Bioconversion of carbon monoxide related volatile compounds into ethanol in bioreactors

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DOCTORAL THESIS

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INFORMAN

Que el trabajo titulado **Bioconversión de monóxido de carbono y compuestos volátiles relacionados en etanol en biorreactores** ha sido realizado por **Haris Nalakath Abubackar** en el Departamento de Química Física e Enxeñería Química I 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 La Coruña, a de de 2015.

Christian Kennes | María del Carmen Veiga Barbazán

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With Love

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Objectives and abstract

The environmental problems associated to the use of fossil fuels as well as their expected scarcity in the near future require a search for new alternative fuels such as bioethanol obtained from waste or renewable sources. Integration of gasification and fermentation technologies, the so-called "Hybrid technology" is a most versatile approach to produce ethanol. It is considered to be an energy-inexpensive and feedstock-flexible technology that utilizes the potential of anaerobic microorganisms to catalyze the conversion of one carbon (C1) compounds to a variety of chemicals and fuels by using the reductive acetyl-CoA pathway. Besides using syngas as feedstock, waste gases containing carbon monoxide and released from industrial processes can also efficiently be utilized for the production of fuel ethanol. The main objective of this doctoral research is the optimization of the fermentation medium and operating parameters for the bioconversion of carbon monoxide to ethanol.

Chapter 1 gives a general introduction of the C1 bioconversion by acetogenic bacteria. The biochemical pathway and the various parameters that may affect the fermentation are also described. A brief note about the various challenges faced by this technology is mentioned in the last section of this chapter.

Chapter 2 describes the materials and methods used for carrying out the research.

In the following chapters (Chapter 3 to Chapter 7), various optimization studies performed with the bacterium *Clostridium autoethanogenum* in bottles and bioreactors and the results and the conclusions are presented.

In Chapter 3, the effect of four factors: initial pH, initial total pressure, cysteine as reducing agent and yeast extract concentrations on cell growth and metabolite distribution is explained. The effect of different nitrogen sources was also evaluated and the results are described in Chapter 4 along with three different bioreactor studies at

varying concentrations of yeast extract and pHs.

The above experimental yielded higher amounts of acetic acid compared to ethanol. In order to understand the effect of trace metals such as tungsten and selenium, and vitamin solutions on bioconversion, studies were performed in bottles as well as in bioreactors. Chapter 5 describes the experiments and results obtained from those studies.

In the following Chapters 6 and 7, the strategy to improve ethanol production through pH shifting and media replacement techniques are explained. In the last part of Chapter 7, the mixotrophic fermentation with xylose and CO is also described.

Objetivos y resumen

Los problemas ambientales asociados al uso de combustibles fósiles, así como su esperada escasez en un futuro próximo, exigen la búsqueda de nuevos combustibles alternativos, como el bioetanol obtenido a partir de residuos o fuentes renovables. El uso de tecnologías de gasificación y de fermentación, la denominada "tecnología híbrida", representa un enfoque más versátil para la producción de etanol. Este último se considera una energía de bajo coste que se obtiene a partir de una gran variedad de materias primas, que utiliza el potencial de los microorganismos anaerobios para catalizar la conversión de compuestos de un único carbono (C1) a una variedad de productos químicos y combustibles mediante el uso de la vía metabólica reductora del acetil-CoA. Además de utilizar gas de síntesis como materia prima, los efluentes gaseosos que contienen monóxido de carbono y liberados en procesos industriales, también pueden ser utilizados de manera eficiente en la producción de etanol como combustible.

El principal objetivo de esta investigación doctoral es la optimización del medio de fermentación y los parámetros de operación para la conversión de monóxido de carbono en etanol.

El Capítulo 1 presenta una introducción general sobre la bioconversión de compuestos de un único carbono por bacterias acetogénicas. También describe la vía metabólica y los diversos parámetros que pueden afectar a la fermentación. En la última sección de dicho capítulo, se incluye una breve descripción de los distintos desafíos que presenta esta tecnología.

El Capítulo 2 describe los materiales y métodos utilizados para llevar a cabo la investigación.

Del Capítulo 3 al 7 se presentan varios estudios de optimización realizados con la

bacteria *Clostridium autoethanogenum* en botellas y en biorreactores continuos, así como sus resultados y conclusiones.

El Capítulo 3 explica el efecto de cuatro factores, - pH inicial, presión total inicial, cisteína como agente reductor y distintas concentraciones de extracto de levadura - , sobre el crecimiento y metabolismo celular. El efecto de las fuentes de nitrógeno también fue evaluado y los resultados se describen en el Capítulo 4, junto con tres estudios diferentes en biorreactores a concentraciones variables de extracto de levadura y distintos pHs.

El ensayo anterior produjo una mayor cantidad de ácido acético en comparación al etanol. Con el fin de entender el efecto de metales traza, tales como el tungsteno y el selenio, y vitaminas sobre la bioconversión, se realizaron estudios en botellas y biorreactores continuos. El Capítulo 5 describe los experimentos y resultados obtenidos a partir de dichos estudios.

En los Capítulos 6 y 7 se explica la estrategia para mejorar la producción de etanol a través de técnicas de variación de pH y sustitución de medio de fermentación. En la última parte del Capítulo 7 se describe la fermentación mixotrófica con xilosa y CO.

Obxectivos e resumo

Os problemas ambientais asociados ao uso de combustibles fósiles, así como a súa esperada insuficiencia nun futuro próximo, esixen a procura de novos combustibles alternativos como o bioetanol, obtido a partir de residuos, ou fontes renovables. O uso de tecnoloxías de gasificación e de fermentación, a denominada "tecnoloxía híbrida", representa un enfoque máis versátil para a produción de etanol. Este último considérase unha enerxía de baixo custo que se pode obter a partir dunha gran variedade de materias primas e que utiliza o potencial dos microorganismos anaerobios para catalizar a conversión de compostos dun único carbono (C1) a unha variedade de produtos químicos e combustibles mediante o uso da vía metabólica redutora do acetil-CoA. Ademais de utilizar gas de síntese como materia prima, os efluentes gaseosos que conteñen monóxido de carbono e liberados en procesos industriais, tamén poden ser utilizados de maneira eficiente na produción de etanol como combustible.

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O Capítulo 1 presenta unha introdución xeral sobre a bioconversión de compostos dun único carbono por bacterias acetoxénicas. Tamén describe a vía metabólica e os diversos parámetros que poden afectar á fermentación. Na última sección do devandito capítulo, inclúese unha breve descrición dos distintos desafíos que presenta esta tecnoloxía.

O Capítulo 2 describe os materiais e métodos empregados para levar a cabo a investigación.

Do Capítulo 3 ao 7 preséntanse varios estudos de optimización realizados coa bacteria *Clostridium autoethanogenum* en botellas e en biorreactores continuos, así como os seus resultados e conclusións.

O Capítulo 3 explica o efecto, sobre o crecemento e metabolismo celular, de catro factores: pH inicial, presión total inicial, cisteína como axente redutor e distintas concentracións de extracto de levadura. Os resultados da avaliación do efecto das fontes de nitróxeno descríbense no Capítulo 4, xunto con tres estudos diferentes en biorreactores a concentracións variables de extracto de levadura e distintos pHs.

O ensaio anterior produciu unha cantidade de ácido acético maior que a de etanol. Co fin de entender o efecto de metais traza, tales como o tungsteno e o selenio, e vitaminas sobre a bioconversión, realizáronse estudos en botellas e biorreactores continuos. O Capítulo 5 describe os experimentos e resultados obtidos a partir dos devanditos estudos.

Nos Capítulos 6 e 7 explícase a estratexia para mellorar a produción de etanol a través de técnicas de variación de pH e sustitución do medio de fermentación en biorreactores co alimentación en continuo de CO. Na última parte do Capítulo 7 descríbese a fermentación mixotrófica cunha mestura de xilosa e CO.

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Chapter 1

GENERAL INTRODUCTION

With the increase in population and growing industrialization of many countries, there is a tremendous rise in the demand for energy in the world. A 17-fold increase in world-wide energy consumption was reported in the last century (Mojović et al., 2009). This energy demand is covered by utilizing primarily the petroleum reserves, which are on the verge of extinction and are estimated to get depleted in less than 50 years at the present consumption rate (Demirbas, 2007). The processing of these fossil fuels and their usage leads to enormous release of hazardous and toxic gases to the environment, which is harmful to man-kind as well as to the environment. The increasing concentrations of these gases has negative impacts such as severe floods and droughts, sea levels rising, and extreme weather conditions (Gullison et al., 2007). Growing concern about global warming leads researchers to search for sustainable and safer alternative renewable fuels.

Ethanol is one of the most promising alternative biofuels. Fuel ethanol is an oxygenated, water-free, high octane (108) alcohol which has been recognized as a potential alternative fuel as well as an additive to gasoline (Balat et al., 2008). As an additive, it can replace methyl tertiary butyl ether (MTBE), which is used as an oxygenate and also to raise the octane number, by which the groundwater pollution due to MTBE usage can be eliminated (Olson et al., 2003). Today, ethanol can be used as blends with mineral gasoline at typical ratios of 10, 15 or 20% (E10, E15 and E20). It can even be used pure or almost pure as an alternative transportation fuel (E85) (Zhang et al., 2010). Since it burns cleaner than petroleum products, by using 10% ethanol blend (E10), a reduction of 25-30%, 6-10%, 7% and 5% respectively of harmful emissions of gases as CO, CO_2, VOCs and NO_x can be achieved (European commission joint research centre, Report EUR 20280 EN, 2002). In addition, ethanol is

biodegradable and contains 35% oxygen, which reduces particulate and NOx emissions upon combustion compared to conventional fuels (Balat and Balat, 2009).

Bioethanol is derived from renewable sources of feedstocks such as sugar, starch, or lignocellulosic materials. Current processes include either direct or indirect fermentation of sugars or catalytic conversion of producer gas. In direct fermentation, feedstocks such as sugar-based crops (e.g. beets, sorghum and cane) or starch based crops (e.g. corn, wheat, barley and potatoes) are converted into alcohols by yeasts or bacteria (Bai et al., 2008; Naik et al., 2010). This technology is well established at industrial level and currently, about 90% of the world bioethanol production comes from fermenting sugars or starch crops, known as first generation technology (Wei et al., 2009). The high value of these crops as food commodity either for human consumption or for feeding livestock and the issue of low utilization efficiency of crop parts per hectare of land used questions the feasibility of this technology. A potential solution for these issues, known as second generation technology, is to utilize lignocellulosic feedstocks such as agricultural or municipal wastes, wood, straw, grasses and crop residues. Lignocellulose is the most abundant renewable organic material on earth, composed of three major components: cellulose (40-50%), hemicelluloses (20-40%) and lignin (10-40%) (Rajagopalan et al., 2002). It is the major structural component of all plants. In indirect fermentation, cellulosic as well as hemicellulosic biomass originating from trees and grasses are hydrolyzed chemically or enzymatically to simple sugars. The available sugars are then fermented to yield ethanol (Hamelinck et al., 2005; Kumar et al., 2009). A large proportion of lignin mostly present in straw and wood, along with cellulose and hemicellulose, is highly resistant to microbial attack. Gasification technology can be used to convert the biomass into a mixture of gases, called producer gas. Producer gas can subsequently be converted to ethanol either by

using a chemical process (Fischer-Tropsch Synthesis, FTS) (Davis, 2001) or by means of anaerobic microbial catalysts.

Bioethanol production is based on rather inexpensive feedstocks, such as biomass and waste organic matter. It in turn reduces the nation's dependency to imported fossil fuels and thus helps the economy. All this biomass based production creates employment opportunities by utilizing trivial lands for the cultivation of inexpensive dedicated feedstocks, and the waste can be considerably regenerated for the production of an eco friendly fuel. Similarly to syngas, CO-rich waste gases can be used as well for bioethanol production.

1.1 ETHANOL PRODUCTION FROM SYNGAS

Syngas, or synthesis gas, a mixture of principally CO, CO_2 and H_2, can be produced by gasification of solid fuels, such as coal, petroleum coke, oil shale, and biomass; by catalytic reforming of natural gas; or by partial oxidation of heavy oils, such as tar-sand oil. The syngas composition mainly depends upon the type of resources used, their moisture content, and the gasification process. The mixture of syngas produced from biomass gasification is called producer gas.

The gasification technology effectively and economically converts biomass into various products through a thermo–chemical process that usually involves partial oxidation of the feedstock in the presence of a controlled amount of oxidant, also called gasifying agent, such as air, O_2, steam, CO_2 or a mixture of each. The gaseous mixture of products formed consists mainly of CO, H_2, CO_2, and N_2; small quantities of NOx, O_2, acetylene, phenol, COS, H_2S, light hydrocarbons such as C_2H_2, C_2H_4, and C_3H_8, ash, char, and tars (Deluga et al., 2009; Bridgwater, 2003). It is considered one of the best alternatives for reuse of waste solids. Air is a comparatively cheaper and widely used gasifying agent compared to other oxidants, but it produces a gas stream that

contains a high percentage of nitrogen, resulting in a low heating value of 3 – 6 MJN/m3. The heating value of producer gas can be increased if pure oxygen is fed into the gasifier, although operating costs will also increase. The H_2 content of the producer gas will be higher if steam is supplied as the gasifying agent. Thereby the heating value of the gas mixture increases to 10–15 MJN/m^3 (Wang et al., 2008). Biomass gasification is a complex conversion process consisting in the following stages: (1) the feed biomass is dried to reduce the moisture content to <5%; the energy to drive this drying process can be obtained by partial combustion of biomass with air or O2, (2) pyrolysis or devolatilisation of dried biomass at 300–500°C in the absence of oxygen or air reduces biomass into solid charcoal and releases gases and bio–oils,(3) in the combustion/oxidation stage, solid carbonaceous materials produced in the pyrolysis stage in the presence of the oxidant (air/O_2) produces CO_2, and the hydrogen from the biomass oxidized produces water, (4) finally, several reduction reactions in the absence or presence of the oxidant at sub-stoichiometric level in the temperature range of 800–1000°C, form CO, CO_2, H_2 as well as CH_4. By controlled supply of the oxidant at sub–stoichiometric level, the composition of the producer gas can be restricted to CO and H_2 (Puig-Arnavat et al., 2010). Gasification takes place in a so–called gasifier. The final composition of the producer gas generated mainly depends on the type of gasifier used (fixed bed, moving bed or fluidized bed, for example), properties of the biomass (including moisture, ash, dust and tar content, particle size), and operational conditions (such as temperature and pressure) (McKendry, 2002). The composition of producer gas using various biomass feedstocks and operating conditions are summarized in Table 1. The most widely used gasifiers in research and industry include the moving–bed, fluidized–bed, and entrained–flow gasifiers.

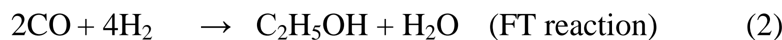
Table 1: Biomass gasification: Operating conditions and producer–gas composition.

Biomass	Gasifier	Gasification Temperature (^0C)	Oxidant	Producer gas compositio				Reference
				CO (%)	CO_2 (%)	H_2 (%)	N_2 (%)	
Switchgrass	Fluidized bed	750 – 800	Air	14.7	16.5	4.4	56.8	Datar et al.,2004
Coffee ground	Dual fluidized bed	800	Steam	38.2	9.5	19.3	Nil	Murakami et al., 2007
Kentucky bluegrass	Novel Gasifier	537	Air	12.96	17.42	2.61	64.23	Boateng et al., 2007
Wood	Downdraft	750 – 1100	Air	26.4	9.2	11	51.3	Erlich and Fransson, 2011
Pine Sawdust	Fluidized bed	780 – 830	Air	9.9 – 22.4	9.0 – 19.4	5.0 – 16.3	41.6 – 61.6	Puig-Arnavat et al., 2010
Miscanthus pellets	CFBGs	820	Air – steam	12.57	16.02	6.00	62.22	Chen et al., 2004
Labee A	Fluidized bed	782	Air	8.0	9.7	5.2	65.1	de Jong et al., 2003
Willow	CFBGs	827	Air	9.4	17.10	7.2	60.47	V.d. drift and Vermeule 2001
L*eucaena* sp.Wood	TLUG	850	Air	15	10	10	48	Saravanakumar et al., 2007
Rice husk	LEFG	1300	O_2	~43	~15	~29	Nr	Zhou et al., 2009
Olive kernel	Fluidized bed	750	Air	14.26	19.42	23.98	~36	B. Z. Alauddin et al., 2010
Larch wood	Fluidized bed	750	Steam	7.71	29.23	55.97	Nil	B. Z. Alauddin et al., 2010
Bagasse	Fluidized bed	800	Air	16	15.9	10.8	50.8	B. Z. Alauddin et al., 2010

Abbreviations: CFBGs: Circulating fluidized bed gasification system; TLUG: Top lit updraft fixed bed gasifier; LEFG: Laminar entrained–flow gasifier; Nr: Not reported

The synthesis gas thus obtained can be converted chemically to ethanol and a variety of chemicals through the Fischer-Tropsch (FT) cycle. A variety of fuels and chemicals such as methanol, acetic acid, methane and heavy waxes can be produced by this technique (Dry, 2002). This method of production is a multi-step energy intensive process carried out at elevated pressure and temperature using different chemical catalysts, which include metal iron, cobalt or rhodium. These conditions make catalytic conversion faster than bioconversion processes (Wei et al., 2009). In this process, the catalytic water gas shift (WGS) reaction takes place, converting CO and H_2O to H_2 and CO_2, thus increases the H_2/CO ratio, which is essential for stoichiometry of reaction as well as for reducing the catalytic deactivation (equation 1). For protecting the sensitive FT catalyst, other products such as tar, oil and water-soluble contaminants present in the producer gas have to be removed. The sulfur contaminants present in the syngas have to be reduced to less than 60 ppb and the limits on level of NO_x and NH_3 to avoid FT catalyst poisoning are in the order of 0.1 and 10 ppm, respectively (USDOE-National Energy Technology Laboratory, Report DE-AC26-99FT40675, 2001). Following the purification, the syngas containing CO and H_2 is converted to ethanol using different catalysts and processing conditions (equation 2).

$$CO + H_2O \rightarrow H_2 + CO_2 \quad \text{(WGS reaction)} \quad (1)$$



$$2CO + 4H_2 \rightarrow C_2H_5OH + H_2O \quad \text{(FT reaction)} \quad (2)$$

Even though this process takes place at high reaction rates, it has got many limitations. Mainly, the various processes such as WGS reaction, FT reaction and purification take place under different process conditions, converting FT synthesis into a complex and expensive method. Moreover, the catalyst used should be specific and will deactivate when the concentration of sulfur as well as carbon deposition increase. The yield of liquid fuels from this process is also not high (Stiles et al., 1991).

An alternative method of converting syngas to ethanol is through bioconversion. Microorganisms, mostly anaerobic, can be used as biocatalysts to produce valuable metabolites such as organic acids and alcohols from syngas. These products include, but are not limited to, acetic, propionic, butyric, formic and lactic acid as well as ethanol, propanol and butanol (Kundiyana et al., 2010a; Munasinghe and Khanal, 2010; Tirado-Acevedo et al., 2010). As a biofuel, ethanol is considered as the desired metabolite and the process has to be optimized to maximize its production. Later the desired product is recovered from the broth either by distillation or extraction or a combination of both or by any other efficient recovery process to yield fuel graded ethanol (Figure 1). Syngas fermentation is a simple process which takes place at near ambient temperature. Although it is characterized by a slower reaction rate, it has got several advantages over the conventional chemical catalytic process. Firstly, it has a high specificity, which leads to a higher yield, simplifies the downstream processing and reduces the concentration of toxic by-products. Secondly, the biocatalyst used is cheap, has high tolerance to sulfur (Vega et al., 1990) and is capable of adapting to contaminants such as tars (Ahmed et al., 2006). Thus the need of costly gas purification steps prior to conversion can be avoided. However, an appropriate filtering system can be used to negate the inhibitory effects of some toxic compounds present in the gas mixture. An advantage of the presence of sulfur compounds is that they can stimulate the growth of anaerobic bacteria by reducing the redox potential of the medium (Vega et al., 1990). Thirdly, bioconversion does not require a fixed H_2/CO ratio. Hence, one reactor vessel is enough to carry out the process by utilizing suitable microorganisms. Finally, the biocatalyst generally dies when exposed to air and the process is odorless, doesn't create any health hazard and generates less environmental pollution (Bioengineering Resources, Inc., 2007). The reaction process is limited by the mass transfer of gaseous

substrates to the medium as well as the need of maintaining rather sterile anaerobic conditions. A continuous supply of nutrients is needed to increase the efficiency of the bioconversion process. Various industries generate CO – rich waste gases in the plant. These waste gases could be captured before their emission into the atmosphere, using conventional techniques. Major industrial processes such as steel milling, non–ferrous products manufacturing, petroleum refining, electric power production, and methods of producing carbon black, ammonia, methanol and coke discharge enormous amounts of waste gases containing CO into the atmosphere either directly or through combustion. Some biocatalysts can then be exploited to convert the CO – rich substrate to ethanol. In such a system, the process occurs at near ambient temperature and pressure with high specificity, using biocatalysts that have the ability to tolerate or adapt to contaminants or impurities that are usually found in some waste gases (Wilkins and Atiyeh, 2011). However, it is highly desirable to treat these waste gases in order to remove any undesirable impurities before feeding them into the fermentor. For example, by making use of scrubbing and filtration methods.

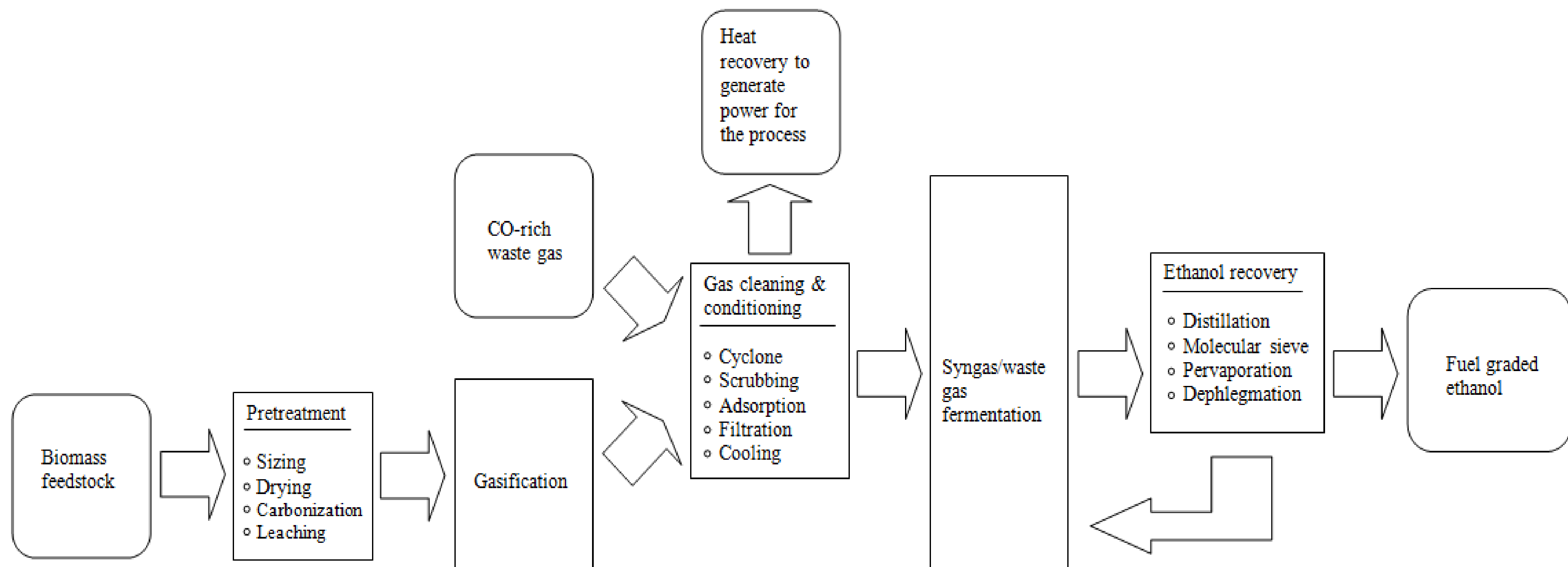


Figure 1: Syngas/CO-rich waste gas bioconversion process overview.

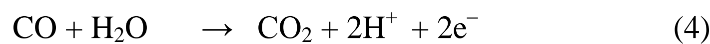
1.2 BIOCHEMICAL PATHWAY FOR ETHANOL PRODUCTION

The pathway which autotrophic anaerobes usually follow for the production of ethanol is the acetyl-CoA biochemical pathway or Wood-Ljungdahl pathway (Figure 2) (Ragsdale and Pierce, 2008; Henstra et al., 2007). This pathway is present in several organisms including homoacetogenic bacteria and methanogenic archaea (Ljungdahl, 2009). It contains an Eastern branch and a Western branch (Ragsdale, 1997). The Eastern branch comprises several reductive steps, where CO_2 is reduced to produce the methyl group of acetyl-CoA. The Western branch, which is unique in anaerobes, either generates CO from CO_2 or directly takes CO from the media which then serves as the carbonyl group for the acetyl-CoA synthesis.

The reducing equivalents for the process are generated from H_2 by hydrogenase enzymes (Hedderich, 2004).

$$H_2 \rightarrow 2H^+ + 2e^- \quad (3)$$

If H_2 is insufficient or inhibition of the hydrogenase enzyme occurs (Acosta et al., 2003; Tibelius and Knowles, 1984) then the reducing equivalents are produced via oxidation of CO to CO_2 using CODH (Ragsdale et al., 1983).



$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^- \quad (4)$$

It is worth observing that the sum of equation (4) and the reverse of equation (3) is the water gas shift reaction used to adjust the H_2:CO ratio during the chemical syngas conversion. The availability of CO as carbon source for ethanol synthesis thus decreases (equation 4) which can be interpreted using the below equations.

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \quad (5)$$

$$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O \quad (6)$$

It can be seen from equation (5), that only one third of the available carbon source (CO) can be theoretically converted to ethanol. This is because CO is used to produce the

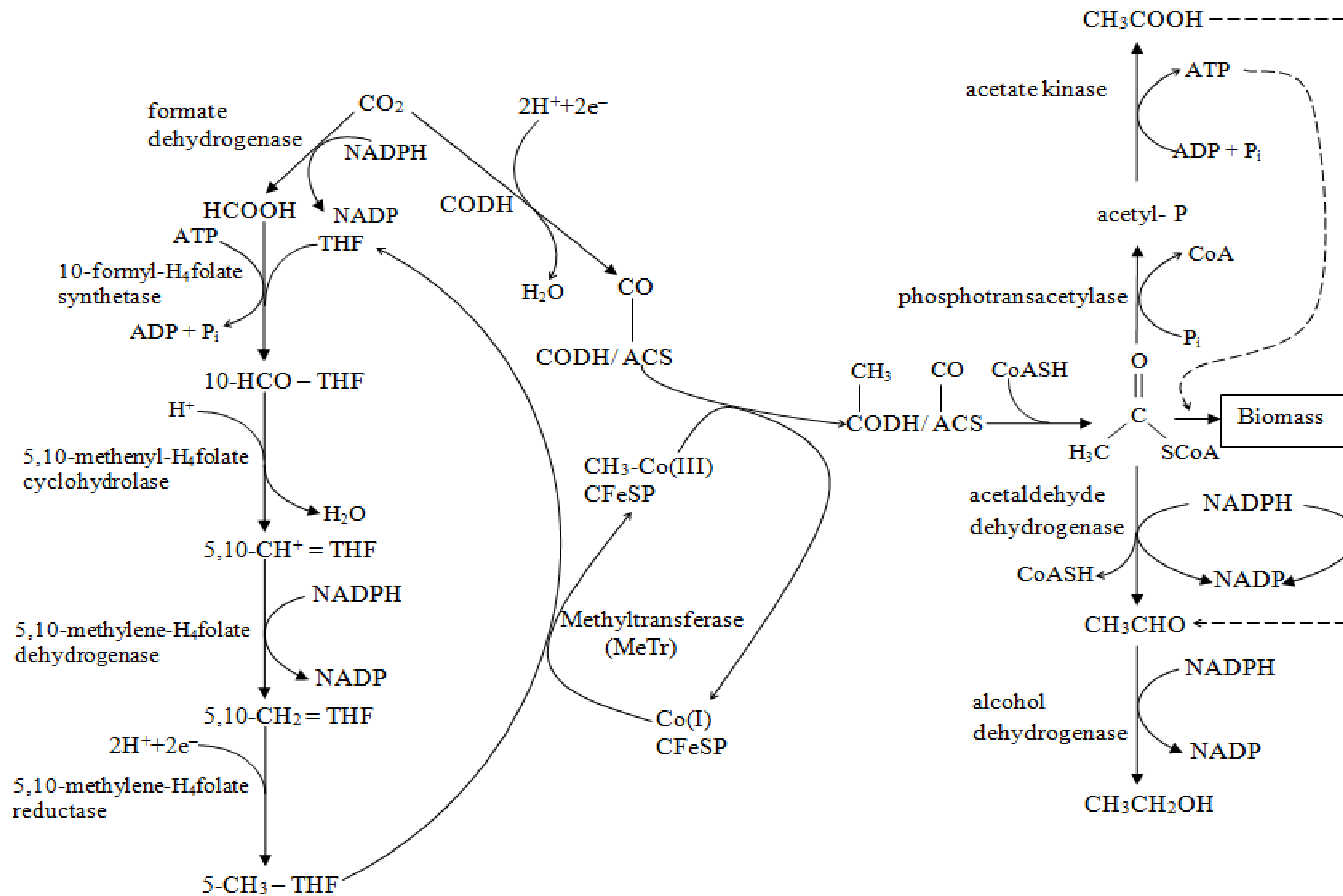


Figure 2: The Wood-Ljungdahl pathway for acetogenic microbes (CO to acetyl CoA) and reduction of acetyl-CoA to ethanol. Abbreviations: THF – Tetrahydrofolate; CFeSP – Corrinoid iron sulfur protein; CODH/ACS – CO dehydrogenase/acetyl-CoA synthase.

reducing equivalents by oxidation to CO_2 via CODH in the absence of H_2 or in the state of inhibition of the hydrogenase enzyme. Moreover from equation (6) it can be deduced that CO_2 can be used to make ethanol if H_2 is present in the syngas.

$$6CO + 6H_2 \rightarrow 2C_2H_5OH + 2CO_2 \qquad (7)$$

Finally from equation (7), for an equimolar mixture of CO and H_2, two-third of the carbon substrate (CO) can be converted to ethanol since sufficient reducing equivalents are provided by hydrogen with the help of hydrogenase enzymes with thus an increased carbon conversion rate.

1.2.1 Eastern branch

The Eastern branch is a H_4folate dependent pathway which involves several reductive steps to convert CO_2 to (6*S*)-5-CH_3-H_4folate. The first step is the conversion of CO_2 by formate dehydrogenase to formate, which is condensed with H_4folate to form 10-formyl-H_4folate catalyzed by 10-formyl-H_4folate synthetase (McGuire and Rabinowitz, 1978). A cyclohydrolase then converts the latter intermediate to 5,10-methenyl-H_4folate (Poe and Benkovic, 1980). The next step is a NAD(P)H-dependent reduction, where the methylene-H_4folate dehydrogenase converts the 5,10-methenyl-H_4folate to 5,10-methylene-H_4folate (Moore et al., 1974), which is reduced to (6*S*)-5-CH_3-H_4folate by methylene-H_4folate reductase (Clark and Ljungdahl, 1984). Thus, the conversion of CO_2 to the precursor of the methyl group of acetyl-CoA involves six electron reductions.

1.2.2 Western branch

The methyl group of the CH_3-H_4folate is transferred into the cobalt centre of the corrinoid/iron-sulfur protein (CFeSP) (Ragsdale et al., 1987) by the action of the methyltransferase (MeTr) (Doukov et al., 2000). This heterodimeric protein CFeSP (Svetlitchnaia et al., 2006) is active when the cobalt centre is in active Co(I) state. The

Co(I) then undergoes transformation into inactive Co(III) state by attaching a methyl group from the CH_3-H_4folate (Zhao et al., 1995). The most important step in the MeTr mechanism is the activation of the methyl group because of the higher stability of CH_3-N bond in CH_3-H_4folate. The most studied mechanism of activation of the methyl group is by protonation of the N5 group of the CH_3-H_4folate thus leading to the electrophilic activation of the methyl group (Seravalli et al., 1999; Smith and Matthews, 2000). Hence the first organometallic intermediate is formed as methyl-Co(III)-CFeSP.

One of the main enzymes in the Wood-Ljungdahl pathway is CO dehydrogenase (Ragsdale, 2008). This Ni-CODH is classified into two groups: a) Monofunctional (Drennan et al., 2001) and b) Bifunctional CODH (Doukov et al., 2002). The monofunctional CODH catalyses the oxidation of CO to CO_2, which is then reduced to formate and finally to the methyl group of acetyl-CoA. The bifunctional CODH converts CO_2 to CO, which then serves the carbonyl group of acetyl-CoA, and also catalyses the formation of acetyl-CoA along with acetyl-CoA synthase (ACS) (Hegg, 2004). Following the synthesis at the C-cluster of CODH, CO then migrates to the proximal Nickel (Ni_p) site ()of A-cluster in ACS forming next organometallic intermediate; Ni-CO (Seravalli and Ragsdale, 2000). The next step in the pathway involves the transfer of the methyl group from the methylated CFeS protein to the CODH/ACS complex. Thus the third organometallic complex, the methyl-Ni complex is formed (Barondeau and Lindahl, 1997; Seravalli et al., 2002). In the next step, condensation of methyl and carbonyl groups at the Ni_p form an acetylmetal, the final organometallic intermediate. Finally, in the Wood-Ljungdahl pathway, CoA together with ACS thiolysis the acetylmetal to form acetyl-CoA (Hu S-I et al., 1982; Roberts et al., 1992). Acetyl-CoA is converted by the cell to cell mass, acetate and ATP during the growth stage and to ethanol and NAD(P) during the non-growth stage.

Several enzymes are involved in the conversion of CO to acetyl-CoA and the subsequent production of metabolites such as acetic acid, ethanol or butanediol. Some of those enzymes have metals at their active sites (Ragsdale and Pierce, 2008). Therefore, the presence or lack of specific trace metals in culture media may affect the syngas bioconversion process. Metalloenzymes involved in biochemical reactions of interest and their physiological functions are described below. The first reaction in the methyl branch is two-electron reduction of CO_2 to formate catalyzed by the enzyme formate dehydrogenase (FDH). FDH in *C. thermoacetica* is NADP-dependent and contains 2 W atoms, 2 Se, 36 Fe and around 50 inorganic S in the form of an FeS cluster. Recently, Wang et al. (2013) reported that FDH of *C. autoethanogenum* forms complexes with FeFe-hydrogenase containing iron, tungsten and selenium. They also discussed that FDH of *C. autoethanogenum* is a tungsten specific protein rather than a molybdenum protein as found in *E. Coli*. The monofunctional CODH that catalyses the reaction equivalent to the water gas shift (WGS) reaction produces two protons and two electrons instead of hydrogen as opposed to that WGS reaction. The bifunctional CODH/ACS contains the metals Fe and Ni at the active sites (Ragsdale and Pierce, 2008). The protein CoFeSP that transfers the methyl group to the CODH/ACS complex is a cobalt containing heterodimeric protein with active Co(I) state (Svetlitchnaia et al., 2006). Another metalloenzyme involved in this pathway is the aldehyde:ferredoxin oxidoreductase (AFOR), which is a tungsten containing enzyme that catalyzes the reduction of acetic acid to acetaldehyde using electrons from the reduced ferredoxin (Fd^{2-}). The AFOR of the hyperthermophilic archaeon, *Pyrococcus furiosus*, is a homodimeric enzyme with 1 W and 4-5 Fe atoms per subunit (Kletzin and Adams, 1996). The sulfate reducing bacterium, *Desulfovibrio gigas* has also been reported to contain 0.68 W, 4.8 Fe and 3.2 S per subunit (L'vov et al., 2002). *C. formicoaceticum*

possesses a molybdenum containing aldehyde oxidoreductase (0.6 Mo atoms per subunit) (Kletzin and Adams, 1996). Moreover, there are evidences that the two tungsten containing enzymes FDH and AFOR have a molybdopterin cofactor (Ragsdale and Pierce, 2008; Wang et al., 2013).

The effect of trace metals on microbial growth and metabolites production has been investigated by some researchers, who studied their effect on the activity of various enzymes involved in the WL pathway. Andreesen and Ljungdahl (1973) reported that the addition of W, Se and Mo in the growth medium enhanced the FDH activity of *C. thermoacetica.* They also demonstrated that tungsten could replace molybdate and stimulate the FDH activity even more. However, not much information has been reported of the effect of trace metals on syngas fermentation to biofuels.

In a recent publication, it was reported that increasing the concentrations of nickel, zinc, selenium and tungsten improved ethanol production in *C. ragsdalei* (Saxena and Tanner, 2011). They also reported that complete elimination of nickel resulted in no growth. Moreover, in that study, activities of FDH, CODH and ADH decreased from 32.25, 38.45 and 0.68 U/mg protein to 7.01, 9.07 and 0.24 U/mg protein when the Fe concentration was reduced from 20.4 µM to 0 µM.

1.3 EFFECTS OF PROCESS PARAMETERS ON THE BIOCONVERSION OF CARBON MONOXIDE AND SYNGAS

In most of the published studies, the production of acetic acid prevailed over ethanol production. During the condition of reduced or non availability of CO, the produced alcohol can convert back to acetic acid in the presence of carbon dioxide (Adams et al., 2010). The acclimation of a microbial culture to low liquid nutrients concentrations results in a poor (ethanol/acetic acid) ratio of less than 1 and that could finally lead to an irreversible low performance of the culture (Adams et al., 2010). In general, various

parameters such as fermentation pH, temperature, syngas composition, CO partial pressure, media redox potential, mass transfer, NAD(P)H to NAD(P) ratio, among others affect the overall ethanol production (Abubackar et al., 2011; Mohammadi et al., 2011). Hence, it is worth optimizing these parameters in order to improve ethanol production relative to the other metabolite's production normally seen during the acetogenic fermentation, and to save on operating costs.

1.3.1 Fermentation medium

The syngas or CO fermentation medium must contain ingredients that can provide essential elements such as nitrogen, phosphorus, trace metals for the growth and production of metabolites. The carbon and energy necessary for the bacteria is obtained either from CO or CO_2/H_2, but in order to achieve a high product titer, there is a need to reach a high cell mass concentration in the bioreactors. A major phenomenon observed in CO/syngas fermentation studies is that nutrient-rich media stimulate cell growth and the simultaneous formation of acetate. This can be linked to the production of ATP during acetate production. Conversely, nutrient-limited conditions promote solventogenesis, i.e. ethanol rather than acetate production. This could be, for example, phosphate-limited media. Since syngas fermentation to ethanol is performed by anaerobic acidogenic bacteria, reduced anaerobic media need to be used, with a negative Redox potential. Therefore, a reducing agent such as cysteine-HCl or $Na_2S.9H_20$ is added to the medium in a way to lower the redox potential (Panneerselvam et al., 2010). However, above a certain concentration, reducing agents may become inhibitory for growth. This was observed in recently published studies in which experiments were performed with *C. aceticum* (Sim and Kamaruddin, 2008).

In a view to reduce the cost of the media used for bioethanol production, some researchers checked the possibility to work with low-cost media that could replace

expensive standard media (Kundiyana et al., 2010b; Liu et al., 2014; Maddipati et al., 2011). Nutrient media such as corn steep liquor (CSL) and cotton seed extract (CSE) have been tested in syngas bioconversion studies in replacement of YE. The industrial price of CSL is 2% of the price of YE (Maddipati et al., 2011). In a study performed in 250-ml bottles to compare the effect of replacing YE with CSL, about 84% more cell mass was obtained with 10 g/L CSL medium than with 1 g/L YE medium. Both media had, otherwise, the same standard composition, except for the replacement of YE by CSL in each of the two different experiments (Maddipati et al., 2011). However, in another study using *Alkalibaculum bacchi*, 38% more cell mass was obtained with 1 g/L YE than with 50 g/L CSL. It was suggested that this might have been due to the presence of minerals added into the YE medium. However, the maximum biomass obtained with 1 g/L YE and 50 g/L CSL, respectively, where 330 mg/L and 240 mg/L (Liu et al., 2014). The limiting nutrients in CSL were identified as NH_4^+, trace metals and the reducing agent. The addition of 2% (w/v) CSL led to the formation of a higher amount biomass compared to the control *i.e.*, standard medium. However, a drastic decrease in growth of *C. ragsdalei* was reported when 5% (50 g/L) and 10% (w/v) CSL were used (Saxena and Tanner, 2012). On the other hand, the cost of CSE is less than 0.5% that of YE (Kundiyana et al., 2010b). Studies were also performed to evaluate the feasibility of incorporating CSE as the complete nutrient medium for syngas fermentation. However, due to accumulation of cell mass onto the CSE solids, the cell concentration could not be measured (Kundiyana et al., 2010b).

Besides the trace metals mentioned above, minerals such as Ca^{2+}, Mg^+, Na^+, K^+, NH_4^+ and PO_4^{3-} have also been reported to have an influence on syngas fermentation (Abubackar et al., 2015b; Guo et al., 2010; Saxena and Tanner, 2012). Also, eliminating Na^{2+} from the medium did not affect the growth of *C. ragsdalei*. Conversely, increasing

the concentration of Na^{2+} from 34.2 mM to 171 mM was found to inhibit its growth by 50% (Saxena and Tanner, 2012). No effect on growth was observed whenever eliminating Ca^{2+} and K^{+} from the standard medium, besides Na^{2+} (Saxena and Tanner, 2012). The effects of the above mentioned media components on metabolite production will be explained below in their respective sections.

1.3.2 Fermentation pH

Fermentation pH has been reported to be an important factor influencing syngas fermentation (Abubackar et al., 2011; Bengelsdorf et al., 2013; Mohammadi et al., 2011). Every organism has its optimum pH allowing to reach its highest metabolic activity. In syngas fermentation, the optimum growth-pH is favorable for acidogenesis (high acetic acid production rate), while a lower pH supports solventogenesis (high rate of production of alcohols). In most cases, applying a low pH as a way to promote solventogenesis will cause a negative impact on cell growth and thereby influence negatively the overall metabolite production. There are various research publications supporting this fact. It is suggested that acetic acid is lipophilic and permeates through the cell membrane into the medium that creates a low internal pH inside the cell because of the conduction of H^{+} ions from the cell. Under these circumstances, along with a low external pH, the cells will be in a stress environment. To overcome this situation, the cell would then start producing alcohols (Bengelsdorf et al., 2013). Some researchers suggested the use of two-stage continuous systems, with two bioreactors in series, in order to obtain high ethanol titers (Gaddy and Clausen, 1992; Richter et al., 2013). Conditions such as an optimum ("high") growth pH and a rich nutrient medium are maintained in the first stage, to promote rapid growth and acidogenesis. In the second stage, different conditions, such as nutrient limitation, are maintained, in order to trigger solventogenesis. Such a set-up allows to convert part of the acetic acid produced in the

first stage into ethanol in the second stage. In a given recent experiment, a 1-L CSTR was used as the first stage followed by a 4-L bubble column reactor equipped with a hollow fiber module as the second stage (Richter et al., 2013). The molar ethanol to acetic acid ratios obtained under steady state conditions were 0.078 and 3 in the first and second stage, respectively.

1.3.3 Effect of gas composition

The producer gas from biomass gasification contains several impurities that interfere in the fermentation process. However, the types of impurities generated during the gasification step will depend upon the feedstock and the gasification technology used and therefore an efficient gas cleaning system would likely need to be setup prior to feeding the syngas to the fermentation process. Accumulation of these impurities in the fermentation media may have inhibitory or stimulatory effects on the fermentation process, including, among others cell dormancy, enzyme inhibition, product redistribution, modified redox potential, osmolarity or pH (Mohammadi et al., 2011). The raw syngas or producer gas mainly composed of CO, CO_2 and H_2, also contains carbonaceous (CH_4, C_2H_2, C_2H_4, C_3H_8, tars), nitrogenous (NH_3, HCN, NO_x) and sulfurous compounds (H_2S, COS, SO_x) (Abubackar et al., 2011). Xu et al. (2011) reported that most of these impurity species are potent inhibitors of enzymes involved in the Wood–Ljungdahl pathway and other pathways followed for ethanol production, including formate dehydrogenase (NO_2), alcohol dehydrogenase (NH_3, NO), hydrogenase (NO) and carbon monoxide dehydrogenase (COS), among others. Studies using the biomass generated producer gas with *C. carboxidivorans* P7 showed that the presence of tar and NO affects cell growth and product redistribution (Ahmed and Lewis, 2007). However, later, it was confirmed that the effects due to the presence of tar can be mitigated by using a cyclone, scrubber (10% acetone) and a 0.025 μm filter prior

to the introduction of syngas into the fermentor. The authors also revealed that *C. carboxidivorans* P7 could overcome those adverse effects after prolonged exposure to it (Ahmed et al., 2006). Ahmed and Lewis (2007), in their studies concluded that levels below 40 ppm NO had no effect on *C. carboxidivorans* activity during syngas fermentation. They also indicated that NO above 40 ppm is a potent inhibitor of hydrogenase enzymes which in turn reduces the available carbon for ethanol production. Preliminary work using *C. ragsdalei* to understand the effect of NH_3 concluded that cell growth, product distribution and hydrogenase activity were negatively affected by the ammonium ions accumulated in the fermentation media. It was attributed to the fact that the inhibition of cell growth was due to the increase in osmolarity due to the build of $NH4^+$ (Xu et al. 2011). However, sulfurous compounds would stimulate the growth of anaerobic bacteria by reducing the redox potential of the fermentation medium as well as scavenging residual oxygen (Hu et al., 2010). Another study with *Rhodospirillum rubrum* showed that the presence of 10% (v/v) acetylene in the gas phase causes a 50% inhibition of CO–linked hydrogenase. As mentioned by Xu et al. (2011), the degree of impact due to these impurities mainly depends on the solubility in liquid media and hence, it is evident that proper clean–up of raw syngas is necessary to alleviate the problem associated with the impurities.

1.3.4 Mass transfer

One potential bottleneck of syngas fermentation is mass transfer limitations (Klasson et al., 1993; Riggs and Heindel, 2006). When the fermentation broth contains a high cell concentration, the system is said to be in a mass transfer limited state, which is due to the low aqueous solubilities of the sparingly soluble gaseous substrates, CO and H_2. Due to these diffusion limitations, availability of gaseous substrates for the microorganisms becomes low, which eventually leads to reduced productivity. The

yield from the process also becomes low when the system is under kinetic-limited conditions, which happens when either the cell concentration or the CO consumption rate is too low (Vega et al., 1989). Both of these two rate limiting conditions may occur during the course of syngas fermentation.

From the theoretical equations of syngas fermentation (equations 5 and 6), it is clearly observed that 6 moles of CO or H_2 have to transfer into the culture medium to produce one mole of ethanol. Moreover, on a molar basis, the solubilities of CO and H_2 are only 77 and 68%, respectively to that of oxygen at 35 ^{0}C (Kapic et al., 2006). Hence, more moles of syngas must be transferred per carbon equivalent consumed in order to enhance the yield and productivity.

Gas-liquid mass transfer is of prime importance and the various gas components present in the bioreactor have to overcome a series of transport resistances before being utilized by the biocatalyst. The overall mass transfer rate of a gaseous substrate to the liquid phase is given by the product of the mass transfer coefficient, available area for mass transfer, and the driving force. The driving force for diffusion in this case is the difference between the actual partial pressure of the substrate in the bulk gas phase, P^g (atm), and the partial pressure of the substrate that would be in equilibrium with the substrate in the bulk liquid phase, P^l (atm). Thus, the overall mass transfer rate can be defined as;

$$\text{Overall mass transfer rate} = \frac{K_L a}{H}\left(P^g - P^l\right) \qquad (8)$$

Where, H is the Henry's constant (L atm mol^{-1}) and $K_L a$ is the volumetric mass transfer coefficient (s^{-1}).

Since the solubility of the substrate in the culture medium or in the biofilm is low, the amount of substrate present in the liquid phase is negligible compared to the substrate in the gas phase. Thus the substrate balance in the gas phase is given by

$$-\frac{1}{V_L}\left(\frac{dN_s}{dt}\right) = \frac{K_L a}{H}\left(P^g - P^l\right) \qquad (9)$$

Where N_s (mol) is the molar substrate concentration in the gas phase and V_L (L) is the volume of the reactor. From the above equation (equation 9), the mass transfer coefficient K_L (m s^{-1}) for the gaseous substrate can be determined.

The Andrew or Haldane model have been used to determine the kinetic substrate utilization and inhibition in syngas fermentation. The specific consumption rate q_s, which is the substrate uptake per dry cell weight, is given by

$$q_s = \frac{q_s^{max} P^l}{K_p + P^l + (P^l)^2 / K_i} \qquad (10)$$

Where q_s is the specific substrate consumption rate (h^{-1}), q_s^{max} is the maximum specific substrate consumption rate (h^{-1}), K_p is constant (atm) and K_i is the substrate inhibition constant (atm).

Ungerman and Heindel (2007) compared CO-water $K_L a$ and power demand in a stirred tank reactor using different impeller designs and schemes and it was found that the highest mass transfer coefficient was obtained with the dual Rushton impeller scheme. Compared with the standard (single) Rushton impeller scheme, the dual Rushton impeller scheme could enhance the mass transfer by up to 27%. However, the impeller performance, which is the measure of volumetric mass transfer coefficient per unit power input, was lowest for the dual Rushton. As discussed later, increasing the

agitation speed as a way to improve the mass transfer consumes more power. Hence this method is not economically feasible for large-scale bioethanol production. Bredwell et al. (1999) reviewed various bioreactor studies on syngas fermentation using conventional stirred tank and columnar reactors and observed that the volumetric mass transfer coefficient in these bioreactors depends mainly on reactor geometry, configuration, process operating conditions and the liquid phase properties. Jin at al. (2009) observed that CO biodegradation in industrial waste gases was higher in packed bed bioreactors with lower amounts of liquid phase compared to suspended growth bioreactors.

Various additives can be added to increase the gas-liquid mass transfer rates which include surfactants, alcohol, salts, catalyst and small particles (Zhu et al., 2008). Ethanol concentration of 1% (w/v) in the fermentation broth was shown to increase the mass transfer rate up to three fold compared to clean water (Hickey, 2009). This is due to the change in surface tension, thereby formation of small gas bubbles and hence better surface area for mass transfer. A new approach to enhance the mass transfer is by using nanoparticles. Zhu et al. (2008) found that surface hydroxyl and functional groups on the nanoparticles have influence in enhancing the CO-water mass transfer coefficient. The highest $K_L a$ enhancement of 1.9 times was obtained when mercaptan groups were grafted on the nanoparticles.

1.3.5 Bioreactors

Different bioreactor configurations have been tested for bioethanol production from syngas. They have been described recently (Abubackar et al., 2011). One of the major bottlenecks of syngas fermentation is low mass transfer efficiency of gaseous substrates (Munasinghe and Khanal, 2010; Ungerman and Heindel, 2007). The most commonly used system is the stirred tank bioreactor (STB) (Kundiyana et al., 2011; Mohammadi et

al., 2012). However, such suspended growth bioreactors are theoretically not the best systems for the bioconversion of poorly soluble, volatile, substrates. Mass transfer from the gas phase to the biocatalyst grown in suspension can be achieved by reducing the bubble size or increasing the stirring speed. However, doing the latter will increase energy needs and the operation costs. Typical ethanol concentrations reached in short term (up to a few weeks or a few months) lab-scale and pilot-scale STB studies reached between a few grams per liter up to about 48 $g_{Ethanol}$/L in the liquid effluent continuously recovered from the system, with ethanol to acetate ratios between less than 1 up to about 20 (Table 2). However, any other type of bioreactor that has commonly been used at full-scale for waste gas treatment can also be used for the bioconversion of volatile substrates. Besides stirred tank systems, bioreactors suitable for waste gas treatment and gas bioconversion, include biofilters and biotrickling filters, membrane bioreactors, fluidized bed bioreactors, moving bed bioreactors, bubble column bioreactors, and monolith bioreactors with Taylor flow (Abubackar et al., 2011; Kennes and Veiga, 2001; Kennes and Veiga, 2013). The stirred tank bioreactor seems to have yielded the best results so far and to have been used in most cases even at demonstration scale, although theoretically packed bed bioreactors, such as biofilters or biotrickling filters would exhibit a lower resistance to mass transfer of poorly water-soluble substrates such as CO, CO_2, H_2 from the gas phase to the microbial biofilm (Jin et al., 2009). Some other bioreactors that have been tested include membrane bioreactors and, more recently, a more novel system, *i.e.* the monolith bioreactor that was originally developed for waste gas treatment and air pollution control (Jin et al, 2006; 2008) In an abiotic study with HFM-BR, the volumetric mass transfer coefficient (K_La) obtained was higher than most of the other reactor's value reported in the literature (Shen et al., 2014a). A continuous HFM-Br study using *C. carboxidivorans* P7 led to achieve 23.93 g/L of

ethanol with a maximum ethanol productivity of 3.44 g/L-day and ethanol to acetic acid ratio of 4.79 (Shen et al., 2014a). Enhanced ethanol production was also reported while using monolith biofilm reactor (MBR) where using a MBR, *C. carboxidivorans* P7 gave 53% enhanced ethanol productivity than with BCR (Shen et al., 2014b).

Table 2: Studies performed with various reactor configurations using acetogenic bacteria in syngas fermentation

Reactor configuration	Syngas composition (%)	Microorganism	Ethanol production (g/L)	EtOH/Ac	Reference
HFM-BR	CO=20, H_2=5, CO_2=15, N_2=60	C. *carboxidivorans* P7	23.93	4.79	Shen et al., 2014a
MBR	CO=20, H_2=5, CO_2=15, N_2=60	C. *carboxidivorans* P7	4.89	2.1	Shen et al., 2014b
STR	CO=55, H_2=20, CO_2=10, Ar=15	C. *ljungdahlii*	6.5	1.53	Mohammadi et al., 2012
STR	CO=20, H_2=5, CO_2=15, N_2=60	C. *ragsdalei* P11	25.26	6.8	Kundiyana et al., 2010a
CSTR-BC	CO=60, H_2=35, CO_2=5	C. *ljungdahlii* ERI-2	19.73	3	Richter et al., 2013
CSTR (Cell recycle)	CO=55, H_2=20, CO_2=10, Ar=15	C. *ljungdahlii*	48	21	Phillips et al., 1993
ICR	CO=13, H_2=14, CO_2=5, N_2=68	C. *ljungdahlii* ERI-2	2.74	0.64	Gaddy, 2000

Abbreviations: HFM-BR, Hollow fiber membrane biofilm reactor; MBR, Monolith biofilm reactor; CSTR; Continuous stirred tank reactor with continuous liquid and gas flow; STR, Stirred tank reactor with liquid batch; CSTR-BC, CSTR-bubble column; ICR, Immobilized cell reactor. Ethanol/acetic ratio is represented in molar concentrations.

1.4 CELL SEPARATION AND ETHANOL RECOVERY

Microorganisms grow either in planktonic form, or as a biofilm on a solid matrix usually on membranes. Cell retention and thereby increase in cell density is possible by the formation of a biofilm attached on a solid support in the bioreactor. Conversely, in suspended-growth reactors, cells grow in suspension and are separated from the product stream by employing solid/liquid separators, which includes membranous ultrafiltration units, hollow fibers or spiral wound filtration systems or centrifuges (Huang et al., 2008). Thus, the cells can return back to the bioreactor.

The concentration of ethanol in the fermentation broth must be kept below a certain level in order to prevent microbial inhibition and to maintain the cells metabolically active. Moreover, biomass derived syngas fermentation usually produces low concentrations of ethanol (below 6%); hence, to economically recover ethanol, an efficient recovery process is required, which includes distillation followed by molecular sieve separation or pervaporation followed by dephlegmation technologies (Gaddy et al., 2007; Vane et al., 2004). Integration of vacuum distillation columns and vapor permeation units has numerous advantages such as amenability to separate ethanol from the fermentation broth even when ethanol concentration is as low as 1% where approximately 99% by weight of dehydrated ethanol can be recovered by this process (Datta et al., 2009). Formation of toxic by-products due to high temperature can be precluded, since vacuum distillation does not require high temperature. Hence, the majority of the distillation column bottoms can be recycled to the fermentor without any prior treatment. Another approach to enhance the concentration of ethanol in the feed to the vacuum distillation column is by flashing the feed before it enters the vacuum distillation column (Datta et al., 2009). Coskata, Inc., Illinois uses a licensed membrane separation technology to separate the ethanol from water thereby a reduction in energy

requirement has been achieved compared to conventional distillation (Datta et al., 2012).

1.5 CHALLENGES AND R&D NEEDS FOR COMMERCIALIZATION OF BIOETHANOL PRODUCTION USING GAS FERMENTATION

1.5.1 Feedstock

The feedstock for syngas production encompasses a wide spectrum of biomass materials such as forest residues, agricultural and organic solid wastes, amongst others. Feedstock properties, for example, a high moisture content has a negative influence on the CO fraction produced in the gasifier. In such case, considerable energy is required for drying the biomass in order to keep the moisture content around 10-15% (Piccolo and Bezzo, 2009). Every biomass contains ash and volatile compounds; the content varies from one feedstock to another. For instance, ash content in rice husk is about 15-25%, whereas in wood it is 2% or less (Deluga et al., 2009). Gasification of such feedstock produces impurities that inhibit the syngas fermentation. Thus extensive gas cleaning steps are required prior to feeding into the bioreactor, which substantially increases the overall production cost. However, the nitrogen and alkali contents of the biomass can be greatly reduced by upstream treatments like fractionation and leaching (McKendry, 2002). It is quite obvious that an appropriate feedstock requires less pretreatment and results in less syngas contaminant production, making ethanol production a process consuming less energy.

1.5.2 Gasification system and syngas purity

Various impurities are produced during gasification of biomass along with CO and H_2 which may cause problems in the subsequent bioconversion steps. The composition of the gas produced in the gasifier is greatly influenced by the gasifier configuration and

the operating conditions. The equipment size can be decreased by feeding the gasifier with pure oxygen. But it will increase the overall cost for the process. The pyrolysis of volatile compounds releases tars, which not only affects the microbial activity during syngas fermentation but also gets deposited on the walls of the gasifier and gas transfer system, which ultimately decreases the performance of the gasifier. Using light hydrocarbons, the tar produced during the gasification can be substantially converted to syngas. About 90% of the tar generated in the gasifier is able to crack by this way (Deluga et al., 2009). On the other hand, the feasibility of using light hydrocarbons derived from renewable energy sources and subsequent use of the produced syngas for microbial utilization to biofuels have yet to be explored.

1.5.3 Microorganisms and media composition

Isolation of high yielding (>25 g L^{-1}) ethanologenic homoacetogens, which have greater tolerance to high ethanol concentrations in the fermentation broth, is necessary for successful commercialization of syngas fermentation. Moreover, culturing of anaerobic microorganisms requires specialized techniques to maintain the system under oxygen-free conditions. Thermophilic microorganisms having the above features might be interesting since less cooling of syngas would be required prior to feeding the bioreactor and an elevated temperature can improve the conversion rate. Another task is to enhance the ethanol production by modifying metabolically the available syngas fermenting microbes through genetic engineering.

There are many factors to be considered while selecting fermentation media for large scale ethanol production such as, but not limited to, media complexity, cost, or presence of chemicals that could improve ethanol productivity. Identifying unique media for specific microorganisms which satisfy the above features is one of the important challenges faced by ethanol producers. Recently, it was reported that cotton

seed extract (CSE) can be used as the sole fermentation medium for culturing *C. ragsdalei* P11 for ethanol production (Kundiyana et al., 2010)

1.5.4 Mass transfer and scale-up

As discussed before, one of the main challenges faced during syngas fermentation is the gas-liquid mass transfer resistance. Various techniques to improve mass transfer of the syngas in STR have been discussed elsewhere (Bredwell et al. 1999; Ungerman and Heindel, 2007). However, for commercial-scale bioreactors more efficient and economical mass transfer systems have to be found.

For scale-up, a clear understanding and estimation of the volumetric mass transfer coefficient (K_La) is required. The achievement of a high syngas mass transfer rate with minimal power consumption and relatively low shear rates, whilst maintaining an anaerobic atmosphere, is a major challenge for syngas fermentation scale-up. More research is still necessary for syngas fermentation scale-up.

1.5.5 Product recovery

The low microbial resistance to ethanol in the fermentation broth is one major obstacle in developing this technology. Furthermore, the fermentation broth also contains other dissolved and undissolved compounds such as cell extracts, unfermented soluble compounds, which also create separation problems during ethanol recovery. For these reasons, *in situ* ethanol separation is considered a better choice by coupling the fermentor vessel with various unit operations (Datta et al., 2009). Still novel separation systems have to be tested to overcome these challenges and thus increasing ethanol volumetric productivity.

1.5.6 Production costs

There are various parameters affecting the techno-economics of syngas fermentation. For instance, the cost of different feedstock regulates the overall production costs. In

one recently published report, feedstock cost has shown to account for about 67% of the total production costs, even when dry biomass wood was used, without considering the depreciation factor (Piccolo and Bezzo, 2009). Besides feedstock, the need to maintain the selected pure biocatalyst can also have a sizable impact on the production costs. Xia and Wiesner (2008) compared the production costs involving two microorganisms, and pointed out that, out of the two acetogens chosen, *C. ljungdahlii* showed better ethanol yield with production costs much lower than for *Moorella sp.* HUC22-1, excluding the operational cost and depreciation terms. This was attributed to the high ethanol production over acetate (3:1) of *C. ljungdahlii* over *Moorella sp.* HUC22-1 (1:28).

Although producing ethanol using syngas fermentation demands very less energy input, process modification and optimization steps are still at the development stage in order to achieve remarkably high process yields (Wei et al., 2009; Piccolo and Bezzo, 2009). From a literature view-point, only very few studies have undertaken a systematic evaluation of the techno-economics involved in the syngas fermentation process, and more detailed studies relating the costs to mass-energy balances, flow sheet modeling and life cycle assessment should be initiated in order to obtain a valuable database.

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Chapter 2

MATERIALS AND METHODS

2.1 MICROBIAL CULTURE

Clostridium autoethanogenum strain was used throughout the experimental studies. The strain (DSM 10061) was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). *C. autoethanogenum* was originally isolated from rabbit feces using CO as sole carbon and energy source. Electron microscopic studies using an old culture revealed that after a long period of incubation the cell morphology changed from rod-shaped to continuous chains of encapsulated filaments having a size of 0.6 x 42.5 μm along with the normal cells (Abrini et al., 1994). It was grown and maintained on DSMZ medium 640 with 0.5% xylose (Table 1). The medium was prepared by boiling for a few minutes, while being degassed, and then cooled continuously under N_2 for 15 minutes to remove oxygen. Cysteine – HCl was added, and the pH of the medium was adjusted to 6.0 by adding either 2M HCl or 2M NaOH.

Table 1: Growth medium (DSMZ 640 medium)

Chemical	Concentration (g/L)	SL-10 trace metal	Concentrations (mg/L)
NH_4Cl	0.9	$FeCl_2 \cdot 4H_2O$	1500
NaCl	0.9	$ZnCl_2$	70
$MgCl_2 \cdot 6H_2O$	0.4	$MnCl_2 \cdot 4H_2O$	100
KH_2PO_4	0.75	H_3BO_3	6
K_2HPO_4	1.5	$CoCl_2 \cdot 2H_2O$	190
$FeCl_3 \cdot 6H_2O$	0.0025	$CuCl_2 \cdot 2H_2O$	2
Trypticase peptone	2.0	$NiCl_2 \cdot 6H_2O$	24
Yeast extract	1.0	$Na_2MoO_4 \cdot 2H_2O$	36
Cysteine-HCl	0.75	7.7 M HCl	10 mL
Resazurin (0.1 %)	0.5 ml		
Xylose	5		
SL-10 solution	1 ml		

The production media used for the experimental studies are described in the corresponding chapters of this thesis.

2.2 BIOCONVERSION STUDIES

2.2.1 Bottle batch experiments

For batch experiments, serum vials with a total volume of 200 mL were used, with 75 mL working volume for experiments described in Chapter 3 and 4. For Chapter 5, studies were carried out in 100 ml serum vials with 30 ml production medium. The experimental set-up and the method used for media preparation are described below.

Respective amount of prepared medium was transferred into experimental vials, boiling for a few minutes, while being degassed, and then cooled continuously under N_2 for 15 minutes to remove oxygen. Cysteine – HCl was added, and the pH of the medium was adjusted by adding either 2M HCl or 2M NaOH. The vials containing the growth medium were then sealed airtight with gas impermeable viton rubber stoppers, and fitted with aluminium crimps. The vials were then sterilized by autoclaving at 120 ^{0}C for 10 min. Two 3-way stop cocks with needle were inserted into the vials through the stopper for sampling purpose. The photographic view of the experimental set-up is shown in Figure 1. The vials were then inoculated with 10% actively growing seed culture, which was grown with CO as sole carbon source. They were pressurized to 1.2 bar with 100% CO using a pressure gauge and were agitated at 150 rpm inside an orbital incubator at 30 ^{o}C. Headspace samples of 0.2 mL were used for CO measurements, and 1 mL of liquid sample was periodically withdrawn from the vials using a push button valve and a syringe (once every 24 h) in order to measure the optical density ($OD_{\lambda=600\ nm}$) related to biomass concentration. The same 1 ml sample was then centrifuged for 10 min (25 ^{0}C, 7000 x g) or filtered using a 0.22 μm PTFE syringe-filter before being used to check metabolite concentrations.

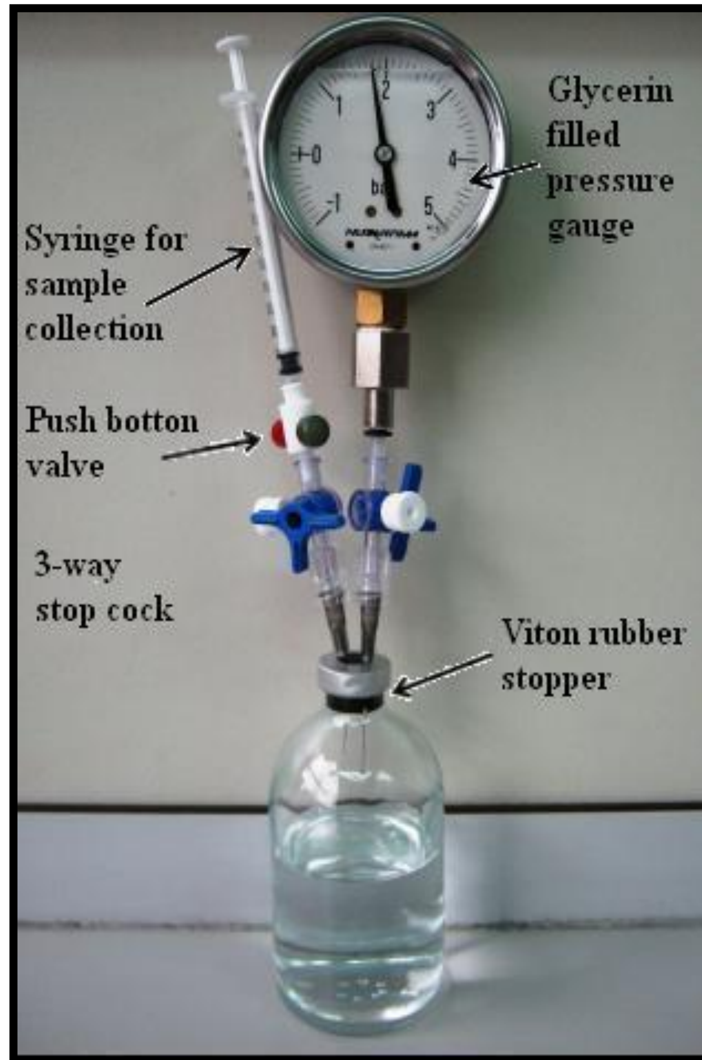


Figure 1: The photographic view of the batch experimental set-up.

2.2.2 Continuous gas-fed bioreactor experiments

The bioreactor experiments were carried out in 2 L BIOFLO 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA). The experiments were done with 1.2 L batch liquid medium and CO (100%) as the gaseous substrate, continuously fed at a rate of 10 or 15 mL/min using a mass flow controller (Aalborg GFC 17, Müllheim, Germany). The bioreactor with the medium was autoclaved and cysteine-HCl (0.75 g/L) was added after cooling, together with nitrogen feeding to ensure anaerobic conditions.

The bioreactor was maintained at a constant temperature of 30 °C with a constant agitation speed of 250 rpm throughout the experiments (Figure 2). 10% of an actively growing culture, which was grown for 48 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 or at 6.0, through addition of either a 2 M NaOH solution or a 2 M HCl solution, fed by means of a peristaltic pump. Gas samples of 0.2 mL were taken from the inlet and outlet sampling ports of the bioreactor to monitor the CO and CO_2 concentrations. Similarly, 2 mL liquid samples were periodically withdrawn from the reactor, once every 24 h, in order to measure the optical density (ODλ = 600 nm), allowing to estimate the biomass concentration. Afterwards the sample was filtered with a syringe using a 0.22 µm PTFE-filter before analyzing the concentrations of water-soluble products.

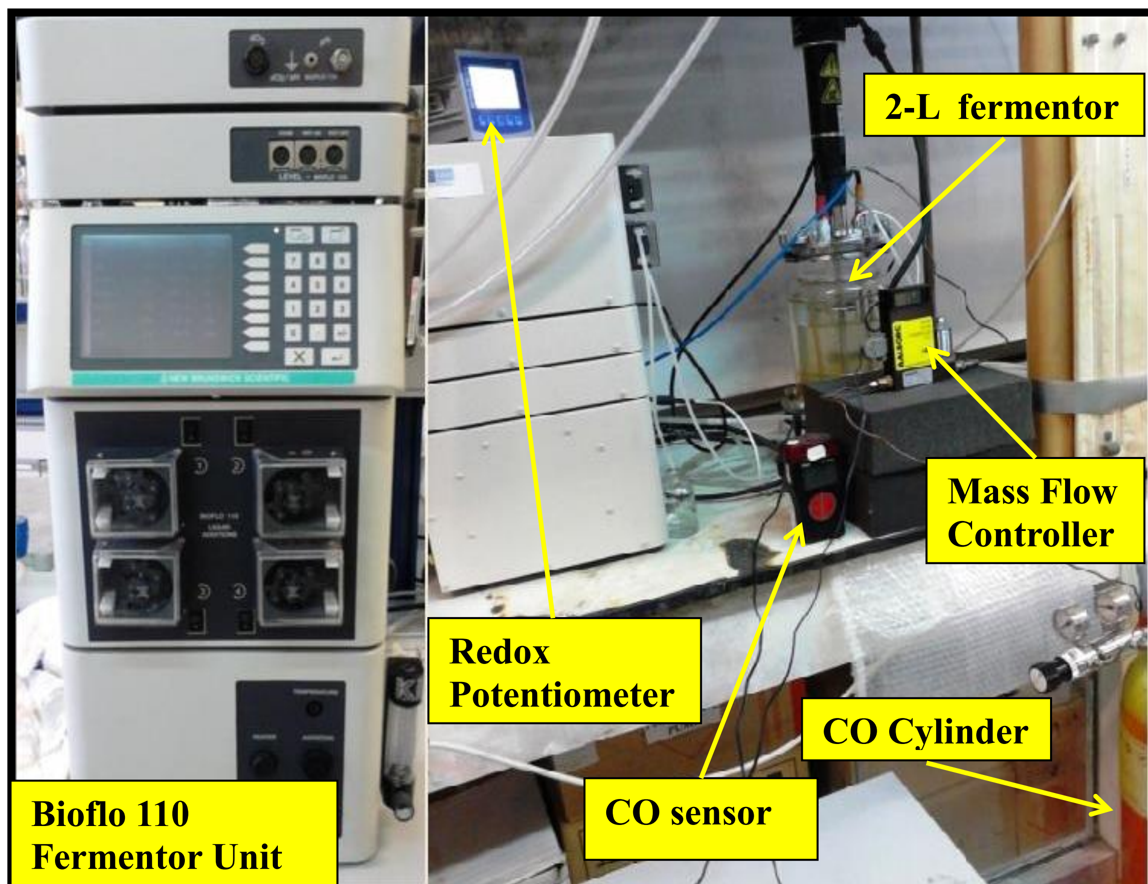


Figure 2: Photographic view of Continuous Gas-Fed Bioreactor

2.2.2.1 Media replacement

In some of the experiments described in Chapter 6 and Chapter 7, part of the fermentation broth (600 ml) was removed aseptically and centrifuged under anaerobic conditions. The cell pellet was then mixed with the same volume of freshly prepared medium (600 ml) and introduced into the bioreactor again. The cell pelleting was performed in a vinyl anaerobic airlock chamber (Coylab Products, Michigan).

2.3 ANALYTICAL EQUIPMENT AND MEASUREMENT PROTOCOLS

2.3.1 Carbon monoxide measurement

Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph (GC) 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 ^{o}C, for 5 min, and then raised by 20 ^{o}C min^{-1} for 2 min, to reach a final temperature of 90 ^{o}C. The temperature of the injection port and the detector were maintained constant at 150 ^{o}C. Helium was used as the carrier gas.

The concentrations in the samples were determined from a calibration curve based on peaks obtained from known concentrations of gas. Gas samples were taken from the reactors by means of a terumo insulin gas tight 1 mL syringe. Figure 3 shows one of the calibration curves for carbon monoxide analysis.

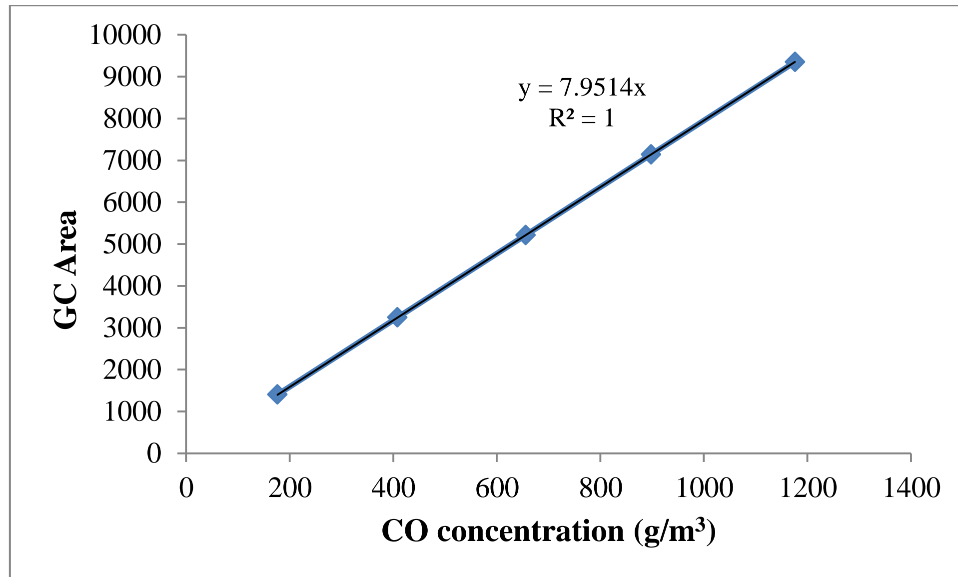


Figure 3: Calibration for carbon monoxide concentrations between 175 and 1175 g/m^3 CO.

2.3.2 Carbon dioxide measurement

CO_2 was analyzed on an HP 5890 gas chromatograph, equipped with a thermal conductivity detector (TCD). 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^{-1}. The injection, oven and detection temperatures were maintained at 90, 30 and 100^0C, respectively. The calibration of carbon dioxide (Figure 4) was performed the same way as for carbon monoxide.

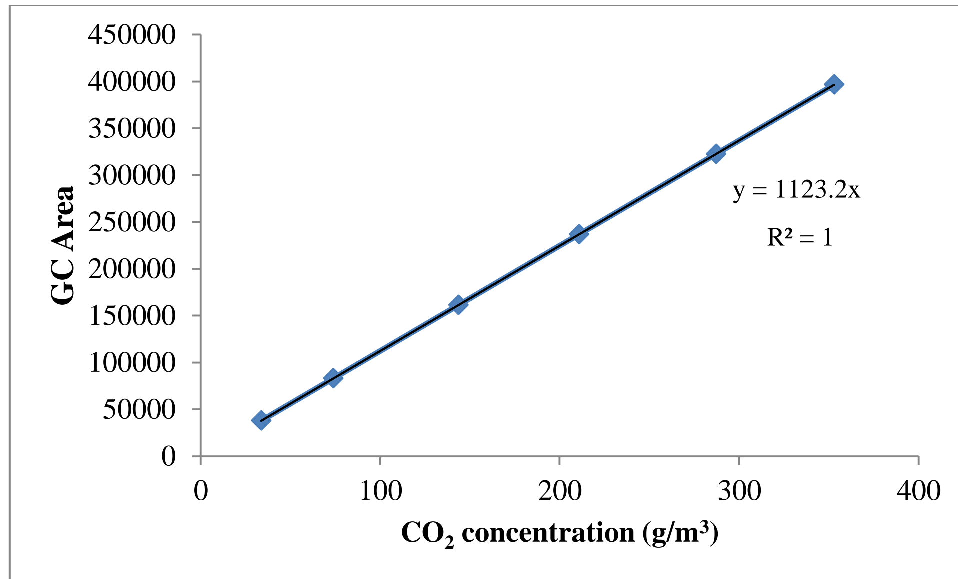
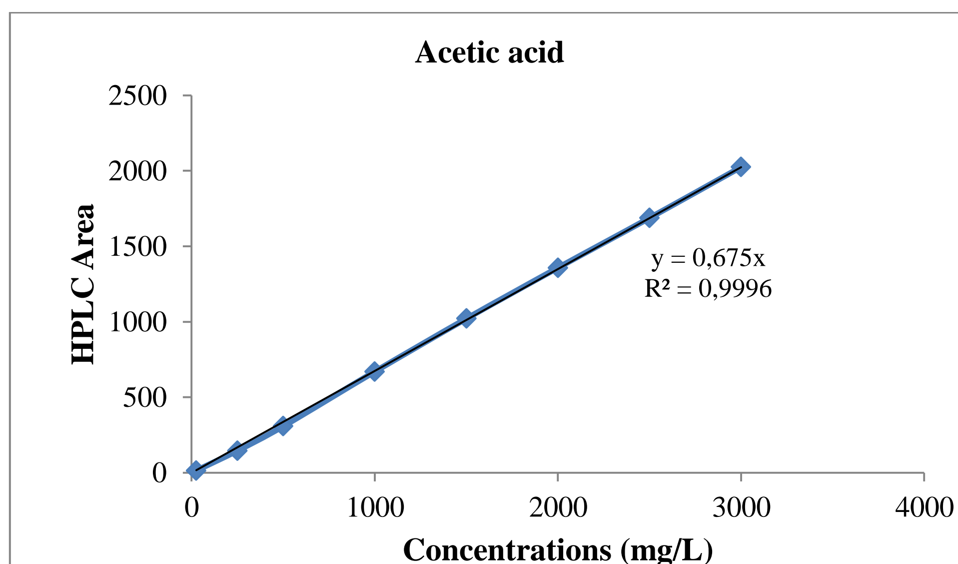


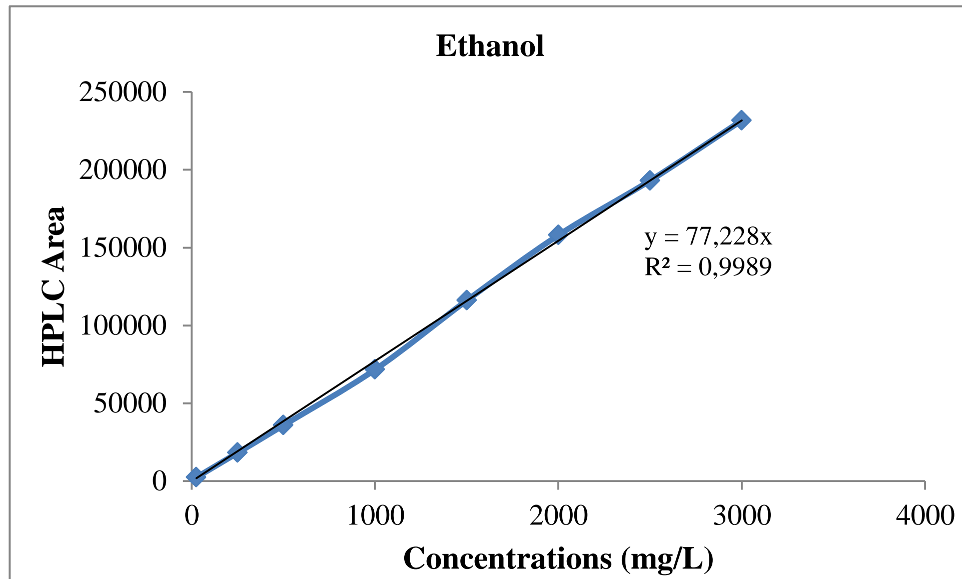
Figure 4: Calibration for carbon dioxide concentrations between 30 and 350 g/m^3 CO_2.

2.3.3 Measurement of metabolite concentrations

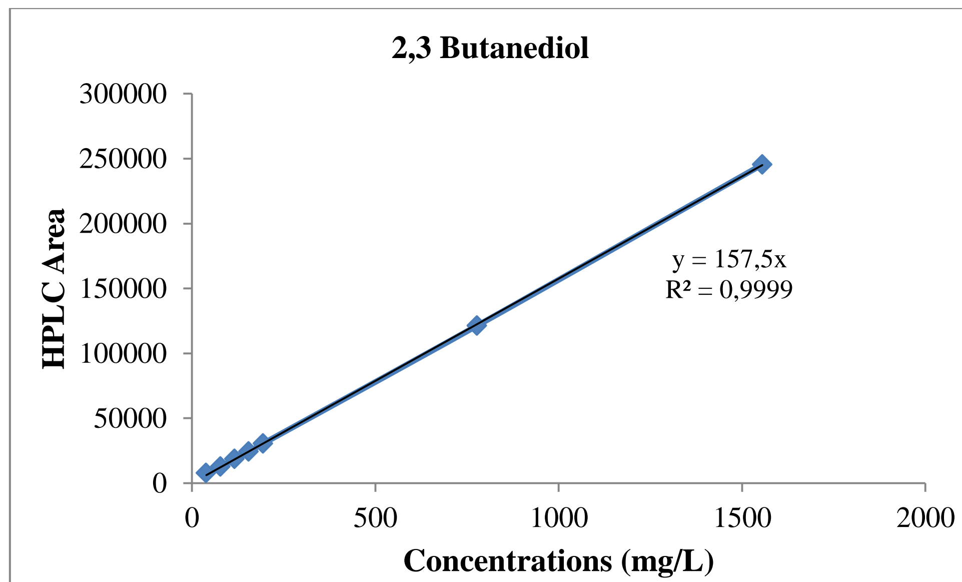
The water-soluble products, acetic acid (Figure 5a), ethanol (Figure 5b), 2,3-butanediol (Figure 5c) and lactic acid (Figure 5d), in the culture broth were analyzed using an HPLC (HP1100, Agilent Co., USA) equipped with a supelcogel C-610 column having a UV detector at a wavelength of 210 nm and a refractive index detector (RID). 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.



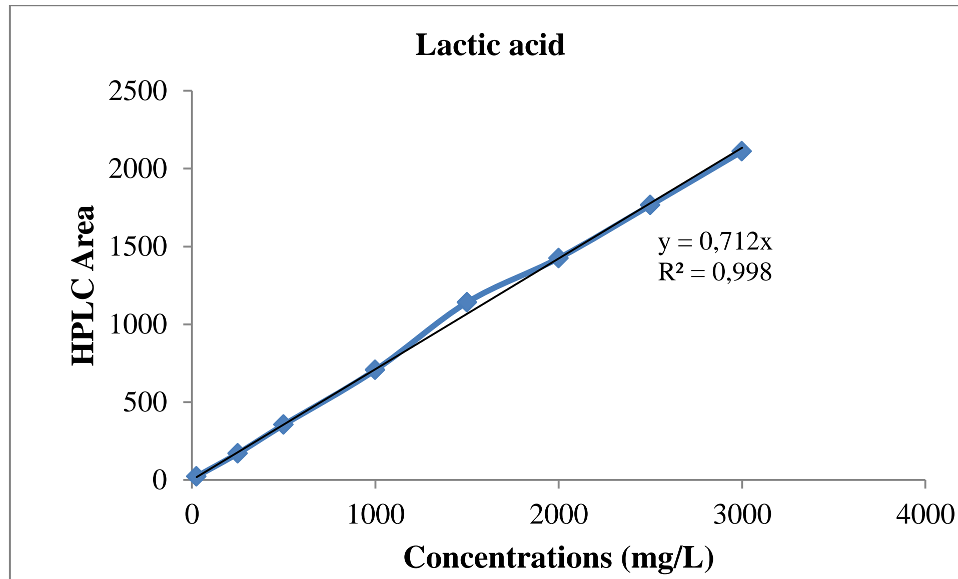
(a)



(b)



(c)

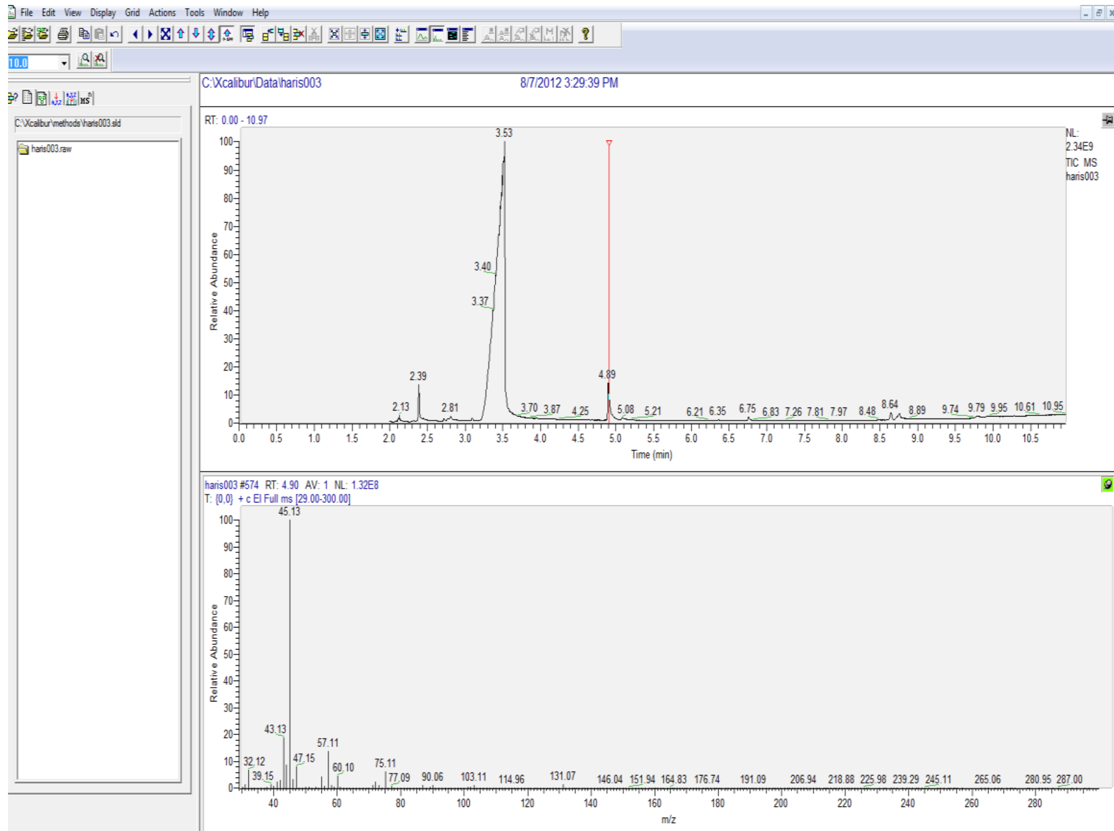


(d)

Figure 5: Calibration for (a) acetic acid, (b) ethanol concentrations between 25 and 3000 mg/L; (c) 2,3 butanediol concentrations between 38 and 1555 mg/L; (d) lactic acid concentrations between 25 and 3000 mg/L

2.3.3.1 2,3-butanediol Identification

For 2,3-butanediol identification, a Thermo Scientific ISQ™ single quadrupole GC-MS system, operated at 70 eV, mounted with a HP-5ms column (30 m × 0.25 mm × 0.25 µm film thickness) was used. The MS transfer line temperature and ion source temperature were both 250 °C. The oven temperature was initially kept constant at 40 °C, for 2 min, and then raised by 10 °C min^{-1} to reach a final temperature of 250 °C. The screenshot of the 2,3-butanediol chromatogram and library search is shown in Figure 6.



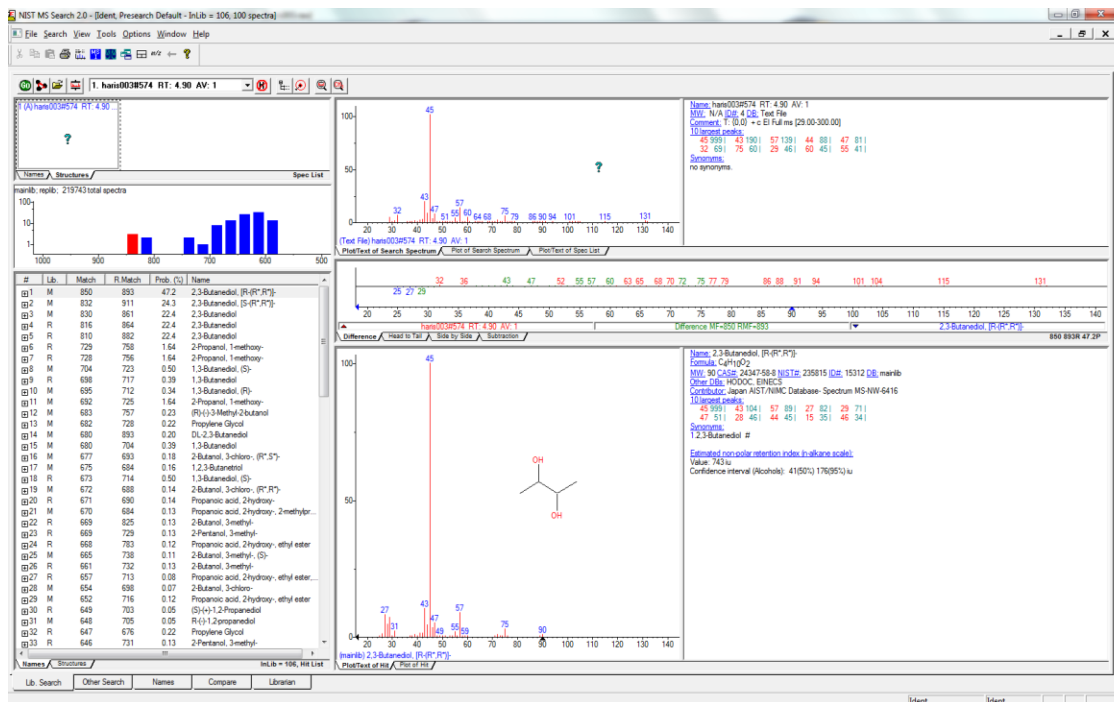


Figure 6: Photographic view of 2,3-butanediol peak and library search in the GC-MS.

2.3.4 Measurement of xylose concentrations

The xylose concentration was measured using an HPLC with the same method as used for the analysis of metabolites (Section 2.3.3). Figure 7 shows the calibration curve to measure xylose.

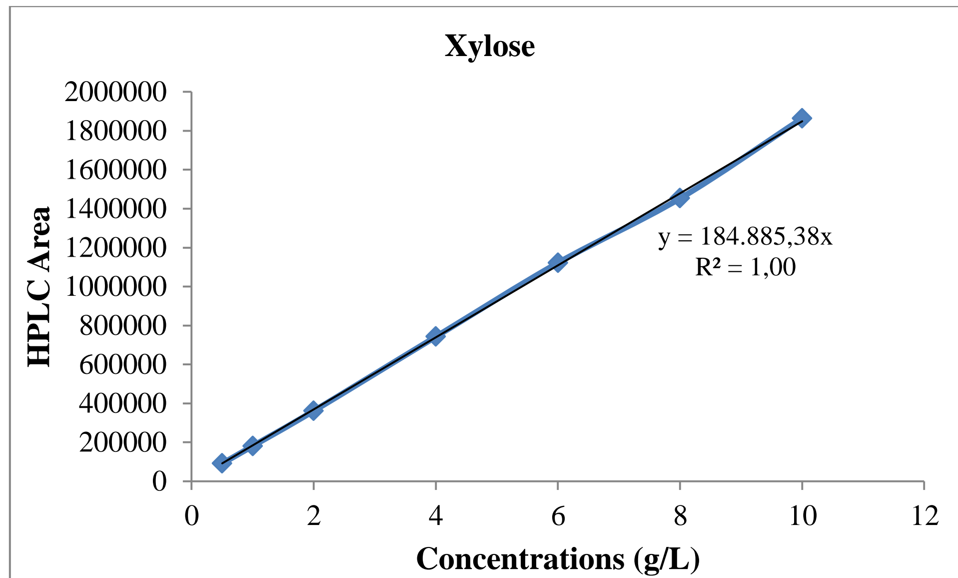


Figure 7: Calibration for xylose concentrations up to 10 g/L.

2.3.5 Biomass quantification

Cell mass was estimated by measuring the absorbance of the sample at a wavelength of 600 nm using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The measured absorbance was then compared to the previously generated calibration curve to calculate the corresponding biomass concentration (mg/L). 1 OD unit corresponds to 305.81 mg of cell mass/ L.

2.3.6 Redox potential

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

2.3.7 16S rRNA analysis of Bioreactor cells

Samples of cells were taken at the end of the CSTR experiments and were analysed during a stay at the Institute of Microbiology and Biotechnology, University of Ulm, Germany as well as at the (Servicios de Apoyo a la Investigación) SAI of the university of La Coruña (UDC). The following techniques were performed to check the purity of the cell mass.

- DNA extraction

The extraction procedure was performed as per the manufacturer protocol using the DNA Isolation Kit. The concentration of 16s rRNA fragment obtained was 17 ng/ul

- PCR run of 16S rRNA fragment

The extracted fragment was then amplified using PCR. Samples were treated with Taq DNA Polymerase, primer, dNTPs and run using the following PCR programme: 95 ^{0}C for 5 min; 32 times (95 ^{0}C for 45 sec; 60 ^{0}C for 1 min; 72 ^{0}C for 1:30 min); 72 ^{0}C for 10 min. The concentration of DNA after PCR run was 169 ng/ul.

- Electrophoresis of PCR product

Electrophoresis of the DNA fragments was performed to identify if the fragments obtained were indeed DNA (Figure 8).

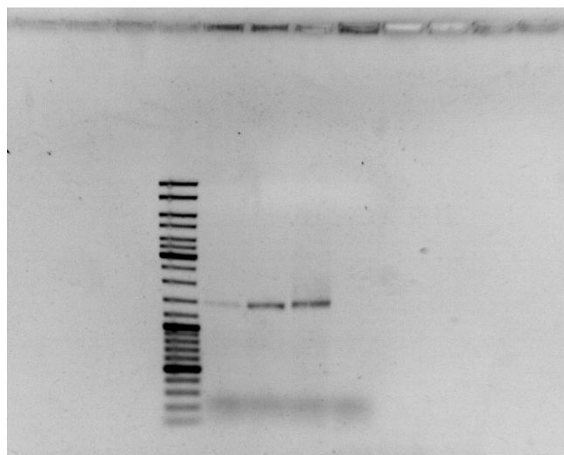


Figure 8: Photographic view of the bands

- Send for sequencing

After confirming that the PCR product was a 16S rRNA fragment, it was then send for sequencing to a private company. The results of sequencing were analyzed using Chromas Lite Software and confirmed that the samples were pure cells of bacteria *C. autoethanogenum*.

2.3.8 Minitab

The software package Minitab 16 (Minitab Inc. State College, PA, USA) was used to design experiments and for data analysis in the form of analysis of variance (ANOVA) for the experiments described in Chapter 3 and Chapter 4. Using the least square technique with Minitab, the individual and interaction effects of the parameters can be approximated to a linear regression model.

2.4 REFERENCES

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Chapter 3

Biological conversion of carbon monoxide to ethanol: Effect of pH, gas pressure, reducing agent and yeast extract

Abstract

A two-level full factorial design was carried out in order to investigate the effect of four factors on the bioconversion of carbon monoxide to ethanol and acetic acid by *Clostridium autoethanogenum*: initial pH (4.75 – 5.75), initial total pressure (0.8 – 1.6 bar), cysteine – HCl.H_2O concentration (0.5 – 1.2 g/L) and yeast extract concentration (0.6 – 1.6 g/L). The maximum ethanol production was enhanced up to 200% when lowering the pH and amount yeast extract from 5.75 to 4.75 and 1.6 to 0.6 g/L, respectively. The regression coefficient, regression model and analysis of variance (ANOVA) were obtained using MINITAB 16 software for ethanol, acetic acid and biomass. For ethanol, it was observed that all the main effects and the interaction effects were found statistically significant ($p < 0.05$). The comparison between the experimental and the predicted values were found to be very satisfactory, indicating the suitability of the predicted model.

Keywords: CO – bioconversion; *Clostridium autoethanogenum*; factorial design; medium optimization; waste gas

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it appears in: *Bioresource Technology*. Published online 21 March 2012. DOI:10.1016/j.biortech.2012.03.027

3.1 INTRODUCTION

Biological conversion of waste gases containing carbon monoxide (CO) using acetogens offers a possibility through which waste can be efficiently utilized for generating valuable fuels like ethanol, butanol and hydrogen (Abubackar et al., 2011a; Mohammadi et al., 2011; Munasinghe and Khanal, 2010). However, one major bottleneck for the commercialization of this technique is the poor aqueous solubility of carbon monoxide gas. Hence, for systems containing CO as sole substrate, the bioconversion process is limited by the CO gas-liquid mass transfer at high cell concentration. Besides, the process is kinetically limited when either the cell concentration or the CO consumption rate is too low (Abubackar et al., 2011a). These rate-limiting conditions would decrease the process yield and CO – bioconversion process and are often encountered at some point in the bioconversion.

Homoacetogens able to produce ethanol from carbon monoxide include *Clostridium ljungdahlii, Clostridium carboxidivorans* P7^T*, Clostridium ragsdalei, Alkalibaculum bacchi* CP11^T*, Clostridium autoethanogenum, Clostridium drakei, Butyribacterium methylotrophicum,* among others (Liu et al., 2011; Mohammadi et al., 2011). These unicarbonotrophic bacteria follow the acetyl-CoA biochemical pathway or Wood-Ljungdahl pathway for cell growth and product formation (Abubackar et al., 2011a). Apart from ethanol, acetic acid is one of the prominent metabolites found during CO conversion using these microorganisms. In most of the previous studies, low ethanol to acetic acid ratios were generally obtained. However, by optimizing the medium composition and operating conditions, this ratio can be increased (Kundiyana et al., 2011a,b). In the present research, a microcosm study was performed using *Clostridium autoethanogenum* as the biocatalyst.

C. autoethanogenum is a strictly anaerobic gram positive rod shaped (0.5 x 3.2 μm) bacterium, originally isolated from rabbit feces using CO as the sole carbon and energy source. (Abrini et al., 1994). In one study, the authors used Plackett–Burman design to screen significant ethanol enhancing factors from the defined medium developed for *C. carboxidivorans*. Optimal levels of these significant factors were evaluated by central composite design (CCD) using a response surface methodology (RSM) and an artificial neural network-genetic algorithm (ANN-GA). It was concluded that an optimal medium containing (g/L) NaCl 1.0, KH_2PO_4 0.1, $CaCl_2$ 0.02, yeast extract 0.15, $MgSO_4$ 0.116 and NH_4Cl 1.694, at pH 4.74 could yield an ethanol concentration of around 0.25 g/L (Guo et al., 2010). Another research reported a concentration of 0.06 – 0.07 g/L with a 1:13 ethanol to acetate ratio in liquid-batch continuous syngas fermentation using a xylose adapted *C. autoethanogenum* culture (Cotter et al., 2009). These studies reveal the importance of medium composition in increasing the overall ethanol production. Hence, the different operating conditions still have to be optimized in order to enhance ethanol production and save on operating costs.

In the present research, *C. autoethanogenum* was used to convert bottled carbon monoxide gas into a valuable fuel product such as ethanol, and to investigate the effect of various process parameters on the bioconversion process, such as the initial pH, initial total pressure, cysteine – $HCl.H_2O$ concentration and yeast extract concentration, and to obtain a reduced regression model that describes the process for products and biomass using a 2^4 full factorial design. In this manuscript, the authors simply called initial total pressure, cysteine – $HCl.H_2O$ and yeast extract as “pressure”, “cysteine – HCl” and “YE”, respectively and in the tables and figures, initial pH as simply “pH”.

3.2 MATERIALS AND METHODS

3.2.1 Microorganism and medium composition

Clostridium autoethanogenum DSM 10061 was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), and was grown and maintained on DSMZ medium 640 with 0.5% xylose. The medium was prepared by boiling for a few minutes, while being degassed, and then cooled continuously under N_2 for 15 minutes to remove oxygen. Cysteine – HCl was added, and the pH of the medium was adjusted to 6.0 by adding either 2M HCl or 2M NaOH.

3.2.2 Bioconversion studies

For batch experiments, serum vials with a total volume of 200 mL were used, with 75 mL working volume. The experimental set-up and the method used for media preparation are described elsewhere (Abubackar et al., 2011b). The culture was maintained under anaerobic conditions and agitated at 150 rpm on an orbital shaker, inside an incubation chamber at 30 ^{o}C. 10% of actively growing culture, which was grown with CO as sole substrate, was used as the inoculum and was aseptically transferred to each experimental vial. Headspace samples of 0.2 mL were used for CO measurements, and 1 mL of liquid sample was periodically withdrawn from the vials (once every 24 h) in order to measure the optical density ($OD_{\lambda=600\ nm}$) related to biomass concentration. The same 1 ml sample was then centrifuged for 10 min (25 ^{0}C, 7000 x g) and the supernatant was used to check both ethanol and acetic acid concentrations.

3.2.3 Analytical equipment and measurement protocols

Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph equipped with a thermal conductivity detector. 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 ^{o}C, for 5 min, and then raised by 20 ^{o}C

min^{-1} for 2 min, to reach a final temperature of 90 ^oC. The temperature of the injection port and the detector were maintained constant at 150 ^oC. Helium was used as the carrier gas. The water-soluble products, acetic acid and ethanol, in the culture broth were analyzed using a HP-5890 Series II GC equipped with a flame ionization detector and a 0.25 mm (ID) × 30 m HP-INNOWax capillary column (Agilent Technologies, Forster, CA, USA). Helium was used as the carrier gas. The oven temperature was held at 80 ^oC for 2 min, then heated to 160 ^oC at a rate of 10 ^oC min^{-1}, and maintained thereafter at 160 ^oC for 1 min. The injector and detector temperatures were kept constant, at 220 and 260 ^oC, respectively. Cell mass was estimated by measuring sample absorbance at a wavelength of 600 nm using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The measured absorbance was then compared with the previously generated calibration curve, to calculate the corresponding cell concentration (mg/L).

3.2.4 Experimental design and statistical analysis

A two level four factor (2^4) full factorial experimental design was used to study the combined effects of initial pH (low 4.75 and high 5.75), initial total pressure (low 0.8 bar and high 1.6 bar), cysteine – HCl.H_2O concentration (low 0.5 g/L and high 1.2 g/L) and yeast extract concentration (low 0.6 g/L and high 1.6 g/L) on products formation (ethanol and acetic acid) and culture stability during the carbon monoxide bioconversion process by *C. autoethanogenum*. Of particular interest for optimizing ethanol production as a biofuel; this study was focused on estimating the optimum range of these parameters that enhances ethanol production.

The software package Minitab 16 (Minitab Inc. State College, PA, USA) was used to design the experiments and for data analysis in the form of analysis of variance (ANOVA). The response variables (Y) that were analyzed were the maximum products

concentrations (g/L) and biomass concentration (mg/L) obtained from the different experimental trials.

Table 1: 2^4 Factorial design of experiments for ethanol, acetic acid and biomass production in the study

Run No	pH	Pressure (Bar)	Cysteine-HCl (g/L)	YE (g/L)	Ethanol production (g/L)		Acetic acid production (g/L)		Biomass production (mg/L)	
					Observed	Predicted	Observed	Predicted	Observed	Predicted
1	4.75	0.8	0.5	0.6	0.115677	0.11568	0.930341	0.9428	141.9	145.99
2	5.75	0.8	0.5	0.6	0.072725	0.07272	0.933748	0.9462	159.02	144.69
3	4.75	1.6	0.5	0.6	0.278010	0.27802	1.950899	1.9558	259.63	253.25
4	5.75	1.6	0.5	0.6	0.080230	0.08026	2.145072	2.15	291.13	302.27
5	4.75	0.8	1.2	0.6	0.141760	0.14176	0.848527	0.8434	161.47	154.47
6	5.75	0.8	1.2	0.6	0.095745	0.09576	1.238078	1.2328	172.17	153.17
7	4.75	1.6	1.2	0.6	0.649213	0.64922	1.66778	1.6552	187.78	216.77
8	5.75	1.6	1.2	0.6	0.090824	0.09082	2.040535	2.0282	263.30	265.79
9	4.75	0.8	0.5	1.6	0.106121	0.10612	0.999329	0.987	175.84	189.71
10	5.75	0.8	0.5	1.6	0.089211	0.0892	1.132418	1.12	221.10	238.73
11	4.75	1.6	0.5	1.6	0.192213	0.19222	2.220619	2.2156	326.00	302.33
12	5.75	1.6	0.5	1.6	0.077787	0.07778	2.330991	2.3258	303.36	301.03
13	4.75	0.8	1.2	1.6	0.155645	0.15564	1.270428	1.2756	153.52	153.23
14	5.75	0.8	1.2	1.6	0.070136	0.07016	1.231282	1.2362	197.25	202.25
15	4.75	1.6	1.2	1.6	0.070065	0.07006	2.521777	2.5342	320.49	310.81
16	5.75	1.6	1.2	1.6	0.130568	0.13058	1.354848	1.3672	310.09	309.51

3.3 RESULTS AND DISCUSSION

Some of the main parameters that affect the CO – bioconversion process are pH, mass transfer, reducing agent concentration and YE concentration (Mohammadi et al., 2011). The design matrix in uncoded values and the observed and predicted values of the responses are presented in Table 1. Three experiments were performed at central points in replication for an estimation of the variance (experimental error) of an effect. Using

the least square technique with Minitab, the individual and interaction effects of the parameters can be approximated to a linear regression model. For 95% confidence level, the p-value, the probability value that is used to determine the statistical significance of the effects in the model should be less than or equal to 0.05 for the effect to be statistically significant.

3.3.1 Main effects plot

Fig. 1 shows the main effects plot for the responses. From the main effects plot for ethanol, it is observed that increasing the initial pH and higher YE concentrations had a negative effect on ethanol production, whereas increasing initial pressure and cysteine – HCl concentration had a positive effect. These fermentation results are consistent with the trend observed in some other CO – bioconversion studies suggesting that lowering the pH and YE concentration results in the production of more reduced compounds such as ethanol (Barik et al., 1988; Phillips et al., 1993). The product spectrum shifted from acidogenic to solventogenic phase when lowering the medium's pH. This was proposed to be due to the following reason: the product, acetic acid, is a lipophilic weak acid and thus permeates through the cell membranes, resulting in a decrease in internal pH due to the conduction of H^+ ions from inside. At low internal pH, the external pH plays a major role in keeping the cell under non-stressed condition (Mohammadi et al., 2011). Hence, at both low external and internal pH, the cells under stress condition overcome the situation by producing solvents. Eliminating YE was found to enhance the ethanol production using *C. ljungdahlii* (Barik et al., 1988). However, for this organism to provide structural integrity, a minimum concentration of 0.01% is said to be necessary (Abubackar et al., 2011a). One potential bottleneck of CO – bioconversion is the mass transfer limitation due to the sparingly soluble nature of that substrate. Hence, one way to overcome this limitation is by increasing the pressure. In batch fermentation, different

CO pressures mean different gaseous substrate concentrations which are directly proportional to the metabolite production and cell density. It was also observed that addition of reducing agents, thereby providing more electrons into the culture medium, will shift the microbial metabolism towards solventogenesis. This occurs due to availability of more reducing equivalents for the conversion of acetyl-CoA to products. For acetic acid, it is evident that pH doesn't exert any effect on acetic acid production. Cysteine – HCl showed only a slight change in response across the studied level. This result is fairly consistent with the observation of Sim and Kamaruddin, (2008), who studied the effect of cysteine – HCl on acetic acid production with *Clostridium aceticum* in a range of 0.1 – 0.5 g/L and found that the cysteine – HCl concentration was less significant. YE had a slightly positive effect on acetic acid production at high concentration. This may be due to the high cell growth achieved at increasing concentrations of yeast extract. Moreover, it has been reported that acetic acid is a growth-related product (Barik et al., 1988).

From the main effect plot for biomass, it is obvious that out of the four parameters studied, only increases in cysteine – HCl showed a slightly negative effect on biomass growth, whereas, increasing the other three factors had a strong positive influence on biomass. Since any organism shows its highest metabolic activity at its optimum pH, stepping down or stepping up in pH has a negative impact on cell growth. The optimum pH for growth of *C. autoethanogenum* is between 5.8 and 6.0 (Abrini et al., 1994). Hence, cell density increases proportionally when the pH is increased from 4.75 to 5.75. The reducing agent, cysteine – HCl, is essential for lowering the redox potential of the growth medium by scavenging the oxygen. However, a high amount of reducing agent is detrimental for cell growth and leads to a lower cell concentration

(Sim and Kamaruddin, 2008). As YE provides nutrients for cell metabolism, an increase in the amount YE therefore increases the cell concentration.

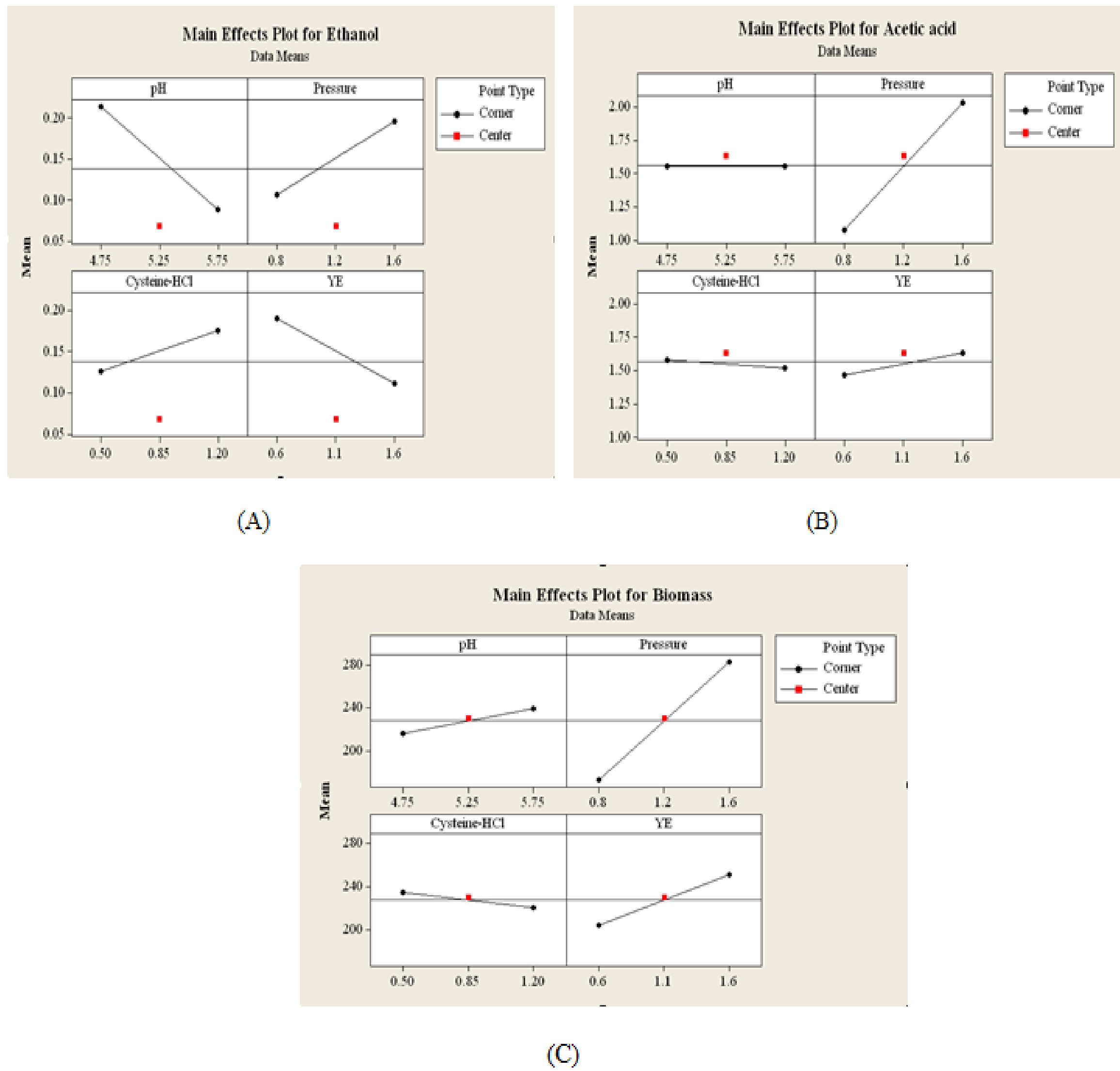


Figure 1: Main effects plot for (A) Ethanol, (B) Acetic acid and (C) Biomass.

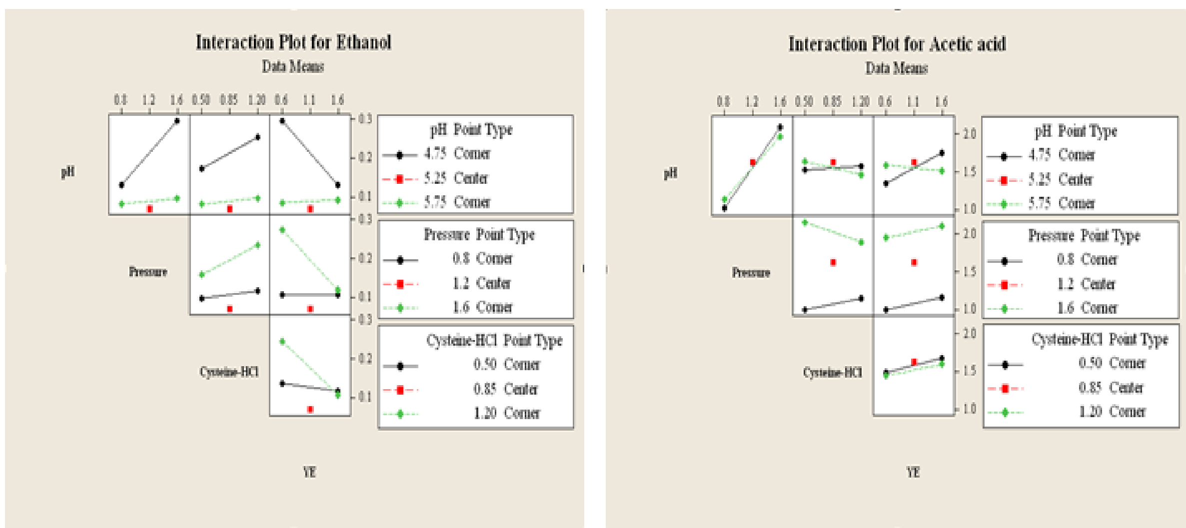
3.3.2 Interaction effects plot

The interaction effects plots are shown in Fig. 2 and represent the mean response at all possible combinations of each two factors studied. If the two lines are non-parallel, it is an indication of interaction between the two factors.

The interaction plot for ethanol showed that there is a strong interaction between each two factors. Whereas for acetic acid, only minor interactions were observed for YE with pressure and with cysteine – HCl. Also, no remarkable interactions between the pairs of factors were seen for biomass production. When the initial medium pH was 5.75, the maximum ethanol production was close to 0.1 g/L, same at low and high level of each other factors, describing the importance of low initial medium pH for increasing ethanol production. It is possible that higher amounts of carbon substrate are channeled towards the cell mass at high (+) level of pH. A higher amount of ethanol was observed at a pressure of 1.6 bar for both concentrations of cysteine – HCl and YE than at a pressure of 0.8 bar. A high amount of ethanol was also found to be produced for a higher cysteine – HCl concentration of 1.2 g/L at both levels of each other factors. In fact a slight reduction in ethanol production was observed at YE concentration of 1.6 g/L compared to ethanol produced for cysteine – HCl concentration of 0.5 g/L at 1.6 g/L of yeast extract.

At high (+) YE concentration level, an increase in pressure from 0.8 to 1.6 bars leads only to a very minor improvement in ethanol production and increases significantly acetic acid and biomass concentrations, showing the importance of lowering the YE concentration for improving ethanol production. Even though growth ceases at low pH and low YE concentration, it can easily be observed from the interaction plot that there is around 200% improvement in ethanol production under such condition. Interaction between total pressure and cysteine – HCl, at their highest

concentrations, has a positive influence on ethanol production and a negative effect on both acetic acid and biomass formation. Also, at low pressure, an increase in cysteine – HCl concentration doesn't make any major difference in their production. This can easily be interpreted by the fact that at a higher pressure, resulting in more supply of carbon substrate, an increment in reducing agent allows the microbes to use the additional carbon for producing highly reduced products.



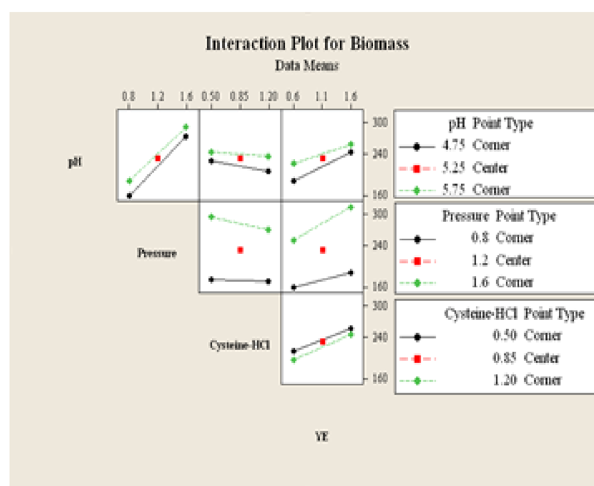


Figure 2: Interaction effects plots for (A) Ethanol, (B) Acetic acid and (C) Biomass.

3.3.3 Regression analysis and prediction of regression model

The statistical software was used to evaluate the observed experimental results to derive a regression function by using ordinary least square method. Regression results determine the statistical significance, direction and magnitude of the relationship between an effect and the response. The sign of each regression coefficient indicates the direction of the relationship. Only the effects with low p-values are said to be statistically significant and can be meaningfully utilized in obtaining the regression function or model (Montgomery, 2005). A comparison between experimental values and the predicted values obtained using the regression equation is performed and satisfactory correlation was found between these values ($R^2 > 0.9$).

The regression models proposed are as follows:

Maximum ethanol production = $0.15100 - 0.06259\ A + 0.04512\ B + 0.02450\ C - 0.03953\ D - 0.03867\ AB - 0.01608\ AC + 0.04305\ AD + 0.01455\ BC - 0.03893\ BD - 0.02936\ CD - 0.00713\ ABC + 0.04473\ ABD + 0.02938\ ACD - 0.02703\ BCD + 0.03757\ ABCD$

Maximum acetic acid production = $1.5510 - 0.0002\ A + 0.4780\ B - 0.0294\ C + 0.0817\ D - 0.0610\ AB - 0.0553\ AC - 0.1202\ AD - 0.1034\ BC - 0.0820\ ABC - 0.0828\ ABD - 0.1259\ ACD - 0.0272\ BCD - 0.0561\ ABCD$

Maximum biomass production = $227.75 + 11.93\ A + 54.97\ B - 7.00\ C + 23.20\ D - 12.58\ ABD + 11.24\ BCD$

These regression models are confined for each variable within the following range: (A) initial pH = 4.75 – 5.75, (B) pressure = 0.8 – 1.6 bars, (C) cysteine – HCl = 0.5 – 1.2 g/L and (D) YE = 0.6 – 1.6 g/L.

3.4 CONCLUSIONS

In this experimental range, higher ethanol production was favored by the lower pH and YE concentration and the higher pressure and cysteine – HCl concentration. A maximum ethanol concentration of 0.65 g/L was obtained under the following conditions: pH = 4.75 (the lowest value tested), pressure = 1.6 bar (the highest value tested), cysteine – HCl = 1.2 g/L (the highest value tested), and YE concentration = 0.6 g/L (the lowest value tested). Such maximum ethanol concentration is considerably higher than that achieved (0.06 and 0.25 g/L) with *C. autoethanogenum* in previous studies (Cotter et al., 2009; Guo et al., 2010).

ACKNOWLEDGEMENTS

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Chapter 4

Ethanol and acetic acid production from carbon monoxide in a *Clostridium* strain in batch and continuous gas-fed bioreactors

Abstract: The effect of different sources of nitrogen as well as their concentrations on the bioconversion of carbon monoxide to metabolic products such as acetic acid and ethanol by *Clostridium autoethanogenum* was studied. In a first set of assays, under batch conditions, either NH_4Cl, trypticase soy broth or yeast extract (YE) were used as sources of nitrogen. The use of YE was found statistically significant ($p < 0.05$) on the product spectrum in such batch assays. In another set of experiments, three bioreactors were operated with continuous CO supply, in order to estimate the effect of running conditions on products and biomass formation. The bioreactors were operated under different conditions, *i.e.*, EXP1 (pH = 5.75, YE 1g/L), EXP2 (pH = 4.75, YE 1 g/L) and EXP3 (pH = 5.75, YE 0.2 g/L). When compared to EXP2 and EXP3, it was found that EXP1 yielded the maximum biomass accumulation (302.4 mg/L) and products concentrations, *i.e.*, acetic acid (2147.1 mg/L) and ethanol (352.6 mg/L). This can be attributed to the fact that the higher pH and higher YE concentration used in EXP1 stimulated cell growth and did, consequently, also enhance metabolite production. However, when ethanol is the desired end-product, as a biofuel, the lower pH used in EXP2 was more favourable for solventogenesis and yielded the highest ethanol/acetic acid ratio, reaching a value of 0.54.

Keywords: acetic acid; bioethanol; carbon monoxide; *Clostridium autoethanogenum*; syngas; waste gas

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it appears in: *International Journal of Environmental Research and Public Health*. Published online 20 January 2015. DOI: 10.3390/ijerph120101029

4.1 INTRODUCTION

Carbon monoxide (CO) is emitted in large amounts in the form of industrial waste gases generated during the incomplete combustion of carbon-containing materials. It is also a major component of synthesis gas (Van Groenestijn et al., 2013). Some anaerobic bacteria have the ability to grow on CO as their sole carbon source and metabolize it to a variety of fuels and chemicals (Abubackar et al., 2011a; Bengelsdorf et al., 2013). These unicarbonotrophs ferment CO into acetyl-CoA, via the acetyl-CoA pathway or Wood-Ljungdahl (WL) pathway, and later into metabolites such as acetic acid, ethanol, hydrogen, *n*-butanol or 2,3-butanediol. In the WL pathway, the net ATP gained by substrate level phosphorylation (SLP) is zero; hence, in order to make bacterial growth on CO possible, the WL pathway must be coupled to energy conservation (Latif et al., 2014; Schuchmann et al., 2014). However, the exact mechanisms involved in energy conservation remain still unclear. Very recently, metabolically engineered acetogens have been used to selectively produce metabolites from CO (Kiriukhin et al., 2013; Banerjee et al., 2014), although it is also possible to produce specific metabolites of interest from CO, in wild type bacteria, through manipulation of the medium composition and/or operating conditions in bioreactors (Hurst and Lewis, 2010; Kundiyana et al., 2011a). Several acetogens are known to produce acetic acid, as major end metabolite, from CO, including *Moorella thermoacetica*, *Acetobacterium woodii*, *Eubacterium limosum* KIST 612, *Peptostreptococcus productus* U-1 and *Clostridium aceticum* (Bengelsdorf et al., 2013); whereas *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Clostridium ragsdalei* and *Alkalibaculum bacchi* are ethanologenic

acetogens, able to produce ethanol besides acetic acid (Abubackar et al., 2011a; Bengelsdorf et al., 2013). Recently *Clostridium ljungdahlii*, *Clostridium autoethanogenum* and *C. ragsdalei* were found to produce 2,3-butanediol and lactic acid as well (Köpke et al., 2011).

In the present work, biological conversion of CO was studied, using *C. autoethanogenum*, in order to produce various metabolites. In most of the CO bioconversion studies to ethanol, co-production of large amounts of acetic acid was observed. Although ethanol is an interesting metabolite as a biofuel, products such as acetic acid have many industrial applications as well, as the key raw material for the manufacture of vinyl acetate monomer, acetic anhydride and acetate esters such as ethyl acetate, *n*-butyl acetate and isopropyl acetate (Sim et al., 2007) Similarly, 2,3-butanediol is another possible by-product, with potential applications in manufacturing industries, such as in the production of food, pharmaceuticals, printing inks, perfumes, fumigants, synthetic rubbers, octane boosters, or plasticizers. Three stereoisomers of 2,3-butanediol exist, comprising the optically active dextro-[L-(+)-] and levo-[D-(−)-] forms and the optically inactive *meso*-form. It has been reported that *C. autoethanogenum* can produce 2,3-butanediol in the form of D(−)-2,3-butanediol (96%) and *meso*-2,3-butanediol (4%) (Köpke et al., 2011). This anaerobic biological route of production of chemicals such as ethanol, acetic acid and 2,3-butanediol from CO is an extremely attractive alternative compared to the traditional chemical route and other biorefinery processes (Bengelsdorf et al., 2013).

Microorganisms require nitrogen for their structural integrity as well as for proteins, and optimization of their concentrations in culture media could improve the productivity of the process and reduce the medium's cost. In some of our previous batch studies, it was found that the nature and the concentration of metabolites produced from

CO depend on the composition of the culture medium as well as on other experimental conditions such as pH and pressure, among others (Abubackar et al., 2012). Guo *et al.* (2010) observed that an optimized medium containing (g/L) NaCl 1.0, KH_2PO_4 0.1, $CaCl_2$ 0.02, yeast extract 0.15, $MgSO_4$ 0.116 and NH_4Cl 1.694, at pH = 4.74 could yield an ethanol concentration of around 0.25 g/L using *C. autoethanogenum* in microcosm studies. Some previous study was done to evaluate the sensitivity of growth and product formation to nitrogen sources and their concentration in clostridia (Cotter et al., 2009). However, xylose was used as the carbon substrate in that study rather than CO. This prompted us to carry-out the present studies with CO, as the xylose fermentation by acetogens exhibits some differences and does also involve the glycolysis and oxidation of pyruvate to acetyl-CoA in addition to the WL pathway. Besides, the few previous studies aimed at estimating the effect of the medium's composition on bacterial growth and production of metabolites in clostridia were generally done in batch assays, in bottles, with no pH regulation. In the present research, bioreactors operated at constant pH, with continuous CO supply, were used. This is a relevant aspect as both pH and the medium's composition affect the metabolism and growth pattern. When both parameters are allowed to vary, it becomes difficult to conclude which one is actually affecting more.

The purpose of this work was to investigate the effect of various sources of nitrogen on the bioconversion of CO to various metabolites, by *C. autoethanogenum*, in bottles as well as in continuous gas-fed bioreactors. In the present study, first, the influence of different sources of nitrogen (NH_4Cl, yeast extract and trypticase soy broth) were compared for their effect on growth and product formation. In the research described in this paper, acetic acid is the major end-product. The adequate selection of the medium and culture conditions would allow ethanol to become the major, or even

single, end metabolite. First, the experiments were carried out in 200 mL serum vials using a 2^3 full factorial design. In the second part of the research the effect of individual sources of nitrogen on growth and metabolites production was studied. In the final part of the research, experiments were performed in laboratory-scale fermentors in continuous mode (continuous gas feed) applying results and conditions previously optimized in batch experiments.

4.2 EXPERIMENTAL SECTION

4.2.1. Microorganism

Clostridium autoethanogenum DSM 10061 was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), and was maintained on medium (pH = 6) with the following composition (per liter distilled water): NH_4Cl, 0.9 g; NaCl, 0.9 g; $MgCl_2 \cdot 6H_2O$, 0.4 g; KH_2PO_4, 0.75 g; K_2HPO_4, 1.5 g; $FeCl_3 \cdot 6H_2O$, 0.0025 g; trypticase peptone, 2.0 g; yeast extract (YE), 1.0 g; cysteine-HCl, 0.75 g; 0.1% resazurin, 0.5 mL; with 0.5% xylose and SL-10 solution, 1.0 mL. The trace metal stock solution SL-10 contained (per liter): 7.7 M HCl, 10 mL; $FeCl_2 \cdot 4H_2O$, 1.5 g; $ZnCl_2$, 70 mg; $MnCl_2 \cdot 4H_2O$, 100 mg; H_3BO_3, 6 mg; $CoCl_2 \cdot 2H_2O$, 190 mg; $CuCl_2 \cdot 2H_2O$, 2 mg; $NiCl_2 \cdot 6H_2O$, 24 mg; and $Na_2MoO_4 \cdot 2H_2O$, 36 mg. For the experimental studies, xylose was omitted from the medium.

4.2.2 Bioconversion Studies

4.2.2.1. Bottle Batch Experiments

A two level three factor (2^3) full factorial experimental design was used to study the combined effects of NH_4Cl (0.2–2 g/L), trypticase (0.2–2 g/L) and YE concentrations (0.1–1 g/L), as sources of nitrogen, on products formation and culture stability during carbon monoxide bioconversion by *C. autoethanogenum*. The software package Minitab 16 (Minitab Inc. State College, PA, USA) was used to design the experiments and for

data analysis in the form of analysis of variance (ANOVA). Table 1 shows the design matrix obtained in uncoded values with the MINITAB software and the observed values of the responses obtained for each experiment as well as the final pH. Factorial design is an important statistical tool that allows to conclude the factors that are most influential in the bioconversion process by carrying out a limited number of experiments. Thus, a total of 18 experimental runs, including the replicate experiments at the central points, were carried out. The individual and interaction effects of the different parameters were studied using the least square technique with the help of a specific software.

Run No	NH_4Cl	Trypticase	YE	Ethanol (g/L)	Acetic Acid (g/L)	Biomass (mg/L)	Final pH
1	0.2	0.2	0.10	0.1733	1.806	152.29	3.88
2	2.0	0.2	0.10	0.3032	1.560	142.81	3.84
3	0.2	2.0	0.10	0.2290	1.855	222.7	4.03
4	2.0	2.0	0.10	0.1959	1.663	244.49	4.00
5	0.2	0.2	1.00	0.0883	2.146	302.90	3.91
6	2.0	0.2	1.00	0.1048	2.101	294.80	3.84
7	0.2	2.0	1.00	0.1061	2.339	335.62	3.93
8	2.0	2.0	1.00	0.1101	2.226	320.03	3.94

Table 1: 2^3 Factorial design table of experiments and responses.

For batch experiments, 10% of actively growing seed culture, grown with CO as sole carbon source, was aseptically transferred into 200 mL serum vials containing 75 mL medium at pH = 6. The medium contained (per liter distilled water): NaCl, 0.9 g; $MgCl_2·6H_2O$, 0.4 g; KH_2PO_4, 0.75 g; K_2HPO_4, 1.5 g; $FeCl_3·6H_2O$, 0.0025 g; 0.1% resazurin, 0.5 mL; and SL-10 solution, 1.0 mL. NH_4Cl, YE or trypticase were added in the same vials as per the experimental design (Table 1). In order to remove oxygen, the medium was boiled and flushed with N_2. After cooling, 0.75 g cysteine-HCl, was added

as reducing agent, and the pH was adjusted to 6 using aqueous solutions of either 2 M HCl or 2 M NaOH. The bottles were then sealed with Viton stoppers and capped with aluminum crimps before autoclaving for 20 min at 121 °C. The experimental set-up and the method used for media preparation are described elsewhere (Abubackar et al., 2011b). 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 30 °C. Headspace samples of 0.2 mL were used for CO measurements, and 1 mL liquid sample was periodically withdrawn from the vials, once every 24 h, in order to measure the optical density ($OD_{\lambda = 600\ nm}$), which is directly related to the biomass concentration. Afterwards, that same 1 mL sample was filtered using a 0.22 µm PTFE syringe-filter and was used to check the concentrations of soluble products. All the bioconversion experiments were conducted in duplicate, reaching statistically highly reproducible results. The response variables (Y) that were analyzed were the maximum products concentrations (g/L) as well as biomass concentrations (mg/L) obtained from the different experimental trials.

Three separate experiments with either NH_4Cl (1.1 g/L), YE (0.55 g/L) or trypticase (1.1 g/L), as sole source of nitrogen, were also performed in duplicate in order to understand the individual effect of each nitrogen source in promoting growth or product formation on CO. Another set of experiments, under the same conditions as above but without any CO, was also performed to check any product formation from YE and trypticase alone. The concentrations of nitrogen sources used in these sets of experiments are the center values of the respective factor ranges considered in the above full factorial design. Experiments and sample analysis were performed in the same way as mentioned above.

4.2.2.2. Continuous Gas-Fed Bioreactor Experiments

Three bioreactor experiments were carried out in 2 L BIOFLO 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA) using the following conditions: (1) pH = 5.75 and YE 1 g/L (referred to as EXP1); (2) pH = 4.75 and YE 1 g/L (EXP2) and (3) pH = 5.75 and YE 0.2 g/L (EXP3). Those experiments were done with 1.2 L batch liquid medium and CO (100%) as the gaseous substrate, continuously fed at a rate of 15 mL/min using a mass flow controller (Aalborg GFC 17, Müllheim, Germany). The bioreactor with the medium was autoclaved and cysteine-HCl (0.75 g/L) was added after cooling, together with nitrogen feeding to ensure anaerobic conditions.

The composition of the medium used in these bioreactor studies was the same as in the bottle experiments, with YE as the sole nitrogen source. The bioreactor was maintained at a constant temperature of 30 °C with a constant agitation speed of 250 rpm throughout the experiments. 10% of an actively growing culture, which was grown for 48 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 addition of either a 2 M NaOH solution or a 2 M HCl solution, fed by means of a peristaltic pump. Gas samples of 0.2 mL were taken from the inlet and outlet sampling ports of the bioreactor to monitor the CO and CO2 concentrations. Similarly, 2 mL liquid samples were periodically withdrawn from the reactor, once every 24 h, in order to measure the optical density (ODλ = 600 nm), allowing to estimate the biomass concentration. Afterwards the sample was filtered with a syringe using a 0.22 µm PTFE-filter before analyzing the concentrations of water-soluble products.

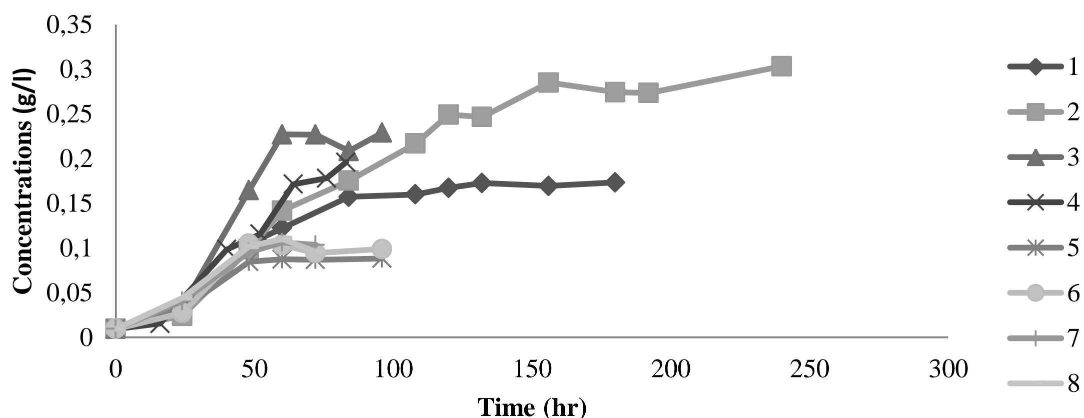
4.2.3. Analytical Equipment and Measurement Protocols

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. Similarly, CO2 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. For 2,3-butanediol identification, a Thermo Scientific ISQ™ single quadrupole GC-MS system (Thermo Fisher Scientific, Madrid, Spain) was used and operated at 70 eV. It was equipped with a HP-5ms column (30 m × 0.25 mm × 0.25 μm film thickness). The water-soluble products in the culture broth, i.e., acetic acid, ethanol and 2,3-butanediol, were analyzed using an HPLC (HP1100, Agilent Technologies, Madrid, Spain) 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. Cell mass was estimated by measuring the absorbance of the sample, at a wavelength of 600 nm, using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The measured absorbance was then compared to a previously generated calibration curve, to calculate the corresponding biomass concentration (mg/L). Besides, the redox potential was monitored continuously using an Ag/AgCl reference electrode connected to a transmitter (M300, Mettler Toledo, Inc., Bedford, MA USA) and maintained inside the bioreactor.

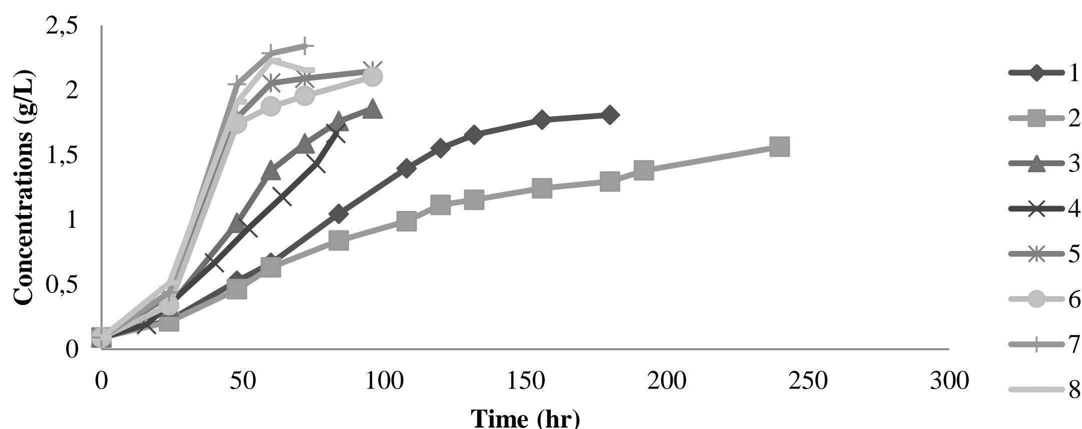
4.3 RESULTS AND DISCUSSION

4.3.1 Bottle Batch Experiments

In the bottle experiments, ethanol and acetic acid production started immediately, without any lag phase (Figure 1). It could be concluded that in these experiments the *Clostridium* strain follows the metabolic route that converts acetyl-CoA to acetaldehyde, followed by reduction to ethanol via a bifunctional acetaldehyde/ethanol dehydrogenase (Figure 2) (Van Groenestijn et al., 2013). Hence, in this CO fermentation, there were no differentiated acetogenic or ethanologenic phases. Maximum biomass (335.6 mg/L) and acetic acid concentrations (2.3 g/L) were produced in run No. 7 (Table 1) when the highest concentrations of YE and trypticase were used. The highest ethanol concentration (0.3 g/L) was obtained in run No. 2. Minor concentrations of by-product, *i.e.*, 2,3-butanediol, were also detected, reaching 0.017–0.101 g/L on the final day of the batch runs. The batch assays were stopped after about 10 days, when all the CO added initially was exhausted and no more biomass nor end-products were formed.



(A)



(B)

Figure 1: Products profile at eight different runs performed in bottle experiments: (A) ethanol profile and (B) acetic acid profile.

4.3.1.1 Main Effects Plot

The main effects plot for the experimental responses is shown in Figure 3. It represents the mean response values at each level of the design parameters. A main effect is considered present when the mean response changes across the level of the factor. From the main effects plot for biomass (Figure 3a), it is clearly observed that NH_4Cl does not exert any significant effect on biomass. However, a slightly higher biomass concentration was observed whenever low NH_4Cl concentrations were used in this study. This effect is in agreement with previously reported studies with *Clostridium aceticum* and *Rhodospirillum rubrum* using CO as the sole carbon substrate (Sim et al., 2008). The presence of both NH_4^+ and acetate could presumably result in the formation of ammonium acetate which is inhibitory to some clostridia, already at low concentrations (Wang and Wang, 1984).

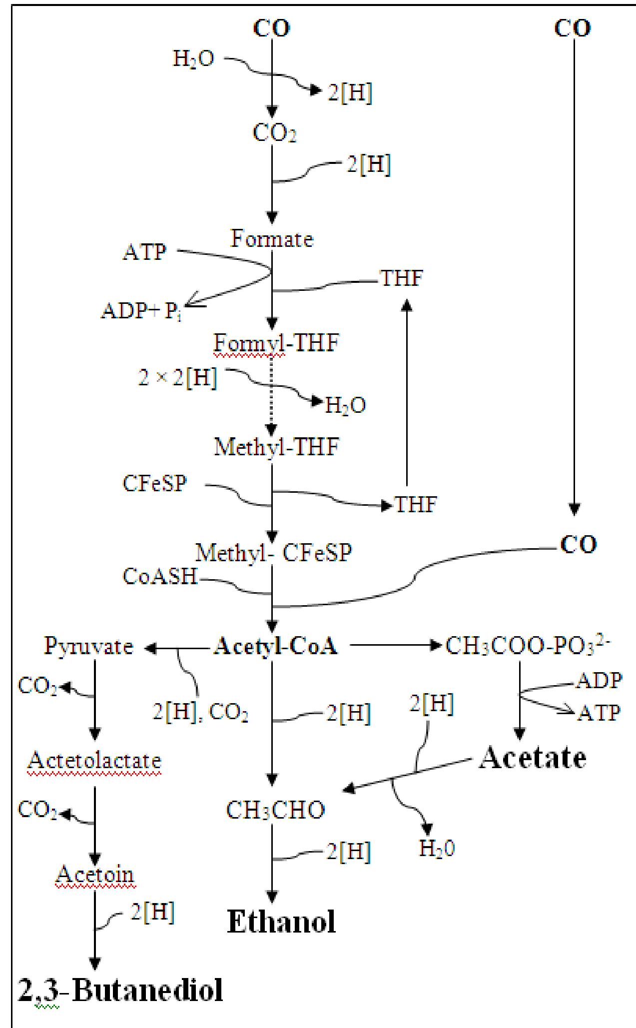
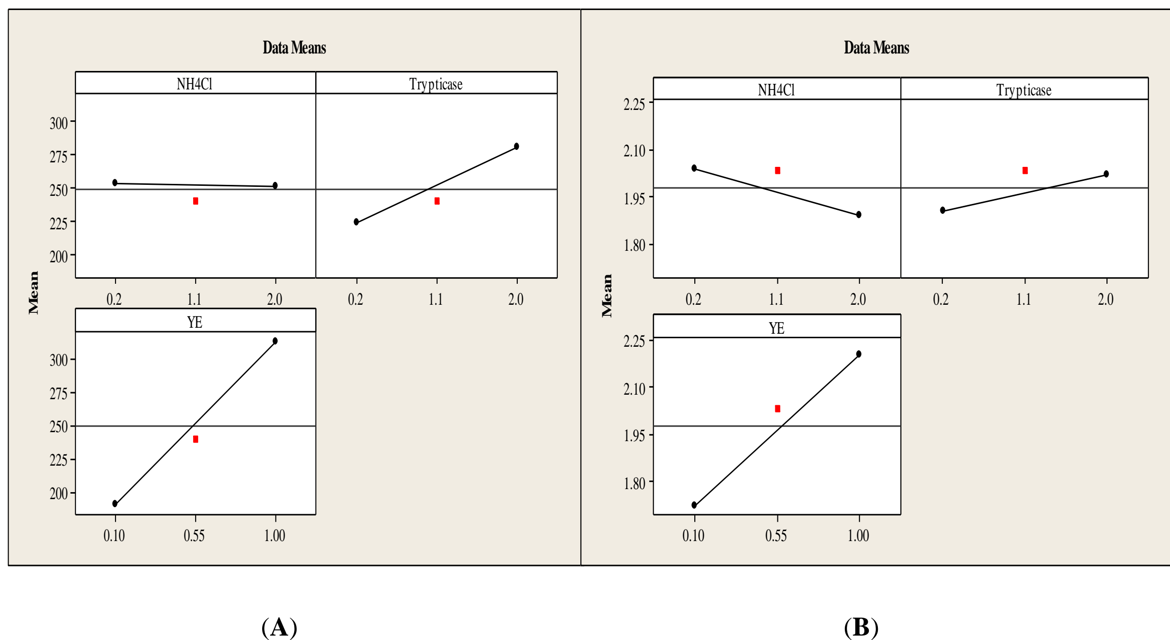


Figure 2: Wood-Ljungdahl pathway and metabolites formation from acetyl-CoA. Abbreviations: THF, tetrahydrofolate; CFeSP, corrinoid iron-sulphur protein.

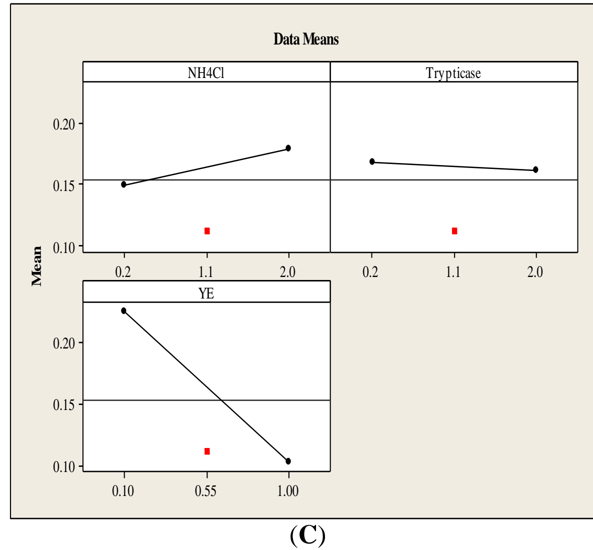
Based on the main effects plot (Figure 3a), cell growth of *C. autoethanogenum* was obviously affected by the initial YE and trypticase concentrations in the medium. The amount biomass increased with an increase of initial YE as well as trypticase concentrations within the range of concentrations studied in this work. This can be attributed to the nutritional value of YE and trypticase soy broth, as both contain various amino acids, vitamins and other growth-stimulating compounds.

From the ANOVA analysis, it was observed that out of all the individual effects of each source of nitrogen, the effects due to the YE concentration was found statistically significant ($p < 0.05$) for ethanol and acetic acid production. For ethanol

production (Figure 3c), the presence of YE showed the highest negative effect, whereas NH_4Cl and trypticase exerted either a slightly positive or a slightly negative effect, respectively. The positive effect of NH_4Cl on ethanol production was also reported by Guo *et al*. (2010). Plackett–Burman design was used in their studies, screening NH_4Cl as one of the significant factors affecting ethanol production, along with $MgSO_4$ and pH (Guo et al., 2010). Enhanced growth in YE-limited media has been reported in previous studies. The presence of YE results in a richer medium, which is favorable for biomass growth. Biomass growth is usually related to acetate formation, while ethanol production is generally not a growth-related metabolite. Barik *et al.* (1988) suggested that a minimum level of approximately 0.01% YE would be essential for providing trace nutrients for cell growth. However, up to 300% improvement in the ethanol/acetate ratio was observed when YE was completely eliminated.



(A) **(B)**



(**C**)

Figure 3: Main effects plot for (a) Biomass, (b) Acetic acid and (c) Ethanol.

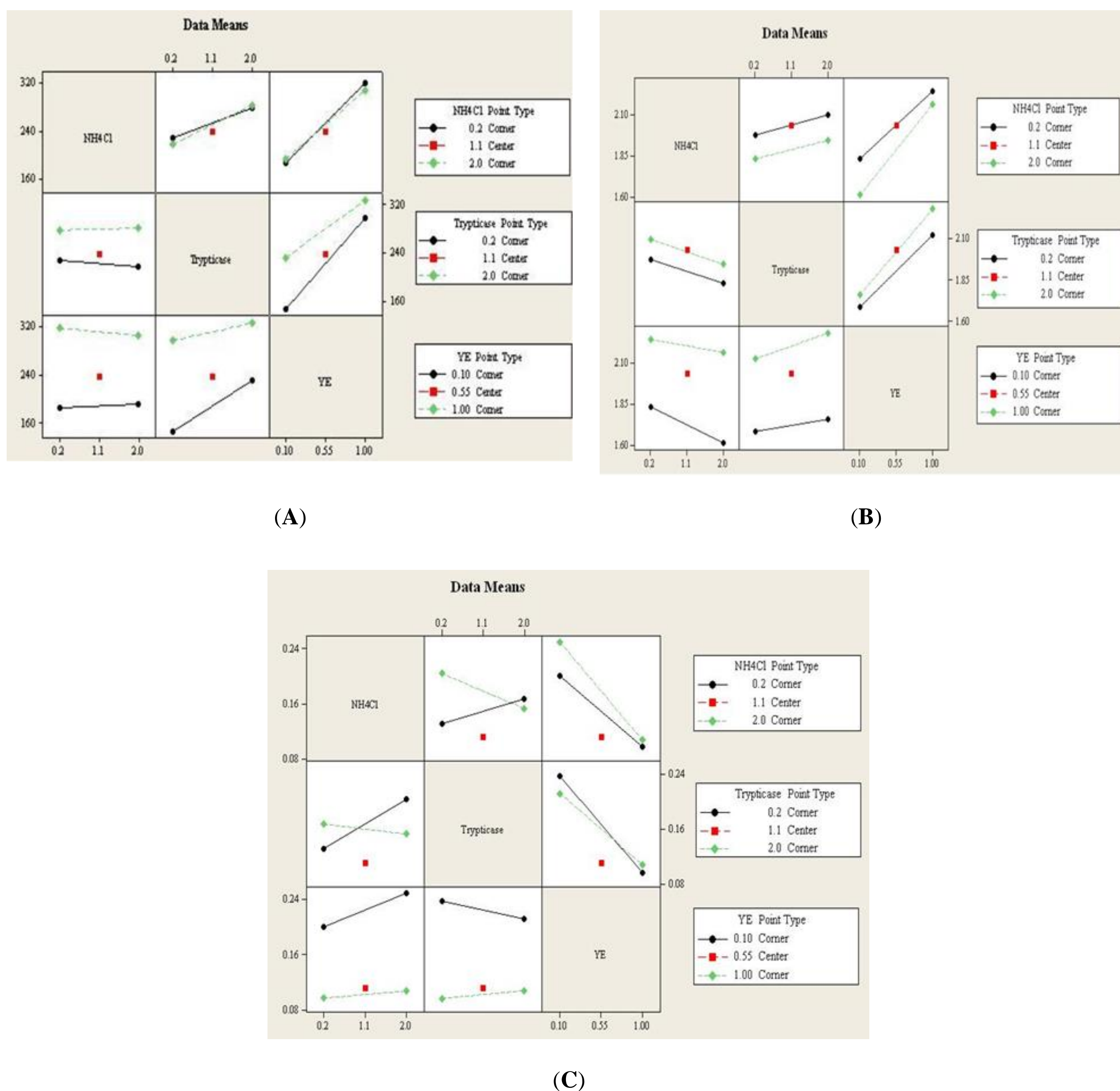
The negative effect of YE on ethanol production is expected to be due to vitamin B12, among others. YE contains vitamin B12, which plays an important role in acetogenic bacteria. Methyl transferase synthase (MeTr) in acetogens is a cobalamin-dependent enzyme and catalyzes the transfer of the methyl group of methyl-H4folate to the cobalt center of the corrinoid iron–sulfur protein (CFeSP). It is proposed that by reducing the H4folate cycle rate, NAD(P)H can build up inside the system with a subsequent increase in ethanol production (Kundiyana et al., 2011a). In another study conducted with *Alkalibaculum bacchi* strain CP15, in a 7-L fermentor, a similar effect was observed; *i.e.*, a YE-free medium produced 13% more ethanol than a YE-containing medium. However, a decreased production of acetic acid and cell mass, reaching up to 40% and 15%, respectively, was observed in the YE-free medium (Liu et al., 2014).

4.3.1.2 Interaction Effects Plot

The interaction effects plot for biomass, ethanol and acetic acid produced from CO is shown in Figure 4 and provides the mean response of all possible combinations from

low to high level of each two factors. That is, the effect of each factor dependent upon the second factor. Non-parallel lines represent an interaction between those two factors (YE, trypticase, and/or NH_4Cl). From the interaction plot for biomass and ethanol (Figures 4a,c), it can be observed that there is a strong interaction between each two factors. However, there is no remarkable interaction between the pairs of factors for acetic acid production (Figure 4b).

The maximum concentrations of biomass and acetic acid achieved were above 290 mg/L and 2.1 g/L, respectively, in all the experiments in which a YE concentration of 1 g/L was used, irrespective of the concentrations of trypticase and NH_4Cl in the medium (Figures 4a,b). The amounts ethanol produced reached their maximum values when YE was present at a low concentration of 0.1 g/L, irrespective of the concentrations of the other two factors (trypticase and NH_4Cl) (Figure 4c). This shows the influence of the YE concentration on the spectrum of products obtained from CO conversion in *C. autoethanogenum*. Considering the interaction between NH_4Cl and trypticase, a higher amount of biomass was found to be produced at a higher trypticase concentration of 2 g/L, at both levels of NH_4Cl, which can be attributed to the complex nutrients present in trypticase (Figure 4a).



(**A**) (**B**)

(**C**)

Figure 4: Interaction effects plots for (a) Biomass, (b) Acetic acid and (c) Ethanol.

4.3.1.3 Effect of Individual Sources of Nitrogen on Growth and Product Formation

Experiments were performed with either NH_4Cl (1.1 g/L), trypticase (1.1 g/L) or YE (0.55 g/L), as the only source of nitrogen. It was observed that there is no growth nor product formation in bottles containing only NH_4Cl. In the bottles with YE or trypticase, similar behaviours were observed, with growth reaching up to approximately 230 mg/L, and product concentrations of around 0.07 g/L for ethanol and 2 g/L for

acetic acid. However, it is also worth recalling that the amount YE used in preparing the medium is half the amount of trypticase. From these observations, in the subsequent studies in continuous bioreactors, YE was chosen as the sole nitrogen source. Since YE and trypticase also contain other compounds besides nitrogen-containing ones, their potential use as substrates for the production of end-metabolites was checked. In that sense, in experiments performed without any CO, it was observed that the presence of YE or trypticase could be involved in approximately up to 10% of the total acetic acid produced in experiments containing CO as carbon source as well as YE and trypticase.

4.3.2 Continuous Gas-Fed Bioreactor Experiments

Bioreactor experiments with continuous gas-flow, *i.e.*, continuous CO supply, were performed for up to two weeks each. Cell growth and the production of different metabolites in three different sets of experiments are shown in Figure 5. The redox potential was constantly monitored for each experimental run. It is related to the electron transfer undergoing inside the cells and hence is very sensitive for even delicate changes in metabolism. Both EXP1 and EXP3 had an instrument reading oxidoreduction potential (ORP) value of −87 ± 10 mV, while it was −43 ± 5 mV for EXP2. The ORP values are directly dependant on the pH of the medium. A lower pH of the liquid phase will result in lower negative values of the redox potential. Oscillations of the redox potential values in the culture medium could be due to microbial growth and variations in the metabolic profile at each point of the experimental run and have also been reported by other researchers in other bioconversion studies (Chen et al., 2012; Liu et al., 2013). Intracellular redox homeostasis is profoundly affected by the ups and downs of the extracellular redox potential which can significantly switch the fermentation type in acidogenic bacteria (Ren et al., 2007).

The biomass in EXP1 (Figure 5a) started growing after a shorter lag phase compared to EXP2 and EXP3, due to the favorable growth conditions (*i.e.*, optimal pH and nutritional value of YE) that prevail inside the bioreactor, attaining a biomass concentration of about 302.4 mg/L in less than 100 h of experimental run. The lag phase was approximately 70 h in both EXP2 and EXP3, reaching maximum biomass concentrations of 113.76 and 151.37 mg/L, respectively; that is 62% and 50% less than in EXP1. This confirms that the pH and YE concentration are important parameters and play a key role in achieving high cell mass concentrations. A drastic decrease in growth occurred after 89 h in EXP1. This could be linked to the accumulation of high amounts of acetic acid (~ 2 g/L) in the fermentation broth. Two enzymes are responsible for the conversion of acetyl CoA during the synthesis of acetate, *i.e.*, phosphotransacetylase (PTA) and acetate kinase (AK). During the acetate production stage, both enzymes are active and ATP is produced as a part of their reaction. However, it was reported that the activity of these enzymes decreases considerably with an increase in acetate concentration in the broth in fermentation with *C. acetobutylicum* (Ballongue et al., 1986). In the latter study, the AK was biosynthesized inside the cell of *C. acetobutylicum*, with buildup of acetate concentrations of up to 3 g/L in the broth, resulting in a rapid decrease in the AK activity with the increase of the amount acetate (Ballongue et al., 1986). However, a clear explanation for stoppage of growth and metabolite production in EXP2 and EXP3 after a certain period of time is yet somehow unclear.

No separate acidogenic and solventogenic phase was observed for *C. autoethanogenum* during these bioreactor studies using the reported media compositions and fermentation conditions. The conversion of acetic acid to ethanol in the late phase of the study was also not observed, although we observed such type of conversion of acetate to ethanol under different operating conditions (manuscript in preparation).

Acetic acid was the predominant metabolite formed during CO fermentation in each of the three experiments described here (Figure 5b). As mentioned above, changing the experimental conditions would allow a shift to ethanol accumulation rather than acetate. A maximum acetic acid concentration of 2.1 g/L was obtained after 137 h in EXP1, which is about 294% and 95% higher than the maximum amounts produced in EXP2 and EXP3, respectively. It is interesting to note that both experiments, EXP1 and EXP3,that were performed at high pH, produced more acetic acid than in studies at lower pH, irrespective of the YE concentrations used. A previous study using *C. ragsdalei* at two different pH values similarly reported a higher acetic acid production at high pH (Kundiyana et al., 2011b).

Although the maximum amount of ethanol was obtained in EXP1, the ratio ethanol/acetic acid was greater in EXP2 characterized by a low pH. Fermentation pH is one the most influential parameters that affects the metabolism of acetogenic bacteria. Lowering the pH appears to cause a shift in the product spectrum from acidogenic to solventogenic phase. The explanation lies in the permeation of the undissociated weak acid, acetic acid, through the cell membranes resulting in a lower internal pH due to the entry of H^+ ions. Bacteria overcome this physiological stress by producing solvents (Mohammadi et al., 2011).

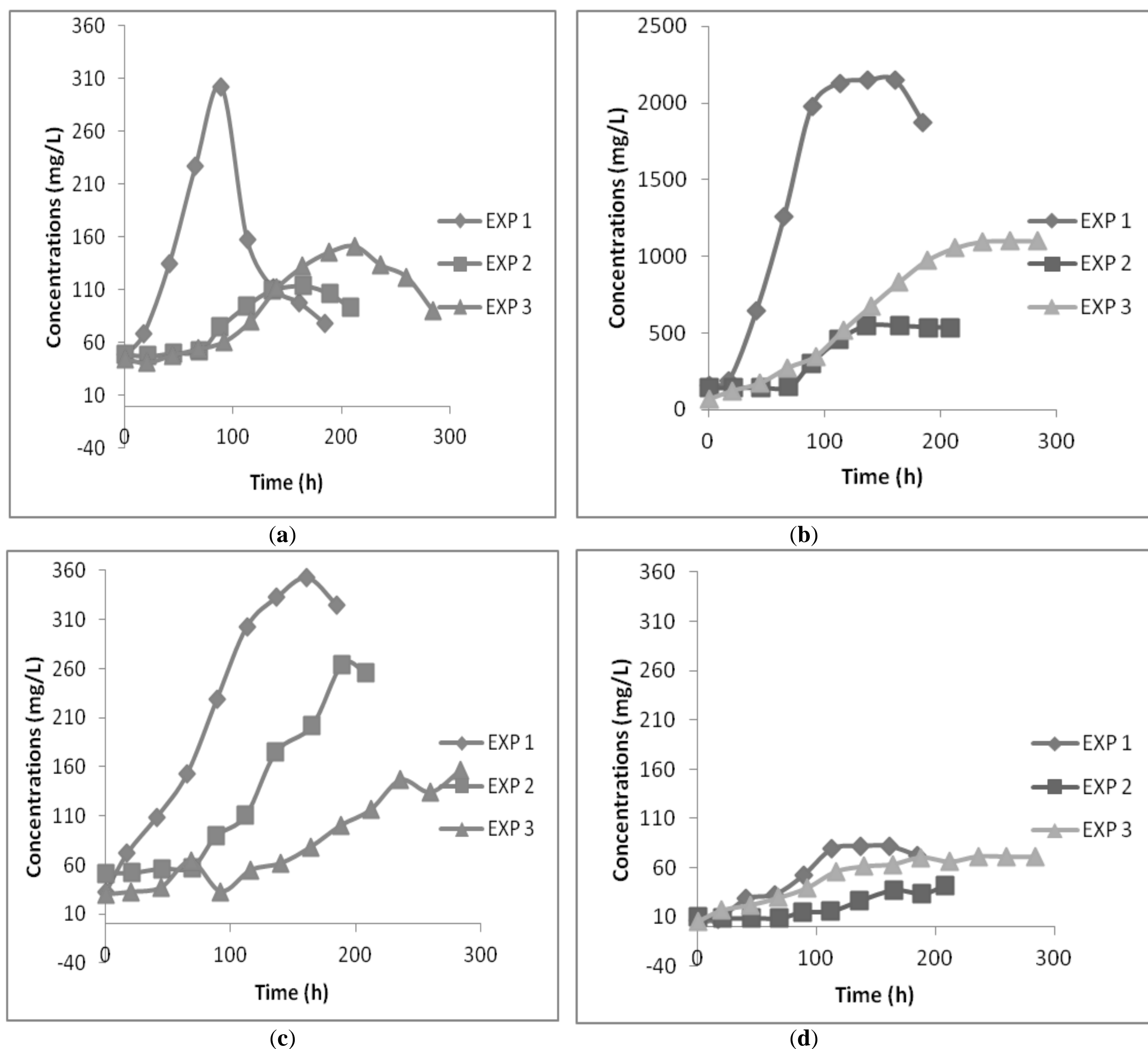


Figure 5: Cell mass (a) and products profiles, Acetic acid (b); Ethanol (c) and Butanediol (d) in three different experiments: EXP1 (pH = 5.75 and YE- 1 g/L); EXP2 (pH = 4.75 and YE 1 g/L); EXP3 (pH = 5.75 and YE 0.2 g/L).

As can be seen in Figure 5c, a higher maximum ethanol production was obtained in EXP1 than in EXP2, although the low external pH induced more solvent production. This could be due to the high biomass concentration achieved in EXP1. The fermentations produced 352.6 mg/L, 264.51 mg/L and 156.95 mg/L ethanol respectively in EXP1, EXP2 and EXP3. On the other hand, a maximum ethanol to acetic acid ratio was obtained for EXP2 with a value of 0.54. It can be seen that a low pH (EXP2) caused

a lengthening of the lag phase and reduced the final biomass concentration, yet it significantly improved the ethanol/acetic acid ratio. Thus, nutrient limitation combined with a low fermentation pH improved such product ratio. Several studies reported that two-stage stirred tank bioreactors, with a different pH in each vessel could improve the ethanol to acetic acid ratio (Gaddy et al., 1992; Klasson et al., 1990). From this study it is observed that using a low initial pH and maintaining it constant could also improve the ethanol/acetic acid ratio, although there is a strong decrease in the overall productivity of metabolites. A major obstacle in CO fermentation, when focussing on ethanol production, is that lowering the pH reduces cell growth; thereby reducing the overall productivity of ethanol in the process. Minor amounts 2,3-butanediol were also produced in all three experiments (Figure 5d). The butanediol concentration increased to a maximum of 81.8, 41.8 and 71.6 mg/L in EXP1, EXP2 and EXP3, respectively.

4.4 CONCLUSIONS

From the experiments it is clearly observed that altering the medium's composition as well as pH alters the product spectrum and biomass growth. From the batch studies, the YE concentration was found to have a significant effect on ethanol production. EXP1, at pH = 5.75 and a YE concentration of 1 g/L, produced a maximum amount of biomass (302.4 mg/L) and maximum concentrations of products, *i.e.*, acetic acid (2147.1 mg/L), ethanol (352.6 mg/L) and butanediol (81.8 mg/L), compared to the other two studies. A maximum ethanol to acetic acid ratio of 0.54 was obtained in EXP2 (pH = 4.75; YE 1 g/L). Though maintaining a low constant pH from the beginning improved the ethanol to acetic acid ratio, it drastically affects the overall productivity of the process as a result of a weaker biomass growth.

ACKNOWLEDGMENTS

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Chapter 5

Carbon monoxide fermentation to ethanol by *Clostridium autoethanogenum* in a bioreactor with no accumulation of acetic acid

ABSTRACT

Fermentation of CO or syngas offers an attractive route to produce bioethanol. However, during the bioconversion, one of the challenges to overcome is to reduce the production of acetic acid in order to minimize recovery costs. Different experiments were done with *Clostridium autoethanogenum*. With the addition of 0.75 µM tungsten, ethanol production from carbon monoxide increased by about 128% compared to the control, without such addition, in batch mode. In bioreactors with continuous carbon monoxide supply, the maximum biomass concentration reached at pH 6.0 was 109% higher than the maximum achieved at pH 4.75 but, interestingly, at pH 4.75, no acetic acid was produced and the ethanol titer reached a maximum of 867 mg/L with minor amounts of 2,3-butanediol (46 mg/L). At the higher pH studied (pH 6.0) in the continuous gas-fed bioreactor, almost equal amounts of ethanol and acetic acid were formed, reaching 907.72 mg/L and 910.69 mg/L respectively.

Keywords: Bioethanol; Carbon monoxide; *Clostridium autoethanogenum*; selenium; syngas; tungsten

With minor editorial changes to fulfill formatting requirements, this chapter is substantially asit appears in: *Bioresource Technology*. Published online 3 March 2015. DOI:10.1016/j.biortech.2015.02.113

5.1 INTRODUCTION

In recent years, growing interest has been found in the use of bio-based fuels as a result of the gradual depletion of global oil reserves and consensus in climate change. By 2020, it will be mandatory for all the Member states (MS) of the EU to reach their assigned targets in terms of energy and to achieve a 20% share of renewable energy (van Groenestijn et al., 2013). Moreover, the use of 10% renewable energy in transportation will be mandatory by then for all MS (Latif et al., 2014). Bioethanol was one of the biofuels which accounted for 28% of the overall biofuels used in the road transport in the EU in 2012. EU bioethanol production was forecasted to reach 5.38 billion liters in 2014 (Flach et al., 2013). Grains such as wheat, corn, barley and rye are currently the prominent feedstocks for bioethanol production in the EU. However, this leads to food-fuel competition. Hence, one way to overcome this situation is to utilize highly available lignocellulosic biomass or even waste as raw material for bioethanol production. However, the conventional way of bioconversion of lignocellulosic biomass to bioethanol is a somewhat complex process (Balat and Balat, 2009). An alternative and promising new generation bioethanol production process is through gasification of biomass in order to generate syngas or producer gas, composed mainly of CO, CO_2 and H_2. It is later introduced into a fermentor that is inoculated with anaerobic bacteria, mainly belonging to genera such as *Clostridium*, under specific process conditions (Abubackar et al., 2011a; Bengelsdorf et al., 2013; Mohammadi et al., 2011). The biocatalysts use these C_1 compounds as sole carbon source, following the reductive acetyl-CoA pathway, leading to the production of ethanol and acetic acid. Trace amounts of 2,3-butanediol, butanol, lactic acid are also reportedly being produced during the fermentation (Bengelsdorf et al., 2013). Recently, some studies were published on syngas fermentation with genetically engineered biocatalysts (Ueki et al.,

2014, Xie et al., 2015). On the other hand, studies are still ongoing with wild type strains of bacteria to improve the ethanol productivity by manipulating several parameters, such as the medium composition and/or fermentor operating conditions (Abubackar et al. 2012; Kundiyana et al. 2011).

The reductive acetyl-CoA pathway, also known as the Wood-Ljungdahl (WL) pathway, comprises an Eastern or methyl branch and a Western or carbonyl branch that uses CO and/or CO_2 as the substrate for the synthesis of acetyl-CoA, the intermediate that serves as precursor for the formation of biomass and metabolites such as ethanol and acetic acid. The proteins that are involved in the WL pathway, require cofactors such as H_4-folate, cobalamin, metal ions or FeS-clusters. For example, Corrinoid FeS proteins contain both cobalamin with a central cobalt atom and an FeS cluster. Carbon monoxide dehydrogenase (CODH) from *Moorella thermoacetica* and *Acetobacterium woodii* contains two Ni-FeS clusters. The formate dehydrogenase (FDH) of *M. thermoacetica* that catalyses the reduction of CO_2 to formate is a tungsten, selenium and FeS cluster containing metalloenzyme (Ragsdale and Pierce 2008).

So far, most studies on the production of ethanol from CO have focused on how various macronutrients (e.g., nitrogen sources) and their concentrations affect the fermentation process. Hardly any research has focused on how trace metals influence ethanol production and none has studied their effects in bioreactors with continuous feed of the gaseous substrate. One study has been published but in batch assays and with no pH control (Saxena and Tanner, 2011). Since tungsten and selenium are components of formate dehydrogenase (FDH), whereas aldehyde:ferredoxin oxidoreductase (AFOR) that catalyzes reduction of carboxylic acids to aldehydes is a tungsten containing enzyme (Figure 1) (Wang et al., 2013), the purpose of this study was to investigate the effects of tungsten and selenium on fermentation of CO by *Clostridium*

autoethanogenum and on product distribution in batch and continuous gas-fed bioreactors. The effect of the presence of vitamins and the influence of pH were also investigated.

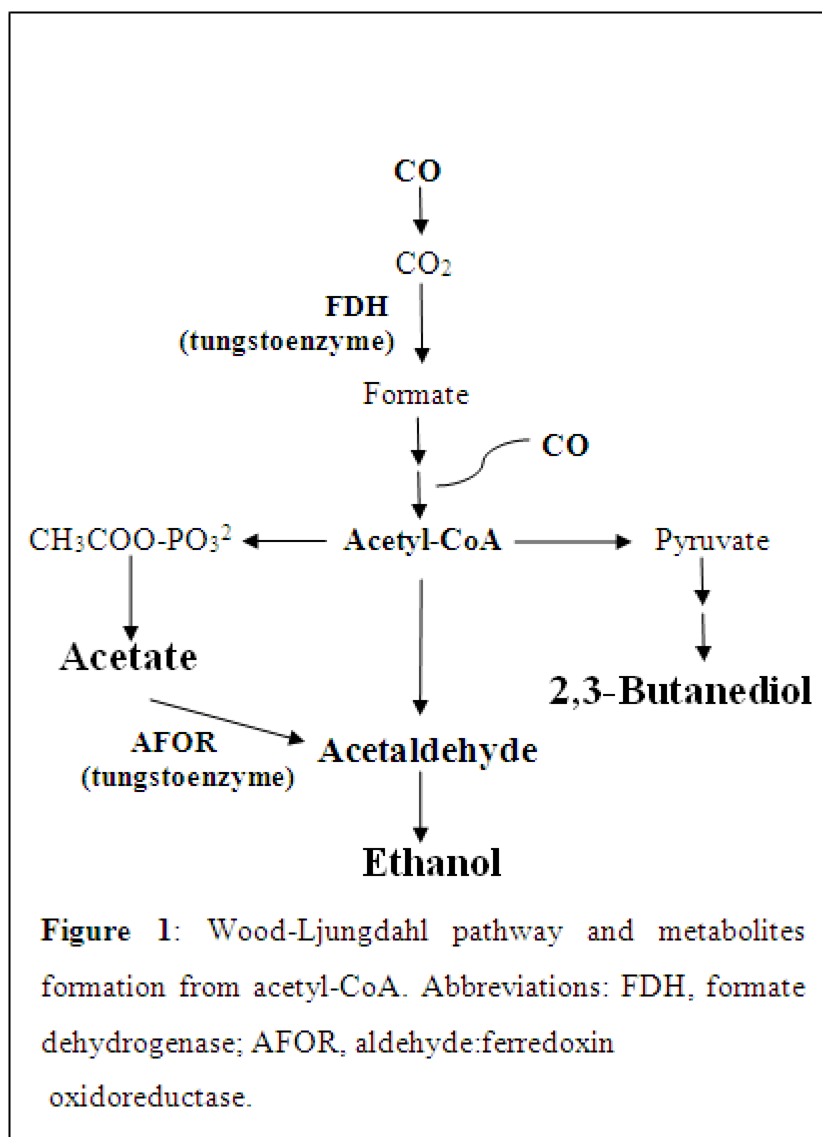


Figure 1: Wood-Ljungdahl pathway and metabolites formation from acetyl-CoA. Abbreviations: FDH, formate dehydrogenase; AFOR, aldehyde:ferredoxin oxidoreductase.

5.2 MATERIALS AND METHODS

5.2.1 Bottle batch experiments

Batch experiments were conducted without pH control to study the effect of trace metals, tungsten (W), selenium (Se) as well as vitamins on growth and product formation in *C. autoethanogenum* DSM 10061. The growth medium to maintain the bacteria as well as the production medium used for the batch experiments is given as supplementary material (Table 1). Five independent tests were performed in duplicate with the production medium having different trace metal compositions, as mentioned hereafter, without vitamins: (1) trace metal SL-10 without W and Se [TM]; (2) SL-10 with 0.075 µM W [Low W]; (3) SL-10 with 0.144 µM Se [Low Se]; (4) SL-10 with 0.75 µM W [High W] and (5) SL-10 with 1.44 µM Se [High Se]. Another set of four experiments was performed in duplicate to check the need and the effect of additional vitamins (Vit) on CO bioconversion: (1) SL-10 and Vit [Vit]; (2) SL-10 with 0.75 µM W and Vit [W-Vit]; (3) SL-10 with 1.44 µM Se and Vit [Se-Vit]; (4) SL-10 with 0.75 µM W and 1.44 µM Se as well as Vit [All]. 1 ml of vitamin solution (Table 1) per liter of production medium was used for the experiments with vitamins (Vit).

Studies were carried out in duplicate at an initial pH of 5.75 in 100 ml serum vials with 30 ml production medium and inoculated with 2.5 ml of actively growing seed culture, which was grown with CO as sole carbon source. 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 ^{o}C. The experimental set-up and the method used for media preparation as well as sampling details are described elsewhere (Abubackar et al., 2011b).

Table 1: Growth and production medium for *C. autoethanogenum*

Growth Medium (pH 6)
The composition (per liter distilled water): NH_4Cl, 0.9 g; NaCl, 0.9 g; $MgCl_2{\cdot}6H_2O$, 0.4 g; KH_2PO_4, 0.75 g; K_2HPO_4, 1.5 g; $FeCl_3{\cdot}6H_2O$, 0.0025 g; trypticase peptone, 2.0 g; yeast extract, 1.0 g; cysteine-HCl, 0.75 g; 0.1 % resazurin, 0.5 mL; with 0.5% xylose and SL-10 solution, 1.0 mL. The trace metal stock solution SL-10 contained (per liter): 7.7 M HCl, 10 mL; $FeCl_2{\cdot}4H_2O$, 1.5 g; $ZnCl_2$, 70 mg; $MnCl_2{\cdot}4H_2O$, 100 mg; H_3BO_3, 6 mg; $CoCl_2{\cdot}2H_2O$, 190 mg; $CuCl_2{\cdot}2H_2O$, 2 mg; $NiCl_2{\cdot}6H_2O$, 24 mg; and $Na_2MoO_4{\cdot}2H_2O$, 36 mg.
Production Medium (pH 5.75)
The composition (per liter distilled water): NaCl, 0.9 g; $MgCl_2{\cdot}6H_2O$, 0.4 g; KH_2PO_4, 0.75 g; K_2HPO_4, 1.5 g; yeast extract 0.5 g; $FeCl_3{\cdot}6H_2O$, 0.0025 g; 0.1 % resazurin, 0.5 mL; cysteine-HCl 0.75 g and SL-10 solution, 1.0 mL.
Vitamins
The vitamin stock solution contained (per liter) 10 mg each of *para*-aminobenzoic acid, calcium pantothenate, nicotinic acid, riboflavin, thiamine, α-lipoic acid, and vitamin B12, 4 mg each of d-biotin, folic acid and 20 mg pyridoxine.
Tungsten and Selenium
The chemicals used were $Na_2WO_4.2H_20$ and Na_2SeO_3

5.2.2 Continuous gas-fed bioreactor experiments with tungsten

Two bioreactor experiments were carried out in a 2-L New Brunswick Scientific BIOFLO 110 bioreactor at either pH 6.0 (High pH) or pH 4.75 (Low pH) with 1.2 L batch liquid medium and CO (100%) as the gaseous substrate, continuously fed at a rate of 10 ml/min using a mass flow controller (Aalborg GFC 17). The medium composition used for the experiments was the same as in batch assays with the trace metal solution

containing 0.75 µM W, as this was shown to favour the desired bioconversion pathway. The bioreactor was maintained at a constant temperature of 30^{0}C, with a constant agitation speed of 250 rpm throughout the experiments. 10% of an actively growing culture, which was grown for 48 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 6.0 or 4.75, through the addition of a 2 M NaOH or 2 M HCl solution, fed by means of a peristaltic pump. Gas samples of 0.2 mL were taken from the inlet and outlet sampling ports of the bioreactor to monitor the CO and CO_2 concentrations. Similarly, 2 mL of liquid sample was periodically withdrawn from the reactor, once every 24 h, in order to measure the optical density ($OD_{\lambda=600\ nm}$) and estimate the biomass concentration. Afterwards the sample was filtered with a syringe using a 0.22 µm PTFE-filter before analyzing the concentrations of soluble products.

5.2.3 Analytical equipment and measurement protocols

Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph (GC) 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 ^oC, for 5 min, and then raised by 20 ^oC min^{-1} for 2 min, to reach a final temperature of 90 ^oC. The temperature of the injection port and the detector were maintained constant at 150 ^oC. 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^{0}C, respectively. For 2,3-butanediol identification, a Thermo Scientific ISQ™ single quadrupole GC-MS system, operated at 70 eV, mounted with a HP-5ms column (30 m × 0.25 mm × 0.25 µm film thickness) was used. The water-soluble

products, acetic acid, ethanol and 2,3-butanediol, in the culture broth were analyzed 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. Cell mass was estimated by measuring the absorbance of the sample at a wavelength of 600 nm using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The measured absorbance was then compared to the previously generated calibration curve to calculate the corresponding biomass concentration (mg/L). Besides, the redox potential was monitored continuously using a Ag/AgCl reference electrode maintained inside the bioreactor and connected to a transmitter (M300, Mettler Toledo, Inc. USA).

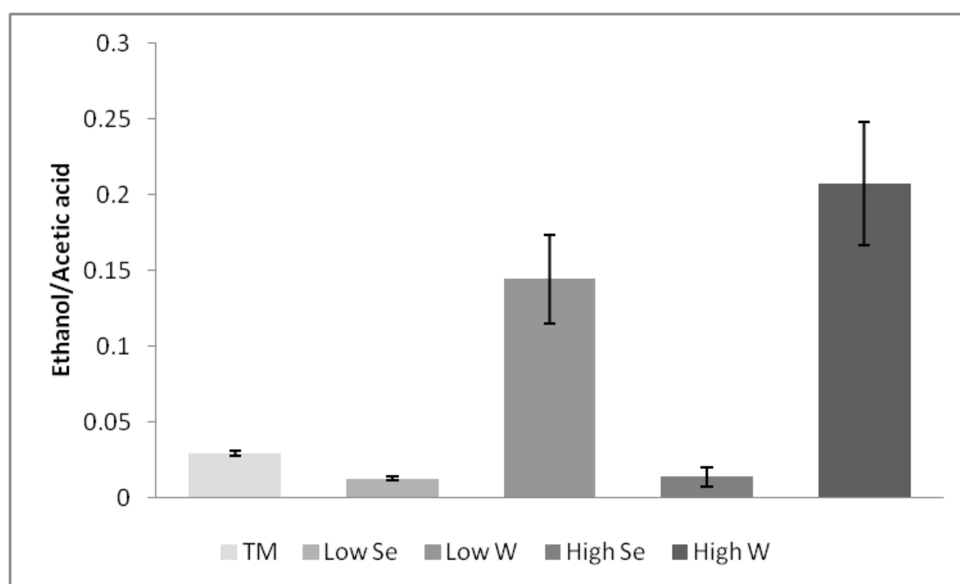
5.3 RESULTS AND DISCUSSION

5.3.1 Bottle batch experiments

Fig. 2 and 3 show the ethanol/acetic acid and butanediol/ acetic acid ratio for the two sets of experiments. The experimental results show that the highest ethanol to acetic acid ratio obtained was 0.19 in the experiment designated as High W; that is 173% higher than the ratio obtained in the experiment with High Se. It is clear from the plot that the ethanol/acetic acid ratio, in batch tests, increased with the presence of tungsten in the medium. In the case of selenium, the ratio obtained was roughly similar in either the Low Se or the High Se experiment, with a value of 0.013 (Fig. 2a), which was even lower than in the control medium (TM). Hence it can be concluded that selenium did not allow to increase the ethanol/acetic acid ratio in *C. autoethanogenum*. It didn’t even favor considerably acetic acid production compared to the control medium. A recent report on a study with another bacterial strain agrees with the present findings, and

suggested no significant change in acetic acid production with or without selenium in the medium (Saxena and Tanner, 2011).

Some tungstoenzymes are involved in the WL pathway and its subsequent routes that lead to metabolites production, include formate dehydrogenase (FDH) and aldehyde:ferredoxin-oxidoreductase (AFOR), having pterin cofactors as their active sites (Fig. 1). FDH catalyzes the first reaction in the WL pathway, that is the two–electron reduction of CO_2 to formate (Ragsdale and Pierce, 2008). The first originally isolated tungstoenzyme is the FDH from *C. thermoaceticum*. It contains 1 tungsten atom, 1 selenium, 18 iron and about 25 inorganic sulfur per dimeric unit, and utilizes NADPH as the physiological electron carrier (Yamamoto et al., 1983). It was reported that the presence of tungsten, selenium, molybdenum and ferrous ions in the growth medium stimulates FDH synthesis (Yamamoto et al., 1983). Recently, it was reported that FDH in *C. autoethanogenum* forms complexes with an electron bifurcating hydrogenase enzyme that is NADP specific (Wang et al., 2013). The chemical analysis of this complex revealed that it contains tungsten. Experiments using *C. ragsdalei* to study the effect of trace metals, when using CO as a substrate, indicated that the presence of tungsten (WO_4^-) at a concentration of 0.681 μM, yielded an ethanol production of 35.73 mM, which improved to 72.3 mM upon increasing the tungsten concentration to 6.81 μM (Saxena and Tanner, 2011). In that study, it was suggested that the presence of both selenium and tungsten in the medium decreases



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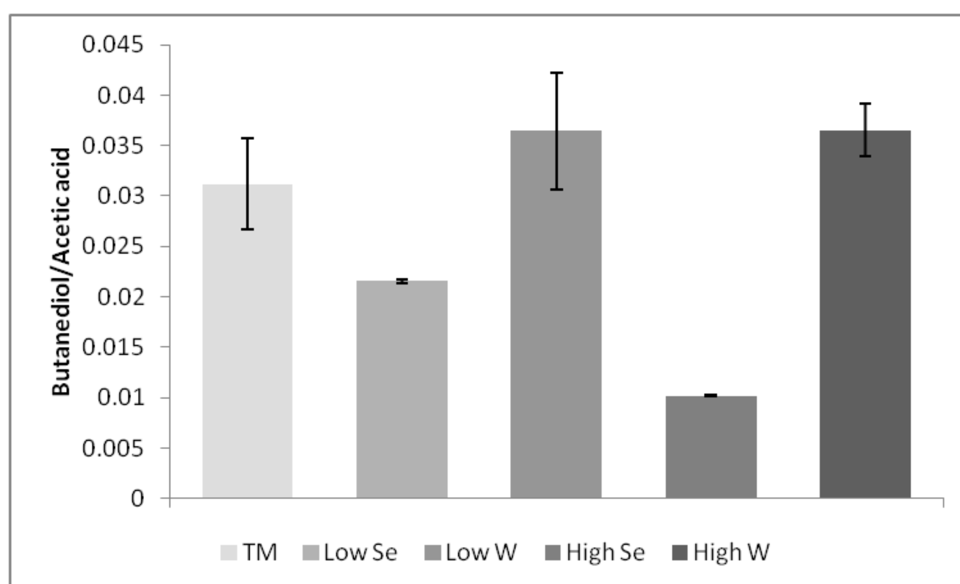


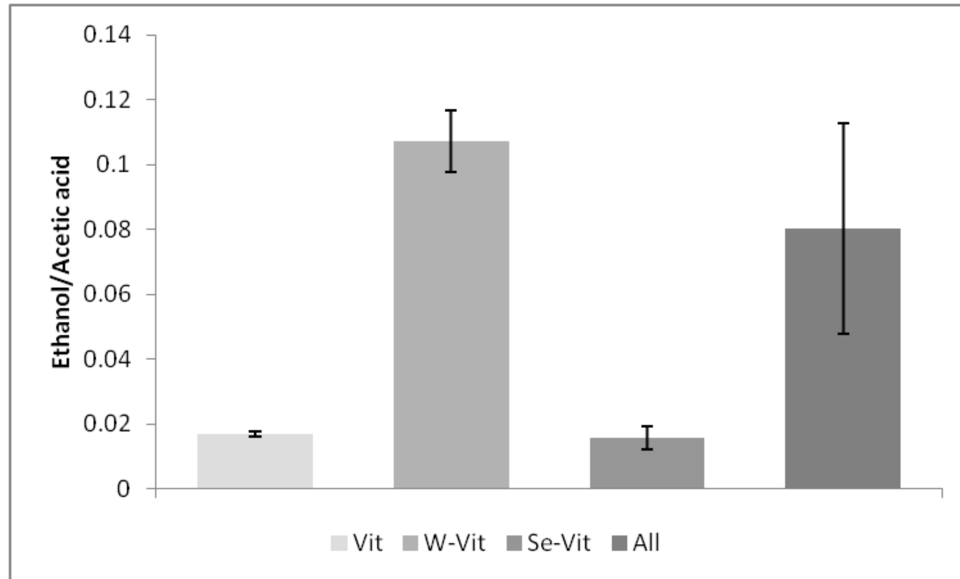
(b)

Figure 2: Ethanol/acetic acid (a) & Butanediol/acetic acid ratio (b) obtained in absence of vitamins. TM = trace metal solution without selenium and tungsten. The error bars represents the standard deviations.

the activity of FDH in *C. ragsdalei* compared to media containing either tungsten or selenium only. AFOR, on the other hand, catalyzes the reduction of acetic acid to acetaldehyde. It was reported that AFOR from the hyper-thermophilic archaeon *Pyrococcus furiosus* is a homodimer with 1 W and 4-5 Fe atoms per molecule (Kletzin and Adams, 1996).

The results from the second set of experiments, aimed at studying the effect of adding a vitamin solution, showed that the presence of additional vitamins did not enhance the ethanol/acetic acid ratio. Interestingly, in the medium containing both selenium and vitamins, besides tungsten ("All", Fig. 3a), the ethanol/acetic acid ratio was twenty five percentage lower than the value obtained in the medium containing both tungsten and vitamins but without any addition of selenium ("W-Vit", Fig. 3a). While many researchers add vitamins in studies on biofuels production (e.g., ethanol) with *clostridia*, the present data questions the need of such addition, which is a relevant cost-related issue. Our *C. autoethanogenum* strain does not need the supply of additional vitamins for ethanol production. To the best of our knowledge, this is different from all other *Clostridium* strains described so far. One possible explanation is that this *C. autoethanogenum* strain has repeatedly been transferred to fresh media without adding vitamins and could therefore have adapted to such conditions. It may be assumed that, in the present study with *C. autoethanogenum*, selenium would inhibit the ethanol production pathway and partly counteract the favorable effect of tungsten. Trace metals might exhibit different effects in different CO-metabolizing strains, but tungsten showed a clear positive effect on ethanol production in our batch assays with *C. autoethanogenum*, while selenium at either no positive effect or even a negative effect depending on the nature of other elements (i.e., trace metals or vitamins) present in the medium.

The production of small amounts of 2,3-butanediol was also observed during CO fermentation in all the batch experiments. The maximum butanediol/acetic acid ratio obtained in the present work was 0.032, and was exactly the same for all the experiments that contained tungsten, irrespective of the tungsten concentration and the presence or not of vitamins (Fig. 2b and 3b). Such data cannot be compared to any other previous experiment as no other study has focused on the effect of trace metals on 2,3-butanediol production from CO in *Clostridia*. From Fig. 2 and 3, it appears that the presence of tungsten increases the butanediol/acetic acid ratio similarly as in the case of the ethanol/acetic acid ratio. However, when compared to the control medium (TM) (with no tungsten nor selenium), the addition of tungsten increased more the ethanol/acetic acid ratio than the butanediol/acetic acid ratio. Indeed the ethanol/acetic acid ratio was 5 to 7 times higher when adding tungsten (either at low or high W concentration), while the butanediol/acetic acid ratio only increased by about 20% when adding tungsten compared to TM. In any case, for both ethanol and 2,3-butanediol, it can be concluded that their relative concentration, compared to acetic acid, decreases under the following conditions, without the addition of a vitamin-solution: presence of tungsten (no selenium) > no tungsten nor selenium > presence of selenium (no tungsten). In the WL pathway and later in 2,3-butanediol production, acetyl-CoA with CO_2 are converted to pyruvate using pyruvate:ferredoxin oxidoreductase (PFOR). Pyruvate gets reduced by acetolactate synthase and acetolactate decarboxylase to acetoin and then later to 2,3-butanediol using 2,3-butanediol dehydrogenase (23BDH) (Köpke et al., 2011). Köpke et al. (2014) recently discovered that *C. autoethanogenum* contains two dehydrogenases that are able to reduce acetoin to 2,3-butanediol, namely 23BDH and primary-secondary alcohol dehydrogenase.



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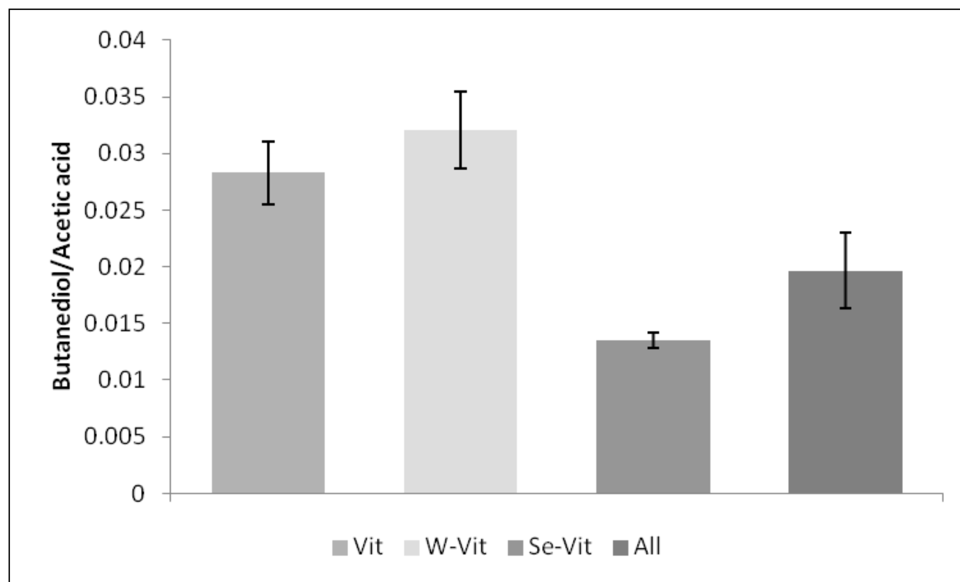


(b)

Figure 3: Ethanol/acetic acid (a) & Butanediol/acetic acid ratio (b) obtained in presence of vitamins. All = presence of selenium, tungsten in addition to vitamins. The error bars represents the standard deviations.

The final pHs after these experimental batch runs were also measured. The production of acetic acid during the growth decreased the pH of the medium significantly and this usually inhibited the bacterial growth and metabolites production. The initial pH of the medium was 5.75 and the initial phosphate concentration was 14 mM. Phosphate in the form of KH_2PO_4/K_2HPO_4 was used as pH-buffering solution. It was observed that the final pH value was in the range of 3.80 – 4.00 for all the experiments.

The above findings in batch bottles confirm that the presence of tungsten improves the ethanol/acetic acid as well as the butanediol/acetic acid ratios, while the addition of selenium and vitamins had no favorable effect for ethanol production. Hence, in further studies in bioreactors with continuous CO supply, trace metal solutions with tungsten were used and vitamins and selenium were omitted, as described below. Since the pH value would affect biomass growth and the production of metabolites, the next experiment was performed using a pH-control unit in order to maintain a constant pH.

5.3.2 Bioreactor experiment with continuous CO supply

5.3.2.1 Biomass profile

Fig. 4 shows that the pH value had a profound effect on biomass production. Although pH could not be maintained constant in the batch bottle assays described above; in the present bioreactor studies pH remained stable throughout the experiments. To the best of our knowledge, no previous other study has been reported on the effect of trace metals and vitamins in continuous CO-fed bioreactors under regulated, constant, pH conditions. pH control is important as it represents an additional parameter expected to affect biomass growth and production of metabolites. Biomass started growing instantly without any lag phase at pH 6.0, while, a 24 h lag phase was observed in the experiment

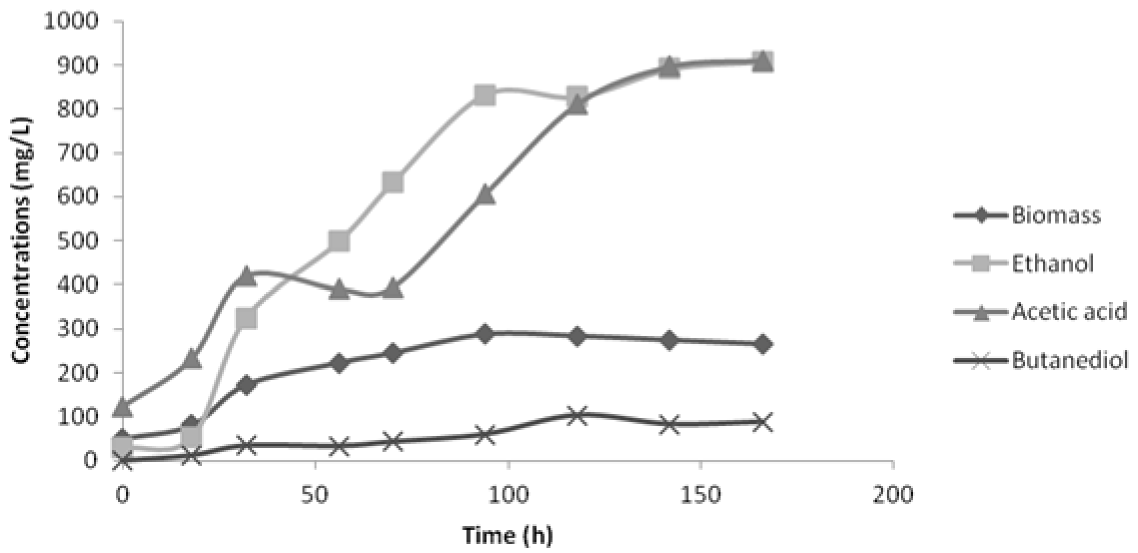
at pH 4.75 (Fig. 4). A maximum biomass concentration of 287.77 mg/L was achieved at pH 6.0 which is 109% higher than the maximum value obtained at pH 4.75 and during the exponential phase, biomass increases at a rate 70% faster at pH 6 than at pH 4.75. This can be attributed to the negative impact of pH deviations from the organism's optimum pH range for growth i.e., between pH 5.8 and 6.0. Hence, the results demonstrated that the growth of *C. autoethanogenum* was limited when pH decreased sharply, under slightly acidic conditions. The biomass entered the stationary phase after 48 h and 96 h, respectively, with the pHs set at either 4.75 or 6.0. The amount biomass achieved during the experimental run is comparatively lower than that obtained for studies with other *Clostridium* strains (Abubackar et al., 2011a; Mohammadi et al., 2011). In one of our previous studies in bioreactor with continuous CO supply and with 1 g/L yeast extract at pH 5.75, the maximum biomass obtained was 302.4 mg/L, which is comparable to the maximum cell mass concentration obtained in this study at pH 6.0 (Abubackar et al., 2015). However, most batch studies with the strain *C. autoethanogenum* usually reported a low level of biomass growth compared to other bacterial species (Cotter et al., 2009; Guo et al., 2010). Cotter et al. (2009) reported a maximum biomass concentration of 150 mg/L in *C. autoethanogenum*, achieved while feeding syngas (20% CO) at a flow rate of 10 ml/min.

The low cell mass concentration in bioreactor studies might be due to two reasons, either to limited nutritional availability in aqueous phase or/and to low availability of gaseous substrate. Biomass yields might also be a strain-linked parameter. Most of our own data, as well as some other published studies, suggest that biomass growth and consequently ethanol production seems to be generally lower in *C. autoethanogenum* than in strains such as *C. ljungdahlii* (Abubackar et al., 2015; Guo et al., 2010; Mohammadi et al., 2011). The solubility of CO in liquid phase is low as well

as its mass transfer into the aqueous medium. Hence, in this state of limited mass transfer, the microorganism could not obtain sufficient substrate for growth and maintenance, which eventually leads to a low growth rate. Since vitamins did not show any effect on biomass accumulation in batch bottle experiments with this specific strain, it was eliminated in the bioreactor study, but to the best of our knowledge, the absence of vitamins would not be an explanation for the low biomass growth. However, a low medium cost is absolutely essential for optimizing the techno-economics of syngas fermentation. In a study using *Alkalibaculum bacchi*, a 50% higher cell mass concentration was reported in YE medium along with vitamins and mineral solutions than with corn steep liquor (CSL) medium, though the maximum cell mass concentration obtained with YE was still only 330 mg/L (Liu et al., 2014).

5.3.2.2 Product formation

As can be observed from the figure (Fig. 4), the metabolic products obtained from CO fermentation were strongly affected by the pH. In some of our previous studies, it was found that *C. autoethanogenum* produced a higher amount of acetic acid than ethanol under the experimental conditions specifically used in that work (Abubackar et al., 2015). In the present work, ethanol and acetic acid were the dominant final fermentation products in the study at pH 6.0 with productions reaching a maximum of 907.72 mg/L and 910.69 mg/L, respectively. A maximum ethanol concentration of 867 mg/L was produced at pH 4.75, together with no acetic acid production and a negligible concentration (< 50 mg/L) of butanediol as the alcohol byproduct. It can be suggested from this study that changing the pH of the medium at a specific stage of the continuous CO fermentation process induces a metabolic shift. In contrast, at pH 6.0, concomitant, continuous, acetic acid and ethanol production was observed, and it could be noted that the ethanol to acetic acid ratio obtained was close to 1. This value is greater than that



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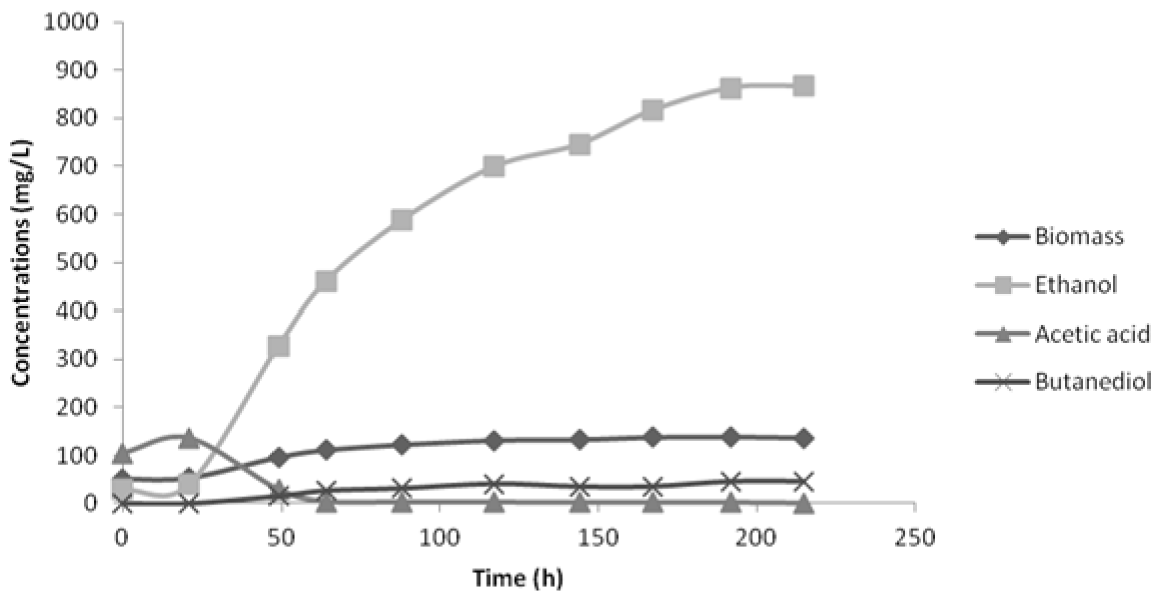

(b)

Figure 4: Cell mass and products profile at two different, constant, pHs studied in bioreactors: pH 6 (a) and pH 4.75 (b)

obtained in our previous experimental studies without tungsten, where the maximum ethanol/acetic acid ratio obtained was 0.54 (Abubackar et al., 2015). Ethanol production is also higher than in the batch bottle assays described above with no pH regulation. A

significant part of the CO fed was directed towards acetic acid production at the branch point of acetyl-CoA in the WL pathway (Fig. 1). Even though ethanol started being produced at the early stage of the biomass growth at both pHs, most of the ethanol titer was produced during the stationary phase. Although the final ethanol concentration was similar both at pH 4.75 and pH 6.0, it took about twice as long to reach such concentration at low pH than at high pH. This is also related to the higher amount biomass found at high pH.

As discovered from the bioreactor study, CO bioconversion by *C. autoethanogenum* changed from a predominant acetate and ethanol production at pH 6.0 to predominant ("single") ethanol production at pH 4.75. An apparent metabolic shift of pathway from acidogenesis to solventogenesis upon decreasing the pH has also been observed previously in ABE (Acetone-Butanol-Ethanol) fermentation by *C. acetobutylicum* (Grupe and Gottschalk, 1992). Solventogenesis in syngas fermentation occurs during unfavorable growth conditions and in the presence of ample reducing equivalents. Using an initial low nutrient medium pH in order to improve the final ethanol titer decreases the cell mass concentration, which might then also decrease the productivity of metabolites. In order to overcome this, some researchers tried to use two stage bioreactors with operating conditions that support growth in the first bioreactor and with the second reactor with reduced pH and conditions that are favorable for ethanol production (Mohammadi et al., 2012; Richter et al., 2013). Here, besides using two reactors in series, another alternative might consist in switching the pH from high (growth conditions) to low (solventogenesis conditions) values. During the fermentation at pH 4.75, the production of acetic acid was not observed and furthermore, the acetic acid initially present in the inoculum was immediately consumed during the experiment. This happens, as discussed above, through the activity of the enzyme AFOR that

converts acetic acid to acetaldehyde and latter to ethanol through an alcohol dehydrogenase (ADH) (Wang et al., 2013). Acetic acid production along with biomass growth and later partial acid conversion to ethanol was recently observed in some studies (Liu et al., 2014). However, to the best of the authors knowledge, there is no previous study that reported syngas fermentation using wild type bacteria without any production or accumulation of acetic acid at all at the end of the fermentation process.

5.4 CONCLUSIONS

In *C. autoethanogenum*, the addition of selenium and/or vitamins did no improve the ethanol/acetic acid ratio compared to a control medium without such additions. Furthermore, it clearly appears that the presence of tungsten improved ethanol production by *C. autoethanogenum*. Enhanced 2,3-butanediol/acetic acid ratio was also obtained with the presence of tungsten, but not with selenium. Results from the bioreactor studies with continuous CO supply revealed that the presence of tungsten together with a shift from high (pH 6) to low pH (pH 4.75) improves ethanol production by *C. autoethanogenum* without any accumulation of acetic acid.

ACKNOWLEDGEMENTS

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Chapter 6

Novel bioreactor operating strategy for continuous ethanol production from carbon monoxide without accumulation of acids

ABSTRACT

Ethanol production from C1 compounds such as carbon monoxide using acetogenic bacteria is an attractive alternative to produce renewable fuels. However, the process is generally associated with acetic acid accumulation, which is often produced in larger quantities than ethanol itself. This study shows the continuous production of ethanol and complete conversion of produced acetic acid to ethanol through pH shifts from high pH to low pH using an optimized medium and one single gas-fed bioreactor. Experiments were performed with pH shifts from high (5.75) to low (4.75) values in a cyclic mode while simultaneously renewing part of the fermentation broth. Such sequencing-batch operating strategy allowed to continuously accumulate increasing amounts of ethanol in each cycle while reaching complete removal of acetic acid which was converted to ethanol. In these studies a final ethanol concentration of 4.3 g/L was reached with just one pH shift and one partial medium renewal.

Keywords: Butanediol; *Clostridium autoethanogenum*; selenium; syngas; tungsten

6.1 INTRODUCTION

Acetogens have rather recently been shown to be able to produce (bio)ethanol by utilizing gaseous substrates. These bacteria use C1 compounds such as CO, H_2-CO_2 or mixtures thereof and convert them into compounds such as acetic acid and ethanol via the Wood-Ljungdahl (WL) pathway (van Groenestijn et al., 2013). Besides, other metabolites such as 2,3-butanediol (2,3-BDO), butanol or butyrate can also be produced by some acetogens (Bengelsdorf et al., 2013). The most common metabolites formed by such acetogens are acids, such as acetic acid, and only very few alcohol producing organisms have been identified so far. Most ethanologenic acetogens belong to the genus *Clostridium***,** including *C. ljungdahlii*, *C. autoethanogenum*, *C. ragsdalei* and *C. coskatii* (Abubackar et al., 2011a; Bengelsdorf et al., 2013). This versatile ethanol production technology allows to utilize lignocellulosic biomass as feedstock and convert it to syngas via gasification or, otherwise, utilize industrial waste gases containing these C1 compounds for their bioconversion to fuels and chemicals. While efficient bioconversion or biodegradation of gaseous substrates is possible in many different types of bioreactors (Kennes and Veiga 2013), the stirred tank bioreactor generally leads to the best results and has so far been the most commonly used bioreactor configuration for the bioconversion of CO-rich gases (Mohammadi et al., 2012; Kundiyana et al., 2011a). Acetogens are facultative autotrophs that can grow either by oxidation of organic substrates through glycolysis or by oxidizing CO to CO_2 and then follow the reductive WL pathway to produce acetyl-CoA (Ragsdale and Pierce, 2008). The overall products from glycolysis through oxidation of 1 mole of hexose sugar are 2 mole of acetyl-CoA, 2 mole of ATP, 2 mole of CO_2 and 8 electrons. The reduction of 2 moles of CO_2 via the WL pathway for producing 1 mole of acetyl-CoA requires 1 mole of ATP and 8 electrons. Since both pathways are present in acetogens, the 2 moles of

CO_2 and 8 electrons produced during glycolysis could be reassimilated to produce 1 mole of additional acetyl-CoA. Thus by combining glycolysis and the WL pathway, a total of 3 moles of acetyl-CoA can be obtained from 1 mole of hexose sugar (Fast et al. 2015). The acetyl-CoA then serves as main intermediate for the production of various metabolites such as ethanol, acetic acid, 2,3-BDO. The enzyme bound acetyl-CoA gets released and converted to acetate by the action of two enzymes, phosphotransacetylase (PTA) and acetate kinase (AK). One mole ATP is generated during acetate production. Ethanol production from acetyl-CoA can occur via two different routes. One route is a two-step reduction via acetaldehyde, which later reduces to ethanol. Another route is through conversion of the produced acetate to acetaldehyde by acetaldehyde:Fd oxidoreductase (AFOR) and then to ethanol by alcohol dehydrogenase (ADHE) (Bengelsdorf et al., 2013). The latter route is beneficial for the bacteria as well as for the industrial sector as it provides the way to produce ethanol by converting acetic acid thereby reducing the complex downstream processing. Using this route helps the bacteria to generate ATP for growth and maintenance. The electron donors for the above reactions are reduced ferredoxin and NADPH. The formation of pyruvate from acetyl-CoA is catalyzed by the enzyme pyruvate:ferredoxin oxidoreductase (PFOR). Pyruvate then gets reduced to 2,3-BDO by the action of three enzymes namely, acetolactate synthase, acetolactate decarboxylase and 2,3-butanediol dehydrogenase (2,3-BDH) (Figure 1) (Köpke et al. 2011).

C. autoethanogenum, which was used in this study, is an anaerobic spore-forming gram positive bacterium that ferments CO into acetate, ethanol and 2,3-butanediol (Abrini et al., 1994; Bengelsdorf et al., 2013). In our laboratory, experiments were previously performed in a 1.2-L continuous stirred tank reactor (CSTR) with continuous CO feed, at pH 5.75, and using a rather rich medium containing 1 g/L yeast

extract. Under such conditions, the *Clostridium* strain produced higher amounts acetic acid than ethanol (Abubackar et al., 2015a). However, other experiments suggested that it is feasible to stimulate ethanol production under optimized conditions such as a low fermentation pH and reduced yeast extract concentration (Abubackar et al., 2012).

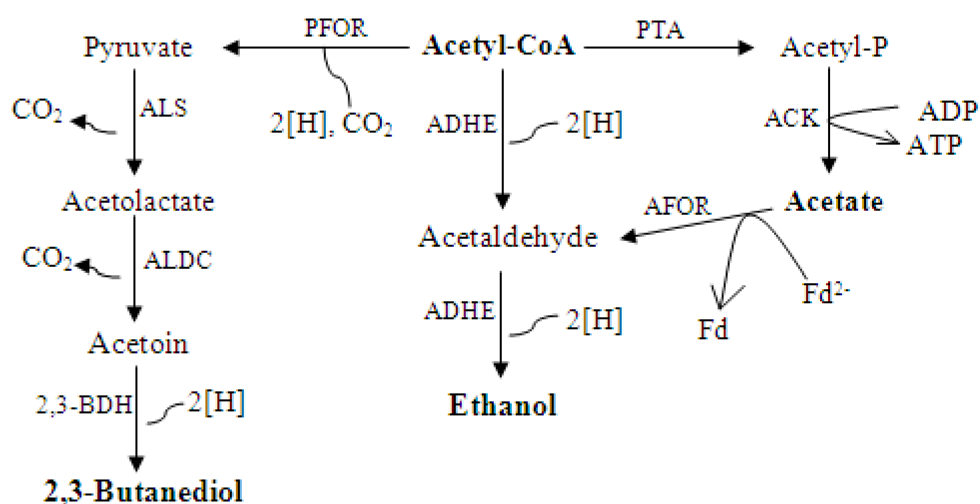


Figure 1: Acetyl-CoA to ethanol, acetic acid and 2,3 butanediol. Abbreviations: PFOR, pyruvate:ferredoxin oxidoreductase; PTA, phosphotransacetylase; ACK, acetate kinase; AFOR, aldehyde:ferredoxin oxidoreductase; ADHE, aldehyde/alcohol dehydrogenase; ALS, Acetolactate synthase; ALDC, acetolactate decarboxylase; 2,3-BDH: 2,3-butanediol dehydrogenase; Fd, ferredoxin; 2 [H], reducing equivalents (either NADH or NADPH)

Furthermore, the enzyme formate dehydrogenase (FDH) that reduces CO_2 to formate in the WL pathway and the enzyme AFOR are tungsten containing enzymes (Kletzin and Adams, 1996). Hence, to understand their collective effect on ethanol production by *C. autoethanogenum*, we performed studies with a yeast extract concentration of 0.5 g/L and 0.75 µM tungsten at a low constant pH of 4.75 throughout the bioreactor experimental study. This allowed to obtain an ethanol concentration of 867 mg/L with no acetic acid accumulation in that study (Abubackar et al., 2015b). This effort to improve the ethanol production by decreasing the pH resulted in less biomass growth (137.9 mg/L), half that obtained under the same experimental conditions but at

pH 6 (Abubackar et al., 2015b). This prompted us, in the present study, to culture the bacteria at optimal growth pH for a certain period of time in order to achieve higher concentrations of cell mass and acetic acid while later shifting to conditions that improve the ethanol production, such as a lower pH. These studies were carried out in a single-stage CSTR system. Furthermore, we also examined the feasibility of a novel sequencing-batch operating strategy with the objective to avoid any acetic acid accumulation while enhancing the overall ethanol titer after cell growth and the metabolite production leveled off.

6.2 EXPERIMENTAL SECTION

6.2.1 Microorganism

Clostridium autoethanogenum DSM 10061 was used in all experiments and was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). It was maintained at pH 6 in a medium with the following composition (per liter distilled water): NH_4Cl, 0.9 g; NaCl, 0.9 g; $MgCl_2·6H_2O$, 0.4 g; KH_2PO_4, 0.75 g; K_2HPO_4, 1.5 g; $FeCl_3·6H_2O$, 0.0025 g; trypticase peptone, 2.0 g; yeast extract, 1.0 g; cysteine-HCl, 0.75 g; 0.1 % resazurin, 0.5 mL; with 100% CO and SL-10 solution, 1.0 mL. The trace metal stock solution SL-10 contained (per liter): 7.7 M HCl, 10 mL; $FeCl_2·4H_2O$, 1.5 g; $ZnCl_2$, 70 mg; $MnCl_2·4H_2O$, 100 mg; H_2BO_3, 6 mg; $CoCl_2·2H_2O$, 190 mg; $CuCl_2·2H_2O$, 2 mg; $NiCl_2·6H_2O$, 24 mg; and $Na_2MoO_4·2H_2O$, 36 mg.

6.2.2 Continuous gas-fed bioreactor experiments

Bioreactor experiments were carried out in a 2-L New Brunswick Scientific BIOFLO 110 bioreactor with 1.2 L aqueous medium and CO (100%) as the gaseous C1 substrate. The bioreactor was continuously fed by means of a microsparger at a rate regulated to 10 ml/min by a mass flow controller (Aalborg GFC 17). The medium

composition used for the experiments was (per liter distilled water): NaCl, 0.9 g; $MgCl_2·6H_2O$, 0.4 g; KH_2PO_4, 0.75 g; K_2HPO_4, 1.5 g; yeast extract 0.5 g; $FeCl_3·6H_2O$, 0.0025 g; 0.1 % resazurin, 0.5 mL; cysteine-HCl 0.75 g and SL-10 solution, 1.0 mL. The trace metal stock solution SL-10 contained (per liter): 7.7 M HCl, 10 mL; $FeCl_2·4H_2O$, 1.5 g; $MnCl_2·4H_2O$, 100 mg; H_3BO_3, 6 mg; $CoCl_2·2H_2O$, 190 mg; $CuCl_2·2H_2O$, 2 mg; $NiCl_2·6H_2O$, 24 mg; $Na_2MoO_4·2H_2O$, 36 mg and Na_2WO_4, 250 mg. The bioreactor with the above medium was autoclaved without cysteine-HCl (0.75 g/L), which was later added after cooling down, with continuous nitrogen feeding. The bioreactor was maintained at a constant temperature of 30^0C with a constant agitation speed of 250 rpm throughout the experiments. 10% of an actively growing culture, which was grown for 48 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 measured on-line and was regulated through the addition of a 2 M NaOH or 2 M HCl solution, fed by means of a peristaltic pump.

Three independent pH shift bioreactor experiments were conducted where pH was shifted from high to low values and out of three experiments, one study was performed to establish the effect of medium replacement on the CO fermentation process. Gas samples of 0.2 mL were taken from the inlet and outlet sampling ports of the bioreactor to monitor the CO and CO_2 concentrations. 2 mL liquid sample was periodically withdrawn from the reactor (once every 24 h) in order to measure the optical density ($OD_{\lambda=600\ nm}$) and estimate the biomass concentration. Afterwards the sample was filtered with a syringe using a 0.22 μm PTFE-filter before analyzing the concentrations of soluble products.

6.2.3 Analytical equipment and measurement protocols

Gas-phase CO concentrations were measured on an HP 6890 gas chromatograph (GC) 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^oC, for 5 min, and then raised by 20^oC min^{-1} for 2 min, to reach a final temperature of 90^oC. The temperature of the injection port and the detector were maintained constant at 150^oC. 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 ^oC, respectively. For 2,3-butanediol identification, a Thermo Scientific ISQ™ single quadrupole GC-MS system operated at 70 eV equipped with a HP-5ms column (30 m × 0.25 mm × 0.25 µm film thickness) was used. The water-soluble products, acetic acid, ethanol and 2,3-butanediol, in the culture broth were analyzed using an HPLC (HP1100, Agilent Co., USA) equipped with a supelcogel C-610 column having UV detector at awavelength of 210 nm and a refractive index detector (RID). 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. Cell mass was estimated by measuring sample absorbance at a wavelength of 600 nm using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The measured absorbance was then compared to the previously generated calibration curve, to calculate the corresponding biomass concentration (mg/L). Besides, the redox potential was monitored continuously using an Ag/AgCl reference electrode connected to a transmitter (M300, Mettler Toledo, Inc. USA) maintained inside the bioreactor. The cell pelleting was performed in a vinyl anaerobic airlock chamber (Coylab Products, Michigan).

6.3 RESULTS AND DISCUSSION

Fermentation pH is a major parameter affects CO and syngas fermentation in terms of cell growth and product distribution (Abubackar et al., 2011; Daniell et al., 2012; Mohammadi et al., 2011). In previous bioreactor studies with *C. autoethanogenum*, we observed that fermentation at a constant low pH of 4.75 throughout the experiment significantly affected the overall biomass production and thereby also affecting the overall productivity of metabolites (Abubackar et al., 2015a, 2015b). Fermentation at optimal growth pH promotes biomass accumulation and acidogenesis and results, therefore, in the production of acids. Generally this occurs at high or less acid pH values, *e.g.* pH 5.75 with *C. autoethanogenum*. Conversely, a decrease from the optimum growth pH will promote solventogenesis and thus the production of higher amounts of reduced alcohols such as ethanol (Daniell et al., 2012). While acidogenesis is usually concomitant with cell growth, biomass growth is most often not related to solventogenesis (Abubackar et al., 2011).

Most bioreactor studies for the conversion of CO-related gases to bioproducts are done either in batch or in two-stage continuous bioreactors with the accumulation of both acids and alcohols in the process. In the present case, *C. autoethanogenum* was used as biocatalyst applying a different, novel, bioreactor operating strategy that takes advantage of the expected effect of a pH shift on the bioconversion pattern, aiming at reaching high alcohol production while avoiding accumulation of acetic acid. Besides playing with the pH and bioreactor configuration in the present study, it is worth recalling that other parameters had already been optimized before in our group and were then applied in this experimental work in order to improve the target bioconversion process (Abubackar et al., 2012, 2015a, 2015b). This is briefly summarized hereafter. Ethanol production by acetogens from CO-rich gases is enhanced by parameters such

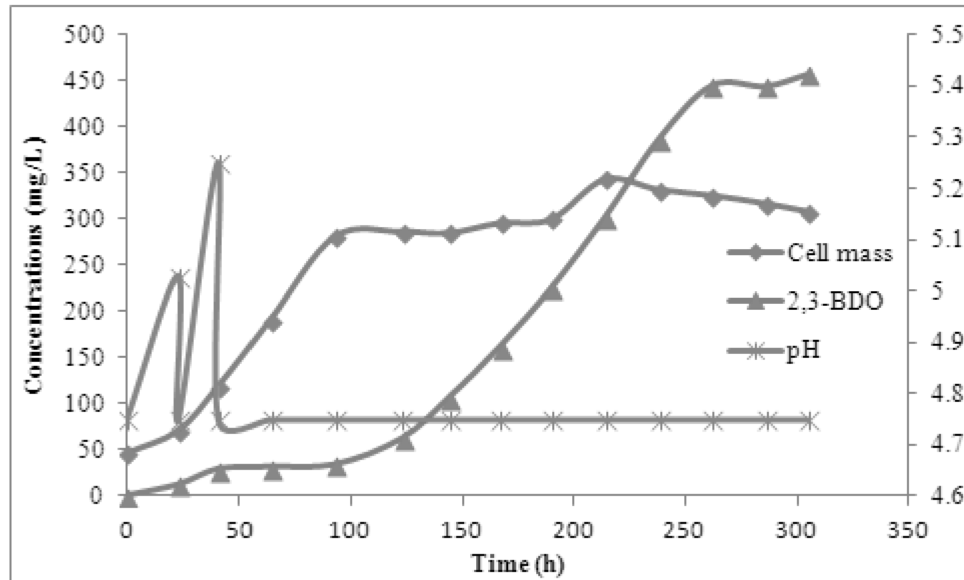
as limited nutrient availability (Abubackar et al., 2012; Philips et al., 2015), presence or absence of certain trace metals (Abubackar et al., 2015b; Saxena and Tanner, 2011), low temperature (Kundiyana et al., 2011b) and low medium pH (Abubackar et al., 2015a,b). The present work was performed at 30°C, which is lower than the optimum growth temperature of 37°C (Abrini et al., 1994), as it is expected to improve solventogenesis but also to improve the water solubility of CO (van Groenestijn et al., 2013). Other authors studying a similar bioconversion process to ethanol, but with *C. ragsdalei*, also reported that ethanol formation was higher at 30°C than at 37°C (Kundiyana et al., 2011b). On the other side, the composition of the fermentation medium is a major parameter having a great influence on the product distribution. An optimized fermentation medium that could eliminate or reduce concentrations of certain metabolites while improving the ethanol productivity would simplify downstream processing and would improve the economics of the process. Previously, we reported that the addition of selenium and/or vitamins did not improve the ethanol/acetic acid ratio in *C. autoethanogenum* in bottle studies with CO in the headspace (Abubackar et al., 2015b). Their presence even reduced the ethanol/acetic acid and butanediol/acetic acid ratios in the fermentation medium (Abubackar et al., 2015b). The effect of medium composition and trace metals on growth and metabolites distribution can be slightly different in different acetogens. The Wood-Ljungdahl pathway and subsequent reduction of acetyl-CoA to ethanol involves enzymes that are metalloproteins (Ragsdale and Pierce, 2008). For example, carbon monoxide dehydrogenase (CODH) contains nickel, iron and sulfur (Drennan et al., 2004); CoFeS-P, a corrinoid iron-sulphur protein contains cobalt, iron and sulphur (Ragsdale and Pierce, 2008); while formate dehydrogenase (FDH) contains tungsten, selenium, iron and sulphur (Yamamoto et al., 1983). FDH of *C. autoethanogenum* has a selenocysteine residue, 4Fe-4S cluster and

molybdopterin cofactor that is tungsten specific (Wang et al., 2013). Besides, AFOR is another tungsten dependent enzyme involved in acetic acid to ethanol conversion in the metabolic pathway (Fig. 1) (Kletzin and Adams, 1996). The presence of tungsten appeared to be important in our strain and was thus added in the medium used in these studies. We suggested that AFOR was stimulated by the presence of tungsten in the fermentation medium, which further stimulates the consumption and conversion of acetic acid to acetaldehyde and later to ethanol. In previous studies, we observed that addition of tungsten improves the ethanol/acetic acid ratio and the conversion of acetic acid to ethanol at an improved rate at low pH (Abubackar et al., 2015b).

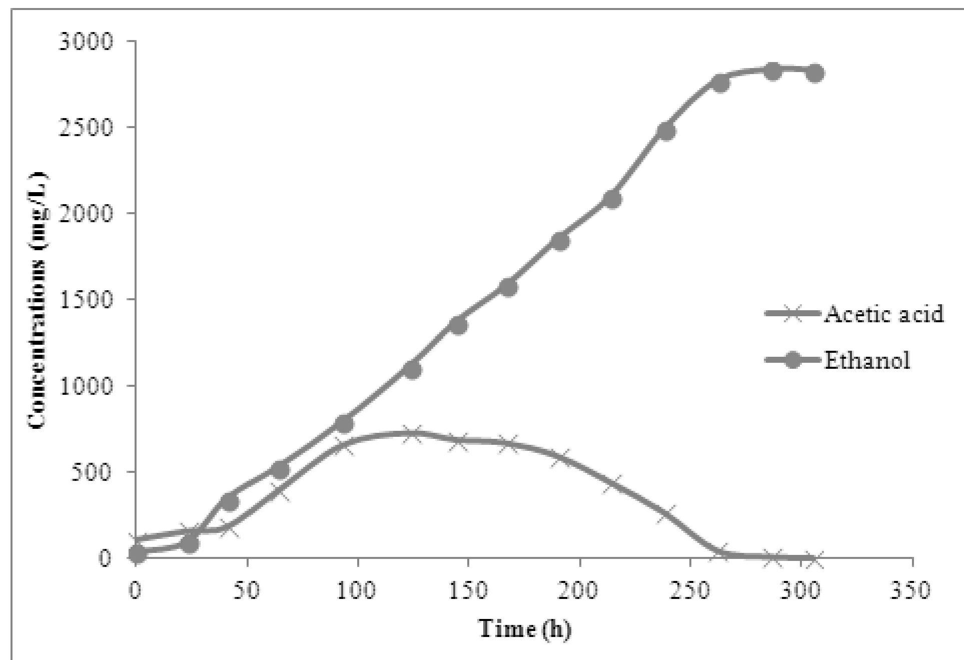
Uncontrolled low initial pH of 4.75 at start-up (experiment I)

This experiment was performed at a low initial pH of 4.75, presumably favourable to solventogenesis, without any pH regulation during the early stages of the study. During the first 24 h, some biomass and cell metabolites were produced and the pH increased naturally to 5.0. At this point, hydrogen chloride was added and the pH was readjusted back to its initial value; but during the second day of operation, it again increased from 4.75 to 5.25. Then, the pH was once again readjusted back to its initial value and, thereafter, automatic pH control was turned-on from the third day in order to maintain it constant for the rest of the experiment. From previous experience it was observed that, without pH regulation, the production of acids results in a pH drop while pH increases when ethanol is formed. Figure 2 depicts pH values, cell growth and the production profile of different metabolites. The pH was kept constant at a low value of 4.75 to stimulate solvent production. A trend observed in acetogenic fermentation is the achievement of high alcohols production rates from CO-related gases and the partial conversion of produced acids, if any, to alcohols, at low pH (Abubackar et al., 2015b).

Though the pH was low, not controlling the pH during the initial period of the experimental run probably helped to reduce the stress that could otherwise lead to lysis or death of the cells. Besides, it is considered to have promoted acidogenesis and cell



(2a)



(2b)

Figure 2: Experiment 1: Cell mass, 2,3-BD and pH profile for the experiment 1 (2a); Production of metabolites, acetic acid and ethanol production profile (2b).

growth during the first 3-4 days of bioreactor operation. Cell mass concentration gradually increased exponentially during the first 93 h up to 281.3 mg/L and reached a maximum of 343.42 mg/L after 214 h. Thereafter cell mass slightly decreased to 308.56 mg/L at 305 h. The continued biomass growth even after the pH changed from the optimum growth to solvent production pH indicated that the cells had acquired the essential enzymes and cofactors during the initial period of the experimental run. Acetic acid production continued, reaching a maximum value of 729 mg/L, indicating that acid-producing cells were active during the first days of the study with no pH regulation and pH increase, even a few hours after automatic pH regulation was turned on. Afterwards, up to 305 h, acetic acid concentration gradually decreased showing that that acid could be fully consumed and converted to alcohols, mainly ethanol, but also some 2,3-butanediol. Maximum ethanol and 2,3-BDO concentrations of 2840 and 457 mg/L, respectively, accumulated at the end of the experiment. The interesting observation and conclusion from this experiment, that was used for the next studies, is that during the initial period of the experiment with no pH regulation and thus pH increase, biomass build-up took place and acids were produced; while maintaining a low, constant pH during the remaining part of the study allowed to completely convert the accumulated acids to alcohols. Together with some preliminary work of our group published very recently (Abubackar et al. 2015b), to the best of our knowledge these are the first reports reaching ethanol accumulation with no acids present at all at the end of the study.

pH shift from high pH 5.75 to a low pH 4.75 (experiment II)

Based on the data of experiment I, in experiment II the pH was first maintained constant at pH 5.75 and later reduced to 4.75, while using one single bioreactor. It was hypothesized that CO conversion would result in biomass growth and accumulation of

acids during the first period of the experiment, at high pH, and that CO and the accumulated acids would then be converted to ethanol during the subsequent period at lower pH; hopefully with complete removal of acetic acid. This was indeed confirmed experimentally. The second experiment was thus performed in two stages in one same bioreactor, first with a constant high pH of 5.75 during the first four days and then, in a second stage (89 h to 258 h), pH was shifted to a lower value of 4.75. All the experimental set up and operating conditions (except the pH) used for the experiment were otherwise the same as in experiment I. The biomass and metabolites production profiles are plotted in Figure 3. During the first four-days, the cell concentration increased gradually from the initial value of 47 mg/L to 306 mg/L. The concentrations of metabolites reached during the first period were 1423, 279 and 49 mg/L, respectively for acetic acid, ethanol and 2,3-BDO. Thus, as foreseen, mainly biomass and acids were formed. The production of a high amount of acetic acid, required feeding NaOH to maintain the pH constant. Once the cell concentration and acetic acid production stabilized, the pH was shifted to a lower value of 4.75 after 89 h (second period). There was a slight decrease in biomass concentration to 287 mg/L due to the sudden change in pH of the fermentation medium, but then the biomass slowly recovered up to a maximum of 376 mg/L at the end of the experiment (Fig. 3). During the second period, at lower pH, basically all the acetic acid produced during the first period was converted to alcohols, mainly ethanol. It can be concluded that lowering the pH of the medium helped the bacteria to consume and convert all acetic acid to ethanol thereby improving the final ethanol titer from the process and helping overcome the complex downstream process to separate acetic acid. The final concentrations of solvents were 2408 mg/L for ethanol and 564 mg/L for 2,3-BDO, while the concentration of acetic acid was negligible (Fig. 3). From this experimental study, it can also be concluded that a low pH

not only produces higher amounts of ethanol, but also some 2,3-BDO, which would also be a commercially interesting platform chemical. The data and conclusions of the first two experiments were then used in order to increase the production of solvents in a third experiment based on the application of sequential pH shifts and cyclic sequencing-batch operation with continuous production of the target metabolites.

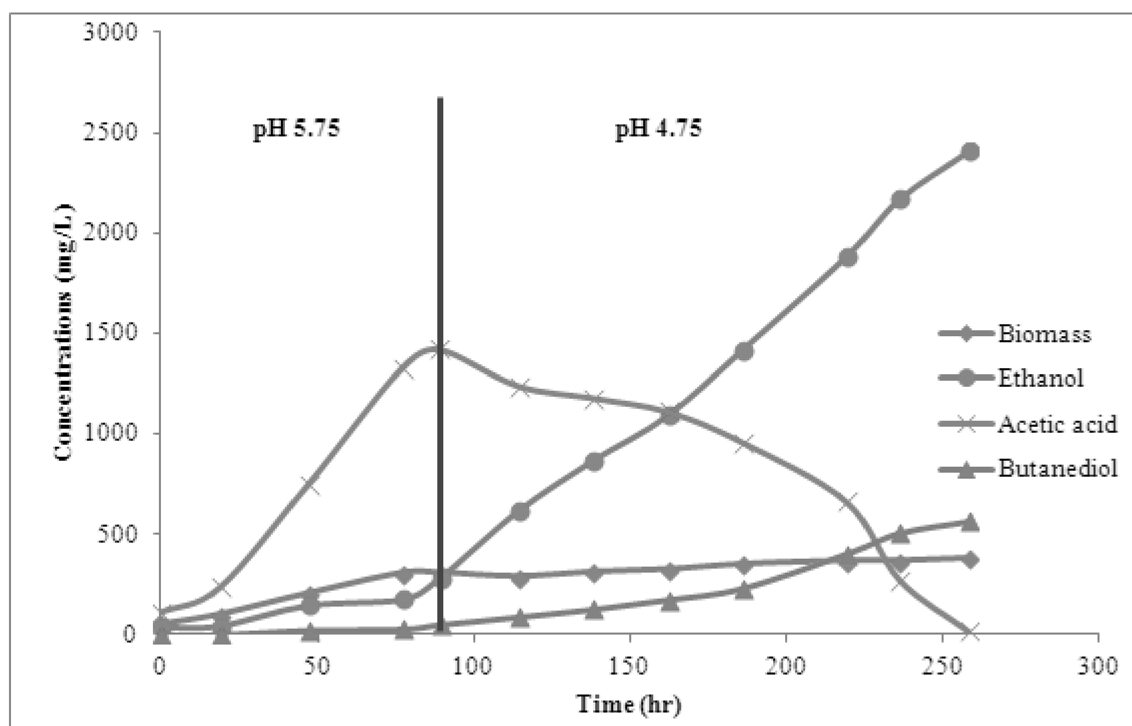


Figure 3: Cell mass and production profile for experiment 2.

Sequencing-batch operation and pH shift from high pH 5.75 to a low pH 4.75(experiment III)

In order to have continuous ethanol production from CO-rich gases, the common alternative already suggested for the first time in the 1990´s (Gaddy and Clausen, 1992), and still used and being optimized nowadays, consists in using two reactors in series, the first one being optimized for acidogenesis and the second one for solventogenesis. Thus, this requires the set-up of two reactors and once steady-state is reached both acids

and alcohols are still found in the outlet stream, although the process is generally optimized in order to reach the most favourable possible ethanol/acetic acid ratio. Although such two stage system was basically not further studied in the nineties, nowadays some researchers are working on the optimization of such two reactor in series set-up for ethanol production. This is by operating the first reactor with conditions of excess nutrients in order to support cell growth, while the second stage promotes the conversion of CO-rich gases and accumulated acetic acid from the first stage to ethanol. In this respect, Gaddy and Clausen (1992) used two CSTR (Completely Stirred Tank Reactors) in series with inclusion of Yeast Extract (YE) and upholding the pH at 5 in the first reactor and eliminating YE and shifting the pH to 4-4.5 in the second reactor. They obtained an ethanol concentration that increased from 1 to 3 g/L and ethanol/acetic acid ratio from 1 to 4 between the first and second reactor respectively. More recently, Richter et al. (2013) also used a similar two-stage system composed of a 1-L CSTR as first stage, as the growth reactor (pH 5.5), and a 4-L bubble column as the production reactor (pH 4.4 - 4.8). In that experiment, an ethanol concentration of 20.7 g/L and ethanol/acetic acid ratio of 2.8 were achieved in the outlet stream of the second reactor. In another recent study, two CSTRs were operated in series to test the effect of calcium pantothenate, vitamin B12 and $CoCl_2$ on syngas fermentation by *C. ragsdalei*. The pH of the growth reactor was maintained above 5.0. In that study the pH of both reactors was uncontrolled once the continuous operation mode was started (Kundiyana et al., 2011a). Some researchers observed also partial conversion of acetic acid during the solventogenesis phase to ethanol during batch fermentation (Hurst and Lewis, 2010; Kundiyana et al., 2010). For example, Liu et al. (2012) reported a partial conversion of acetic acid to ethanol in studies using *Alkalibaculum bacchi* CP15 in the late stage of serum bottle experiments where pH was uncontrolled, although the bioconversion

conditions were not optimized. The starting hypothesis of experiment III, based on the results of the previous two experiments, was that it should be feasible to use one single bioreactor for production of ethanol but no accumulation at all of acids, by performing cyclic pH shifts and cyclic feeds of medium rather than continuous ones. The results of experiment III, detailed hereafter, showed that this is indeed feasible.

Thus, the next experiment was performed in order to evaluate the effect of cyclic pH shifts and medium replacement during the fermentation. Figure 4 shows the production of cells and metabolites during the experiment. A total of three periods with varying conditions characterized the experimental run. During the first period, the fermentation pH was maintained at higher level (pH 5.75). Commencement of the second period consisted in removing part of bioreactor liquid (600 ml), centrifuging the biomass, and thereafter introducing the centrifuged cell pellets back into reactor together with 600 ml fresh medium. The pH was maintained constant at 5.75 during this period. In the third stage, pH was shifted to a low value of 4.75. Table 1 explains the fermentation operating conditions and metabolites production for the different stages of the experiment.

First period: During this period the biomass started increasing gradually from 54.74 to 243.42 mg/L at the end of period I (Fig. 4). The pH was maintained constant at 5.75 to promote cell growth and acetic acid production. However, from the acetic acid production profile, between time 0 and 120 h, acetic acid production was not observed and in addition, the acetic acid initially present (150.54 mg/L) from the inoculum was consumed, reaching a final concentration of 10.7 mg/L. . After four days, acetic acid concentration started increasing slightly to reach a value of 35.43 mg/L. Although the reason for this was not totally clear, one possible explanation for the consumption of acetic acid at pH 5.75 followed by an only low production rate could be the presence of

more solventogenic cells than acidogenic ones during start-up, just after inoculation. This was concluded from the presence of more biomass, ethanol and acetic acid in the inoculum compared to all previous experiments. Besides, the inoculum is grown in batch bottles with no pH regulation and the medium usually gets acidified to different extents which is favorable for the accumulation of solventogenic cells. However, as the biomass started to increase, the acidogenic cells still proliferated and some acetic acid production started to be observed in the bioreactor as already explained above, up to 35.43 mg/L.

Second period: 600 ml of the fermentation broth was removed aseptically and centrifuged under anaerobic conditions. The cell pellet was then mixed with the same volume of freshly prepared medium (600 ml) and introduced into the bioreactor again. Initially, just after replacing the medium, the cell concentration first decreased from 200 mg/L to 94 mg/L after 380 h, before it started growing again to reach a value of 307.64 mg/L at the end of this second period. Although anaerobic conditions and an anaerobic glove box were used, the decrease in biomass concentration and the lag phase lasting a few days at the beginning of this second period was most probably due to some contact with oxygen while centrifuging and reintroducing the cells and fresh medium. Once the system recovered, acetic acid production gradually increased from 16.83 to 796.9 mg/L at the time point 576 h; after which it remained almost constant until the end of period II (Table 1). On the other hand, some ethanol and 2,3-BDO were also already formed.

Third period: At the end of period II, acetic acid production got stabilized and even started being consumed at a slow rate of 0.49 mg/L.h. In order to facilitate the conversion of acetic acid into alcohols at a fast rate, the pH was then shifted from 5.75 to 4.75. Basically, all acetic acid was converted to ethanol and its concentration

decreased from 763.6 mg/L to a final, near negligible, value of 49 mg/L, when the experiment was stopped. It can be observed that after maintaining a

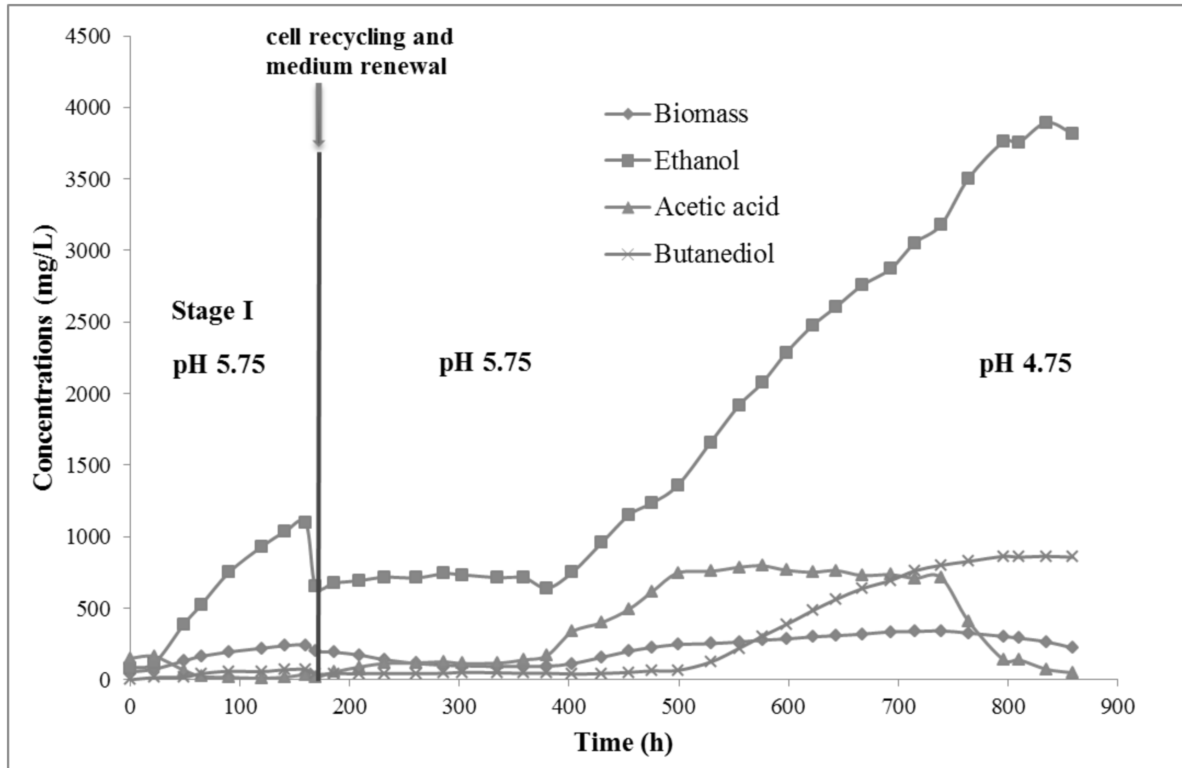


Figure 4: Cell mass and metabolites production profile for experiment 3.

low pH of 4.75, the conversion of acetic acid to alcohols occurred at a relatively high rate of 3.3 mg/L.h. Along the observation from the previous two experiments, the complete conversion of acetic acid in this experimental case was expected as well and did indeed occur. Biomass started decreasing after reaching the maximum concentration of 341.89 mg/L after 729

Table 1: Fermentation conditions and productions at various stages of the experimental run.

	Bioreactor fermentation medium and conditions	**Reactor performance**			**Inference**
			Initial (mg/L)	Final (mg/L)	
Period I (0-169 h)	pH 5.75; Medium mentioned in the experimental section	Cell concentration	54.74	243.42	Acetic acid got consumed and ethanol production continues. This can be attributed to the stage of cells inoculated into the bioreactor
		Acetic acid	150.54	35.43	
		Ethanol	82.27	1094.42	
		2,3-BDO	0	71.04	
			Initial (mg/L)	Final (mg/L)	
Period II (169-643 h)	pH 5.75; At 169 h, 600 ml of the fermentation liquid is removed and centrifuged and cell pellets were introduced into the bioreactor and in addition 600 ml of fresh medium was added; At 303 h, 1 g of trypticase peptone	Cell concentration	200	307.64	
		Acetic acid	16.83	763.6	
		Ethanol	651.37	2604.46	

	and 0.45 g of NaCl were added.	2,3-BDO	38.45	560.69	
Period III **(643-859 h)**	pH 4.75		Initial (mg/L)	Final (mg/L)	Basically all acetic acid got converted to ethanol at low pH at high rate.
		Cell concentration	307.64	222.62	
		Acetic acid	763.6	49	
		Ethanol	2604.46	3816.8	
		2,3-BDO	560.69	856.05	

h to 222.62 mg/L after 859 h. This decrease in biomass can be assumed to be due to two reasons. On one side the low pH is not optimum for growth and on the other side, because of the lack of production of ATP during the oxidation of acetate to acetaldehyde and its subsequent reduction to ethanol. However, taking into account the amount of ethanol produced at this stage, it can be confirmed that *C. autoethanogenum* not only followed the acetate to ethanol reduction route but did also follow the pathway that reduces acetyl-CoA directly to acetaldehyde and then to ethanol. The amounts of ethanol and 2,3-BDO produced during this third period reached respectively 1212.34 mg/L and 295.35 mg/L. The overall concentrations of the end metabolites obtained from the study were 4259.76 mg ethanol; 888.6 mg 2,3-BDO and 49 mg acetic acid per liter. Acetic acid would presumably have dropped down to zero if the experiment had been stopped a few hours later. This third experiment shows that using cyclic pH shifts and partial medium renewal together with biomass recycling in one single same bioreactor allows to accumulate increasing amounts of ethanol in each successive sequencing-batch cycle while avoiding build up of any acids at the end of the study. The complete absence of acetic acid makes downstream processing simpler and more cost-effective. Additionally, the use of one same bioreactor instead of two reactors in series would reduce investment and operating costs as well as costs related to the use of pumps and power for the continuous feed of fermentation broth.

6.4 CONCLUSIONS

The effect of pH shift in CO fermentation were studied using a single-reactor system. Basically all the produced acetic acid got successfully converted to ethanol. Enhanced ethanol and 2,3BDO production was observed at low fermentation pH. A novel strategy of fed batch system by replacing part of fermentation medium with fresh medium was successfully tested. Conditions such as presence of tungsten, elimination of selenium

and vitamins and pH shift from high (pH 5.75) to low (pH 4.75) resulted in complete conversion of acetic acid to ethanol during CO fermentation using *C. autoethanogenum.*

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Chapter 7

A strategy to improve ethanol production from CO fermentation by *Clostridium autoethanogenum*: modification of fermentation conditions and medium composition

ABSTRACT

The effect of various chemical components and fermentation conditions on cell mass and metabolite production was studied in a continuously CO-fed bioreactor. Cyclic pH shifts from high to low level along with partial medium replenishment, resulted in increased ethanol production reaching a maximum of 7143 mg/L, together with 2,3-BDO reaching a concentration of 1603 mg/L. Due to a decreasing biomass activity at the end of the experiment, complete conversion of acetic acid to ethanol was not reached leaving behind 2032 mg/L of acetic acid. Vitamin addition helped to improve acetic acid production. In order to try increasing the biomass concentration and activity, a mixotrophic fermentation using xylose as additional carbon source were performed as well. Results obtained from the study concluded that xylose is a preferred carbon source compared to CO and improved the cell mass concentration (420.79 mg/L).

Keywords: pH; *Clostridium autoethanogenum*; mixotrophic fermentation; xylose; tungsten

7.1 INTRODUCTION

Syngas/carbon monoxide fermentation into ethanol and other products using acetogens is considered a promising technology for converting pollutants to value added products such as, -but not limited to-, ethanol, acetic acid, 2,3-butanediol, butanol (van Groenestijn et al., 2013). In addition, carbon monoxide rich off-gases from metal industries, refineries and power plants and waste incinerators can also be utilized for the production of biofuel and other chemicals. There are only few acetogens known to produce ethanol as one of their main products from syngas/CO, including *C. ljungdahlii*, *C. autoethanogenum*, *C. ragsdalei* and *A. bacchi*. From reported studies with these organisms, it can be concluded that factors such as a low pH and limited nutrient concentrations, presence or absence of certain trace elements could improve ethanol production (Abubackar et al., 2015a; Mohammadi et al., 2011; Saxena and Tanner, 2011). However, higher amounts of acetic acid besides ethanol is reported in most of the studies (Abubackar et al., 2011). This puts a damper on biofuel industry as more carbon flows towards acetic acid production rather than towards ethanol. Moreover, below pH 7, the product acetate is present in the form of free acetic acid. It was reported that at lower pH, producing acetic acid, may be inhibitory during homoacetic fermentation (Schiel-Bengelsdorf and Dürre, 2012).

Concerning *C. autoethanogenum*, it is an anaerobic spore-forming gram positive bacterium that can grown on CO as sole carbon and energy source. It follows the Wood-Ljungdahl (WL) pathway and produces ethanol and acetic acid directly from acetyl-CoA; and 2,3 butanediol and lactate through pyruvate (Bengelsdorf et al., 2013). This acetogenic bacterium while growing on CO as the sole carbon source, obtains reducing equivalents for the pathway via a biological water gas shift reaction ($CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-$) catalyzed by carbon monoxide dehydrogenase (CODH).

From a batch bottle study performed in our laboratory using *C. autoethanogenum*, an enhancement of 200% in the ethanol concentration was observed when the initial pH and YE concentration were lowered from 5.75 to 4.75 and 1.6 to 0.6 g/L, respectively (Abubackar et al., 2012). We also observed, in a bioreactor study, a 143.5% lower ethanol production compared to acetic acid production, at pH 5.75 (Abubackar et al., 2015a). Working at the optimum growth pH improves the products yield due to higher biomass production; however, it reduces the ethanol/acetic acid ratio. Under certain fermentation conditions such as a pH value of 6, the presence of tungsten, and selenium and vitamins omission, acetic acid and ethanol productions equalize, while the same fermentation conditions at a reduced pH of 4.75 results in the production of ethanol as major fermentation metabolite (Abubackar et al., 2015b).

Recently, we found that *C. autoethanogenum* is able to consume and convert all the accumulated acetic acid produced during growth in to ethanol upon reducing the pH to a low level of 4.75 (Abubackar et al., 2015c). The ability of *C. autoethanogenum* to produce ethanol from acetic acid relies on the activities of two enzymes; acetaldehyde:Fd oxidoreductase (AFOR) that converts acetate to acetaldehyde and alcohol dehydrogenase (ADHE) that converts acetaldehyde later to ethanol. In this study, the influence of a pH shift between high level (5.75) and a low level (4.75) in a cyclic mode with simultaneous renewal of part of the fermentation broth was performed in a stirred tank bioreactor fed carbon monoxide, using the bacterium *C. autoethanogenum*. A strategy to improve the biomass concentration was also tested by addition of certain chemicals such as trypticase peptone, vitamins and NH_4Cl, during the experimental run. An additional study was also performed with xylose and CO as carbon sources in-order to study the mixotrophic fermentation and its effect on improving the productivity.

7.2 EXPERIMENTAL SECTION

7.2.1 Microorganism

Clostridium autoethanogenum DSM 10061 was used in all experiments and was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). It was maintained at pH 6 in a medium with the following composition (per liter distilled water): NH_4Cl, 0.9 g; NaCl, 0.9 g; $MgCl_2 \cdot 6H_2O$, 0.4 g; KH_2PO_4, 0.75 g; K_2HPO_4, 1.5 g; $FeCl_3 \cdot 6H_2O$, 0.0025 g; trypticase peptone, 2.0 g; yeast extract, 1.0 g; cysteine-HCl, 0.75 g; 0.1 % resazurin, 0.5 mL; with 100% CO and SL-10 solution, 1.0 mL. The trace metal stock solution SL-10 contained (per liter): 7.7 M HCl, 10 mL; $FeCl_2 \cdot 4H_2O$, 1.5 g; $ZnCl_2$, 70 mg; $MnCl_2 \cdot 4H_2O$, 100 mg; H_3BO_3, 6 mg; $CoCl_2 \cdot 2H_2O$, 190 mg; $CuCl_2 \cdot 2H_2O$, 2 mg; $NiCl_2 \cdot 6H_2O$, 24 mg; and $Na_2MoO_4 \cdot 2H_2O$, 36 mg.

7.2.2 Continuous gas-fed bioreactor experiments

Bioreactor experiments were carried out in a 2-L New Brunswick Scientific BIOFLO 110 bioreactor with 1.2 L aqueous medium and CO (100%) as the gaseous C1 substrate. The bioreactor was continuously fed by means of a microsparger at a rate regulated at 10 ml/min by a mass flow controller (Aalborg GFC 17). The medium composition used for the experiments was (per liter distilled water): NaCl, 0.9 g; $MgCl_2 \cdot 6H_2O$, 0.4 g; KH_2PO_4, 0.75 g; K_2HPO_4, 1.5 g; yeast extract 0.5 g; $FeCl_3 \cdot 6H_2O$, 0.0025 g; 0.1 % resazurin, 0.5 mL; cysteine-HCl 0.75 g and SL-10 solution, 1.0 mL. The trace metal stock solution SL-10 contained (per liter): 7.7 M HCl, 10 mL; $FeCl_2 \cdot 4H_2O$, 1.5 g; $ZnCl_2$, 70 mg; $MnCl_2 \cdot 4H_2O$, 100 mg; H_3BO_3, 6 mg; $CoCl_2 \cdot 2H_2O$, 190 mg; $CuCl_2 \cdot 2H_2O$, 2 mg; $NiCl_2 \cdot 6H_2O$, 24 mg; $Na_2MoO_4 \cdot 2H_2O$, 36 mg and Na_2WO_4, 250 mg. The bioreactor with the above medium was autoclaved without cysteine-HCl (0.75 g/L), which was later added after cooling down, with continuous nitrogen feeding. The

vitamin stock solution contained (per liter) 10 mg each of *para*-aminobenzoic acid, calcium pantothenate, nicotinic acid, riboflavin, thiamine, α-lipoic acid, and vitamin B12, 4 mg each of d-biotin, folic acid and 20 mg pyridoxine. The bioreactor was maintained at a constant temperature of 30^0C with a constant agitation speed of 250 rpm throughout the experiments. 10% of an actively growing culture, which was grown for 48 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 measured on-line and was regulated through the addition of a 2 M NaOH or 2 M HCl solution, fed by means of a peristaltic pump.

Mixotrophic experiments were performed to test the ability of the stain to use xylose and CO simultaneously as the carbon sources. The pH was maintained constant at 5.75 throughout the experiment. The initial concentration of xylose present in the bioreactor is the amount measured by HPLC, after autoclaving. Gas samples of 1 mL were taken from the inlet and outlet sampling ports of the bioreactor to monitor the CO and CO_2 concentrations. 2 mL liquid sample was periodically withdrawn from the reactor (once every 24 h) in order to measure the optical density ($OD_{\lambda=600\ nm}$) allowing to estimate the biomass concentration. Afterwards the sample was filtered with a syringe using a 0.22 µm PTFE-filter before analyzing the concentrations of soluble products as well as xylose.

7.2.3 Analytical equipment and measurement protocols

Gas-phase CO concentrations were measured on an HP 6890 gas chromatograph (GC) 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. 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. For 2,3-butanediol identification, a Thermo Scientific ISQ™ single quadrupole GC-MS system operated at 70 eV mounted by a HP-5ms column (30 m × 0.25 mm × 0.25 µm film thickness) was used. The water-soluble products, acetic acid, ethanol and 2,3-butanediol, in the culture broth were analyzed using an HPLC (HP1100, Agilent Co., USA) equipped with a supelcogel C-610 column having a UV detector at a wavelength of 210 nm a refractive index detector (RID). 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. Cell mass was estimated by measuring sample absorbance at a wavelength of 600 nm using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The measured absorbance was then compared to the previously generated calibration curve, to calculate the corresponding biomass concentration (mg/L). Besides, the redox potential was monitored continuously inside the bioreactor using an Ag/AgCl reference electrode connected to a transmitter (M300, Mettler Toledo, Inc. USA). The cell pelleting was performed in a vinyl anaerobic airlock chamber (Coylab Products, Michigan).

7.3 RESULTS AND DISCUSSION

Fermentation medium and fermentation pH are among the main parameters that affect the biomass and product distribution during CO bioconversion (Abubackar et al., 2011; Mohammadi et al., 2011). The nitrogen source and the trace metals composition are major parameters affecting metabolites production and growth under CO. An enriched medium promotes acidogenesis, i.e., a metabolic phase in acetogenic bacteria where profound cell growth and production of acetate are found. This can be clearly

understood from the acetate production route where ATP is generated during the conversion of acetyl-phosphate to acetate. This ATP could be used for growth and maintenance. On other hand, a nutrient limited medium stimulates solventogenesis, the phase where the bacteria produce higher amounts of reduced compounds such as ethanol and in some occasions partial or complete conversion of produced acids to alcohols, with no observed growth. For example, increasing or decreasing the YE concentration in the medium does either promote acidogenesis or solventogenesis, respectively, in ethanologenic bacteria such as *C. ljungdahlii* (Barik et al., 1988). This is also consistent with the observations, from our previous batch and bioreactor studies with *C. autoethanogenum* (Abubackar et al., 2012; 2015b). Besides the influence of the nitrogen sources, trace minerals such as as Ca^{2+}, Mg^{+}, Na^{+}, K^{+}, and PO_4^{3-} have been reportedly to have either positive or negative effects on ethanol production. Saxena and Tanner, (2012) examined the effects of trace minerals on ethanol production in *C. ragsdalei* using standard media containing 0.5 g YE and observed a decrease in ethanol production upon elimination of Mg^{2+} and PO_4^{3-}. Besides, they observed no effects on ethanol production while eliminating Na^{+}, Ca^{2+} and K^{+} or increasing Ca^{2+}, Mg^{2+}, K^{+}, NH^{4+} and PO_4^{3-}. Trace metal compositions and their concentrations vary considerably the activity of various enzymes involved in the WL pathway and the subsequent reductive steps that lead to product formation (Saxena and Tanner, 2011). For example, acetic acid reduction to acetaldehyde is catalyzed by the enzyme aldehyde:ferredoxin oxidoreductase (AFOR) using electrons from the reduced ferredoxin (Fd^{2-}). The AFOR of the hyperthermophilic archaeon *Pyrococcus furiosus* is reported to contain tungsten and iron atoms (Kletzin and Adams, 1996). Wang et al. (2013) reported that the enzyme formate dehydrogenase (FDH) in *C. autoethanogenum* that catalyzes the reduction of CO_2 to formate is a tungsten containing enzyme. Studies with *C. ragsdalei* indicated

that nickel, zinc, selenium and tungsten improve ethanol production Saxena and Tanner (2011). Recently we observed that presence of selenium and vitamins doesn't improve the ethanol/acetic acid ratio in bottle studies performed with the strain *C. autoethanogenum* (Abubackar et al., 2015b). However, addition of tungsten enhanced ethanol production significantly (Abubackar et al., 2015b). As all reported studies use vitamin solution in their experiments, the non essentiality for our strain of vitamins might be because of the adaptation to the nutrient medium without vitamins during repeated subculturing. Hence, one of the objectives of this study was to understand their effect in a long-term continuous CO fermentation experiment.

Another important factor is the fermentation pH. Various studies support the fact that lowering the pH from the optimum growth pH is favorable for solventogenesis (Abubackar et al., 2011; Daniell et al., 2012; Mohammadi et al., 2011). It is hypothesized that bacteria overcome the stress from their surrounding as well as internal low pHs by producing alcohols. However, reducing the pH causes a negative impact on cell growth and thereby on the overall metabolite productivity. In one of our studies at two different pHs, 62% less cell mass was observed at pH 4.75 compared to the study at a higher pH of 5.75 (Abubackar et al., 2015a). This reduces significantly the products concentration in the experiment performed at pH 4.75. However, when focusing on the ethanol/acetic acid ratio, it appears that a low pH allows reaching an approximately three times higher ratio than at higher pH (Abubackar et al., 2015a).

Cyclic pH shifts and medium replacement (experiment I)

In a previous studies, we tested the feasibility of applying a pH shift from high to low value, in order to obtain higher amount of cell mass and acetic acid during the period of high pH followed by the consumption and conversion of almost all acetic acid to ethanol at a lower pH. Besides, medium replacement in continuous CO fed fermentation

was also decided in order to improve ethanol production (Abubackar et al., 2015c). A maximum alcohol concentration of 2408 mg ethanol/L, together with 564 mg 2,3-BDO/L and a negligible amount of acetic acid, was obtained in the study with pH shift. The overall concentrations of products obtained in the study with one pH shift and medium replacement were 4259.76 mg ethanol; 888.6 mg 2,3-BDO and 49 mg acetic acid/liter together with (Abubackar et al., 2015c). These findings prompted us to perform a cyclic pH shifts medium replacement. Several periods with varying fermentation conditions characterized the experimental run: different pHs (5.75 and 4.75); addition of chemicals (NH_4Cl, trypticase peptone and vitamin solutions) and medium replacement.

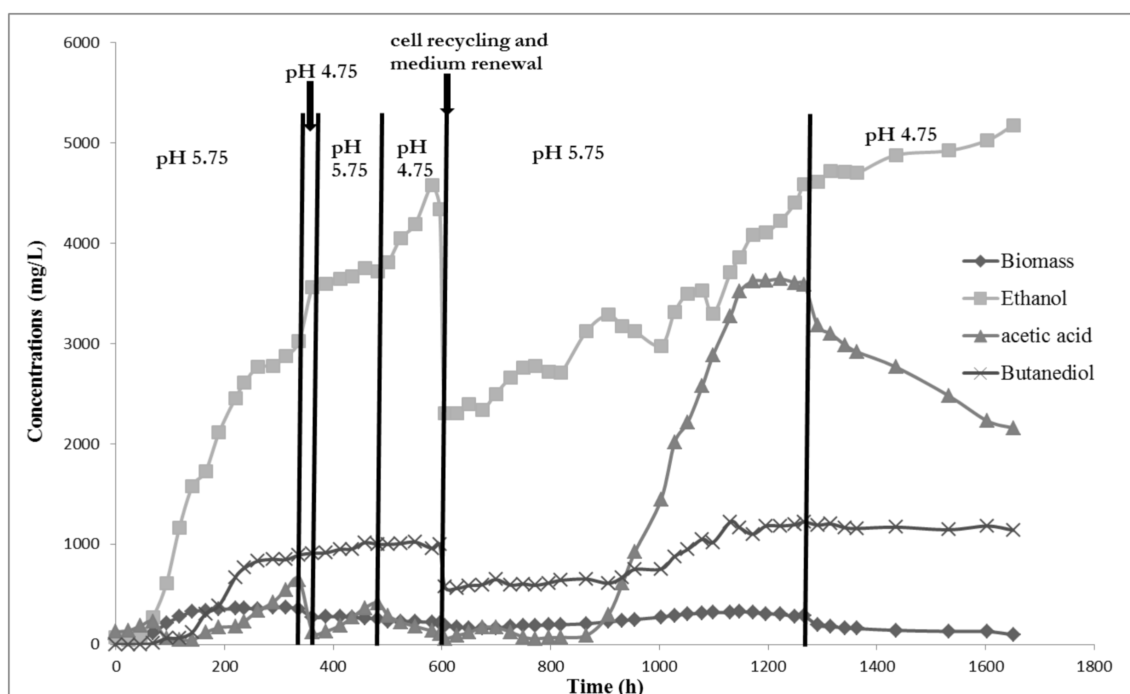

Figure 1: Cell mass and production profile for experiment 1.

Period I (0 -337 h): In addition to 0.5 g/L of yeast extract normally used in the medium for our bioreactor studies, an additional 0.5 g/L of trypticase peptone was included as a way to provide the bacteria a nutrient rich medium. However, the maximum amount of cell mass obtained in this respect was similar to the amount achieved in the absence of

trypticase peptone (Abubackar et al., 2015b). The cell mass gradually increased from 46.17 mg/L to 369.41 mg/L at 261 h and then started slightly decreasing to reach 352.59 mg/L at the end of period I. However, the metabolites reaches their maximum concentrations of 3020.87 mg ethanol, 891.05 mg 2,3-BDO and 644.84 mg acetic acid per liter (Figure 1).

Period II (337 -365 h): As the cell mass got stabilized during Period I, the pH was shifted from the initial value of 5.75 to a lower value of 4.75 at 337 h in order to stimulate the bacteria to consume and convert the acetic acid produced during Period I. The concentration of acetic acid did indeed decrease, reaching a value of 124.09 mg/L. Due to the sudden decrease in fermentation pH, the cell mass decreased down to 269.72 mg/L. This decrease in biomass when the pH was shifted to a low value was observed in our previous studies as well (Abubackar et al., 2015c). The sudden decrease in cell mass might be due to the sudden drop of pH from its optimum growth value. It can be concluded that the rise in ethanol concentration resulted from acetic acid consumption and its conversion to ethanol that reached a value of 3562.3 mg/L. However, no significant production of 2,3-BDO was observed. The concentration of 2,3-BDO reached 909.12 mg/L.

Period III (365- 484 h): In order to facilitate cell mass growth again, the pH was shift back from 4.75 to the bacteria optimum growth pH of 5.75. The higher pH could allow to initiate acetic acid production thereby generating more ATP that might support bacterial growth (Bengelsdorf et al., 2013). However, there was no production of acetic acid, contrary to what was expected. Another parameter that helps to support acetic acid production is a nutrient rich medium (Abubackar et al., 2011). In this respect, at 386 h, 0.6 g trypticase peptone and at 436 h, 1.08 g NH_4Cl were added. At the end of the period, acetic acid concentration reached 414.12 mg/L. The concentrations of solvent

were 3715.2 mg ethanol and 998.25 mg 2,3-BDO per liter. However, the cell mass didn't increase; it rather slightly decreased to 252.90 mg/L at 484 h. The possible explanation for the absence of cell growth might be due to the insufficient amount of ATP generated during acetic acid production.

Period IV (484- 605 h): Since the cell mass kept decreasing, the pH was shift back to 4.75 to facilitate the conversion of acetic acid produced to ethanol before replacing the fermentation medium at the next period (period V). As expected almost all acetic acid got converted to ethanol. The final concentration of products at the end of the period was 4338.7, 108 and 993.38 mg/L respectively of ethanol, acetic acid and 2,3-BDO. The biomass further decreased to 212.23 mg/L at the end of this stage, which could be expected considering the low pH value.

Period V (605- 1268 h): At this period, medium replacement and vitamins additions were used to support acetogenesis, i.e., to support cell growth and acetic acid production. An amount of 600 ml of the fermentation broth was aseptically transferred and centrifuged to separate the cell under strict anaerobic condition. The cell pelleting was performed inside the anaerobic chamber and the cell were then introduced into the bioreactor along with freshly prepared medium. After replacing the medium, the pH was adjusted to 5.75. The biomass started decreasing from the initial value of 185.62 mg/L to 157.79 mg/L before gradually increasing again to 189.29 mg/L. Whereas, ethanol and 2,3-BDO concentrations improved from 2304.1 to 2772 mg/L and from 572.45 to 588.24 mg/L in 168 h. However, acetic acid started also increasing somewhat, from 57.58 to 169.14 mg/L at 699 h and thereafter it got consumed and reached a low value of 55.98 mg/L. The possible explanation for the conversion of acetic acid to ethanol might be due to the availability of increased amounts of reduced ferredoxin that helps to convert acetate to acetaldehyde and latter to ethanol. To understand the effect of a

vitamin solution in this bioreactor study, 20 ml of a vitamin solution was added at 773 h. A significant difference from the experiment without vitamin addition (period I) is that in this case, an increased amount of acetic acid is found in the fermentation broth. A total of 3587.23 mg/L of acetic acid was produced at this stage. At the late stage of this period (1223 -1268 h), acetic acid got slightly consumed. Other products concentrations were 4588.59 mg ethanol and 1220.52 mg 2,3-BDO per liter. The biomass continued to increase gradually from 189.29 to 326.91 mg/L at 1148 h and thereafter decreased to 282.26 mg/L at 1268 hr. The maximum amount of biomass obtained after vitamin addition was similar to the amount obtained without such addition. This concludes that the presence of vitamins improves acetic acid production but it doesn't increase the final amount of biomass.

Period V (1268- 1651 h): During this period the pH was maintained at a low level of pH 4.75. As expected acetic acid got consumed and converted to ethanol. However, probably due to the lack of enough active biomass, conversion of acetic acid was slow and the experiment was stopped before complete consumption.

The overall concentrations of the solvents obtained from the study were 7142.87 mg/L and 1620.71 mg/L of ethanol and 2,3-BDO, respectively, taking into account the final concentrations present at the end of the experiment in the bioreactor (Figure 1) as well as the concentration withdrawn from the from the system during medium renewal. A summary of the experiments performed with the *C. autoethanogenum* in our laboratory, their results and conclusions are tabulated in Table 1.The metalloenzyme aldehyde:ferredoxin oxidoreductase (AFOR) that converts acetic acid to acetaldehyde is a tungsten containing enzyme. We believe that the presence of tungsten in the medium stimulated the activity of AFOR and helps to consume and convert acetic acid to ethanol. Some researchers used a two stage continuous system in series to reach a high

ethanol titer by maintaining conditions that support growth and acidogenesis in the first reactor and with conditions that trigger solventogenesis in the second reactor (Gaddy and Clausen, 1992; Richter et al. 2013). One such study was reported by Richter et al. (2013) where they use a 1-L CSTR as the first stage and a 4-L bubble column reactor equipped with a hollow fiber module in the second stage. The molar ethanol to acetic acid ratio obtained under steady state conditions were 0.078 and 3 in the first and second stage respectively. Such a two- stage system was also previously tested by Klasson et al. (1990). We believe that using a pH shift and medium replacement, it is possible to convert acetic acid to ethanol provided there should be sufficient actively biomass inside the bioreactor, thus helping to enhance the overall ethanol production using a single reactor. Based on the results obtained from this study, we performed the next experiment using xylose as carbon sugar and continuously feeding CO, in order to try to improve the biomass growth and concentration.

Mixotrophic fermentation with xylose and CO (Experiment II)

Experiments were performed to understand the effect of having both CO and xylose as carbon substrates on *C. autoethanogenum* metabolism. A CO-adapted *C. autoethanogenum* was used for this study in a bioreactor containing 4.18 g/L of xylose (concentration after autoclaving) and simultaneously feeding 100% CO. *C. autoethanogenum* readily consumed CO or xylose when they are present alone. However, here, we observed that in the presence of both xylose and CO, less amount of CO was consumed than usual. Furthermore, xylose was consumed instantly. When xylose was almost completely consumed, the CO consumption increased as can be observed from the CO outlet profile (Figure 2), during the time from 255 to 303 h. At 303 h, cell mass reached the stationary stage and consumption of CO decreased. Due to unexpected problems with the GC, the last three data points for the CO concentration

could not be measured. The maximum amount of biomass concentration obtained was 420.79 mg/L which is approximately 33% higher than the average maximum (300 mg/L) obtained with only CO in all our previous studies (Abubackar et al., 2015a,b). According to the result, it seemed that *C. autoethanogenum* could produce more biomass.

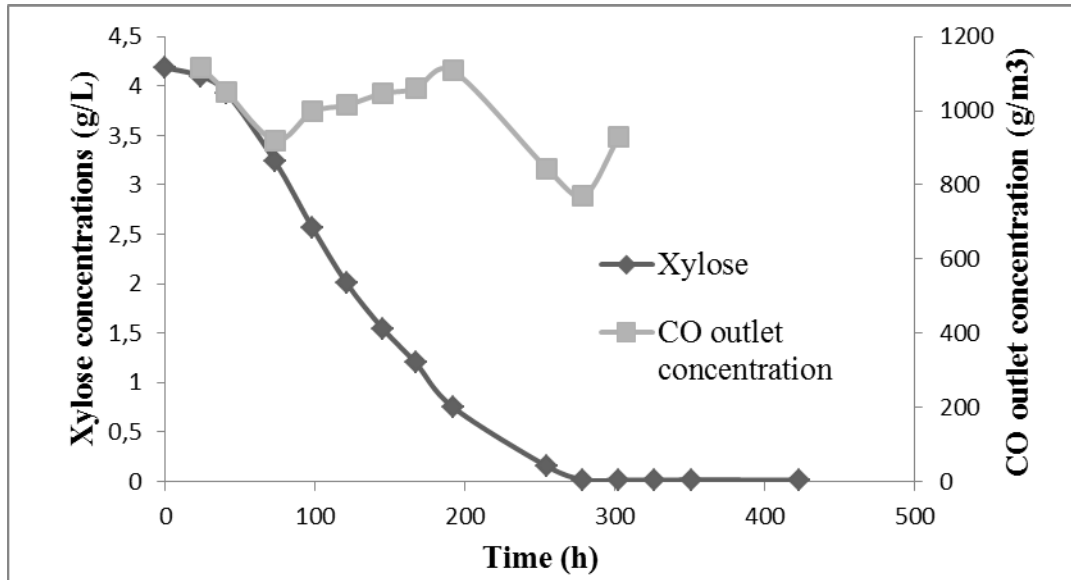

Figure 2: xylose and CO outlet concentrations profile

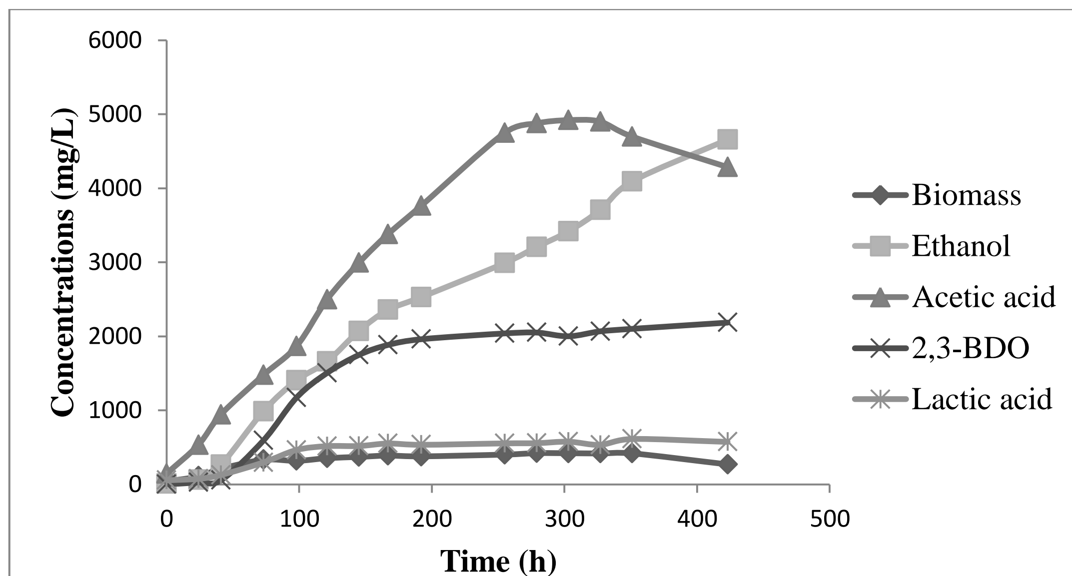

Figure 3: Cell mass and production profile for mixotrophic fermentation.

Table 1: Various experiments performed with *C. autoethanogenum* in CO-fed bioreactor in our laboratory

Experiment	Fermentation condition	Results (mg/L)	Conclusions	Reference
Continuous fed-batch experiment at two different pHs without tungsten addition	pH 5.75 and 4.75, YE= 1 g/L, No addition of tungsten, selenium and vitamins	**pH 5.75** Cell mass= 302.4; Acetic acid= 2147.1; Ethanol=352.6 23-BDO=81.8 **pH 4.75** Cell mass= 113.7; Acetic acid= 536.41; Ethanol=264.5; 23-BDO= 41.8	Low pH reduces the cell mass as well as products concentrations, but helps to obtain higher ethanol/ acetic acid ratio.	Abubackar et al., 2015a
Continuous fed-batch experiment at two different pHs with tungsten addition	pH 6 and 4.75, YE=0.5 g/L, Addition of tungsten	**pH 6** Cell mass= 287.77; Acetic acid= 910.69; Ethanol= 907.72; 23-BDO=103.3 **pH 4.75** Cell mass= 137.9; Acetic acid= Nil; Ethanol= 867; 23-BDO= 46	Low pH and tungsten addition leads to no accumulation of acetic acid.	Abubackar et al., 2015b
Continuous fed-batch experiment with pH shift and tungsten addition	pH shift from 5.75 to 4.75 Presence of tungsten	Cell mass= 376; Acetic acid= Nil; Ethanol= 2408 23-BDO= 564	Ethanol and 2,3-BD production got improved; pH shift from high to low value helps to consume and convert all the acetic acid to ethanol.	Submitted
Continuous fed-batch experiment with one cycle of pH shift and media replacement	pH shift from 5.75 to 4.75; Presence of tungsten; 600 ml of the fermentation broth was replaced with fresh new medium	Acetic acid= 49; Ethanol= 4259.76; 23-BDO= 888.6	Ethanol and 2,3-BD production got further improved; Almost all acetic acid got converted to ethanol; The negligible amount of acetic acid got further be converted if the experiments continues	Submitted

Continuous fed-batch experiment with two cycle of pH shift and media replacement	Cyclic pH shift between 5.75 and 4.75. Presence of tungsten and vitamins; 600 ml of the broth was replaced with fresh new medium	Acetic acid= 2031.75; Ethanol = 7142.87; 23-BDO= 1620.71	Partial conversion of acetic acid was possible due to the lack of active biomass. Addition of Vitamins improves acetic acid not cell mass concentrations	Submitted

At 255 h, acetic acid production stabilized reaching a value of 4749.91 mg/L and thereafter it increased to a value of 4921.31 mg/L at 303 hr. Afterwards, it got consumed and converted to ethanol during the stationary growth phase of the bacteria. The final titer of acetic acid left was 4287.08 mg/L. An improved ethanol production was observed during the late stage of the experiment. The maximum concentrations of acetic acid and ethanol obtained were 4921.31 and 4659.26 mg/L respectively. In addition, 2185.74 mg/L of 2,3-BDO and 573.77 mg/L of lactic acid were also obtained. The production of pyruvate side products, i.e. lactic acid and 2,3-BDO, could have been formed due to saturation of ferredoxin that helps the carbon flow towards pyruvate (Fast et al. 2015). The ability of *C. autoethanogenum* to produce lactic acid was previously reported by Köpke et al. (2011).

7.4 CONCLUSIONS

Two cycles of pH shift from high level of 5.75 to 4.75 was successfully conducted in a bioreactor using *C. autoethanogenum*. The higher pH value of 5.75 supports the growth and product formation. However a decrease in pH value to 4.75 helped to consume and convert the produced acetic acid to ethanol. The strategy of pH shifts and media replacement allowed to improve the final ethanol titer from the process. During growth with both xylose and CO, *C. autoethanogenum* preferably consumed xylose instantly and CO consumption started once xylose got consumed.

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GENERAL DISCUSSION AND CONCLUSIONS

Carbon monoxide fermentation using the acetogenic bacterium *C. autoethanogenum* was studied under different conditions. From the various studies performed in our laboratory we found that the metabolic products during CO fermentation by the bacterium were acetic acid, ethanol and 2,3-butanediol. As an alternative promising biofuel, ethanol is the interesting end product for our study. So far, most published studies reported large amounts of acetic acid production compared to ethanol. Those results were presented in the Introduction section (Chapter 1). Furthermore, acetic acid accumulation leads to the necessity of complex downstream processing. The objective of this thesis was to optimize the fermentation medium and conditions that could enhance the productivity of ethanol. In addition to completely eliminate the accumulation of acetic acid. In this respect studies were performed under various fermentation conditions such as varying pHs, reducing agent concentrations, CO partial pressure, nitrogen sources and their concentrations in order to understand their effects on growth and product formation (Chapter 3 and Chapter 4). The WL pathway that bacteria used involves various metalloenzymes (Chapter 1). Changing the concentrations of some trace metals might affect the activity of these enzymes. The effect of trace metals such as tungsten and selenium were studied in this respect. The effect of addition of vitamins was also studied as low medium cost is absolutely essential for every fermentation industry. Experiments were then performed using a novel strategy of cyclic pH shift and partial medium replacement with the objective of enhancing the overall ethanol productivity. All the works were performed either in serum bottles for batch studies or CSTR for continuous gas-fed reactors. In addition to CO as the sole carbon source, the effect of a combination of a sugar source along with CO was also studied. A summary of the work and the most significant conclusions are detailed in this section.

- A two-level full factorial design approach was employed to optimize following range of process parameters: initial pH = 4.75 to 5.75, initial pressure = 0.8 to 1.6 bar, cysteine-HCl concentration = 0.5 to 1.2 g/L, and yeast extract concentration = 0.6 to 1.6 g/L. Batch microcosm experiments were performed under strictly anaerobic conditions. The results from this microcosm study shows that, a prior understanding of the effect of parameters such as initial fermentation pH, initial pressure, cysteine-HCl and yeast extract concentration is essential for designing well-optimized continuous bioreactors in order to enhance ethanol production (Chapter 3). Furthermore, it was observed that a low initial pH (pH = 4.75) and low yeast extract concentrations (0.6 g/L) favor high ethanol production by *Clostridium autoethanogenum*. Regarding total pressure and cysteine-HCl, optimal values (1.6 bar and 1.2 g/L, respectively) of these parameters that are not toxic and inhibitory to the microorganism would yield high ethanol concentrations. A maximum ethanol concentration of 0.65 g/L was obtained in those study under the condition: pH = 4.75 (the lowest value tested), pressure = 1.6 bar (the highest value tested), cysteine–HCl = 1.2 g/L (the highest value tested), and YE concentration = 0.6 g/L (the lowest value tested).
- Other microcosm studies were performed to examine the effects of nitrogen sources and concentrations on the bioconversion of carbon monoxide to ethanol by *Clostridium autoethanogenum* (Chapter 4). A two level three factor (2^3) full factorial experimental design was performed to examine the combined effects of NH_4Cl (0.2–2 g/L), trypticase (0.2–2 g/L) and YE concentrations (0.1–1 g/L). A maximum ethanol concentration of 0.30 g/L was obtained under the following nitrogen concentrations: NH_4Cl = 2 g/L (the highest value tested), trypticase soy

broth = 0.2 g/L (the lowest value tested) and yeast extract concentrations = 0.1 g/L (the lowest value tested).

- In order to determine the individual effect of nitrogen sources, separate experiments with either NH_4Cl (1.1 g/L), trypticase (1.1 g/L) or YE (0.55 g/L) were conducted in bottles. Those studies help us to conclude that for obtaining a certain amount of biomass and metabolites, the amount of YE required was half the amount of trypticase (Chapter 4).
- In order to translate the conditions optimized with batch bottle experiments to a continuous study, three CO-fed continuous CSTR experiments were performed. The bioreactors were operated under the following conditions: EXP1 (pH = 5.75, YE 1g/L), EXP2 (pH = 4.75, YE 1 g/L) and EXP3 (pH = 5.75, YE 0.2 g/L). From the bottle experiments (Chapter 3), we observed that a low pH improves ethanol production. However, in the bioreactor study with constant low pH (EXP2), we observed a drastic effect on the overall productivity of the process, though this condition gave the maximum ethanol/acetic acid ratio. EXP1 gave the maximum cell mass concentrations of 302.4 mg/L and product concentrations of 2147.1 mg acetic acid and 352.6 mg ethanol/L. Although conditions such as low pH and low YE concentrations improves the ethanol/acetic acid ratio, the acetic acid concentration obtained was comparatively much higher than the ethanol.
- From the set of batch experiments performed to understand the effect of tungsten, selenium and vitamins on ethanol, acetic acid and 2,3-butanediol, the following conclusions are made (Chapter 5):
  - The presence of tungsten improved ethanol production from CO fermentation.

- Selenium and vitamins addition did not improve the ethanol/acetic acid ratio.
- The 2,3-butanediol/acetic acid ratio increased with the addition of tungsten.

❖ Two set of bioreactor experiments at constant high pH (pH 6.0) and low pH (4.75) with a trace metal solution containing 0.75 µM tungsten, without selenium and vitamins were performed to translate the optimized conditions obtained from the batch study (Chapter 5). At high pH of 6.0, 109% higher cell mass than the maximum achieved at pH 4.75 and almost equal amounts (900 mg/L) of ethanol and acetic acid were obtained in that study. Interestingly, at a low pH of 4.75, no accumulation of acetic acid was observed reaching an ethanol concentration of 867 mg/L. These findings prompted us to perform the next set of bioreactor study at optimal growth pH for a certain time period to achieve a higher cell concentration and acetic acid and later shifting to a lower pH.

❖ A bioreactor experiment was performed under previously optimized conditions (low initial pH of 4.75; YE concentration of 0.5 g/L; addition of tungsten (0.75 µM W) (Chapter 6). In that study, pH was not regulated during the early stage of the study. After a certain period pH was then maintained constant at pH 4.75. A maximum acetic acid concentration of 729 m/L was reached and a cell mass of 343.3 mg/L. After a certain time period, acetic acid got gradually consumed and converted to ethanol. The maximum concentrations of ethanol and 2,3-butanediol obtained from that study were 2840 and 457 mg/L, respectively.

- Another bioreactor study was performed at a constant high pH of 5.75 for a certain period until the cell mass and acetic acid production got stabilized, while later shifting the pH to a low value of 4.75 in order to consume and convert all acetic acid produced to ethanol (Chapter 6). The maximum cell mass concentration reached was 376 mg/L, whereas ethanol and 2,3-butanediol concentrations were 2408 mg/L and 564 mg/L, respectively.
- A novel strategy consisting in a sequencing feed of fresh aqueous fermentation medium was successfully tested (Chapter 6). In that study 600 ml fermentation broth was replaced with fresh nutrient medium along with the cell recycling obtained from centrifuging 600ml fermentation broth. The overall amount of products obtained from that study were 4259.76 mg ethanol; 888.6 mg 2,3-butanediol and 49 mg acetic acid per liter. This small amount of acetic acid would presumably have dropped down to zero if the experiment had been stopped a few hours later.
- A continuous bioreactor study which lasted 1651 h was performed in a CSTR with two cyclic pH shifts and partial medium replacement (Chapter 7). The effects of various chemicals such as NH_4Cl, trypticase peptone as well as vitamin solutions were tested in that study. It was found that the presence of a vitamin solution improves acetic acid production. However, all these above mentioned chemicals addition didn't maximize the cell mass. The overall concentrations of the products obtained from the study were 2100.25, 7142.87 and 1620.71 mg/L of acetic acid, ethanol and 2,3-BDO, respectively.
- Mixotrophic growth of C. autoethanogenum was tested with both xylose and CO as carbon sources (Chapter 7). The results show that the bacteria used comparatively less amount of CO when xylose was present in the medium.

However, bacteria used the xylose instantly. An improved cell mass concentration of 420.79 mg/L was obtained which is comparatively higher than that obtained while feeding only CO.

As any fermentation outcomes depend on various factors such as biocatalyst, fermentation medium, operating conditions and gas composition, general conclusions made from the results obtained from the various above mentioned studies are that:

- Simultaneous ethanol and acetic acid production could be observed at the early stage of the fermentation study performed at optimal growth pH.
- Acetic acid conversion to ethanol could be observed during the stationary stage.
- The rate of conversion of acetic acid to ethanol could be increased by reducing the fermentation pH.

However, it was not possible to reach higher ethanol productivity due to the lack of high concentration of cell mass. In this respect, future studies will focus on improving the cell concentrations by a continuous liquid feeding and recycling the cells using membrane modules as the studies reported in the thesis are liquid-batch system and sequential liquid-feeds. Through the optimized fermentation medium and operating conditions, it is indeed possible to produce the desired metabolites of interest from CO using a wild type strain, although some researchers have recently started using metabolically engineered acetogens as well.

RESUMEN DE LA TESIS EN CASTELLANO

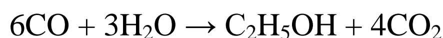
El precio del crudo ha aumentado de forma considerable en los últimos años. La producción de combustibles fósiles y su uso para satisfacer la demanda de energía conduce a la liberación de gases peligrosos y tóxicos, perjudiciales para la humanidad, así como para el medioambiente. Además las reservas de combustibles convencionales, fósiles, son cada vez más escasas. El uso de etanol como fuente alternativa de energía permite disminuir nuestra dependencia de los combustibles fósiles importados de otros países. Además, es más ecológico que otras fuentes de energía derivadas del petróleo.

En la actualidad, la única vía de producción de bioetanol, comercializada, es mediante la fermentación microbiana de azúcares presentes en materias primas que se utilizan también para la alimentación humana y animal (caña de azúcar, tubérculos, etc.). Otra via alternativa,, en fase de desarrollo, consiste en producir bioetanol a partir de biomasa lignocelulósica, de la cual se extrae azúcares simples, fermentables en etanol. La biomasa está compuesta por celulosa, hemicelulosa, y lignina. Los azúcares simples se obtienen a partir de la celulosa y la hemicelulosa, mediante unas etapas de hidrólisis y tratamiento enzimático, seguido por un proceso de fermentación aerobia de azúcares. Esta tecnología tiene limitaciones debido a la compleja estructura polimérica que forman los materiales lignocelulósicos de las plantas. La lignina representa una fracción importante de la biomasa pero, a diferencia de la celulosa y la hemicelulosa, no permite obtener azúcares fermentables; es decir que limita seriamente la eficiencia de aprovechamiento y conversión de la biomasa en etanol. El costo de las enzimas utilizadas en las etapas de pretratamiento sigue siendo relativamente alto y la estabilidad de las diversas enzimas hidrolíticas, por ejemplo la celulosa, es demasiado baja en presencia de lignina. Además, los distintos azúcares simples derivados de la biomasa suelen ser una mezcla de pentosas (monosacáridos formados por una cadena de 5 átomos de carbono) y hexosas (monosacáridos con 6 átomos de carbono). No son

utilizados en su totalidad en el proceso de producción de bioetanol, dado que la mayoría de los microorganismos son capaces de fermentar las hexosas pero no pueden metabolizar las pentosas que constituyen gran parte de los polímeros de la hemicelulosa. La combustión parcial de la biomasa lignocelulósica mediante gasificación rompe todos los polímeros, complejos, convirtiéndolos en gases simples y molécula de un único átomo de carbono. Esta mezcla gaseosa es conocida como "gas de síntesis". La gasificación es un proceso de pirólisis y reducción controlada en el que la biomasa se puede convertir en gas (gas de síntesis), compuesto principalmente de CO y otros gases simples como el CO_2 y el H_2. La conversión química de este gas de síntesis en etanol a través del proceso de Fischer-Tropsch es conocida y ha sido estudiada. Sin embargo, hay varias limitaciones para el uso de catalizadores químicos, incluyendo su desactivación debido a los componentes de azufre presentes en el gas en forma de H_2S y CS a bajas concentraciones.

Un método alternativo de conversión del gas de síntesis a etanol es a través de una reacción de bioconversión con bacterias acetogénicas. Estos microorganismos anaerobios, en su mayoría, pueden ser utilizados como biocatalizadores para producir metabolitos valiosos, como el etanol a partir de gas de síntesis. La fermentación del gas de síntesis es un proceso simple que tiene lugar a temperatura cercana a la ambiente (a diferencia del proceso químico, Fischer-Tropsch). El proceso biológico tiene varias ventajas, tales como una alta especificidad, lo que conduce a un rendimiento más alto, simplifica la separación de los productos, y reduce la concentración de subproductos tóxicos. El biocatalizador utilizado es barato, tiene una alta tolerancia al azufre, y es capaz de adaptarse a los contaminantes, tales como los alquitranes. Por lo tanto, la necesidad de una purificación costosa del gas antes de la conversión es reducida.

La estequiometría de la formación de etanol (C_2H_5OH) a partir de CO es:



$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2$$

Los microorganismos acetogénicos, en su mayoría, utilizan el CO como única fuente de carbono y energía para el crecimiento y la producción de biocombustibles. Por otra parte, el proceso de gasificación permite la conversión de prácticamente cualquier material a base de carbono, como por ejemplo la biomasa, los residuos municipales e industriales, residuos agrícolas, productos forestales, cultivos energéticos, o los productos de desechos orgánicos (por ejemplo, neumáticos) en gas de síntesis que contiene CO. Ciertos procesos industriales, tales como el refinado de petróleo, fresado de acero, y los métodos para la producción de coque, amoníaco, o metanol, descargan enormes cantidades de gases residuales, que contienen principalmente CO, a la atmósfera, ya sea directamente o por medio de combustión. Así, mediante este proceso novedoso, eficiente y económico, todos los residuos orgánicos pueden ser posteriormente transformados en productos útiles, tales como los biocombustibles. La fermentación del gas de síntesis produce también otros productos tales como ácidos orgánicos, a partir de CO. Estos productos incluyen, pero no están limitados a, ácidos acético, propiónico, butírico, fórmico, láctico así como metanol, etanol, propanol, butanol, butanodiol.

En esta tesis, la fermentación del monóxido de carbono mediante el uso de la bacteria acetogénica *C. autoethanogenum* (DSM 10061) se estudió en diferentes condiciones. De los diversos estudios realizados en nuestro laboratorio, los productos metabólicos obtenidos en la fermentación del CO fueron ácido acético, etanol y 2,3-butanediol. Como una alternativa prometedora como biocombustible, el etanol es el producto final más interesante para nuestro estudio. Hasta ahora, la mayoría de los estudios publicados describen la producción de grandes cantidades de ácido acético

durante la fermentación en comparación al etanol. Estos resultados se presentan en la Introducción en el Capítulo 1. Además, la acumulación de ácido acético requiere el uso de procesos de separación de metabolitos más complejos. El objetivo de esta tesis fue optimizar el medio de fermentación y condiciones que mejoren la productividad de etanol, así como eliminar completamente la acumulación de ácido acético. En ese sentido, se han realizado estudios en diversas condiciones de fermentación, tales como diferentes pHs, concentraciones de agente reductor, presiones parciales de CO, fuentes y concentraciones de nitrógeno, con el fin de comprender sus efectos sobre el crecimiento y formación de productos (Capítulos 3 y 4). La vía metabólica de Wood-Ljungdahl (WL) que utilizan las bacterias involucra diversas metaloenzimas (Capítulo 1). Variaciones en las concentraciones de algunos metales traza podrían afectar a la actividad de dichas enzimas. En ese sentido, se estudió el efecto de metales traza, tales como el tungsteno y el selenio. También se estudió el efecto de la adición de vitaminas dado que el desarrollo de medios de bajo coste es algo absolutamente esencial en industrias de fermentación. Los experimentos se realizaron utilizando una nueva estrategia de operación de biorreactores basada en cambios cíclicos de pH y renovación parcial del medio de fermentación, con el objetivo de mejorar la producción global de etanol. Todos los trabajos se llevaron a cabo en botellas para estudios en discontinuo (batch) o en RCTA para reactores con alimentación continua de gas. La concentración celular se estima mediante la medición de la absorbancia de la muestra a una longitud de onda de 600 nm utilizando un espectrofotómetro UV-visible (Hitachi, U-200). La concentración en CO en la fase gaseosa se mide usando un cromatografo de gases HP 6890 (GC) equipado con un detector de conductividad térmica (Thermal Conductivity Detector, TCD) y el producto líquido se analiza mediante HPLC (HP de la serie 1100). El análisis cualitativo de los productos de fermentación se realiza mediante GC/MS

(TRACE GC Ultra-ISQ). El diseño experimental y análisis de datos se realiza mediante ANOVA, utilizando el paquete de software de Minitab 16. En algunos de los experimentos descritos en el Capítulo 6 y el Capítulo 7, parte del de fermentación (600 ml) se retiró asépticamente y se centrifugo en condiciones anaeróbicas. A continuación, el precipitado celular se mezcla con el mismo volumen de medio recién preparado (600 ml) y se introduce en el biorreactor de nuevo. La resuspensión de células se llevó a cabo en una cámara anaeróbica de vinilo (Coylab productos, Michigan). El análisis de 16S rRNA de las células del biorreactor se realizaro para comprobar la pureza de las células. Los diferentes pasos y protocolos utilizados se encuentran descritos en el Capítulo 2. Los materiales y métodos utilizados para llevar a cabo la investigación se describe en el Capítulo 2.

Además de CO como única fuente de carbono, se estudió también el efecto de una combinación de una fuente de azúcar junto con el CO. Un resumen de los trabajos y las conclusiones más significativas se detallan a continuación.

- ❖ Se empleó un enfoque de diseño factorial de dos niveles completos para optimizar la siguiente gama de parámetros de proceso: pH inicial = 4,75 a 5,75, presión inicial = 0,8 a 1,6 bar, concentración de cisteína.HCl = 0,5 a 1,2 g/L, y la concentración de extracto de levadura = 0,6 a 1,6 g/L. Las tandas de experimentos a pequeña escala se realizaron bajo condiciones estrictamente anaerobias. Los resultados de este estudio de microcosmos muestran que, una comprensión previa del efecto de parámetros tales como el pH inicial de la fermentación, la presión inicial, la concentración de cisteína-HCl y la concentración de extracto de levadura es esencial para el diseño de biorreactores continuos bien optimizados con el fin de mejorar la producción de etanol (Capítulo 3). Además, se observó que un pH inicial bajo (pH = 4,75) y

bajas concentraciones de extracto de levadura (0.6 g/L) favorecen una alta producción de etanol por *Clostridium autoethanogenum*. En cuanto a la presión total y la concentración de cisteína-HCl, se producen altas concentraciones de etanol para valores óptimos de estos parámetros (1,6 bar y 1,2 g/L, respectivamente) que no son tóxicos ni inhibitorios para el microorganismo. Se consiguió una concentración máxima de etanol de 0,65 g/L en los estudios bajo las siguientes condiciones: pH = 4,75 (el valor más bajo estudiado), presión = 1,6 bar (el valor más alto probado), cisteína-HCl = 1,2 g/L (el más alto valor probado), y la concentración de YE = 0,6 g/L (el valor más bajo ensayado).

- Se realizaron otros estudios a pequeña escala para examinar los efectos de fuentes de nitrógeno y sus concentraciones en la bioconversión de monóxido de carbono a etanol por *Clostridium autoethanogenum* (Capítulo 4). Se realizó un diseño experimental factorial de tres factores de dos niveles completos (2^3) para examinar los efectos combinados de NH_4Cl (0,2–2 g/L), tripticasa (0,2–2 g/L) y las concentraciones de YE (0,1–1 g/L). Se obtuvo una concentración máxima de etanol de 0,30 g/L bajo las siguientes concentraciones de nitrógeno: NH_4Cl = 2 g/L (el valor más alto estudiado), tripticasa de soja = 0,2 g/L (el valor más bajo probado) y la concentración de extracto de levadura = 0,1 g/L (el valor más bajo ensayado).
- Para determinar el efecto individual de diversas fuentes de nitrógeno se llevaron a cabo experimentos separados en botellas, ya sea con NH_4Cl (1,1 g/L), tripticasa (1,1 g/L) o YE (0,55 g/L). Estos estudios nos ayudan a concluir que para la obtención de una cierta cantidad de biomasa y metabolitos, la cantidad de YE requerida era la mitad de la cantidad de tripticasa (capítulo 4).
- Para utilizar las condiciones optimizadas en los experimentos en botellas en

discontinuo a estudios en continuo, se realizaron tres experimentos con RCTA alimentados en continuo con CO. Los biorreactores operaron bajo las siguientes condiciones: EXP1 (pH = 5,75, YE 1 g/L), EXP2 (pH = 4,75, YE 1 g/L) y EXP3 (pH = 5,75, YE 0,2 g/L). A partir de los experimentos en botella (Capítulo 3), se observó que un pH bajo mejora la producción de etanol. Sin embargo, en el estudio del biorreactor con un pH bajo constante (EXP2), se observó un efecto drástico en la productividad global del proceso, aunque esta condición dio lugar a la máxima relación etanol/ácido acético. El EXP1 dio las máximas concentraciones de masa celular, 302,4 mg/L, y de productos: 2147.1 mg/L de ácido acético y 352,6 mg/L de etanol. Aunque condiciones tales como pH bajo y bajas concentraciones de YE mejoran la relación etanol/ácido acético, la concentración de ácido acético obtenido era comparativamente mucho más alta que la del etanol.

❖ En el conjunto de las tandas de experimentos llevados a cabo para comprender el efecto del tungsteno, selenio y vitaminas sobre la producción de etanol, ácido acético y 2,3-butanediol, se llega a las siguientes conclusiones (capítulo 5):

- La presencia de tungsteno mejora de la producción de etanol a partir de la fermentación de CO.
- La adición de selenio y vitaminas no mejoró la relación etanol/ácido acético.
- La relación 2,3-butanediol/ácido acético aumentó con la adición de tungsteno.
- En cualquier caso, tanto para el etanol y el 2,3-butanodiol, se puede concluir que su concentración relativa, en comparación con el ácido acético, disminuye bajo las siguientes condiciones, sin la adición de una solución de

vitaminas: presencia de tungsteno (sin selenio)> ausencia tanto de tungsteno como de selenio> presencia de selenio (sin tungsteno).

- Se realizaron dos grupos de experimentos en biorreactores con un pH alto constante (pH 6,0) y con un pH bajo (4.75), y con una solución de metales traza conteniendo 0,75 µM de tungsteno, sin selenio ni vitaminas para reproducir las condiciones optimizadas en los estudio en botellas en discontinuo (capítulo 5). A un pH alto de 6,0, la masa celular fue 109 % mayor comparado con el valor máximo alcanzado a pH 4,75 y con la obtención de cantidades casi iguales (900 mg/L) de etanol y ácido acético. Curiosamente, a un pH bajo de 4,75, no se observó acumulación de ácido acético alcanzando una concentración de etanol de 867 mg/L. Estos resultados nos llevaron a realizar la siguiente serie de estudios en biorreactor a un pH óptimo para el crecimiento celular en una primera fase, durante un cierto período de tiempo, para alcanzar una concentración celular y de ácido acético más alta, seguida de una da fase en la que se cambio el pH a un valor inferior.
- Un experimento en biorreactor se realizó bajo condiciones previamente optimizadas (pH inicial bajo de 4,75; concentración de YE de 0,5 g/L; adición de tungsteno (0,75 µM como W) (Capítulo 6). En ese estudio, el pH no fue regulado durante las primeras etapas del mismo, pero después de un determinado período, el pH se mantiene constante a pH 4,75. Se alcanzó una concentración máxima de ácido acético de 729 mg/L y una masa celular de 343,3 mg/L. Después de un cierto período de tiempo, el ácido acético se consumió gradualmente y se convirtió en etanol. Las concentraciones máximas de etanol y 2,3-butanediol obtenidas a partir de ese estudio fueron 2840 y 457 mg/L, respectivamente.

- Otro estudio en biorreactor se llevó a cabo a un pH alto constante de 5,75 durante un cierto período hasta que la producción de masa celular y la de ácido acético se estabilizaron, mientras que más tarde se modificó el pH a un valor bajo de 4,75 con el fin de consumir y convertir todo el ácido acético producido a etanol (Capítulo 6). La concentración máxima de masa celular alcanzada fue de 376 mg/L, mientras que las concentraciones de etanol y 2,3-butanediol fueron 2408 mg/L y 564 mg/L, respectivamente.
- Una nueva estrategia que consiste en una alimentación secuenciada del medio acuoso fresco de fermentación fue probado con éxito (capítulo 6). En ese estudio 600 ml de medio de fermentación fue reemplazado con medio nutritivo fresco junto con un reciclado celular obtenido a partir de la centrifugación de 600 ml del medio de fermentación. La cantidad global de los productos obtenidos a partir de ese estudio fueron 4259,76 mg/L de etanol; 888,6 mg/L de 2,3-butanediol y 49 mg/L de ácido acético. Esta pequeña cantidad de ácido acético presumiblemente habría caído a cero si el experimento hubiese sido detenido unas horas más tarde.
- Un estudio en biorreactor continuo que se prolongó durante 1651 h se realizó en un RCTA con dos cambios de pH cíclicos y con renovación parcial del medio (Capítulo 7). Los efectos de diversos productos químicos tales como NH_4Cl, peptona tripticasa, así como soluciones de vitaminas fueron probados en ese estudio. Se encontró que la presencia de una solución de vitamina mejora la producción de ácido acético. Sin embargo, todos estos productos químicos anteriormente mencionados no maximizaron la masa celular. Las concentraciones totales de los productos obtenidos a partir de este estudio fueron 2100,25, 7142,87 y 1620,71 mg/L de ácido acético, etanol y 2,3-BDO,

respectivamente.

- El crecimiento mixotrófico de *C. autoethanogenum* fue probado con xilosa y CO como fuentes de carbono (Capítulo 7). Los resultados muestran que las bacterias utilizaban comparativamente menos cantidad de CO cuando la xilosa estaba presente en el medio. Sin embargo, las bacterias utilizaban la xilosa inmediatamente. Se obtuvo una mejora en la concentración de masa celular alcanzando 420,79 mg/L la cual es comparativamente mayor que la obtenida mientras se alimentaba sólo con CO.
- Como en cualquier fermentación los resultados dependen de varios factores tales como el biocatalizador, el medio de fermentación, las condiciones de operación y la composición del gas. Las conclusiones generales elaboradas a partir de los resultados obtenidos en los diversos estudios mencionados anteriormente son las siguientes:
    - La producción de etanol y de ácido acético simultáneas se pudieron observar en la fase temprana del estudio de fermentación realizado a un pH óptimo para el crecimiento.
    - La conversión de ácido acético a etanol se pudo observar durante la fase estacionaria.
    - La tasa de conversión de ácido acético a etanol puede aumentar mediante la reducción del pH de fermentación.

Sin embargo, no fue posible alcanzar una mayor productividad de etanol debido a la falta de una alta concentración de masa celular. En este sentido, los estudios futuros se centrarán en la mejora de las concentraciones de células mediante una alimentación líquida continua y el reciclaje de las células utilizando unidades de membrana Utilizando una composición optimizada para el medio de fermentación así como

condiciones de operación óptimas para los biorreactores, es posible producir los metabolitos deseados a partir de CO usando una cepa bacteriana salvaje, aunque algunos investigadores han también comenzado recientemente a utilizar bacterias acetógenicas manipuladas genéticamente.

APPENDIX

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Biological conversion of carbon monoxide: rich syngas or waste gases to bioethanol

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Abstract: Bioconversion of syngas/waste gas components to produce ethanol appears to be a promising alternative compared to the existing chemical techniques. Recently, several laboratory-scale studies have demonstrated the use of acetogens that have the ability to convert various syngas components (CO, CO_2, and H_2) to multicarbon compounds, such as acetate, butyrate, butanol, lactate, and ethanol, in which ethanol is often produced as a minor end-product. This bioconversion process has several advantages, such as its high specificity, the fact that it does not require a highly specific H_2/CO ratio, and that biocatalysts are less susceptible to metal poisoning. Furthermore, this process occurs under mild temperature and pressure and does not require any costly pre-treatment of the feed gas or costly metal catalysts, making the process superior over the conventional chemical catalytic conversion process. The main challenge faced for commercializing this technology is the poor aqueous solubility of the gaseous substrates (mainly CO and H_2). In this paper, a critical review of CO-rich gas fermentation to produce ethanol has been analyzed systematically and published results have been compared. Special emphasis has been given to understand the microbial aspects of the conversion process, by highlighting the role of different micro-organisms used, pathways, and parameters affecting the bioconversion. An analysis of the process fundamentals of various bioreactors used for the biological conversion of CO-rich gases, mainly syngas to ethanol, has been made and reported in this paper. Various challenges faced by the syngas fermentation process for commercialization and future research requirements are also discussed. © 2011 Society of Chemical Industry and John Wiley & Sons, Ltd

Keywords: syngas; CO-rich waste gas; biofuel; ethanol; homoacetogens; bioreactors

Introduction

With the increase in population and growing industrialization of many countries, there has been a tremendous rise in the demand for energy in the world. A 17-fold increase in world-wide energy consumption was reported in the last century.[1] This energy demand is overcome by utilizing primarily the petroleum reserves, which are on the verge of extinction and are estimated to be depleted in less than 50 years at the present consumption rate.[2] The processing of these fossil fuels and their usage

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leads to enormous release of hazardous and toxic gases to the environment, which is harmful to mankind as well as to the environment. The increasing concentrations of these gases has negative impacts such as severe floods and droughts, rising sea levels, and extreme weather conditions.[3] Growing concern about global warming leads researchers to search for sustainable and safer alternative renewable fuels.

Ethanol is one of the most promising alternative biofuels. Fuel ethanol is an oxygenated, water-free, high octane (108) alcohol which has been recognized as a potential alternative fuel as well as an additive to gasoline.[4] As an additive, it can replace methyl tertiary butyl ether (MTBE), which is used as an oxygenate and also to raise the octane number, by which the groundwater pollution due to MTBE usage can be eliminated.[5] Today, ethanol can be used as blends with mineral gasoline at typical ratios of 10, 15, or 20% (E10, E15, and E20). It can even be used pure or almost pure as an alternative transportation fuel (E85).[6] Since it burns cleaner than petroleum products, by using 10% ethanol blend (E10), a reduction of 25–30%, 6–10%, 7% and 5% respectively of harmful emissions of gases as CO, CO_2, VOCs, and NO_x can be achieved.[7] In addition, ethanol is biodegradable and contains 35% oxygen, which reduces particulate and NOx emissions upon combustion compared to conventional fuels.[8]

Bioethanol is derived from renewable sources of feedstock such as sugar, starch, or lignocellulosic materials. Current processes include either direct or indirect fermentation of sugars or catalytic conversion of producer gas. In direct fermentation, feedstocks such as sugar-based crops (e.g. beets, sorghum, and cane) or starch-based crops (e.g. corn, wheat, barley, and potatoes) are converted into alcohols by yeasts or bacteria.[9,10] This technology is well established at industrial level and currently, about 90% of the world bioethanol production comes from fermenting sugars or starch crops, known as first-generation technology.[11] The high value of these crops as a food commodity either for human consumption or for feeding livestock and the issue of low utilization efficiency of crop parts per hectare of land used questions the feasibility of this technology. A potential solution for these issues, known as second-generation technology, is to utilize lignocellulosic feedstocks, such as agricultural or municipal wastes, wood, straw, grasses and crop residues. Lignocellulose is the most abundant renewable organic material on earth, composed of three major components: cellulose (40–50%), hemicelluloses (20–40%), and lignin (10–40%).[12] It is the major structural component of all plants. In indirect fermentation, cellulosic as well as hemicellulosic biomass originating from trees and grasses are hydrolyzed chemically or enzymatically to simple sugars. The available sugars are then fermented to yield ethanol.[13,14] A large proportion of lignin mostly present in straw and wood, along with cellulose and hemicellulose, is highly resistant to microbial attack. Gasification technology can be used to convert the biomass into a mixture of gases, called producer gas. Producer gas can subsequently be converted to ethanol either by using a chemical process (Fischer-Tropsch Synthesis, FTS)[15] or by means of anaerobic microbial catalysts.

Bioethanol production is based on rather inexpensive feedstocks, such as biomass and waste organic matter. It in turn reduces the nation's dependency on imported fossil fuels and thus helps the economy. All this biomass-based production creates employment opportunities by utilizing trivial lands for the cultivation of inexpensive dedicated feedstocks, and the waste can be considerably regenerated for the production of an ecofriendly fuel. Similarly to syngas, CO-rich waste gases can also be used for bioethanol production. The paper summarizes the microbial aspects of ethanol production, ethanologenic homoacetogens, parameters affecting the syngas fermentation and various bioreactors reported in literature. Challenges and R&D needs for syngas fermentation processes are also explained.

Ethanol production from syngas

Syngas, or synthesis gas, a mixture of principally CO and H_2, can be produced by gasification of solid fuels, such as coal, petroleum coke, oil shale, and biomass; by catalytic reforming of natural gas; or by partial oxidation of heavy oils, such as tar-sand oil. The syngas composition mainly depends upon the type of resources used, their moisture content, and the gasification process.[16]

Gasification is the thermochemical process of converting carbonaceous materials, such as coal, petroleum or biomass, in the presence of a controlled amount of oxidant (air/O_2), into a gas mixture consisting mainly of CO, H_2, CH_4, CO_2, and N_2. The intrinsic chemical energy of the solid feedstock

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is thereby extracted and converted into both the thermal and chemical energy of the gas. It is a flexible and well commercialized efficient technology. Usually the process takes place in a gasifier and the composition of the syngas depends mainly on factors such as gasifier type (fixed bed, fluidized bed, etc.), feedstock properties (moisture, ash, dust and tar content, particle size, etc.), and operational conditions (temperature, pressure, etc.).[17] Gasification of biomass involves three steps: (1) drying step to remove the moisture out of the feedstock; (2) pyrolysis at 300–500°C in the absence of oxidizing agents to produce gases, tars, bio-oils, and solid char; and finally (3) gasification of the products of pyrolysis in the presence of an oxidizing agent to yield the various components of producer gas.[18] By optimizing the gasification operation, the composition of the producer gas can be narrowed to mainly CO and H_2, which are the main components for the ethanol production.[6] Also by maintaining adiabatic conversion, the resource energy can be conserved to a higher extent. For example, gasifying at temperatures of 1500–1800°C and 1100°C, respectively of coal and biomass, produces syngas with CO and H_2 as main products.[19] In addition, by using pure oxygen to feed the gasifier, the nitrogen concentration in the producer gas can be reduced.[20]

The synthesis gas thus obtained can be converted chemically to ethanol and a variety of chemicals through the Fischer-Tropsch (FT) cycle. A variety of fuels and chemicals such as methanol, acetic acid, methane and heavy waxes can be produced by this technique.[21] This method of production is a multistep, energy-intensive process carried out at elevated pressure and temperature using different chemical catalysts, which include metal iron, cobalt, or rhodium. These conditions make catalytic conversion faster than bioconversion processes.[11] In this process, the catalytic water gas shift (WGS) reaction takes place, converting CO and H_2O to H_2 and CO_2, thus increasing the H_2/CO ratio, which is essential for the stoichiometry of reaction as well as for reducing the catalytic deactivation (Eqn 1). For protecting the sensitive FT catalyst, other products, such as tar, oil, and water-soluble contaminants, present in the producer gas have to be removed. The sulfur contaminants present in the syngas have to be reduced to less than 60 ppb and the limits on level of NO_x and NH_3 to avoid FT catalyst poisoning are in the order of 0.1 and 10 ppm, respectively.[22] Following the purification, the syngas containing CO and H_2 is converted to ethanol using different catalysts and processing conditions (Eqn 2).

$$CO + H_2O \rightarrow H_2 + CO_2 \qquad \text{(WGS reaction)} \qquad (1)$$

$$2CO + 4H_2 \rightarrow C_2H_5OH + H_2O \qquad \text{(FT reaction)} \qquad (2)$$

Even though this process takes place at high reaction rates, it has many limitations. Mainly, the various processes such as WGS reaction, FT reaction, and purification take place under different process conditions, converting FT synthesis into a complex and expensive method. Moreover, the catalyst used should be specific and will deactivate when the concentration of sulfur, as well as carbon deposition, increases. The yield of liquid fuels from this process is also not high.[23]

An alternative method of converting syngas to ethanol is through bioconversion. Micro-organisms, mostly anaerobic, can be used as biocatalysts to produce valuable metabolites, such as organic acids and alcohols, from syngas. These products include, but are not limited to, acetic, propionic, butyric, formic, and lactic acids as well as methanol, ethanol, propanol, and butanol.[24,25] As a biofuel, ethanol is considered the desired metabolite and the process has to be optimized to maximize its production. Later the desired product is recovered from the broth either by distillation or extraction or a combination of both or by any other efficient recovery process to yield fuel graded ethanol (Fig. 1). Syngas fermentation is a simple process which takes place at near ambient temperature. Although it is characterized by a slower reaction rate, it has several advantages over the conventional chemical catalytic process. First, it has a high specificity, which leads to a higher yield, simplifies the downstream processing, and reduces the concentration of toxic byproducts. Secondly, the biocatalyst used is cheap, has high tolerance to sulfur,[26] and is capable of adapting to contaminants, such as tars.[27] Thus, the need of costly gas purification steps prior to conversion can be avoided. However, an appropriate filtering system can be used to negate the inhibitory effects of some toxic compounds present in the gas mixture. An advantage of the presence of sulfur compounds is that they can stimulate the growth of anaerobic bacteria by reducing the redox potential of the medium.[26] Thirdly, bioconversion does not require a fixed H_2/CO ratio. Hence, one reactor vessel is enough to carry out the process by utilizing suitable micro-organisms. Finally, the

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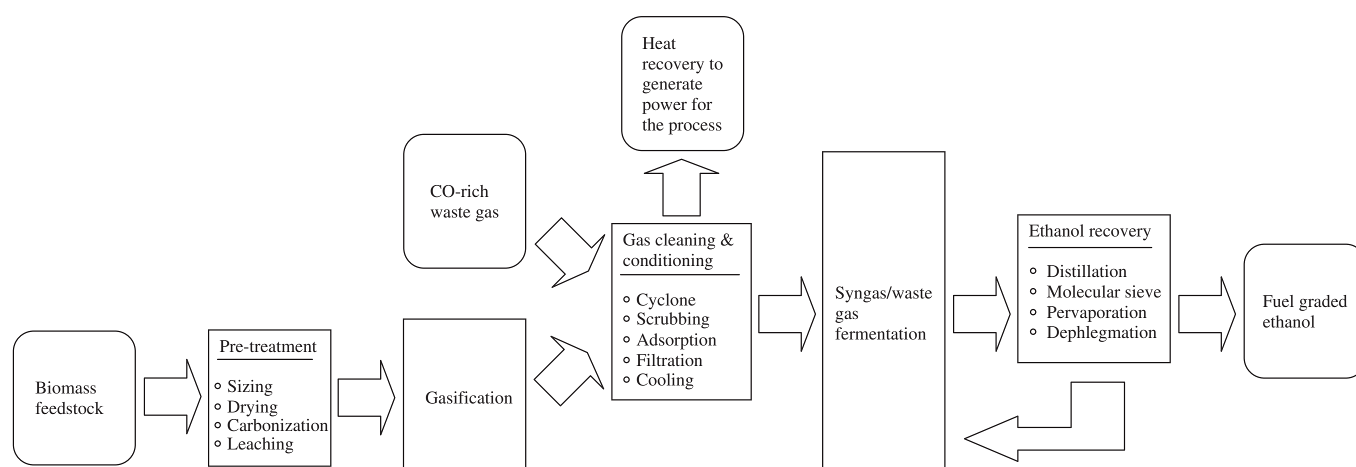


Figure 1. Syngas/CO-rich waste gas bioconversion process overview.

biocatalyst generally dies when exposed to air, the process is odorless, doesn't create any health hazard, and generates less environmental pollution.[28] The reaction process is limited by the mass transfer of gaseous substrates to the medium as well as the need of maintaining rather sterile anaerobic conditions. A continuous supply of nutrients is needed to increase the efficiency of the bioconversion process. Certain industrial processes, such as petroleum refining, steel milling, and methods for producing carbon black, coke, ammonia, and methanol discharge enormous amounts of waste gases containing mainly CO and H_2 into the atmosphere either directly or through combustion. Biocatalysts can be exploited to convert these waste gases to chemicals and fuels as, for example, ethanol, in a similar way as in the case of syngas fermentation.[29]

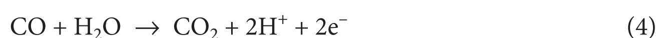
Biochemical pathway for ethanol production

The pathway which autotrophic anaerobes usually follow for the production of ethanol is the acetyl-CoA biochemical pathway or Wood-Ljungdahl pathway (Fig. 2).[30,31] This pathway is present in several organisms including homoacetogenic bacteria and methanogenic archea.[32] It contains an eastern branch and a western branch.[33] The eastern branch comprises several reductive steps, where CO_2 is reduced to produce the methyl group of acetyl-CoA. The western branch, which is unique in anaerobes, either generates CO from CO_2 or directly takes CO from the media which then serves as the carbonyl group for the acetyl-CoA synthesis.

The reducing equivalents for the process are generated from H_2 by hydrogenase enzymes.[34]

$$H_2 \rightarrow 2H^+ + 2e^- \quad (3)$$

If H_2 is insufficient or inhibition of the hydrogenase enzyme occurs,[35,36] then the reducing equivalents are produced via oxidation of CO to CO_2 using CODH.[37]



$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^- \quad (4)$$

It is worth observing that the sum of Eqn 4 and the reverse of Eqn 3 is the water gas shift reaction used to adjust the H_2/CO ratio during the chemical syngas conversion. The availability of CO as carbon source for ethanol synthesis thus decreases (Eqn 4) which can be interpreted using the Eqns 5 and 6.

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \quad (5)$$

$$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O \quad (6)$$

It can be seen from Eqn 5 that only one-third of the available carbon source (CO) can be theoretically converted to ethanol. This is because CO is used to produce the reducing equivalents by oxidation to CO_2 via CODH in the absence of H_2 or in the state of inhibition of the hydrogenase enzyme. Moreover from Eqn 6 it can be deduced that CO_2 can be used to make ethanol if H_2 is present in the syngas.

$$6CO + 6H_2 \rightarrow 2C_2H_5OH + 2CO_2 \quad (7)$$

Finally from Eqn 7, for an equimolar mixture of CO and H_2, two-thirds of the carbon substrate (CO) can be

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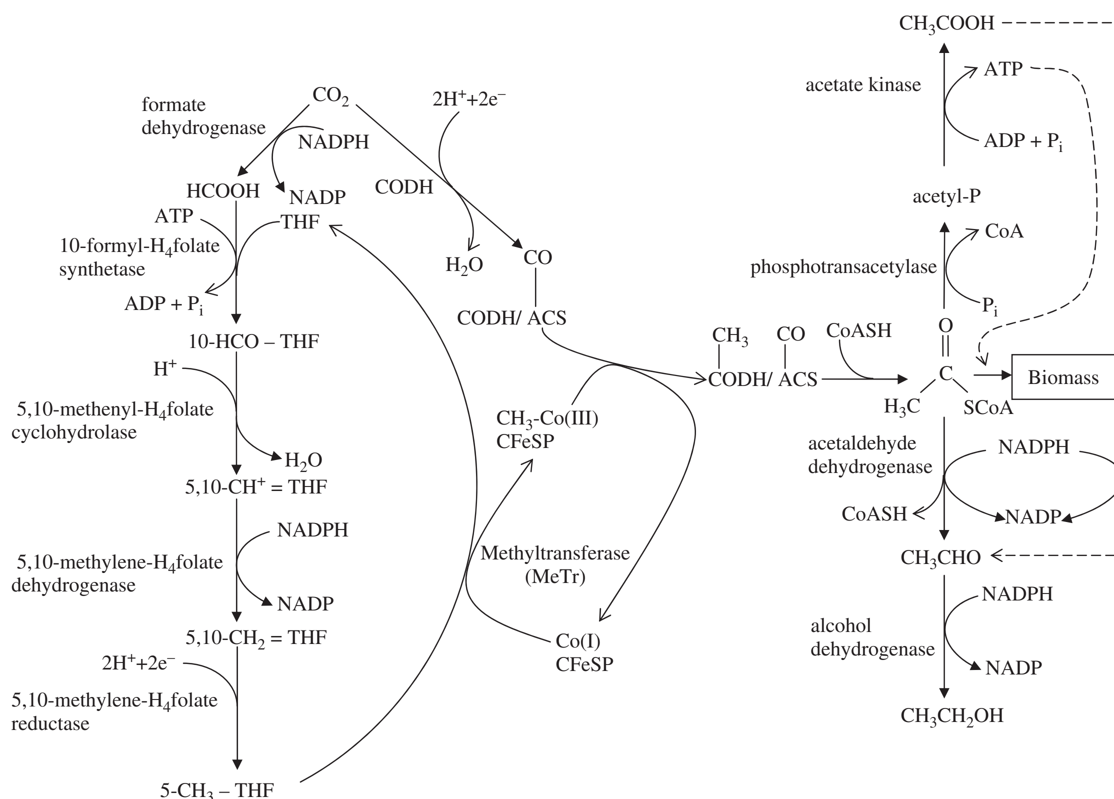

Figure 2. The Wood-Ljungdahl pathway for acetogenic microbes (CO to acetyl CoA) and reduction of acetyl-CoA to ethanol. Abbreviations: THF – Tetrahydrofolate; CFeSP – Corrinoid iron sulfur protein; CODH/ACS – CO dehydrogenase/ acetyl-CoA synthase.

converted to ethanol since sufficient reducing equivalents are provided by hydrogen with the help of hydrogenase enzymes with a subsequent increased carbon conversion rate.

Eastern branch

The eastern branch is an H_4folate-dependent pathway which involves several reductive steps to convert CO_2 to (6*S*)-5-CH_3-H_4folate. The first step is the conversion of CO_2 by formate dehydrogenase to formate, which is condensed with H_4folate to form 10-formyl-H_4folate catalyzed by 10-formyl-H_4folate synthetase.[38] A cyclohydrolase then converts the latter intermediate to 5,10-methenyl-H_4folate.[39] The next step is an NAD(P)H-dependent reduction, where the methylene-H_4folate dehydrogenase converts the 5,10-methenyl-H_4folate to 5,10-methylene-H_4folate,[40] which is reduced to (6*S*)-5-CH_3-H_4folate by methylene-H_4folate reductase.[41] Thus, the conversion of CO_2 to the precursor of the methyl group of acetyl-CoA involves six electron reductions.

Western branch

The methyl group of the CH_3-H_4folate is transferred into the cobalt centre of the corrinoid/iron-sulfur protein (CFeSP)[42] by the action of the methyltransferase (MeTr).[43] This heterodimeric protein CFeSP[44] is active when the cobalt centre is in active Co(I) state. The Co(I) then undergoes transformation into inactive Co(III) state by attaching a methyl group from the CH_3-H_4folate.[45] The most important step in the MeTr mechanism is the activation of the methyl group because of the higher stability of CH_3-N bond in CH_3-H_4folate. The most studied mechanism of activation of the methyl group is by protonation of the N5 group of the CH_3-H_4folate thus leading to the electrophilic activation of the methyl group.[46,47] Hence the first organometallic intermediate is formed as methyl-Co(III)-CFeSP.

One of the main enzymes in the Wood-Ljungdahl pathway is CO dehydrogenase.[48] This Ni-CODH is classified into two groups: (1) Monofunctional;[49] and (2) Bifunctional CODH.[50] Monofunctional CODH catalyses the oxidation of CO to CO_2,

which is then reduced to formate and finally to the methyl group of acetyl-CoA. The bifunctional CODH converts CO_2 to CO, which serves the carbonyl group of acetyl-CoA, and also catalyses the formation of acetyl-CoA along with acetyl-CoA synthase (ACS).[51] Following the synthesis at the C-cluster of CODH, CO then migrates to the Ni_p site of A-cluster in ACS forming organometallic intermediate; Ni-CO.[52] The next step in the pathway involves the transfer of the methyl group from the methylated CFeS protein to the CODH/ACS complex. Thus the third organometallic complex, the methyl-Ni complex is formed.[53,54] In the next step, condensation of methyl and carbonyl groups at the Ni_p form an acetylmetal, the final organometallic intermediate. Finally, in the Wood-Ljungdahl pathway, CoA together with ACS thiolysis the acetylmetal to form acetyl-CoA.[55,56] Acetyl-CoA is converted by the cell to cell mass, acetate and ATP during the growth stage and to ethanol and NAD(P) during the non-growth stage.

Autotrophic bacteria for the conversion of syngas or waste gas to ethanol

The production of ethanol by anaerobic bacteria using syngas was first reported around the 1990s.[57–59] However, chemicals such as acetic acid, butanol, and in some cases butyric acid and lactic acid were also produced along with ethanol. Various ethanol producing homoacetogens and their characteristics are listed in Table 1. These unicarbonotrophic microbes exhibit great potential for use in the conversion of syngas and follow the acetyl-CoA pathway for cell growth and product formation. Though these micro-organisms grow well on multicarbon compounds, their potential to utilize CO, CO_2, and H_2 gases without additional feedstocks as co-substrates to produce various chemicals and fuels is important and well documented in the literature.[60,61]

The majority of the original works have focused on increasing the culture stability throughout the growth on syngas and increasing the alcohol production over acids. In most cases, a general trend in the shift from acetogenic to solventogenic product spectrum was observed as the fermentation pH is reduced. Hence, fermentation pH was found to have great influence in the regulation of syngas metabolism. Since most of the ethanol producing micro-organisms mentioned here were isolated recently, most of the research work with those microbes was done by only few research groups in the 1990s (Table 2).

Clostridium ljungdahlii

C. ljungdahlii is, by far, the most widely studied ethanol-producing homoacetogen. Various research works have been completed using this organism, mainly focusing on increasing the ethanol yield or improving the ethanol to

Table 1. Characteristics of different syngas/CO-rich waste gas fermenting bacteria for ethanol production.

Characteristics	*C. ljungdahlii*	*C. carboxidivorans P7*[T]	*B. methylotrophicum*[a]	*C. autoethanogenum*	*C. drakei*	*C. ragsdalei P11*	*A. bacchi CP11*[T]
Origin	Chicken yard waste	Agriculture settling lagoon	Sewage digestor	Rabbit feces	Acidic sediment	Duck pond sediment	Saturated soil
Size (μm)	0.6 x 2 – 3	0.5 x 3	0.8 ± 0.2 x 2.7 ± 0.54	0.5 x 3.2	0.6 x 3 – 4	0.7 – 0.8 x 4 – 5	0.5 – 0.8 x 1.5 – 2.2
Temperature range (°C)	30 – 40	24 – 42	10 – 50	20 – 44	18 – 42	18 – 37	15 – 40
Optimum temperature (°C)	37	37 – 40	37 – 40	37	30 – 37	37	37
pH range	4.0 – 7.0	4.4 – 7.6	6 – 9	4.5 – 6.5	4.6 – 7.8	4.0 – 8.5	6.5 – 10.5
Optimum pH	6.0	5.0 – 7.0	7.5	5.8 – 6.0	5.5 – 7.5	6.3	8.0 – 8.5
G + C (mol %)	22 – 23	31 – 32	49.8 ± 0.2	26 ± 0.6	30 – 32	29 – 30	34
Doubling time on H_2/CO_2 (h)	2.7	5.8	19		3.5	4[b]	
Reference	Tanner *et al.*[114]	Liou *et al.*[62]	Zeikus *et al.*[64]	Abrini *et al.*[70]	Liou *et al.*[62]	Huhnke *et al.*[77]	Allen *et al.*[76]

[a]Marburg strain;
[b]Doubling time on CO.

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acetate ratio. Vega *et al.*[57] demonstrated the effects of yeast extract concentration in syngas fermentation on the stability of the process in both batch and continuous mode. Furthermore, with various initial substrate pressures in batch studies, the variation in the uptake and utilization of different substrates (CO or H_2) was briefly explained. In a continuous process with constant gas flow rate of 3.5 mL min^{-1} (18.5% H_2, 15.4% Ar, 56.1% CO and 10% CO_2), a molar ratio of ethanol to acetate of 1:0.8 was obtained with a liquid flow rate of 10.85 mL h^{-1} and by reducing the yeast extract concentration (0.01%).[57] Using *E. coli* as a model, medium composition was optimized by Philips *et al.*[58] where B-vitamin concentration was reduced and yeast extract was eliminated for *C. ljungdahlii*. Ethanol concentrations of 50 and 25 g L^{-1} were then obtained respectively with and without cell recycle. An ethanol-to-acetate ratio ranging from 1.6 to 21 mol mol^{-1} was reported in the latter research during CSTR studies with cell recycle.[58] Using two-CSTR in series, Klasson *et al.*[59] achieved a 30-fold increase in ethanol production rate (250–300 mmol g^{-1}cell d^{-1}) compared to a single CSTR. By promoting cell growth in the first reactor followed by an increase in the ethanol production in the second reactor, a product ratio

Table 2. Ethanol production using gaseous substrate by various homoacetogenic bacteria.

Micro-organism	Bioprocess mode	Culture elapsed time (h)	Syngas composition (v/v%)	pH	Ethanol concentration (g L^{-1})	Alcohol/acid (mol mol^{-1})	Reference
Clostridium ljungdahlii	Continuous stirred tank bioreactor with cell recycle	560	CO = 55, H_2 = 20, CO_2 = 10, Ar = 15	4.5	48	21	58
	Two CSTR in series	16[a]	CO = 55.25, H_2 = 18.11, CO_2 = 10.61, Ar = 15.78	4.0	3[b]	1.5[b]	59
Butyribacterium methylotrophicum	Continuous stirred tank bioreactor		CO = 100	6[c]	0.056	0.131	68
	Batch experiments with serum bottles	144	CO = 35, H_2 = 40, CO_2 = 25	7.3	0.02	0.018[d]	69
Eubacterium limosum KIST612	Continuous bubble column reactor with cell recycle	233[e]	CO = 100	6.8	0.092[b]	0.061[f]	75
Clostridium carboxidivorans P7[T]	Continuous bubble column reactor	10[a]	CO = 14.7, CO_2 = 16.5, N_2 = 56.8, H_2 = 4.4[g]	6[h]	1.6		61
	Batch experiments with cell culture flask	6.5[a]	CO = 20, CO_2 = 15, H_2 = 5, N_2 = 60[i]	5.7	0.337	0.392	63
Clostridium autoethanogenum	Continuous modified bioreactor[j]	72	CO = 20, CO_2 = 20, N_2 = 50, H_2 = 10	6	0.066[k]	0.062[k]	73
Moorella sp. HUC22–1[l]	Batch experiments with serum bottles	156	H_2 = 80, CO_2 = 20	6.3[m]	0.069	0.026	74
	Repeated batch experiments with cell recycle using fermentor	430	H_2 = 80, CO_2 = 20	5.8	0.317	0.023	115

[a]Culture elapsed time in days (d);
[b]Approximate value in the reactor;
[c]Other products at pH 6: butyrate, acetate and butanol;
[d]Other products: acetic acid, butyric acid and lactic acid;
[e]Dilution rate of 0.15 h^{-1};
[f]Other products: butyrate 6 mM, acetate 16.5 mM;
[g]Rest contains CH_2 = 4.2%, C_2H_4 = 2.4%, C_2H_6 = 0.8%;
[h]The broth pH at the ethanol concentration of 1.6 g L^{-1};
[i]130 ppm of NO was added into the medium;
[j]Modified spinner flask: the spinner is replaced with stainless steel tube with stainless steel porous gas dispersion cylinder connected to it;
[k]At a flow rate of 10 mL min^{-1};
[l]Thermophile which grows at a temperature of 55 °C;
[m]Initial pH.

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of 4 mol ethanol mol^{-1} acetate was attained. The influence of nutrient limitation, pH, and dilution rate was studied to improve the product distribution.[59] One major limitation in the overall CO bioconversion rate is the very low water solubility of carbon monoxide at ambient temperature and pressure. The influence of using a pressurized system in decreasing the gas-liquid mass transfer resistance in syngas fermentation and thus obtaining high CO uptake rate was postulated by Younesi *et al.*[60] The CO_2 concentration profile during the study revealed that CO was the preferred inorganic carbon source for *C. ljungdahlii*. A maximum product ratio of 0.54 mmol ethanol $mmol^{-1}$ acetate was obtained at syngas (55% CO, 20% H_2, 10% CO_2 and 15% Ar) total pressure of 1.8 atm in batch study using Wheaton serum bottles.[60]

Clostridium carboxidivorans P7^T

C. carboxidivorans P7^T, named on the basis of its ability to readily utilize CO, is an obligate anaerobe isolated from an agricultural settling lagoon.[62] It was shown that this strain is able to produce ethanol, acetic acid, and butanol when grow on 'clean' bottled syngas without hydrogen in a bubble column.[12] The effects of biomass-generated producer gas on cell stability, hydrogen utilization, and acid/alcohol production in a 4-L bubble column reactor were also assessed by Datar *et al.*[61] It was observed that cells are very sensitive to chemical species, such as nitric oxide and acetylene, produced along with the syngas generated from switchgrass. A minor amount of butyrate (max. conc. 1.2 g L^{-1}) was also reported along with the usually observed products mentioned above.[61] The stoppage of hydrogen uptake as well as a decline in cell concentration after introduction of biomass-generated syngas reveal the need for further studies to identify chemicals contributing to the above findings. By using a 0.025 µm filter instead of 0.2 µm, the previously observed decline in cell concentration was able to negate.[27] But hydrogen consumption ceased irrespective of the filter size. Further studies revealed that the presence of NO below 40 ppm in syngas will not cause any negative impact on cell growth, hydrogenase enzyme activity, and product re-distribution in *C. carboxidivorans* P7^T.[63]

Butyribacterium methylotrophicum

B. methylotrophicum is able to use methyl radicals and was first isolated from a sewage sludge digestor in Marburg, Federal Republic of Germany.[64] The neotype strain of the species is called the Marburg strain and grows on multicarbon compounds in addition to one-carbon compounds and acetate, typically used by other methylotrophs. A comparison of the efficiency of cell synthesis during the growth of *B. methylotrophicum* on heterotrophic (glucose) and unicarbonotrophic (H_2:CO_2, and methanol) substrates has been done by Lynd and Zeikus.[65] A final acetate concentration of 16 mM was produced during the growth on H_2:CO_2 (2:1) and a minor amount of butyrate was detected after the growth, with a doubling time of 9.0 h.[65] Another strain of *B. methylotrophicum*, the CO strain, was the first anaerobic microbe to show its ability to grow unicarbonotrophically on CO as the sole carbon and energy source. It was designated CO strain for its growth on 100% CO.[66] This strain, when grown in batch culture with a continuous supply of CO in the headspace, yielded an acetate/butyrate ratio of 32:1 at a pH of 6.8.[67] A general trend in the formation of more reduced products like alcohols was observed when the fermentation pH was reduced from 6.8 to 6 in the continuous study with a dilution rate of 0.015 h^{-1}. A gradual increase in the ethanol concentration in the continuous culture from 0.028 to 0.056 g L^{-1} was observed with a pH shift from 6.8 to 6 with a doubling time of 12 h.[68] Nearly half of the available carbon in the substrate was found to be lost via CO_2 formation and acetate was the main reduced product formed in both the batch and continuous studies mentioned above. A more recent study investigated on the effect of supplementing CO with CO_2 and H_2, using a CO adapted strain. The study revealed that bottles supplemented with CO_2 showed increased final product concentrations.[69] During this batch study, an ethanol concentration of 0.02 g L^{-1} and a total carbon yield to products of 110% was obtained using syngas ($CO:CO_2:H_2$ = 35:25:40) along with other products such as acetic acid (1.1 g L^{-1}), butyric acid (0.3 g L^{-1}) and a minor amount of lactic acid.

Clostridium autoethanogenum

C. autoethanogenum was originally isolated from rabbit feces using CO as the sole carbon and energy source. Electron microscopic studies using an old culture revealed that after a long period of incubation, the cell morphology changed from rod-shaped to continuous chains of encapsulated filaments having a size of 0.6 x 42.5 µm along with the normal cells.[70] Less syngas fermentation research has been completed using this micro-organism. With the objective of

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examining the effects of nitrogen-limited media on resting cells of *C. autoethanogenum* in ethanol production, Cotter *et al.*[71] formulated six different non-growing media by varying or excluding some of the following nitrogen sources: yeast extract, trypticase peptone, and/or NH_4Cl and using xylose as substrate. In that study, a high ethanol production of 9.43 mM and ethanol to acetate ratio of 1:4.5 was reported in yeast extract excluded media, which is greater than the values obtained in growing cultures (5.11 mM and 1:7.8, respectively). This result supports the findings that yeast extract limitation can enhance ethanol production in homoacetogenic bacteria.[57,72] Importantly, a high level of culture stability was observed throughout the experiment in the medium containing 0.1 g L^{-1} yeast extract.[71] The same research group achieved a 1:13 ethanol to acetate ratio when using syngas as substrate in liquid-batch continuous gas fermentation with a xylose adapted culture. Three different flow rates of 5, 7.5, and 10 mL min^{-1} of bottled synthesis gas were used in that study allowing the conclusion that despite increasing growth and product formation, the gas flow rate has no role in the product distribution in *C. autoethanogenum*.[73]

Other strains

Isolates of *Moorella* species HUC22-1 were demonstrated to produce ethanol and acetate as main products formed from 130 mM CO_2 and 270 mM H_2 at 55°C, with an ethanol concentration of 1.5 mM after 156 h study. Moreover, this is the first example of an ethanol-producing thermophile that converts H_2 and CO_2 during growth.[74] Another strain, *Eubacterium limosum* KIST612, isolated from an anaerobic digester, which was found to have high ability to grow at elevated CO partial pressure, was able to produce trace amounts of ethanol along with butyrate and acetate using 100% CO as substrate.[75] A number of other anaerobic bacteria were also shown to utilize syngas as carbon and energy source and in particular, to convert syngas mainly to ethanol along with some other metabolites. Some of them are new isolates, including *Alkalibaculum bacchi* CP11^T, *Clostridium drakei,* and *Clostridium ragsdalei* P11.[62,76,77]

Parameters affecting the bioconversion of syngas to ethanol

If one wants to produce bioethanol as a fuel product, conditions should be optimized to form that metabolite over any other during the bioconversion of CO-containing syngas or waste gases.

Effect of pH

As with other biochemical processes, fermentation pH is found to have a strong influence in regulating the metabolism of the substrate, namely CO-containing syngas or waste gas. There is a significant relationship between pH and the product composition. A general trend observed in most of the syngas fermentation studies is the shift in the product spectrum from acidogenic to solventogenic phase when lowering the fermentation pH. Since every organism is metabolically active over a limited range of pH, decreasing the pH has a negative impact on the cell growth. This is one major obstacle in the optimal conversion of syngas to fuel ethanol, as lowering the pH to produce highly reduced products as ethanol will also reduce the overall productivity of the process. In most studies, it was observed that lowering the pH causes a decrease in electron and carbon flow from the substrate toward the cell mass. At the same time, a decrease in acid production and an increase in alcohol production at the expense of the acid were observed.[58,67,68] In a more recent study with *C. ljungdahlii*, an expected increase in ethanol production by lowering the pH of the broth from 6.8 to 5.5 was not observed. The culture in more acidic medium (pH 5.5) reached overall cell and ethanol concentrations of 388 mg L^{-1} and 1.81 mM, respectively, which was lower than at pH 6.8 (562 mg L^{-1} and 3.81 mM, respectively).[73] Nevertheless, fermentation pH is one of the important factors to be considered for the overall success of the syngas fermentation process.

Effect of media composition

During syngas fermentation, micro-organisms consume syngas constituents as carbon and energy sources; however, they also need various mineral nutrients to maintain a high metabolic activity. Special compounds, such as vitamins, may also be needed. Earlier reports suggest that even though growth ceases, a reduction in B-vitamins concentration along with eliminating yeast extract favored an increase in ethanol to acetate ratio.[58] Eliminating yeast extract causes an improvement in product ratio of up to 300%; however, a minimum concentration of 0.01% yeast extract is necessary to provide the required trace nutrients for the structural

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integrity in *C. ljungdahlii*.[78] Some studies have been reported on different nutrient sources, which induce sporulation along with an improvement in solvent production. It was found that compared to yeast extract and other nutrients, cellulobiose-containing culture media show an increase in cell concentration of greater than 20%, as well as ethanol concentration and ethanol to acetate ratio values greater than 4 and 3 times respectively compared to the values obtained in the presence of yeast extract.[72]

The provision of more electrons by the addition of reducing agents into the culture medium will help the metabolism of microbes to shift toward solventogenesis.[57] This occurs due to the presence of more reducing equivalents for the microbes to convert acetyl-CoA to ethanol. Klasson *et al*.[79] examined the feasibility of increasing the ethanol concentration as well as product ratio in *C. ljungdahlii* by using different concentrations of reducing agents such as sodium thioglycolate, ascorbic acid, methyl viologen, and benzyl viologen. The authors found that even though the growth ceased, a high ethanol concentration (3.7 mmol) and a high product ratio (1.1) were found in bottles containing 30 ppm benzyl viologen. The most commonly recommended reducing agents for various acetogens by ATCC and DSMZ are cysteine-HCl and Na_2S x $9H_2O$. The effect of various trace metal ions in the fermentation media on growth and ethanol production by *C. ragsdalei* was investigated and it was observed that increasing the concentration of Ni^{2+}, Zn^{2+}, SeO_4^- and WO_4^- positively affected ethanol production.[80]

Effect of gas composition

Gasification of biomass generates primarily CO, CO_2, CH_4, H_2, N_2 and small amounts of NO_x, O_2, acetylene, phenol, COS, H_2S, light hydrocarbons such as C_2H_2, C_2H_4, and C_3H_8, ash, char, and tars. Autotrophic microbes are capable of growing well on bottled synthesis gas composed of CO, CO_2, and H_2. However, biomass-generated producer gas fermentation may sometimes face problems in maintaining the culture stability and the efficiency of carbon conversion due to the presence of trace amounts of additional constituents, such as acetylene or NO.[61,63] Acetylene and NO are known to be potent inhibitors of hydrogenase enzyme activity.[81,82] Since hydrogenase activity is essential for the reaction with hydrogen to obtain electrons for the CO conversion process, the inhibition of the hydrogenase enzyme will force the cell to obtain electrons from CO using CODH enzymes. Thus, available carbon for ethanol production will be greatly reduced. Hence, a decrease in carbon conversion efficiency will be seen during the process. In a study using *Rhodospirillum rubrum*, it was found that CO-linked hydrogenase enzymes show a 50% inhibition in the presence of 10% (v/v) C_2H_2.[82] Recent studies with *C. carboxidivorans* P7^T showed that product redistribution also happened due to the presence of NO[63] and the effects due to the presence of tar towards the cell dormancy was eliminated by cleaning the syngas using a cyclone, scrubber (10% acetone) and a 0.025 μm gas cleaning filter prior to the introduction into the fermentor.[27]

Effect of substrate pressure

The partial pressure of the various constituents in syngas or waste gases plays a crucial role in the metabolism of the microbes. Partial pressures of both CO (P_{co}) and CO_2 (P_{co_2}) significantly influence microbial growth and product distribution. CO is used as a carbon source and sometimes oxidized to produce reducing equivalents via carbon monoxide dehydrogenase in the absence of H_2. Moreover, since CO is usually the least soluble gas among the syngas or waste gas components, more attention needs to be given to overcome mass transfer limitation due to this gas. Hence, one way of reducing the gas–liquid mass transfer limitations is by increasing the initial pressure of the gaseous substrates. The net electron production from CO by CODH increases with an increase in P_{co} and decreases with an increase in P_{co_2}. In a study done with *C. carboxidivorans* P7^T,[83] it was shown that the maximum cell concentration increased when increasing the P_{co}. A decrease in acetic acid concentration with an increase in ethanol concentration was also reported in the later stages of experiments conducted at high P_{co} (1.35 and 2 atm). This is due to the utilization of excess electrons produced at high P_{co} for the conversion of acetic acid to ethanol. An increase of 440% in cell concentration in that study was reported for an increase in P_{co} from 0.35 to 2.0 atm. But some microorganisms are also reported to be less resistant to high P_{co} resulting in an increase in their doubling time when increasing P_{co}.[84,85] In batch experiments using *C. ljungdahlii*, it was reported that increasing the initial pressure of the syngas will cause lengthening of the lag-phase period[57]

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and significantly improve the substrate utilization while yielding high ethanol/acetate ratios.[86]

Mass transfer

One potential bottleneck of syngas fermentation is mass transfer limitations.[87,88] When the fermentation broth contains a high cell concentration, the system is said to be in a mass transfer limited state, which is due to the low aqueous solubilities of the sparingly soluble gaseous substrates, CO and H_2. Due to these diffusion limitations, availability of gaseous substrates for the micro-organisms becomes low, which eventually leads to reduced productivity. The yield from the process also becomes low when the system is under kinetic-limited conditions, which happens when either the cell concentration or the CO consumption rate is too low.[89] Both of these two rate-limiting conditions may occur during the course of syngas fermentation.

From the theoretical equations of syngas fermentation (Eqns 5 and 6), it is clearly observed that 6 moles of CO or H_2 have to transfer into the culture medium to produce 1 mole of ethanol. Moreover, on a molar basis, the solubilities of CO and H_2 are only 77 and 68%, respectively to that of oxygen at 35°C.[90] Hence, more moles of syngas must be transferred per carbon equivalent consumed in order to enhance the yield and productivity.

Gas–liquid mass transfer is of prime importance and the various gas components present in the bioreactor have to overcome a series of transport resistances before being utilized by the biocatalyst. The overall mass transfer rate of a gaseous substrate to the liquid phase is given by the product of the mass transfer coefficient, available area for mass transfer, and the driving force. The driving force for diffusion in this case is the difference between the actual partial pressure of the substrate in the bulk gas phase, P^g (atm), and the partial pressure of the substrate that would be in equilibrium with the substrate in the bulk liquid phase, P^l (atm). Thus, the overall mass transfer rate can be defined as;

$$\text{Overall mass transfer rate} = \frac{K_L a}{H}(P^g - P^l) \quad (8)$$

where H is the Henry's constant (L atm mol^{-1}) and K_La is the volumetric mass transfer coefficient (s^{-1}).

Since the solubility of the substrate in the culture medium or in the biofilm is low, the amount of substrate present in the liquid phase is negligible compared to the substrate in the gas phase. Thus the substrate balance in the gas phase is given by

$$-\frac{1}{V_L}\left(\frac{dN_s}{dt}\right) = \frac{K_L a}{H}(P^g - P^l) \quad (9)$$

where N_S (mol) is the molar substrate concentration in the gas phase and V_L (L) is the volume of the reactor. From Eqn 9, the mass transfer coefficient K_L (m s^{-1}) for the gaseous substrate can be determined.

The Andrew or Haldane model has been used to determine the kinetic substrate utilization and inhibition in syngas fermentation. The specific consumption rate q_s, which is the substrate uptake per dry cell weight, is given by

$$q_s = \frac{q_s^{max} P^l}{K_p + P^l + (P^l)^2/K_i} \quad (10)$$

where q_s is the specific substrate consumption rate (h^{-1}), q_s^{max} is the maximum specific substrate consumption rate (h^{-1}), K_p is constant (atm) and K_i is the substrate inhibition constant (atm).

Ungerman and Heindel[91] compared CO-water K_La and power demand in a stirred tank reactor using different impeller designs and schemes and it was found that the highest mass transfer coefficient was obtained with the dual Rushton impeller scheme. Compared with the standard (single) Rushton impeller scheme, the dual Rushton impeller scheme could enhance the mass transfer by up to 27%. However, the impeller performance, which is the measure of volumetric mass transfer coefficient per unit power input, was lowest for the dual Rushton. As discussed later, increasing the agitation speed as a way to improve the mass transfer consumes more power. Hence this method is not economically feasible for large-scale bioethanol production. Bredwell *et al.*[92] reviewed various bioreactor studies on syngas fermentation using conventional stirred tank and columnar reactors and observed that the volumetric mass transfer coefficient in these bioreactors depends mainly on reactor geometry, configuration, process operating conditions and the liquid phase properties.

Various additives can be added to increase the gas–liquid mass transfer rates which include surfactants, alcohol, salts, catalyst and small particles.[93] Ethanol concentration of 1% (*w/v*) in the fermentation broth was shown to increase the

mass transfer rate up to 3-fold compared to clean water.[94] This is due to the change in surface tension, thereby formation of small gas bubbles and hence better surface area for mass transfer. A new approach to enhance the mass transfer is by using nanoparticles. Zhu *et al.*[93] found that surface hydroxyl and functional groups on the nanoparticles have influence in enhancing the CO–water mass transfer coefficient. The highest K_La enhancement of 1.9 times was obtained when mercaptan groups were grafted on the nanoparticles.

Bioreactors for syngas fermentation

The selection of an appropriate bioreactor configuration is important for efficient syngas fermentation, especially configurations that could overcome mass transfer limitations and achieve high cell density. Transfer of syngas components mainly CO and H_2 is a major concern due to their low aqueous solubility.[88,91]

To obtain a high syngas conversion, a good bioreactor should provide a high specific surface area for the reaction to occur, and favor high mass transfer rates. The bubble diameter will be one of the key parameters in gas–liquid mass transfer in suspended growth bioreactors. The specific surface area for mass transfer is inversely proportional to the bubble diameter under mass transfer limited condition.[92] Hence, dispersing a sparingly soluble substrate using microbubble dispersion would offer a high area for mass transfer and the decreased bubble size during this process would allow longer gas hold-up times in the reactor, due to its slow rise. Microbubbles or colloidal gas aphrons are surfactant-stabilized small bubbles of diameter 50–60 μm, created by intense stirring using a high shear impeller in a separate reactor.[95] Sebba[96] proposed that these bubbles are composed of a gas bubble surrounded by a surfactant-stabilized shell of water. They are comparatively stable and offer high surface areas. The multiple surfactant shell prevents the adjacent bubbles from coalescence by imparting electric repulsion between them. A 6-fold increase in the overall mass transfer coefficient of CO has been reported by Bredwell and Worden,[95] with microbubble sparging compared to conventional sparging, using *B. methylotrophicum*. Furthermore, the power requirement to generate the microbubbles for syngas fermentation was estimated to be very low, of 0.01 kW m^{-3} of fermentation capacity.[95]

The most commonly used bioreactor configurations reported for conversion of syngas to ethanol include conventional stirred tank bioreactors, bubble columns, and membrane reactors; their schematics are illustrated in Fig. 3. Maximum cell and product concentrations obtained in various bioreactor studies are summarized in Table 3.

Stirred tank bioreactor (STB)

It has so far been one of the most studied reactor configurations for ethanol production,[68,74] where the syngas or the gaseous substrate ultimately breaks into smaller bubbles, well dispersed in the liquid medium by the mechanical agitation caused by the rotating impeller. One way to increase the mass transfer of sparingly soluble gases like CO and H_2 is by increasing the impeller speed. Increasing the speed can increase the bubble break-up, but this requires a relatively high input of energy per unit volume. Consequently, this method of increasing the speed is not economically viable for large-scale production processes due to the excessive operational cost.

Continuous fermentation studies using a 750-mL stirred tank reactor without cell recycling, at different liquid dilution rates and yeast extract concentrations, resulted in achieving different cell densities and product distributions.[57] The highest ethanol concentration (2 g L^{-1}) and molar product ratio of 1.2 was achieved in that study at a dilution rate of 0.031 h^{-1} and at 0.01% yeast extract concentration. With cell recycling in a STB (13.5 L) using the strain *C. ljungdahlii*, maximum cell and ethanol concentrations of 4 and 48 g L^{-1} were achieved, respectively, after 560 h of continuous operation.[58] These values are much higher compared to any other ethanol production studies using syngas.[27,74] Recently, a successful installation and operation of a pilot scale fermentor (100 L) was reported by Kundiyana *et al.*[97] In that study, a 6-fold increase in ethanol production from syngas using *Clostridium* strain P11 was achieved by microbubble sparging.

Bubble column reactor (BCR)

BCRs are considered to be a potential alternative to the conventional STBs, in which mixing of gaseous substrates is achieved by gas sparging without mechanical agitation, and are considered to be economically viable in terms of saving energy costs. Some advantages of bubble columns include low capital and operational costs, lack of moving parts, and

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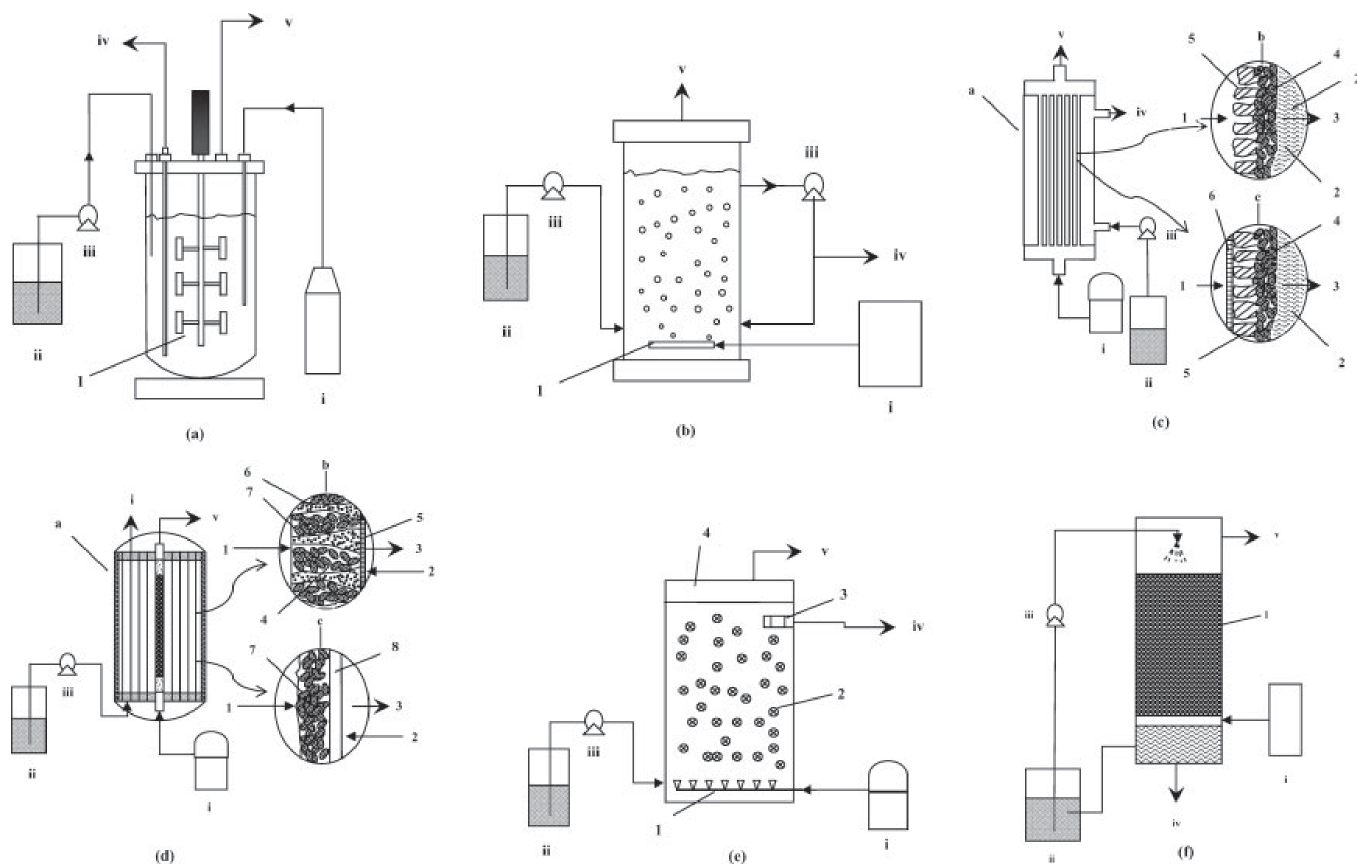

Figure 3. Schematic representation of various bioreactors for the conversion of syngas/CO-rich waste gas into ethanol. (**a**) Stirred tank bioreactor (STB): 1 – Agitator; (**b**) Bubble column reactor (BCR): 1 – Gas sparger; (**c**) Membrane bioreactor (MBR) with gas fed through the hollow fiber lumens while the liquid flows through the outer surface: a – Bioreactor vessel having plurality of membrane modules, b – Cross section of a microporous membrane present in modular membrane supported bioreactor (MMSB), c – Cross section of membrane present in membrane supported bioreactor (MSB), 1 – Gas inlet to the membrane, 2 – Liquid phase, 3 – Liquid products from the membrane, 4 – Microorganism (biofilm), 5 – Microporous membrane, 6 – Liquid impermeable layer; (**d**) Membrane bioreactor (MBR) with gas fed through the outer surface of the membrane fibers and the liquid flowing through the hollow fiber lumens: a – Bioreactor vessel having plurality of membrane modules, b – Cross section of an asymmetric membrane present in stacked array bioreactor (SAB) and horizontal array bioreactor (HAB), c – Cross section of an hydrophilic membrane having biofilm growth on the membrane surface, 1 – Gas inlet to the membrane, 2 – Medium inlet, 3 – Liquid products from the membrane, 4 – Biopores, 5 – Hydration layer, 6 – Biolayer, 7 – Microorganism (biofilm), 8 – Hydrophilic membrane; (**e**) Moving bed biofilm reactor (MBBR): 1 – Gas sparger, 2 – Biomass carrier, 3 – Carrier retainer, 4 – Gas recovery chamber; and (**f**) Trickling bed reactor (TBR): 1 – Packed bed. i – Gaseous feed into the reactor; ii – Nutrient feed into the reactor; iii – Pump; iv – Liquid products from the reactor and v – Gas outlet from the reactor.

Source: Figure (c) is adapted from Tsai *et al*.,[98] Hickey *et al*.,[99] and Datta *et al*.,[100] ; figure (d) from Tsai *et al*.,[101] Tsai *et al*.,[102] and Hickey *et al*.[104] and figure (e) from Hickey *et al*.[94]

satisfactory high heat and mass transfer rates. Increasing the flow rate for enhancing mixing will cause a heterogeneous flow to occur. Such a condition will eventually lead to back mixing of the gaseous components. Less research has been done using BCRs for ethanol production compared to STBs.

Ethanol production by *E. limosum* KIST612 using CO was carried out in a 200- mL bubble column reactor in batch and continuous mode by Chang *et al.*[75] In that study, a membrane module of pore size 0.2 μm was connected to the reactor for cell recycling. High ethanol yields were easily obtained from

Table 3. Ethanol production using various components of syngas in bioreactors.

Bioreactors	Organism	Culture elapsed time (d)	Culture volume (L)	Stirring speed (rpm)	Gas retention time (min)	Dilution rate (h^{-1})	Cell concentration ($g\ L^{-1}$)	Ethanol concentration ($g\ L^{-1}$)	Reference
STB	*C. ljungdahlii*	1	0.6[a]	1000	1.4	0.208	7.1	12	102
	C. carboxidivorans P7^T	17	3	400	18.75	0.0069	0.215[b]	0.75[b]	27
	Clostridium strain P11	59	70	150	77.78	NA	0.87	25.26	107
	B. methylotrophicum	9	1.25	50	25	0.015	0.286	0.056	68
	B. methylotrophicum	56	1.5[a]	200	NR	NR	9[b]	TA	95
	Moorella Sp. HUC22–1	220[c]	0.5	500	8.34	NA	0.28[b]	0.221	74
	C. carboxidivorans P7^T	100[c]	0.123	120	NR	NA	1.08	2	83
BCR	*E. limosum* KIST612	233[c]	0.2	NA	2.5	0.15	4.01	0.092	75
	C. carboxidivorans P7^T	10	4.5	NA	22.5	0.027	NR	0.16[d]	12
	C. carboxidivorans P7^T	20	4	NA	22.22	0.023	0.215[b]	2.75[b]	61
MBBR	*C. ragsdalei*	30	18000	NA	5.14	1.33	NR	30	94
MBR	*C. ragsdalei*	20	0.18[a]	NA	NR	NR	NR	15	116

Abbreviations: STB – Stirred tank bioreactor; BCR – Bubble column reactor; MBBR – Moving bed biofilm reactor; MBR – Membrane bioreactor with asymmetric hydrophilic membranes; TA – Trace amount; NA – Not applicable; NR – Not reported or not sufficient data to calculate.
[a]Reactor volume.
[b]Approximate values.
[c]Culture elapsed time in h.
[d]Ethanol concentration mentioned in wt%.
Note: Maximum cell concentration and maximum ethanol concentration reported during the respective studies is quoted here.

CO in a 4.5 L BCR, and these values were, respectively 6 and 2 times higher than for acetic acid and butanol for *C. carboxidivorans* P7^T.[12]

Membrane bioreactor (MBR)

Various membrane-based bioreactors have recently been studied and/or patented for the conversion of syngas to soluble products. In these bioreactors, microbial cells are attached to the membrane surface to form a biofilm, thereby achieving a high cell retention and high cell concentration.

Modular membrane supported bioreactors (MMSB), also known as submerged membrane supported bioreactors (SMSR), consist of plurality of membrane modules having either microporous or non-porous or composite membranes made into hollow fibers.[98,99] The syngas components are introduced into hollow fiber lumens and the biofilm containing micro-organisms is maintained on the outer surface, i.e. on the liquid contacting side of the membrane fibers. The process gas passes across the hollow fiber wall toward the biofilm, where the micro-organisms convert the gaseous substrates into ethanol, which is then mixed with the process liquids. Ethanol is recovered by using suitable recovery systems. One of the major disadvantages of this system is that the liquid may enter the pores owing to variation in pressure across the membrane, thus leading to a phenomenon known as pore-wetting. Performance of this type of membrane bioreactor to produce ethanol was studied and it was found that using microporous membranes with a biofilm of *C. ragsdalei* produced a concentration of 10 g L^{-1} after a 20-day continuous operation.[99]

Membrane supported bioreactors (MSB) comprise membranes having a microporous layer to support a biofilm at the liquid contacting side (outer surface), while the gas

contacting side (lumen) of the membrane is having a liquid impermeable layer, which may be a silicone coating.[100] To maintain a stable gas–liquid transfer in this system configuration, it is not necessary to maintain a very precise pressure difference across the membranes as required for systems having only microporous membranes. In addition, the impermeable layer provides higher gas transfer across the membrane than offered by a composite membrane. In MSB, a sandwiched type combination of membranes having a liquid impermeable layer between two microporous membranes was used as an alternative to the double layer construction. In one study with *C. ragsdalei* using this sandwiched type membranes, ethanol production increased to a maximum (13.3 g L^{-1}) and then ceased due to pore-wetting.[100]

The stacked array bioreactor (SAB) and the horizontal array bioreactor (HAB) make use of hydrophilic asymmetric membranes with biopores having an effective diameter greater than 1 μm.[101,102] As the name indicates, SAB consist of membrane modules in axially stacked arrangement, whereas in HAB, plurality of modules is arranged in a horizontal plane inside the bioreactor. Each membrane module consists of asymmetric membranes made into hollow fibers with a biolayer that retains micro-organisms on the outside and the hydration layer in contact with the liquid on the lumen side. Hollow fibers are packed to form membrane modules. The fermentation liquid flows through the inner side of the hollow fiber and permeates the biolayer. The syngas stream passes through the outer surface of the hollow fiber; contact with the immobilized cells inside the biopores is provided by the biolayer. The liquid products flow from the gas contacting side toward the lumen which is ultimately recovered from the process liquid. An approach to enhance the ethanol production is by periodically laving the biolayer by decreasing the pressure on the shell side relative to the lumen side of the asymmetric membrane module. This was studied by growing *C. ragsdalei* inside the biopores. Datta *et al.*[103] were able to enhance the ethanol production from 1.6 g L^{-1} to 4.2 g L^{-1} in that study.

In another membrane supported biofilm bioreactor, biofilm is retained on the biofilm exclusion surface present at the gas contacting side (outer surface) of the hydrophilic membrane.[104] This biofilm exclusion surface has a pore size not greater than 0.5 μm, preventing the biofilm from flowing across the membrane to the liquid contacting side. In one study, by using this approach, *C. ragsdalei* produced an ethanol concentration of 10 g L^{-1} after 20 days of continuous operation.[104]

Moving bed biofilm reactor (MBBR)

The moving bed biofilm reactor (MBBR) employs the state of the art of cell retention on an inert biomass carrier, promoting greater gas dissolution and utilization of syngas components by using eminent gas transfer systems.[94] The MBBR comprises (1) a vessel for maintaining the culture broth and liquid product; (2) a gas injection system for delivering syngas into the vessel and also for providing additional mixing by creating eddy currents in the surrounding liquid; (3) an inert biomass carrier for supporting microbial growth; and (4) a carrier retainer for hindering biomass carrier to flow out through the outlet. Gas bubbles rise through the fermentation broth and convert into liquid products using the microbes attached on the suspended carrier. By using a slot or jet gas transfer system, the necessary syngas pre-treatment step to remove small particulates can be avoided. Studies using an active culture of *C. ragsdalei* in a MBBR having a fermentor vessel of 36 m^3 reported an ethanol concentration of 30 g L^{-1} after 30 days of continuous operation.[94]

Trickling bed reactor (TBR)

The trickling bed reactor (TBR) or biotrickling filter (BTF) is a commonly used reactor design for various gas treatments.[105] Reactor packing material size, liquid recirculation rate, and gas flow rate are the main parameters which greatly influence the mass transfer rate in TBR.[92,106] In this columnar reactor, plug flow is most readily achieved. In a study to compare the performance of three different types of bioreactors for syngas fermentation, Klasson *et al.*[72] concluded that higher CO conversion rates (>80%) and higher productivities were achieved in a TBR than in a continuous STB and BCR. To our knowledge, no studies have been reported using this bioreactor for the fermentation of syngas to produce ethanol as one among the main products.

Product yield

A major advantage of microbial processes, as stated before, is the product specificity, yielding few byproducts and

increased process yield. To get high productivity and yield, the cell concentration in the bioreactor has to be high; this is achieved by either cell recycling or by cell retention. Membrane-based bioreactor systems have recently been used, wherein the biofilm grows and attaches to the surface of the membrane as a biopolymer matrix, thereby preventing cell washout.[94] As a fuel, ethanol is the most desired product of the syngas fermentation, while in most of the fermentation studies acetate productivity prevails over the ethanol production. Hence, in order to improve ethanol productivity or to increase the ethanol-to-acetate ratio, it is necessary to manipulate various fermentation parameters. Once a stable cell density is achieved, the following parameters can be adjusted individually or in combination to improve the ethanol productivity and to limit the acetic acid production: alteration of the medium constituents, liquid and gas feed rates, operating pH, temperature, pressure, and agitation rate or by providing excess H_2. By these ways, a reduction in redox potential and increased NADPH-to-NADP ratio in the fermentation broth is maintained, thereby promoting the reduction of acetic acid production compared to ethanol. Excess supply of H_2 means that ratio of H_2 fed to the sum of twice the CO converted and three times the CO_2 converted should be greater than 1 to promote ethanol production.[107] In a patented study using *C. ljungdahlii*, it was observed that the biological pathway is directed in favor of ethanol production and less acetic acid production by first feeding gaseous H_2 in excess and then limiting the calcium pantothenate and cobalt concentrations in the nutrient medium.[107] A doubling of ethanol concentration and reduction in acetate production in the fermentation broth was also reported when the iron concentration was increased 10-fold.[77] Hence medium optimization is a prerequisite to favor ethanol over acetate production.

Cell separation and ethanol recovery

Micro-organisms grow either in planktonic form, or as a biofilm on a solid matrix usually on membranes. Cell retention, and thereby an increase in cell density, is possible by the formation of a biofilm attached on a solid support in the bioreactor. Conversely, in suspended-growth reactors, cells grow in suspension and are separated from the product stream by employing solid/liquid separators, which includes membranous ultrafiltration units, hollow fibers, or spiral wound filtration systems or centrifuges.[108] Thus, the cells can return to the bioreactor.

The concentration of ethanol in the fermentation broth must be kept below a certain level in order to prevent microbial inhibition and to keep the cells metabolically active. Moreover, biomass-derived syngas fermentation usually produces low concentrations of ethanol (below 6%); hence, to economically recover ethanol, an efficient recovery process is required, which includes distillation followed by molecular sieve separation or pervaporation followed by dephlegmation technologies.[107,109] Integration of vacuum distillation columns and vapor permeation units has numerous advantages, such as amenability to separate ethanol from the fermentation broth even when ethanol concentration is as low as 1% where approximately 99% by weight of dehydrated ethanol can be recovered by this process.[110] Formation of toxic byproducts due to high temperature can be precluded, since vacuum distillation does not require high temperature. Hence, the majority of the distillation column bottoms can be recycled to the fermentor without any prior treatment. Another approach to enhance the concentration of ethanol in the feed to the vacuum distillation column is by flashing the feed before it enters the vacuum distillation column.[110] Coskata Inc., Illinois uses a licensed membrane separation technology to separate the ethanol from water; thereby a reduction of 50% in energy requirement has been achieved compared to conventional distillation (www.coskata.com).

Survey on syngas bioconversion to ethanol in industry

Gasification of biomass followed by syngas fermentation to produce bioethanol is a developing technology. Very few companies have scaled up bioconversion technology at pilot scale. Coskata Inc., a US bioethanol company, developed bioethanol, known as FlexEthanol^{TM} from biomass derived syngas via biofermentation. The proprietary process produces approximately 100 gallons of ethanol per ton of dry input material. A study by Argonne National Laboratory, Illinois has determined that Coskata's process can achieve a net energy balance of 7.7 and offer up to 80–90% reduction in lifecycle greenhouse gas emissions when compared to conventional gasoline. Its technology has been scaled

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up to a semi-commercial-scale plant located in Madison, Pennsylvania and the first commercial-scale plant will start operation by 2011. A New-Zealand-based clean technology company, LanzaTech, uses proprietary bacteria to convert industrial waste gases, i.e. mainly off-gas from steel industries, or biomass syngas into high octane premium fuel (www.lanzatech.co.nz). Using its proprietary technology, a pilot plant has been commissioned in 2008 at BlueScope steel plant, Glenbrook, to produce ethanol from steel mill flue gases. LanzaTech uses low-cost media as the sole fermentation media component and the process has been carried out with minimum waste gas conditioning. INEOS Bio, a UK/US-based bioenergy company uses a proprietary bioconversion process for converting a wide range of organic wastes, including household and commercial wastes into bioethanol (www.ineosbio.com). INEOS's bio pilot-scale facility in Fayetteville, Arkansas, has been in operation since 2003.

Challenges and R&D needs for commercialization of bioethanol production using gas fermentation

Feedstock

The feedstock for syngas production encompasses a wide spectrum of biomass materials, such as forest residues, agricultural and organic solid wastes, amongst others. Feedstock properties, for example, a high moisture content, have a negative influence on the CO fraction produced in the gasifier. In such cases, considerable energy is required for drying the biomass in order to keep the moisture content around 10–15%.[111] Every biomass contains ash and volatile compounds; the content varies from one feedstock to another. For instance, ash content in rice husk is about 15–25%, whereas in wood it is 2% or less.[17] Gasification of such feedstock produces impurities that inhibit the syngas fermentation. Thus extensive gas-cleaning steps are required prior to feeding into the bioreactor, which substantially increases the overall production cost. However, the nitrogen and alkali contents of the biomass can be greatly reduced by upstream treatments, such as fractionation and leaching.[16] It is quite obvious that an appropriate feedstock requires less pre-treatment and results in less syngas contaminant production, making ethanol production a process consuming less energy.

Gasification system and syngas purity

Various impurities are produced during gasification of biomass along with CO and H_2 which may cause problems in the subsequent bioconversion steps. The composition of the gas produced in the gasifier is greatly influenced by the gasifier configuration and the operating conditions. The equipment size can be decreased by feeding the gasifier with pure oxygen. But it will increase the overall cost of the process. The pyrolysis of volatile compounds releases tars, which not only affects the microbial activity during syngas fermentation but also gets deposited on the walls of the gasifier and gas transfer system, which ultimately decreases the performance of the gasifier. Using light hydrocarbons, the tar produced during the gasification can be substantially converted to syngas. About 90% of the tar generated in the gasifier is able to crack by this way.[17] On the other hand, the feasibility of using light hydrocarbons derived from renewable energy sources and subsequent use of the produced syngas for microbial utilization to biofuels have yet to be explored.

Micro-organisms and media composition

Isolation of high yielding (>25 g L^{-1}) ethanologenic homoacetogens, which have greater tolerance to high ethanol concentrations in the fermentation broth, is necessary for successful commercialization of syngas fermentation. Moreover, culturing of anaerobic micro-organisms requires specialized techniques to maintain the system under oxygen-free conditions. Thermophilic micro-organisms having the above features might be interesting since less cooling of syngas would be required prior to feeding the bioreactor and an elevated temperature can improve the conversion rate. Another task is to enhance the ethanol production by modifying metabolically the available syngas fermenting microbes through genetic engineering.

There are many factors to be considered while selecting fermentation media for large-scale ethanol production such as, but not limited to, media complexity, cost, or presence of chemicals that could improve ethanol productivity. Identifying unique media for specific micro-organisms which satisfy the above features is one of the important challenges faced by ethanol producers. Recently, it was reported that cotton seed extract (CSE) can be used as the sole fermentation medium for culturing *C. ragsdalei* P11 for ethanol production.[112]

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Mass transfer and scale-up

As discussed before, one of the main challenges faced during syngas fermentation is the gas–liquid mass transfer resistance. Various techniques to improve mass transfer of the syngas in STR have been discussed elsewhere.[91,92] For commercial-scale bioreactors, however, more efficient and economical mass transfer systems have to be found.

For scale-up, a clear understanding and estimation of the volumetric mass transfer coefficient (K_La) is required. The achievement of a high syngas mass transfer rate with minimal power consumption and relatively low shear rates, whilst maintaining an anaerobic atmosphere, is a major challenge for syngas fermentation scale-up. More research is still necessary for syngas fermentation scale-up.

Product recovery

The low microbial resistance to ethanol in the fermentation broth is one major obstacle in developing this technology. Furthermore, the fermentation broth also contains other dissolved and undissolved compounds, such as cell extracts and unfermented soluble compounds, which also create separation problems during ethanol recovery. For these reasons, *in situ* ethanol separation is considered a better choice by coupling the fermentor vessel with various unit operations.[110] Novel separation systems have still to be tested to overcome these challenges and thus increasing ethanol volumetric productivity.

Production costs

There are various parameters affecting the techno-economics of syngas fermentation. For instance, the cost of different feedstock regulates the overall production costs. In one recently published report, feedstock cost was shown to account for about 67% of the total production costs, even when dry biomass wood was used, without considering the depreciation factor.[111] Besides feedstock, the need to maintain the selected pure biocatalyst can also have a sizable impact on the production costs. Xia and Wiesner[113] compared the production costs involving two micro-organisms, and pointed out that, out of the two acetogens chosen, *C. ljungdahlii* showed better ethanol yield with production costs much lower than for *Moorella sp.* HUC22-1, excluding the operational cost and depreciation terms. This was attributed to the high ethanol production over acetate (3:1) of *C. ljungdahlii* over *Moorella sp.* HUC22-1 (1:28).

Although producing ethanol using syngas fermentation demands substantially less energy input, process modification and optimization steps are still at the development stage in order to achieve remarkably high process yields.[11,111] From a literature viewpoint, very few studies have undertaken a systematic evaluation of the techno-economics involved in the syngas fermentation process, and more detailed studies relating the costs to mass-energy balances, flow sheet modeling, and life cycle assessment should be initiated in order to obtain a valuable database.

Conclusions

Bioethanol production from biomass as well as from CO-rich waste gases or syngas fermentation is potentially viable. The presence of specific impurities, NO and acetylene, in syngas can have a severe antagonistic effect on the enzyme activity and its conversion pathway, and advanced filtration systems can be used as a pre-treatment step to remove these impurities. Literature reports on gene manipulation in the syngas fermenting microbes have been initiated only very recently. Alteration in the properties, such as ethanol tolerance level and production rate at the gene level, by recombinant DNA technology could improve the overall performance of this technology. The use of membrane-based bioreactors for syngas fermentation offers several advantages, in terms of providing a large surface area for both gas–liquid mass transfer and cell attachment, over conventional bioreactors. Yet, there is also a need to develop and evaluate hybrid and multistage bioreactor configurations keeping in view the low aqueous solubility of the syngas components and required high ethanol productivity. A systematic improvement, through retro-fitting and implementation of current technologies in these industries would guarantee investors and financial providers to reach their business goals without making risky investments.

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Biological conversion of carbon monoxide to ethanol: Effect of pH, gas pressure, reducing agent and yeast extract

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ABSTRACT

A two-level full factorial design was carried out in order to investigate the effect of four factors on the bioconversion of carbon monoxide to ethanol and acetic acid by *Clostridium autoethanogenum*: initial pH (4.75–5.75), initial total pressure (0.8–1.6 bar), cysteine–HCl·H_2O concentration (0.5–1.2 g/L) and yeast extract concentration (0.6–1.6 g/L). The maximum ethanol production was enhanced up to 200% when lowering the pH and amount yeast extract from 5.75 to 4.75 g/L and 1.6 to 0.6 g/L, respectively. The regression coefficient, regression model and analysis of variance (ANOVA) were obtained using MINITAB 16 software for ethanol, acetic acid and biomass. For ethanol, it was observed that all the main effects and the interaction effects were found statistically significant ($p < 0.05$). The comparison between the experimental and the predicted values was found to be very satisfactory, indicating the suitability of the predicted model.

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1. Introduction

Biological conversion of waste gases containing carbon monoxide (CO) using acetogens offers a possibility through which waste can be efficiently utilized for generating valuable fuels like ethanol, butanol and hydrogen (Mohammadi et al., 2011; Munasinghe and Khanal, 2010). Different bioreactors can be used for (waste) gas treatment or bioconversion (Abubackar et al., 2011a; Kennes et al., 2009). However, one major bottleneck for the commercialization of this technique is the poor aqueous solubility of carbon monoxide gas. Hence, for systems containing CO as sole substrate, the bioconversion process is limited by the CO gas–liquid mass transfer at high cell concentration. Besides, the process is kinetically limited when either the cell concentration or the CO consumption rate is too low (Abubackar et al., 2011a). These rate-limiting conditions would decrease the process yield and CO–bioconversion process and are often encountered at some point in the bioconversion.

Homoacetogens able to produce ethanol from carbon monoxide include *Clostridium ljungdahlii*, *Clostridium carboxidivorans* P7^T, *Clostridium ragsdalei*, *Alkalibaculum bacchi*, *C. autoethanogenum*, *Clostridium drakei*, and *Butyribacterium methylotrophicum*, among others (Liu et al., 2012 ; Mohammadi et al., 2011, 2012). These unicarbonotrophic bacteria follow the acetyl-CoA biochemical pathway or Wood-Ljungdahl pathway for cell growth and product formation (Abubackar et al., 2011a). Apart from ethanol, acetic acid is one of the prominent metabolites found during CO conversion using these microorganisms. In most of the previous studies, low ethanol to acetic acid ratios were generally obtained. However, by optimizing the medium composition and operating conditions, this ratio can be increased (Kundiyana et al., 2011a,b). In the present research, a microcosm study was performed using *C. autoethanogenum* as the biocatalyst.

C. autoethanogenum is a strictly anaerobic gram positive rod shaped (0.5 × 3.2 μm) bacterium, originally isolated from rabbit faeces using CO as the sole carbon and energy source. (Abrini et al., 1994). In one study, the authors used Plackett–Burman design to screen significant ethanol enhancing factors from the defined medium developed for *C. carboxidivorans*. Optimal levels of these significant factors were evaluated by central composite design (CCD) using a response surface methodology (RSM) and an artificial neural network-genetic algorithm (ANN-GA). It was concluded that an optimal medium containing (g/L) NaCl 1.0, KH_2PO_4 0.1, $CaCl_2$ 0.02, yeast extract 0.15, $MgSO_4$ 0.116 and NH_4Cl 1.694, at pH 4.74 could yield an ethanol concentration of around 0.25 g/L (Guo et al., 2010). Another research reported a concentration of 0.06–0.07 g/L with a 1:13 ethanol to acetate ratio in liquid-batch continuous syngas fermentation using a xylose adapted *C. autoethanogenum* culture (Cotter et al., 2009). These studies reveal the importance of medium composition in increasing the overall ethanol production. Hence, the different operating conditions still have to be optimized in order to enhance ethanol production and save on operating costs.

In the present research, *C. autoethanogenum* was used to convert bottled carbon monoxide gas into a valuable fuel product such as

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ethanol, and to investigate the effect of various process parameters on the bioconversion process, such as the initial pH, initial total pressure, cysteine–HCl·H_2O concentration and yeast extract concentration, and to obtain a reduced regression model that describes the process for products and biomass using a 2^4 full factorial design. In this manuscript, the authors simply called initial total pressure, cysteine–HCl·H_2O and yeast extract as "pressure", "cysteine–HCl" and "YE", respectively and in the tables and figures, initial pH as simply "pH".

2. Methods

2.1. Microorganism and medium composition

C. autoethanogenum DSM 10061 was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), and was grown and maintained on DSMZ medium 640 with 0.5% xylose. The medium was prepared by boiling for a few minutes, while being degassed, and then cooled continuously under N_2 for 15 min to remove oxygen. Cysteine–HCl was added, and the pH of the medium was adjusted to 6.0, by adding a small volume of either 2 M HCl or 2 M NaOH solutions.

2.2. Bioconversion studies

For batch experiments, serum vials with a total volume of 200 mL were used, with 75 mL working volume. The experimental set-up and the method used for media preparation are described elsewhere (Abubackar et al., 2011b). The culture was maintained under anaerobic conditions and agitated at 150 rpm on an orbital shaker, inside an incubation chamber at 30 °C. 10% of actively growing culture, which was grown with CO as sole substrate, was used as the inoculum and was aseptically transferred to each experimental vial. Headspace samples of 0.2 mL were used for CO measurements, and 1 mL of liquid sample was periodically withdrawn from the vials (once every 24 h) in order to measure the optical density ($OD_{\lambda=600\ nm}$) related to biomass concentration. The same 1 ml sample was then centrifuged for 10 min (25 °C, 7000×*g*) and the supernatant was used to check both ethanol and acetic acid concentrations.

2.3. Analytical equipment and measurement protocols

Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph equipped with a thermal conductivity detector. 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. The water-soluble products, acetic acid and ethanol, in the culture broth were analyzed using a HP-5890 Series II GC equipped with a flame ionization detector and a 0.25 mm (ID) × 30 m HP-INNOWax capillary column (Agilent Technologies, Forster, CA, USA). Helium was used as the carrier gas. The oven temperature was held at 80 °C for 2 min, then heated to 160 °C at a rate of 10 °C min^{-1}, and maintained thereafter at 160 °C for 1 min. The injector and detector temperatures were kept constant, at 220 and 260 °C, respectively. Cell mass was estimated by measuring sample absorbance at a wavelength of 600 nm using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The measured absorbance was then compared to the previously generated calibration curve, to calculate the corresponding biomass concentration (mg/L).

2.4. Experimental design and statistical analysis

A two level four factor (2^4) full factorial experimental design was used to study the combined effects of initial pH (low 4.75 and high 5.75), initial total pressure (low 0.8 bar and high 1.6 bar), cysteine–HCl·H_2O concentration (low 0.5 g/L and high 1.2 g/L) and yeast extract concentration (low 0.6 g/L and high1.6 g/L) on products formation (ethanol and acetic acid) and culture stability during the carbon monoxide bioconversion process by *C. autoethanogenum*. Of particular interest for optimizing ethanol production as a biofuel; this study was focused on estimating the optimum range of these parameters that enhances ethanol production.

The software package Minitab 16 (Minitab Inc. State College, PA, USA) was used to design the experiments and for data analysis in the form of analysis of variance (ANOVA). The response variables (*Y*) that were analyzed were the maximum products concentrations (g/L) and biomass concentration (mg/L) obtained from the different experimental trials.

3. Results and discussion

Some of the main parameters that affect the CO–bioconversion process are pH, mass transfer, reducing agent concentration and YE concentration (Mohammadi et al., 2011). The design matrix in uncoded values and the observed and predicted values of the responses are presented in Table 1. Three experiments were performed at central points in replication for an estimation of the variance (experimental error) of an effect. Using the least square technique with Minitab, the individual and interaction effects of the parameters can be approximated to a linear regression model. For 95% confidence level, the *p*-value, the probability value that is used to determine the statistical significance of the effects in the model should be less than or equal to 0.05 for the effect to be statistically significant.

3.1. Main effects plot

Fig. 1 shows the main effects plot for the responses. From the main effects plot for ethanol, it is observed that increasing the initial pH and higher YE concentrations had a negative effect on ethanol production, whereas increasing initial pressure and cysteine–HCl concentration had a positive effect. These fermentation results are consistent with the trend observed in some other CO–bioconversion studies suggesting that lowering the pH and YE concentration results in the production of more reduced compounds such as ethanol (Barik et al., 1988; Phillips et al., 1993). The product spectrum shifted from acidogenic to solventogenic phase when lowering the medium's pH. This was proposed to be due to the following reason: the product, acetic acid, is a lipophilic weak acid and thus permeates through the cell membranes, resulting in a decrease in internal pH due to the conduction of H^+ ions from inside. At low internal pH, the external pH plays a major role in keeping the cell under non-stressed condition (Mohammadi et al., 2011). Hence, at both low external and internal pH, the cells under stress condition overcome the situation by producing solvents. Eliminating YE was found to enhance the ethanol production using *C. ljungdahlii* (Barik et al., 1988). However, for this organism to provide structural integrity, a minimum concentration of 0.01% is said to be necessary (Abubackar et al., 2011a). One potential bottleneck of CO–bioconversion is the mass transfer limitation due to the sparingly soluble nature of that substrate. Hence, one way to

Table 1
2^4 Factorial design of experiments for ethanol, acetic acid and biomass production in the study.

Run No.	pH	Pressure (Bar)	Cysteine–HCl (g/L)	YE (g/L)	Ethanol production (g/L)		Acetic acid production (g/L)		Biomass production (mg/L)	
					Observed	Predicted	Observed	Predicted	Observed	Predicted
1	4.75	0.8	0.5	0.6	0.115677	0.11568	0.930341	0.9428	141.9	145.99
2	5.75	0.8	0.5	0.6	0.072725	0.07272	0.933748	0.9462	159.02	144.69
3	4.75	1.6	0.5	0.6	0.278010	0.27802	1.950899	1.9558	259.63	253.25
4	5.75	1.6	0.5	0.6	0.080230	0.08026	2.145072	2.15	291.13	302.27
5	4.75	0.8	1.2	0.6	0.141760	0.14176	0.848527	0.8434	161.47	154.47
6	5.75	0.8	1.2	0.6	0.095745	0.09576	1.238078	1.2328	172.17	153.17
7	4.75	1.6	1.2	0.6	0.649213	0.64922	1.66778	1.6552	187.78	216.77
8	5.75	1.6	1.2	0.6	0.090824	0.09082	2.040535	2.0282	263.30	265.79
9	4.75	0.8	0.5	1.6	0.106121	0.10612	0.999329	0.987	175.84	189.71
10	5.75	0.8	0.5	1.6	0.089211	0.0892	1.132418	1.12	221.10	238.73
11	4.75	1.6	0.5	1.6	0.192213	0.19222	2.220619	2.2156	326.00	302.33
12	5.75	1.6	0.5	1.6	0.077787	0.07778	2.330991	2.3258	303.36	301.03
13	4.75	0.8	1.2	1.6	0.155645	0.15564	1.270428	1.2756	153.52	153.23
14	5.75	0.8	1.2	1.6	0.070136	0.07016	1.231282	1.2362	197.25	202.25
15	4.75	1.6	1.2	1.6	0.070065	0.07006	2.521777	2.5342	320.49	310.81
16	5.75	1.6	1.2	1.6	0.130568	0.13058	1.354848	1.3672	310.09	309.51

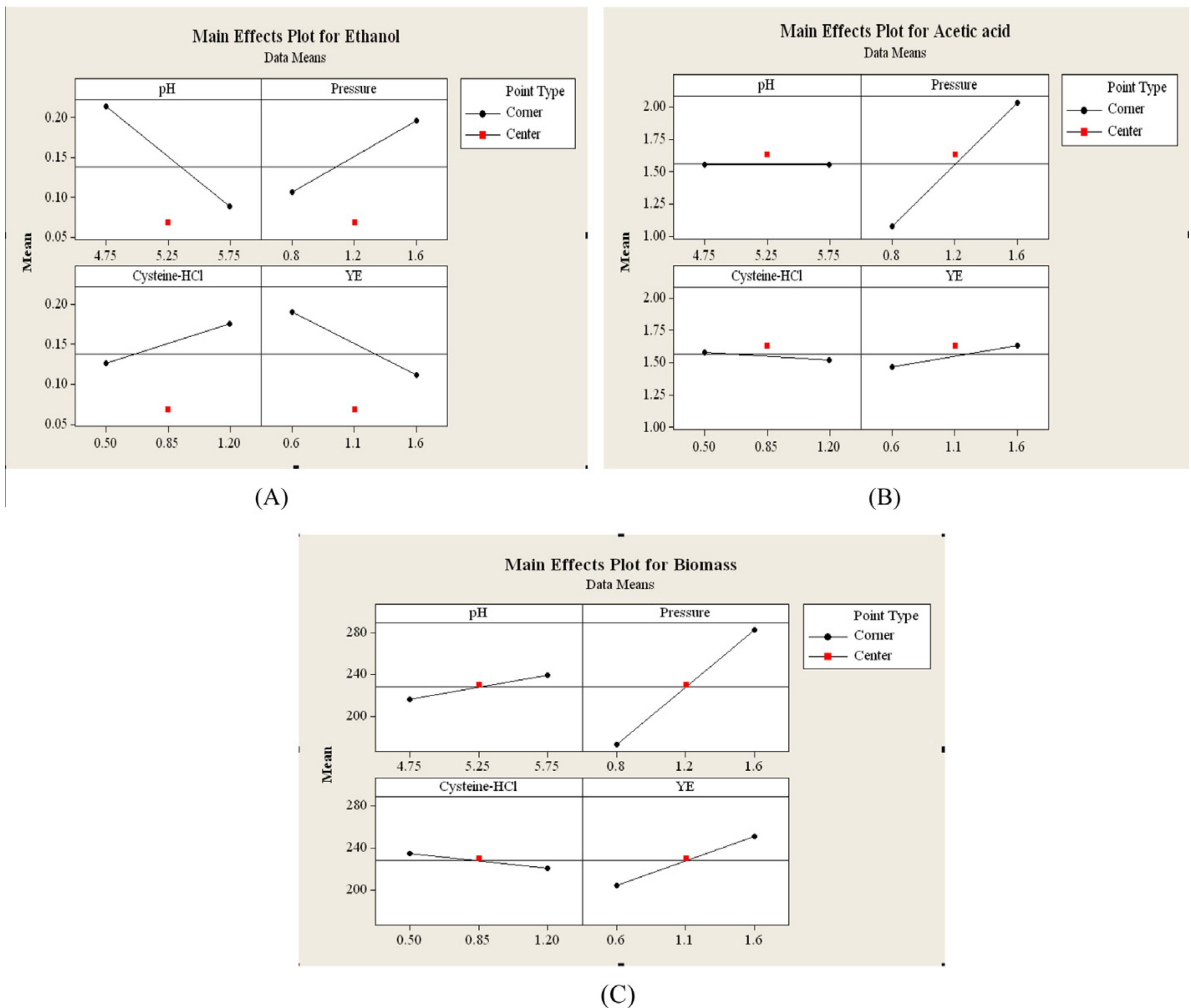

Fig. 1. Main effects plot for (A) Ethanol, (B) Acetic acid and (C) Biomass.

overcome this limitation is by increasing the pressure. In batch fermentation, different CO pressures mean different gaseous substrate concentrations which are directly proportional to the metabolite production and cell density. It was also observed that addition of reducing agents, thereby providing more electrons into the culture medium, will shift the microbial metabolism towards solventogenesis. This occurs due to availability of more reducing equivalents for the conversion of acetyl-CoA to products.

For acetic acid, it is evident that pH does not exert any effect on acetic acid production. Cysteine–HCl showed only a slight change in response across the studied level. This result is fairly consistent with the observation of Sim and Kamaruddin (2008), who studied the effect of cysteine–HCl on acetic acid production with *Clostridium aceticum* in a range of 0.1–0.5 g/L and found that the cysteine–HCl concentration was less significant. YE had a slightly positive effect on acetic acid production at high concentration. This may be due to the high cell growth achieved at increasing concentrations of yeast extract. Moreover, it has been reported that acetic acid is a growth-related product (Barik et al., 1988).

From the main effect plot for biomass, it is obvious that out of the four parameters studied, only increases in cysteine–HCl showed a slightly negative effect on biomass growth, whereas, increasing the other three factors had a strong positive influence on biomass. Since any organism shows its highest metabolic activity at its optimum pH, stepping down or stepping up in pH has a negative impact on cell growth. The optimum pH for growth of *C. autoethanogenum* is between 5.8 and 6.0 (Abrini et al., 1994). Hence, cell density increases proportionally when the pH is increased from 4.75 to 5.75. The reducing agent, cysteine–HCl, is essential for lowering the redox potential of the growth medium by scavenging the oxygen. However, a high amount of reducing agent is detrimental for cell growth and leads to a lower cell concentration (Sim and Kamaruddin, 2008). As YE provides nutrients for cell metabolism, an increase in the amount YE therefore increases the cell concentration.

3.2. Interaction effects plot

The interaction effects plots are shown in Fig. 2 and represent the mean response at all possible combinations of each two factors studied. If the two lines are non-parallel, it is an indication of interaction between the two factors.

The interaction plot for ethanol showed that there is a strong interaction between each two factors. Whereas for acetic acid, only

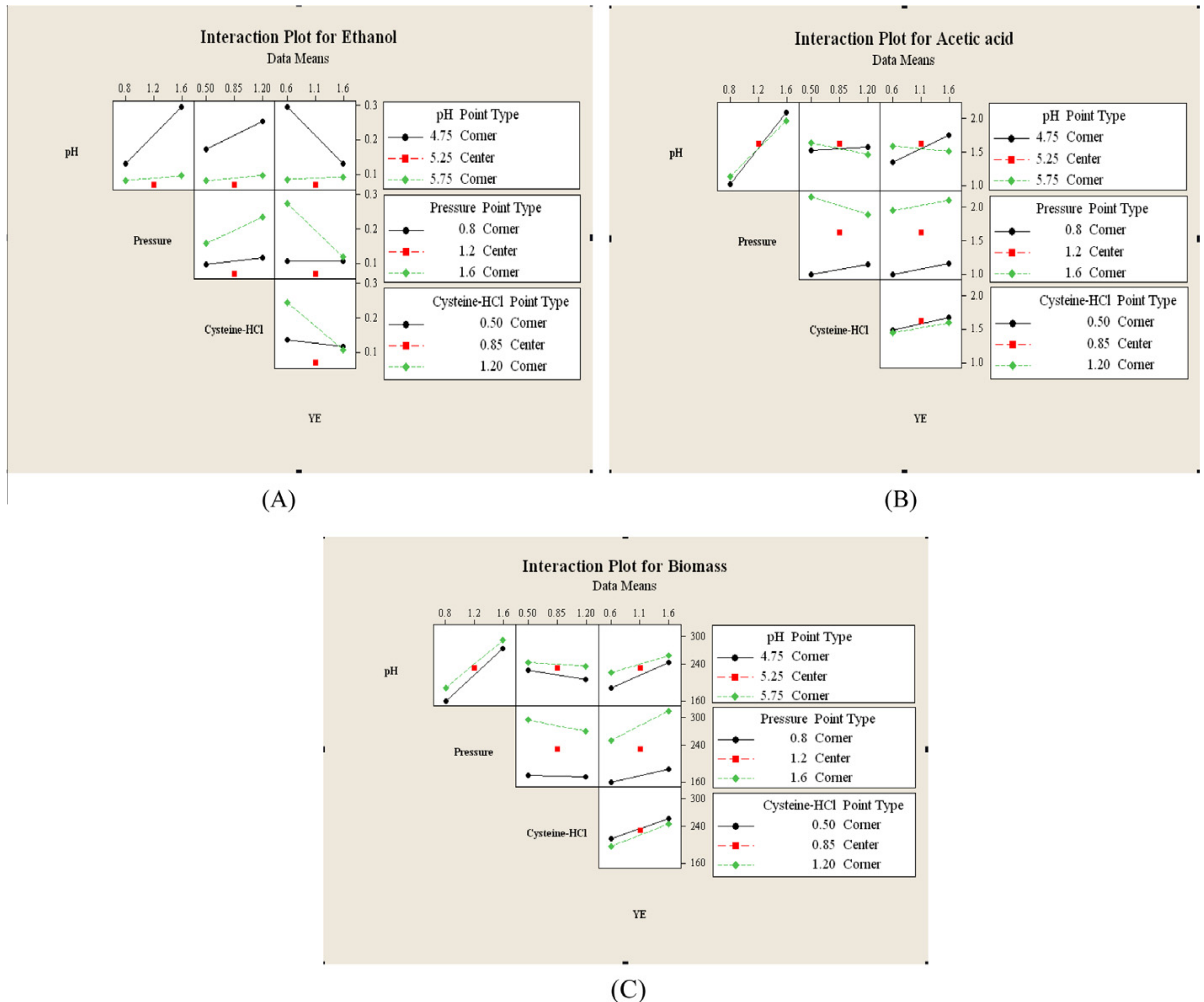

Fig. 2. Interaction effects plots for (A) Ethanol, (B) Acetic acid and (C) Biomass.

minor interactions were observed for YE with pressure and with cysteine–HCl. Also, no remarkable interactions between the pairs of factors were seen for biomass production. When the initial medium pH was 5.75, the maximum ethanol production was close to 0.1 g/L, same at low and high level of each other factors, describing the importance of low initial medium pH for increasing ethanol production. It is possible that higher amounts of carbon substrate are channeled towards the cell mass at high (+) level of pH. A higher amount of ethanol was observed at a pressure of 1.6 bar, for both concentrations of cysteine–HCl and YE, than at a pressure of 0.8 bar. A high amount of ethanol was also found to be produced for a higher cysteine–HCl concentration of 1.2 g/L at both levels of each other factors. In fact a slight reduction in ethanol production was observed at a YE concentration of 1.6 g/L compared to ethanol produced for a cysteine–HCl concentration of 0.5 g/L at 1.6 g/L of yeast extract.

At high (+) YE concentration level, an increase in pressure from 0.8 to 1.6 bars leads only to a very minor improvement in ethanol production and increases significantly acetic acid and biomass concentrations, showing the importance of lowering the YE concentration for improving ethanol production. Even though growth ceases at low pH and low YE concentration, it can easily be observed from the interaction plot that there is around 200% improvement in ethanol production under such condition. Interaction between total pressure and cysteine–HCl, at their highest concentrations, has a positive influence on ethanol production and a negative effect on both acetic acid and biomass formation. Also, at low pressure, an increase in cysteine–HCl concentration does not make any major difference in their production. This can easily be interpreted by the fact that at a higher pressure, resulting in more supply of carbon substrate, an increment in reducing agent allows the microbes to use the additional carbon for producing highly reduced products.

3.3. Regression analysis and prediction of regression model

The statistical software was used to evaluate the observed experimental results to derive a regression function by using an ordinary least square method. Regression results determine the statistical significance, direction and magnitude of the relationship between an effect and the response. The sign of each regression coefficient indicates the direction of the relationship. Only the effects with low *p*-values are said to be statistically significant and can be meaningfully utilized in obtaining the regression function or model (Montgomery, 2005). A comparison between experimental values and the predicted values obtained using the regression equation is performed and satisfactory correlation was found between these values ($R^2 > 0.9$).

The regression models proposed are as follows:

Maximum ethanol production = $0.15100 - 0.06259\,A + 0.04512\,B + 0.02450\,C - 0.03953\,D - 0.03867\,AB - 0.01608\,AC + 0.04305\,AD + 0.01455\,BC - 0.03893\,BD - 0.02936\,CD - 0.00713\,ABC + 0.04473\,ABD + 0.02938\,ACD - 0.02703\,BCD + 0.03757\,ABCD$.

Maximum acetic acid production = $1.5510 - 0.0002\,A + 0.4780\,B - 0.0294\,C + 0.0817\,D - 0.0610\,AB - 0.0553\,AC - 0.1202\,AD - 0.1034\,BC - 0.0820\,ABC - 0.0828\,ABD - 0.1259\,ACD - 0.0272\,BCD - 0.0561\,ABCD$.

Maximum biomass production = $227.75 + 11.93\,A + 54.97\,B - 7.00\,C + 23.20\,D - 12.58\,ABD + 11.24\,BCD$.

These regression models are confined for each variable within the following range: (*A*) initial pH = 4.75–5.75, (*B*) pressure = 0.8–1.6 bars, (*C*) cysteine–HCl = 0.5–1.2 g/L and (*D*) YE = 0.6–1.6 g/L.

4. Conclusion

In this experimental range, higher ethanol production was favored by a lower pH and YE concentration and a higher pressure and cysteine–HCl concentration. A maximum ethanol concentration of 0.65 g/L was obtained under the following conditions: pH = 4.75 (the lowest value tested), pressure = 1.6 bar (the highest value tested), cysteine–HCl = 1.2 g/L (the highest value tested), and YE concentration = 0.6 g/L (the lowest value tested). Such maximum ethanol concentration is considerably higher than that achieved (0.06 and 0.25 g/L) with *C. autoethanogenum* in previous studies (Cotter et al., 2009; Guo et al., 2010).

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Article

Ethanol and Acetic Acid Production from Carbon Monoxide in a *Clostridium* Strain in Batch and Continuous Gas-Fed Bioreactors

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Abstract: The effect of different sources of nitrogen as well as their concentrations on the bioconversion of carbon monoxide to metabolic products such as acetic acid and ethanol by *Clostridium autoethanogenum* was studied. In a first set of assays, under batch conditions, either NH_4Cl, trypticase soy broth or yeast extract (YE) were used as sources of nitrogen. The use of YE was found statistically significant ($p < 0.05$) on the product spectrum in such batch assays. In another set of experiments, three bioreactors were operated with continuous CO supply, in order to estimate the effect of running conditions on products and biomass formation. The bioreactors were operated under different conditions, *i.e.*, EXP1 (pH = 5.75, YE 1g/L), EXP2 (pH = 4.75, YE 1 g/L) and EXP3 (pH = 5.75, YE 0.2 g/L). When compared to EXP2 and EXP3, it was found that EXP1 yielded the maximum biomass accumulation (302.4 mg/L) and products concentrations, *i.e.*, acetic acid (2147.1 mg/L) and ethanol (352.6 mg/L). This can be attributed to the fact that the higher pH and higher YE concentration used in EXP1 stimulated cell growth and did, consequently, also enhance metabolite production. However, when ethanol is the desired end-product, as a biofuel, the lower pH used in EXP2 was more favourable for solventogenesis and yielded the highest ethanol/acetic acid ratio, reaching a value of 0.54.

Keywords: acetic acid; bioethanol; carbon monoxide; *Clostridium autoethanogenum*; syngas; waste gas

1. Introduction

Carbon monoxide (CO) is emitted in large amounts in the form of industrial waste gases generated during the incomplete combustion of carbon-containing materials. It is also a major component of synthesis gas [1]. Some anaerobic bacteria have the ability to grow on CO as their sole carbon source and metabolize it to a variety of fuels and chemicals [2,3]. These unicarbonotrophs ferment CO into acetyl-CoA, via the acetyl-CoA pathway or Wood-Ljungdahl (WL) pathway, and later into metabolites such as acetic acid, ethanol, hydrogen, *n*-butanol or 2,3-butanediol. In the WL pathway, the net ATP gained by substrate level phosphorylation (SLP) is zero; hence, in order to make bacterial growth on CO possible, the WL pathway must be coupled to energy conservation [4,5]. However, the exact mechanisms involved in energy conservation remain still unclear. Very recently, metabolically engineered acetogens have been used to selectively produce metabolites from CO [6,7], although it is also possible to produce specific metabolites of interest from CO, in wild type bacteria, through manipulation of the medium composition and/or operating conditions in bioreactors [8,9]. Several acetogens are known to produce acetic acid, as major end metabolite, from CO, including *Moorella thermoacetica*, *Acetobacterium woodii*, *Eubacterium limosum* KIST 612, *Peptostreptococcus productus* U-1 and *Clostridium aceticum* [3]; whereas *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Clostridium ragsdalei* and *Alkalibaculum bacchi* are ethanologenic acetogens, able to produce ethanol besides acetic acid [2,3]. Recently *Clostridium ljungdahlii*, *Clostridium autoethanogenum* and *Clostridium ragsdalei* were found to produce 2,3-butanediol and lactic acid as well [10].

In the present work, biological conversion of CO was studied, using *C. autoethanogenum*, in order to produce various metabolites. In most of the CO bioconversion studies to ethanol, co-production of large amounts of acetic acid was observed. Although ethanol is an interesting metabolite as a biofuel, products such as acetic acid have many industrial applications as well, as the key raw material for the manufacture of vinyl acetate monomer, acetic anhydride and acetate esters such as ethyl acetate, *n*-butyl acetate and isopropyl acetate [11]. Similarly, 2,3-butanediol is another possible by-product, with potential applications in manufacturing industries, such as in the production of food, pharmaceuticals, printing inks, perfumes, fumigants, synthetic rubbers, octane boosters, or plasticizers. Three stereoisomers of 2,3-butanediol exist, comprising the optically active dextro-[L-(+)-] and levo-[D-(−)-] forms and the optically inactive *meso*-form. It has been reported that *C. autoethanogenum* can produce 2,3-butanediol in the form of D(−)-2,3-butanediol (96%) and *meso*-2,3-butanediol (4%) [10]. This anaerobic biological route of production of chemicals such as ethanol, acetic acid and 2,3-butanediol from CO is an extremely attractive alternative compared to the traditional chemical route and other biorefinery processes [3].

Microorganisms require nitrogen for their structural integrity as well as for proteins, and optimization of their concentrations in culture media could improve the productivity of the process and reduce the medium's cost. In some of our previous batch studies, it was found that the nature and the concentration of metabolites produced from CO depend on the composition of the culture medium as well as on other

experimental conditions such as pH and pressure, among others [12]. Guo *et al*. observed that an optimized medium containing (g/L) NaCl 1.0, KH_2PO_4 0.1, $CaCl_2$ 0.02, yeast extract 0.15, $MgSO_4$ 0.116 and NH_4Cl 1.694, at pH = 4.74 could yield an ethanol concentration of around 0.25 g/L using *C. autoethanogenum* in microcosm studies [13]. Some previous study was done to evaluate the sensitivity of growth and product formation to nitrogen sources and their concentration in clostridia [14]. However, xylose was used as the carbon substrate in that study rather than CO. This prompted us to carry-out the present studies with CO, as the xylose fermentation by acetogens exhibits some differences and does also involve the glycolysis and oxidation of pyruvate to acetyl-CoA in addition to the WL pathway. Besides, the few previous studies aimed at estimating the effect of the medium's composition on bacterial growth and production of metabolites in clostridia were generally done in batch assays, in bottles, with no pH regulation. In the present research, bioreactors operated at constant pH, with continuous CO supply, were used. This is a relevant aspect as both pH and the medium's composition affect the metabolism and growth pattern. When both parameters are allowed to vary, it becomes difficult to conclude which one is actually affecting more.

The purpose of this work was to investigate the effect of various sources of nitrogen on the bioconversion of CO to various metabolites, by *C. autoethanogenum,* in bottles as well as in continuous gas-fed bioreactors. In the present study, first, the influence of different sources of nitrogen (NH_4Cl, yeast extract and trypticase soy broth) were compared for their effect on growth and product formation. In the research described in this paper, acetic acid is the major end-product. The adequate selection of the medium and culture conditions would allow ethanol to become the major, or even single, end metabolite. First, the experiments were carried out in 200 mL serum vials using a 2^3 full factorial design. In the second part of the research the effect of individual sources of nitrogen on growth and metabolites production was studied. In the final part of the research, experiments were performed in laboratory-scale fermentors in continuous mode (continuous gas feed) applying results and conditions previously optimized in batch experiments.

2. Experimental Section

2.1. Microorganism

Clostridium autoethanogenum DSM 10061 was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), and was maintained on medium (pH = 6) with the following composition (per liter distilled water): NH_4Cl, 0.9 g; NaCl, 0.9 g; $MgCl_2 \cdot 6H_2O$, 0.4 g; KH_2PO_4, 0.75 g; K_2HPO_4, 1.5 g; $FeCl_3 \cdot 6H_2O$, 0.0025 g; trypticase peptone, 2.0 g; yeast extract (YE), 1.0 g; cysteine-HCl, 0.75 g; 0.1% resazurin, 0.5 mL; with 0.5% xylose and SL-10 solution, 1.0 mL. The trace metal stock solution SL-10 contained (per liter): 7.7 M HCl, 10 mL; $FeCl_2 \cdot 4H_2O$, 1.5 g; $ZnCl_2$, 70 mg; $MnCl_2 \cdot 4H_2O$, 100 mg; H_3BO_3, 6 mg; $CoCl_2 \cdot 2H_2O$, 190 mg; $CuCl_2 \cdot 2H_2O$, 2 mg; $NiCl_2 \cdot 6H_2O$, 24 mg; and $Na_2MoO_4 \cdot 2H_2O$, 36 mg. For the experimental studies, xylose was omitted from the medium.

2.2. Bioconversion Studies

2.2.1. Bottle Batch Experiments

A two level three factor (2^3) full factorial experimental design was used to study the combined effects of NH_4Cl (0.2–2 g/L), trypticase (0.2–2 g/L) and YE concentrations (0.1–1 g/L), as sources of nitrogen, on products formation and culture stability during carbon monoxide bioconversion by *C. autoethanogenum*. The software package Minitab 16 (Minitab Inc. State College, PA, USA) was used to design the experiments and for data analysis in the form of analysis of variance (ANOVA). Table 1 shows the design matrix obtained in uncoded values with the MINITAB software and the observed values of the responses obtained for each experiment as well as the final pH. Factorial design is an important statistical tool that allows to conclude the factors that are most influential in the bioconversion process by carrying out a limited number of experiments. Thus, a total of 18 experimental runs, including the replicate experiments at the central points, were carried out. The individual and interaction effects of the different parameters were studied using the least square technique with the help of a specific software.

Table 1. 2^3 Factorial design table of experiments and responses.

Run No	NH_4Cl	Trypticase	YE	Ethanol (g/L)	Acetic Acid (g/L)	Biomass (mg/L)	Final pH
1	0.2	0.2	0.10	0.1733	1.806	152.29	3.88
2	2.0	0.2	0.10	0.3032	1.560	142.81	3.84
3	0.2	2.0	0.10	0.2290	1.855	222.7	4.03
4	2.0	2.0	0.10	0.1959	1.663	244.49	4.00
5	0.2	0.2	1.00	0.0883	2.146	302.90	3.91
6	2.0	0.2	1.00	0.1048	2.101	294.80	3.84
7	0.2	2.0	1.00	0.1061	2.339	335.62	3.93
8	2.0	2.0	1.00	0.1101	2.226	320.03	3.94

For batch experiments, 10% of actively growing seed culture, grown with CO as sole carbon source, was aseptically transferred into 200 mL serum vials containing 75 mL medium at pH = 6. The medium contained (per liter distilled water): NaCl, 0.9 g; $MgCl_2 \cdot 6H_2O$, 0.4 g; KH_2PO_4, 0.75 g; K_2HPO_4, 1.5 g; $FeCl_3 \cdot 6H_2O$, 0.0025 g; 0.1% resazurin, 0.5 mL; and SL-10 solution, 1.0 mL. NH_4Cl, YE or trypticase were added in the same vials as per the experimental design (Table 1). In order to remove oxygen, the medium was boiled and flushed with N_2. After cooling, 0.75 g cysteine-HCl, was added as reducing agent, and the pH was adjusted to 6 using aqueous solutions of either 2 M HCl or 2 M NaOH. The bottles were then sealed with Viton stoppers and capped with aluminum crimps before autoclaving for 20 min at 121 °C. The experimental set-up and the method used for media preparation are described elsewhere [15]. 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 30 °C. Headspace samples of 0.2 mL were used for CO measurements, and 1 mL liquid sample was periodically withdrawn from the vials, once every 24 h, in order to measure the optical density ($OD_{\lambda = 600 \text{ nm}}$), which is directly related to the biomass concentration. Afterwards, that same 1 mL sample was filtered using a 0.22 µm PTFE syringe-filter and was used to check the concentrations of soluble products. All the bioconversion experiments were conducted in duplicate, reaching statistically highly reproducible results. The response variables (Y) that were analyzed were the maximum products concentrations (g/L) as well as biomass concentrations (mg/L) obtained from the different experimental trials.

Three separate experiments with either NH_4Cl (1.1 g/L), YE (0.55 g/L) or trypticase (1.1 g/L), as sole source of nitrogen, were also performed in duplicate in order to understand the individual effect of each nitrogen source in promoting growth or product formation on CO. Another set of experiments, under the same conditions as above but without any CO, was also performed to check any product formation from YE and trypticase alone. The concentrations of nitrogen sources used in these sets of experiments are the center values of the respective factor ranges considered in the above full factorial design. Experiments and sample analysis were performed in the same way as mentioned above.

2.2.2. Continuous Gas-Fed Bioreactor Experiments

Three bioreactor experiments were carried out in 2 L BIOFLO 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA) using the following conditions: (1) pH = 5.75 and YE 1 g/L (called EXP1); (2) pH = 4.75 and YE 1 g/L (EXP2) and (3) pH = 5.75 and YE 0.2 g/L (EXP3). Those experiments were done with 1.2 L batch liquid medium and CO (100%) as the gaseous substrate, continuously fed at a rate of 15 mL/min using a mass flow controller (Aalborg GFC 17, Müllheim, Germany). The bioreactor with the medium was autoclaved and cysteine-HCl (0.75 g/L) was added after cooling, together with nitrogen feeding to ensure anaerobic conditions. The composition of the medium used in these bioreactor studies was the same as in the bottle experiments, with YE as the sole nitrogen source. The bioreactor was maintained at a constant temperature of 30 °C with a constant agitation speed of 250 rpm throughout the experiments. 10% of an actively growing culture, which was grown for 48 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 addition of either a 2 M NaOH solution or a 2 M HCl solution, fed by means of a peristaltic pump. Gas samples of 0.2 mL were taken from the inlet and outlet sampling ports of the bioreactor to monitor the CO and CO_2 concentrations. Similarly, 2 mL liquid samples were periodically withdrawn from the reactor, once every 24 h, in order to measure the optical density ($OD_{\lambda = 600 \text{ nm}}$), allowing to estimate the biomass concentration. Afterwards the sample was filtered with a syringe using a 0.22 µm PTFE-filter before analyzing the concentrations of water-soluble products.

2.3. Analytical Equipment and Measurement Protocols

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. 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. For 2,3-butanediol identification, a Thermo Scientific ISQ™ single quadrupole GC-MS system (Thermo Fischer Scientific, Madrid, Spain) was used and operated at 70 eV. It was equipped with a HP-5ms column (30 m × 0.25 mm × 0.25 µm film thickness). The water-soluble products in the culture broth, *i.e.*, acetic acid, ethanol and 2,3-butanediol, were analyzed using an HPLC (HP1100, Agilent Technologies, Madrid, Spain) 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. Cell mass was estimated by measuring the absorbance of the sample, at a wavelength of 600 nm, using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The measured absorbance was then compared to a previously generated calibration curve, to calculate the corresponding biomass concentration (mg/L). Besides, the redox potential was monitored continuously using an Ag/AgCl reference electrode connected to a transmitter (M300, Mettler Toledo, Inc., Bedford, MA, USA) and maintained inside the bioreactor.

3. Results and Discussion

3.1. Bottle Batch Experiments

In the bottle experiments, ethanol and acetic acid production started immediately, without any lag phase (Figure S1). It could be concluded that in these experiments the *Clostridium* strain follows the metabolic route that converts acetyl-CoA to acetaldehyde, followed by reduction to ethanol via a bifunctional acetaldehyde/ethanol dehydrogenase (Figure 1) [1]. Hence, in this CO fermentation, there were no differentiated acetogenic or ethanologenic phases. Maximum biomass (335.6 mg/L) and acetic acid concentrations (2.3 g/L) were produced in run No. 7 (Table 1) when the highest concentrations of YE and trypticase were used. The highest ethanol concentration (0.3 g/L) was obtained in run No. 2. Minor concentrations of by-product, *i.e.*, 2,3-butanediol, were also detected, reaching 0.017–0.101 g/L on the final day of the batch runs. The batch assays were stopped after about 10 days, when all the CO added initially was exhausted and no more biomass nor end-products were formed.

3.1.1. Main Effects Plot

The main effects plot for the experimental responses is shown in Figure 2. It represents the mean response values at each level of the design parameters. A main effect is considered present when the mean response changes across the level of the factor. From the main effects plot for biomass (Figure 2a), it is clearly

observed that NH_4Cl does not exert any significant effect on biomass. However, a slightly higher biomass concentration was observed whenever low NH_4Cl concentrations were used in this study. This effect is in agreement with previously reported studies with *Clostridium aceticum* and *Rhodospirillum rubrum* using CO as the sole carbon substrate [16]. The presence of both NH_4^+ and acetate could presumably result in the formation of ammonium acetate which is inhibitory to some clostridia, already at low concentrations [17].

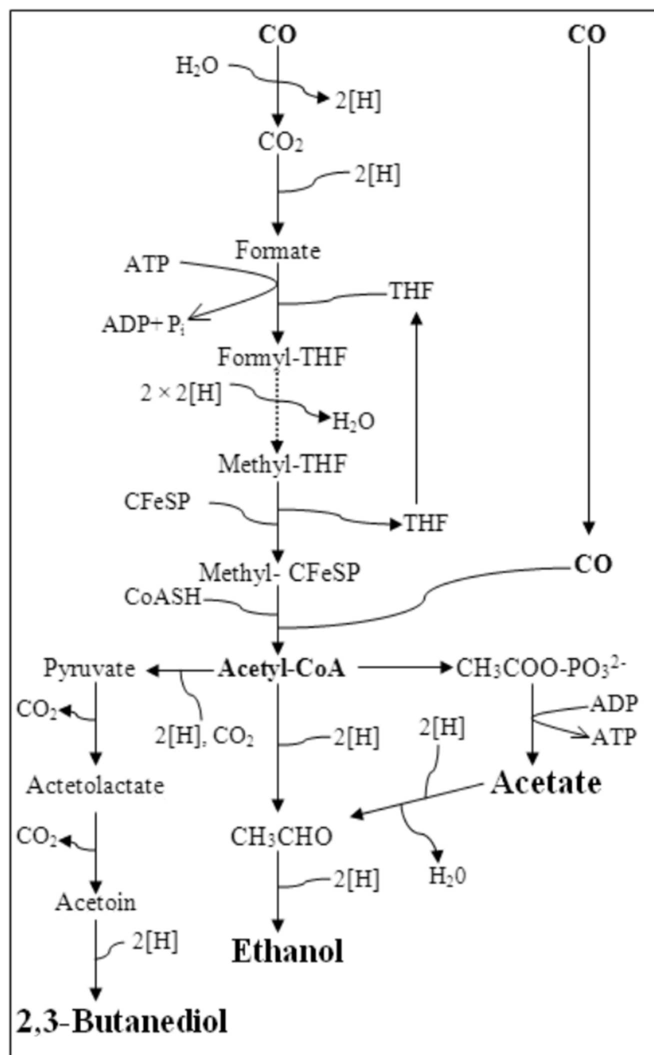

Figure 1. Wood-Ljungdahl pathway and metabolites formation from acetyl-CoA. Abbreviations: THF, tetrahydrofolate; CFeSP, corrinoid iron-sulphur protein.

Based on the main effects plot (Figure 2a), cell growth of *C. autoethanogenum* was obviously affected by the initial YE and trypticase concentrations in the medium. The amount biomass increased with an increase of initial YE as well as trypticase concentrations within the range of concentrations studied in this work. This can be attributed to the nutritional value of YE and trypticase soy broth, as both contain various amino acids, vitamins and other growth-stimulating compounds.

From the ANOVA analysis, it was observed that out of all the individual effects of each source of nitrogen, the effects due to the YE concentration was found statistically significant ($p < 0.05$) for ethanol and acetic acid production. For ethanol production (Figure 2c), the presence of YE showed the highest

negative effect, whereas NH_4Cl and trypticase exerted either a slightly positive or a slightly negative effect, respectively. The positive effect of NH_4Cl on ethanol production was also reported by Guo *et al.*, Plackett–Burman design was used in their studies, screening NH_4Cl as one of the significant factors affecting ethanol production, along with $MgSO_4$ and pH [13]. Enhanced growth in YE-limited media has been reported in previous studies. The presence of YE results in a richer medium, which is favorable for biomass growth. Biomass growth is usually related to acetate formation, while ethanol production is generally not a growth-related metabolite. Barik *et al.* suggested that a minimum level of approximately 0.01% YE would be essential for providing trace nutrients for cell growth. However, up to 300% improvement in the ethanol/acetate ratio was observed when YE was completely eliminated [18].

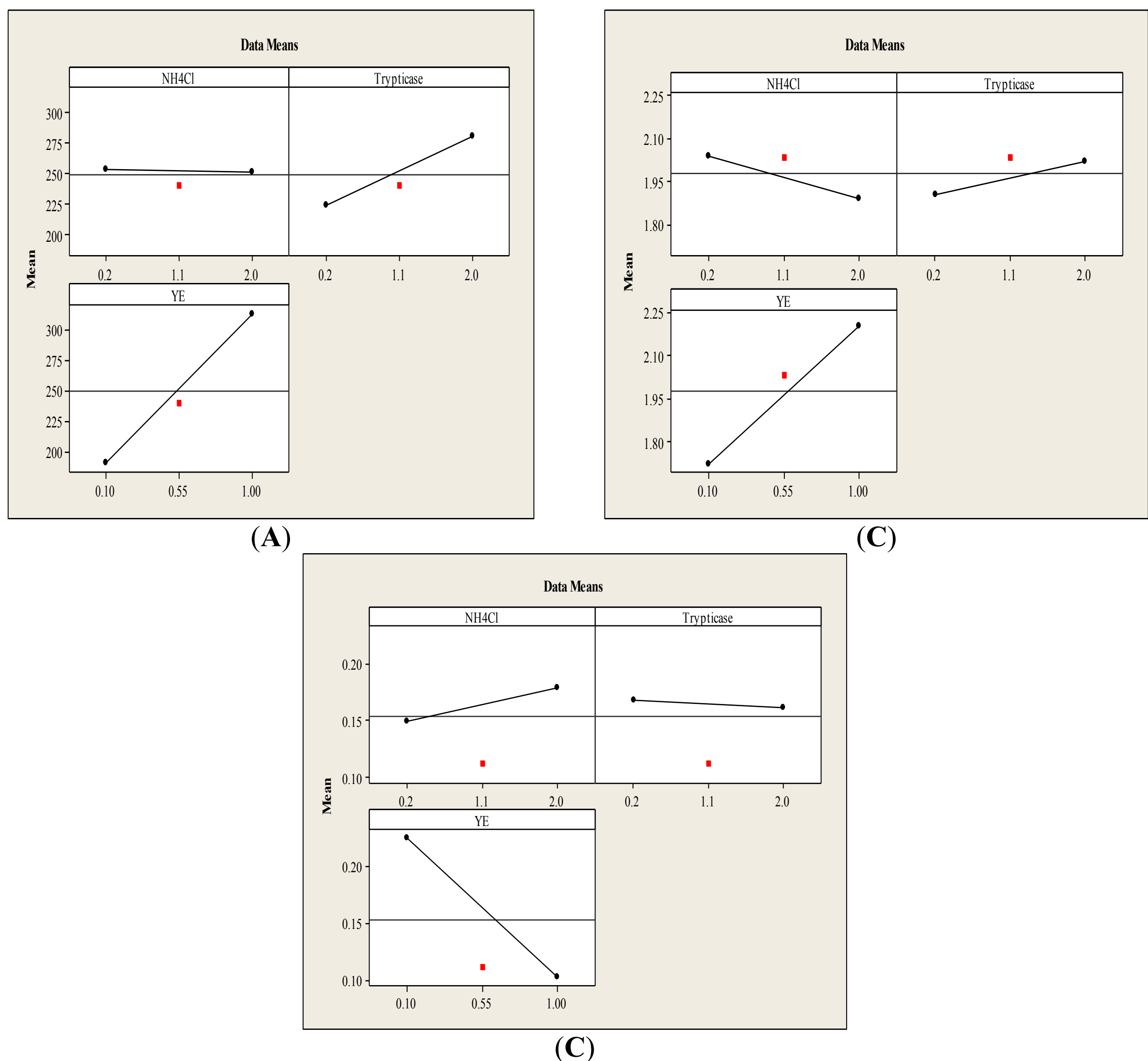

Figure 2. Main effects plot for (**a**) Biomass, (**b**) Acetic acid and (**c**) Ethanol.

The negative effect of YE on ethanol production is expected to be due to vitamin B12, among others. YE contains vitamin B12, which plays an important role in acetogenic bacteria. Methyl transferase synthase (MeTr) in acetogens is a cobalamin-dependent enzyme and catalyzes the transfer of the methyl

group of methyl-H4folate to the cobalt center of the corrinoid iron–sulfur protein (CFeSP). It is proposed that by reducing the H4folate cycle rate, NAD(P)H can build up inside the system with a subsequent increase in ethanol production [9]. In another study conducted with *Alkalibaculum bacchi* strain CP15, in a 7-L fermentor, a similar effect was observed; *i.e.*, a YE-free medium produced 13% more ethanol than a YE-containing medium. However, a decreased production of acetic acid and cell mass, reaching up to 40% and 15%, respectively, was observed in the YE-free medium [19].

3.1.2. Interaction Effects Plot

The interaction effects plot for biomass, ethanol and acetic acid produced from CO is shown in Figure 3 and provides the mean response of all possible combinations from low to high level of each two factors. That is, the effect of each factor dependent upon the second factor. Non-parallel lines represent an interaction between those two factors (YE, trypticase, and/or NH_4Cl). From the interaction plot for biomass and ethanol (Figure 3a,c), it can be observed that there is a strong interaction between each two factors. However, there is no remarkable interaction between the pairs of factors for acetic acid production (Figure 3b).

The maximum concentrations of biomass and acetic acid achieved were above 290 mg/L and 2.1 g/L, respectively, in all the experiments in which a YE concentration of 1 g/L was used, irrespective of the concentrations of trypticase and NH_4Cl in the medium (Figure 3a,b). The amounts ethanol produced reached their maximum values when YE was present at a low concentration of 0.1 g/L, irrespective of the concentrations of the other two factors (trypticase and NH_4Cl) (Figure 3c). This shows the influence of the YE concentration on the spectrum of products obtained from CO conversion in *C. autoethanogenum*. Considering the interaction between NH_4Cl and trypticase, a higher amount of biomass was found to be produced at a higher trypticase concentration of 2 g/L, at both levels of NH_4Cl, which can be attributed to the complex nutrients present in trypticase (Figure 3a).

3.1.3. Effect of Individual Sources of Nitrogen on Growth and Product Formation

Experiments were performed with either NH_4Cl (1.1 g/L), trypticase (1.1 g/L) or YE (0.55 g/L), as the only source of nitrogen. It was observed that there is no growth nor product formation in bottles containing only NH_4Cl. In the bottles with YE or trypticase, similar behaviours were observed, with growth reaching up to approximately 230 mg/L, and product concentrations of around 0.07 g/L for ethanol and 2 g/L for acetic acid. However, it is also worth recalling that the amount YE used in preparing the medium is half the amount of trypticase. From these observations, in the subsequent studies in continuous bioreactors, YE was chosen as the sole nitrogen source. Since YE and trypticase also contain other compounds besides nitrogen-containing ones, their potential use as substrates for the production of end-metabolites was checked. In that sense, in experiments performed without any CO, it was observed that the presence of YE or trypticase could be involved in approximately up to 10% of the total acetic acid produced in experiments containing CO as carbon source as well as YE and trypticase.

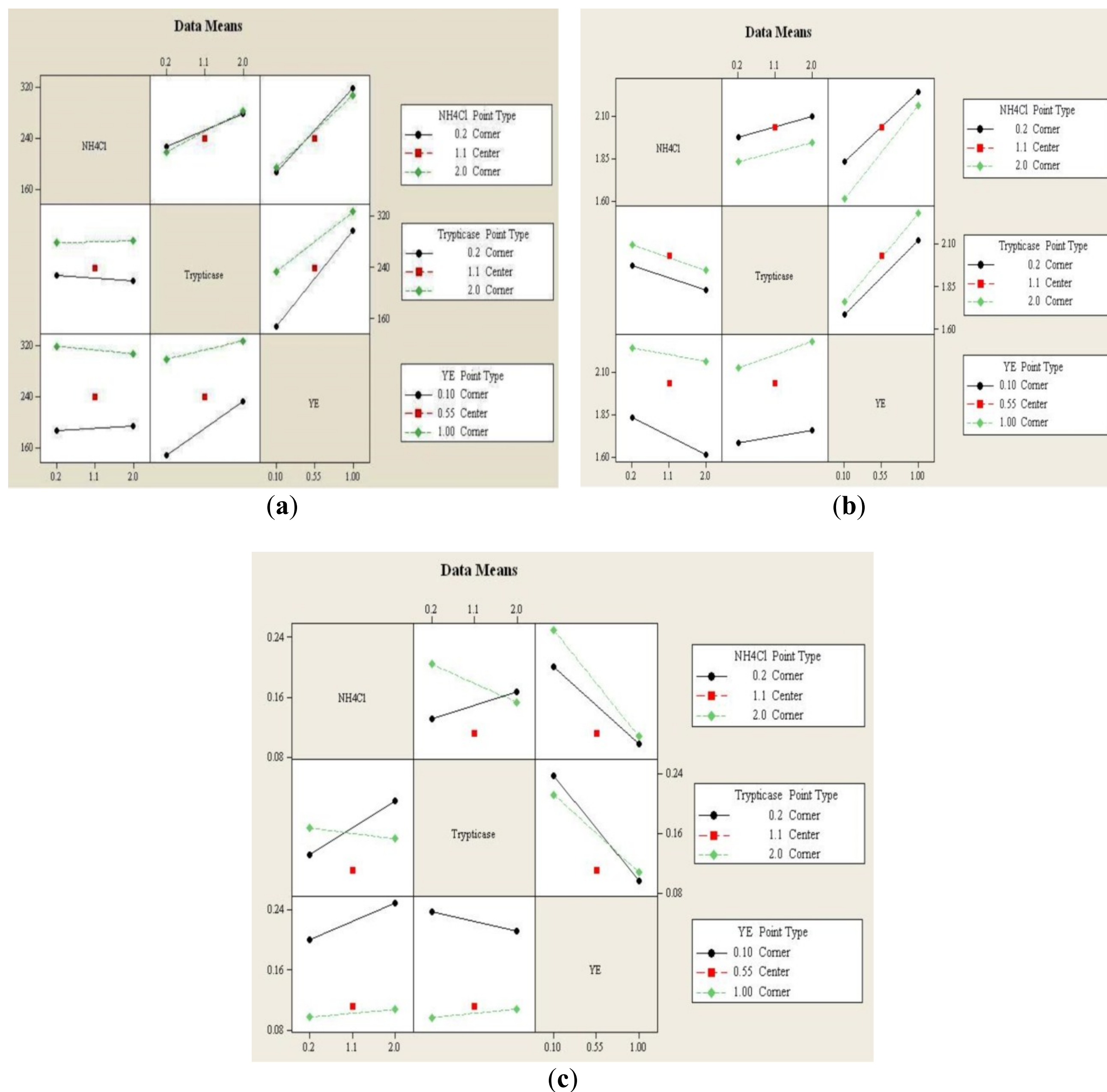

Figure 3. Interaction effects plots for (**a**) Biomass, (**b**) Acetic acid and (**c**) Ethanol.

3.2. Continuous Gas-Fed Bioreactor Experiments

Bioreactor experiments with continuous gas-flow, *i.e.*, continuous CO supply, were performed for up to two weeks each. Cell growth and the production of different metabolites in three different sets of experiments are shown in Figure 4. The redox potential was constantly monitored for each experimental run. It is related to the electron transfer undergoing inside the cells and hence is very sensitive for even delicate changes in metabolism. Both EXP1 and EXP3 had an instrument reading oxidoreduction potential (ORP) value of −87 ± 10 mV, while it was −43 ± 5 mV for EXP2. The ORP values are directly dependant on the pH of the medium. A lower pH of the liquid phase will result in lower negative values of the redox potential. Oscillations of the redox potential values in the culture medium could be due to microbial growth and variations in the metabolic profile at each point of the experimental run and have also been reported by other researchers in other bioconversion studies [20,21]. Intracellular redox

homeostasis is profoundly affected by the ups and downs of the extracellular redox potential which can significantly switch the fermentation type in acidogenic bacteria [22].

The biomass in EXP1 (Figure 4a) started growing after a shorter lag phase compared to EXP2 and EXP3, due to the favorable growth conditions (*i.e.*, optimal pH and nutritional value of YE) that prevail inside the bioreactor, attaining a biomass concentration of about 302.4 mg/L in less than 100 h of experimental run. The lag phase was approximately 70 h in both EXP2 and EXP3, reaching maximum biomass concentrations of 113.76 and 151.37 mg/L, respectively; that is 62% and 50% less than in EXP1. This confirms that the pH and YE concentration are important parameters and play a key role in achieving high cell mass concentrations. A drastic decrease in growth occurred after 89 h in EXP1. This could be linked to the accumulation of high amounts of acetic acid (~ 2 g/L) in the fermentation broth. Two enzymes are responsible for the conversion of acetyl CoA during the synthesis of acetate, *i.e.*, phosphotransacetylase (PTA) and acetate kinase (AK). During the acetate production stage, both enzymes are active and ATP is produced as a part of their reaction. However, it was reported that the activity of these enzymes decreases considerably with an increase in acetate concentration in the broth in fermentation with *C. acetobutylicum* [23]. In the latter study, the AK was biosynthesized inside the cell of *C. acetobutylicum*, with buildup of acetate concentrations of up to 3 g/L in the broth, resulting in a rapid decrease in the AK activity with the increase of the amount acetate [23]. However, a clear explanation for stoppage of growth and metabolite production in EXP2 and EXP3 after a certain period of time is yet somehow unclear.

No separate acidogenic and solventogenic phase was observed for *C. autoethanogenum* during these bioreactor studies using the reported media compositions and fermentation conditions. The conversion of acetic acid to ethanol in the late phase of the study was also not observed, although we observed such type of conversion of acetate to ethanol under different operating conditions (manuscript in preparation). Acetic acid was the predominant metabolite formed during CO fermentation in each of the three experiments described here (Figure 4b). As mentioned above, changing the experimental conditions would allow a shift to ethanol accumulation rather than acetate. A maximum acetic acid concentration of 2.1 g/L was obtained after 137 h in EXP1, which is about 294% and 95% higher than the maximum amounts produced in EXP2 and EXP3, respectively. It is interesting to note that both experiments, EXP1 and EXP3,that were performed at high pH, produced more acetic acid than in studies at lower pH, irrespective of the YE concentrations used. A previous study using *C. ragsdalei* at two different pH values similarly reported a higher acetic acid production at high pH [24].

Although the maximum amount of ethanol was obtained in EXP1, the ratio ethanol/acetic acid was greater in EXP2 characterized by a low pH. Fermentation pH is one the most influential parameters that affects the metabolism of acetogenic bacteria. Lowering the pH appears to cause a shift in the product spectrum from acidogenic to solventogenic phase. The explanation lies in the permeation of the undissociated weak acid, acetic acid, through the cell membranes resulting in a lower internal pH due to the entry of H^+ ions. Bacteria overcome this physiological stress by producing solvents [25].

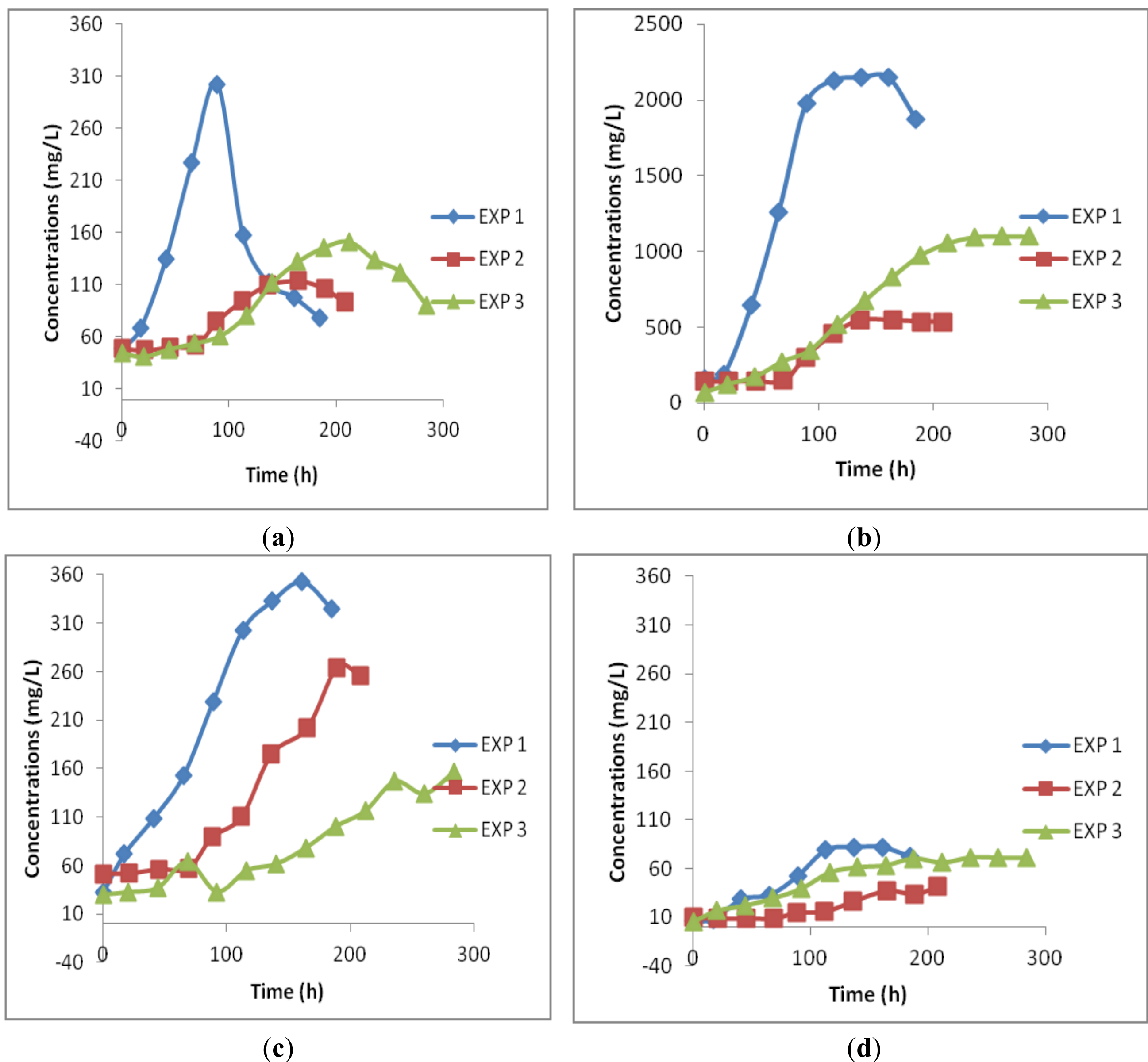

Figure 4. Cell mass (**a**) and products profiles, Acetic acid (**b**); Ethanol (**c**) and Butanediol (**d**) in three different experiments: EXP1 (pH = 5.75 and YE 1 g/L); EXP2 (pH = 4.75 and YE 1 g/L); EXP3 (pH = 5.75 and YE 0.2 g/L).

As can be seen in Figure 4c, a higher maximum ethanol production was obtained in EXP1 than in EXP2, although the low external pH induced more solvent production. This could be due to the high biomass concentration achieved in EXP1. The fermentations produced 352.6 mg/L, 264.51 mg/L and 156.95 mg/L ethanol respectively in EXP1, EXP2 and EXP3. On the other hand, a maximum ethanol to acetic acid ratio was obtained for EXP2 with a value of 0.54. It can be seen that a low pH (EXP2) caused a lengthening of the lag phase and reduced the final biomass concentration, yet it significantly improved the ethanol/acetic acid ratio. Thus, nutrient limitation combined with a low fermentation pH improved such product ratio. Several studies reported that two-stage stirred tank bioreactors, with a different pH in each vessel could improve the ethanol to acetic acid ratio [26,27]. From this study it is observed that using a low initial pH and maintaining it constant could also improve the ethanol/acetic acid ratio, although there is a strong decrease in the overall productivity of metabolites. A major obstacle in CO

fermentation, when focussing on ethanol production, is that lowering the pH reduces cell growth; thereby reducing the overall productivity of ethanol in the process. Minor amounts 2,3-butanediol were also produced in all three experiments (Figure 4d). The butanediol concentration increased to a maxium of 81.8, 41.8 and 71.6 mg/L in EXP1, EXP2 and EXP3, respectively.

4. Conclusions

From the experiments it is clearly observed that altering the medium's composition as well as pH alters the product spectrum and biomass growth. From the batch studies, the YE concentration was found to have a significant effect on ethanol production. EXP1, at pH = 5.75 and a YE concentration of 1 g/L, produced a maximum amount of biomass (302.4 mg/L) and maximum concentrations of products, *i.e.*, acetic acid (2147.1 mg/L), ethanol (352.6 mg/L) and butanediol (81.8 mg/L), compared to the other two studies. A maximum ethanol to acetic acid ratio of 0.54 was obtained in EXP2 (pH = 4.75; YE 1 g/L). Though maintaining a low constant pH from the beginning improved the ethanol to acetic acid ratio, it drastically affects the overall productivity of the process as a result of a weaker biomass growth.

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Author Contributions

Haris Nalakath Abubackar performed the experimental studies. Christian Kennes and María C. Veiga obtained financial support to undertake the research and supervised the experimental work. Haris Nalakath Abubackar prepared the first draft of the paper. All authors contributed to the final writing and revision of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Carbon monoxide fermentation to ethanol by *Clostridium autoethanogenum* in a bioreactor with no accumulation of acetic acid

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HIGHLIGHTS

- The presence of tungsten improved ethanol production from CO fermentation.
- Selenium and vitamins addition did not improve the ethanol/acetic acid ratio.
- The 2,3-butanediol/acetic acid ratio increased with the addition of tungsten.
- The addition of tungsten, at low pH, resulted in no accumulation of acetic acid.

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ABSTRACT

Fermentation of CO or syngas offers an attractive route to produce bioethanol. However, during the bioconversion, one of the challenges to overcome is to reduce the production of acetic acid in order to minimize recovery costs. Different experiments were done with *Clostridium autoethanogenum*. With the addition of 0.75 μM tungsten, ethanol production from carbon monoxide increased by about 128% compared to the control, without such addition, in batch mode. In bioreactors with continuous carbon monoxide supply, the maximum biomass concentration reached at pH 6.0 was 109% higher than the maximum achieved at pH 4.75 but, interestingly, at pH 4.75, no acetic acid was produced and the ethanol titer reached a maximum of 867 mg/L with minor amounts of 2,3-butanediol (46 mg/L). At the higher pH studied (pH 6.0) in the continuous gas-fed bioreactor, almost equal amounts of ethanol and acetic acid were formed, reaching 907.72 mg/L and 910.69 mg/L respectively.

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1. Introduction

In recent years, growing interest has been found in the use of bio-based fuels as a result of the gradual depletion of global oil reserves and consensus on climate change. By 2020, it will be mandatory for all the Member States (MS) of the EU to reach their assigned targets in terms of energy and to achieve a 20% share of renewable energy (van Groenestijn et al., 2013). Moreover, the use of 10% renewable energy in transportation will be mandatory by then for all MS (Latif et al., 2014). Bioethanol was one of the biofuels which accounted for 28% of the overall biofuels used in the road transport in the EU in 2012. EU bioethanol production was forecasted to reach 5.38 billion liters in 2014 (Flach et al., 2013). Grains such as wheat, corn, barley and rye are currently the prominent feedstocks for bioethanol production in the EU. However, this leads to food-fuel competition. Hence, one way to overcome this situation is to utilize highly available lignocellulosic biomass or even waste as raw material for bioethanol production. However, the conventional way of bioconversion of lignocellulosic biomass to bioethanol is a somewhat complex process (Balat and Balat, 2009). An alternative and promising new generation bioethanol production process is through gasification of biomass in order to generate syngas or producer gas, composed mainly of CO, CO_2 and H_2. It is later introduced into a fermentor that is inoculated with anaerobic bacteria, mainly belonging to genera such as *Clostridium*, under specific process conditions (Abubackar et al., 2011a; Bengelsdorf et al., 2013; Mohammadi et al., 2011). The biocatalysts use these C_1 compounds as sole carbon source, following the reductive acetyl-CoA pathway, leading to the production of ethanol and acetic acid. Trace amounts of 2,3-butanediol, butanol, lactic acid are also reportedly being produced during the fermentation (Bengelsdorf et al., 2013). Recently, some studies were published on syngas fermentation with genetically engineered biocatalysts as well (Ueki et al., 2014; Xie et al., 2015). On the other hand, studies are still ongoing with wild type strains of bacteria

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to improve the ethanol productivity by manipulating several parameters, such as the medium composition and/or fermentor operating conditions (Abubackar et al., 2012; Kundiyana et al., 2011).

The reductive acetyl-CoA pathway, also known as the Wood–Ljungdahl (WL) pathway, comprises an Eastern or methyl branch and a Western or carbonyl branch that use CO and/or CO_2 as the substrate for the synthesis of acetyl-CoA, the intermediate that serves as precursor for the formation of biomass and metabolites such as ethanol and acetic acid. The proteins that are involved in the WL pathway, require cofactors such as H_4-folate, cobalamin, metal ions or FeS-clusters. For example, Corrinoid FeS proteins contain both cobalamin with a central cobalt atom and an FeS cluster. Carbon monoxide dehydrogenase (CODH) from *Moorella thermoacetica* and *Acetobacterium woodii* contains two Ni-FeS clusters. The formate dehydrogenase (FDH) of *M. thermoacetica* that catalyzes the reduction of CO_2 to formate is a tungsten, selenium and FeS cluster containing metalloenzyme (Ragsdale and Pierce, 2008).

So far, most studies on the production of ethanol from CO have focused on how various macronutrients (e.g., nitrogen sources) and their concentrations affect the fermentation process. Hardly any research has focused on how trace metals influence ethanol production and none has studied their effects in bioreactors with continuous feed of the gaseous substrate. One study has been published but in batch assays and with no pH control (Saxena and Tanner, 2011). Since tungsten and selenium are components of formate dehydrogenase (FDH), whereas aldehyde:ferredoxin oxidoreductase (AFOR) that catalyzes reduction of carboxylic acids to aldehydes is a tungsten containing enzyme (Fig. 1) (Wang et al., 2013), the purpose of this study was to investigate the effects of tungsten and selenium on fermentation of CO by *Clostridium autoethanogenum* and on product distribution in batch and continuous gas-fed bioreactors. The effect of the presence of vitamins and the influence of pH were also investigated.

2. Methods

2.1. Bottle batch experiments

Batch experiments were conducted without pH control to study the effect of trace metals, tungsten (W), selenium (Se) as well as vitamins on growth and product formation in *C. autoethanogenum* DSM 10061. The growth medium to maintain the bacteria as well as the production medium used for the batch experiments is given

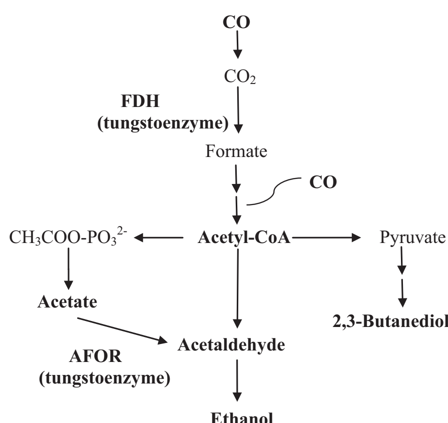

Fig. 1. Wood–Ljungdahl pathway and metabolites formation from acetyl-CoA, with the corresponding metalloenzymes. *Abbreviations:* FDH, formate dehydrogenase; AFOR, aldehyde:ferredoxin oxidoreductase.

in Table 1. Five independent tests were performed in duplicate with the production medium having different trace metal compositions, as mentioned hereafter, without vitamins: (1) trace metal SL-10 without W and Se [TM]; (2) SL-10 with 0.075 μM W (refers to the W concentration in the test vials) [Low W]; (3) SL-10 with 0.144 μM Se [Low Se]; (4) SL-10 with 0.75 μM W [High W] and (5) SL-10 with 1.44 μM Se [High Se]. Another set of four experiments was performed in duplicate to check the need and the effect of additional vitamins (Vit) on CO bioconversion: (1) SL-10 and Vit [Vit]; (2) SL-10 with 0.75 μM W and Vit [W-Vit]; (3) SL-10 with 1.44 μM Se and Vit [Se-Vit]; (4) SL-10 with 0.75 μM W and 1.44 μM Se as well as Vit [All]. 1 ml of vitamin solution (Table 1) per liter of production medium was used for the experiments with vitamins (Vit).

Studies were carried out in duplicate at an initial pH of 5.75 in 100 ml serum vials with 30 ml production medium and inoculated with 2.5 ml of actively growing seed culture, which was grown with CO as sole carbon source. 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 set-up and the method used for media preparation as well as sampling details are described elsewhere (Abubackar et al., 2011b).

2.2. Continuous gas-fed bioreactor experiments with tungsten

Two bioreactor experiments were carried out in a 2-L New Brunswick Scientific BIOFLO 110 bioreactor at either pH 6.0 (High pH) or pH 4.75 (Low pH) with 1.2 L batch liquid medium and CO (100%) as the gaseous substrate, continuously fed at a rate of 10 ml/min using a mass flow controller (Aalborg GFC 17). The medium composition used for the experiments was the same as in batch assays with the trace metal solution containing 0.75 μM W, as this was shown to favor the desired bioconversion pathway in the batch assays. The bioreactor was maintained at a constant temperature of 30 °C, with a constant agitation speed of 250 rpm throughout the experiments. 10% of an actively growing culture, which was grown for 48 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 6.0 or 4.75, through the addition of a 2 M NaOH or a 2 M HCl solution, fed by means of a peristaltic pump. Gas samples of 0.2 mL were taken from the inlet and outlet sampling ports of the bioreactor to monitor the CO and CO_2 concentrations. Similarly, 2 mL of liquid sample was periodically withdrawn from the reactor, once every 24 h, in order to measure the optical density (OD_λ = 600 nm) and estimate the biomass concentration. Afterwards the sample was filtered with a syringe using a 0.22 μm PTFE-filter before analyzing the concentrations of soluble products.

2.3. Analytical equipment and measurement protocols

Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph (GC) 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. 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. For 2,3-butanediol identification, a Thermo Scientific ISQ™ single quadrupole GC–MS system, operated at 70 eV,

Table 1
Composition of culture media used in the experiments.

Growth medium (pH 6)
Composition (per liter distilled water): NH_4Cl, 0.9 g; NaCl, 0.9 g; $MgCl_2 \cdot 6H_2O$, 0.4 g; KH_2PO_4, 0.75 g; K_2HPO_4, 1.5 g; $FeCl_3 \cdot 6H_2O$, 0.0025 g; trypticase peptone, 2.0 g; yeast extract, 1.0 g; cysteine-HCl, 0.75 g; 0.1% resazurin, 0.5 mL; with 0.5% xylose and SL-10 solution, 1.0 mL. The trace metal stock solution SL-10 contained (per liter): 7.7 M HCl, 10 mL; $FeCl_2 \cdot 4H_2O$, 1.5 g; $MnCl_2 \cdot 4H_2O$, 100 mg; H_2BO_3, 6 mg; $CoCl_2 \cdot 2H_2O$, 190 mg; $CuCl_2 \cdot 2H_2O$, 2 mg; $NiCl_2 \cdot 6H_2O$, 24 mg; and $Na_2MoO_4 \cdot 2H_2O$, 36 mg
Production medium (pH 5.75)
Composition (per liter distilled water): NaCl, 0.9 g; $MgCl_2 \cdot 6H_2O$, 0.4 g; KH_2PO_4, 0.75 g; K_2HPO_4, 1.5 g; yeast extract 0.5 g; $FeCl_3 \cdot 6H_2O$, 0.0025 g; 0.1% resazurin, 0.5 mL; cysteine-HCl 0.75 g and SL-10 solution, 1.0 mL
Vitamins
The vitamin stock solution contained (per liter) 10 mg each of *para*-aminobenzoic acid, calcium pantothenate, nicotinic acid, riboflavin, thiamine, α-lipoic acid, and vitamin B12, 4 mg each of d-biotin, folic acid and 20 mg pyridoxine
Tungsten and selenium
The chemicals used were $Na_2WO_4 \cdot 2H_20$ and Na_2SeO_3

mounted with a HP-5 ms column (30 m × 0.25 mm × 0.25 μm film thickness) was used. The water-soluble products, acetic acid, ethanol and 2,3-butanediol, in the culture broth were analyzed 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. Cell mass was estimated by measuring the absorbance of the sample at a wavelength of 600 nm using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The measured absorbance was then compared to the previously generated calibration curve to calculate the corresponding biomass concentration (mg/L). Besides, the redox potential was monitored continuously using a Ag/AgCl reference electrode maintained inside the bioreactor and connected to a transmitter (M300, Mettler Toledo, Inc. USA).

3. Results and discussion

3.1. Bottle batch experiments

Figs. 2 and 3 show the ethanol/acetic acid and butanediol/acetic acid ratio for the two sets of experiments. The experimental results show that the highest ethanol to acetic acid ratio obtained was 0.19 in the experiment designated as High W; that is 173% higher than the ratio obtained in the experiment with High Se. It is clear from the plot that the ethanol/acetic acid ratio, in batch tests, increased with the presence of tungsten in the medium. In the case of selenium, the ratio obtained was roughly similar in either the Low Se or the High Se experiment, with a value of 0.013 (Fig. 2a), which was even lower than in the control medium (TM). Hence it can be concluded that selenium did not allow to increase the ethanol/acetic acid ratio in *C. autoethanogenum*. It did not even favor considerably acetic acid production compared to the control medium. A recent report on a study with another bacterial strain in batch assays agrees with the present findings, and suggested no significant change in acetic acid production with or without selenium in the medium (Saxena and Tanner, 2011).

Some tungstoenzymes involved in the WL pathway and its subsequent routes that lead to metabolites production, include formate dehydrogenase (FDH) and aldehyde:ferredoxin-oxidoreductase (AFOR), having pterin cofactors as their active sites (Fig. 1). FDH catalyzes the first reaction in the WL pathway, that is the two–electron reduction of CO_2 to formate (Ragsdale and Pierce, 2008). The first originally isolated tungstoenzyme is the FDH from *Clostridium thermoaceticum*. It contains 1 tungsten atom, 1 selenium, 18 iron and about 25 inorganic sulfur per dimeric unit, and utilizes NADPH as the physiological electron carrier (Yamamoto et al., 1983). It was reported that the presence of tungsten, selenium, molybdenum and ferrous ions in the growth medium

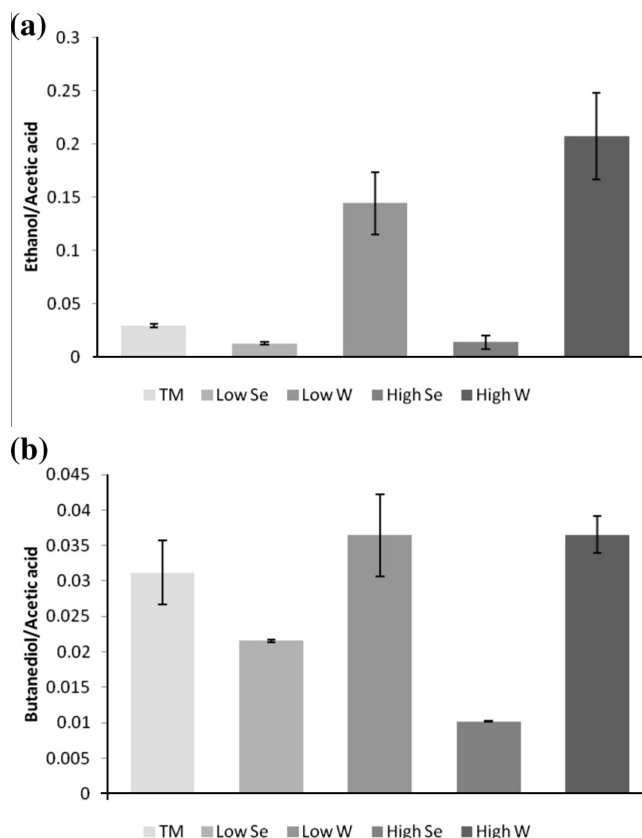

Fig. 2. (a) Ethanol/acetic acid ratio and (b) Butanediol/acetic acid ratio, obtained in absence of vitamins. TM = trace metal solution without selenium and tungsten. The error bars represent the standard deviations.

stimulates FDH synthesis (Yamamoto et al., 1983). Recently, it was reported that FDH in *C. autoethanogenum* forms complexes with an electron bifurcating hydrogenase enzyme that is NADP specific (Wang et al., 2013). The chemical analysis of this complex revealed that it contains tungsten. Experiments using *Clostridium ragsdalei* to study the effect of trace metals, when using CO as a substrate, indicated that the presence of tungsten (WO_4^-) at a concentration of 0.68 l μM, yielded an ethanol production of 35.73 mM, which improved to 72.3 mM upon increasing the tungsten concentration to 6.81 μM (Saxena and Tanner, 2011). In that study, it was suggested that the presence of both selenium and tungsten in the medium decreases the activity of FDH in *C. ragsdalei* compared to media containing either tungsten or selenium only. AFOR, on the other hand, catalyzes the reduction of acetic acid to acetaldehyde. It was reported that AFOR from the hyper-

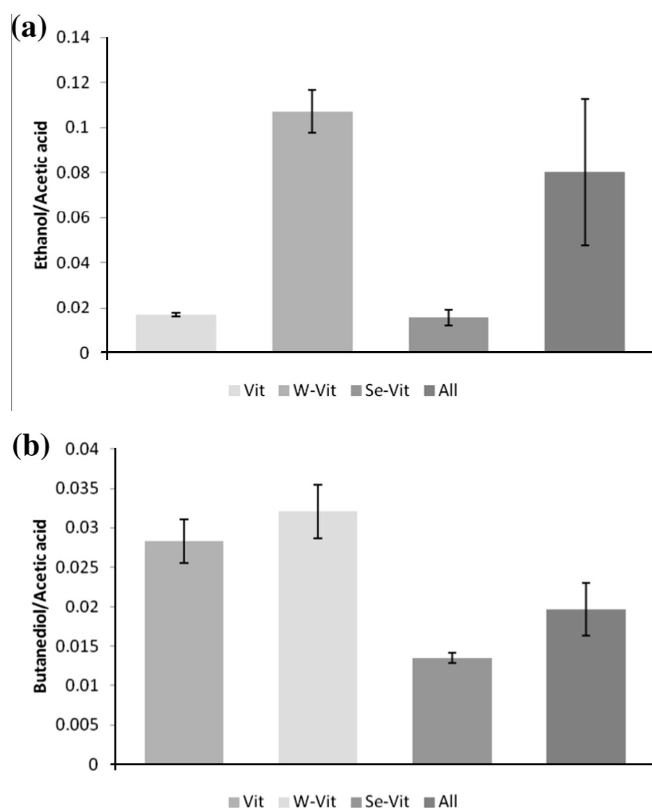

Fig. 3. (a) Ethanol/acetic acid and (b) Butanediol/acetic acid ratio, obtained in presence of vitamins. All = presence of selenium, tungsten in addition to vitamins. The error bars represent the standard deviations.

thermophilic archaeon *Pyrococcus furiosus* is a homodimer with 1 W and 4–5 Fe atoms per molecule (Kletzin and Adams, 1996).

The results from the second set of experiments, aimed at studying the effect of adding a vitamin solution, showed that the presence of additional vitamins did not enhance the ethanol/acetic acid ratio. Interestingly, in the medium containing both selenium and vitamins, besides tungsten ("All", Fig. 3a), the ethanol/acetic acid ratio was twenty-five percent lower than the value obtained in the medium containing both tungsten and vitamins but without any addition of selenium ("W-Vit", Fig. 3a). While many researchers add vitamins in studies on biofuels production (e.g., ethanol) with *clostridia*, the present data questions the need of such addition, which is a relevant cost-related issue. Our *C. autoethanogenum* strain did not need the supply of additional vitamins for ethanol production. To the best of our knowledge, this is different from other *Clostridium* strains described so far. One possible explanation is that this *C. autoethanogenum* strain has repeatedly been transferred to fresh media without adding vitamins and could therefore have adapted to such conditions. It may be assumed that, in the present study with *C. autoethanogenum*, selenium would inhibit the ethanol production pathway and partly counteract the favorable effect of tungsten. Trace metals might exhibit different effects in different CO-metabolizing strains, but tungsten showed a clear positive effect on ethanol production in our batch assays with *C. autoethanogenum*, while selenium at either no positive effect or even a negative effect depending on the nature of other elements (i.e., trace metals or vitamins) present in the medium.

The production of small amounts of 2,3-butanediol was also observed during CO fermentation in all the batch experiments. The maximum butanediol/acetic acid ratio obtained in the present work was 0.032, and was exactly the same for all the experiments that contained tungsten, irrespective of the tungsten concentration and the presence or not of vitamins (Figs. 2b and 3b). Such data cannot be compared to any other previous experiment as no other study has focused on the effect of trace metals on 2,3-butanediol production from CO in *Clostridia*. From Figs. 2 and 3, it appears that the presence of tungsten increases the butanediol/acetic acid ratio similarly as in the case of the ethanol/acetic acid ratio. However, when compared to the control medium (TM) (with no tungsten nor selenium), the addition of tungsten increased more the ethanol/acetic acid ratio than the butanediol/acetic acid ratio. Indeed, the ethanol/acetic acid ratio was 5 to 7 times higher when adding tungsten (either at low or high W concentration), while the butanediol/acetic acid ratio increased by only about 20% when adding tungsten compared to TM. In any case, for both ethanol and 2,3-butanediol, it can be concluded that their relative concentration, compared to acetic acid, decreases under the following conditions, without the addition of a vitamin-solution: presence of tungsten (no selenium) > no tungsten nor selenium > presence of selenium (no tungsten). In the WL pathway and later in 2,3-butanediol production, acetyl-CoA with CO_2 are converted to pyruvate using pyruvate:ferredoxin oxidoreductase (PFOR). Pyruvate gets reduced by acetolactate synthase and acetolactate decarboxylase to acetoin and then later to 2,3-butanediol using 2,3-butanediol dehydrogenase (23BDH) (Köpke et al., 2011). Köpke et al. (2014) recently discovered that *C. autoethanogenum* contains two dehydrogenases that are able to reduce acetoin to 2,3-butanediol, namely 23BDH and primary-secondary alcohol dehydrogenase.

The final pHs after these experimental batch runs were also measured. The production of acetic acid during the growth decreased the pH of the medium significantly and this usually inhibited the bacterial growth and metabolites production. The initial pH of the medium was 5.75 and the initial phosphate concentration was 14 mM. Phosphate in the form of KH_2PO_4/K_2HPO_4 was used as pH-buffering solution. It was observed that the final pH value was in the range of 3.80–4.00 for all the experiments.

The above findings in batch bottles confirm that the presence of tungsten improves the ethanol/acetic acid as well as the butanediol/acetic acid ratios, while the addition of selenium and vitamins had no favorable effect for ethanol production. Hence, in further studies in bioreactors with continuous CO supply, a trace metal solution with tungsten was used and vitamins and selenium were omitted, as described below. Since the pH value would affect biomass growth and the production of metabolites, the next experiment was performed using a pH-control unit in order to maintain a constant pH.

3.2. Bioreactor experiment with continuous CO supply

3.2.1. Biomass profile

Fig. 4 shows that the pH value had a profound effect on biomass production. Although pH could not be maintained constant in the batch bottle assays described above; in the present bioreactor studies pH remained stable throughout the experiments. To the best of our knowledge, no previous other study has been reported on the effect of trace metals and vitamins in continuous CO-fed bioreactors under regulated, constant, pH conditions. pH control is important as it represents an additional parameter expected to affect biomass growth and production of metabolites. Biomass started growing instantly without any lag phase at pH 6.0, while, a 24 h lag phase was observed in the experiment at pH 4.75 (Fig. 4). A maximum biomass concentration of 287.77 mg/L was achieved at pH 6.0 which is 109% higher than the maximum value obtained at pH 4.75 and, during the exponential phase, biomass was found to increase at a rate 70% faster at pH 6 than at pH 4.75. This can be attributed to the negative impact of pH deviations from the organism's optimum pH range for growth i.e., between pH 5.8 and 6.0. Hence, the results demonstrated that the growth of *C.*

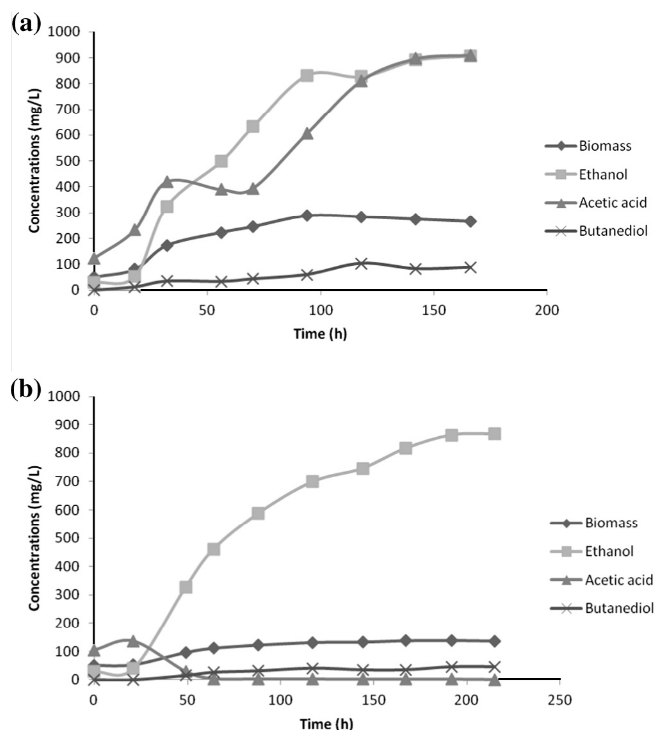

Fig. 4. Cell mass and products profile at two different pHs studied in bioreactors: (a) pH 6 and (b) pH 4.75.

autoethanogenum was limited when pH decreased sharply, under slightly acidic conditions. The biomass entered the stationary phase after 48 h and 96 h, respectively, with the pHs set at either 4.75 or 6.0. The amount biomass achieved during the experimental run is comparatively lower than that obtained for studies with other *Clostridium* strains (Abubackar et al., 2011a; Mohammadi et al., 2011). In one of our previous studies in bioreactor with continuous CO supply and with 1 g/L yeast extract at pH 5.75, the maximum biomass obtained was 302.4 mg/L, which is comparable to the maximum cell mass concentration obtained in this study at pH 6.0 (Abubackar et al., 2015). However, most batch studies with the strain *C. autoethanogenum* usually reported a low level of biomass growth compared to other bacterial species (Cotter et al., 2009; Guo et al., 2010). Cotter et al. (2009) reported a maximum biomass concentration of 150 mg/L in *C. autoethanogenum*, achieved while feeding syngas (20% CO) at a flow rate of 10 ml/min.

The low cell mass concentration in bioreactor studies might be due to two reasons, either to limited nutritional availability in aqueous phase or/and to low availability of gaseous substrate. Biomass yields might also be a strain-linked parameter. Most of our own data, as well as some other published studies, suggest that biomass growth and consequently ethanol production from CO-related substrates seems to be generally lower in *C. autoethanogenum* than in strains such as *Clostridium. ljungdahlii* (Abubackar et al., 2015; Guo et al., 2010; Mohammadi et al., 2011). The solubility of CO in liquid phase is low as well as its mass transfer into the aqueous medium. Hence, in this state of limited mass transfer, the microorganism could not obtain sufficient substrate for growth and maintenance, which eventually leads to a low growth rate. Since vitamins did not show any effect on biomass accumulation in batch bottle experiments with this specific strain, it was eliminated in the bioreactor study, but to the best of our knowledge, the absence of vitamins would not be an explanation for the low biomass growth. However, a low medium cost is absolutely essential for optimizing the techno-economics of syngas fermentation. In a study using *Alkalibaculum bacchi*, a 50% higher cell mass concentration was reported in YE medium along with vitamins and mineral solutions than with corn steep liquor (CSL) medium, though the maximum cell mass concentration obtained with YE was still only 330 mg/L (Liu et al., 2014).

3.2.2. Product formation

As can be observed from the figure (Fig. 4), the metabolic products obtained from CO fermentation were strongly affected by the pH. In some of our previous studies, it was found that *C. autoethanogenum* produced a higher amount of acetic acid than ethanol under the experimental conditions specifically used in that work (Abubackar et al., 2015). In the present work, ethanol and acetic acid were the dominant final fermentation products in the study at pH 6.0 with productions reaching a maximum of 907.72 mg/L and 910.69 mg/L, respectively. A maximum ethanol concentration of 867 mg/L was produced at pH 4.75, together with no acetic acid production and a negligible concentration (<50 mg/L) of butanediol as the alcohol byproduct. It can be suggested from this study that changing the pH of the medium at a specific stage of the continuous CO fermentation process induces a metabolic shift. In contrast, at pH 6.0, concomitant, continuous, acetic acid and ethanol production was observed, and it could be noted that the ethanol to acetic acid ratio obtained was close to 1. This value is greater than that obtained in our previous experimental studies without tungsten, where the maximum ethanol/acetic acid ratio obtained was 0.54 (Abubackar et al., 2015). Ethanol production is also higher than in the batch bottle assays described above with no pH regulation. A significant part of the CO fed was directed towards acetic acid production at the branch point of acetyl-CoA in the WL pathway (Fig. 1). Even though ethanol started being produced at the early stage of the biomass growth at both pHs, most of the ethanol titer was produced during the stationary phase. Although the final ethanol concentration was similar both at pH 4.75 and pH 6.0, it took about twice as long to reach such concentration at low pH than at high pH. This is also related to the higher amount biomass found at high pH.

As discovered from the bioreactor study, CO bioconversion by *C. autoethanogenum* changed from a predominant acetate and ethanol production at pH 6.0 to predominant ("single") ethanol production at pH 4.75. An apparent metabolic shift of pathway from acidogenesis to solventogenesis upon decreasing the pH has also been observed previously in ABE (Acetone-Butanol-Ethanol) fermentation by *C. acetobutylicum* (Grupe and Gottschalk, 1992). Solventogenesis in syngas fermentation occurs during unfavorable growth conditions and in the presence of ample reducing equivalents. Using an initial low nutrient medium pH in order to improve the final ethanol titer decreases the cell mass concentration, which might then also decrease the productivity of metabolites. In order to overcome this, some researchers tried to use two stage bioreactors with operating conditions that support growth in the first bioreactor and with the second reactor with reduced pH and conditions that are favorable for ethanol production (Mohammadi et al., 2012; Richter et al., 2013). Here, besides using two reactors in series, another alternative might consist in switching the pH from high (growth conditions) to low (solventogenesis conditions) values. During the fermentation at pH 4.75, the production of acetic acid was not observed and furthermore, the acetic acid initially present in the inoculum was immediately consumed during the experiment. This happens, as discussed above, through the activity of the enzyme AFOR that converts acetic acid to acetaldehyde and latter to ethanol through an alcohol dehydrogenase (ADH) (Wang et al., 2013). Acetic acid production along with biomass growth and later partial acid conversion to ethanol was recently observed in some studies (Liu et al., 2014). However, to the best of the authors knowledge, there is no previous study that

reported carbon monoxide or syngas fermentation using wild type bacteria without any production or accumulation of acetic acid at all at the end of the fermentation process.

4. Conclusions

In *C. autoethanogenum*, the addition of selenium and/or vitamins did no improve the ethanol/acetic acid ratio compared to a control medium without such additions. Furthermore, it clearly appears that the presence of tungsten improved ethanol production by *C. autoethanogenum*. Enhanced 2,3-butanediol/acetic acid ratio was also obtained with the presence of tungsten, but not with selenium. Results from the bioreactor studies with continuous CO supply revealed that the presence of tungsten together with a shift from high (pH 6) to low pH (pH 4.75) improves ethanol production by *C. autoethanogenum* without any accumulation of acetic acid.

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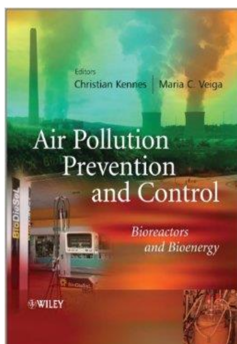

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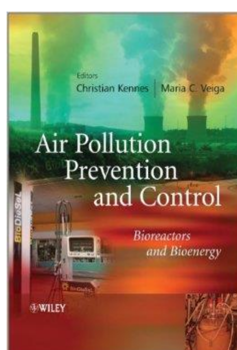

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