\left(CH_2\right)_2COOH + 6CO_2 \quad (2)$$

Once the production of acids stops, no more carbon dioxide is generated, resulting in net consumption of that gaseous C1 substrate to produce ethanol, butanol or eventually hexanol as shown in Equations (3) to (5):[3]

$$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O \quad (3)$$

$$12H_2 + 4CO_2 \rightarrow C_4H_9OH + 7H_2O \quad (4)$$

$$18H_2 + 6CO_2 \rightarrow C_6H_{13}OH + 11H_2O \quad (5)$$

Moreover, typical reactions of bioconversion of organic acids to alcohols do not produce any carbon dioxide either, as can be seen in Equations (6), (7) and (8), for ethanol, butanol and hexanol, respectively:[3]

$$CH_3COOH + 2H_2 \rightarrow C_2H_5OH + H_2O \quad (6)$$

$$CH_3\left(CH_2\right)_2COOH + 2H_2 \rightarrow C_4H_9OH + H_2O \quad (7)$$

$$CH_3\left(CH_2\right)_4COOH + 2H_2 \rightarrow C_6H_{13}OH + H_2O \quad (8)$$

Continuous gas-fed bioreactor at constant pH

Biomass growth and production of metabolites

This experiment was started in a similar way to the previous one, except that a high pH value was maintained constant throughout the study in order to check how this would affect the production of acids and if their concentration might reach higher values, without any inhibition, eventually resulting in increased accumulation of alcohols. Again, the bacteria started growing immediately after inoculation, without any lag phase. The maximum biomass concentration was observed 91 h after inoculation (Fig. 3(a)). Growth data appearing in Fig. 3(a) confirm the trend already observed in the previous experiment in Fig. 1(a), with high growth rates (0.056 h^{-1}) and fast biomass production at high pH (5.75) (Fig. 1(a) and 3(a)), compared with slower kinetics at pH 4.75 (Fig. 1(a)). In the previous experiment, acidification and omission of pH regulation resulted in slow growth with maximum biomass concentration reached after 236 h, whereas the constant higher pH of 5.75 in this new experiment allowed the bacteria to grow faster, reaching a maximum bacterial concentration after 91 h.

The first acid produced in this experiment, under pH regulated conditions, was acetic acid followed by butyric acid and finally hexanoic acid, similarly to the previous experiment with natural acidification. The highest concentrations of acetic and butyric acids were found, in both cases, after 116 h, although acetic acid started being produced earlier than butyric acid. Those maximum values were 6.20 g L^{-1} for acetic acid and 1.40 g L^{-1} for butyric acid, whereas the maximum hexanoic acid concentration was 0.40 g L^{-1} and was reached later, after 258 h (Fig. 3(b)). Hexanoic acid production started only on the fourth day, after 100 h. Maintaining a constant high pH value of 5.75 in this experiment allowed higher total concentrations of acids than in the experiment with the pH naturally dropping down to a value of 4.75. This was also observed in another experiment in which CO was used as substrate rather than syngas.[26] The maximum amount of organic acids accumulating in the case of natural acidification was about half the amount obtained at a constant pH of 5.75 as it corresponded to 3.45 g L^{-1} acetic acid, 0.72 g L^{-1} butyric acid and 0.18 g L^{-1} hexanoic acid (Fig. 1(b)) at low pH (4.75) compared with 6.20 g L^{-1} acetic acid, 1.40 g L^{-1} butyric acid and 0.40 g L^{-1} hexanoic acid at this higher pH (5.75).

In the previous experiment, the production of alcohols took place as soon as acidification started, almost immediately after inoculation. This means that, at such low pH, there is coexistence of acidogenesis and solventogenesis in *C. carboxidivorans* and it indicates that, under such experimental conditions, both bioconversion processes can take place simultaneously. Conversely, in this new experiment at higher pH, the exponential production of ethanol appeared later, several hours after inoculation (around t = 96 h), while butanol and hexanol production started after 116 and 163 h, respectively. The maximum alcohol concentrations in this assay at constant pH were 2.7 g L^{-1} ethanol, 1.9 g L^{-1} butanol

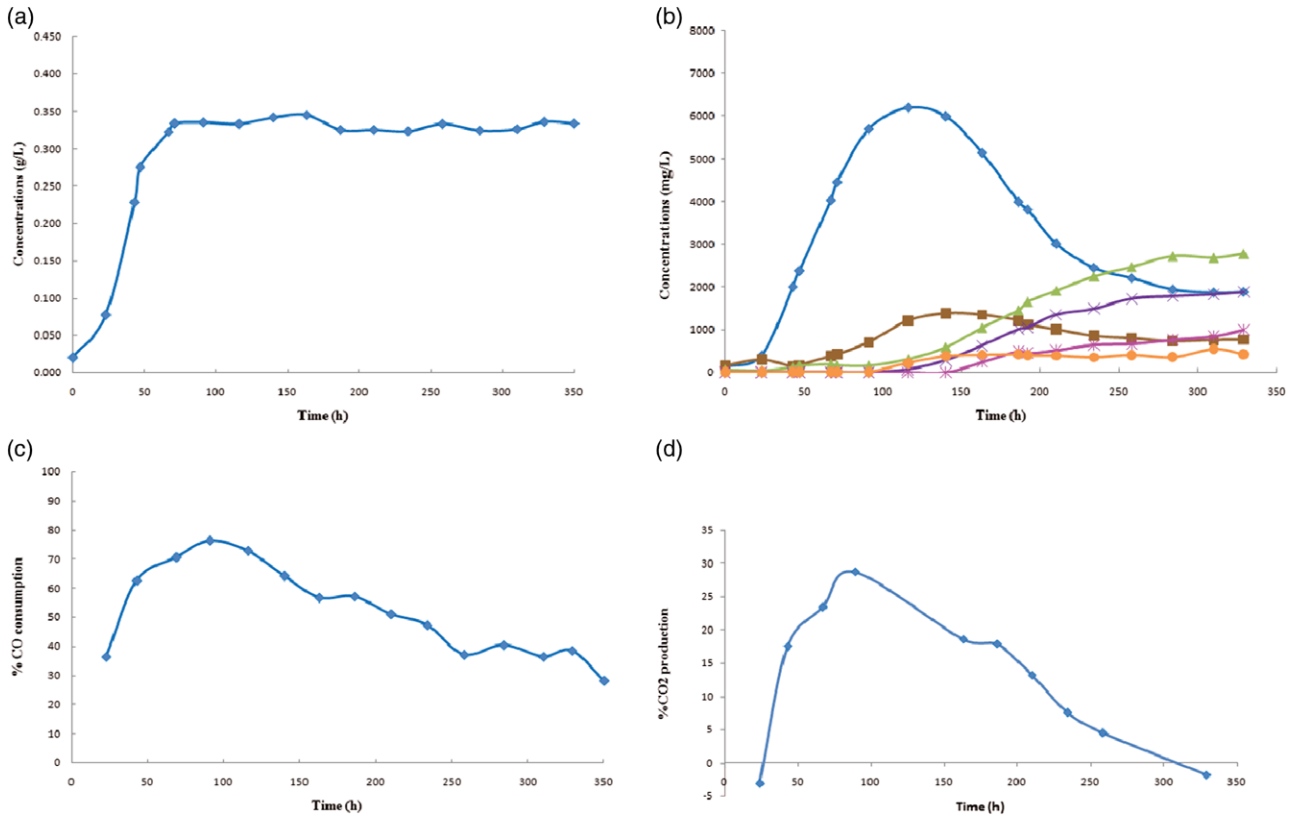

Figure 3. Continuous gas-fed bioreactor experiment at constant pH. (a) measured growth expressed in g L^{-1} over time (♦); (**b**) production of metabolites: acetic acid (♦), butyric acid (■), hexanoic acid (●), ethanol (▲), butanol (X), hexanol (*) expressed in mg L^{-1} over time; (**c**) percentage of CO consumption over time (♦); (**d**) percentage of CO_2 production over time (♦).

and 0.85 g L^{-1} hexanol, which were reached after 310 h (Fig. 3(b)). It is agreed that the gas (i.e. substrate) composition may have an effect on the bioconversion pattern. In the present study with syngas (CO, CO_2, H_2) a somewhat lower conversion of acids to alcohols was observed compared with results for pure CO as substrate, reported recently.[26] In both experiments described in the present paper, the pH remained constant during the solventogenic phase. Indeed, a low pH value of 4.75 was quickly reached in the first experiment, through natural acidification, and it was then maintained constant during solvent production in order to avoid any inhibitory effect. Similarly, in this new experiment the pH remained constant during solventogenesis but at a higher value of 5.75. In the latter experiment (pH 5.75), as already indicated above, the maximum concentrations of ethanol, butanol and hexanol were 2.7 g L^{-1}, 1.9 g L^{-1} and 0.85 g L^{-1}, respectively (Fig. 3(b)), which were higher values than in the experiment at pH 4.75, where the maximum concentrations of ethanol, butanol and hexanol were 2.25 g L^{-1}, 1.43 g L^{-1}, and 0.72 g L^{-1}, respectively (Fig. 1(b)). The higher concentrations of solvents at higher pH can thus be assumed to be related to the higher production of acids at constant high pH. When the production rates of each alcohol are analyzed, different patterns are observed in the two experiments, as summarized in Table 1. In all cases, the production rates of alcohols were higher in the first experiment than in the second one. This can be attributed to the fact that a lower pH simulates a more effective solventogenic phase and, consequently, the production rates of alcohols are higher.[7,25] A similar pattern was observed in a previous study in which pure CO was fermented, rather than syngas, by the same microbial strain and at two different pH values.[26] In that experiment ethanol and butanol production rates were higher at pH 4.75 than at pH 5.75.

Table 1. Comparison of the different production rates of alcohols in the experiment at high pH (5.75) and the experiment with natural medium acidification (4.75). The rates are expressed in g h^{-1} g^{-1}-biomass

	Ethanol production rate	Butanol production rate	Hexanol production rate
Experiment 1 (pH 4.75)	0.048	0.037	0.026
Experiment 2 (pH 5.75)	0.044	0.035	0.014

In the present experiment, at constant high pH, the following total net productions of alcohols were found: ethanol, 3.24 g; butanol, 2.28 g; and hexanol, 1.02 g; in a 1.2 L bioreactor. The production rates, at pH 5.75, were calculated for each alcohol. The ethanol production rate reached 0.044 g h^{-1} g^{-1}-biomass between 116 h and 284 h; the butanol production rate reached 0.035 g h^{-1} g^{-1}-biomass over that same period; and the hexanol production rate was 0.014 g h^{-1} g^{-1}-biomass between 163 h and 330 h. Besides, the total net production of butanol and ethanol can also be compared between the CO fermentation study reported previously[26] and this syngas fermentation at pH 4.75, corresponding to the first experiment described in the previous section. In the CO fermentation process, the total net production of ethanol and butanol was 4.21 g and 2.29 g, respectively,[26] whereas 3.88 g ethanol and 2.18 g butanol were obtained at the end of this

syngas fermentation process. Thus both values are quite similar. It is worth mentioning that in our previous recent study on CO fermentation,[26] the presence of hexanol was not reported, as it had not been measured at that time, although subsequent analysis revealed that hexanol was actually also produced, in similar amounts to that in syngas fermentation. The redox potential was also measured throughout the study. When the pH was maintained at 5.75 the redox potential remained stable at -85 ± 3 mV.

Consumption of gaseous C1-substrates

Concerning CO consumption in this second experiment, at constant pH, the results can be seen in Fig. 3(c). When the pH was maintained at 5.75, such higher pH seemed to favour higher levels of CO assimilation. The highest percentage CO consumption was observed during the first stages of the experiment reaching a value of 76% after 91 h, which is higher than the maximum value found in the previous experiment with acidification of the culture medium. During the rest of the process, mainly during the solventogenic stage, CO consumption gradually decayed, reaching a minimum value of 38% at the end of the experiment. On average, CO consumption levels were also somewhat higher in this experiment at constant high pH (5.75) compared with the previous experiment with culture medium acidification down to pH 4.75.

A similar behaviour is observed in this experiment to that in the previous one, leading to similar explanations and confirming the conclusions hypothesized in the first fermentation run. High carbon monoxide consumption levels are observed initially, after start-up. This leads to fast biomass growth as well as exponential production of organic acids, mainly acetic acid. As explained earlier and as shown in Equations (1) and (2), the accumulation of acids leads to the simultaneous net production of carbon dioxide, reaching its highest peak during the early stages of the fermentation process (Fig. 3(d)). This is also related to the Wood–Ljungdahl (WL) pathway in which CO can be converted into CO_2. Both C1 gases can later on also be metabolized through the WL pathway to generate acetyl-CoA which is an intermediate metabolite in the subsequent bioconversion to organic acids and/or alcohols (Fig. 2). After 100 h, the concentration of carbon dioxide decays and, at the same time, organic acids are metabolized and converted to alcohols (Equations (6) to (8)), with carbon dioxide consumption. Once the fermentation of organic acids stops, net CO_2 consumption is observed (Fig. 3(d)).

CONCLUSIONS

It can be concluded that: (a) a high pH (5.75) value was more favorable to syngas/waste gas bioconversion to organic acids than a low pH (4.75), thus reaching a higher transient accumulation of such acids; (b) faster production rates of alcohols were observed at lower pH (favourable to solventogenesis), while a higher total amount of alcohols accumulated at higher pH as a result of the presence of higher transient concentrations of organic acids; (c) organic acids and alcohols are produced in the following order in all cases: C2 > C4 > C6; (d) although it stimulates solventogenesis, a lower pH has a negative effect on bacterial growth, reaching near inhibition below pH 4.75; and (e) H–B–E fermentation allows net removal of volatile pollutants having a greenhouse effect, such as CO_2 or CO, while producing ethanol and higher alcohols.

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Glucose bioconversion profile in the syngas-metabolizing species *Clostridium carboxidivorans*

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ABSTRACT

Some clostridia produce alcohols (ethanol, butanol, hexanol) from gases (CO, CO_2, H_2) and others from carbohydrates (*e.g.*, glucose). *C. carboxidivorans* can metabolize both gases as well as glucose. However, its bioconversion profile on glucose had not been reported. It was observed that *C. carboxidivorans* does not follow a typical solventogenic stage when grown on glucose. Indeed, at pH 6.2, it produced first a broad range of acids (acetic, butyric, hexanoic, formic, and lactic acids), several of which are generally not found, under similar conditions, during gas fermentation. Medium acidification did not allow the conversion of fatty acids into solvents. Production of some alcohols from glucose was observed in *C. carboxidivorans* but at high pH rather than under acidic conditions, and the total concentration of those solvents was low. At high pH, formic acid was produced first and later converted to acetic acid, but organic acids were not metabolized at low pH.

1. Introduction

Many fuels and platform chemicals are, nowadays, still largely being obtained from crude oil as starting material in conventional refineries. Because of the instability of oil prices, the environmental impact and the increasing scarcity of crude oil, scientists and industries are being forced to look for new alternative feedstocks to produce metabolites of interest in a more sustainable way in biorefineries (Gowen and Fong, 2011; Abdehagh et al., 2014; Liu et al., 2016). Some examples of such metabolites are acetone, methanol, ethanol, butanol and hexanol, to cite just few. The latter compounds can be produced from renewable materials such as lignocellulosic biomass, but also from municipal or agricultural wastes. They can be used to obtain either simple fermentable sugars or, otherwise, syngas, which can be fermented by clostridia into biofuels and other bioproducts.

Lignocellulosic materials are composed of cellulose (35–50%), hemicellulose (20–35%), and lignin (15–20%) (Anwar et al., 2014). Cellulose is mainly a glucose polymer, while hemicellulose is largely composed of hexoses and pentoses, *e.g.* glucose, xylose, arabinose, mannose, galactose (Karimi et al., 2006; Saha, 2003). Lignin does unfortunately not yield any sugars. Pretreatments are necessary in order to extract carbohydrates from the cellulose and hemicellulose polymers; those carbohydrates can then be fermented by different microorganisms (Hendriks and Zeeman, 2009). The most studied organism is *Clostridium acetobutylicum*. There are two major different types of pretreatments of lignocellulosic materials, enzymatic hydrolysis and chemical hydrolysis. The first one is largely used due to the low production of by-products which could inhibit the fermentation process compared to chemical hydrolysis (Qureshi and Manderson, 1995; Rabinovich et al., 2002; Jönsson et al., 2013). Several studies have focussed on ABE fermentation, which is the bioconversion of carbohydrates by clostridia in order to obtain a mixture of acetone, butanol and ethanol (Jones and Woods, 1986). All three metabolites are not necessarily found, depending on the strain.

On the other side, the above mentioned feedstocks can be gasified. This will yield a volatile product called synthetic gas or syngas (Kennes et al., 2016). In case of lignocellulosic materials, all the polymeric structure, *i.e.* cellulose, hemicellulose, but also lignin, can be gasified and converted to syngas, resulting in a better, full, use of the starting material. This gas mixture can then also be fermented by clostridia. In case of species such as *C. autoethanogenum* or *C. ljungdahlii*, the main products which are formed are acetic acid first and then ethanol (Abubackar et al., 2011; van Groenestijn et al., 2013). In other species, such as *Clostridium carboxidivorans*, a mixture of organic acids appears initially (acetic, butyric, hexanoic acids), followed by the accumulation of the corresponding C2, C4, and C6 alcohols (ethanol, butanol, hexanol), in the novel HBE fermentation process (Fernández-Naveira et al., 2017a,b). It is a two-steps process characterized by exponential bacterial growth first and the production of different organic acids as major products. When the conditions become favourable, then the second step

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takes place, in which the acids are converted into ethanol and higher alcohols by the same bacteria (Fernández-Naveira et al., 2017b; Phillips et al., 2015). Alternatively, the accumulation of fatty acids rather than solvents could be stimulated, with further bioconversion of such acids into other products by different microorganisms in a two-stage process (Lagoa-Costa et al., 2017).

Since *C. carboxidivorans* is able to ferment gases and produce acids and then alcohols (HBE fermentation); in the present study, the main goal was to check if that species would also be able to use sugars, such as glucose, as carbon source while following a similar bioconversion profile as in the ABE fermentation, or otherwise what would be its metabolic profile with sugars compared to the HBE and the ABE fermentation processes in clostridia.

The experiments were carried out in three bioreactors in order to elucidate the metabolic profile of glucose fermentation by *C. carboxidivorans*. Besides, another major objective of this research was to study the effect of different pH and operating conditions in order to try to improve the production of alcohols or solvents. Three bioreactors were run with different operational conditions, the first one with constant pH, the second one without pH regulation and the last one with a change of pH at the end of the acidogenic stage; the idea being to find how the operational conditions affect sugar fermentation in *C. carboxidivorans* and to check to what extent the production of solvents from carbohydrates is possible and if it can be optimized.

2. Material and methods

2.1. Microorganism and culture media

C. carboxidivorans DSM 15243 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was maintained anaerobically on modified basal medium (Liou et al., 2005; Tanner, 2007) at pH 5.75 with glucose as the sole carbon source. The basal medium was composed of (per liter distilled water): 30 g glucose; 1 g yeast extract; 25 mL mineral solution; 10 mL trace metal solution; 10 mL of vitamins stock solution; 1 mL resazurin; 0.60 g cysteine-HCl.

The composition of the mineral stock solution was (per liter) 80 g sodium chloride, 100 g ammonium chloride, 10 g potassium chloride, 10 g potassium monophosphate, 20 g magnesium sulphate, and 4 g calcium chloride.

The composition of vitamin stock solution was (per liter) 10 mg pyridoxine, 5 mg each of thiamine, riboflavin, calcium pantothenate, thioctic acid, paraamino benzoic acid, nicotinic acid, and vitamin B12, and 2 mg each of d-biotin, folic acid, and 2-mercaptoethanesulphonic acid.

The composition of trace metal stock solution was (per liter) 2 g nitrilotriacetic acid, 1 g manganese sulphate, 0.80 g ferrous ammonium sulphate, 0.20 g cobalt chloride, 0.20 g zinc sulphate, and 20 mg each of cupric chloride, nickel chloride, sodium molybdate, sodium selenate, and sodium tungstate.

2.2. Continuous bioreactor experiments

Three bioreactor experiments were carried out in 2 L BIOFLO 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA). The final working volume in the three experiments was 1.2 L; using in all cases the same medium composition and glucose concentrations.

The medium with all the compounds, except glucose, vitamins and cysteine-HCl, was introduced in the bioreactor and autoclaved at 120 °C for 20 min. Once autoclaving finalized, the bioreactor was kept under an extraction hood where it was flushed with N_2 in order to ensure anaerobic conditions. The vitamins solution, glucose stock solution and cysteine-HCl were added when the bioreactor reached a temperature below 40 °C. Once the bioreactor medium was completely anaerobic, N_2 flushing was stopped. Then, 10% seed culture, in the early exponential growth phase (through previously growing the strain for 72 h with glucose) was inoculated in the bioreactor. The experimental conditions of the three bioreactors were the same, with the temperature of the medium maintained at 33 °C and a constant agitation speed of 250 rpm. Inside the bioreactor, four baffles avoided vortex formation and allowed thus to improve liquid mixing. The pH was automatically maintained constant through the addition of either 2M NaOH or 2M HCl solutions, by means of a peristaltic pump.

In the first experiment, the pH was maintained constant at 6.20 throughout the study. In the second experiment, the pH was maintained constant at 6.20 during the first 52 h, but afterwards automatic pH regulation was stopped. That way natural acidification took place, as a result of the production of acids, reaching a pH value of 5.20, which was then maintained constant. In the third experiment, the pH was maintained constant at 6.20 during the first hours, and when the maximum concentration of acids was reached, the pH regulation was changed to 5.20 adding HCl 2M in a gradual way.

2.3. Growth measurement

The optical density ($OD_{\lambda=600nm}$) was measured daily on a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain) removing 1 mL liquid samples from the bioreactors. The biomass concentration (g/L) was estimated using a previously generated calibration curve.

2.4. Fermentation products

At least every 24 h, 1 mL liquid sample was taken from each bioreactor and then centrifuged (7000*g*, 3 min) using a bench-centrifuge (ELMI Skyline ltd CM 70M07). The samples were then filtered through a filter with a pore size of 0.22 μm. They were then analyzed on an HPLC (HP1100, Agilent Co., USA) in order to determinate the concentrations of acids and alcohols present in liquid phase in the bioreactors. The HPLC was equipped with a 5 μm × 4 mm × 250 mm Hypersil ODS column and a UV detector at a wavelength of 284 nm. The mobile phase was a 0.1% ortho-phosphoric acid solution fed at a flow rate of 0.5 mL/min.

2.5. Redox potential

The redox potential was constantly monitored in each bioreactor using an Ag/AgCl reference electrode connected to a transmitter (M300, Mettler Toledo, Inc., Bedford, MA, USA) and maintained inside the bioreactor.

2.6. 16S rDNA analysis of bioreactor cells

Samples from the different bioreactors were taken during the experiments as well as at the end of each assay and were analyzed using 16S rDNA gene sequencing in order to confirm the stability and purity of the inoculated strain in each experiment. The DNA extraction procedure was performed as per the manufacturer's protocol using E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek). After the DNA extraction, the quality of the DNA samples was analyzed using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE). A sequence of 1500 bp was amplified to obtain the 16S rDNA using a PCR. To obtain the complete sequence, a genetic analyzer 313XL Apply Biosystem was used using the Sanger method, in order to obtain different fragments of the complete sequence. Later, these sequence fragments were used in the program CLUSTAL W, in order to determinate the query sequence of the 16S rDNA of each sample. The query sequence allows to identify the microbial strain with help of the BLAST (Basic Local Alignment Search Tool) program, using the database 16S ribosomal RNA sequences (Bacteria and Archaea).

3. Results and discussion

3.1. Glucose bioreactor at constant pH

A common characteristic of some *Clostridium spp.* is their ability to produce both organic acids and alcohols or other solvents (*e.g.* acetone), either through the ABE fermentation with carbohydrates as carbon sources or through the HBE fermentation with gaseous substrates. Those are two-step bioconversion processes in which organic acids are produced first (acidogenesis), at near-neutral or slightly acidic pH, together with biomass growth; followed by a second step consisting in the production of solvents (mainly alcohols, acetone) (solventogenesis) after acidification of the medium as a result of the production and subsequent bioconversion of acids into those solvents. Organisms such as *Clostridium acetobutylicum* convert sugars into volatile fatty acids and produce later acetone, butanol and ethanol (ABE fermentation). In a similar way, *C. carboxidivorans* is a bacterial species able to perform the HBE fermentation using substrates such as CO (Fernández-Naveira et al., 2016) or syngas (CO, CO_2, H_2) (Fernández-Naveira et al., 2017c; Phillips et al., 2015) following the above described two-step bioconversion process. Previous recent studies (Fernández-Naveira et al., 2017c) have shown that both acidogenesis and solventogenesis can take place at slightly acidic pH (around pH 6), before any stronger acidification. Experiments were performed here under different pH conditions, in order to check the bioconversion characteristics of *C. carboxidivorans* and its potential to produce acids and alcohols from glucose, in a similar way as in the ABE fermentation in other clostridia. A first experiment was performed at a constant, slightly acidic, pH of 6.20 and the results were compared to the ABE and HBE fermentation profiles.

In this first experiment at constant pH, after bioreactor inoculation, *C. carboxidivorans* started growing and entered the exponential growth phase quite soon (Fig. 1A). A maximum biomass concentration of 0.502 g/L was reached 71 h after inoculation, with its bacterial growth rate reaching 0.080 h^{-1} in the log phase. This value is very similar to the ones found previously when growing that strain either on CO (Fernández-Naveira et al., 2016) or on syngas (Fernández-Naveira et al., 2017c) at a similar pH (pH 5.75).

3.1.1. Glucose bioconversion profile

The exponential consumption of glucose started about 24 h after inoculation and ended approximately 80 h after starting the experiment, concomitant with the log-growth; followed by a lower residual substrate consumption up to about 100 h. At the end of the assay the total net glucose consumption was 26.73 g/L, which corresponds to an overall substrate assimilation of 81%. However, most of it was consumed during the exponential growth phase, corresponding to 78% of that overall consumption (Fig. 1B).

Contrary to the HBE fermentation profile of *C. carboxidivorans* in which a limited number of organic acids (acetic, butyric and hexanoic acids, mainly) and alcohols (ethanol, butanol, hexanol) are produced as sole metabolites, in the present case with glucose as carbon source, a wider range of acids was found in the culture medium, at non-negligible concentrations. Similarly, in the ABE fermentation, with species such as *C. acetobutylicum*, acetic acid and butyric acid are the only major acids produced (Millat and Winzer, 2017). In glucose bioconversion by *C. carboxidivorans*, the production of acids started simultaneously to sugar consumption. Acetic acid and formic acid were the first metabolites appearing in the fermentation broth, which is different from the bioconversion of CO or syngas by that organism, in which acetic acid is also the first metabolite to be detected but no formic acid is found. Butyric acid and lactic acid appeared a few hours later and their concentrations increased quite fast. Almost at the same time, isobutyric acid and propionic acid were found as well but in minor concentrations. Hexanoic acid started being produced still later, 71 h after bioreactor start-up (Fig. 1C). This is another difference with the HBE gas-fermentation in *C. carboxidivorans*, in which hexanoic acid is the third most

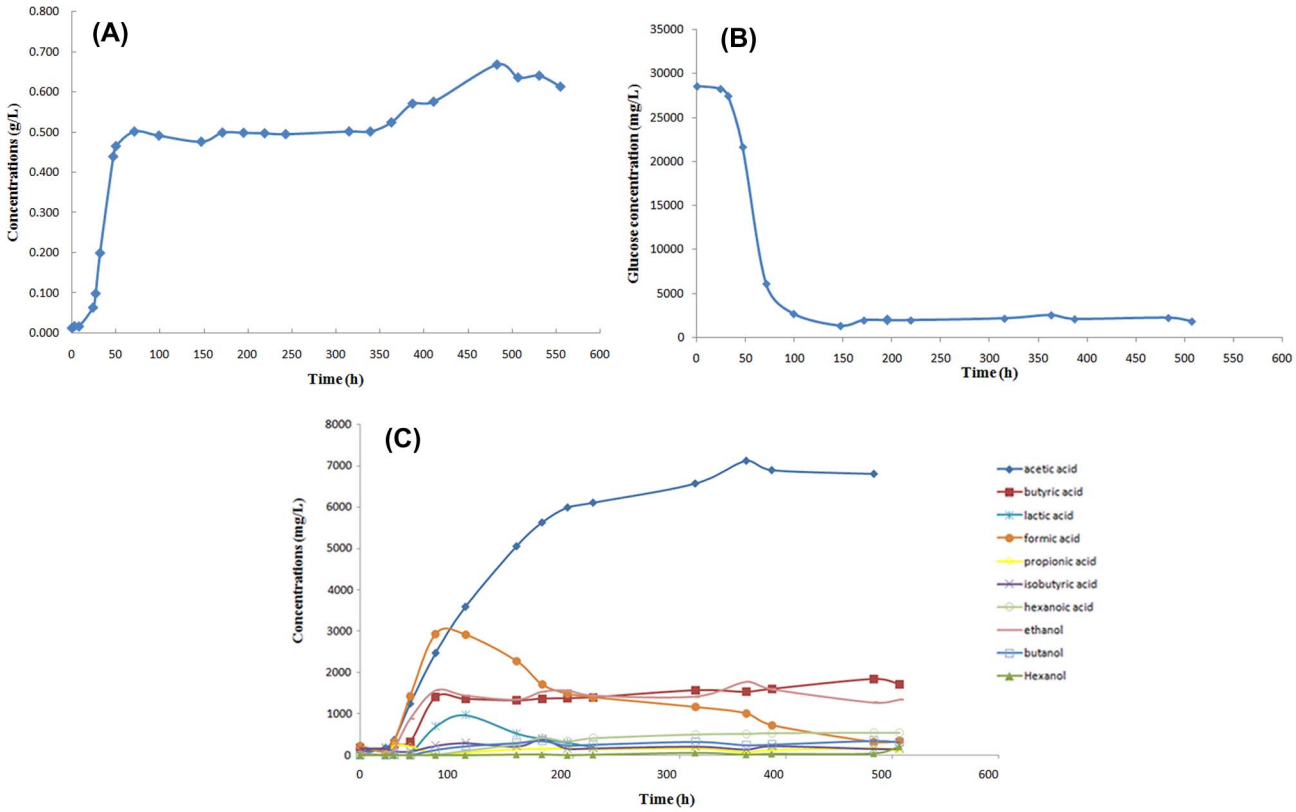

Fig. 1. Glucose bioreactor at constant pH. **(A)**: measured growth expressed in g/L over time. **(B)**: glucose consumption over time with concentrations expressed in g/L. **(C)**: product formation over time (acetic acid, butyric acid, isobutyric acid, propionic acid, lactic acid, formic acid, hexanoic acid, ethanol, butanol and hexanol).

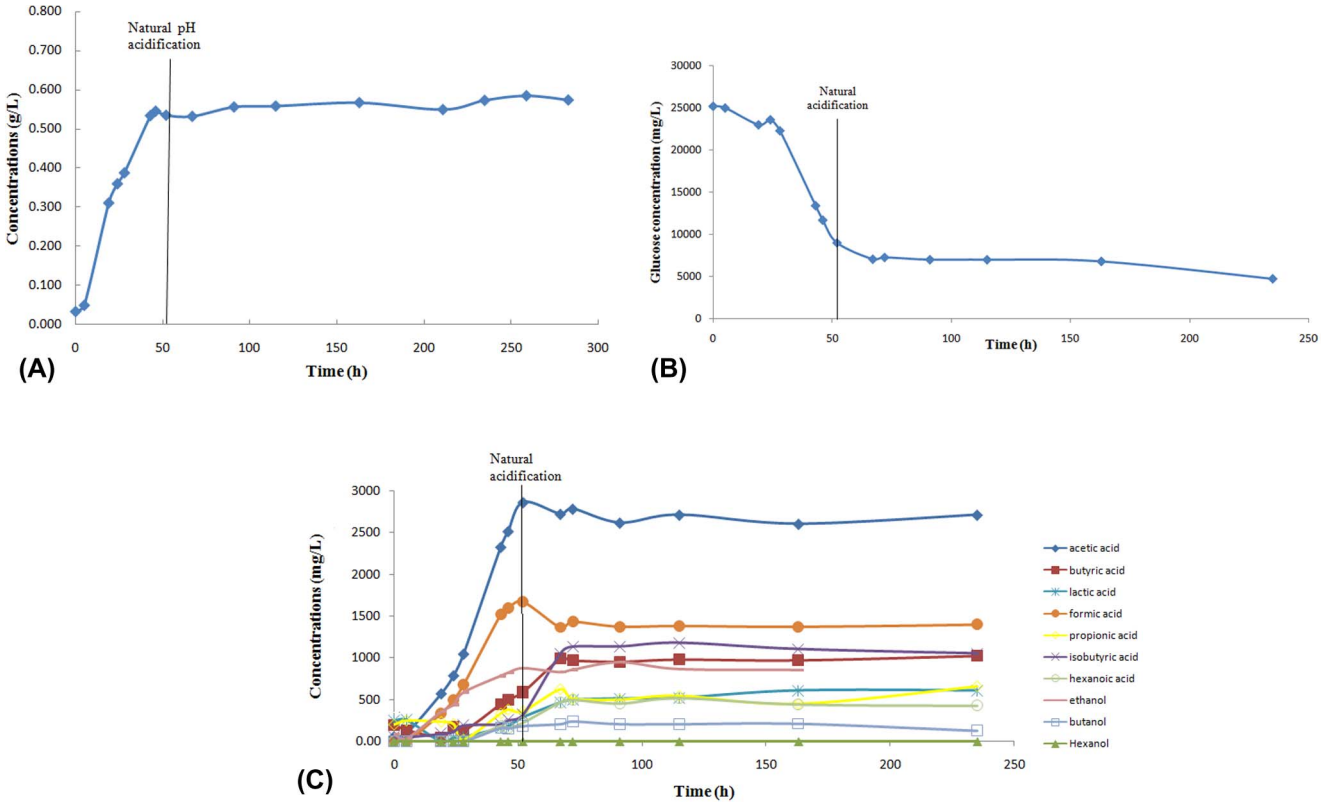

Fig. 2. Glucose bioreactor with natural acidification. **(A)**: measured growth expressed in g/L over time. **(B)**: glucose consumption over time with concentrations expressed in g/L. **(C)**: product formation over time (acetic acid, butyric acid, isobutyric acid, propionic acid, lactic acid, formic acid, hexanoic acid, ethanol, butanol and hexanol).

abundant acid after acetic and butyric acids with basically no other acids detected. The presence of formic acid in the culture medium has never been reported in the gas fermentation process, but it is the second most abundant acid in this glucose fermentation. Lactic acid and formic acid reached their maximum concentrations of 0.97 g/L and 2.92 g/L, respectively, after 99 h already. After 180 h, the maximum concentrations of isobutyric acid and propionic acid were found, with values of 0.38 g/L and 0.17 g/L, respectively. Interestingly, the accumulation of some acids, mainly acetic acid went on for a quite long period of time still after glucose consumption had stopped (after approximately 80–100 h, as explained earlier). The maximum concentration of acetic acid was reached after 363 h, with a value of 7.2 g/L, which is almost twice the concentration reached at the end of glucose consumption, suggesting that acetic acid could be produced from another compound than glucose. The concentration of formic acid started decreasing once glucose consumption leveled off, while the acetic acid concentration kept increasing. Overall, 2.60 g/L of the produced formic acid was consumed, leaving only 0.34 g/L in the medium by the end of the experiment. Therefore, a relationship between formic acid elimination and further acetic acid accumulation can reasonably be hypothesized, as will be discussed in next experiments. Maximum butyric acid and hexanoic acid concentrations of 1.85 g/L and 0.54 g/L, respectively, were reached after 483 h (Fig. 1C). That way the main acids present in the fermentation broth at the end of the experiment were: acetic acid > butyric acid > lactic acid > hexanoic acid > isobutyric acid > formic acid > propionic acid.

In terms of solvents, despite working at a relatively high pH (6.20), ethanol started being produced soon, 24–32 h after start-up. Butanol was detected somewhat later but after 47 h already, when the concentration of acids was still increasing. A similar behavior was reported in the HBE fermentation with *C. carboxidivorans* and CO or syngas as substrates, at pH 5.75 (Fernández-Naveira et al., 2016, 2017c). With all three substrates (CO, syngas, glucose), ethanol appears soon during the acidification stage, and butanol is always detected around the 44–50 h after inoculation with the three different carbon sources. The production of hexanol started later, after 150 h. The maximum concentrations of alcohols were 1.78 g/L ethanol, 0.33 g/L butanol and 0.06 g/L hexanol (Fig. 1C), but contrary to the ABE fermentation with glucose or the HBE fermentation with gases, here most of the alcohols appeared during the consumption of the original substrate (glucose) (first 3–4 days) and no clear correlation could be found between alcohol production and any possible consumption of organic fatty acids. Although simultaneous production and consumption of acetic acid could have taken place, its net concentration did never decrease, suggesting that alcohols such as ethanol and butanol could have been produced directly from glucose rather than from the organic acids. No acetone was found with *C. carboxidivorans*, although this is a common metabolite in ABE fermentation with other clostridia. It was recently shown that organism lacks genes for acetone production (Bruant et al., 2010; Fernández-Naveira et al., 2017b).

The maximum ethanol production rate was much higher than for other alcohols, reaching 0.072 g/h*g biomass, between 32 h and 71 h. The butanol production rate was 0.008 g/h*g biomass, between 47 h and 147 h; and the hexanol production rate was only 0.001 g/h*g biomass, between 219 and 315 h.

The RedOx potential was monitored. On starting-up the bioreactor and during the first hours, organic acids were largely produced and automatic addition of sodium hydroxide was required in order to maintain a constant pH of 6.20. The RedOx potential was then −145.0 ± 5 mV. Interestingly, when the bioreactor started to consume HCl, the RedOx decreased to values of −320.0 ± 5 mV.

3.2. Glucose bioreactor with natural acidification

High amounts of acids were produced in the previous experiment at a high, constant, pH of 6.20. In *C. carboxidivorans*, alcohols have been

found to be produced (solventogenesis) both at high (*e.g.*, 6 or above) as well as lower pH (*e.g.*, 5 or below), during HBE fermentation (Fernández-Naveira et al., 2017c). However, in ABE fermentation with glucose as substrate, solventogenesis has frequently been reported to be stimulated during medium acidification, after the production of acids (Schiel-Bengelsdorf et al., 2013; Fernández-Naveira et al., 2017b). Therefore, because of the absence of significant amounts of solvents in the first experiment at a constant pH of 6.20, compared to the HBE fermentation, a new assay was set-up with natural acidification, in order to check if this would change the metabolic profile of *C. carboxidivorans* and if it could stimulate solventogenesis.

The pH value was initially maintained constant at 6.20 for the first two days to ensure good bacterial growth. After 52 h, natural acidification was allowed to take place in order to check its possible stimulating effect on solventogenesis. Similarly as in the previous experiment and since the bioreactor start-up conditions were the same, *C. carboxidivorans* started growing without any lag phase, reaching a maximum biomass concentration of 0.546 g/L 46 h after inoculation (Fig. 2A). The bacterial growth rate was 0.087 h^{-1} measured during the exponential growth phase, up to 30 h. This value is similar as in the previous assay; with a slight difference which can be explained by the limited number of data points available for calculations. pH was allowed to drop naturally just after the maximum amount biomass was reached; that way the final maximum biomass concentration and the biomass growth rate were not affected by the possible inhibitory effect of the pH decrease.

3.2.1. Glucose bioconversion profile

Exponential consumption of glucose started soon after inoculation, but its consumption rate decreased dramatically once natural acidification took place, after 52 h. No more substrate was used at all 67 h after inoculation, at low pH. The total net glucose consumption reached 20.46 g/L by the end of the experiment which corresponds to a total sugar consumption of 82%, but with the major part of it (72% of the total consumption) being assimilated during the first 67 h (Fig. 2B). The near instantaneous inhibition of glucose assimilation once pH dropped to 5.20, suggests that it cannot be metabolized at such a low pH. The first acids detected in this experiment were acetic and formic acids, followed by butyric acid, lactic acid, isobutyric acid and propionic acid and finally hexanoic acid (Fig. 2C), similarly as in the previous experiment. This is because the acids appeared during the first 43 h, before natural acidification, when the conditions are the same as in the previous experiment. 52 h after inoculation the pH was allowed to drop down to 5.20 in a natural way as a result of the bacterial production of acids. Only 15 h were necessary to see the pH decrease to 5.20.

In this case, the maximum concentrations of acetic and formic acids were found after 52 h, reaching respectively 2.86 g/L and 1.68 g/L. After 67 h, the production of butyric acid, isobutyric acid and propionic acid stopped, with maximum concentrations of 1.00 g/L, 1.05 g/L and 0.63 g/L, respectively. Finally, hexanoic acid and lactic acid reached their maximum concentrations after 72 h and 163 h, respectively, with values of 0.52 g/L and 0.61 g/L (Fig. 2C). Suitable pH values for *C. carboxidivorans* have been reported to range from 4.4 to 7.6, with optimal conditions between 5.0 and 7.0 (Liou et al., 2005); however, in this experiment, the production of any metabolites did significantly slowdown after natural acidification down to pH 5.20. Although acidification could have been expected to stimulate solventogenesis, the opposite occurred. There was no consumption of acids at all, at pH 5.20, and no production of solvents. Even formic acid consumption was inhibited at such low pH, while it had almost completely been used up at high pH, in the previous assay. That way the main acids present in the fermentation broth at the end of this experiment were: acetic acid > formic acid > butyric acid = isobutyric acid > propionic acid > lactic acid > hexanoic acid. The proportions of acids are then also different from the previous assay. Since formic acid was not metabolized, its final concentration was higher than that of butyric acid at the end of the experiment. In the previous assay, the maximum concentration of formic acid, after 99 h, was also higher than for butyric acid, but it was then used up at constant high pH. Therefore, at the end of the experiment there was only a low, residual, concentration of formic acid when maintaining a high pH of 6.20, while it remained present at its maximum concentration when acidification takes place.

Concerning the concentrations of solvents, ethanol was the first alcohol appearing in the fermentation broth. The presence of some ethanol was already detected soon after bioreactor inoculation and its concentration continued immediately increasing, meaning that this solvent appeared during the high pH period while it was not produced anymore after natural acidification. The maximum ethanol concentration was reached after 52 h with a value of 0.88 g/L. There was some delay before butanol was produced and it first appeared 43 h after inoculation. It was mainly produced at high pH (close to 90% of its final concentration), although its maximum concentration was reached after 72 h, slightly after the pH decreased, with a value of 0.24 g/L. No hexanol was found in this experiment. Thus, the solvents, ethanol and butanol, appeared before any pH drop, when acids were still produced by the bacteria, following a similar pattern as in the previous experiment. This is different from the HBE fermentation with *C. carboxidivorans* (Fernández-Naveira et al., 2016, 2017a,b), as the strain grown on CO or syngas is still active at pH 5.20 and even below pH 5, and it produces solvents at somewhat increased rates at such low pH values. Solventogenesis is stimulated under acidic conditions in HBE fermentation with gaseous substrates while it is not, in the present study, when the strain is grown on glucose.

The alcohols production rates were calculated at pH 6.20. The ethanol and butanol production rates were 0.039 g-ethanol/h*g biomass (between 5 h and 52 h) and 0.007 g-butanol/h*g biomass (between 28 h and 52 h). The rates were not calculated at low pH as no significant production of alcohols was observed. In this case, the production rate of ethanol was lower than in the previous experiment. The reason was the inhibition caused by the pH drop on the production of alcohols. However, the production rate for butanol was similar in both experiments.

In assays using gases as substrates, CO or syngas, the highest production rates of alcohols were observed at low pH, during the solventogenic stage, whereas, in the present experiment, with glucose as a carbon source, the production of alcohols at low pH was not significant; that way the highest production rate was obtained at higher pH.

The RedOx potential was also monitored. When the pH was maintained at 6.20 (first 52 h), the RedOx potential was –150 ± 10 mV, as in the first experiment. However, when the pH dropped to 5.20, the RedOx potential was higher, with a value of –90 ± 10 mV. The RedOx potential is known to be affected by the pH value and to become less negative at lower pH.

3.3. Glucose bioreactor with artificial pH change

It is known and accepted, from ABE and HBE fermentations, that maintaining a high pH, optimal for acidogenesis, for a longer period of time, will increase the production of acids during that first stage; which will, afterwards, stimulate solventogenesis and increase the amount of solvents produced during the second stage, as a result of the higher concentrations and availability of fermentable organic acids. In order to check such possible behavior in the case of glucose bioconversion by *C. carboxidivorans*, in this experiment, a high pH was maintained constant for several days in order to produce higher amounts of organic acids, before artificially decreasing the pH and check if this would more efficiently stimulate solventogenesis and result in the accumulation of higher amounts of alcohols. Therefore, the pH was maintained constant until the maximum concentrations of acids were reached (211 h). Thereafter, the pH was decreased to 5.20, through the addition of HCl 2M. *C. carboxidivorans* started growing immediately after inoculation, without any lag phase (Fig. 3A), and a maximum biomass concentration

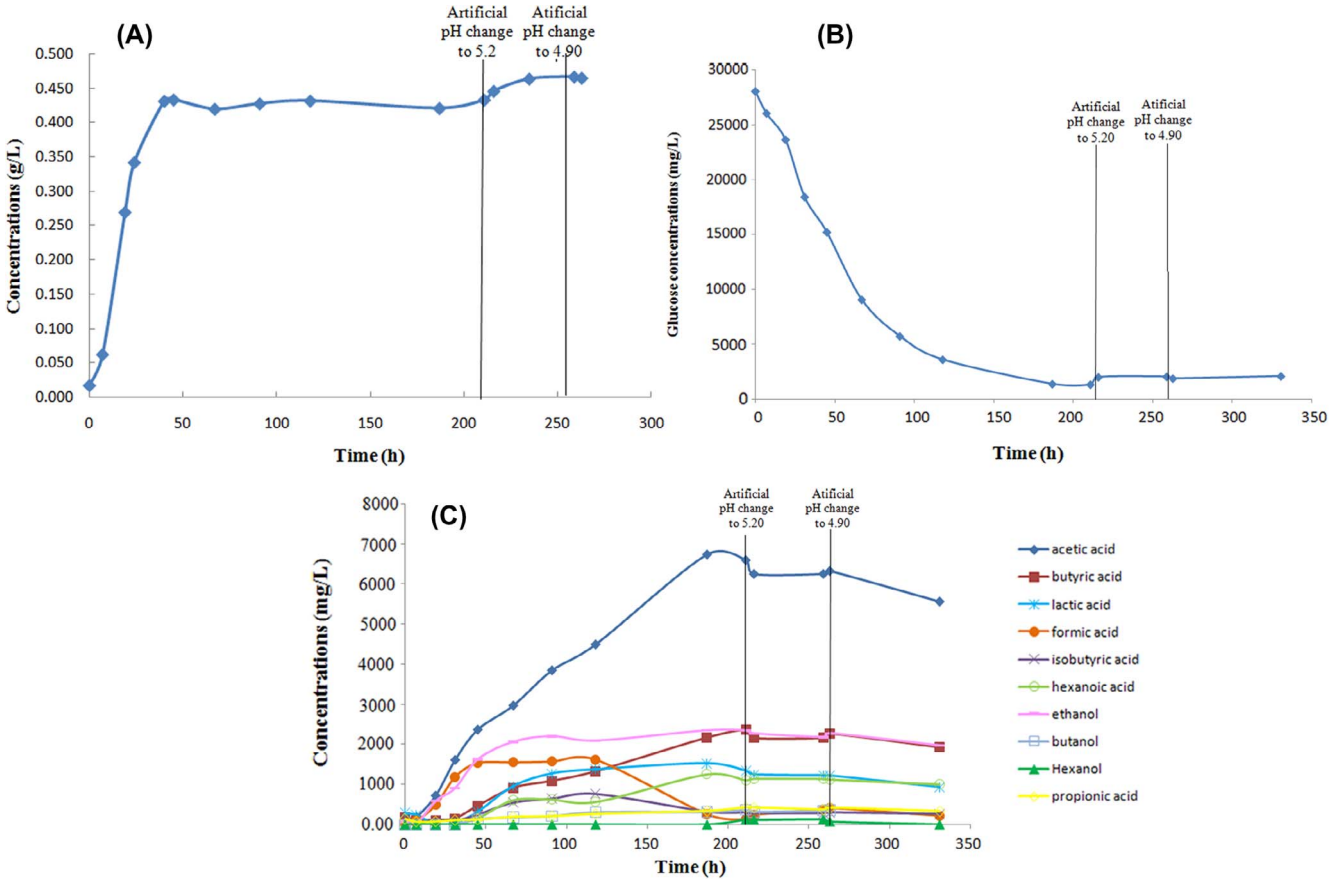

Fig. 3. Glucose bioreactor with artificial pH change. **(A)**: measured growth expressed in g/L over time. **(B)**: glucose consumption over time with concentrations expressed in g/L. **(C)**: product formation over time (acetic acid, butyric acid, isobutyric acid, propionic acid, lactic acid, formic acid, hexanoic acid, ethanol, butanol and hexanol).

of 0.432 g/L was reached within 40h, with a bacterial growth rate of 0.081 h^{-1} during those first 40 h of exponential phase. The pH was modified artificially after the maximum biomass concentration had been reached; that way possible growth inhibition at low pH was avoided.

3.3.1. Glucose bioconversion profile

C. carboxidivorans started to consume glucose soon after inoculation and its consumption stabilized 120 h after bioreactor start-up. The total net glucose consumption was 26 g/L at the end of the experiment, which represents 95% of the initial concentration. No more glucose was metabolized after the artificial pH change (211 h) (Fig. 3B).

As in all cases, acetic acid and formic acid were produced first, as soon as the bioreactor was inoculated. A few hours later, butyric acid, lactic acid, isobutyric acid and propionic acid appeared. Hexanoic acid was the last acid to be detected (Fig. 3C) similarly as in the previous two experiments. All those acids reached their maximum concentrations before the pH was decreased. The first acids reaching their maximum concentrations were lactic acid, formic acid and isobutyric acid, with values of 1.40 g/L, 1.62 g/L and 0.77 g/L, respectively, 118 h after inoculation. The next ones were propionic acid, acetic acid, butyric acid, and hexanoic acid, with maximum values of 0.42 g/L for propionic acid, 6.73 g/L for acetic acid, 2.2 g/L for butyric acid and 1.25 g/L for hexanoic acid (Fig. 3C). Except for formic acid, for all other organic acids their concentrations kept gradually increasing until the pH change. The behavior was reproducible under similar conditions and comparable trends are observed in Fig. 3C and Fig. 1C when the pH was maintained constant at a high value of 6.20.

Before the pH change, 1.40 g/L formic acid was consumed, but not anymore after the pH change towards the end of the experiment. There was some, but only very slight, consumption of the other acids after lowering the pH: 0.5 g/L isobutyric acid, 0.4 g/L lactic acid, 0.7 g/L acetic acid, 0.3 g/L butyric acid and 0.2 g/L hexanoic acid. The most abundant acid was acetic acid and the concentrations of all different acids followed the following pattern from the highest to the lowest concentrations: acetic acid > butyric acid > hexanoic acid > lactic acid > isobutyric acid > propionic acid > formic acid. This is the same pattern as observed in the experiment at constant pH (first experiment above).

Concerning solvents, those were produced, in this case again, at high pH during glucose assimilation, but not anymore after acidification, contrary to the typical successive acidogenic-solventogenic steps in ABE and HBE fermentations (Fernández-Naveira et al., 2017b). Ethanol was produced as soon as the experiment started, and butanol appeared for the first time 45 h after inoculation. This is similar as in the first two experiments, at high pH (6.20), with production of alcohols when the concentrations of acids were still increasing. Hexanol production started after 211 h, just a few hours before the pH was changed. Maximum concentrations of ethanol, butanol and hexanol were reached just before the pH change from 6.20 to 5.20 (211 h after inoculation) with values of 2.34 g/L, 0.36 g/L and 0.13 g/L, respectively (Fig. 3C). 50 h after the pH change to 5.20, a second artificial pH change was decided, down to 4.90, to see if a lower pH would better stimulate the production of alcohols. However, higher concentrations of alcohols were not reached with any of both pH changes. Thus, most of the production took place at high pH, contrary to the common solventogenic stages in ABE and HBE fermentation. This is then also different from the trend observed with gaseous substrates metabolized by *C. carboxidivorans*, where higher productions rates were observed at low pH (4.75) than at high pH (5.75) for all three alcohols.

The alcohols production rates were also calculated, but only at pH 6.20, as no more alcohols were produced after the pH drop. The ethanol

production rate was 0.085 g/h*g biomass between 7 h and 67 h. The butanol production rate was 0.009 g/h*g biomass between 31 h and 118 h; and the hexanol production rate was 0.005 g/h*g biomass between 187 and 211 h.

Although the production of fatty acids is ubiquitous in clostridia, their further conversion to solvents is much less widespread. Still, *C. carboxidivorans* is known to possess the required enzymes to metabolize gaseous substrates (CO, syngas) and convert acids obtained from gas assimilation into alcohols. Under any of the three experimental conditions described above, the production of solvents was limited and occurred at high, rather than low, pH. Acid crash is a phenomenon that can explain the absence of a clear and efficient solventogenic stage and the lack of conversion of acids into alcohols in clostridia. However, the concentration of fatty acids accumulating in the present experiments are similar or lower than found under similar conditions (*e.g.* pH) in gas fermentation with the same strain. Thus, a possible inhibition of solventogenesis by fatty acids, such as acetic and butyric acids, is not expected. Other potential inhibitory condition is the accumulation of formic acid (Qi et al., 2016). That compound is observed to accumulate transiently in this glucose fermentation, but it was not found in gas fermentation processes (Fernández-Naveira et al., 2017b). Some studies have reported that formic acid might be more inhibitory than the above mentioned fatty acids and trigger acid crash in *C. acetobutylicum* (Wang et al., 2011). On the other side, some authors did also suggest that formic acid could have a stimulating effect on species such as *C. acetobutylicum* or *C. beijerinckii*, but only when present at low concentrations (Cho et al., 2012).

Formic acid is produced during the early stages of the fermentation process in *C. carboxidivorans*, concomitant to the consumption of glucose. In the experiment at constant pH 6.2, after glucose consumption, formic acid starts disappearing while the acetic acid concentration keeps increasing. Once glucose is roughly exhausted, it sounds reasonable to assume that the increase in concentration of the C2-fatty acid results from the conversion of formic acid. This agrees with data reported in a recent study suggesting that *C. carboxidivorans* would convert formic acid into acetic acid, to different extents, depending on conditions such as the pH of the medium, such bioconversion would mainly occur at higher pH (*e.g.* pH 6.0, but not pH 5.0) (Ramió-Pujol et al., 2014). Although some authors reported that formic acid does not support growth (Liou et al., 2005), others hypothesized that some growth could simultaneously take place at high pH (*e.g.*, 6.0), but not a low pH (Ramió-Pujol et al., 2014), which might explain formic acid consumption and a concomitant slight increase in biomass concentration observed here in the late stage of the experiment at constant pH 6.2, as shown in Fig. 1A and C, while formic acid consumption and additional growth were not observed in the experiment with natural acidification down to pH 5.2 (Fig. 2A and C). Such two-phase growth on mixtures of organic acids and gaseous substrates such as CO has also been reported in other anaerobic bacteria (Haddad et al., 2013). There is no evidence from any of the experiments that formic acid might significantly have inhibited solventogenesis. From Fig. 3C, it appears that, even several days after complete elimination of that acid, no additional relevant increase in concentrations of alcohols (ethanol, butanol, hexanol) was found. *C. carboxidivorans* should posses all the required enzymatic machinery to produce alcohols and does indeed produce some of them, mainly ethanol (up to 2 g/L, depending on culture conditions), but mainly at the beginning of the experiments during glucose consumption, usually at high pH (6.2), rather than through any possible conversion of acids into alcohols during a specific solventogenic stage at lower pH.

3.4. 16S rDNA analysis

The experiments were carried out under sterile operating conditions. However, glucose is a suitable carbon source for many microorganisms. Therefore, the 16S rDNA sequence was analyzed at the end of each experiment, showing a query sequence with a similarity of 99% to *C. carboxidivorans* in all three experiments. This analysis confirms the purity and stability of the inoculated culture.

4. Conclusions

It is known that, when grown on $CO/CO_2/H_2$, *C. carboxidivorans* produces first a mixture of acetic, butyric and hexanoic acids, resulting in medium acidification and the concomitant conversion of such acids into hexanol, butanol, ethanol (HBE fermentation). It can also grow on glucose, but has not a typical ABE fermentation pattern, which would consist in the production of acids followed by the production of solvents after acidification similarly as in HBE fermentation. Instead, organic acids and low amounts of alcohols are found at high pH, although this is not followed by solventogenesis after the pH drops down to lower values.

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BIOENERGY AND BIOFUELS

Carbon monoxide bioconversion to butanol-ethanol by *Clostridium carboxidivorans*: kinetics and toxicity of alcohols

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Abstract Butanol production from carbon monoxide-rich waste gases or syngas is an attractive novel alternative to the conventional acetone-butanol-ethanol (ABE) fermentation. Solvent toxicity is a key factor reported in ABE fermentation with carbohydrates as substrates. However, in the gas-fermentation process, kinetic aspects and the inhibition effect of solvents have not thoroughly been studied. Therefore, different batch bottle experiments were carried out with the bacterial species *Clostridium carboxidivorans* using CO as carbon source for butanol-ethanol fermentation. A maximum specific growth rate of 0.086 ± 0.004 h^{-1} and a biomass yield of 0.011 $g_{biomass}/g_{CO}$ were found, which is significantly lower than in other clostridia grown on sugars. Besides, three assays were carried out to check the inhibitory effect of butanol, ethanol, and their mixtures. Butanol had a higher inhibitory effect on the cells than ethanol and showed a lower IC_{50}, reduced growth rate, and slower CO consumption with increasing alcohol concentrations. A concentration of 14–14.50 g/L butanol caused 50 % growth inhibition in *C. carboxidivorans*, and 20 g/L butanol resulted in complete inhibition, with a growth rate of 0 h^{-1}. Conversely, 35 g/L ethanol decreased by 50 % the final biomass concentration respect to the control and yielded the lowest growth rate of 0.024 h^{-1}. The inhibitory effect of mixtures of both alcohols was also checked adding similar, near identical, concentrations of each one. Growth decreased by 50 % in the presence of a total concentration of alcohols of 16.22 g/L, consisting of similar amounts of each alcohol. Occasional differences in initially added concentrations of alcohols were minimal. The lowest growth rate (0.014 h^{-1}) was observed at the highest concentration assayed (25 g/L).

Keywords *Clostridium carboxidivorans* · Butanol · Ethanol · Inhibitory effect · Batch experiment · IC_{50}

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Introduction

Nowadays, the instability of the oil price as well as recent concerns about the increasing scarcity of fossil fuels and their negative environmental impact have led to a growing interest in biofuels such as ethanol and butanol (Gowen and Fong 2011; Abdehagh et al. 2014). Butanol has recently been recognized as a highly promising biofuel (Bellido et al. 2014) and has several advantages. It is less hygroscopic and less corrosive and has a higher caloric content than ethanol (Qureshi et al. 2001; Wallner et al. 2009). For those reasons, in recent years, butanol has been considered a chemical of great industrial importance with a high potential to replace gasoline (Dürre 2007; Lee et al. 2008).

Ethanol and butanol can be obtained through fermentation of different sugars available in food crops, which is the conventional and most common commercial technology nowadays, known as first generation process. However, this process leads to food-fuel competition. Recently, in order to avoid such drawback, a new alternative has been developed using lignocellulosic feedstocks from agricultural waste and energy crops, which are inexpensive and renewable starting materials for biofuels production and do not adversely affect food supplies (Gowen and Fong 2011). Extracting simple, carbohydrates from the polymeric lignocellulosic structure is a complex process (Balat and

Balat 2009) requiring physical, chemical, or enzymatic pretreatments in order to hydrolyze the biomass into fermentable sugars in the so-called second generation process. Carbohydrates can be converted to butanol by clostridial strains, together with other side products, i.e., ethanol and acetone, through the acetone-butanol-ethanol (ABE) fermentation. Based on advances in biotechnology and process engineering, new fermentation processes are being developed, using renewable carbon sources, for a more efficient production of butanol (Dürre 2007; Lee et al. 2008; Papoutsakis 2008).

Besides butanol production from carbohydrates, a novel production route has been suggested, consisting of converting biomass or any other carbonaceous feedstocks into CO-rich gases, such as syngas. The gaseous substrate can then be fermented into ethanol and/or butanol by some bacterial species, mainly clostridia. Interestingly, this alternative route can also use CO-rich waste gases as substrates (Abubackar et al. 2011; Kennes and Veiga 2013). Under optimized conditions, a mixture of butanol and ethanol (B-E) is obtained as end-products (Fernández-Naveira et al. 2016). Only very few bacteria have been isolated so far and shown to produce butanol from carbon monoxide. *Clostridium carboxidivorans* is one such bacterium able to grow on synthesis gas, by using CO, CO_2, and H_2 to produce the liquid biofuels ethanol and butanol using a variation of the classical Wood-Ljungdahl pathway (Ukpong et al. 2012).

In the more extensively studied conventional ABE fermentation from carbohydrates, solvent toxicity is a critical problem. Under normal conditions, the clostridial cellular activity decreases significantly in the presence of 20 g/L or more solvents (Woods 1995). This is one of the most important factors to be considered in butanol fermentation as bacterial cells rarely tolerate concentrations exceeding 2 % butanol (Liu and Qureshi 2009). This should thus be taken into account in order to get a more efficient production process. Although studies have been performed on the inhibitory effect of butanol on cell growth of specific bacteria with sugars as a carbon source in the conventional ABE fermentation (Moreira et al. 1981), no data are yet available in the literature on the kinetics and the inhibition effect of solvents (butanol, ethanol) in the more recently developed clostridial butanol-ethanol (B-E) fermentation from CO-rich gases as carbon source.

In order to increase butanol production from gaseous substrates, it is necessary to know its inhibitory effect as well as the inhibitory effect of other end-product solvents (i.e. ethanol) on the B-E fermentation process. Therefore, this work focussed on evaluating the inhibitory effect of end-products of the B-E fermentation on the growth kinetics and bioconversion of CO to valuable metabolites, by *C. carboxidivorans* in batch bottle experiments.

Material and methods

Microorganism and culture media

C. carboxidivorans P7 DSM 15243 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was maintained anaerobically on modified basal medium (Liou et al. 2005; Tanner 2007) at pH 5.75 with CO (100 %) as the sole gaseous substrate.

The medium composition (per liter distilled water) was as follows: Yeast extract, 1 g; mineral solution 25 mL; trace metal solution, 10 mL; resazurin, 1 mL; cysteine-HCl, 0.60 g.

The mineral stock solution contained (per liter distilled water): 80 g sodium chloride, 100 g ammonium chloride, 10 g potassium chloride, 10 g potassium monophosphate, 20 g magnesium sulfate, and 4 g calcium chloride.

The trace metal stock solution contained (per liter distilled water) the following: 2 g nitrilotriacetic acid, 1 g manganese sulfate, 0.80 g ferrous ammonium sulfate, 0.20 g cobalt chloride, 0.20 g zinc sulfate, and 20 mg each of cupric chloride, nickel chloride, sodium molybdate, sodium selenate, and sodium tungstate.

Bottle batch experiments

Batch experiments were carried out in order to check the inhibitory effect of ethanol, butanol, and mixtures of both ethanol and butanol.

All media used for the batch experiments were prepared with the same methods and under the same conditions. For the experiments, 10 % seed culture in the early exponential growth phase, grown with CO as sole carbon source, was aseptically inoculated into 200-mL serum vials containing 100 mL medium at pH = 5.0 ± 0.1. The bottles were maintained under anaerobic conditions. They were pressurized to 1.2 bar with 100 % CO and were agitated at 150 rpm inside an orbital incubator at 30 °C. The experimental setup and the method used for media preparation as well as sampling details are described elsewhere (Abubackar et al. 2011; Abubackar et al. 2015; Fernández-Naveira et al. 2016). All the batch experiments were carried out in duplicate, reaching statistically highly reproducible results, and some were even repeated in order to confirm data whenever needed.

Three separate experiments were carried out in order to analyze the inhibitory effect of each compound separately. Butanol was checked at the following concentrations: control (0 g/L), 1, 5, 10, 15, and 20 g/L. The effect of ethanol, which appeared to be somewhat less inhibitory, was checked at the following concentrations: control (0 g/L), 1, 5, 20, 25, and 35 g/L. Similarly, the combined inhibitory effect of both alcohols together was analyzed, using the following total final concentrations: control (0 g/L), 2, 6, 15, and 25 g/L, using

identical concentrations of each alcohol. There was only a slight difference in the initial concentrations at the highest values assayed of 15 and 25 g/L, but always below 10 %.

Growth measurement

One milliliter liquid sample was withdrawn daily in order to avoid affecting too much the total liquid volume. Two daily samples were occasionally taken, mainly during the exponential growth phase, in order to have more data points allowing to calculate the exponential growth rate. The optical density ($OD_{\lambda = 600\ nm}$) was measured for each sample, in order to estimate the biomass concentration, using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa and Giralt, Madrid, Spain). The measured absorbance allowed to estimate the biomass concentration (mg/L) by comparing it with a previously generated calibration curve.

Growth rates, (μ), expressed in hour^{-1}, were calculated using the following formula:

$$\mu = [\mathrm{Ln}(N_t) - \mathrm{Ln}(N_0)]/(t - t_0)$$

Where Nt is the cell density (g/L) at time t (expressed in hours) and N_0 is the cell density at time 0 (t_0).

Such maximum growth rate was estimated during the exponential growth phase based on the best slope fit to the experimental data and making sure reproducible results were obtained. Whenever needed, an additional experiment was performed to confirm reproducibility.

One of the most common parameters used in toxicity assays is the IC_{50} (Leboulanger et al. 2001), i.e., the concentration of the tested substance that decreases the growth by 50 %. IC_{50} values were calculated using non-linear regression analysis (four parameters sigmoidal) of transformed alcohol concentration as natural logarithm data versus percentage of growth inhibition. The regression analysis was performed using the regression Wizard software (Sigma-Plot 12.5, SPSS Inc.).

Cell yield coefficient ($Y_{X/S}$) is defined as the amount of cell mass produced per amount of substrate consumed (CO). It was estimated through the following equation:

$$Y_{x/s} = \frac{N - N_o}{S - S_o}$$

Where N is the cell density at the end of the exponential growth phase (g/L), N_0 is the cell density at time 0, S is the final substrate concentration at the end of the exponential growth phase, and S_0 is the substrate concentration at time 0.

Gas-phase CO concentrations

Gas samples of 1 mL were taken from the headspace of the bottles to monitor the CO concentrations.

Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD). The GC was fitted with a 15-m HP-PLOT Molecular Sieve 5A column (ID 0.53 mm, film thickness 50 μm). The oven temperature was initially kept constant at 50 °C, for 5 min, and then raised by 20 °C·min^{-1} for 2 min, to reach a final temperature of 90 °C. The temperature of the injection port and the detector were maintained constant at 150 °C. Helium was used as the carrier gas.

Ethanol and butanol concentrations

The concentrations of ethanol and butanol were analyzed for each bottle from liquid samples (1 mL) using an HPLC (HP1100, Agilent Co., USA) equipped with a 5 μm × 4 mm × 250 mm Hypersil ODS column and a UV detector at a wavelength of 284 nm. The mobile phase was a 0.1 % ortho-phosphoric acid solution fed at a flow rate of 0.5 ml/min. The column temperature was set at 30 °C. The samples were centrifuged (7000*g*, 3 min) using a centrifuge (ELMI Skyline Ltd. CM 70M07) before analyzing the concentration of water-soluble compounds by HPLC.

Results

Growth parameters of *C. carboxidivorans*

Growth parameters of *C. carboxidivorans* were estimated on pure carbon monoxide in experiments repeated in sextuplicate. The maximum specific growth rate (μ) was found to reach 0.086 ± 0.004 h^{-1}. This value is significantly lower than for clostridial strains grown on sugars in ABE fermentation (Table 1). Each experiment corresponds to the duplicate controls of the three alcohols inhibition experiments (i.e., a total of six assays) described in the next section and summarized in Tables 2, 3, and 4. Detailed experimental data can thus be found in the next sections. Besides, the biomass yield ($Y_{X/S}$) was also estimated based on the amount substrate consumed and generated biomass and appeared to reach 0.011 g biomass per gram carbon monoxide.

Butanol toxicity experiment

In the experiments with added butanol, *C. carboxidivorans* started growing immediately after inoculation (Fig. 1a) at all the concentrations assayed, with the exception of the bottle with the highest butanol concentration (20 g/L) in which no growth at all was observed. Butanol affected the growth of *C. carboxidivorans* and the negative effect on growth was concentration-dependent (Fig. 1a, Table 2). The control reached its maximum biomass concentration (0.135 g/L) after

Table 1 Specific growth rates of *Clostridium carboxidivorans* and *Clostridium acetobutylicum* grown, respectively, on CO or carbohydrates (glucose, lactose)

Carbon source	Specific growth rate (h^{-1})	Microorganism	References
Carbon monoxide	0.086 ± 0.004	*C. carboxidivorans*	This study
Glucose	0.48	*C. acetobutylicum*	Srivastava and Volesky 1990
Lactose	0.23–0.28	*C. acetobutylicum*	Napoli et al. 2012

30 h (Fig 1a), and the maximum growth rate was reached in the control bottles, in the absence of any added butanol, with a value of 0.084 h^{-1} (Table 2). All butanol concentrations assayed provoked a decrease in the growth rate of that strain (Table 2). The highest concentration assayed (20 g/L) completely inhibited the bacterial growth, with a growth rate of 0 h^{-1}.

The IC_{50} of butanol for growth was 14.50 g/L after 48 h of butanol exposure and 14.20 g/L after 72 h of exposure.

Carbon monoxide consumption was monitored during the experiment and is shown in Fig. 1b. In the control bottles and with 1 g/L of butanol, 50 % CO was already consumed after 24 h and it was totally consumed after 77 h. In the case of butanol concentrations of 5 and 10 g/L, 50 % CO consumption was reached after 28 h of butanol exposure, and total CO consumption was observed after 92 h exposure. Only in the bottles with the highest alcohol concentrations (15 and 20 g/L) was total substrate removal not possible, observing 20 % CO consumption, after 99 h of butanol exposure. This was related to the fact that in those bottles, biomass had scarcely grown and could not consume CO, whereas significant growth was found in the control bottles as well as in the presence of butanol at 1, 5, and 10 g/L, where growth took place at different rates, and reaching different final biomass concentrations. The highest final, total amount biomass was reached in the control bottle. The total maximum biomass concentration gradually decreased at increasing added butanol concentrations.

Ethanol toxicity experiment

In case of the ethanol inhibition study, ethanol showed a negative effect on the bacterial growth of *C. carboxidivorans*, and this effect was here also concentration-dependent (Fig. 2a and Table 3) similarly as for butanol. The maximum biomass concentration was observed in the bottles with 1 g/L of ethanol (0.153 g/L) as well as in the control bottles (0.150 g/L), resulting in statistically similar values.

It is noteworthy that in bottles with an added ethanol concentration of 1 g/L, in assays performed under exactly the same conditions, and with the same preculture inoculum, a somewhat higher growth rate was found than in the control bottles, with growth rates of 0.090 and 0.082 h^{-1}, respectively. The study was repeated and that effect was observed again in a second experiment, where the growth rate in bottles with 1 g/L added ethanol was slightly higher than the value found in the control bottles. Although the highest ethanol concentration added (35 g/L) was higher than for butanol (20 g/L), growth was detected in all the bottles with ethanol and reached growth rates higher than 0.024 h^{-1} in all cases, even in the most concentrated bottles (25 and 35 g/L) (Table 3).

The IC_{50} of ethanol for growth could not be estimated with the statistical software (SigmaPlot) because the most concentrated bottle had only reached 51 % growth inhibition at the end of the experiment, suggesting a much lower inhibitory effect of ethanol compared to butanol. However, a rather accurate estimation of the IC_{50} could be done based on the experimental data obtained, that way the IC_{50} of ethanol for growth appeared to be very close to 35 g/L, where 51 % inhibition was found.

Carbon monoxide consumption is shown in Fig. 2b. Maximum fast CO consumption was observed after a similar time period in all the assays up to an ethanol concentration of 5 g/L. In the bottles with 20 g/L of ethanol, carbon monoxide did not disappear completely, and the maximum consumption was 84 % after 329 h of ethanol exposure. Similarly, in the

Table 2 Batch experiments with butanol. Maximum specific growth rates in the presence of different butanol concentrations, expressed in h^{-1}

Butanol concentration (g/L)	Growth rate (h^{-1})
0	0.084
1	0.076
5	0.043
10	0.026
15	0.019
20	0.000

Table 3 Batch experiments with ethanol. Maximum specific growth rates in the presence of different ethanol concentrations, expressed in h^{-1}

Ethanol concentration (g/L)	Growth rate (h^{-1})
0	0.082
1	0.090
5	0.066
20	0.055
25	0.028
35	0.024

Table 4 Batch experiments with mixtures of alcohols. Maximum specific growth rates in the presence of different total concentrations of butanol and ethanol (1:1), expressed in h^{-1}

Total concentration of alcohols (1:1 mixtures) (g/L)	Growth rate (h^{-1})
Control	0.090
2	0.072
7	0.033
15	0.018
25	0.014

bottles with the highest alcohol concentrations (25 and 35 g/L), total substrate consumption was not possible either, observing 42 and 39 % CO consumption, respectively, at the end of the experiment after 230 h of ethanol exposure. This was related to the fact that in those bottles, biomass had scarcely grown and could thus not consume CO, whereas in the control bottles and with 1, 5, and 20 g/L added ethanol, growth took place at different rates, and different final biomass concentrations were reached. The final total amount accumulated biomass gradually decreased at increasing ethanol concentrations.

Toxicity experiment with mixtures of both alcohols

In this experiment, the bacteria started growing soon after inoculation (Fig. 3a). The negative effect of the mixture of ethanol and butanol on the growth of *C. carboxidivorans* is shown in Fig. 3a and in Table 4. This negative effect of the ethanol-butanol mixture is here also concentration-dependent (Fig. 3a and Table 4). The maximum biomass concentration (0.170 g/L) was reached after 48 h in the control bottle. Growth took place in all the bottles and reached growth rates higher than at least 0.014 h^{-1}. In the bottles with the highest alcohol concentration (25 g/L), growth started initially and

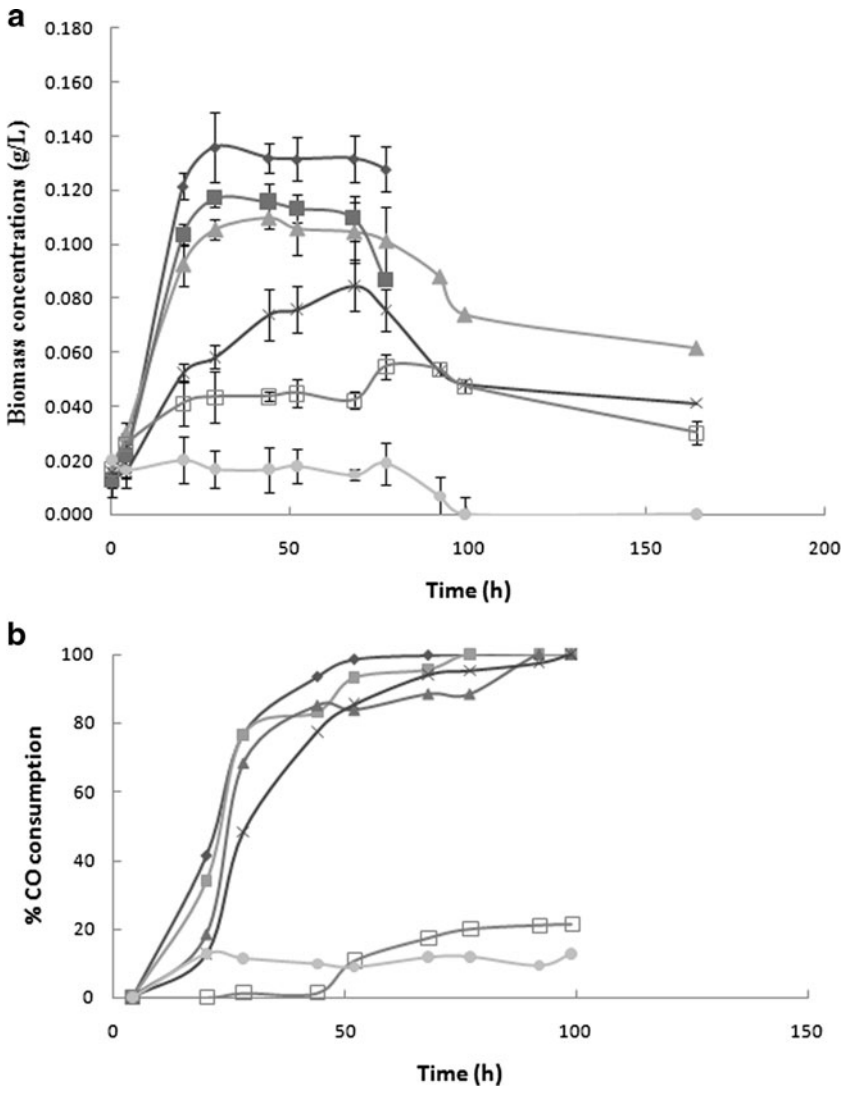

Fig. 1 **a** Batch experiments with butanol. Measured biomass accumulation over time, expressed in g/L. Data are given as mean values ± standard deviation of the means (control bottles represented as *filled diamond*, 1 g/L butanol represented as *filled square*, 5 g/L butanol represented as *filled triangle*, 10 g/L butanol represented as X, 15 g/L butanol represented as *square*, and 20 g/L butanol represented as *filled circle*). **b** Batch experiments with butanol. Percentage CO consumption (control bottles represented as *filled diamond*, 1 g/L butanol represented as *filled square*, 5 g/L butanol represented as *filled triangle*, 10 g/L butanol represented as X, 15 g/L butanol represented as *square*, and 20 g/L butanol represented as *filled circle*)

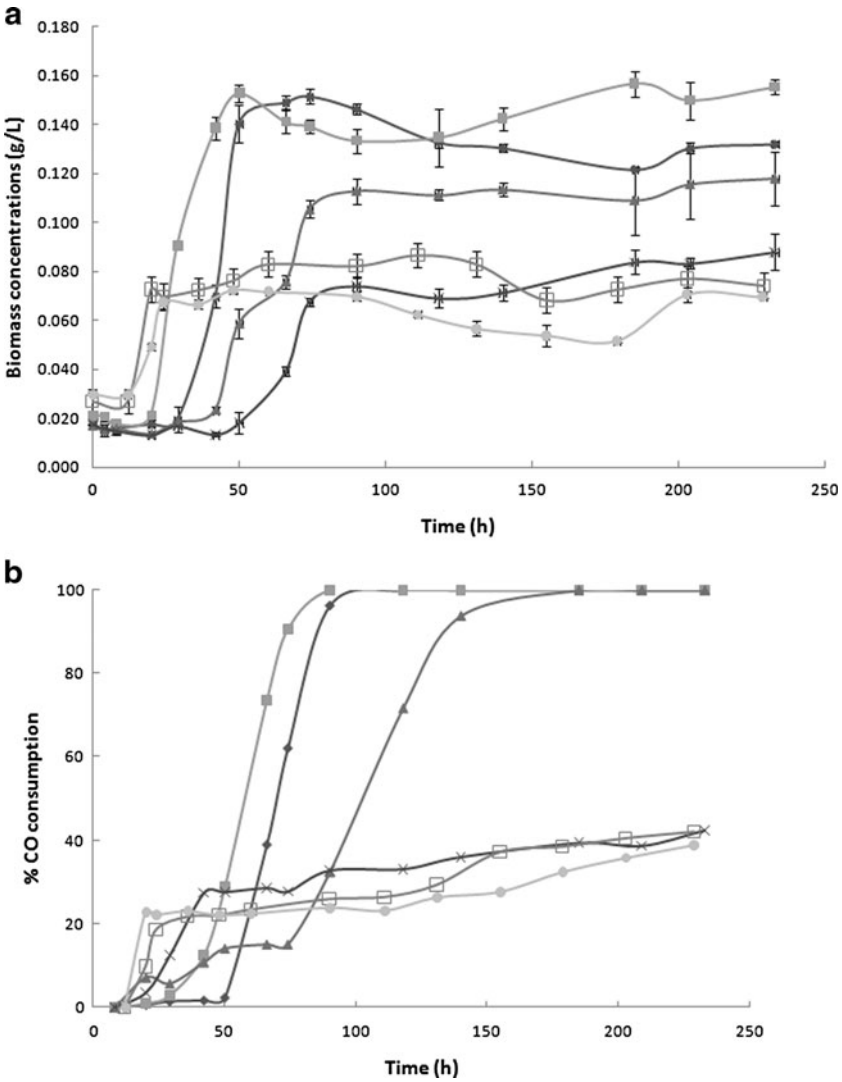

Fig. 2 **a** Batch experiments with ethanol. Measured biomass accumulation over time, expressed in g/L. Data are given as mean values ± standard deviation of the means (control bottles represented as *filled diamond*, 1 g/L ethanol represented as *filled square*, 5 g/L ethanol represented as *filled triangle*, 20 g/L ethanol represented as X, 25 g/L ethanol represented as *square*, and 35 g/L ethanol represented as *filled circle*). **b** Batch experiments with ethanol. Percentage CO consumption (control bottles represented as *filled diamond*, 1 g/L ethanol represented as *filled square*, 5 g/L ethanol represented as *filled triangle*, 20 g/L ethanol represented as X, 25 g/L ethanol represented as *square*, and 35 g/L ethanol represented as *filled circle*)

reached a maximum biomass concentration of 0.053 g/L after 60 h of alcohols exposure. However, after that time of alcohols exposure, growth stopped and the biomass started slightly decaying.

The IC_{50} for growth was 16.22 g/L after 111 h of alcohols exposure, which is similar, but slightly higher than in the assays with pure butanol, and can be explained by the lower toxicity of ethanol in the mixture of both alcohols.

Carbon monoxide consumption was monitored during all the experiment and is shown in Fig. 3b. In the control bottles, 50 % CO was consumed after 24 h and it was totally consumed after 60 h. In the case of 2 g/L, complete CO consumption was found after 130 h of alcohols exposure. In the bottles with 7 g/L, the maximum final consumption was 63 % after 60 h of alcohols exposure, and in the bottles with the highest concentrations of alcohols (15 and 25 g/L), only 30 and 22 % CO consumption were respectively observed, after 230 h of alcohols exposure.

Comparison of inhibitory effects of alcohols

Figure 4 compares the inhibitory effect on growth rates of ethanol, butanol, and mixtures of both alcohols. It shows that pure ethanol is the least toxic to bacterial growth, followed by the mixture of alcohols and pure butanol with the highest inhibitory effects. A similar trend can be found for the inhibitory effects of the different alcohols on substrate consumption as well as IC_{50} data.

Discussion

Bioalcohols such as butanol can be produced through the conversion of lignocellulosic feedstocks into carbohydrates which are then fermented into biofuels. Alternatively, another recent approach consists in using CO-rich gases (i.e., syngas, waste gases) which can also be fermented to butanol and ethanol. Both the carbohydrate and the carbon monoxide routes use

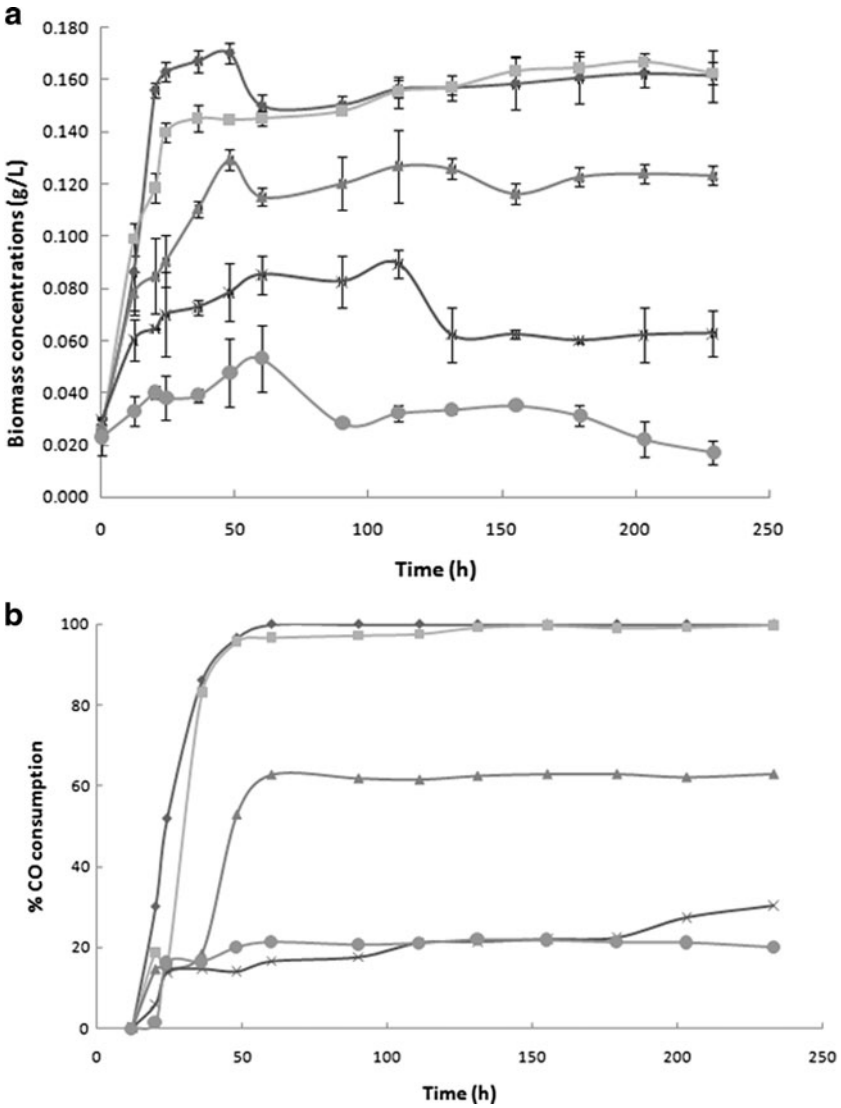

Fig. 3 **a** Batch experiments with both butanol and ethanol (1:1, *w/w*). Measured biomass accumulation over time, expressed in g/L. Data are given as mean values ± standard deviation of the means (control bottles represented as *filled diamond*, 2 g/L represented as *filled square*, 7 g/L represented as *filled triangle*, 15 g/L represented as X, 25 g/L represented as *filled circle*). **b** Batch experiments with both butanol and ethanol (1:1, *w/w*). Percentage CO consumption (control bottles represented as *filled diamond*, 2 g/L represented as *filled square*, 7 g/L represented as *filled triangle*, 15 g/L represented as X, 25 g/L represented as *filled circle*)

clostridia or acetogens in general as biocatalysts and present each their own advantages and drawbacks (Kennes et al. 2016). The lower biomass yield and slower bacterial growth rate is a typical drawback of the syngas approach, studied in this paper, reaching specific growth rates close to hardly 0.086 h^{-1}, to be compared to values of 0.23–0.48 h^{-1} when clostridia are grown on sugars (Table 1). Thus, growth rates appear to be about 4–5 times higher on carbohydrates than on carbon monoxide. Similarly, our data show that the biomass yield on CO is 0.011$g_{biomass}/g_{CO}$, while it reaches 0.36–0.53 $g_{biomass}/g_{carbohydrate}$ when growing clostridia on sugars such as lactose (Napoli et al. 2012). Specific strategies are thus needed to overcome the low biomass production on such gaseous substrates in continuous bioreactors for B-E fermentation, such as cell recycling.

Another aspect to be taken into account, common to both the carbohydrate and the carbon monoxide (waste gas, syngas) approach, is the potential toxicity of end-metabolites, i.e., butanol and ethanol, on growth and substrate conversion itself. Toxicity studies have been performed and reported in the literature for clostridial strains converting sugars to solvents (ABE fermentation), but, to the best of our knowledge, no previous report is available on the effect of alcohols on clostridia fermenting CO-rich gases; although, in a recent patent application, it has been shown that recombinant strains of CO-fermenting clostridia might tolerate ethanol concentrations of up to around 50 g/L (Koepke et al. 2012).

The IC_{50} value of butanol obtained for *C. carboxidivorans* grown on CO in the present work (14.50 g/L), and reported here for the first time, is rather close to the values available from studies with other *Clostridium* species grown on carbohydrates. Similar results in terms of butanol inhibition were obtained by Moreira et al. (1981) using sugars as carbon source in *C. acetobutylicum*. They found that 0.10–0.15 M (7.41–11.12 g/L) butanol caused 50 % inhibition of cell growth and sugar uptake rate by negatively affecting the

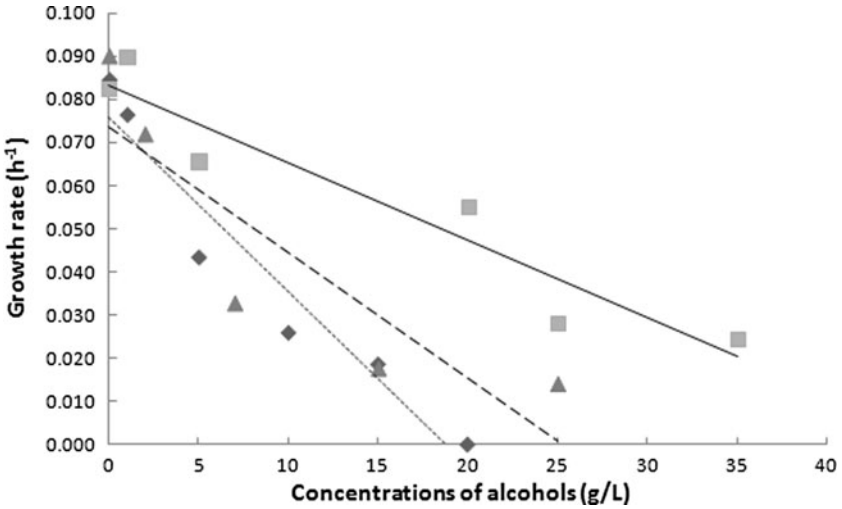

Fig. 4 Comparison of growth rates in each experiment. Maximum specific growth rates (GR) of each treatment in the three experiments, expressed in h^{-1}. Butanol experiment represented as *filled diamond*, ethanol experiment represented as *filled square*, mixture of alcohols experiment represented as *filled triangle*. The lines represent the general trend of variation of the growth rates as a function of the concentration of alcohols (butanol experiment represented as *dotted line*, ethanol experiment represented as *solid line*, mixture of alcohols experiment represented as *dashed line*)

ATPase activity. A comparable effect was reported by Jones and Woods (1986), who found that 7–13 g/L butanol caused 50 % inhibition of cell growth.

The mechanism of butanol toxicity seems to be related to its hydrophobic nature, as this alcohol is a lipophilic solvent which can disrupt the phospholipid and fatty acid composition of the cell membrane causing an increase in membrane fluidity (Bowles and Ellefson 1985). This increase in the membrane fluidity would cause destabilization of the membrane and disruption of membrane functions such as transport processes, substrate (glucose) uptake, and membrane ATPase activity (Bowles and Ellefson 1985). On the other hand, Gottwald and Gottschalk (1985) also found that butanol can inhibit the ability of *C. acetobutylicum* to maintain its internal pH and abolishes the membrane pH gradient. Butanol toxicity has also been related to the autolytic degradation of the solventogenic cells in *C. acetobutylicum* P262 (Van der Westhuizen et al. 1982), and it was suggested that concentrations of butanol near the inhibitory concentration were involved in the release of autolysin during the solventogenic phase (Barber et al. 1979).

The effect of ethanol was also studied in the present work and it was shown that concentrations of 35 g/L could reduce the growth of *C. carboxidivorans* by 50 % after 200 h of ethanol exposure. That value is similar to the values obtained in the fermentation of sugars, for which it was suggested that the addition of acetone and ethanol up to 40 g/L reduced growth by 50 % (Jones and Woods 1986), and total growth inhibition appeared at concentrations of about 50–60 g/L of ethanol (Leung and Wang 1981; Costa and Moreira 1983).

In the butanol toxicity experiment with *C. carboxidivorans*, the IC_{50} (14.50 g/L) was much lower the value of IC_{50} observed in the ethanol toxicity experiment (35 g/L). A similar trend was observed for the growth rate. In the assay on ethanol toxicity, in the bottle with 35 g/L of ethanol (the most concentrated one), a growth rate of 0.024 h^{-1} was reached, which is a value close to the one obtained in the assays with 10 g/L of butanol. These results show that ethanol has a quite weaker inhibitory effect on *C. carboxidivorans* than butanol, as more ethanol is required to observe the same toxic effect as with butanol.

In both experiments with individual alcohols, the % CO consumed was monitored. This parameter shows that the bottles with ethanol reached higher percentages of CO consumption than the bottles with butanol. The assays with the highest butanol concentrations (20 g/L) did not even reach 20 % CO consumption, whereas in the bottles with the same concentration of ethanol, as much as 83 % CO was consumed. Besides, the bottles with the highest amount ethanol (35 g/L) still reached as much as 40 % CO consumption. There was basically no growth at all of *C. carboxidivorans* in the presence of a concentration of 20 g/L butanol; that way, this concentration would thus be near the concentration of full inhibition. As described above, the effect of alcohols on the level of CO consumption could be related to the fact that butanol disrupts the membrane fluidity, so the uptake of CO is a function of the characteristics of the membrane, which can get damaged as a result of butanol toxicity (Bowles and Ellefson 1985). Also, butanol at high concentrations could be involved in the release of autolysin, with an effect on the autolytic degradation of the cell (Van der Westhuizen et al. 1982; Barber et al. 1979).

Ethanol added at low concentration (up to 1 g/L) appeared not to cause any negative effect on the cells, and a somewhat

faster biomass accumulation than in control bottles was even observed. This might be related to the fact that ethanol may favor the uptake of cholesterol or saturated fatty acids into membranes (Goldstein 1986). Goldstein (1986) suggested that the bacteria he studied could be able to uptake a higher quantity of molecules such as fatty acids or other compounds, which could favor their growth.

In the last experiment, the effect of similar amounts of both alcohols in mixtures was analyzed. In that experiment, the IC_{50} was found to reach a value of 16.22 g/L of the ethanol and butanol mixture. This value is higher than the value obtained in the butanol toxicity assay, and it can most probably be related to the lower inhibitory effect of ethanol in the mixture. The growth rates in that experiment were higher than in the butanol experiment, at all the concentrations assayed. However, compared with the ethanol toxicity experiment, the growth rate was lower in the mixture than in the ethanol experiment. That difference is related to the strong toxic effect of butanol in the mixture in comparison with ethanol.

Also, differences were observed regarding the % CO consumed. A rather similar effect in terms of CO consumption was observed between the butanol experiment and the assay with mixtures of both alcohols, again as a result of the more significant inhibitory effect of butanol compared to ethanol. The bottles with the highest concentrations of alcohols only consumed 20–30 % CO in both cases, whereas in the bottles with ethanol only, the % CO consumption was always higher and exceeded at least 40 % in all cases.

It can be concluded that: (a) The alcohol with lowest inhibitory effect has the highest IC_{50}, meaning that butanol had the highest toxic effect on CO fermentation by *C. carboxidivorans*, followed by the 1:1 mixture of alcohols and finally ethanol; (b) small quantities of ethanol (around up to 1 g/L) have no toxic effect and seem even to exhibit a slightly positive effect on biomass growth and accumulation compared to the control cultures; (c) the alcohols produce a negative effect on the growth rate, on biomass accumulation as well as on the CO consumption rate in all the experiments (except at low ethanol concentrations).

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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