INSIGHTS INTO KEY PARAMETERS FOR BIO-ALCOHOL PRODUCTION IN SYNGAS FERMENTATION USING MODEL CARBOXYDOTROPHIC BACTERIA

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DOCTORAL THESIS

Insights into key parameters for bio-alcohol production in syngas fermentation using model carboxydotrophic bacteria

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2016

Doctoral program in Water Science and Technology

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That this doctoral thesis entitled "***Insights into key parameters for bio-alcohol production in syngas fermentation using model carboxydotrophic bacteria***", that Sara Ramió Pujol submitted to obtain the doctoral degree from Universitat de Girona, has been completed under their supervision and meets the requirements to opt for the International Doctor mention.

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Girona, 2016

La ciència segueix el mateix cicle que l'aigua. Hem de conèixer unes muntanyes nevades perquè l'aigua que se'n vingui quan aquestes es desglacin ens portin uns nous objectius. Necessitem que aquesta aigua comenci a brollar per la posterior formació del riu per dur a terme els experiments necessaris. Una vegada el riu ja hagi nascut, nosaltres també tindrem els nostres resultats que, imitant a un riu, tindran moments crítics (de sequera), resultats que costen entendre (ràpids) i alguns una mica rebuscats (meandres). Trobar les explicacions a aquests resultats és alliberador, de la mateixa manera que un riu desemboca el mar. Les conclusions que se'n deriven venen representades per la mateixa aigua que s'evapora a alta mar per formar uns núvols nous que descarregaran neu a dalt d'una muntanya per reprendre el cicle. Igual que una altra persona encaminarà la seva recerca amb les referències que deixem al nostre pas.

Agraïments/Acknowledgements

Sou moltes les persones que heu passat per la meva vida durant aquests procés i que hi heu deixat petjada. Primer de tot haig d'agrair a les persones que han fet aquesta tesi possible.

Catxo has estat una de les primeres persones que ha confiat i apostat per mi. M'has "cuidat" moltíssim i això ho valoraré sempre. Per totes les estones que hem passat, pels riures, per tot el que m'has arribat a ensenyar, per ser sincer, per donar-me confiança i per mil coses més, em quedo curta amb un Gràcies.

Jesús C, Gràcies per haver-me donat l'oportunitat, per haver-me obert les portes d'un món totalment nou per mi, per portar-me de congrés, per procurar per mi, per les nostres converses moltes vegades reveladores i per haver confiat amb mi.

Ramon, t'haig d'agrair tota la feina feta, sé que hi has posat molt de temps. També vull agrair-te tots els bons moments i dir-te que valoro tot el que has fet per mi.

A tots els companys de feina que he tingut, que no són pocs. Ari, tu la primera, has estat allà i t'has preocupat per mi des del meu primer dia de pràctiques en empresa. Tinc un carinyo especial a totes les hores de conversa que hem tingut, ja fos al laboratori, relaxant-nos després d'un congrés o fora de la feina. Mireia L, hem compartit moltes coses i també molts moments, m'encanta que tinguem "telepatia" i els mateixos gustos musicals. Elena, han estat moltes hores, molts trajectes, molta xerrameca, molta confiança i molts riures. Imma, m'agrada la teva energia, fins i tot en els teus mals moments desprens bones vibracions. A la resta de micros també tinc coses a dir-vos, he gaudit molt de la vostra companyia i després de cada un de vosaltres: Carla C, Júlia C, Àlex, Mireia F, Rosalia, Marga, Marc LL, Tri, Arantxa, Anna P, Gela, Laia, Olga, Sergi i Jess. També a en Bo, m'encanta el teu sentit de l'humor; en Gich, en Xavi V, en Carles A i en Jesús G.

No em puc oblidar del LEQUIA. Pau B, no sé perquè però des del primer moment les nostres converses eren molt necessàries i alliberadores. Alba C, sempre has estat allà amb un somriure i amb positivisme, no canviïs. Sebas, ets molt gran. Tampoc puc oblidar-me d'en Narcís, de l'Anna V, l'Elena T, en Tiago, l'Eric, en Ramiro, l'Alexandre, la Teresa, la Gemma R, l'Alba A, en Jordi G, en Tico, en Maël, la Patricia G... i tota la resta que no sou pocs.

També hi ha moltes altres persones de la universitat de qui no vull oblidar-me i amb qui he passat bons moments, les hores de dinar, converses al despatx, moments de procrastinació... Pau B; Sandra F; Roger V; Roger P; Maño, des del màster fins ara són una colla d'anys, però continuo sense cansar-me del teu sentit de l'humor, no el perdis mai; David D, se't troba a faltar, ara ningú vol cantar amb mi a l'hora de dinar; Dani; Pau C; Laia P; Vicky; Vero; Anna V; Marta P; Marta G; Mariona; Pere; Montse; Santi, podemos con todo; Cris; Dolors; Clara; David S i Gloria. No m'oblido de tu Txell, hem passat moltes coses i encara espero passar-ne moltes més.

Haig, també, de donar les gràcies al meu exèrcit d'estudiants, tots, qui més qui menys, han col·laborat en aquesta tesi: Eric A, Txell F, Sandra B, Eduard P, Marc P, Clara D, Andrea P, Cristina B, Marina G, Ester C i Gregori M.

I would also like to thank to Professor Miriam Rosembaum to host me in her lab. To Bastian, Babo, I had a great time with you in the lab. To all the people in my research group when I was in Aachen: Simone, Salome, Erik, Tatjana, Ivan, Malte, Thomas, and Carola. Carola, you made it a great and funny experience! Thank you for everything! And of course, I also have to thank to Kerstin, Win Jing, and Henrick to be always there. To you, Christoph L. To Mathias, Eik, Elena, Bernd, Jannis, Timo, Dario, Alana, Patrick, Hellen, Promi, Elvira, Hamed, Fritz, and company! Till, pase grandes momentos contigo, fuiste un gran amigo y apoyo para mí, aunque nuestras celebraciones se echan de menos aun nos quedan muchas que hacer. Àngels vas ser la meva salvació en un món desconegut! Moltes gràcies a tots!

Però aquesta tesi també la vull dedicar i agrair a molta gent de fora de la universitat que m'ha donat molt recolzament.

A les "Xurris", tot i que últimament hagi estat monotemàtica m'heu fet més suport que mai. Laura, admiro la força que tens; Carla, gaudeixo molt de les nostres estones, encomanes la teva alegria; Gemma N, sento una gran complicitat amb tu; Gemma M, desprens tranquil·litat i positivisme; i Maria, la teva energia no deixa a ningú indiferent.

A les meves princeses del màster la Maria R i la Gina O, amb vosaltres la vida és de color rosa.

A les meves nenes d'Edinburgh, d'això ja fa temps però sovint penso en vosaltres, en les diferents anècdotes que vam passar i m'apareix un somriure a la boca. Ho recordo amb carinyo i nostàlgia i us vull donar les gràcies a totes vosaltres: Lucy, Montse, Maria B., Laura C. i

especialment a tu Laura F, espero que siguis a on siguis ens tornem a veure. Ellen, I could not forget you, thanks to you too, for your entertained classes.

A l'Elisabeth T i la Laura S, sense vosaltres les meves cervicals haguessin guanyat la batalla.

Avi, m'agradava molt venir-te a veure els dissabtes al matí, recordo moments molt especials amb tu, sobretot abans de marxar d'estada i quan vaig tornar. Sé que si va anar bé, va ser gràcies a tu.

I finalment, a la meva família més propera. A Lydia i al Nen perquè les estones que passem junts m'agraden i em relaxen. Papa, sé que alguns dies no ha estat fàcil aguantar-me però això ens passa perquè ens assemblem massa, gràcies Ramirin. Mama, aconsegueixes il·luminar-me els dies més foscos amb una de les teves abraçades. Diuen que la família no la tries, si a mi m'haguessin deixat, no ho hagués pogut fer millor. Vosaltres sou la meva loteria.

This thesis was financially supported by the Catalan Government (2013 FI-DGR – ref33, pre-doctoral grant), the University of Girona (MOB2014-ref27, mobility grant), and the Spanish Ministry through the following projects: CTQ2014-53718-R and CTM2013-43454-R). LEQUIA and EcoAqua have been recognized as consolidated groups by the Catalan Government with code SGR-1168 and SGR-484, respectively.

List of publications

Some results of this thesis has been published or submitted to ISI journals. Peer reviewed paper publications obtained as parts of this PhD thesis are listed below:

1. **Ramió-Pujol, S.;** Ganigué, R.; Bañeras, L.; Colprim, J., 2014. Impact of formate on the growth and productivity of *Clostridium ljungdahlii* PETC and *Clostridium carboxidivorans* P7 grown on syngas. *International Microbiology* 17: 195-204. DOI: 10.2436/20.1501.01.222
   Biotechnology and Applied Microbiology Q3.

2. **Ramió-Pujol, S.;** Ganigué, R.; Bañeras, L.; Colprim, J., 2015. How can alcohol production be improved in carboxydotrophic clostridia? *Process Biochemistry* 50 (7): 1047-1055. DOI: 10.1016/j.procbio.2015.03.019
   Chemical Engineering Q1. Review written by editors' invitation.

3. **Ramió-Pujol, S.;** Ganigué, R.; Bañeras, L.; Colprim, J., 2015. Incubation at 25 ºC prevents acid crash and enhances alcohol production in *Clostridium carboxidivorans* P7. *Bioresource Technology* 192: 296-303. DOI: 10.1016/j.biortech.2015.05.077
   Biotechnology and Applied Microbiology Q1. Paper awarded as a cover in this volume.

4. Ganigué, R.; **Ramió-Pujol, S.;** Sánchez, P.; Bañeras, L.; Colprim, J., 2015. Conversion of sewage sludge to commodity chemicals via syngas fermentation. *Water Science & Technology* 72.3: 415-420. DOI: 10.2166/wst.2015.222
   Environmental Sciences Q3.

Chapters of this PhD submitted as journal article are listed below:

5. **Ramió-Pujol, S.**; Ganigué, R.; Bañeras, L.; Colprim, J., 2015. Media supplements are not essential for the growth and production of *Clostridium carboxidivorans* P7 on syngas fermentation. Research note (Unpublished).

6. **Ramió-Pujol, S.**; Ganigué, R.; Bañeras, L.; Colprim, J., 2015. Inhibition effects of ethanol and butanol on growth of three alcohol-producing model carboxydotrophic bacteria. Original research (Unpublished).

List of abbreviations

ABE	acetone-butanol-ethanol
au	Absorbance units
ACS	acetyl-CoA synthase
ADH	Alcohol dehydrogenase
ADHE	Acetaldehyde/ethanol dehydrogenase
AOR	Aldehyde:ferredoxin oxidoreductase
BCR	bubble column reactor
BES	bioelectrochemical systems
CoA	coenzyme A
CODH	carbon monoxide dehydrogenase
CSE	cotton seed extract
CSL	cotton steep liquor
CW	Constructed wetland
DGGE	denaturing gradient gel electrophoresis
DMP	Duck manure pond
Fd	ferredoxin
FDH	formate dehydrogenase
FTP	Fischer-Tropsch process
FW	fresh weight
H_2ASE	hydrogenase
HydABC	bifurcating hydrogenase
IC50	Half inhibitory constant
MBBR	moving bed biofilm reactor
MBR	membrane reactor
MES	2-(N-morpholino)ethanesulfonic acid
PCR	Polymerase chain reaction
RCM	Reinforced Clostridia Media
Rnf	Ferredoxin:NAD reductase
RM	Rabbit manure
SM	Sheep manure from a long term storage deposit
SMSR	Submerged membrane supported bioreactor
STR	Stirred tank bioreactor
syngas	synthesis gas

TF Trickling filters

THF Tetrahydrofolate

TL Temporal lagoon

UA Undissociated acids

WLP Wood Ljungdahl pathway

YE Yeast extract

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SUMMARY

Alcohols, such as ethanol and butanol, are non-conventional biofuels that can be obtained from bacterial fermentation. Carbon sources for microbial conversion into alcohols include organic compounds, mainly carbohydrates, and inorganic compounds, CO and CO_2, as preferred substrates. Inorganic carbon, in the form of synthesis gas (syngas), can be obtained from a large series of urban and agricultural wastes via gasification. Syngas can be subsequently fermented to biofuels by carboxydotrophic bacteria, which are able to use CO_2/H_2 or CO as the sole carbon and reducing power sources for growth and maintenance. Inorganic carbon is fixed through the Wood-Ljungdahl pathway, a non-cyclic reaction that converts either CO or CO_2+H_2 into acetyl-CoA. Acetyl-CoA can be used as a precursor of cellular biomass or further converted into organic acids and alcohols, depending on the metabolic demand of the cell. Therefore, imbalances in cell metabolism may lead to increase or decrease formation of desired products. Alcohols and acids can be used either as biofuels or feedstock chemicals for many industrial processes. In this light, the control of carboxydotrophic clostridia metabolism is one of the most challenging scientific ambitions nowadays.

The present thesis focuses on identifying key factors that control alcohol production in syngas fermentation by carboxydotrophic clostridia. For that, we have used three carboxydotrophic bacteria: *Clostridium ljungdahlii* PETC, *Clostridium carboxidivorans* P7, and *Butyribacterium methylotrophicum* DSM3468; in syngas lab-scale fermentation experiments in batch conditions.

We observed that formate, as a partially reduced carbon source, has the potential to reduce the hydrogen/CO demand for formate dehydrogenase activity in the Wood-Ljungdahl pathway in *C. ljungdahlii* PETC cultures. The results show the ability of *C. ljungdahlii* PETC to use formate diminishing the energy demand of the cell, and enhancing strain productivity. This is of interest from the biotechnological point of view as the ability of *C. ljungdahlii* PETC to use formate opens up potential upgrading of carboxydotrophic fermentation using additional soluble carbon supplies.

Besides, our results showed that the optimal conditions for carboxydotrophs growth 37 ºC may not be desirable for alcohol production. The fast accumulation of organic acids, specifically

acetate, during the exponential growth phase often results in the end of alcohol production and cell growth due to a phenomenon known as acid crash. Our results proved that sub-optimal incubation temperatures prevented acid crash, minimizing any adverse effect of organic acid accumulation on *C. carboxidivorans* P7 growth. Additionally, low temperatures increased gas solubility and enhanced the alcohol production of *C. carboxidivorans* P7.

Finally, although alcohols are mainly produced during the stationary phase, several studies have reported their production during the growth phase. This thesis investigated the inhibitory effects of ethanol and butanol on the growth of *C. ljungdahlii* PETC, *C. carboxidivorans* P7, and *B. methylotrophicum* DSM3468, showing a higher sensitivity to butanol than ethanol for the three strains. However, inhibitory thresholds are well above current product concentrations and therefore tackling alcohols inhibition does not seem to be an urgent need of syngas fermentation.

Taking all the results together, we provided new insights at the cell physiology level, which could help to improve syngas fermentation and its full-scale application. Although all experiments were conducted using lab-scale reactors, most results are directed to central aspects of cell physiology and can be adopted for future considerations of industrial-scale fermentations. Nevertheless, despite the advantages found in this technology, we must be aware that the physiological state of the inoculum cells is an important issue that must be controlled to ensure an effective fermentation. Although not studied in detail here, inoculum maintenance and control appears as a determinant factor determining alcohol production and must be studied in the future.

RESUM

Els alcohols, com l'etanol i el butanol, són biocombustibles poc tradicionals que poden obtenir-se mitjançant la fermentació bacteriana. Existeixen diferents fonts de carboni les quals poden ser utilitzades pels microorganismes per a la seva conversió a alcohols, majoritàriament parlem de carbohidrats, tot i que també ens trobem davant d'alguns compostos inorgànics com el CO i el CO_2. El carboni inorgànic en forma de gas de síntesi es pot obtenir mitjançant la gasificació d'una gran varietat de deixalles tant urbanes com agrícoles. El gas de síntesis pot ser fermentat a biocombustibles mitjançant bacteris carboxidotròfics, els quals són capaços d'utilitzar CO_2/H_2 o CO com a única font de carboni i poder reductor pel seu creixement i manteniment. El carboni inorgànic es fixa a través de la ruta metabòlica Wood-Ljungdahl, una ruta no cíclica que converteix tan el CO com el CO_2 + H_2 en acetil-CoA. Aquest metabòlit intermediari pot ser utilitzat com a precursor de la biomassa cel·lular o convertir-se en alcohols o àcids orgànics, depenent de la demanda metabòlica de la cèl·lula. Per això, el desequilibri del metabolisme cel·lular pot portar a augmentar o disminuir la formació dels productes desitjats. Els alcohols i els àcids es poden utilitzar com a biocombustibles, a més de matèria primera per processos químics industrials. Així doncs, el coneixement i control del metabolisme de clostridis carboxidotròfics és una de les ambicions científiques més desafiant avui dia.

Aquesta tesi s'enfoca en la identificació dels factors claus que poden potenciar la producció d'alcohols durant la fermentació de gas de síntesi feta per clostridis carboxidotròfics. Per aconseguir-ho, vam dissenyar diferents experiments en "batch" a escala de laboratori, on la fermentació de tres soques de clostridis carboxidotròfiques: *Clostridium ljungdahlii* PETC, *Clostridium carboxidivorans* P7 i *Butyribacterium methylotrophicum* DSM3468 hi tenia lloc en condicions autotròfiques.

Vam observar que el formiat, com a font de carboni parcialment reduïda podia disminuir la demanda d'hidrogen o CO per l'activitat de la "formiat deshidrogenasa" que té lloc dins la ruta metabòlica de Wood-Ljungdahl en els cultius de *C. ljungdahlii* PETC. Els resultats mostren l'habilitat de *C. ljungdahlii* PETC d'utilitzar formiat disminuint la demanda d'energia necessària pel creixement i potenciant la productivitat de la soca. Aquest fet és d'interès des del punt de vista

biotecnològic ja que l'habilitat d'aquesta soca en vers la utilització del formiat potencia el procés de fermentació carboxidotròfica amb una font de carboni addicional.

A més a més, els nostres resultats demostraven que les condicions òptimes pel creixement dels bacteris carboxidotròfics (37 ºC), poden no ser les desitjades per la producció d'alcohols. La ràpida acumulació d'àcids orgànics, especialment l'acetat, durant la fase exponencial de creixement sovint acaba amb la producció d'alcohols i el creixement per culpa d'un fenomen conegut com "acid crash". Els nostres resultats mostren que incubar els bacteris a temperatures més baixes prevé l' "acid crash", minimitzant qualsevol afecta advers provocat per l'acumulació d'àcids orgànics en el creixement de *C. carboxidivorans* P7. S'ha d'afegir que baixes temperatures augmenten la solubilitat dels gasos i potencien la producció d'alcohols de *C. carboxidivorans* P7.

Finalment, encara que els alcohols es produeixin majoritàriament a la fase estacionaria, molts estudis han descrit la seva producció durant la fase de creixement. Aquesta tesi ha investigat els efectes inhibitoris de l'etanol i el butanol en el creixement de *C. ljungdahlii* PETC, *C. carboxidivorans* P7 i *B. methylotrophicum* DSM3468. Les tres soques van mostrar una sensibilitat més elevada cap al butanol que cap a l'etanol. No obstant, els límits inhibitoris estan molt per sobre de la concentració d'alcohols produïda actualment i, ara mateix, la inhibició dels alcohols no sembla ser una necessitat urgent a la qual fer front.

Resumint, hem aportat nous coneixements a nivell de la fisiologia cel·lular que poden ajudar a millorar la fermentació del gas de síntesi i les seves aplicacions a escala real. Encara que els experiments es van fer a escala de laboratori, molts dels resultats obtinguts són dirigits a aspectes fisiològics de la cèl·lula i poden adoptar-se en processos industrials. Tot i això, malgrat els avantatges d'aquesta tecnologia, hem d'estar al cas que l'estat fisiològic de les cèl·lules de l'inòcul és un tema important i que s'ha de controlar per assegurar una fermentació exitosa. Encara que no ha estat estudiat en detall en aquesta tesi, el manteniment i control de l'inòcul sembla ser un factor determinant que governarà la producció d'alcohols durant la fermentació i s'ha d'estudiar en el futur.

RESUMEN

El etanol y el butanol, entre otros alcoholes, son biocombustibles poco tradicionales que pueden obtenerse mediante la fermentación bacteriana. Existen diferentes fuentes de carbono que pueden ser utilizadas por los microrganismos para su conversión a alcoholes, mayoritariamente carbohidratos, aunque también pueden serlo compuestos inorgánicos como CO y CO_2. El carbono inorgánico en forma de gas de síntesis se puede obtener mediante la gasificación de una gran variedad de desechos urbanos y agrícolas. El gas de síntesis puede fermentarse a biocombustibles mediante bacterias carboxidotróficas, las cuales son capaces de utilizar CO_2/H_2 y CO como fuentes únicas de carbono y poder reductor para su crecimiento y mantenimiento. El carbono inorgánico se incorpora mediante la ruta metabólica Wood-Ljungdahl, es una ruta no cíclica que convierte el CO y/o CO_2 + H_2 en acetil-CoA. Este metabolito intermediario puede usarse como precursor de la biomasa celular o convertirse en alcoholes o ácidos orgánicos, dependiendo de la demanda metabólica de la célula. Por consiguiente, el desequilibrio en el metabolismo celular puede llevar a un aumento o reducción de la formación de los productos deseados. Los alcoholes y los ácidos se pueden utilizar como biocombustibles, además de materia prima para diferentes procesos químicos industriales. Así pues, el conocimiento y control del metabolismo de bacterias carboxidotróficas es una de las ambiciones científicas más desafiante de hoy en día.

Ésta tesis pone su atención en la identificación de los factores clave que pueden potenciar la producción de alcoholes durante la fermentación de gases de síntesis hechas por bacterias carboxidotróficas que pertenecen a la familia Clostridiaceae. Así pues, diseñamos diferentes experimentos en "batch" a escala de laboratorio, donde la fermentación de tres cepas carboxidotróficas: *Clostridium ljungdahlii* PETC, *Clostridium carboxidivorans* P7 y *Butyribacterium methylotrophicum* DSM3468 ocurría en condiciones autotróficas.

Observamos que el formiato, como fuente de carbono parcialmente reducida, tenía la capacidad de disminuir la demanda de hidrogeno o CO debido a la actividad de la "formiato deshidrogenasa" que sucede en la ruta metabólica Wood-Ljungdahl en los cultivos de *C. ljungdahlii* PETC. Los resultados muestran la habilidad de *C. ljungdahlii* PETC de utilizar formiato disminuyendo la demanda de energía necesaria para el crecimiento y potenciando la

productividad de la cepa. Estos resultados son de interés desde el punto de vista biotecnológico ya que la habilidad de esta cepa con lo que respeta a la utilización de formiato potencia el proceso de fermentación carboxidotrófica con una fuente de carbono adicional.

Nuestros resultados también demostraron que las condiciones óptimas de crecimiento de las bacterias carboxidotróficas (37 ºC) pueden no ser las deseadas para la producción de alcoholes. La rápida acumulación de ácidos orgánicos, especialmente el acetato, durante la fase exponencial del crecimiento a menudo termina con la producción de alcoholes y el crecimiento debido a un fenómeno conocido como "acid crash". Nuestros resultados prueban que incubar las bacterias a temperaturas más bajas impide el "acid crash", minimizando cualquier afecto adverso provocado por la acumulación de ácidos orgánicos en el crecimiento de *C. carboxidivorans* P7. Asimismo, a bajas temperaturas aumenta la solubilidad de los gases potenciando la producción de alcoholes en *C. carboxidivorans* P7.

Por último, aunque los alcoholes se producen mayoritariamente durante la fase estacionaria, varios estudios describieron su producción durante la fase de crecimiento. Esta tesis ha investigado los efectos inhibitorios del etanol y el butanol durante el crecimiento de *C. ljungdahlii* PETC, *C. carboxidivorans* P7 y *B. methylotrophicum* DSM3468. Las tres cepas mostraron una mayor sensibilidad hacia el butanol que hacia el etanol. No obstante, los límites inhibitorios se encuentran muy por encima de la concentración de alcoholes producida actualmente, así pues, la inhibición de los alcoholes no parece ser una necesidad urgente con la que trabajar hoy en día.

Conjuntamente, nuestros resultados contribuyen con nuevos conocimientos a nivel de fisiología celular que pueden ayudar a mejorar la fermentación de gas de síntesis y sus aplicaciones a escala real. Aunque los experimentos realizados se hicieron a escala de laboratorio, muchos de los resultados obtenidos son dirigidos a la fisiología de la célula y pueden adoptarse en procesos industriales. Aun así, a pesar de las ventajas de esta tecnología, debemos tener en cuenta que el estado fisiológico de las células del inoculo es un tema importante y se debe controlar para asegurar una fermentación exitosa. Y, aunque no se haya estudiado detalladamente en esta tesis, el mantenimiento y control del inoculo parece ser un factor determinante que dirigirá la producción de alcoholes durante la fermentación y se debe estudiar en el futuro.

INTRODUCTION:

Carboxydotrophic clostridia

Clostridia are well-known organisms to the scientific community. They have been identified as an ancient lineage that likely evolved in an early oxygen-free earth (Paredes et al., 2005), and have served as model bacteria for research on organisms living in "extreme" environmental conditions (i.e., high temperatures and strict anareobiosis). In addition, the potential infectious threat to humanity of some *Clostridium* species (i.e., *C. botulinum*, *C. tetani*, and *C. perfringens*, among others) has deepened our knowledge of the genus at the physiological and genetic level (Kubiak et al., 2012). Clostridia metabolism have been thoroughly studied and, whenever possible, harnessed to benefit humankind. As an example, the onset of the first industrial bioproduction of organic solvents (acetone and butanol), far in the late 1920s, would have never been possible without the use of clostridia (the "Weizmann organism", *C. acetobutylicum*) (Spivey, 1978).

However, until very recently, less attention has been paid to one of the most unique features of some clostridia, their ability to fix carbon using a non-cyclic metabolism, the acetyl-CoA pathway (described in depth in Section 1.2). Most clostridia have been isolated from organic matter rich environments, such as anaerobic sediments, animal manure, sewage or sludge (Liou et al., 2005; Tanner et al., 1993). Despite being abundant in organic matter rich environments, some isolates possess two desirable traits from an industrial perspective: the ability to grow in strict autotrophic conditions independently of light (non-photosynthetic metabolism), and the capacity to produce added-value compounds (i.e., acids and/or alcohols) as end-metabolites. Due to these and other key factors of their physiology, clostridia have recently raised significant industrial interest as potential platforms for the sustainable production of carbon neutral biofuels (Abubackar et al., 2011; Daniell et al., 2012). However, the successful production of alcohols in clostridia relies on controlling factors that govern the metabolic shift from acidogenesis (production of acids) to solventogenesis (production of alcohols), a mandatory physiological change for an effective production of added-value compounds at cellular level. The mechanisms governing this shift have been extensively investigated, especially in acetone–

butanol–ethanol (ABE) fermenting clostridia, but are far from being completely understood.

1.1 The need for a change in the energy model: alternatives involving *Clostridium* spp.

One key scientific challenge of the early 21st century is the development of new technologies that allow for the progressive transition from the current energy model, based on fossil fuels, to a more sustainable model, based on renewable and carbon-neutral fuels as energy sources. To date, many alternatives have been experimentally explored to achieve this goal, i.e., the generation of electricity from renewable sources, and the transformation of renewable feedstock materials into fuels. Among the latter, the use of biologically produced alcohols as substitutes for oil-based energy sources has achieved great acceptance in the transportation industry because the use of alcohols requires simple and economically feasible adaptation of existing engines. In this context, the utilization of clostridia for the production of ethanol and butanol, two of the most promising alternative biofuels (Farrell et al., 2006; Lee et al., 2008), has been extensively studied over the last three decades (Dürre, 1998; Holt et al., 1984; Jiang et al., 2014; Liou et al., 2005).

Initial efforts focused on production of "first generation biofuels", mainly obtained through biological transformations of starch, corn or molasses. Although successful, this approach relied on the utilization of food feedstocks, which resulted in a subsequent increase in crop prices, and reduced food security in developing countries. In recent years, there has been a growing interest in the development of a "second generation" of biofuels, which utilize non-food feedstocks, such as forestry, agricultural, and municipal solid wastes (McKendry, 2002; Puig-Arnavat et al., 2010). However, the direct fermentation of these substrates into biofuels is critically hampered by the low conversion efficiency of cellulose and lignin into readily fermentable substrates (Naik et al., 2010).

An alternative to circumvent the low biological degradation efficiencies of cellulose- and lignin-enriched wastes is the syngas platform. This process relies on an initial gasification step of wastes into synthesis gas or syngas (a mixture of primarily CO, CO_2, and H_2), and the subsequent transformation of the gaseous carbon into organic acids and alcohols by carboxydotrophic acetogenic bacteria (Mohammadi et al., 2011). As of today, some international companies (i.e., Lanzatech and INEOS Bio, among others) are commercializing and licensing the syngas fermentation process for the production of ethanol and other added-value chemicals from various feedstocks (Daniell et al., 2012; Munasinghe and Khanal, 2010a).

Conversion of syngas into biofuels can also take place using the Fischer-Tropsch process (FTP). FTP was developed over 90 years ago and has been already applied into large scale processes from coal to liquid fuel conversion in South Africa (Tijmensen, 2002). It consists of a surface catalyzed polymerization process, in which hydrocarbons are produced from CH_x monomers generated by the hydrogenation of CO (Iglesia, 1997). Metal catalysts such as Co, Ru, Rh, and Fe have been used in FTP (Tirado-Acevedo et al., 2010). Compared to FTP, the conversion of syngas to biofuels via biocatalysts has several advantages, such as (1) low operating temperature and pressure, (2) high tolerance of toxic gas compared to chemical catalysts, (3) high specificity to substrate, and (4) independent of H_2:CO ratio.

Acetogenic bacteria use the acetyl-CoA pathway (also named as Wood–Ljungdahl pathway, WLP) for the reduction of CO_2 to acetyl-CoA, energy conservation and assimilation of CO_2 into cell carbon. Acetic acid is the main product of fermentation, although, under certain conditions, some bacteria can also produce significant concentrations of alcohols. To date, over 100 bacterial isolates, belonging to distantly related phylogenetic groups, are known to be acetogenic. Among them, only a few are able to reduce organic acids into alcohols and these mostly belong to class *Clostridia* (Drake et al., 2008) (Table 1.1).

Table 1.1 Carboxydotrophic clostridia able to ferment inorganic carbon CO_2/H_2 or/and CO to alcohols

Species	Isolation source	Alcohol produced	Reference
Acetogenium (Thermoanaerobacter) kivui	Lake Kivui	Ethanol	(Leigh et al., 1981)
Alkalibaculum bacchi	Livestock impacted soil	Ethanol	(Allen et al., 2010)
Butyribacterium methylotrophicum	Sewage digester sludge	Ethanol, butanol	(Grethlein et al., 1991; Zeikus et al., 1980)
Clostridium autoethanogenum	Rabbit feces	Ethanol	(Abrini et al., 1994)
Clostridium carboxidivorans **P7**	Agricultural settling lagoon sediment	Ethanol, butanol, hexanol	(Liou et al., 2005; Ramachandriya et al., 2013)
Clostridium glycolicum **RD-1**	Sea-grass roots	Ethanol	(Kusel et al., 2001)
Clostridium ljungdahlii	Chicken waste	Ethanol	(Tanner et al., 1993)
Clostridium methozybenzovorans	Olive oil mill waste water	Ethanol	(Mechichi et al., 1999)
"Clostridium ragsdalei P11"	Duck pond sediment	Ethanol, butanol	(Kundiyana et al., 2010a)
Eubacterium limosum	Sheep rumen	Ethanol, butanol	(Genthner and Bryant, 1982; Mohammadi et al., 2011)
Morella **sp. HUC22-1**	Mud from underground hot water	Ethanol	(Sakai et al., 2004)
Peptostreptococcus productus **U-1**	Anaerobic sewage digester sludge	Ethanol	(Lorowitz and Bryant, 1984)

1.2 Conversion of syngas to alcohols by clostridia

Acetyl-CoA is the end product of the Wood-Ljungdahl Pathway (WLP). Acetyl-CoA is a key metabolite in many anabolic and catabolic reactions in bacteria. Mainly, it can be further transformed into cell building blocks through successive incorporation of carbon atoms, or converted to fermentation end products that are excreted from the cell. The latter are of industrial interest because ethanol, butanol, and longer carbon-chain alcohols can be obtained.

1.2.1 Carbon fixation in carboxydotrophic clostridia

WLP is an example of non-cyclic carbon fixation pathway in which no intermediate metabolites are furnished for additional carbon assimilation. WLP is dispersed among phylogenetically distant groups and can function in both, the oxidative and reductive

directions. However, it only takes place under strictly anaerobic conditions (Mohammadi et al., 2011). In acetogens, the pathway is a terminal electron accepting process used in the reductive direction for energy conservation, as well as for autotrophic carbon assimilation into biomass and cell components (Drake et al., 2002; Ragsdale and Pierce, 2008).

Two separate branches, the Eastern or methyl branch and the Western or carbonyl branch, are present in WLP (Figure 1.1). In the methyl branch, one molecule of CO_2 is stepwise reduced to a methyl group at the expense of six electrons. The much shorter carbonyl branch catalyzes the incorporation of one mole of carbon monoxide. The latter is condensed with the methyl group formed in the methyl branch and coenzyme A (CoA) into acetyl-CoA (Abubackar et al., 2011; Ljungdahl, 1986) (Eq. (1)).

$$[CH_3\text{-}CFeSP] + CO + HS\text{-}CoA \rightarrow CH_3COS\text{–}CoA + [Co\text{-}E] \quad (1)$$

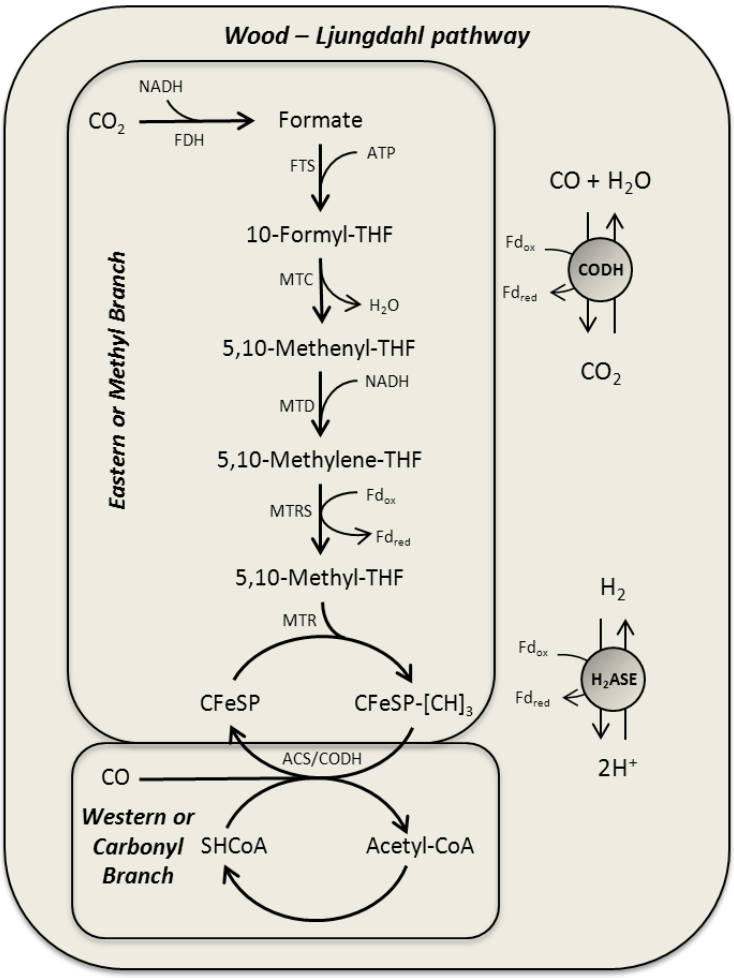

Figure 1.1 The Wood-Ljungdahl pathway. Abbreviations of the different enzymes are: ACS, acetyl-CoA synthase; CODH, CO dehydrogenase; FDH, formate dehydrogenase; FTS, formyl-THF synthase; H_2ASE, hydrogenase; MTC, methenyl-THF cyclohydrolase; MTD, methylene-THF dehydrogenase; MTR, methyl transferase; and MTRS, methylene-THF reductase.

According to the currently available information on bacterial genomes, the genes encoding the methyl branch are present in many anaerobic bacteria (e.g., strict anaerobes, methanogens, and autotrophic sulfate reducers), although those genes do not form a contiguous operon in all strains, revealing the complex evolutionary history of this metabolic pathway. Contrarily, the genes coding for enzymes of the carbonyl branch, acetyl-CoA synthase (ACS), are unique to carboxydotrophic clostridia (Roberts et al., 1989). Acetyl-CoA synthase is a multifunctional enzyme that is also responsible for the reversible conversion of CO into CO_2, showing carbon monoxide dehydrogenase (CODH) activity (Ragsdale and Pierce, 2008). Therefore, the enzyme complex is often referred to as ACS/CODH. CO oxidation is coupled to the transfer of electrons to ferredoxin (Fd) and constitutes the unique source of reducing equivalents in the absence of H_2 (Ragsdale, 2004; Ragsdale et al., 1983) (Eq. (2)).

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

Acetyl-CoA is further oxidized to acetate in a two-step reaction involving a phosphotransacetylase and an acetate kinase. The latter is responsible for the main reaction from which carboxydotrophic clostridia derive energy in a substrate level phosphorylation reaction. However, no net ATP gain is obtained in WLP because one molecule of ATP is consumed to activate formate. As the synthesis of acetate from two moles of CO_2 or CO enables the growth of acetogens, the pathway must be coupled to net ATP formation, which is accomplished by ion gradient-driven phosphorylation. Chemiosmotic energy conservation couples an exergonic reaction (in most cases, an electron-transfer reaction) to the translocation of ions, which results in the generation of an ion gradient across the membrane, driving ATP synthesis by a membrane-bound ATP synthase (Schuchmann and Müller, 2014). Three different mechanisms for chemiosmotic driven energy conservation have been proposed in model carboxydotrophic microorganisms, namely: cytochrome containing, H^+-gradient dependent organisms (*Moorella thermoacetica*) (Muller, 2003); cytochrome free, Na^+-dependent acetogens (*Acetobacterium woodii*) (Heise et al., 1992); and no cytochrome, no sodium dependent

organisms (*Clostridium ljungdahlii*) (Köpke et al., 2010). Although the dependence on an H^+ or Na^+ gradient holds true, recent advances and thorough inspection of the genome information of these three species predict fairly similar energy conservation mechanisms in most acetogenic bacteria (Schuchmann and Müller, 2014).

The main metabolic difference among acetogenic bacteria rely on the mechanisms that are involved in the formation of reduced compounds that may be coupled to the generation of an ion gradient. During autotrophic growth, the production of reduced NAD and ferredoxin is restricted to the oxidation of H_2 or CO. Although distinct protein structures have been elucidated for different microorganisms, in the presence of H_2, Fd^{2-}, and NADH are mainly produced by an electron bifurcation hydrogenase (HydABC), and three or four H_2 molecules are split into six or eight H^+. Electrons are concomitantly used for the simultaneous reduction of Fd and NAD. By contrast, in the presence of CO as the only source for reducing equivalents, Fd^{2-} formation is exclusively coupled to ACS/CODH activity (Ragsdale, 2004).

Reduced NADH and Fd^{2-} are essential for CO_2 reduction to either formate or CO, sequential reduction of the C group in the methyl branch, production of NADP in the cytoplasm, ion translocation (H^+ or Na^+) at the membrane bound complex, and biosynthesis requirements during the growth and maintenance of cellular structures. In clostridia, the Rnf redox-driven ion pump complex catalyzes electron transfer from Fd^{2-} to NAD^+ while protons are pumped out of the cell, compensating for the disequilibrium in Fd^{2-}/NADH from autotrophic growth at the expense of CO_2 + H_2. ATP is finally generated at a membrane bound ATPase (Schuchmann and Müller, 2014).

The use of hydrogen and carbon monoxide as sources of reducing equivalents is maximized during autotrophic growth. Therefore, economization of reducing power is an important factor to consider in such conditions. Owing to the first steps in the methyl and carbonyl branches of the WLP, formate synthesis and the carbon monoxide formation, are recognized as reducing equivalents sinks that may diminish the growth

capacity of cells and the conversion of inorganic carbon into valuable chemicals (Daniell et al., 2012). In this condition, the assimilation of a partially reduced carbon source, such as formate, has the potential to reduce the hydrogen/CO demand for formate dehydrogenase activity in the WLP (Brown et al., 2014; Schuchmann and Müller, 2013).

1.2.2 Phases of clostridial growth in the laboratory

Carboxydotrophic clostridial growth in the laboratory can be divided in the following three phases: first, exponential growth and the formation of acids; second, the transition to the stationary growth phase with a re-assimilation of acids and the concomitant formation of solvents; and third, the sporulation phase (Lütke-Eversloh and Bahl, 2011). Controlling the experimental conditions that govern the transition from the exponential growth phase (mainly acidogenic) to the stationary phase (mainly solventogenic) is essential to develop an effective syngas platform for biofuel production. Acidogenesis is linked to the generation of ATP via acetyl-phosphate (and butyryl-phosphate) dephosphorylation (Figure 1.2). Under favorable growth conditions, organic acids are the major end products; acetate production is favored over butyrate because it yields twice as much ATP per mole of acetyl-CoA and requires less than half of the reducing equivalents.

In growth limiting conditions (i.e., substrate limitation or acidification), bacteria transit from exponential growth to stationary phase. Alcohol producing strains activate solventogenesis during this transfer. Solventogenesis has two main physiological functions for the cell: first, it contributes to NAD recycling and second, avoids excessive acidification of the media. The production of one mole of ethanol from acetyl-CoA requires two moles of NADH. The same occurs for the production of butanol from butyryl-CoA. Provided that, in autotrophic growth conditions, CO and/or H_2 are the only sources of reducing equivalents, special care must be taken to ensure that those substrates have not been exhausted before the solventogenic phase commences.

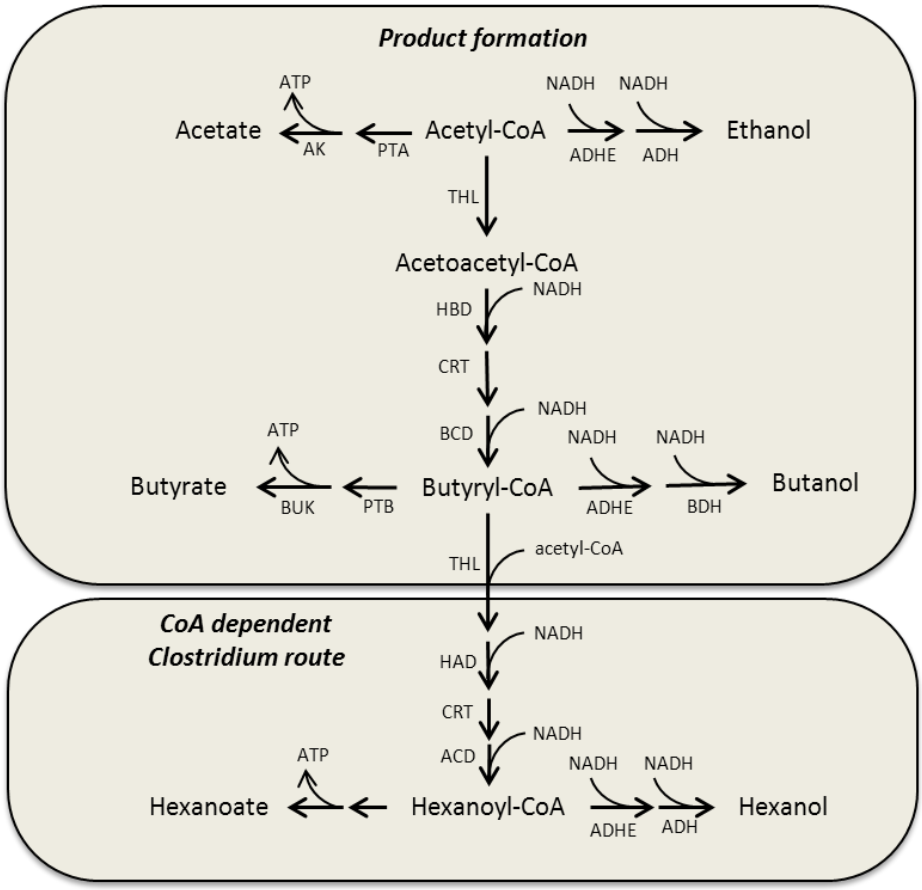

Figure 1.2 Acetyl-CoA reductive pathway and CoA dependent *Clostridium* route. Abbreviations of the different enzymes are as follows: ACD, acyl-CoA dehydrogenase; ADH, alcohol dehydrogenase; ADHE, aldehyde/alcohol dehydrogenase; AK, acetate kinase; BCD, butyryl-CoA dehydrogenase; BDH, butanol dehydrogenase; BUK, butyrate kinase; CRT, crotonase; HAD, 3-hydroxyacyl-CoA dehydrogenase; HABD, 3-hydroxybutyryl-CoA dehydrogenase; PTA, phosphotransacetylase; PTB, phosphotransbutyrylase; and THL, thiolase.

To date, little is known about the regulatory circuits and molecular mechanisms for the transition to the solventogenesis, and it is not yet possible to draw a detailed picture on the regulation of solvent production in carboxydotrophic clostridia. The transcriptomic profiling of *Clostridium beijerinckii* NCIMB8052 at different times of the growth curve revealed a differential expression between the genes related to acetic and butyric acid formation compared to those genes participating in alcohol production (Wang et al., 2012). Although no such data exist for carboxydotrophic clostridia thus far, the results obtained for heterotrophic clostridia suggest that different regulatory mechanisms may act in the acidogenic and solventogenic phases, opening new options for genetic manipulation of strains.

Sporulation is a common trait in most clostridia and, although less studied, it is also present in carboxydotrophic bacteria. Most information on how solventogenesis is affected during the sporulation phase comes from physiological studies of ABE fermenting clostridia. For example, in *C. acetobutylicum*, granulose synthesis and sporulation initiation are regulated through a quorum sensing dependent system (*agr* gene cluster), which has also been found in *Clostridium carboxidivorans* P7 and *Clostridium kluyveri*, among others (Steiner et al., 2012). Induced changes and knock-out mutations of the *agr* regulatory circuit do not seem to affect solvent production in *C. acetobutylicum*, when grown on sugar compounds (Steiner et al., 2012); similarly, solventogenesis related genes where not down-regulated during the sporulation phase in *C. beijerinckii* (Wang et al., 2012). Thus, based on what has been observed for species similar to carboxydotrophic bacteria, it can be hypothesized that solventogenesis and sporulation may co-occur during growth, but they may follow differential regulatory circuits. However, this is yet to be confirmed for carboxydotrophic clostridia.

1.2.3 Product spectrum

The main products of solvent-producing carboxydotrophic clostridia are two-carbon (C2) compounds, acetate and ethanol, although some strains have the capability to also produce four-carbon (C4) compounds, such as butyrate and butanol, or longer-chain (C6–C8) metabolites through the CoA-dependent *Clostridium* route. For instance, Ramachandriya and co-workers reported the production of 5.1 mM of hexanol and 3.6 mM of caproic acid in syngas fermentation using *C. carboxidivorans* (Ramachandriya et al., 2013). Longer chain alcohols (C6–C8) are interesting from the industrial standpoint because they can be used as valuable chemical feedstocks as well as alternative fuels (Machado et al., 2012). Nevertheless, their production in carboxydotrophic bacteria is rather uncommon, which is mainly due to the higher demand of reducing equivalents compared to those needed for the production of C2 and C4 compounds (Ramachandriya et al., 2013).

1.3 Factors affecting alcohol production

Operational variables not only impact cell growth, but also affect product yield and speciation. In this light, the key factors governing alcohol production in carboxydotrophic clostridia will be discussed in this section including operational conditions, media composition, and the physiological state of the cells.

1.3.1 Medium composition

The medium composition has a substantial impact on growth and product formation in carboxydotrophic clostridia. Carbon and reducing equivalent sources for autotrophic growth are usually restricted to CO_2, CO and H_2, and their impact on the energy yield and, consequently, product formation will be further discussed in Section 1.3.4. However, other components of the culture media, such as the inorganic salts content (nitrogen and phosphate), trace elements (mainly metal ions), and the presence of enzyme cofactors, also have an effect on growth and, more importantly, on product speciation in clostridia.

Nitrogen is a key nutrient for life. It is usually taken up by clostridia in the form of inorganic nitrogen. Initial descriptions of *C. ljungdahlii* growth conditions revealed that the limitation of nitrogen sources increased the relative ethanol formation and minimized growth (Barik et al., 1988; Klasson et al., 1992). Saxena and Tanner studied the impact of different concentrations of ammonia on syngas-fermenting "Clostridium ragsdalei" cultured in a medium containing corn steep liquor (Saxena and Tanner, 2012). The experimental results showed that high concentrations of ammonia (93.4 mM) did not have any effect on ethanol production. In contrast, productivity was diminished by 40% in the absence of ammonia. It is important to bear in mind that corn steep liquor contains organic nitrogen, which might have been used by "C. ragsdalei" in the reported experimental conditions. To the best of our knowledge, the effect of nitrogen salts in completely autotrophic conditions, which is in the absence of any source of organic matter, has yet to be established. In another study, Cotter *et al.* examined the effect of

various nitrogen sources on the ethanol and acetate production in non-growing cultures of *Clostridium autoethanogenum* and *C. ljungdahlii* (Cotter et al., 2009). Nitrogen was required for proper culture maintenance over a long time period; however, nitrogen limitation did not have a significant impact on the product formation in resting cultures. In this light, the use of complex organic nitrogen sources, i.e., yeast extract and protein extracts, in media formulations seems to provide stable cultures for longer times.

The availability of other inorganic ions, such as sodium, may be crucial to certain acetogens, especially those harboring a Na^+-dependent translocation ATPase for energy generation. The presence of sodium may affect the growth and final cell density by regulating energy conservation, projecting an effect on solvent production. Most media formulations contain approximately 35 mM sodium, and a negative effect on end-products formation has only been observed at concentrations lower than 5 mM (Heise et al., 1989). As expected, the negative effect is less pronounced in strains that do not rely on Na^+-dependent ATPase for energy generation; for those, the sodium concentration can be lowered to 1.4 mM without significant effects (Köpke et al., 2010).

Most key enzymes of the acetyl-CoA pathway, including ACS/CODH, formate dehydrogenase (FDH), hydrogenase (H_2ASE), and alcohol dehydrogenase (ADH), are metalloproteins that contain essential metal cofactors, such as nickel, iron or zinc (Ragsdale and Pierce, 2008). Saxena and Tanner studied the effect of multiple trace elements on growth and ethanol production in "C. ragsdalei" and showed that an increased concentration of iron, nickel, zinc, selenium, and wolfram salts resulted in higher activities of CODH, H_2ASE, and FDH (Saxena and Tanner, 2011), leading to an enhanced growth rate (44.1%) and ethanol production (up to 425.6%). Copper had the opposite effect, and its presence in the medium reduced ethanol production by 40%, which was most likely to the negative effect on acetyl-CoA synthase activity (Saxena and Tanner, 2011). The work of Adams *et al.* reported on cobalt limitation as a way to favor ethanol production (Adams et al., 2010). Cobalt limitation hampers the transfer of a methyl group from THF to acetyl-CoA, reducing its function, affecting the NAD(P)H to

NAD(P) ratio and, subsequently, enhancing ethanol production. In a similar way, Huhnke and co-workers observed for a "C. ragsdalei" culture that solvent to acid ratio increased when all metals, except Mo, were eliminated from the medium (Huhnke et al., 2010). In the case of Fe and Ni elimination, there was close to a 300 and 400 percent increase in the ratio. The elimination of Co and Cu decreased acetate production, increasing the solvent to acid ratio, while Mo elimination increased acetate production. According to this, Phillip *et al.* reported one of the highest butanol and hexanol concentration produced by *C. carboxidivorans* when Cu was eliminated and Mo was increased ten folds in the media (Phillips et al., 2015).

The addition of complex vitamin solutions is required in some specific strains due to the vitamin limitation in those strains can lead to adverse growth conditions and induce a non-growing or resting cell state, which will shift metabolism to the solventogenic phase. However, the effect of trace compounds in media formulations has yet to be comprehensively studied.

1.3.2 Organic carbon sources

The addition of media supplements (i.e., yeast extract (YE), corn steep liquor (CSL) or cotton seed extract (CSE)) is a common practice in syngas fermentation experiments with carboxydotrophic bacteria. Media supplements are considered to be a source of micronutrients that may substitute or supplement the use of purified vitamin solutions (Kundiyana et al., 2010a; Maddipati et al., 2011; Ramachandriya et al., 2013). In addition, they contain a large variety of organic molecules (i.e., carbohydrates, amino acids, and nucleotides), which may be incorporated into biological structures as building blocks. Many experiments report on the impact of these supplements on alcohol production, although their effects are, in some cases, controversial (Abubackar et al., 2012; Gaddy and Clausen, 1992; Sato et al., 1992). Barik *et al.* (1988) and Gaddy and Clausen (1992) experimented on the effect of different YE concentrations on the product distribution during a batch fermentation using *C. ljungdahlii* and observed that the highest ethanol

concentration was achieved at the low YE concentration range (from 0 to 0.1 g/L). The same results were also obtained with *C. autoethanogenum* DSM10061 (Abubackar et al., 2012). The effect of YE on non-carboxydotrophic *Clostridium thermocellum* strains I-1-B, ATCC27405, and JW20 was tested in the range of 1 to 20 g YE/L, diminishing growth and ethanol productions at both low and high YE concentrations (Sato et al., 1992).

CSL and CSE have been preferred alternatives as additive in media for large-scale fermentation processes because those components are industrial by-products that can be obtained at reasonably lower market prices (Kundiyana et al., 2010a; Saxena and Tanner, 2012). Additionally, experimental studies have shown that most trace elements and vitamins needed for carboxydotrophic bacteria are contained in CSE formulas and can not only sustain cell growth almost completely, but they can also improve their productivity (Kundiyana et al., 2010a). The impact of CSL on alcohol production by carboxydotrophic bacteria was studied by Saxena and Tanner (2012), who observed that CSL can be used as an inexpensive source of nutrients for ethanol production from syngas fermentation by "C. ragsdalei". Experiments from Maddipati and co-workers using the same strain and conditions showed enhanced ethanol (31% increase) and butanol (7-fold increase) production in the presence of 2% (w/v) CSL compared to YE (Maddipati et al., 2011). Similarly, Kundiyana *et al.* (2010a) observed an increase of 28% in the ethanol production using low concentrations of CSE (0.5 g/L) compared to the use of standard media with YE in *Clostridium* strain P11 cultures. Finally, Ramachandriya *et al.* reported that CSL medium promoted the production of C4 and C6 alcohols and acids, while CSE medium supported ethanol formation with "C. ragsdalei" and *C. carboxidivorans* (Ramachandriya et al., 2013).

Actual research is performed with the presence of media supplements. However, it has never been reported that the use of these compounds is essential or indispensable during the syngas fermentation for most carboxydotrophic clostridia. Perhaps, the addition of media supplements is not a justified cost.

1.3.3 pH changes

Most researchers convey that pH is the most important factor regulating the metabolism and physiology of carboxydotrophic bacteria, including the shift between acidogenesis and solventogenesis. Acidification of the external medium due to acid production is crucial for regulating the cell physiological processes, leading to pH homeostasis, the establishment of transmembrane potential and the use of a proton-motive force (Mohammadi et al., 2011). As a result, changes in the outer pH affect by-product exportation and catabolic reactions, leading to changes in the product composition (Munasinghe and Khanal, 2010b). The lipophilicity of un-dissociated organic acids allows them to permeate through the cell membrane, resulting in an increase in the H^+ ions inside the cytoplasm. This can significantly affect the cell fitness because at low internal pH, the external pH plays a major role in keeping the cells at non-stress conditions (Mohammadi et al., 2011). In this light, at low external and internal pH, cells under stress may overcome the situation by re-assimilating acids and converting them into alcohols (Abubackar et al., 2012), which will require extra reducing equivalents. Under strict autotrophy; solventogenesis will basically rely on the CO or H_2 in the gas phase. Commonly, in most carboxydotrophs, the shift to solventogenesis occurs between pH 4.5 and 5.0 (Grethlein et al., 1990; Klasson et al., 1993). Abubackar *et al.* (2012) observed that an increase of the initial fermentation pH had a negative effect on the ethanol production of *C. autoethanogenum*, diminishing the concentration of alcohols in the broth. For instance, an increase in the pH from 4.75 to pH 5.75 decreased the final ethanol concentration by 38%.

Grethlein and co-workers (1991, 1990) developed a continuous fermentation system with cell recycling for the production of high concentrations of alcohols. The rationale behind this approach was that maintaining high cell densities in the reactor allowed mitigating the significant decrease of specific growth rates at pH lower than 6. In their study, the bioreactor was continuously operated with *Butyribacterium methylotrophicum* at

pH 5.5, producing concentrations of ethanol two folds higher and thirty times more butanol, than batch fermentation experiments conducted with the same strain at pH 6.0.

A sudden accumulation of organic acids in the media may lead to the rapid termination (or complete absence) of the solventogenic phase, which invariably causes cessation of substrate consumption and alcohol production. This phenomenon, named acid crash, has been widely described in typical solventogenic species (Maddox et al., 2000; Russell and Diez-Gonzalez, 1997), but it is hypothesized to also occur in carboxydotrophic strains (Mohammadi et al., 2014; Richter et al., 2013). Maddox *et al.* concluded that the accumulation of undissociated acids in the culture broth to over 57 mM could be responsible for acid crash events and low alcohol production in *C. beijerinckii* NRRLB592 (Maddox et al., 2000). However, despite being a commonly observed phenomenon, the specific clues that induce cultures to acid crash events cannot be generalized for all strains and growing conditions. Two methods have been explored to prevent the acid crash, both involving the minimization of the accumulation of undissociated acids as follows: (i) To provide reactors with pH control, maintaining relatively low concentrations of undissociated acids, regardless of the total concentration of organic acids (Maddox et al., 2000; Richter et al., 2013). (ii) To diminish the metabolic rates and prevent fast accumulation of organic acids outside the cells by conducting fermentations at suboptimal temperatures, such as at 28°C (Kashket and Cao, 1995). For both strategies, once solventogenesis has started, the control of process conditions, either pH or temperature, can be relaxed to enhance the solvent productivity.

1.3.4 Gaseous sources of carbon and reducing equivalents

The composition of syngas is also an important point to consider when aiming at alcohol production because it has been hypothesized that an excess of electrons in the culture medium favors solventogenesis (Abubackar et al., 2012; Hurst and Lewis, 2010). In carboxydotrophic bacteria, and especially in *Clostridium* species, ferredoxin acts as the principal electron carrier to ensure ion gradient generation at the membrane bound Rnf

complex. Reduced equivalents are derived from either CO oxidation, due to ACS/CODH activity, or from H_2, due to the activity of an electron HydABC. However, the two activities are unlikely to occur simultaneously at high rates due to the inhibition of hydrogenase by CO. In pure extracts of *A. woodii* hydrogenase, the protein activity was reduced to less than 50% even when trace levels of CO (7 nM) were present (Ragsdale and Ljungdahl, 1984).

Additionally, it is important to bear in mind that solubility of CO is almost ten times higher than that of H_2 (7.4 and 0.78 mmol/L·atm at 25◦C, respectively) (Sander, 1999). In this light, differences in the CO and H_2 content of the syngas will not have a proportional effect on the metabolic production and cell density (Abubackar et al., 2012), and they will likely play a role in the production of alcohols. For example, Hurst and Lewis (2010) observed an increase in the solvent production, from 1.7 to 2.6 g of ethanol per gram cell, in *C. carboxidivorans* P7 syngas fermentation, when the partial pressures of CO were increased from 1.35 to 2.0 atm.

1.3.5 Product inhibition

As commented above, the two major fermentation products of carboxydotrophic clostridia are organic acids and alcohols. An excessively high concentration of acids or alcohols may end up with a failure of culture growth and, therefore, a decrease in the solvent production. Section 1.3.3 described the acid crash phenomenon, caused by the fast accumulation of high concentrations of undissociated organic acids. On top of that, the toxic nature of solvents on bacteria is, also, one of the major limiting factors for industrial carboxydotrophic alcohol production. The accumulation of organic solvents has been shown to disrupt the cell membrane and inhibit cellular processes (Baer et al., 1987). Ethanol is known to fluidize the cell membrane, resulting in an increased diffusion of solutes in an out the cell. This uncontrolled transport may cause a disruption of the proton gradient across the membrane, which can lead to a leakage of important cofactors such as Mg^{2+} and the cessation of growth (Cartwright et al., 1986; Ingram, 1976). Ethanol

can also inactivate membrane and cytosolic enzymes. For example, ATPase and some glycolytic enzymes are inhibited by ethanol in a non-competitive manner (Banat et al., 1998; Ding et al., 2009; Ingram, 1976). Alcohols with longer carbon chains have further negative effects. Butanol can insert into the cell membrane and break hydrogen bonds between lipid tails increasing its toxicity as compared to ethanol (Ly and Longo, 2004). Some possibilities to ease toxic effects of alcohols on producing strains include in-line product extraction to detoxify alcohols of the environment where the cultures grow, or the continuous recycling of the cells culturing the bacteria in a fresh media (Gaddy et al., 2007; Phillips, Klasson, Clausen, & Gaddy, 1993). However, these approaches have also a negative impact on the overall economics of the process. Apart from these, genetic modification of microbial strains has the potential to improve the alcohol tolerance, as well as, other metabolic features according to the desired needs (Tomas et al., 2003).

1.4 Bioreactors for syngas fermentation

Different bioreactor configurations have been reported for its use in conversion of syngas to alcohols, including: conventional stirred tank bioReactors (STR), moving bed biofilm reactor (MBBR), bubble columns reactors (BCR), trickling filters (TF), and membrane reactors (MBR) (Figure 1.3). For efficient syngas fermentation, the selection of the bioreactor configuration is important for production purposes both at the lab and pilot scale (Table 1.2).

STRs have been widely used for alcohol production (Grethlein et al., 1990; Sakai et al., 2004). Mass transfer of gaseous substances to the bulk liquid is dependent on the rotation impeller. Despite STRs are easily maintained, their function require an energy input proportional to the reactor volume, significantly increasing costs that may hamper large-scale development. Continuous fermentation performed with *C. ljungdahlii* in a 13.5 L STR achieved the highest ethanol concentration (48 g/L) reporting the highest productivity of ethanol (3.57 g/L·h) when cell recycling was used in the STR (Phillips et al., 1993). Although in a smaller quimiostate and a low productivity (0.041 g/Lh) by

Butyribacterium methylotrophicum, butanol concentration was also among the highest value ever achieved (2.7 g/L) (Grethlein et al., 1991).

MBBR are characterized to retain biomass on an inert carrier supporting microbial growth and hindering biomass to flow out through the outlet (Hickey, 2009). The gas injection system in these reactors provides mixing by creating eddy currents in the fermentation broth. A study using an active culture of "C. ragsdalei" in a medium scale MBBR (36 m^3) reported an ethanol concentration of 30 g/L after 30 days of operation with a productivity of 2.2 g/Lh (Hickey, 2009).

Gas in BCRs is sparged in the bulk liquid and no mechanical agitation is needed. BCRs may allow satisfactory heat and mass transfer rates (Abubackar et al., 2011), although these may be limited by coalescence or back mixing of gaseous components, even at high flow rates (Acharya et al., 2015; Munasinghe and Khanal, 2010b). This technology was primarily developed for industrial use with large working volumes. Alcohol production by *C. carboxidivorans* P7 was observed in a 4 L BCR in a batch mode with a producer gas at production rates lower than 0.042 g/Lh, obtaining concentrations of 5.00 and 0.75 g/L of ethanol and butanol, respectively (Datar et al., 2004).

Trickling filters (TF) characterized by a continuous feed of a liquid trickling phase and the use of inert packing materials are commonly used for different gas treatments (Kennes et al., 2009). The main parameters influencing the mass transfer in TF are reactor packing and material size, liquid recirculation rate, and gas flow rate (Bredwell et al., 1999). In TF, plug flow is easily achieved and there is no need of mechanical agitation (Ahmed and Lewis, 2007). A study comparing the performance of continuous STR, BCR, and TF for syngas fermentation concluded that higher CO conversion rates (>80%) and higher productivities were achieved in a TF (Klasson et al., 1992).

In MBRs, microbial cells are attached to the membrane surface in the form of a biofilm. Syngas is injected into the hollow fiber and the process gas flows across the membrane toward the biofilm, enhancing the mass transfer. Different kinds of MBR

containing microporous layer membranes to support a biofilm on the outer surface have been described (Datta et al., 2009; Hickey et al., 2011; Tsai et al., 2011). One of the major disadvantages of this system is that the liquid may enter the pores owing to variation in pressure across the membrane, leading to a phenomenon known as pore-wetting. The use of microporous membranes with a biofilm of "C. ragsdalei" produced an ethanol concentration of 10 g/L after a 20 days continuous operation with an ethanol productivity of 0.1 g/Lh (Hickey et al., 2011). The productivity of ethanol by the same species was increased to 0.28 g/Lh, reaching ethanol concentrations of 13.3 g/L, when a sandwiched type combination of membranes having a liquid impermeable layer between two microporous membranes was used (Datta et al., 2009). Some other MBRs use hydrophilic membranes instead to separate the gas to the liquid phase. By using this approach "C. ragsdalei" presented the same productivity as in microporous membrane (0.05 g/Lh) (Hickey et al., 2010). Although the use of hydrophilic asymmetric membranes has also been tested for the ethanol production (4.2 g/L), its productivity reached values of 0.08 g/Lh using "C. ragsdalei" (Datta et al., 2010).

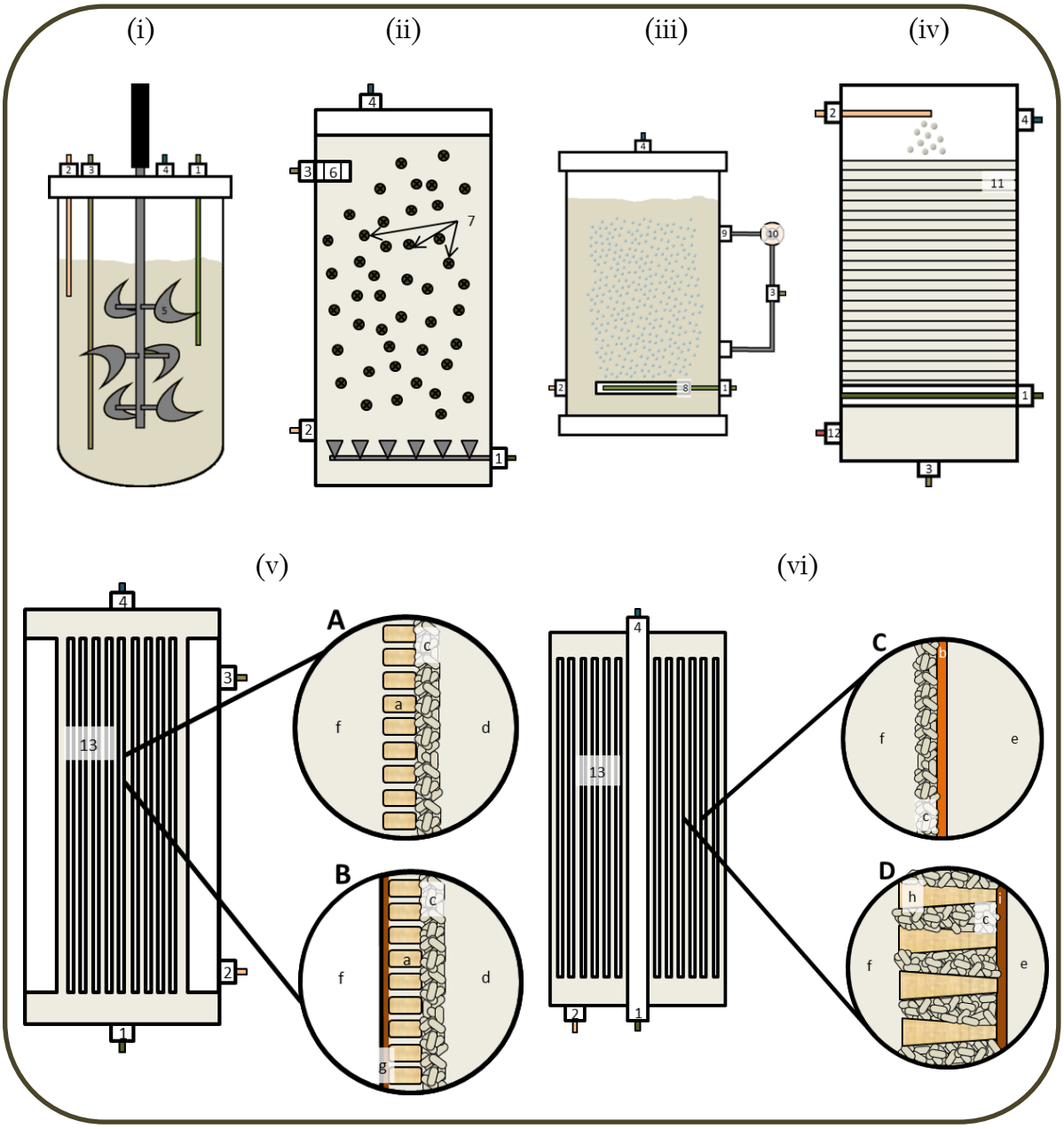

Figure 1.3 Schematic representations of STR (i), MBBR (ii), BCR (iii), TF (iv), and MBR (v-vi). Gas (1) and nutrient (2) feed into the reactor, liquid products from the reactor (3), gas outlet (4), agitator (5), carrier retainer (6), biomass retainer (7), sparger (8), recirculation (9), pump (10), packed bed (11), nutrient recirculation (12), and membrane modules (13). **A**: cross section of a microporous membrane present in modular membrane supported bioreactor and nutrient recirculation, **B**: cross section of microporous membrane present in membrane supported bioreactor, **C**: cross section of a hydrophilic membrane having biofilm growth on the membrane surface, **D**: cross section of an asymmetric membrane present in stacked array bioreactor and horizontal array bioreactor. a: microporous membrane, b: hydrophilic membrane, c: microorganism (biofilm), d: liquid phase, e: medium inlet and liquid products from the membrane, f: gas inlet to the membrane, g: liquid impermeable layer, h: biolayer, i: hydration layer. Adapted from Abubackar *et al* (Abubackar et al., 2011).

Table 1.2 Ethanol and butanol production using different bioreactors, bacteria, volume, and ethanol and butanol productivity and concentration using syngas fermentation.

Bioreactor	Bioreactor characteristics	Microorganisms	Culture volume	Productivity (g/Lh)		(g/L)		References
				Ethanol	Butanol	Ethanol	Butanol	
STR	Quimiostate	*Clostridium ljungdahlii*	1	3.570	na	48.00	(na)	(Phillips et al., 1993)
STR	Quimiostate	*Clostridium ljungdahlii*	2.0	0.049	na	6.50	(na)	(Mohammadi et al., 2012)
STR	Quimiostate	*Clostridium ljungdahlii*	2.5	0.650	na	26.00	(na)	(Gaddy et al., 2007)
STR	Quimiostate	Mix culture	3.3	0.088	0.017	8.0	(1.0)	(Liu et al., 2014)
STR	Quimiostate	*Butyribacterium methylotrophicum*	1.2	0.005	0.041	0.33	(2.7)	(Grethlein et al., 1991)
STR	Batch	*Clostridium carboxidivorans*	0.35	0.020	na	2.00	(nr)	(Hurst and Lewis, 2010)
STR	Batch	"Clostridium ragsdalei"	70.0	0.036	nr	25.26	(0.47)	(Kundiyana et al., 2010b)
STR	Batch	"Clostridium ragsdalei"	3.0	0.027	0.027	9.60	(0.00)	(Maddipati et al., 2011)
BCR	Continuous	*Eubacterium limosum*	0.2	0.950	na	0.09	(0.00)	(Chang et al., 2001)
BCR	Batch (first 96 h)	*Clostridium carboxidivorans*	4.5	0.014	0.012	1.30	1.10	(Rajagopalan et al., 2002)
BCR	Batch with gas producer	*Clostridium carboxidivorans*	4.0	0.042	0.006	5.00	0.75	(Datar et al., 2004)
STR and BCR	Batch STR for 2 days switched to BCR	*Clostridium ljungdahlii*	1.0	0.370	na	21.00	na	(Richter et al., 2013)
MBRa	Continuous	*Clostridium carboxidivorans*	8.0	0.120	nr	23.93	<0.45	(Shen et al., 2014)
MBRa	Continuous	"Clostridium ragsdalei"	3.0	0.125	na	15.00	nr	(Tsai et al., 2012)
SMSR	Batch mode for 2 days switched to continuous	"Clostridium ragsdalei"	3.0	0.050	na	10.00	nr	(Hickey et al., 2010)
MSBb	Continuous	"Clostridium ragsdalei"	2.0	0.100	na	10.00	nr	(Hickey et al., 2011)
MSBa	Continuous	"Clostridium ragsdalei"	nr	0.277	na	13.30	nr	(Datta et al., 2009)
SAB	Batch mode for 46.5 h switched to continuous	"Clostridium ragsdalei"	3.0	0.084	na	4.20	nr	(Datta et al., 2010)
MBBR	Continuous	"Clostridium ragsdalei"	$1.8{\cdot}10^4$	2.2	na	30.0	nr	(Hickey, 2009)

Abbreviations: STR: Stirred tank bioreactor; BCR: Bubble column reactor; MBR: Membrane bioreactor; SMSR: submerged membrane supported bioreactors; MSB: membrane supported bioreactor; SAB: Membrane modules in axially stacked array bioreactor (MBR); MBBR: Moving bed biofilm reactor.
a: hollow fiber membrane; b: hydrophilic membrane; na: not applicable; nr: not reported

The development of new bioreactor designs for syngas fermentation and alcohol production was out of the scope of the present PhD dissertation, and has been covered here just briefly. We found it important to review how the use of different bioreactors and operation configurations has been targeted as a research objective to increase alcohol productivity. However, the work in this thesis focuses mainly to study some of key physiological properties of model carboxydotorphic bacteria and anaerobic hungate tubes and bottles, all operated in batch conditions, have been chosen for all experiments.

OBJECTIVES

The aim of this PhD was to provide a deeper insight into key physiological aspects of syngas fermentation by carboxydotrophic clostridia, with special focus on clarifying key factors about molecular mechanisms that enhance alcohol production.

Specific objectives:

1. To understand the impact of added formate on kinetic parameters for growth and alcohol production. The reducing power demand for formate dehydrogenase activity in WLP has the potential to diminish due to the assimilation of formate as a partially reduced carbon source.

2. To test the effect on growth rate and alcohol production of pH, temperature, and organic matter supplements as key factors capable of controlling growth and shift from acidogenesis to solventogenesis in carboxydotrophic species.

3. To determine inhibitory effects of ethanol and butanol during syngas fermentation. Alcohol toxicity is not known in detail and it could be useful especially in fermentation modeling using continuous reactors.

4. To discover new isolates with the potential to transform inorganic carbon to ethanol or butanol as biofuel in order to increase the small number of syngas-fermenting bacterial strains known to date and, may be also, the range of operational conditions in which bio-ethanol and bio-butanol can be produced from inorganic carbon.

MATERIALS & METHODS

3.1 Bacterial strains.

All experiments were conducted using *Clostridium ljungdahlii* PETC (DSM13528^T), *Clostridium carboxidivorans* P7 (DSM15243^T), and/or *Butyribacterium methylotrophicum* DSM3468. The three strains were obtained from the DSMZ culture collection as a freeze dried cultures.

3.2 Media and culture conditions.

Bacteria were cultured in an anaerobic mineral medium modified from ATCC 1754 PETC (Table 3.1). Differences from ATCC 1754 PETC were: (1) all soluble carbon sources, i.e. yeast extract, fructose, and $NaHCO_3$ were excluded from the original formulation; (ii) 2-(N-morpholino)ethanesulfonic acid (MES) (100 mM, final concentration) was used instead of bicarbonate as pH buffer.

Liquid medium was prepared with the mineral medium, Wolfe's vitamins solution, trace elements solution, and resazurin. The pH of the medium was initially adjusted to 6.0 with 1M NaOH. After that, the medium was boiled to remove dissolved oxygen and distributed anaerobically in butyl rubber 125 mL glass bottles (for culture maintenance) and 25 mL Hungate tubes (for fermentation experiments). Tubes and bottles were flushed with N_2 and sealed before sterilization in the autoclave. MES buffer and reducing agents were added to the autoclaved medium from sterile anaerobic stock solutions.

Headspace was flushed with synthetic syngas ($CO:H_2:N_2:CO_2$ [32:32:28:8]) of high purity (Praxair Technology Ltd, Spain). All culture manipulations and inoculation of freshly prepared media were done in an anaerobic chamber (gas mixture $N_2:H_2:CO_2$ [90:5:5], Coy Lab Products, Michigan, USA).

Cultures were incubated in gas-tight closed bottles outside the anaerobic chamber in a Stuart incubator SI500 (Bibby Scientific Limited, OSA, UK), and were maintained active by weekly transfers of 1 mL of culture (1:25 ratio) into serum bottles containing

25 mL of modified ATCC 1754 PETC medium (Figure 3.1A). The headspace of bottles was filled with syngas at an overpressure of 100 kPa at the beginning of the incubation period.

Table 3.1. Composition of the mineral medium, reducing agents, trace elements solution, and the Wolfe's vitamin solution for the preparation of modified ATCC 1754 PETC medium.

Mineral medium		Reducing agents	
Element	Final concentration (g/L)	Element	Final concentration (g/L)
NH_4Cl	1.0	L-Cysteine·HCl	0.4
KCl	0.1	$Na_2S·9H_2O$	0.4
$MgSO_4·7H_2O$	0.2		
NaCl	0.8		
KH_2PO_4	0.1		
$CaCl_2·2H_2O$	0.02		
MES	19.52		
Resazurin	0.001		

Wolfe's Vitamin Solution		Trace elements solution	
Element	Final concentration (mg/L)	Element	Final concentration (mg/L)
Biotin	0.02	Nitrilotriacetic acid	20
Folic acid	0.02	$MnSO_4·H_2O$	10
Pyridoxine-HCl	0.10	$Fe(SO_4)_2(NH_4)_2·6H_2O$	8.0
Thiamine-HCl·$2H_2O$	0.05	$CoCl_2·6H_2O$	2.0
Riboflavin	0.05	$ZnSO_4·7H_2O$	0.002
Nicotinic acid	0.05	$CuCl_2·2H_2O$	0.2
D-Ca-pantothenate	0.05	$NiCl_2·6H_2O$	0.2
Vitamin B12	0.001	$Na_2MoO_4·2H_2O$	0.2
p-Aminobenzoic acid	0.05	Na_2SeO_4	0.2
Thioctic acid	0.05	Na_2WO_4	0.2

3.3 Fermentation experiments.

Cultures in the exponential growth phase were used as inoculum for batch experiments. Fermentation experiments were conducted in 25 mL Hungate tubes containing 6 mL of modified ATCC 1754 PETC medium (Figure 3.1B). A 10% inoculum was used in all experiments. Culture tubes were thoroughly flushed with syngas mixture for at least 1 min. Headspace overpressure was adjusted to 100 kPa. Depending on the experiment syngas was injected only at the beginning of the experiment, or once a day to ensure replenishment of gas substrates. The incubation was done under mild agitation (100 rpm) in a Stuart incubator SI500 shaker. Tubes were incubated horizontally to enhance gas-

liquid mass transfer. The same parent culture was used to inoculate all tubes used in a single kinetic experiment to prevent possible biases due to differences in the inoculum.

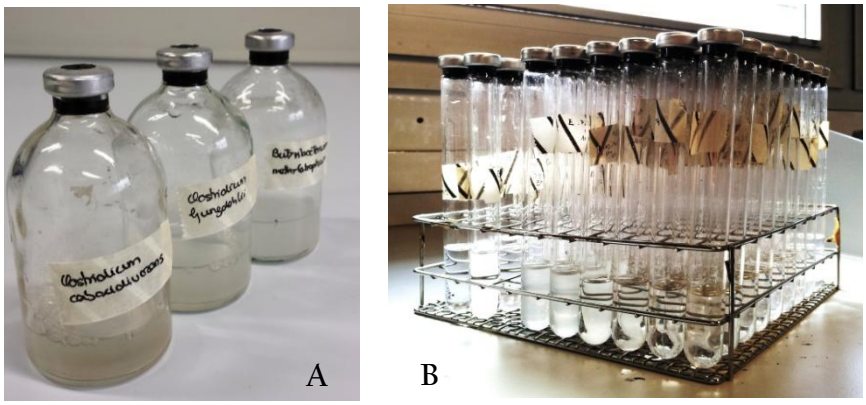

Figure 3.1. A: Image of 125 mL serum bottles used to maintain cultures of the three carboxydotrophic bacteria in active state. **B:** Fermentation experiments conducted in 25 mL Hungate tubes.

Growth was monitored on a daily basis by measuring the absorbance at 600 nm using a spectrophotometer. Samples for the determination of organic acids (formate, acetate, butyrate, and caproate) and alcohols (ethanol, butanol, and hexanol) concentrations were obtained regularly from the liquid media. Liquid samples were filtered using nylon filters (0.2 µm pore size) to remove cells, and stored at 4 ºC until analyzed. Headspace samples for the analysis of gaseous substrates (CO, CO_2, and H_2) were also collected with a syringe and distributed in BD Vacutainer tubes. Vacutainer tubes (Sangüesa SA, Spain) were stored at room temperature. The pH of the media was measured using a BASIC 20 pH meter.

3.4 Determination of growth variables

Linear regression of natural logarithm transformed absorbance readings (lnA_t) at time intervals (t) were used to estimate growth rate, according to equation (Eq. (3)):

$$lnA_t = lnA_{init} + \mu \cdot t \qquad (3)$$

Growth rates (µ, h^{-1}) were calculated for each incubation experiment as a measure of the growth capacity of the bacterial cultures at the conditions set in the different ex-

periments. Duration of the lag phase was estimated as the time interval between the inoculation of tubes and the time at which the maximum growth rate was observed.

3.5 Analytical methods

3.5.1 Protein concentration

Concentration of proteins was determined following a protocol adapted from Scott *et al.* (2011). Frozen pellets of 1 mL bacterial culture were suspended in 1 mL of 40 mM Tris at pH 7.0. Cells were disrupted by sonication. Six sonication cycles, each cycle including 30 s of sonication at maximum intensity and one minute on ice, were performed using a tip probe (5 mm) sonicator (B.Braun Labsonic 2000). Cellular debris were removed by centrifugation at 16000 rcf for 30 min. Protein quantification was performed using a QubitTM kit (Qubit® 2.0 Fluorometer, Invitrogen) and the recommended method.

3.5.2 Gas composition

Composition of CO_2, CO, H_2 and N_2 (% vol) in gas samples was analyzed using a gas chromatograph (Agilent 7890A GC system, Agilent Technologies, Spain) equipped with a fused zeolite capillary column (HP-Molesieve, 30 m x 0.53 mm x 50 µm) and thermal conductivity detector. Helium was used as the carrier gas. The injector and detector temperatures were set to 115 ºC and 275 ºC, respectively. The oven temperature was initially kept at 45 ºC for 6 min, and subsequently increased following a ramp of 8 ºC·min^{-1} until 70 ºC, holding the temperature 2 min, 5 ºC·min^{-1} from 70 ºC to 130 ºC and 35 ºC·min^{-1} from 130 ºC to 220 ºC. The cycle finished at temperature of 220 ºC for 5 min.

3.5.3 Organic acids and alcohols

Concentrations of organic acids and alcohols were determined using an Agilent 7890A GC system (Agilent Technologies, Spain) equipped with a fused-silica capillary column (DB-FFAP, 30 m x 0.32 mm x 0.5 µm) and a flame ionization detector. The injector and detector temperatures were set to 250 ºC and 275 ºC, respectively. The oven temperature

was initially kept at 40 ºC for 1 min, and subsequently increased following a ramp of 5 ºC·min^{-1} until 70 ºC, 10 ºC·min^{-1} from 70 ºC to 180 ºC and 35 ºC·min^{-1} from 180 ºC to 250 ºC. Finally, temperature was kept at 250 ºC for 5 min.

3.6 Calculations

Concentrations of undissociated acids (UA) were estimated based on actual pH of media and the total concentrations of acid (the salt and the acid form) according to the equilibrium equation (Eq. (4)).

$$UA = C_t - (10^{(pH-pKa)} \cdot C_t)/(10^{(pH-pKa)} + 1) \quad (4)$$

Where C_t is the concentration of acid measured in the GC and pKa is the logarithmic of the acid dissociation constant. The formate pKa value used was 3.76 corresponding to the equilibrium constant at 35 ºC (Kim et al., 1996). The pKa values for acetic acid were 4.76 and 4.77 at 25 ºC and 37 ºC, respectively, pKa for butyric acid were 4.82 and 4.88 at corresponding temperatures and pKa for caproic acid were 4.88 at both temperatures (Creager and Clarke, 1994; Harned and Ehlers, 1933; Zigová et al., 1999).

3.7 Statistical analyses

All statistical analyses were conducted using SPSS 15.0 statistical package for Windows (LEAD Technologies Inc., EEUU). Significance levels were established for $p \leq 0.05$. ANOVA test were used to analyze differences of growth parameters or alcohols and acid production to initial different variable among treatments. When multiple comparisons were required, a T3 of Dunnet post-hoc test or Bonferroni post-hoc test were used assuming non equal variances or equal variances between treatments, respectively. Kruskal-Wallis test was used when non-parametric tests were required. Pearson correlation tests were used to analyze possible correlations between experimental data.

RESULTS

The results of this PhD dissertation have been organized in six different chapters outlined in the form of scientific contributions to research journals. Most of these chapters have already been published or had been submitted to ISI journals at the time this dissertation was written.

Impact of formate on growth and productivity of *Clostridium ljungdahlii* PETC and *Clostridium carboxidivorans* P7 grown on syngas

4.1

Part of this chapter has been published as:

Ramió-Pujol, S.; Ganigué, R.; Bañeras, L.; Colprim, J., 2014. Impact of formate on the growth and productivity of *Clostridium ljungdahlii* PETC and *Clostridium carboxidivorans* P7 grown on syngas. *International Microbiology* 17: 195-204. DOI: 10.2436/20.1501.01.222

4.1.1 Background

The first steps in the methyl and carbonyl branches of the WLP, formate synthesis and the carbon monoxide formation, are recognized as reducing equivalent sinks that may diminish the growth capacity of cells and the conversion of inorganic carbon into valuable chemicals (Daniell et al., 2012). The use of hydrogen and carbon monoxide as sources of reducing equivalents is maximized during autotrophic growth. In this condition, the assimilation of formate as a partially reduced carbon source has the potential to reduce the hydrogen/CO demand for formate dehydrogenase activity in the WLP (Brown et al., 2014; Schuchmann and Müller, 2013). We hypothesize that the excess hydrogen can further be diverted to acetate reduction, increasing biofuel production. The synthesis of formic acid from CO_2 has been accomplished in a bioelectrochemical system (BES) using a purified formate dehydrogenase enzyme (Srikanth et al., 2014). Electrosynthesis coupled to fermentation by carboxydotrophic bacteria, has been proven but not studied in detail (Nevin et al., 2010). However, the concept of enzymatic electrocatalysis involving energy applications is gaining in prominence, especially in the direction of enzymatic electrosynthesis of desired chemicals and fuels under non-limiting reducing power supply.

Formate has been reported as an inducer of acetate production in *Clostridium acetobutylicum*. Maximum effects of formate on acetate production in *C. acetobutylicum* were obtained under acidic conditions (at pH=4.8) (Ballongue et al., 1985). Despite this example, growth on weak organic acids is rather difficult for most microorganisms and inhibition occurs at very low concentrations. Inhibition effects are higher at low pH values where higher concentrations of the undissociated acid forms exist, which can freely diffuse to the cytoplasm of the cell eventually causing the dissipation of energy gradients built across the cell membrane (Jones and Woods, 1986). Additionally, formate can cause sub-lethal damage in some bacteria and has been used as an antibacterial agent (Thompson and Hinton, 1997).

PETC has been grown chemoorganotrophically in a medium containing 5 g/L of formate and 1 g/L of yeast extract (Tanner et al., 1993). However, similar experiments have never been done autotrophically with this strain. Moreover, *B. methylotrophicum* can also use formate as substrate for growth, but it is unclear whether other acetogenic bacteria, including P7, can use formate when growing either organo- or autotrophically (Kerby and Zeikus, 1987; Liou et al., 2005). In this light, the present chapter hypothesizes that the addition of formate, as a partially reduced C1 compound, would positively impact kinetic parameters for growth and alcohol production in *C. ljungdahlii* PETC and *C. carboxidivorans* P7 by diminishing the need for external reducing equivalents. The aim of this chapter was to provide experimental evidence to evaluate formate addition as a potential enhancer of alcohol production in *C. ljungdahlii* PETC and *C. carboxidivorans* P7.

4.1.2 Experimental design

4.1.2.1 Fermentation experiments.

Exponentially growing *C. ljungdahlii* PETC and *C. carboxidivorans* P7 cultures were used as inocula for batch experiments to test for formate effects on growth and alcohol production. Syngas was injected only at the beginning of the experiment. Sodium formate solutions adjusted at the desired pH (5.0, 6.0 or 7.0) were aseptically added to the medium at final concentrations of: 0.1, 1.0, 2.2, 5.5, 7.6, 10.9, 15.0, 20.0, 27.2, 54.5, and 109.0 mM. In all batch tests, tubes containing no sodium formate were included as controls for growth kinetics under fully autotrophic conditions. Experiments were carried out for the two bacterial species at three pH values, 5.0, 6.0, and 7.0. The cultures were incubated at 35 ºC under mild agitation on a rotary shaker. All experimental conditions were assayed in triplicate using three independent inoculated cultures.

Growth experiments finished once cultures reached the stationary growth phase, which was considered to occur 48 to 72 h after growth cessation. Samples for the determination of organic acids (formate, acetate, and butyrate) and alcohols (ethanol and

butanol) concentrations were obtained at the beginning and at the end of the incubation experiments.

Additionally, an independent experiment was conducted at five sodium formate concentrations: 13.7, 17.2, 21.9, 30.0, and 97.5 mM at pH 6.0 using PETC strain to test the consumption of formate throughout growth. The samples for the determination of formate concentration were obtained every 48–72 h, as well as, at the beginning and at the end of the incubation experiment.

4.1.2.2 Analytical methods

The total amount of formic acid/formate (the acid and the salt form) was measured by using a spectrophotometric method (Sleat and Mah, 1984). Formate concentrations were measured at the beginning and the end of incubation experiments. The equilibrium constant corresponding at 35 ºC used to calculate the undissociated formic acid concentration at initial conditions was 3.76 (Kim et al., 1996).

4.1.3 Results and Discussion

4.1.3.1 Growth in the presence of formate

Clostridium ljungdahlii PETC was able to grow under all experimental conditions tested except at sodium formate concentrations of 54.5 and 109.0 mM at pH 6.0 (Table 4.1.1). On the contrary, *C. carboxidivorans* P7 showed a more restricted range of growth conditions and no increase in absorbance was observed in many of the experimental conditions, especially at low pH values. Growth of P7 was restricted to formate concentrations lower than 10.9 mM at pH 6.0, and no growth was observed at any of the formate concentrations tested at pH 5.0. Main differences observed in growth curves for the two strains were the decrease in the optical density at the stationary phase and the duration of the lag phase depending on the formate concentration (Figure 4.1.1).

Table 4.1.1. Concentration of formate and formic acid at the beginning of the experiments; mean values ± SD (n = 3) of lag phase, formate concentration consumed during the experiment and pH reduction after growth of different experiments of *Clostridium ljungdahlii* PETC and *Clostridium carboxidivorans* P7.

Initial pH	Sodium formate (mM)	Formic acid (mM)[a] (x10[2])	*C. ljungdahlii* PETC			*C. carboxidivorans* P7		
			Lag phase (days)	Δ formate concentration (mM)	Δ pH	Lag phase (days)	Δ formate concentration (mM)	Δ pH
5.0	0.0	0	1.7 ± 0.6	0.76 ± 0.42	-0.41 ± 0.04	7.0 ± 3.0	nm	-0.32 ± 0.03
	1.0	6	1.0 ± 0.0	-1.19 ± 0.21	-0.49 ± 0.01	ng	na	na
	2.2	10	1.0 ± 0.0	-1.64 ± 0.13	-0.44 ± 0.03	ng	na	na
	5..5	30	2.3 ± 1.2	-5.26 ± 0.42	-0.25 ± 0.01	ng	na	na
	7.6	40	3.0 ± 0.0	-5.95 ± 0.30	-0.14 ± 0.01	ng	na	na
	10.9	60	6.5 ± 0.7	-8.83 ± 0.11	-0.22 ± 0.02	ng	na	na
6.0	0.0[b]	0	2.2 ± 0.4	1.10 ± 0.30	-0.69 ± 0.08	1.0 ± 0.0	1.26 ± 0.30	-0.57 ± 0.06
	1.0	0.1	3.0 ± 0.0	0.09 ± 0.24	-0.72 ± 0.07	1.7 ± 1.2	0.18 ± 0.08	-0.39 ± 0.18
	2.2[b]	1.1	1.0 ± 0.0	-0.70 ± 0.20	-0.64 ± 010	1.0 ± 0.0	-0.41 ± 0.22	-0.59 ± 0.06
	5.5[b]	2	1.6 ± 0.6	nm	-0.71 ± 0.08	1.0 ± 0.0	-1.73 ± 0.15	-0.46 ± 0.05
	7.6	5.7	1.8 ± 0.8	nm	-0.66 ± 0.10	5.8 ± 2.9	nm	-0.33 ± 0.04
	10.9[b]	6.5	1.7 ± 0.6	-7.71 ± 0.32	-0.50 ± 0.11	4.0 ± 1.4	-7.34 ±0.52	-0.24 ± 0.26
	15.0	11	6.0 ± 0.0	nm	-0.27 ± 0.09	ng	na	na
	15.0	15	7.0 ± 4.4	-13.21 ± 0.08	-0.56 ± 0.05	ng	na	na
	20.0	19	9.5 ± 0.7	-16.75 ± 0.05	-0.43 ± 0.04	ng	na	na
	27.2	30	9.0 ± 1.4	-21.29 ± 0.00	-0.34 ± 0.00	ng	na	na
	54.5	60	ng	na	na	ng	na	na
	109.0	120	ng	na	na	ng	na	na
7.0	0.0	0	1.0 ± 0.0	0.23 ± 0.14	-0.69 ± 0.07	1.0 ± 0.0	0.36 ± 0.49	-0.58 ± 0.02
	1.0	0.081	1.0 ± 0.0	-0.95 ± 0.35	-0.76 ± 0.06	1.0 ± 0.0	-0.87 ± 0.07	-0.55 ± 0.10
	2.2	0.17	2.0 ± 0.0	-2.11 ± 0.08	-0.59 ± 0.18	1.0 ± 0.0	-2.31 ± 0.04	-0.56 ± 0.02
	5.5	0.4	1.0 ± 0.0	-5.23 ± 0.19	-0.69 ± 0.06	1.0 ± 0.0	-4.92 ± 0.39	-0.58 ± 0.01
	7.6	0.6	2.3 ± 1.2	-6.57 ± 0.27	-0.46 ± 0.03	1.0 ± 0.0	-6.67 ± 0.34	-0.47 ± 0.05
	10.9	0.8	1.7 ± 1.2	-7.59 ± 0.12	-0.31 ± 0.01	1.0 ± 0.0	-7.01 ± 1.21	-0.52 ± 0.02

na: not applicable; **nm:** not measured; **ng:** no growth; **a:** values calculated according to equation 2, **b:** n=6

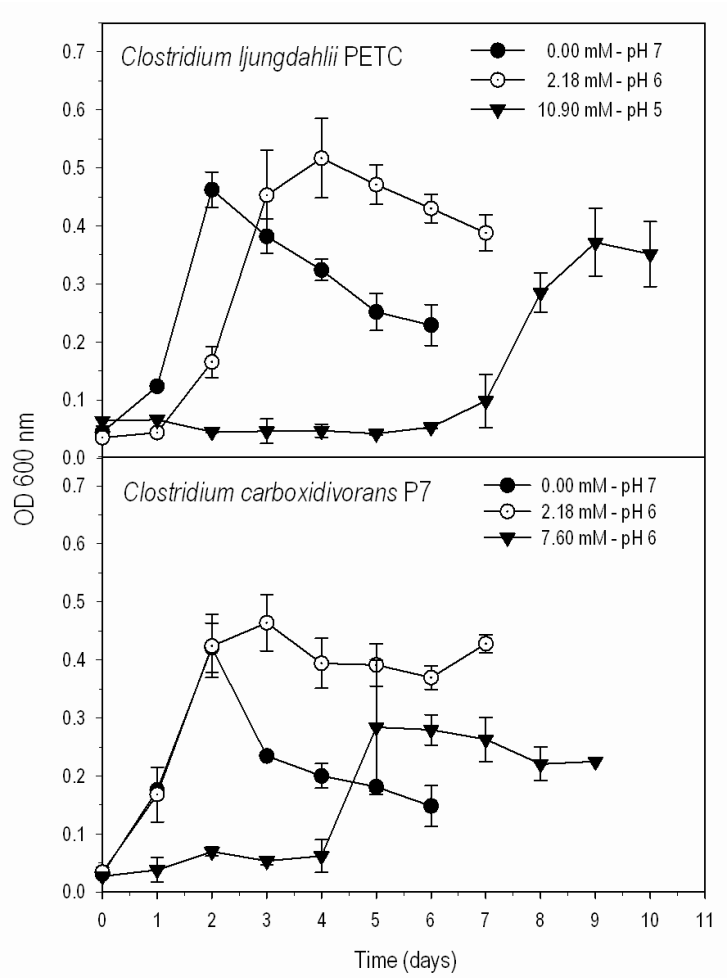

Figure 4.1.1 Selected growth curves (mean values and SD, n≥3) of *Clostridium ljungdahlii* PETC **(top)** and *Clostridium carboxidivorans* P7 **(bottom)** at different pH and formate concentrations.

The rapid decrease in absorbance values during the stationary phase was observed for both *Clostridium* species when incubated at pH 7.0. Reasons for this decrease were not investigated in detail but a thorough inspection of those cultures under phase-contrast microscopy revealed the presence of cell clumps and lysed cells (results not shown), both contributing to the decrease in the absorbance.

The duration of the lag phase varied from 1 to 12 days for both P7 and PETC, and was directly correlated with the increase in the sodium formate concentration of the culture, especially at low pH values (Table 4.1.1). The increase on the lag phase of bacteria is generally recognized as an adaptation phase, during which bacteria stimulate transcription of new genes to resume growth under the new environmental conditions (Rolfe et al., 2012). For instance, it has been reported that 10 mM of formic acid at pH 5.0 caused bacteriostasis in *Escherichia coli*, and growth resumed only after a 2 h incubation period

proving its adaptation to formic acid (Cherrington et al., 1990). However, adaptation to increasing formate concentrations may be complex since organic acids can serve as both additional carbon substrates and inhibitory compounds, depending on the concentration, pH of the media and/or the cell resistance to the acid, which could explain such long lag phases. Moreover, the addition of sodium formate, particularly at high concentrations, could have caused a significant increase in the ionic strength of the culture medium thus causing an additional stress for cell growth. The effect of sodium chloride concentration on the growth and alcohol production of *Clostridium autoethanogenum* in a completely autotrophic medium has been previously tested using a Plakett-Burmann experimental design; even if that work reports a positive effect of NaCl in ethanol production, it is not significant in the range of 0.4 to 1.0 g/L (Guo et al., 2010).

The maximum estimated growth rates for PETC and P7 were 0.063 ± 0.020 h^{-1} and 0.063 ± 0.010 h^{-1}, respectively (Table 4.1.1). The calculated maximum growth rates agree with the values obtained in previous works using the same strains (Kerby and Zeikus, 1987; Köpke et al., 2010; Perez et al., 2013). In both strains at low pH values, low concentrations of sodium formate (<2.2 mM) resulted in a slight increase in the growth rate compared to the formate free media, although the observed differences were only significant for PETC cultures at pH 5.0 ($p<0.05$, Bonferroni test, $n\geq3$). According to the observed growing capacity, PETC was more tolerant to formate concentration than P7, and complete growth inhibition was only observed at 54.5 mM at pH 6.0. As stated above, a potential effect of added salt concentration could also contribute to growth inhibition. However, this is not expected to occur at sodium formate concentrations lower than 10.9 mM, according to the results obtained at pH 7.0, at which no significant growth inhibition occurred in any of both strains. This observation suggests that low sodium formate concentrations at pH 6.0 and 5.0 might enhance the growth of PETC.

Extra-cellular formic acid diffuses across the lipid bilayers and dissociates inside the cell based on the intra-cellular pH (Walter and Gutknecht, 1984). The distribution of dissociated and undissociated forms on the two sides of the cell membrane is propor-

tional to the pH (Hirshfield et al., 2003). The most common metabolic processes to circumvent organic acid diffusion into cells includes the use of specific transporters functioning as efflux pumps (Kanjee and Houry, 2013; Nakano et al., 2006). However, and at least for enterobacteriaceae, several other strategies exist including aminoacid decarboxylases and other protective mechanisms (Bearson et al., 2006). An inspection of public genome sequences of PETC and P7 have confirmed the presence of putative formate transporters, although with differences in the two bacterial species (Köpke et al., 2010; Paul et al., 2010). One single gene encoding for a hypothetical formate/nitrite transporter (WP_013240353) was identified in the PETC genome (NC_014328), whereas, the P7 draft genome (PRJNA48985; PRJNA29495; PRJNA55755; PRJNA33115) contains at least three genes coding for formate transporters, two formate/nitrite transporters (WP_007062507 and WP_007063385) and one oxalate/formate antiport (WP_007061997). The alignment of the four retrieved amino acid sequences revealed that the unique nitrite/formate transporter found in PETC had a highly similar homolog (>80%, Blosum62 matrix) in *C. carboxidivorans* P7 (results not shown). Note that PETC showed a much faster adaptation and higher tolerance to formate, which might be explained to some extent by differences in the other two transport proteins detected.

4.1.3.2 Formate consumption

Formate concentrations were measured once growth stopped and were compared to the initial concentration to assess its net consumption or production (Table 4.1.1). A net production was detected for both strains when incubated under completely autotrophic conditions or at low formate concentration (<1.0 mM). This production was probably due to the activity of formate dehydrogenase (FDH), which converts CO_2 into formate in the first step of the WLP. Net production ranged from 1.1 to 0.09 mM in PETC and from 1.26 to 0.18 mM in P7. On the contrary, formic acid consumption was observed in most of the treatments where formate had been added and growth resumed after the lag phase. This net formate consumption was significant for both strains, accounting for more than 80% of the added sodium formate. Time course experiments were carried out to elucidate

whether formate consumption occurred during the lag or the exponential phase. No net formate consumption was observed during the lag phase, it being mostly consumed during the exponential growth phase at any of the concentrations tested (Figure 4.1.2).

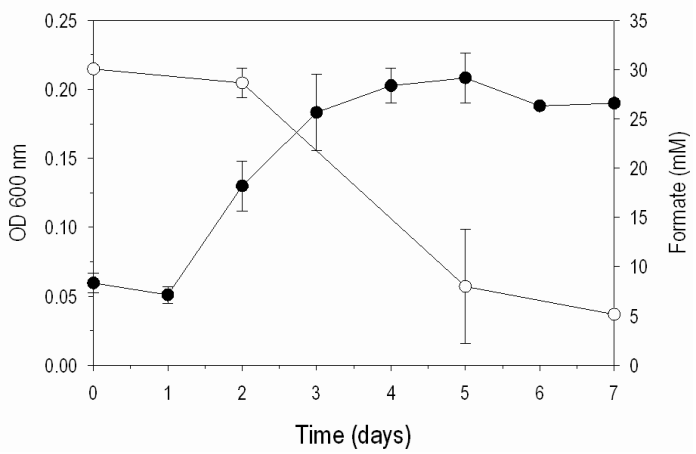

Figure 4.1.2 Formate consumption during growth of *Clostridium ljungdahlii* PETC. Optical density (black dots) and formate concentration (white dots) are shown as mean values of two replicates. Error bars indicate SD.

The observed formate consumption could be related to its use as an alternative carbon or energy substrate in addition to H_2, CO_2, and CO, as has been proven for some acetogenic bacteria. Theoretically, formate uptake would partially circumvent the use of hydrogen/CO and make the first step in the WLP unnecessary. Moreover, formate oxidation to CO_2 via FDH would provide an additional reducing power source, which would diminish the total energy requirements to incorporate new carbon molecules (Ragsdale and Pierce, 2008). The most favorable reaction to obtain reducing power in acetogenic bacteria is the oxidation of CO to CO_2 by the carbon monoxide dehydrogenase (CODH) (Hu et al., 2011), but no analyses of the composition of the gas phase were done to confirm this hypothesis in this experiment. Cultivation of PETC and P7 in the same media composition as the used here, but no formate added, resulted in a complete depletion of CO, which was mostly converted into CO_2 and used as the main source of reducing power instead of H_2 (results not shown). In this respect, the incorporation of formate would considerably lower the CO oxidation as a reducing power source and increase the carbon available for fixation into cellular biomass. This probably occurred at low formate concentrations, at which an increase in the growth rate and the growth yield of *C. ljungdahlii* was observed. Most presumably, the positive effect of

formate addition on growth was masked by the activation of resistance mechanisms to circumvent potential inhibition effects of either increased formic acid concentration or ionic strength.

4.1.3.3 Production of acids and alcohols

The concentration of acids (acetate and butyrate) and alcohols (ethanol and butanol) produced by *C. ljungdahlii* PETC and *C. carboxidivorans* P7 measured at the end of the incubation experiments is shown in Figure 4.1.3.

Acetate production by PETC increased with increasing concentrations of formate at pH 5.0 and 6.0. Maximum acetate production (337.9 mg C/L and 584.7 mg C/L, respectively) was observed at 2.18 mM of formate, representing an increase of 104.5% and 52.4% compared to the control. On the contrary, acetate production was negatively affected by increasing concentration of sodium formate at pH 7.0 ($p<0.05$, Pearson correlation test, $n=18$). Acetate production of PETC decreased from 546.1 mg C/L (control test) to 116.3 mg C/L (10.9 mM formate). Regarding ethanol, the highest production occurred at pH 5.0, with maximum concentrations slightly over 230.0 mg C/L. Nevertheless, even if differences in alcohol production were observed at different pH values, changes were not related to the initial formate concentration ($p>0.05$, ANOVA test, $n\geq3$). Formate concentrations higher than 2.18 mM can positively affect the ethanol production due to the changes in salt concentrations (Guo et al., 2010).

Acetate and ethanol production in *C. carboxidivorans* P7 was lower than in PETC, but different formate dependence trends were observed depending on pH. At pH 6.0, acetate production remained almost invariable between 0 and 10.9 mM. However, acetate concentration showed a negative correlation ($p<0.05$, Pearson correlation test, $n=18$) with added formate at pH 7.0. Maximum butyrate was 14.9 mg C/L, and was obtained at a sodium formate concentration of 2.18 mM and pH 6.0. Neither pH nor initial formate concentration did play a major role in butyrate production, except at pH 7.0 where production decreased more than 50% in the presence of formate. Alcohols production of

PETC at pH 6.0 showed some significant differences, although no correlation to the sodium formate addition was observed ($p>0.05$, ANOVA test, $n\geq3$) (Figure 4.1.3).

Overall, the addition of formate increased the acid production of *C. ljungdahlii* PETC at pH 5.0 and 6.0, although solvent production remained unaffected. This could be explained by the acid crash effect, during which the fast accumulation of acids results in

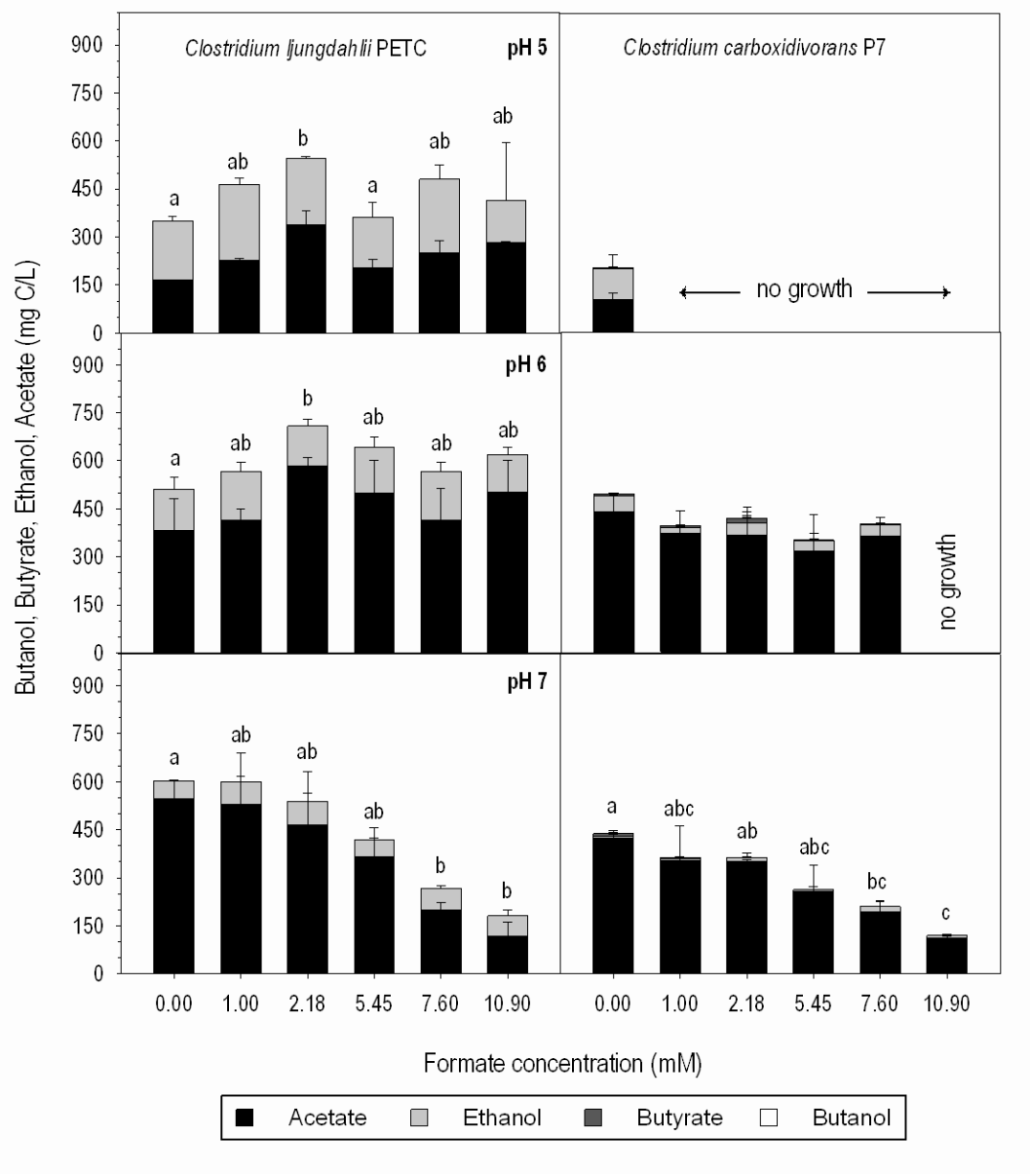

Figure 4.1.3 Concentrations of organic acids (acetate and butyrate) and alcohols (ethanol and butanol) produced by *Clostridium ljungdahlii* PETC (left) and *Clostridium carboxidivorans* P7 (right) according to initial formate concentration. Incubations at different pH values are shown. Different letters above bars indicate significant differences of acetate production between experiments within each bacterial species according to Bonferroni or T3 of Dunnet post-hoc test assuming equal or not equal variance respectively. Bars show mean value of 3 replicates. Error bars indicate SD.

a failure of the switch from acidogenic phase to solventogenic phase to occur, and no solvent are produced by clostridia. Formic acid has been reported to play a major role in triggering the acid crash of ABE fermentations (Maddox et al., 2000; Wang et al., 2011). This phenomenon was not observed for *C. carboxidivorans*, as the concentrations of acids produced in the different experiments were never higher than that of the control. The addition of formate at pH 7.0 caused the opposite effect in both strains, and accumulation of acids decreased with increasing formate concentrations. These observations seem to be in disagreement with the measure OD, lag phases, and growth rates (Table 4.1.1), which shows that, at pH 7.0, undissociated formic acid concentration remained low and inhibitory effects were clearly diminished in both PETC and P7 strains. Ideally, the energy saved by the use of formate as a substrate could be utilized to increase cellular ATP production. It has been long recognized that autotrophic growth by the WLP must be linked to an energy generating anaerobic respiratory process, since during autotrophic growth there is no net ATP synthesis by substrate-level phosphorylation (Ragsdale and Pierce, 2008). Thus, the use of partially reduced compounds could have also allowed higher available reducing power and/or ATP, so reducing the need of acetate production. However, this was not clearly confirmed and further work would be needed to test this hypothesis.

The highest alcohol to total product ratios was obtained at pH 5.0 for both strains, and they decreased significantly at higher pH values. The statistical tests ($p>0.05$, Dunnet T3 test, $n\geq3$) proved that such differences were not linked to the presence of sodium formate at P7 strain, but to incubation pH. The highest alcohol to total product ratio through all the experiment was 0.53, corresponding to the PETC control experiment at pH 5.0. This ratio was negatively influenced by the addition of formate because the productivity enhancement led to the production of mainly acetate. Finally, alcohols/products ratio at pH 7.0 significantly increased from 0.09 to 0.36 ($p<0.05$, Dunnet T3 test, $n\geq3$) in PETC. However, the reason for such an increase was related to the reduction of acetate production rather than to an increase in the net alcohol

production. Although the total amount of carbon fixed into synthesized products was lower, such operational conditions could be beneficial when aiming at alcohol production. Downstream separation processes account for a large part of operational costs, therefore the decrease in acetate production in the fermentation medium could ease alcohol separation (Ramachandriya et al., 2013).

4.1.3.4 Implications and future prospects

The present chapter assessed the impact of the addition of formate on *C. ljungdahlii* PETC and *C. carboxidivorans* P7. Results showed the higher tolerance of PETC to formate, in addition to the enhancement of its growth rate and productivity at low formate concentrations at pH 5.0 and 6.0. This is of interest from the biotechnological point of view as the ability of the PETC strain to use formate as a feedstock opens up potential to upgrade carboxydotrophic fermentation process with external formate supply. Of special interest could be the combination of enzymatic electrocatalysis to produce formic acid (Srikanth et al., 2014) and microbial electrosynthesis (Ganigué et al., 2015; Marshall et al., 2013). In fact this could be a major breakthrough in the production of added-value compounds from carbon dioxide via bio-electrochemical fermentation. In this light, PETC could be cultivated at moderately acid pH in a BES with low formic acid production to accelerate its metabolism and enhance carbon fixation into products. However, further studies are required to elucidate the metabolic fate of formate, and to understand the impact of formate assimilation on the cell energy and reducing power balances.

Conversion of sewage sludge to commodity chemicals via syngas fermentation

4.2

Part of this chapter has been published as:

Ganigué, R.; **Ramió-Pujol, S.**; Sánchez, P.; Bañeras, L.; Colprim, J., 2015. Conversion of sewage sludge to commodity chemicals via syngas fermentation. Water Science & Technology 72.3: 415-420. DOI: 10.2166/wst.2015.222

4.2.1 Background

Fermenation products are dependent on the operational conditions (pH, temperature, concentration of biomass, and products) and the microbial species involved in the transformation (Daniell et al., 2012). Up to date, most of the studies on syngas fermentation have focused on pure cultures (Köpke et al., 2010; Liu et al., 2012), reporting the production of mainly two-carbon (C2) compounds (acetate and ethanol). Butyrate and butanol (C4 compounds) can be also produced by some homoacetogens, although their proportion and concentration are usually lower than C2 compounds (Bruant et al., 2010; Perez et al., 2013). Recently, the use of mixed cultures for syngas fermentation has been also explored, being organic acids the main end-product (Alves et al., 2013; Zhang et al., 2013).

Depending on their concentration, fermentation products can serve as industrial feedstock, biofuels or even external carbon source to enhance biological nitrogen and phosphorous removal in wastewater treatment plants when there is a shortage of biodegradable organic matter. Taking syngas as a starting point, this chapter presents the results of the production of added-value products using pure and mixed cultures of bacteria, both in bench-scale batch experiments and during continuous reactor operation.

4.2.2 Experimental design

4.2.2.1 Inoculum and mineral media

Pure culture experiments were conducted using *Clostridium carboxidivorans* P7. Mixed culture experiments were carried out using a carboxydotrophic inoculum enriched from sludge of the anaerobic digester of Girona Wastewater treatment plant (Spain). Analysis of the community by PCR of the 16S rRNA gene DGGE showed that enriched cultures were dominated by a phylotype close to *C. ljungdahlii*, "C. ragsdalei" and *C. autoethanogenum*

(100% similarity). Unfortunately, the exact identification could not be elucidated with the applied technique because the three species have an identical 16S rRNA gene according to sequences available in the GenBank database. Both pure and mixed cultures were grown anaerobically, solely on modified ATCC 1754 PETC medium and syngas using two different pH buffer: MES (100 mM) and $NaHCO_3$ (23.8 mM) for pure culture and mixed culture experiments, respectively. The mixed culture medium contained also 20 mM of 2-bromoethanesulfonate to inhibit methanogenesis.

4.2.2.2 Fermentation experiments

Kinetic experiments were conducted to characterize the growth and production of *C. carboxidivorans* P7. The kinetic experiments consisted in 36 sacrificial tubes incubated at 35 ºC and 100 rpm. Every 8-10 hours three tubes were sampled. The composition of the gas headspace was analyzed at the end of the experiment. Additionally, the impact of initial pH on the production of alcohols was assessed at pH values of 5.0, 6.0, and 7.0, following the previously described procedure. Production of the different compounds per unit of fresh weight (FW) was calculated based on the equivalence of 1 unit of OD equals 1.18 g FW/mL for *Clostridium carboxidivorans* P7.

4.2.2.3 Production of acids and alcohols using mixed cultures: Enrichment and production of organic acids in batch reactors

Experiments were conducted in a Biostat B Plus (Sartorius AG, Germany) fermentor. The reactor (with a total volume of 3 L and liquid to headspace ratio of 1:1) was equipped with pH, dissolved oxygen, redox and temperature probes, a two blade segment impeller and a microsparger. Syngas (32%H_2-32%CO-28%N_2- 8%CO_2) was sparged in the liquid phase every 4 hours for a period of one minute. Temperature and agitation were controlled at 37 ºC and 150 rpm, respectively. The set-up was inoculated with an enriched carboxydotrophic culture. The reactor was operated in batch mode to favor the accumulation of high concentrations of added-value products, with 80% of the

fermentation broth being replaced with fresh media at the beginning of each batch. The system was operated on a stable way for two replicate batch experiments (40 days) and samples for pH and liquid composition analysis were drawn regularly.

4.2.3 Results and Discussion

4.2.3.1 Kinetics of pure cultures

Figure 4.2.1 depicts the results of the kinetic experiments conducted with *C. carboxidivorans* P7. During the first two days, OD increased from 0.02 to 0.4, and pH decreased 0.45 pH units due to the production of organic acids. Over that period, acetate concentration increased from about 40 mg C/L to 454 mg C/L and levelled off. Maximum ethanol concentration, 167 mg C/L, was transiently obtained after 1 day of incubation. Concentration of C4 compounds (butyrate and butanol) was lower. Butyrate concentration reached 8 mg C/L after 1.5 days and remained almost constant for the rest of the experiment. Regarding butanol, its maximum concentration peaked at 5 mg C/L. At the end of the experiment, all CO had been consumed from the headspace of the tubes, together with 15.3 ± 0.07% of the initial hydrogen, whereas the percentage of CO_2 had increased from 8% to 23.8 ± 0.73%. This pointed out that CO was used as the main source

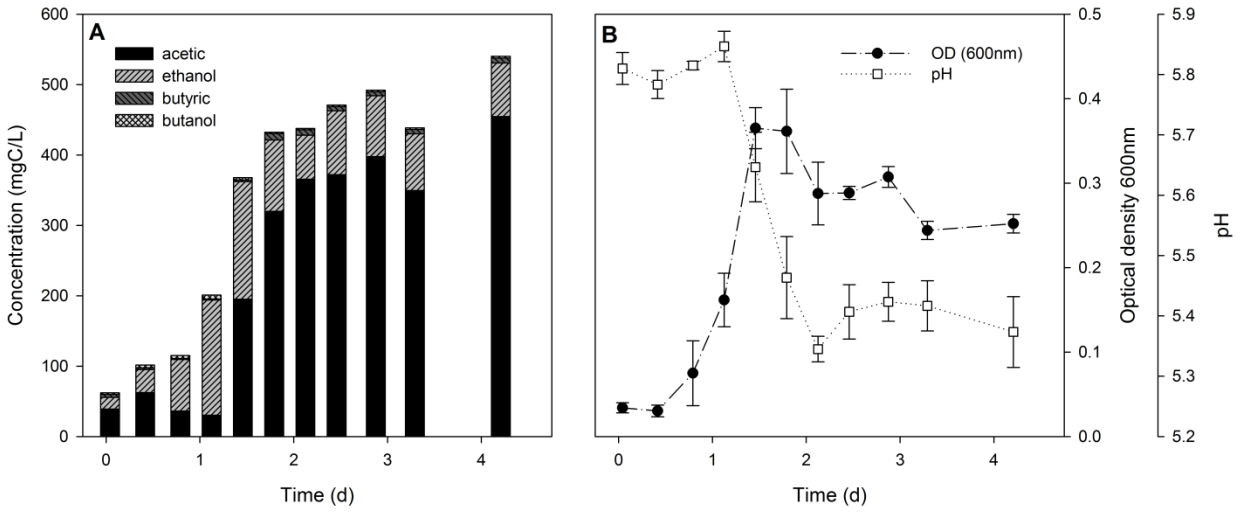

Figure 4.2.1 Left: Product concentration during the 100 h of kinetic experiment of *C. carboxidivorans* P7. **Right**: Optical density and pH measurements of *C. carboxidivorans* P7 culture.

of reducing power via CODH enzyme (Hurst and Lewis, 2010), and had been most likely depleted around day 2, limiting the growth of *C. carboxidivorans* P7. Growth rate (μ_{obs}) at pH 5.6 - 5.9 was determined to be 0.073 h^{-1}, about half of the maximum growth rate (μ_{max}, 0.16 h^{-1} at pH 6.2 (Liou et al., 2005)), indicating that growth had been substrate-limited. Low carbon fixation rates (480 mg C/d) were observed during the experiment due to the low concentration of biomass. Finally, the carbon-to-products yield at the end of the batch was 41.8%, with part of the carbon monoxide being used as a source of reducing power.

4.2.3.2 Impact of media pH on product speciation

Experiments were conducted to elucidate how initial media pH affected the carbon flux (Figure 4.2.2). Results proved that pH played a significant role on product speciation, in particular on alcohols to total products molar ratios. Overall, total amount of carbon fixed into products was very similar for pH 5.0, 6.0, and 7.0, being slightly higher at the later -1236 mg C/g of culture FW - over pH 5.0 and 6.0, both around 1068 mg C/g of culture FW. However, at lower initial pH (5.0) ethanol production was favored over acetic acid, with 46% of the total concentration of products being alcohols. The relative alcohol production decreased to 14% and 2% at initial pH values of 6.0 and 7.0, respec-

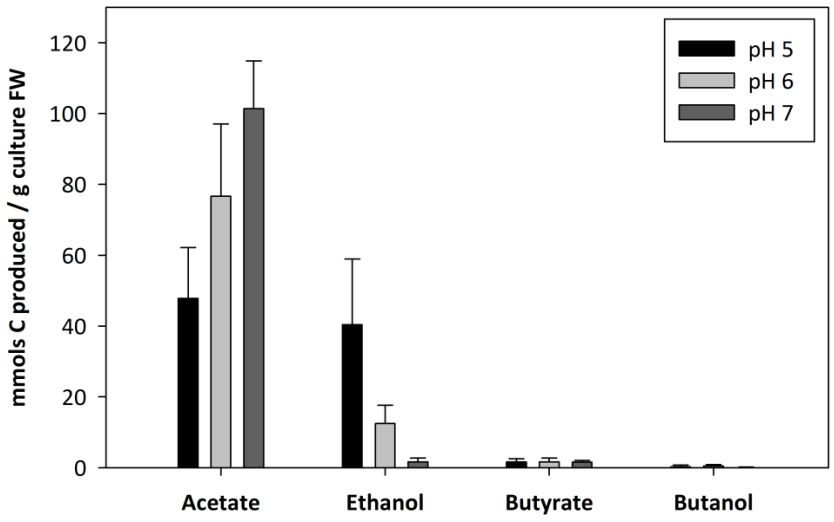

Figure 4.2.2 Specific production of acetate, ethanol, butyrate, and butanol by *C. carboxidivorans* P7 at different initial medium pH.

tively. The enhancement of the solventogenesis at low pHs was probably due to the inhibitory effect of high concentrations of undissociated organic acids (mainly acetic acid) on homoacetogenic bacteria. Under such conditions, direct production of solvents and/or the re-assimilation of the already produced and its subsequent transformation into ethanol are favored over acid production to avoid further inhibition (Jones and Woods, 1986). On the other hand, pH did not affect the production of C4 compounds, which was in all three cases very low, about 24 mg C/g of culture FW.

4.2.3.3 Production of organic acids and alcohols by mixed cultures in batch reactors

Figure 4.2.3 presents the evolution of the concentration of organic acids and alcohols in a 3 L batch reactor for a period of 40 days, in two consecutive batches. Results showed that mixed cultures were capable of transforming gaseous inorganic carbon into products with added value, although in contrast with pure cultures, longer-chain metabolites were the main end-product representing at the end of the batch 75-90% of the total carbon as products. Butyrate was the dominant fermentation product, with a maximum concentration of 1184 mg C/L. High concentrations of butanol (up to 277 mg C/L) were also observed, revealing enriched carboxydotrophic cultures obtained from anaerobic digester

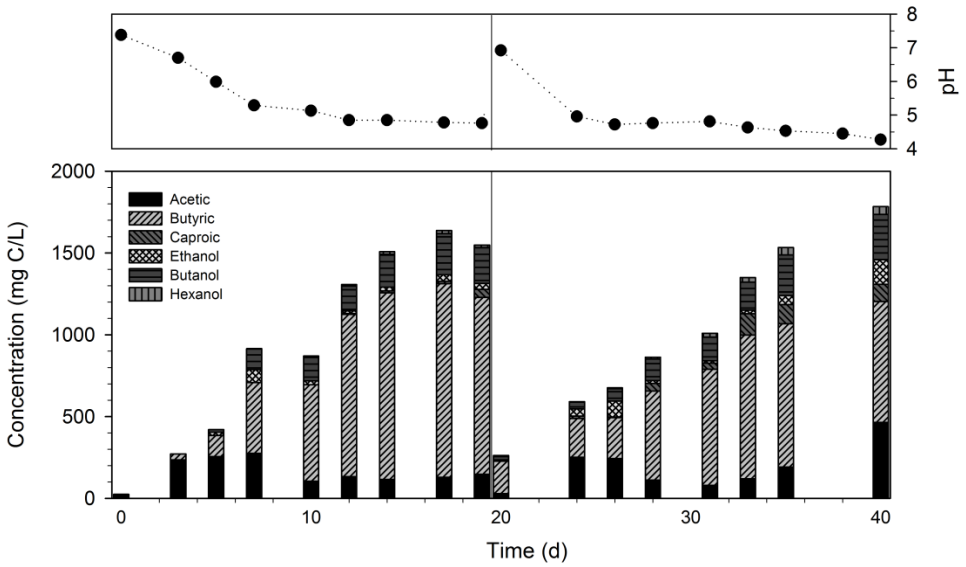

Figure 4.2.3 Mixed culture syngas fermentation. **Top**: pH of the fermentation broth. **Bottom**: Product concentration. Vertical lines indicate new batch.

sludge as a suitable platform for its production. This is very interesting since butanol has 10-30% higher energy density compared to ethanol, and can be more easily extracted from the fermentation broth (Dürre, 2007). Acetate and ethanol were also produced but in a lower titer (464 mg C/L and 150 mg C/L, respectively).

Interestingly, the percentage of C4 compounds produced during mixed culture fermentation was significantly higher than in the pure culture experiments. One possible explanation for this could be that low pH favors, not only the production of alcohols, but also longer carbon chains compounds. This is supported by Figure 4.2.4, which depicts the production rate of different metabolites as a function of fermentation broths.

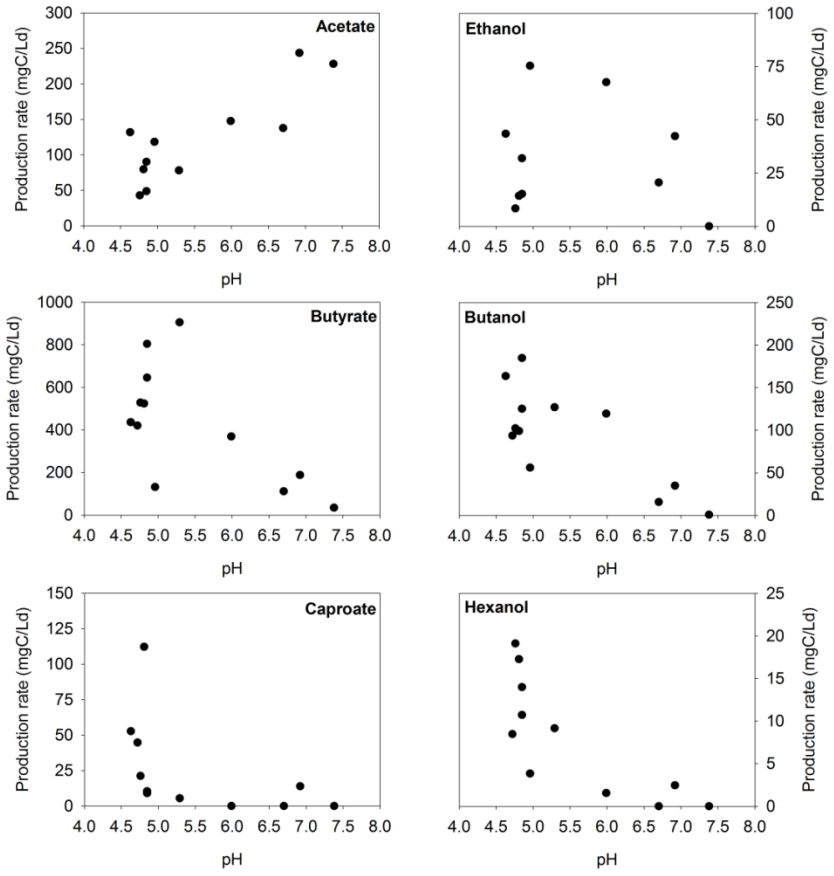

Figure 4.2.4 Production rate of the different metabolites by the carboxydotrophic mixed culture at different pH.

Another hypothesis could be the occurrence of the so-called chain elongation reactions were acetate and ethanol can further be used to produce n-butyrate (Agler et al., 2011). These reactions can be catalyzed by *Clostridium kluyveri*, which was detected during early stages of enrichment process. This rationale would be supported by the production of C6 compounds (up to 110 mg C/L·d of caproate and 20 mg C/L·d of hexanol), although these can be also produced by *C. carboxidivorans* P7, as proven by Ramachandriya and co-workers (2013).

Incubation at 25 ºC prevents acid crash and enhances alcohol production in *Clostridium carboxidivorans* P7

4.3

Part of this chapter has been published as:

Ramió-Pujol, S.; Ganigué, R.; Bañeras, L.; Colprim, J., 2015. Incubation at 25 ºC prevents acid crash and enhances alcohol production in *Clostridium carboxidivorans* P7. Bioresource Technology 192: 296-303. DOI: 10.1016/j.biortech.2015.05.077

4.3.1 Background

Butanol and ethanol production by homoacetogenic Clostridia is negatively affected by high concentrations of undissociated organic acids and low pH values. Thus, uncontrolled fermentation conditions may lead to lower ethanol and/or butanol productivity. For instance, the so-called acid crash effect is the early termination of solventogenesis in Clostridia due to the fast accumulation of organic acids in the medium, resulting in small or null solvent production (Maddox et al., 2000; Mohammadi et al., 2014; Wang et al., 2011). As earlier proposed by Maddox and co-workers (Maddox et al., 2000), this risk can be mitigated by several practices including: i) fine-tuning of fermentation pH, and ii) reduction of acid accumulation rates, which can be achieved by slowing down the metabolic rate of the cells. The latter may be accomplished by lowering the nutrient availability in growth media, or by operating bioreactors at sub-optimal values for key variables controlling growth, such as temperature. Temperature may be the first choice for this purpose since its control is simple and inexpensive, and is relevant as an effector on key cell processes, such as growth, maintenance, and metabolites synthesis, all impacting the accumulation of alcohols (Price and Sowers, 2004).

Most acetogens are mesophilic bacteria and grow at temperatures ranging from 20 to 45 ºC, with 37 ºC being the optimal growth temperature for the majority of strains (Munasinghe and Khanal, 2010a). Consequently, most of the studies on syngas fermentation have been conducted at 37 ºC (Köpke et al., 2010; Kundiyana et al., 2010a; Mohammadi et al., 2014). As of today, only a limited number of studies have investigated the fermentation of syngas at temperatures lower than the optimal (Abubackar et al., 2015, 2012; Kundiyana et al., 2011b). Kundiyana and co-workers reported an increase of cell growth and ethanol production by "C. ragsdalei" when temperature decreased from 37 to 32 ºC (Kundiyana et al., 2011b). Observed higher production rates were explained by the higher solubility of the CO, CO_2 and H_2 at lower temperatures.

The present chapter hypothesizes that a decrease in the incubation temperature would also have a positive effect on alcohol production on *Clostridium carboxidivorans* P7 fermenting syngas by i) decreasing metabolic rates; and ii) by increasing solubility (and hence the availability) of CO and H_2. The aim of this chapter was to provide experimental evidence to evaluate the effect of two temperatures, 25 ºC and 37 ºC, on growth and alcohol production of the type strain *C. carboxidivorans* P7 (DSM15243T) grown on syngas under strict autotrophic conditions.

4.3.2 Experimental design

4.3.2.1 Fermentation experiments

C. carboxidivorans P7 cultures in their exponential growth phase were used as inoculum for batch experiments to test for temperature effects on growth and alcohol production. Culture tubes were thoroughly flushed with syngas mixture for at least 1 minute. During the batch tests, the headspace of each tube was flushed with syngas once a day for 1 minute to ensure replenishment of gas substrates. Incubation of cultures was done at 25 ºC and 37 ºC under mild agitation (100 rpm) on a rotary shaker.

Three independent culture tubes were sampled for gas and liquid media composition at regular intervals: every 8 hours from 11 to 107 h, every 12 hours from 107 to 167 h and every 24 hours from that point until the end of the experiment (15 days).

4.3.2.2 Analytical methods

The total amount of undissociated acids was measured. The pKa values for acetic acid were 4.76 and 4.77 at 25 ºC and 37 ºC, respectively, pKa for butyric acid were 4.82 and 4.88 at corresponding temperatures and pKa for caproic acid were 4.88 at both temperatures (Creager and Clarke, 1994; Harned and Ehlers, 1933; Zigová et al., 1999).

A mass balance of the carbon uptaken was conducted according to concentrations of substrates and products in both, liquid and gas phases. Consumption of substrates (mainly CO), and production of CO_2, end-metabolites (alcohol and acids), and cells (measured as protein concentration) were calculated experimentally of each data point interval. The amount of carbon diverted to maintenance purposes for living cells was estimated according to equation (Eq. (5)):

$$\Delta CO_{consumption} = \Delta CO_{2production} + \Delta (Acid + Alcohol)_{production} + Cells + Maintenance \quad (5)$$

A comprehensive description of these procedures is presented in Supplementary materials A.

4.3.3 Results and Discussion

4.3.3.1 Growth and production of C. carboxidivorans P7 at 37 ºC

During the course of the experiment at 37 ºC, *C. carboxidivorans* P7 exhibited a two-step increase of the OD600 from 0 to 125 hours, before reaching the stationary phase (125-355 hours) (Figure 4.3.1A). Maximum optical densities of the culture were above 0.550 (187 hours). The concentration of proteins was higher during the first step of growth achieving values of 52.8 µg/mL and dropped down to 12.4 µg/mL during the stationary phase (Table 4.3.1). Although it was not proven experimentally, the decrease in the protein concentration was hypothesized to be due to the minimization of growth rate during unfavorable environmental conditions at the stationary phase such as product toxicity or acidification. This may have caused partial cell lysis and solubilization of proteins, which were not analyzed (Finkel, 2006; Navarro Llorens et al., 2010). The pH of the fermentation broth decreased from 5.9 to a mean value of 4.7 during the stationary phase. Growth was concomitant with the decrease in pH and the production of acetic acid in the culture (Pearson correlation, p-value<0.05). Maximum concentrations of acetate, butyrate, and caproate; 58.2 mM, 4.11 mM, and 0.419 mM, respectively, were reached during the stationary phase (Figure 4.3.1B). The production of ethanol was low,

with maximum concentrations of 1.56 mM. Longer chain alcohols were not detected at significant concentrations (Figure 4.3.1C).

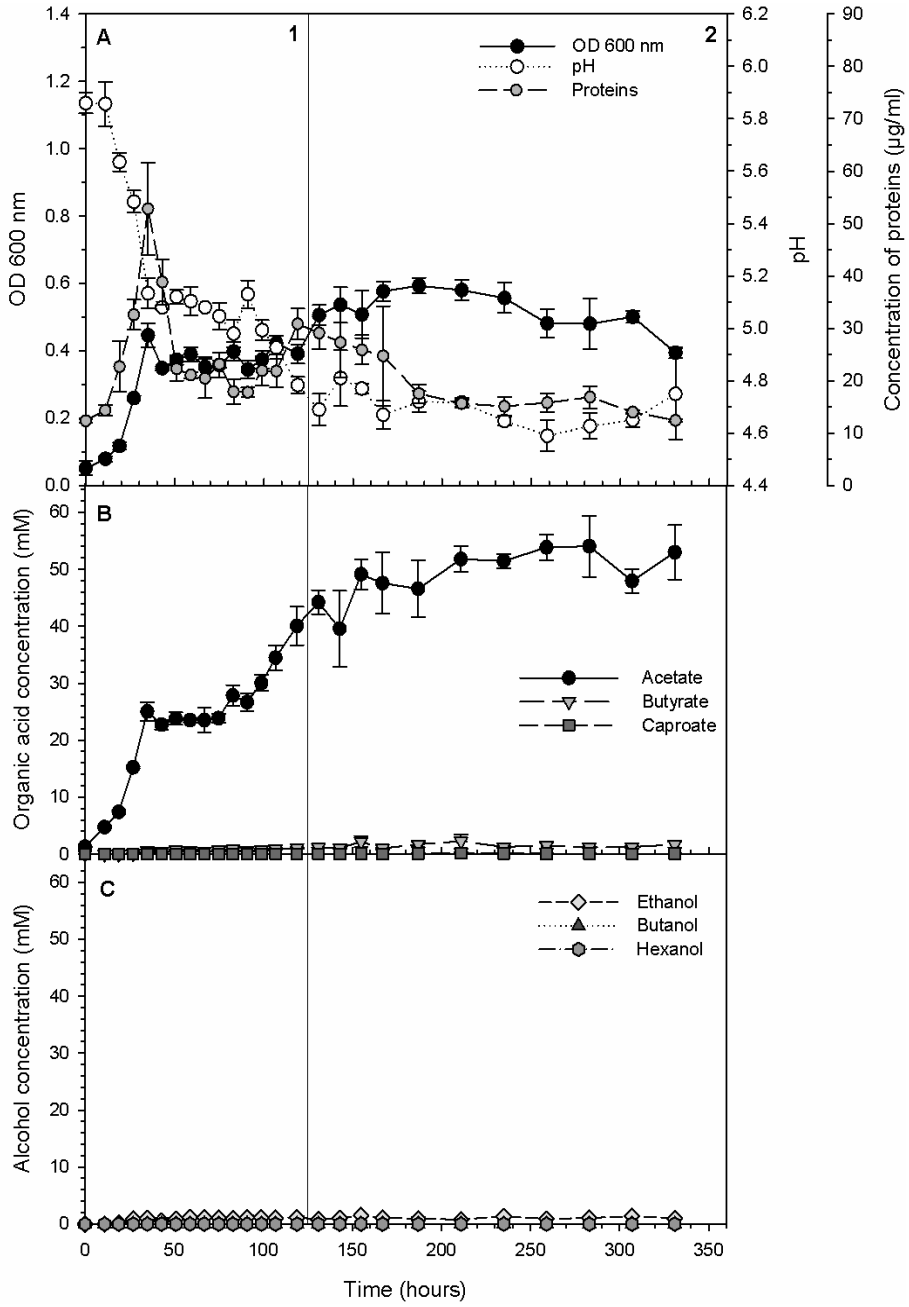

Figure 4.3.1 Growth and production of *Clostridium carboxidivorans* P7 at 37 ºC. Mean values and standard errors of three replicates are shown in all graphs. (**A**) Optical density at 600 nm (black dots), pH (white dots), and concentration of proteins (grey dots). (**B**) Concentrations (mM) of acetate (black dots), butyrate (light grey inverted triangles) and caproate (dark grey squares). (**C**) Concentrations (mM) of ethanol (light grey rhombus), n-butanol (black triangles) and 1-hexanol (dark grey dots). Line shows the end of the growth phase (**1**) and the beginning of the stationary phase (**2**).

Table 4.3.1 Summary of the maximum and average (in brackets) growth rate, concentration of protein, acids and solvents production rates, and gas consumption rate during the different phases (detailed described in section 3.1 and 3.2) of growth at 25 and 37 ºC incubation temperatures of *Clostridium carboxidivorans* P7.

		Maximum growth rate (h^{-1})	Maximum concentration of protein ($\mu g \cdot mL^{-1}$)	Acid production ($mmols \cdot g \cdot prot^{-1} \cdot h^{-1}$)			Solvent production ($mmols \cdot g \cdot prot^{-1} \cdot h^{-1}$)			Gas consumption ($mmols \cdot g \cdot prot^{-1} \cdot h^{-1}$)		
				Acetate	Butyrate	Caproate	Ethanol	Butanol	Hexanol	CO	CO_2	H_2
37 ºC	Growth phase (0 -125 hours)	0.082 (0.068)	52.8 (25.3)	35.4 (13.0)	1.54 (0.284)	0.068 (0.001)	3.09 (0.361)	-	-	107.7 (51.6)	-39.8 (-30.5)	16.5 (1.50)
	Stationary phase (125 – 355 hours)	-	16.8 (19.0)	29.9 (7.89)	1.71 (0.058)	0.289 (0.004)	1.35 (0.160)	-	-	82.3 (18.0)	-29.7 (-5.81)	60.8 (9.52)
25 ºC	Growth phase (0 -125 hours)	0.079 (0.052)	73.2 (52.9)	26.8 (1.79)	3.60 (0.704)	2.61 (0.380)	4.62 (1.24)	1.52 (0.416)	1.22 (0.198)	67.2 (52.2)	-48.3 (-30.9)	19.0 (-0.92)
	Stationary phase (125 – 355 hours)	-	65.7 (50.3)	2.85 (0.002)	1.14 (0.117)	2.13 (0.412)	3.17 (0.886)	1.79 (0.348)	1.61 (0.238)	35.9 (31.5)	-20.4 (-8.52)	7.102 (2.24)

Production of alcohols in classical ABE fermenters using carbohydrates usually occurs at pH ranging from 4.5 to 5.0, when their metabolism shifts from acidogenesis to solventogenesis (Jones and Woods, 1986). This also holds true for syngas fermenting carboxydotrophic bacteria (Grethlein et al., 1990; Phillips et al., 1993). Poor alcohol production in such conditions is usually attributed to the acid crash phenomenon (Maddox et al., 2000), which has been also reported in acetogenic *Clostridium ljungdahlii* ATCC 55383 (Mohammadi et al., 2014). Our experimental results at 37 ºC suggested that acid crash could be the main effect leading to poor alcohol productivity in *C. carboxidivorans* P7. This phenomenon was observed in 70 independent batch cultures inoculated and maintained at 37 ºC, presenting pH values below 4.8. A thorough investigation of the culturing conditions in *Clostridium beijerinckii* NRRL B592 concluded that this phenomenon was triggered at concentrations of undissociated acids (acetate and butyrate) over 57 mM in the culture broth (Maddox et al., 2000). Mohammadi *et al.* (2014) reported also low ethanol production for *Clostridium ljungdahlii* at lower concentrations of undissociated acids (34.5 – 33.2 mM). This discrepancy in results implies that inhibition thresholds are likely to be strain-specific. The maximum concentration of undissociated acids found in the present study, 36.4 mM (Supplementary material, Figure B1), is in agreement with the previously reported figures for *C. ljungdahlii*. A second factor that could explain the low alcohol production is the rate of accumulation of acids. A fast accumulation of undissociated organic acids contributes to the acid crash in acetogenic bacteria, which invariably leads to a decreased growth capacity and productivity (Maddox et al., 2000; Russell and Diez-Gonzalez, 1997; Wang et al., 2011). In the present chapter, the acetate accumulation during the exponential phase was high (40.1 mM), although the culture reached maximum concentration (58.2 mM).

4.3.3.2 Growth and production of *C. carboxidivorans* P7 at 25 ºC

C. carboxidivorans P7 exhibited also a typical growth curve at 25 ºC, with clearly defined lag phase (0-50 hours), exponential growth phase (50-200 hours), and a stationary phase (200-355 hours). Production of acids mainly occurred during exponential growth, whereas production of alcohols sequentially started during the late exponential growth phase and spanned down to the end of the experiment. Strain P7 reached maximum OD600 values of 1.2, doubling those measured at 37 ºC (Figure 4.3.2A). As expected, the average growth rate during the exponential growth phase was lower at 25 ºC when compared to 37 ºC (0.052 ± 0.020 h^{-1} and 0.068 ± 0.013 h^{-1}, respectively). Observed growth rates for the two experimental conditions fall within the range reported by other authors at similar growth conditions (0.053 h^{-1} and 0.063 h^{-1}) (Bruant et al., 2010; Ukpong et al., 2012). Lower temperatures also caused an increase in the duration of the lag phase of about 50 hours, thus confirming a lower metabolic activity. Regarding the concentration of proteins, its value increased up to 73.2 µg/mL, which was about 40% higher than at 37 ºC. Similarly to what was observed at 37 ºC, the concentration of proteins decreased gradually to about 30 µg/mL during the stationary phase while OD600 remained constant.

Acetic acid was the main product during the exponential growth phase at 25 ºC, reaching a maximum concentration of 27.4 mM at time 91 hours (Figure 4.3.2B). Production of butyric and caproic acids started also during the exponential growth phase, although their onset was not observed until 59 and 67 hours, respectively. The maximum concentrations of these compounds during the stationary phase were 7.90 mM and 9.02 mM, respectively. At 25 ºC, the maximum production rate of acetate was lower than at 37 ºC (26.8 vs.35.4 mmol·g $prot^{-1}$·h^{-1}). On the contrary, the maximum butyrate production rate doubled its value to 3.60 mmol·g $prot^{-1}$·h^{-1} at 25 ºC. The maximum production rates for acetate and butyrate coincided with the exponential growth phase, whereas maximum caproate production rate was 2.61 mmol·g $prot^{-1}$·h^{-1} at the end of the growth phase (Table 4.3.1). It is hypothesized that lower acid production rates and lower

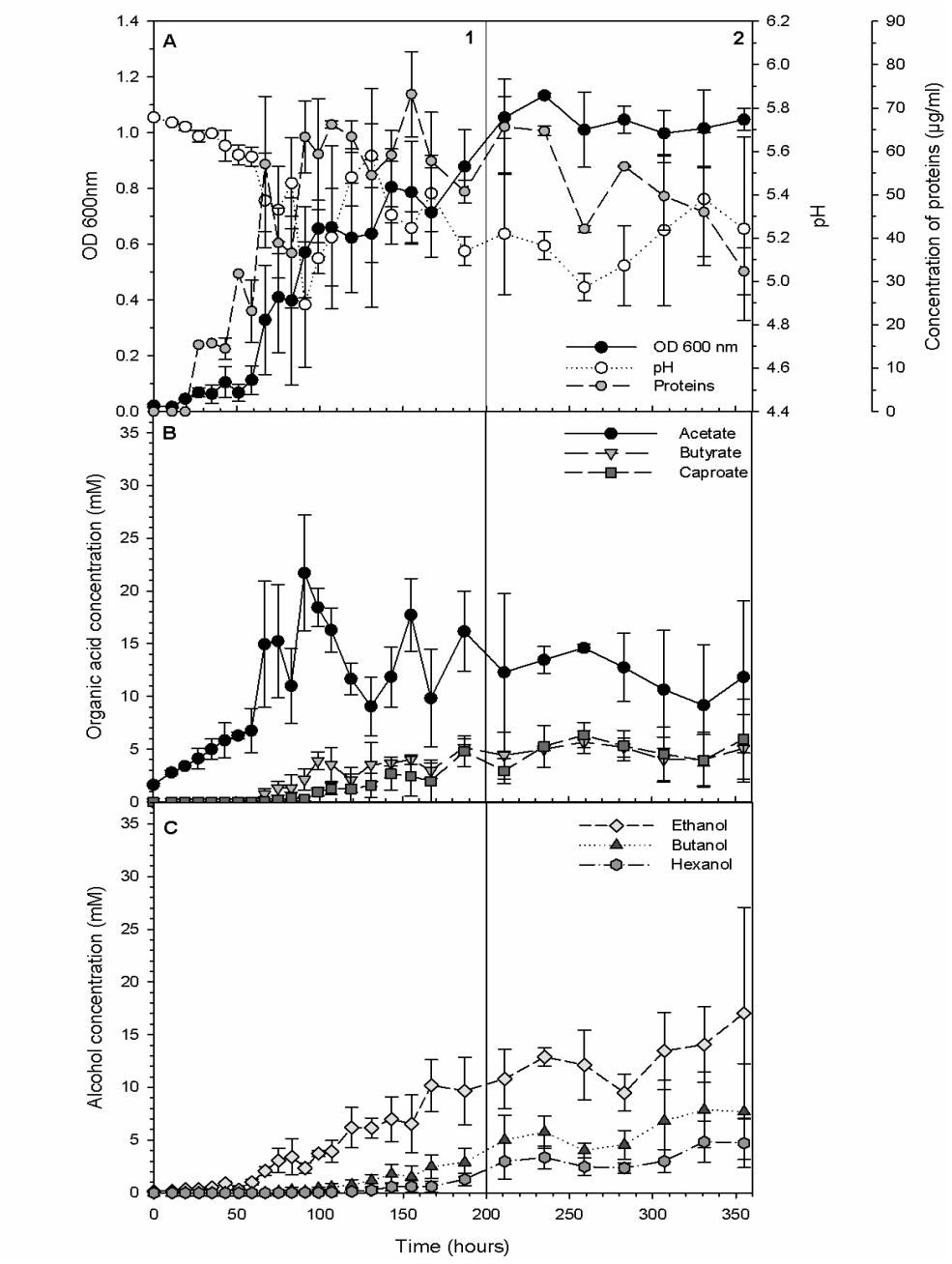

Figure 4.3.2 Growth and production of *Clostridium carboxidivorans* P7 at 25 ºC. Mean values and standard errors of three replicates are shown in all graphs. (**A**) Optical density at 600 nm (black dots), pH (white squares), and concentration of proteins (grey dots). (**B**) Concentrations (mM) of acetate (black dots), butyrate (light grey inverted triangles) and caproate (dark grey squares). (**C**) Concentrations (mM) of ethanol (light grey rhombus), n-butanol (black triangles) and 1-hexanol (dark grey dots). Line shows the end of the growth phase (**1**) and the beginning of the stationary phase (**2**).

concentration of undissociated acids at 25 ºC (20.9 mM, Supplementary material Figure B1) contributed to prevention of the acid crash at 25 ºC.

Alcohol production started during the exponential growth phase, in concomitance with acidogenesis. Production of ethanol, butanol and hexanol initiated after 50, 100 and 120 hours of incubation, respectively. The pH decreased from 5.76 up to 4.89 before alcohol production started, and subsequently increased to pH 5.38. The highest concentrations of alcohols were observed during the stationary phase, being 32.1 mM of ethanol (17.0 ± 10.0), 14.5 mM of butanol (7.70 ± 4.54), and 8.21 mM of hexanol (4.72 ± 2.33). Butanol titers observed in this study were among the highest reported for *C. carboxidivorans* grown in strict autotrophic conditions. Comparable butanol concentrations have recently been reported by Phillips *et al.* in a batch culture incubated at 37 ºC (14.7 mM) (Phillips et al., 2015) and by Rajagopalan and co-workers in a continuously operated bioreactor at 37 ºC and pH 5.3 (14.8 mM) (Rajagopalan et al., 2002). The maximum specific alcohol production rates of the present study were 4.62 mmol·g $prot^{-1}$·h^{-1} for ethanol, 1.79 mmol·g $prot^{-1}$·h^{-1} for butanol, and 1.61 mmol·g $prot^{-1}$·h^{-1} for hexanol. Longer carbon chain alcohols (C4 and C6) were mainly produced during the stationary phase.

4.3.3.3 Substrate consumption and carbon balance

Experiments were conducted under strict autotrophic conditions, with a syngas mixture of CO, CO_2, and H_2 as sources of carbon and reducing equivalents. At both experimental conditions, CO was consumed while a net cumulative production of CO_2 was observed (Figure 4.3.3). Interestingly, H_2 concentrations remained almost invariable, revealing that CO was preferentially consumed over H_2 as a source of reducing power. In acetogenic bacteria, CO is oxidized to CO_2 by CO dehydrogenase (CODH) and electrons are transferred mainly to a ferredoxin carrier, whereas hydrogenase activity (H_2/hydrogenase) catalyzes the H_2 conversion into protons and electrons, which are transferred to an electron carrier (ferredoxin (Fd^{2-}) and/or NADH) (Ragsdale, 2004). However, the two activities are not likely to occur simultaneously at high rates since trace amounts of CO inhibits hydrogenase activity. In *A. woodii* the threshold for 50% inhibition was determined to be as low as 7 nM (Ragsdale and Ljungdahl, 1984). Regard-

ing the experimental conditions used here, CO concentration in solution was increased after daily flushing up to 26 mM, and therefore H_2/hydrogenase remained inactive, leaving CO as the sole source of reducing equivalents.

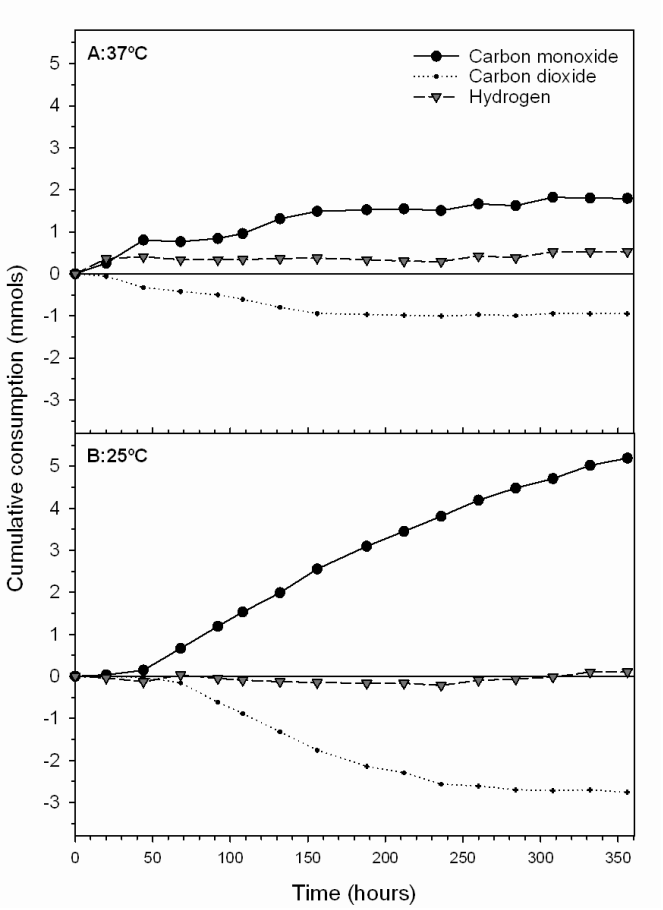

Figure 4.3.3 Cumulative consumption of the different syngas components (mmols) over time (hours) in batch tubes of *Clostridium carboxidivorans* P7 at 37 ºC (**A**) and 25 ºC (**B**). CO (black dots), CO_2 (small black dots), and H_2 (dark grey inverted triangles).

Solubility of gases is higher at lower temperatures, thus resulting in an increased availability when used as substrates for bacterial growth. This property may not be relevant for CO_2, which is a relatively soluble gas (24.9 mmol·L^{-1}·atm^{-1}) at 37 ºC. However, CO is far less soluble (6.25 mmol·L^{-1}·atm^{-1}) at the same temperature and solubility changes due to temperature decrease may have an impact, and thus should be considered. Based on the Henry's law, the amount of CO in the liquid phase of each tube after syngas flushing was calculated to be 31.3 and 26.4 nmols at 25 ºC and 37 ºC, respectively. Although the solubility of H_2 may also be highly influenced by temperature (0.731 mmol·L^{-1}·atm^{-1}), this effect was not considered here since no significant consumption of H_2 was observed.

In the experiment at 37 ºC, the consumption of CO (and concomitant production of CO_2) was mainly observed during the initial growth (0-150 hours). On the contrary, carbon fixation to products at 25 ºC took place during the whole incubation period (Figure 4.3.4). Maximum CO consumption rates at 37 ºC and 25 ºC were 107.7 mmols CO·g prot^{-1}·h^{-1} and 67.2 mmols CO·g prot^{-1}·h^{-1}, respectively. The high rate of CO

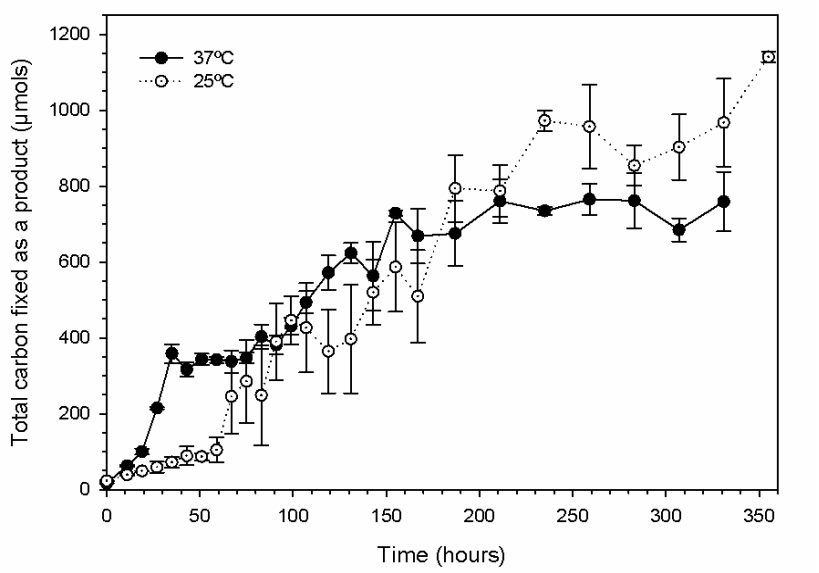

Figure 4.3.4 Carbon fixed (µmols) as products over time (hours) in batch tubes of *Clostridium carboxidivorans* P7 at 37 ºC (black dots) and 25 ºC (white dots).

consumption found during the exponential phase at 37 ºC caused a complete depletion of the preferred reducing power source (CO) before syngas was replaced. This limitation could have also favored the trigger of the acid crash event since alcohol synthesis demands for a higher amount of reducing power equivalents in comparison with organic acids.

When looking at the carbon balance, the total amount of CO consumed at 25 ºC was almost three times higher than at 37 ºC, being 4.85 and 1.80 mmols C, respectively. Most of the consumed carbon was directly converted into CO_2, 2.83 (58.3%) and 0.95 mmols C (52.8%) at 25 and 37 ºC, respectively, providing reducing equivalents to the cells. In addition, the total amount of carbon fixed as product was different in both cultures being 1.12 (23.1%) and 0.74 mmols C (41.1%) at 25 and 37 ºC. Finally, 0.90 and 0.11 mmols of C were estimated to have been used for growth and maintenance of bacterial cells at 25 and 37 ºC, respectively. A higher amount of carbon was incorporated into the bacterial biomass at 25 ºC due to the higher number of cells active in the culture during a longer time period.

4.3.3.4 Production of longer carbon chain fermentation products

The main products of acetogenic bacteria are molecules of two or four carbon chains, although *C. carboxidivorans* P7 is able to produce longer carbons chains using the CoA-dependent *Clostridium* route (Zhang et al., 2012). In this study, relatively high concentration of C6 compounds were obtained in *C. carboxidivorans* P7 at 25 ºC but not at 37 ºC, supporting the studies on classical ABE fermentation which reported enhanced production of longer carbon chain molecules at sub-optimal temperatures (Dwidar et al., 2012; Li et al., 2014). Maximum concentrations of hexanol and caproic acid achieved in this study were 8.21 mM and 9.02 mM, respectively. Production of C6 compounds from a mixture of H_2:CO_2:N_2 by *C. carboxidivorans* was previously reported by Ramachandriya and co-workers, with total maximum concentration of hexanol and caproic around 5.09 mM and 3.62 mM, respectively, significantly lower than those obtained in the present chapter (Ramachandriya et al., 2013). In that study, authors suggested that the production of C6 compounds is not usually present in autotrophic fermentations due to the higher demand of reducing equivalents compared to those needed for the production of C4 and C2 compounds (Ramachandriya et al., 2013). The present experimental set-up, with frequent supply of fresh syngas (and thus CO and H_2) likely allowed circumventing this limitation. Recently, Phillips *et al.* reported the development of a new medium and culture techniques, obtaining for strain P7 similar concentrations of hexanol (9.20 mM) as the present study (Phillips et al., 2015). In this case the authors triggered the production of hexanol by using minimal medium and controlling the supply of CO.

Condensation of C1 substrates into C4 and C6 alcohols in Clostridia can be done in two ways. C4 and C6 alcohols (either linear or branched) can be produced de novo via the CoA-dependent *Clostridium* route (Zhang et al., 2012) or as metabolic intermediates (generally the corresponding acids), which are excreted outside the cell during acidogenesis and latter, re-assimilated and reduced to alcohols by carboxylic acid reductases (Jones and Woods, 1986). The latter is common in many bacteria and has been proven to

be effective in acid conversion to alcohols in Clostridia (Perez et al., 2013). To elucidate whether one or the other synthesis route prevails at given working conditions is biotechnologically relevant because a re-assimilation-reduction route can be artificially sustained as long as reducing enzymes are active and substrate added to the culture. In the present study both mechanisms may have occurred concomitantly during the growth phase and the early stationary phase since alcohols and acids were simultaneously produced. However, during late stationary phase C2, C4 and C6 alcohols were produced while acid concentrations decreased suggesting that alcohol production took place at the expense of acids (Figure 4.3.5). Significant negative correlations ($p<0.01$) were found between the acid and alcohol concentrations for acetic/ethanol, butyric/butanol, and caproic/hexanol pairs, at 25 ºC. Linear regressions yielded slopes higher than 0.6 for all three alcohols, and approached to acid:alcohol ratio close to 1:1 for C2 and C4 compounds (Pearson correlation test, $n=20$). The reassimilation of acids and their reduction into alcohols was also observed in *C. carboxidivorans* and "C. ragsdalei" (ATCC PTA-7826) in syngas fermentation (Ramachandriya et al., 2013). Acid re-assimilation and conversion

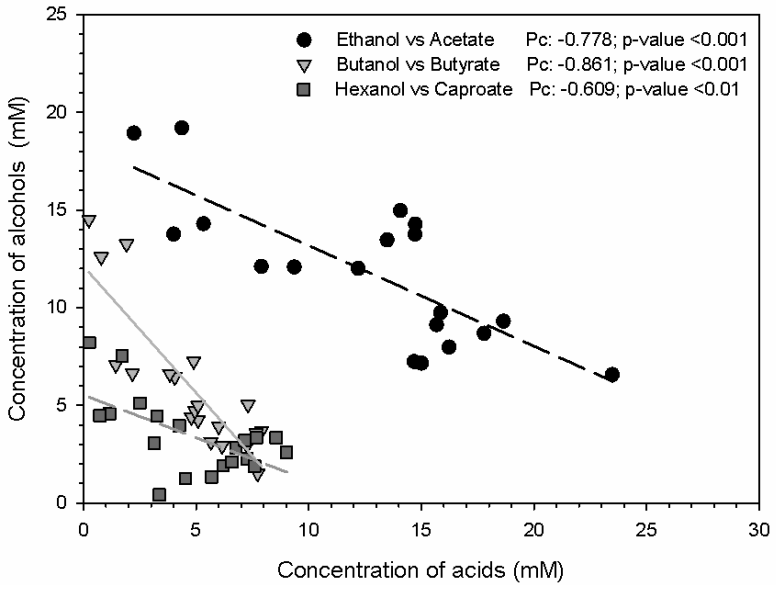

Figure 4.3.5 Correlations between acetate/ethanol, butyrate/butanol, and caproate/hexanol produced by *Clostridium carboxidivorans* P7 at 25 ºC in stationary phase. C2, black dots and short dash line; C4, light grey inverted triangles and solid; C6, dark grey squares and long dash line. **Pc** identifies the Pearson correlation coefficient.

into alcohols is postulated as a detoxification mechanism to prevent cell damage and activity decline at excessively low pH values and high concentrations of undissociated organic acids. However, according to the results obtained at 37 ºC, there seem to be a "point of no return" in which the effects of low pH cannot be reversed. In addition, the identification of the molecular mechanisms leading to this would help in describing the acid crash phenomenon and provide knowledge on how to avoid it during fermentations.

4.3.4 Supporting information:

A. Carbon balance

A carbon balance was conducted to determine the fraction of carbon consumed that was converted into products. For that, samples for the analysis of gas phase composition and concentration of products in the fermentation broth were periodically taken.

A.1 Consumption of the different components

Consumption of gas components was calculated throughout the experiment from measured concentrations. Provided that nitrogen gas is an inert gas, neither consumed nor produced *by C. carboxidivorans*, its total amount (and thus, its partial pressure) was assumed to remain constant over time. Therefore pressure losses in incubation tubes due to gas consumption were estimated according to measured partial pressures of N_2. Syngas composition was 32% CO - 32% H_2 - 28% N_2 - 8% CO_2, and was used at 1 atm overpressure. The partial pressure of N_2 (P_{N2}) at 1 atm in each tube was after syngas flushing was 0.56 atm.

Total pressure (P_T) in each vial was determined according to equation A1.

$$P_{N2} = P_T \cdot X_{N2} \qquad \text{(A1)}$$

X_{N2} is the molar fraction of N_2, determined by gas chromatography in every sample.

The partial pressure of each component present in the gas phase (Pi) was calculated for each sample according to Raoult's law (Eq. (A2)).

$$P_i = P_T \cdot X_i \qquad \text{(A2)}$$

Where P_T is the headspace pressure calculated according to Equation A1 and X_i is the molar fraction of each gas, determined by gas chromatography.

The quantity of each component in the gas phase (*ni*, mols) was determined for each sample according to Ideal gas law (Eq. (A3)).

$$n_i = P_i \cdot V_g / R \cdot T \quad \text{(A3)}$$

Where V_g was the total headspace volume (18.4 mL).

Based on these, the concentration of the different components in the liquid phase at equilibrium conditions was calculated for each sample following Henry's law (Eq. (A4)).

$$L_i = P_i \cdot K_H \quad \text{(A4)}$$

Where L_i is the concentration of the dissolved gas (mols/L) and K_H is the Henry's constant. Henry's constants used were 0.0074; 0.034; 0.00078 mol/L·atm at 25°C and 0.00625; 0.024893; 0.000731 mol/L·atm at 37°C for CO, CO_2, and H_2 respectively (Sander, 1999).

Finally, the accumulated consumption of each component (C_i) throughout the experiment was calculated by subtracting the amount of the component *i* (both in the gas and liquid phase) at a given time (*t*) with the quantity of each component in the gas and liquid phase after the flushing period (*t-n*, where n is the previous flushing period, 24 hours). This is presented in Equation A5.

$$C_i = n_{i(t)} - n_{i(t-n)} \quad \text{(A5)}$$

A.2 Carbon in products

To calculate the amount of carbon transformed into extracellular organic products (i.e. organic acids and alcohols, *Ei*), the total concentration of each product were transformed from mg per liter to mmols according to their respective molecular weight and the volume of the liquid phase, *Vl*, which was 6.6 mL. These values were transformed

into mmols of carbon as product by taking into consideration the length of the carbon chain of each compound.

A.3 Overall carbon balance

For each tube, the quantity of the different components of syngas in the liquid and gas phase was added up (*Zi*). The accumulated consumption and/or production (*Bi*) for each sample was calculated following the Equation A6.

$$Bi = Zi + Zi_{(t-1)} \quad \text{(A6)}$$

The carbon amount used for the maintenance and the growth of the culture (*Gi*) was calculated adding the CO_2 and CO consumption to obtain the quantity of carbon fixated and subtracting the quantity of carbon transformed into extracellular organic products (i.e. organic acids and alcohols) (Eq. (A7)).

$$B_{CO} + B_{CO2} - (\sum Ei) = Gi \quad \text{(A7)}$$

B. Concentration of undissociated organic acids in the fermentation broth

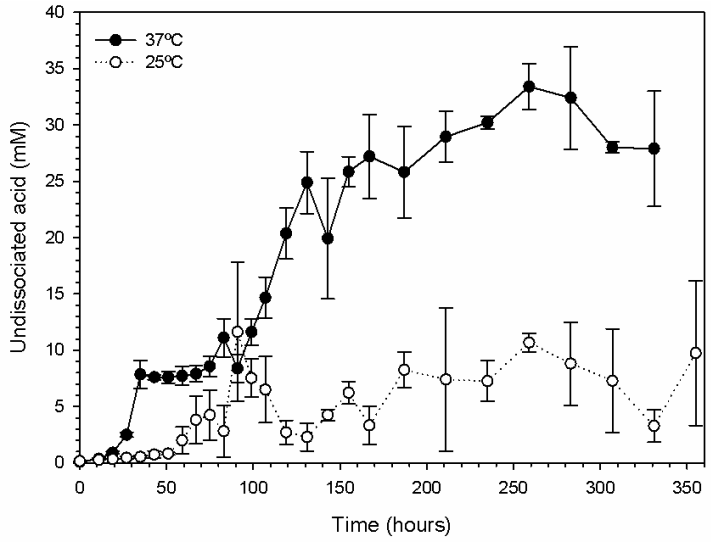

Figure B1 Concentration of undissociated organic acids in the fermentation broth during the fermentation of syngas by *Clostridium carboxidivorans* P7 at 37 ºC (black dots) and 25 ºC (white dots). Mean values and SE of three replicates are shown in the graph.

4.4 Yeast extract supplementation does not improve growth or productivity of *Clostridium carboxidivorans* P7 grown on syngas

Part of this chapter has been submitted as:

Ramió-Pujol, S.; Ganigué, R.; Bañeras, L.; Colprim, J., 2015. Media supplements are not essential for the growth and production of *Clostridium carboxidivorans* P7 on syngas fermentation. Research note (unpublished data).

4.4.1 Background

Media formulation plays an important role in growth and product yield of acetogens (Ramachandriya et al., 2013; Ramió-Pujol et al., 2015a). The development of low-cost fermentation medium formulae containing all essential nutrients required for cell growth and product formation has been in the spotlight to reduce the overall costs of the fermentation process. Standard medium for acetogenic bacteria reported for solvent production is composed of vitamins, minerals, trace metals, and reducing agents. In addition, supplements such as yeast extract (YE), corn steep liquor (CSL), or cotton seed extract (CSE), are used in most media formulations (Ramió-Pujol et al., 2015a). This complex organic matrices are considered as sources of micronutrients that may substitute or supplement the use of purified vitamin solutions (Kundiyana et al., 2010a; Maddipati et al., 2011; Ramachandriya et al., 2013). They also contain a large variety of organic molecules, which may be directly incorporated by active cells as building blocks into biological structures (Ramió-Pujol et al., 2015a), thus decreasing carbon fixation needs through the WLP. Nevertheless, it was reported that CO and CO_2 remain the main carbon source during syngas fermentation (Kundiyana et al., 2010a; Maddipati et al., 2011; Ramachandriya et al., 2013).

Despite the extended use of YE on syngas fermentation, experiments in our lab showed that this may not be indispensable for the growth and production of several acetogenic bacteria (i.e. *Clostridium ljungdahlii* PETC, *Clostridium carboxidivorans* P7, and *Butyribacterium methylotrophicum* DSM3468 (Ramió-Pujol et al., 2015b, 2015c)). In contrast, the use of YE enhances contamination risks of fermentative bacteria, which although fully controlled in lab scale reactors, can be a serious issue for larger scale reactors. The main objective of this study was to investigate whether YE supplementation had any positive effects on growth and production of *Clostridium carboxidivorans* P7 during syngas fermentation, and to provide experimental data to support or dismiss a widely extended belief among researchers in the field.

4.4.2 Experimental design

4.4.2.1 Fermentation experiments

A 10 % inoculum from *C. carboxidivorans* P7 (P7) at middle exponential growth phase was used for batch experiments. Culture tubes were thoroughly flushed with syngas mixture (syngas, 32% CO - 32% H_2 - 28% N_2 - 8% CO_2) of high purity (Praxair Technology Ltd, Spain) every 24 hours to ensure replenishment of gas substrates. Headspace overpressure was 100 kPa. YE was added at final concentrations of 0.5 g/L and 1.0 g/L. Tubes containing no YE (0.0 g/L experiment) were included as controls under fully autotrophic conditions. Tubes were incubated horizontally to enhance gas-liquid mass transfer on a rotatory shaker at 100 rpm and 25 ºC (Ramió-Pujol et al., 2015b).

Three independent culture tubes were sampled at 0, 71, 142, 239, and 335 hours for analysis of product formation. Liquid samples were filtered using nylon filters (0.2 µm pore size, Millipore, Germany) to remove cells and stored at 4 ºC until analyzed. The pH of the media was measured using a BASIC 20 pH meter (Crison, Spain). Growth was monitored on a daily basis by measuring the absorbance at 600 nm using a CE1021 spectrophotometer (CECIL, Cambridge, UK).

4.4.3 Results and Discussion

4.4.3.1 Effect of YE concentration on growth of Closridium carboxidivorans P7

Figure 4.4.1 presents the growth curves of P7 at 0.0, 0.5, and 1.0 g YE /L. Growth of P7 was similar for the three tested concentrations, despite the presence of a 24 hours lag phase in treatments without YE.

The maximum growth rates during the exponential growth for P7 under completely autotrophic conditions (0.0 g/L of YE), with 0.5 g YE/L, and with 1.0 g YE/L were 0.047 ± 0.002 h^{-1}, 0.055 ± 0.005 h^{-1}, and 0.045 ± 0.003 h^{-1}, respectively, and are in

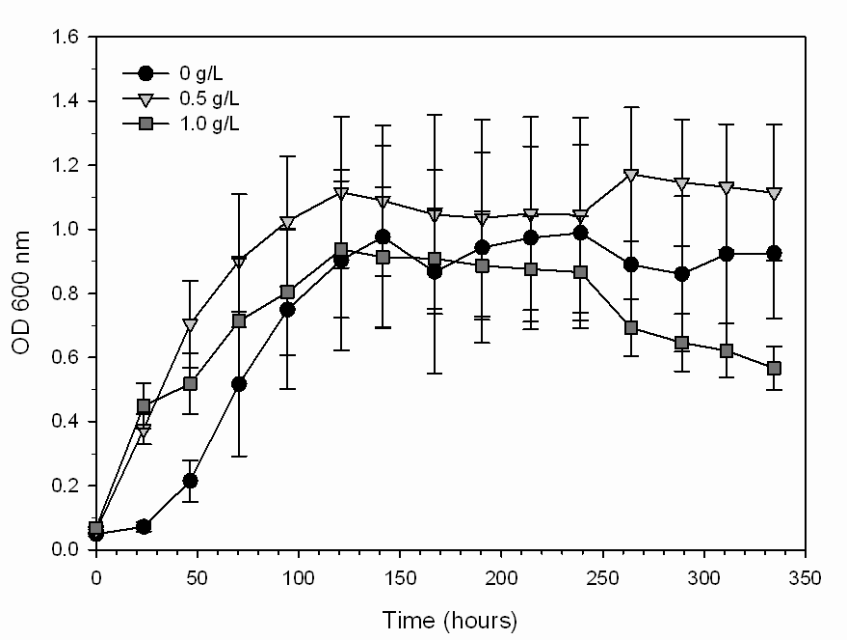

Figure 4.4.1 Growth curves (mean values and SD, n=3) of *C. carboxidivorans* P7 at different yeast extract concentrations.

agreement with values reported in previous works using the same strain (Ramió-Pujol et al., 2015b; Ukpong et al., 2012). The growth rate of the experiments at 0.5 g YE/L was significantly higher compared to the other conditions ($p<0.05$, Kruskal-Wallis test, n=22). Besides, the highest cell density (1.24 ± 0.10 au) was obtained for a concentration of YE of 0.5 g YE/L, which was similar to that obtained in the experiments without YE addition (1.22 ± 0.10 au). On the contrary, the maximum concentration of cells of the experiment supplemented with 1.0 g YE/L, 1.02 ± 0.16 au, was significantly lower than those obtained for the other two treatments ($p<0.05$, Kruskal-Wallis test, n=22). In a similar set of experiments, the growth of three non carboxydotrophic *Clostridium thermocellum* strains (I-1-B, ATCC27405, and JW20) was significantly reduced in media supplemented with low and high concentrations of YE (1.0 to 4.0 and 18.0 to 20.0 g YE/L) (Sato et al., 1992).

4.4.3.2 Effect of YE on syngas fermentation production

The concentration of acids and alcohols produced by P7 were measured at the beginning of the experiment (0 h); during the exponential growth phase (71 h); at the end of the growth phase (142 h); during the stationary phase (239 h); and at the end of the experiment (335 h) (Figure 4.4.2).

Acetic acid was the main end-metabolite in all the incubation conditions and was mostly produced during the growth phase. The maximum concentration of acetic acid was 2.5 g/L, corresponding to the cultures amended with 0.5 g YE/L. Butyric and caproic acid were also detected in all culturing conditions, with maximum concentration of 1.5 g/L and 1.2 g/L, respectively. No significant differences were observed for the production of any acid across the different YE concentration ($p>0.05$, Kruskal-Wallis test, $n=28$), which took place throughout the whole experiment.

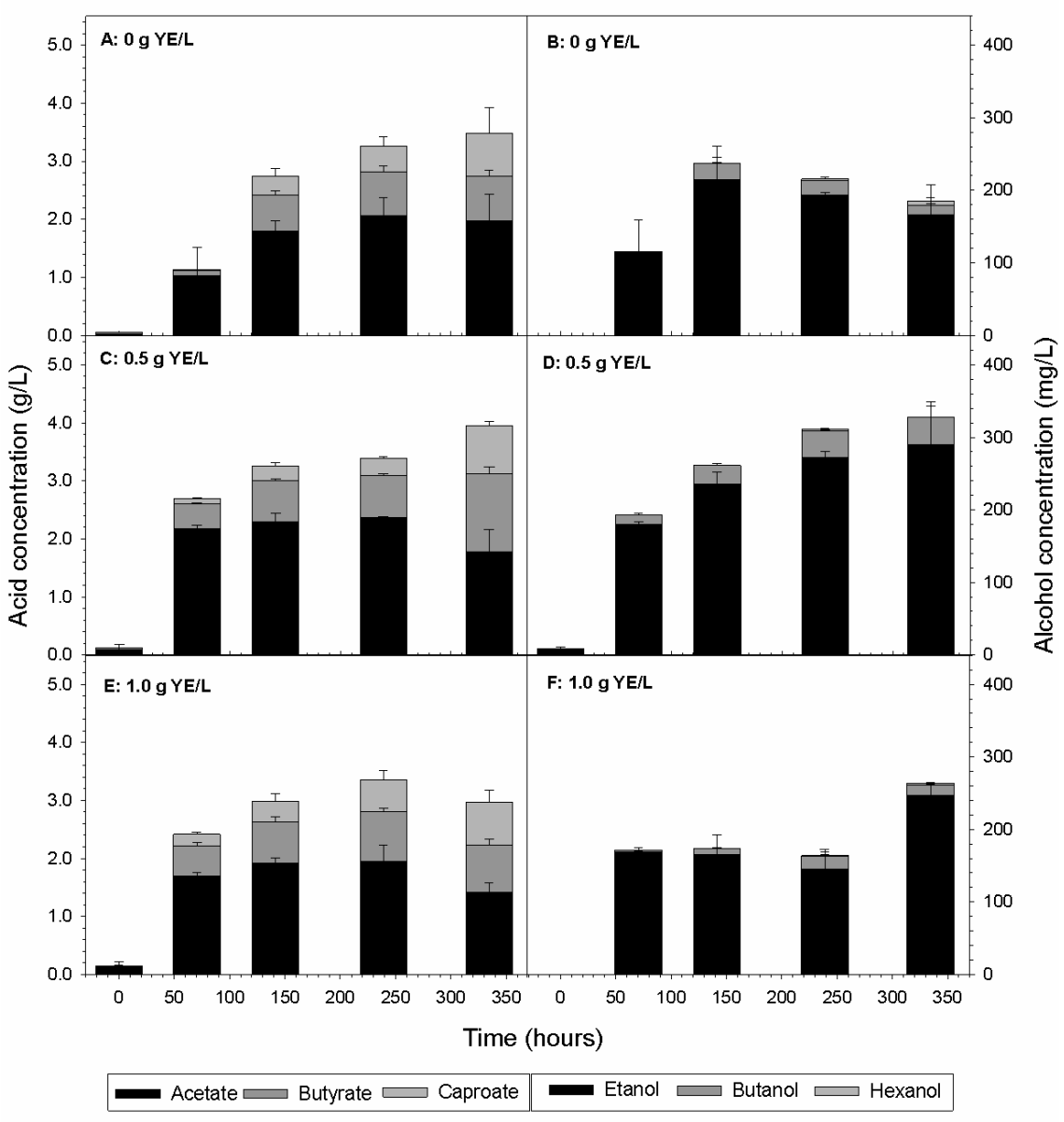

Figure 4.4.2 Concentrations of organic acids (acetate, butyrate, and caproate) (**A, C, E**) and alcohols (ethanol, butanol, and hexanol) (**B, D, F**) at different yeast extract concentration over the time (hours) in batch tubes of *C. carboxidivorans* P7.

Regarding alcohols production, maximum concentration of ethanol and butanol were 0.35 g/L and 0.05 g/L, respectively, obtained for cultures incubated at 0.5 g YE/L. Hexanol production was rather low in all culture conditions. Maximum recorded concentrations were 0.01 g/L when no YE was added to the medium. However, despite the higher concentrations of alcohols in the absence of YE, these differences were not significant across the different YE concentration ($p>0.05$, Kruskal-Wallis test, $n=28$). Similarly to what is observed here, some previous studies reported of an increase on ethanol production with other carboxydotrophic bacterial species in conditions were YE was completely removed from the media (Liu et al., 2014; Phillips et al., 1993).

4.4.3.3 Concluding remark

Although YE has not a specific formulation and significant differences may be found on amino acids, nucleotides, and other organic molecules, relative composition when different producers are compared, it is generally included in media formulations as a source of vitamins, protein building blocks, carbohydrates and some micronutrients. In this chapter we show that low YE concentrations suitable to be used in large scale syngas fermentation have limited benefits on syngas fermentation by *C. carboxidivorans* P7 and likely do not fall beyond a "priming effect" for bacterial growth.

As of today, YE has been used as an essential component of formulated liquid media on syngas fermentation for carboxydotrophic bacteria, usually presupposed to increase the fermentation yield (product formation per unit C). However, in this study, no experimental evidence could be obtained to support this assumption. At the tested concentrations, YE was not strictly necessary for growth or alcohol production. In contrast, the addition of 1.0 g YE/L, although reducing the duration of the lag phase, had a negative effect on growth rate and delayed alcohol production. This effect was not observed at 0.5 g YE/L compared to cultures in complete autotrophic conditions. In conclusion, the outcomes of this study support the thesis that the concentration of YE from media formulations for P7 syngas fermentation can be reduced (or even completely

removed) without significant negative impacts on fermentation performance. Ideally, this practice will lower fermentation cost, and indirectly will facilitate downstream processing since lower complexity of exhausted media is expected for YE free formulations.

Inhibition effects of ethanol and butanol on growth of three alcohol-producing model carboxydotrophic bacteria

4.5

Part of this chapter has been submitted as:

Ramió-Pujol, S.; Ganigué, R.; Bañeras, L.; Colprim, J., 2015. Inhibition effects of ethanol and butanol on growth of three alcohol-producing model carboxydotrophic bacteria. Original research *(Unpublished data)*

4.5.1 Background

The two major fermentation products of carboxydotrophic clostridia are organic acids (i.e. acetic, butyric, and caproic acid) and alcohols (i.e. ethanol, butanol, and hexanol). Acidogenesis and solventogenesis have long been thought to occur separately during growth of clostridia since only the former is linked to substrate energy level generation. Transient stages from the acidogenesis to the solventogenesis phases are not clearly defined and depend on many variables, among which the concentrations of undissociated organic acids is thought to play a preponderant role. High concentrations of these can affect the proton gradient across the membrane, collapsing the ΔpH and affecting growth and production (Terracciano and Kashket, 1986). In extreme cases, the fast accumulation of acids may result in a failure of the switch from acidogenic to solventogenic phase, resulting in poor or the complete lack of solvent production (Maddox et al., 2000; Mohammadi et al., 2014; Ramió-Pujol et al., 2015b). Although the production of alcohols takes place mainly during the stationary phase, several studies have reported the production of both acids and alcohols during the growth phase (Bruant et al., 2010; Köpke et al., 2010; Phillips et al., 2015; Ramió-Pujol et al., 2015b), reaching concentrations as high as 1500 mg/L of ethanol and 300 mg/L of butanol even before the onset of the stationary phase (Phillips et al., 2015). Additionally, several reports have also proven the feasibility of syngas fermentation to alcohols in continuous reactors, in which cell growth is concomitant to solvent production (Gaddy et al., 2007; Kundiyana et al., 2011a; Liu et al., 2014; Mohammadi et al., 2012; Phillips et al., 1993). Among them, the highest concentration of ethanol in the fermentation broth was 8.0 g/L, on the other side concentrations of butanol were up to 1.0 g/L (Liu et al., 2014).

Alcohols are toxic to bacteria because they disrupt the cell membrane and inhibit cellular processes (Baer et al., 1987). Ethanol is known to fluidize the cell membrane, resulting in an increased diffusion of solutes in and out the cell. This uncontrolled transport may cause a disruption of the proton gradient across the membrane, which can

lead to a leakage of important cofactors such as Mg^{2+}, and the cessation of growth (Cartwright et al., 1986; Huffer et al., 2011; Ingram, 1976). Ethanol can also inactivate membrane and cytosolic enzymes. For example, ATPase and some glycolytic enzymes are inhibited by ethanol in a non-competitive manner (Banat et al., 1998; Ding et al., 2009; Ingram, 1976). Alcohols with longer carbon chains, such as butanol, have further negative effects. Butanol can insert into the cell membrane and break hydrogen bonds between lipid tails increasing its toxicity effects as compared to ethanol (Ly and Longo, 2004).

The low ethanol and butanol productivity of syngas-fermenting clostridia is one of the key bottlenecks or industrial scale-up of the process, especially in continuous fermentation reactors. Current research efforts target improved solvent production for industrial fermentation (Ramió-Pujol et al., 2015a). However, although reports have shown that most of the carboxydotrophic syngas fermentation strains (i.e. *C. carboxidivorans*, *C. ljungdahlii*) can grow in low alcohol concentrations, alcohol toxicity is not known in detail and, to the best of authors' knowledge, no reliable estimation of kinetic parameters for alcohol inhibition exist for these bacterial species. The aim of this chapter was to investigate the inhibitory effects of ethanol and butanol on the growth of three key carboxydotrophic bacteria used in syngas fermentation: *Clostridium carboxidivorans* P7, *Clostridium ljungdahlii* PETC, and *Butyribacterium methylotrophicum* DSM3468, and to provide with kinetic parameters that may eventually be used in production modelling.

4.5.2. Experimental design

4.5.2.1. Toxicity experiments

P7, PETC, and DSM3468 cultures at middle exponential growth phase were used as inoculum for batch experiments to investigate alcohol toxicity. During batch tests, the headspace of each tube was flushed with syngas for 1 minute every 24 hours to ensure replenishment of substrates. Ethanol and butanol were added to the medium at the

beginning of the experiment to final concentrations of: 2, 5, 10, and 15 g/L. In all batch tests, tubes with no-added alcohol were included as controls for growth kinetics under strictly autotrophic non-inhibitory conditions. Negative, abiotic controls were also prepared as tubes without inoculum containing alcohol at concentrations of 5 and 15 g/L, and used to estimate alcohol losses due to volatilization during repetitive syngas flushing. Incubation of cultures was done at 25 ºC under mild agitation (100 rpm) on a rotary shaker.

Growth experiments were finished once cultures reached the stationary growth phase, and no significant increase in OD 600 nm was observed during the following 24 hours. Samples for the determination of ethanol and butanol concentrations were obtained at the beginning and at the end of the incubation experiments. Growth rates (μ, h^{-1}) were calculated during the initial growth for each incubation experiment (from 35 to 83 hours for PETC and P7, and from 0 to 141 hours for DSM3468) as a measure of the growth capacity of the bacterial cultures at the conditions set in the experiment.

A 1 mL aliquot from cultures not showing a significant increase in OD readings after 279 h in contact with the added alcohol were further re-inoculated in an alcohol-free medium and incubated at 25 ºC under mild agitation (100 rpm). This procedure was done to check for a putative irreversible inhibition of cell activity due to the presence of increased alcohol concentration.

4.5.2.2. Inhibition model

A generalization of the Monod expression that has been used in products suspected to have multiple inhibition effects on cells was used for the determination of inhibition effects on growth (Lin et al., 2008; Yang and Tsao, 1994; Zeng and Deckwer, 1991) (Eq. (6)):

$$\mu = \mu_{max}[\sum(1-(Cp/Cpi)^{ni})] \quad (6)$$

Where μ_{max} is the maximum specific growth rate (calculated at zero alcohol concentration with the given incubation conditions for each strain), *Cp* is the concentration of inhibitor, *Cpi* is an estimate of the critical concentration of the inhibitor above which growth is completely inhibited, and *ni* is a constant coefficient representing the extent of the inhibition effect. *ni* values close to 1 indicate a linear decrease of growth rate as a function of the inhibitor concentration.

The kinetic parameters of Eq. (6) were estimated using SigmaPlot 11.0 (Systat Software, San Jose, California, USA). Constraints to the estimation of *ni* were imposed ($ni>1$) if experimental μ vs *Cp* plots revealed a convex shape.

Half inhibitory constants (IC50) were also estimated as the concentration of toxic at which $\mu=\mu_{max}/2$, and used as an indicator of the degree of toxicity.

4.5.3. Results

4.5.3.1. Control batch experiments of strain P7, PETC, and DSM3468

A control batch experiment under strict autotrophic conditions was run in ATCC 1754 PETC modified medium with no added alcohols using *Clostridium carboxidivorans* P7 (P7), *Clostridium ljungdahlii* PETC (PETC), and *Butyribacterium methylotrophicum* DSM3468 (DSM3468) strains. Over the course of 179 hours P7 and PETC reached the stationary phase, on the other hand DSM3468 presented a much lower growth rate and stationary phase was only reached after 470 hours of incubation. The maximum estimated growth rates for P7, PETC, and DSM3468 under strictly autotrophic conditions and 25 ºC were 0.035 ± 0.002 h^{-1}, 0.022 ± 0.002 h^{-1}, and 0.008 ± 0.000 h^{-1}, respectively. Ethanol was produced by the three strains at expected concentrations for the conditions used here. Butanol production was also detected in P7 and DSM3468 cultures. The latter result is remarkable since DSM3468 has been usually described as a poor alcohol producer (Heiskanen et al., 2007). As expected, no butanol production was observed in PETC.

4.5.3.2. *Effect of the alcohols on cell biomass*

Growth of the three strains occurred at all tested ethanol concentrations (from 0.1 to 15 g/L), although the initial concentration of the toxic affected the growth curve of the three bacteria in different ways (Figure 4.5.1). Growth of P7 and DSM3468 was negatively influenced by increased concentrations of ethanol (Table 4.5.1). The maximum biomass achieved by P7 in the culture was negatively affected by the increasing concentrations of ethanol (Pearson correlation, $p<0.05$, $n\geq12$). Contrarily, PETC biomass cultures were positively affected by the presence of ethanol (Pearson correlation, $p<0.05$, $n= 14$), and DSM3468 remained unaffected.

Butanol presented higher toxicity and a significant reduction of growth was observed for all the three strains at butanol concentrations above 5 g/L (Figure 4.5.2). None of the

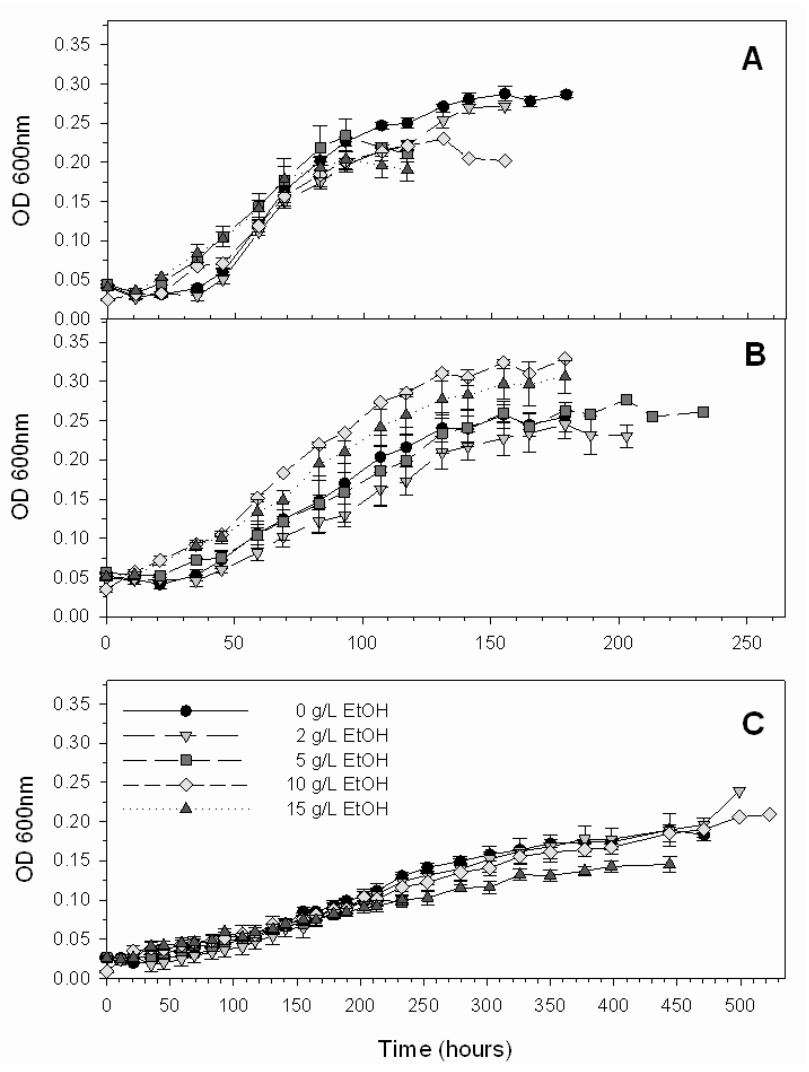

Figure 4.5.1. Growth curves (mean values and SD, n=3) of *C. carboxidivorans* P7 (**A**), *C. ljungdahlii* PETC (**B**), and *B. methylotrophicum* (**C**) at different ethanol concentrations. Note differences in the x axis scale of graphs.

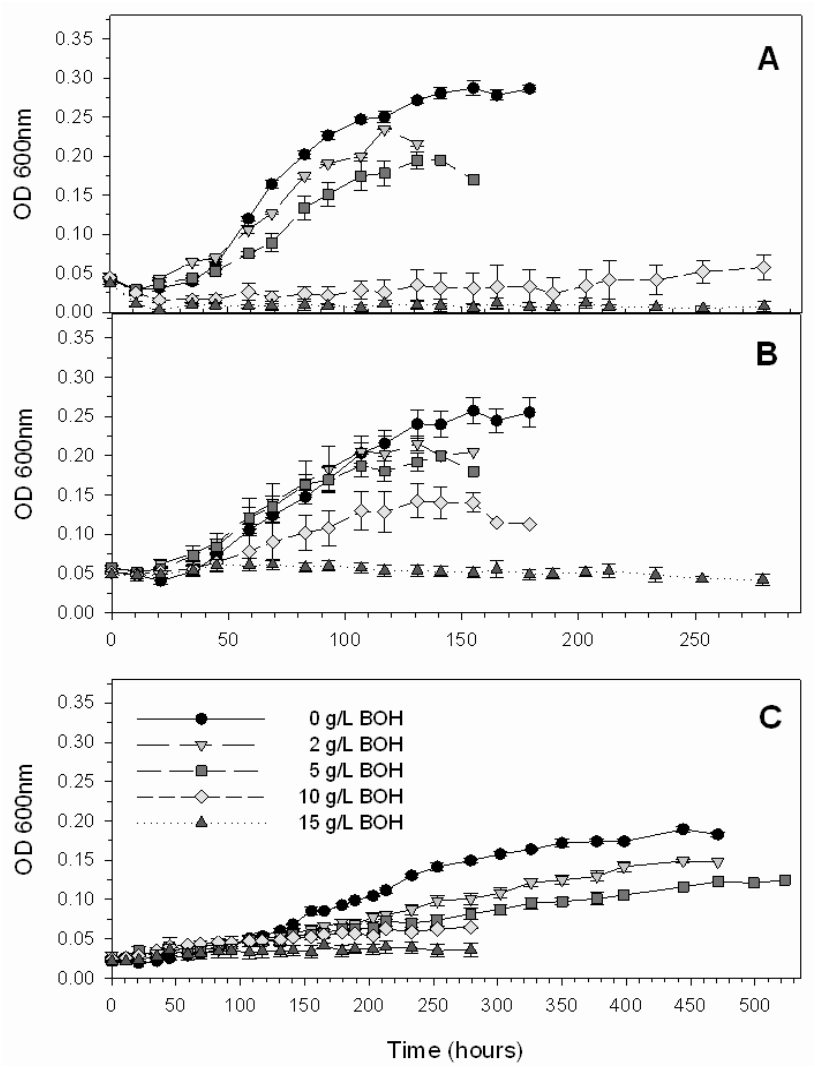

Figure 4.5.2 Growth curves (mean values and SD, n=3) of *C. carboxidivorans* P7 (**A**), *C. ljungdahlii* PETC (**B**), and *B. methylotrophicum* (**C**) at different ethanol concentrations. Note differences in the x axis scale of graphs.

bacteria was able to grow at concentrations of 15 g/L of butanol. However, growth was resumed for PETC and DSM3468 after a re-inoculation of affected cells in butanol-free modified ATCC 1754 PETC medium. An exception to this was P7, which did not present any activity after 58 days of incubation in butanol-free media. This points out to non-reversible toxicity effects of butanol for P7 at concentrations above 10 g/L. The maximum biomass attained at the end of the incubation period by the three species was lower at higher concentrations of butanol, exhibiting clear negative effects (Pearson correlation, $p<0.05$, $n\leq14$) (Table 4.5.1).

The concentration of ethanol and butanol at the end of the growth experiments decreased significantly for all the three strains (Table 4.5.1). Provided that no significant alcohol loss (neither ethanol nor butanol) was measured in the control abiotic

Table 4.5.1 Estimated cultures growth parameters for ethanol and butanol incubation experiments of *Clostridium carboxidivorans* P7, *Clostridium ljungdahlii* PETC and *Butyribacterium methylotrophicum* DSM 3468.

			Ethanol (g/L)				Butanol (g/L)			
		0.00	2.00	5.00	10.00	15.00	2.00	5.00	10.00	15.00
Clostridium carboxidivorans P7	µmax (h^{-1})	0.035±0.002	0.039±0.003	0.021±0.001	0.021±0.004	0.017±0.003	0.025±0.005	0.023±0.004	0.000±0.000	0.000±0.000
	DO max[a]	0.291±0.006	0.275±0.004	0.218±0.027	0.196±0.043	0.192±0.015	0.226±0.011	0.197±0.009	0.060±0.014	0.037±0.001
	(%) Alcohol consumption	na	19.3±2.4	19.4±4.2	29.9±7.6	13.0±2.5	0.0±0.0	11.8±7.4	10.6±7.1	28.0±4.6
Clostridium ljungdahlii PETC	µmax (h^{-1})	0.022±0.002	0.021±0.002	0.011±0.005	0.019±0.001	0.015±0.000	0.017±0.001	0.018±0.003	0.012±0.002	0.000±0.000
	DO max[a]	0.258±0.017	0.246±0.019	0.236±0.051	0.325±0.005	0.307±0.022	0.217±0.009	0.198±0.009	0.146±0.018	0.064±0.009
	(%) Alcohol consumption	na	3.1±4.2	0.0±0.0	18.7±0.4	8.7±2.9	7.2±9.6	13.1±4.9	6.3±6.6	19.8±4.1
Butyribacterium methylotrophicum DSM3468	µmax (h^{-1})	0.011±0.001	0.013±0.003	0.008±0.001	0.006±0.000	0.005±0.001	0.005±0.001	0.005±0.000	0.003±0.000	0.000±0.000
	DO max[a]	0.186±0.005	0.201±0.026	0.101±0.001	0.188±0.014	0.145±0.007	0.140±0.013	0.113±0.008	0.066±0.001	0.043±0.004
	(%) Alcohol consumption	na	33.8±1.5	8.1±0.2	29.5±3.7	10.2±1.0	14.0±6.7	21.0±3.5	14.6±3.0	22.0±2.4

[a]: DO max refers to the maximum optical density reached by the culture; **na**: not applicable.

experiments at any of the concentrations tested (5 and 15 g/L), it was hypothesized that alcohols were actively consumed during growth. Unfortunately, concentration of ethanol and butanol was not measured at all sampling time intervals, which hampers the determination of any insights on the kinetics of ethanol and butanol consumption for these strains. Similarly, the addition of relatively large quantities of alcohols at the beginning of the experiment difficult the calculation of production rates for all assayed conditions.

4.5.3.3. Effect of toxics on growth rate

The effect of ethanol and butanol on the three strains was further investigated by evaluating its impact on the specific growth rate (μ). An inhibition model was used to describe the potential effect of product over growth rate and allowed estimating the kinetic inhibition parameters. Values of maximum μ were fixed at zero alcohol concentration incubation for each strain. The parameter *ni*, accounting for the extent of the inhibition effect, was set up at values higher than 1 to adjust the model with a positive curve as shown in the plots (Figure 4.5.3 and 4.5.4).

Growth rate decreased almost linearly for P7 with increasing ethanol concentrations. Poor fitting of experimental data to the proposed model was obtained (Figure 4.5.3). Lesser effects of ethanol were observed on PETC and DSM3468 growth when compared to P7. Consequently, *Cpi* values for the three strains were set over 15 g/L of ethanol (maximum concentration assayed in this experiment) as no complete inhibition of growth was observed within the concentration range used here.

The higher toxicity of butanol on the three strains was confirmed by adjusting experimental data to the inhibition model (Figure 4.5.4). Good model fittings were obtained for the three strains. Similar predicted *Cpi* values were obtained 12.94, 14.79, and 15.00 g/L for P7, PETC, and DSM3468, respectively. The data sets allowed also the

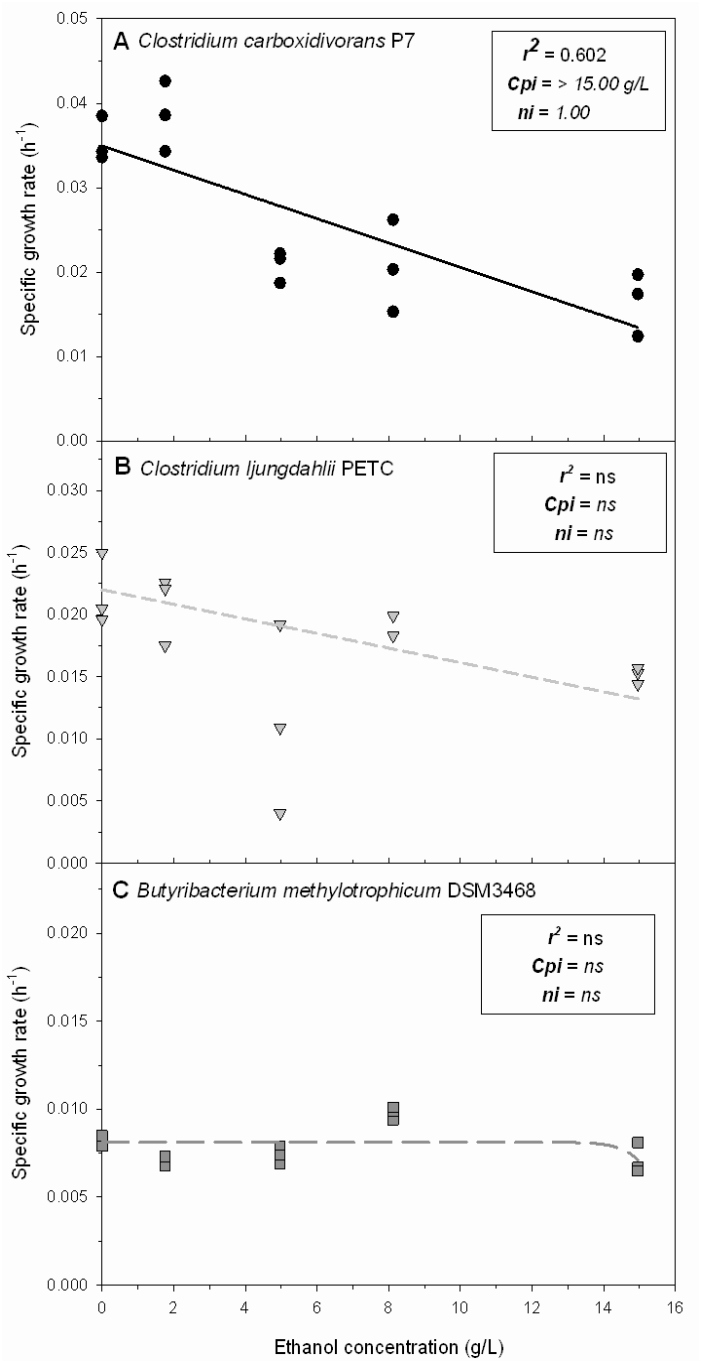

Figure 4.5.3. Experimental data and model fit of the normalized specific growth rate of *C. carboxidivorans* P7 (A), *C. ljungdahlii* PETC (B), and *B. methylotrophicum* DSM3468 (C) at different ethanol concentrations. Ns: not significant.

calculation of the IC50 for butanol, which was also similar among the three species (6.47, 8.03, and 8.22 g/L for P7, DSM3468, and PETC, respectively).

4.5.4. Discussion

Despite the assumed biphasic fermentation metabolism of acetogenic bacteria, recent reports have shown that solvent production can also take place during growth phases (Bruant et al., 2010; Köpke et al., 2010; Phillips et al., 2015; Ramió-Pujol et al., 2015b). This

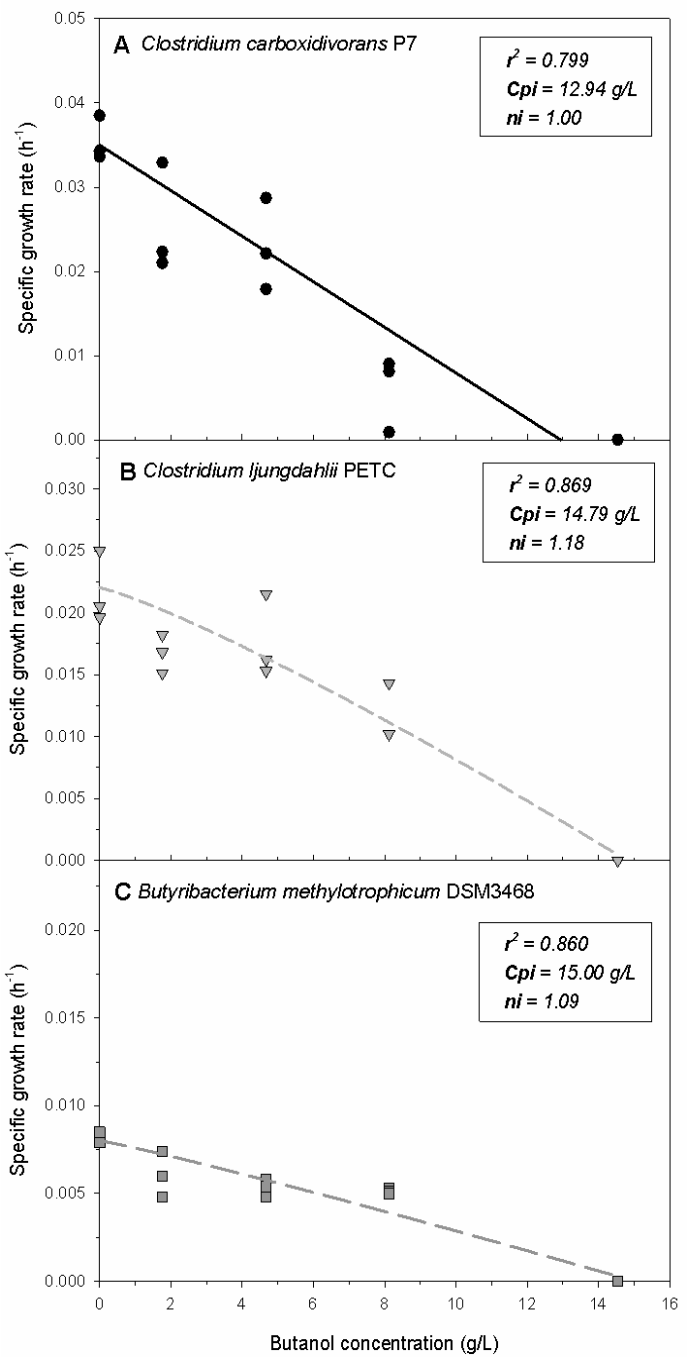

Figure 4.5.4. Experimental data and model fit of the normalized specific growth rate of *C. carboxidivorans* P7 (A), *C. ljungdahlii* PETC (B), and *B. methylotrophicum* DSM3468 (C) at different butanol concentrations.

is of special relevance in continuous syngas fermentation systems, where cell growth and solvent production (and accumulation) occur concomitantly (Gaddy et al., 2007; Kundiyana et al., 2011a; Liu et al., 2014; Mohammadi et al., 2012; Phillips et al., 1993). Concentrations as high as 8 g/L of ethanol and 1.0 g/L of butanol have been reported in continuous reactors (Liu et al., 2014). In view of further development of syngas fermentation for bio-alcohol production in continuous systems, this chapter investigated the inhibitory effects of ethanol and butanol on the growth of three key

carboxydotrophic bacteria used in syngas fermentation: i) *C. carboxidivorans* P7, which has been proven to produce concentrations of ethanol, butanol and even hexanol up to 3.25 g/L, 1.0 g/L and 0.9 g/L, respectively; ii) *C. ljungdahlii* PETC which has been reported to produce maximum ethanol concentrations of 6.5 g/L in a continuous stirred tank reactor (Mohammadi et al., 2012). The resistance of this strain to increasing concentrations of butanol was tested despite its lack of the homologous genes encoding for crotonase and butyryl-CoA dehydrogenase (Bengelsdorf et al., 2013; Köpke et al., 2010) because it has been successfully genetically modified. Specifically, Köpke and co-workers proved it could be engineered to produce butanol (Köpke et al., 2010); and iii) *B. methylotrophicum* DSM3468, one of the few non-clostridial acetogen to date proven capable of producing C2 and C4 compounds, its phylogenetic distance with the other two strains could lead to significantly different tolerance to alcohols making it attractive from a genetic engineering perspective. Experiments were conducted at 25 ºC because sub-optimal incubation temperatures have been proven to prevent acid crash, minimizing any adverse effects of organic acids on strain growth. Additionally, such low temperatures have also proven to decrease the metabolic rate of the cells, increase gas solubility and enhance the alcohol production of both P7 and "Clostridium ragsdalei" (Kundiyana et al., 2011b; Ramió-Pujol et al., 2015b).

None of the ethanol concentration completely inhibited the growth of any of the three strains tested. On the contrary, some of the conditions seemed to improve PETC growth. Tanner *et al.* reported that medium containing 5 g/L of ethanol and 1 g/L of yeast extract could support growth of PETC (1993). Such observations led to the conclusion that PETC has the ability to grow mixotrophically at the expenses of ethanol, and would explain the increase of biomass of PETC in the experiments at higher ethanol concentrations. In a similar way, recent reports also demonstrated the ability of P7 to grow in the presence of ethanol or butanol (Liou et al., 2005). However, despite its capacity, the consumption of alcohol in the present study did not seem to have an impact on the culture growth parameters. Likewise, experiments of DSM3468 showed

consumption of ethanol and butanol, an ability which has not yet been reported in literature with butanol by this strain. The present results contrast with the observations of Zeikus *et al.* in their initial description of the Marburg strain, for which they reported that growth was not detectable on a mix of ethanol and acetate as energy sources (Zeikus et al., 1980). Nevertheless, such consumption of alcohols did not have any positive impact on the maximum concentration of biomass of DSM3468 reached in the experiments. While ethanol results did not show a conclusive effect, maximum biomass concentration was clearly negatively affected at higher butanol concentrations.

The inhibition model used to estimate the kinetic inhibition parameters for ethanol and butanol showed that the *Cpi* values obtained in the present experiment for the three tested strains are in line with the threshold toxicity of different ethanol and butanol producer species. In agreement with our results *Clostridium beijerinckii*, *Escherichia coli*, and *Zymomonas mobilis* also showed lower tolerance to butanol than ethanol (Huffer et al., 2011). Huffer *et al.* reported that both *C. beijerinckii* and *E. coli* K12 can grow in a medium with a concentration up to 45 g/L of ethanol. Contrarily, the former tolerates growth at concentrations up to 12 g/L of butanol whereas the latter only grew at butanol concentrations lower than 4 g/L. These differences between species can be partially explained by the fact that *C. beijerinckii* is a butanol producer whereas butanol is not one of *E. coli* K12 fermentation products (Clark, 1989). One of the species reported with a significant tolerance to ethanol is *Z. mobilis* which can grow at concentrations up to 65 g/L (Huffer et al., 2011). However, its tolerance to butanol is much lower, presenting the same toxicity level at 12 g/L of butanol and at 45 g/L of ethanol. Finally, it is also important to highlight the existence of some bacteria which present higher tolerance to butanol than to ethanol such as *Thermoanaerobacterium saccharolyticum* (Huffer et al., 2011).

To sum up, the results of the present chapter point out that alcohol toxicity is not a barrier impeding the current development for the industrial production of alcohols from syngas fermentation, as product titers are still far below the inhibitory/toxic thresholds

of both ethanol and butanol. Production yields can still be improved in several ways, i.e. modifying medium composition, amending additional organic carbon sources, controlling pH changes, adjusting composition of syngas, and, also, by genetic modification of gene clusters that may turn non-producers into producer strains (Green, 2011; Ramió-Pujol et al., 2015a). Finally, should alcohol concentrations in the broth reach inhibitory/toxic levels, some possibilities to ease toxic effects of alcohols on producing strains include in-line product extraction to detoxify alcohols of the environment where the cultures grow, or the continuous recycling of the cells culturing the bacteria in a fresh media (Gaddy et al., 2007; Phillips et al., 1993).

4.6 Isolation of new carboxydotrophic strains

4.6.1 Background

Although there has been a recent increase in the number of published reports on syngas fermentation, the number of carboxydotrophic strains able to follow WLP is still quite limited (Ramió-Pujol et al., 2015a). Solvent production by carboxydotrophs has mostly been described for *Clostridium* species. The relatively small number of syngas-fermenting bacterial strains known to date limits the range of operational conditions in which bio-ethanol or bio-butanol can be produced from syngas. Most, if not all, carboxydotrophic clostridia have been isolated from organic matter rich environments, such as anaerobic sediments, animal manure, and sewage (Liou et al., 2005; Tanner et al., 1993). Taking these environments as a starting point, we aimed at the discovery of new isolates with the potential to ferment syngas and produce ethanol or butanol as biofuel.

4.6.2 Experimental design

4.6.2.1 Sample preparation and enrichment process

Samples for enrichment purposes were taken from different anaerobic organic matter rich environments. Five samples were used including, the sediment from a duck manure stabilization pond (DMP), the sediment of an open air temporary lagoon located beside a farm (TL), sheep manure pellets from a long term storage deposit (SM), fresh rabbit manure pellets (RM), and a sediment sample from a constructed wetland at the Empuriabrava WWTP (CW). All samples were taken in the area of Girona (Girona, Spain). No specific physicochemical characterization of samples was performed.

Samples were collected manually using common aseptic practices and transported to the laboratory in a cooled container (4 ºC). Once in the lab, about 1.9 to 2.5 gram of each sample were suspended in a modified ATCC 1754 PETC medium (Ramió-Pujol et al., 2015c). Initial pH was adjusted to 6.0. Sediment and manure slurries were prepared under anaerobic conditions within an anaerobic glove chamber (Coy Lab), and were

dispensed (200 mL) in Pyrex bottles of a nominal capacity of 500 mL. Headspaces were thoroughly flushed with syngas (5% H_2 – 15% CO_2 – 30% CO – 50% N_2) for at least five minutes. Bottles were closed and maintained at an overpressure of 100 KPa. No additional sources of organic carbon were added to sediment or manure slurries. All bottles were incubated at 37 ºC under mild agitation in a rotary incubator at 100 rpm.

Enrichments were maintained for at least 30 days until cell growth was observed in the liquid culture. After this initial period, 3 mL aliquots were aseptically transferred to freshly prepared medium, and incubated using the same conditions above. At least 4 transfers to new media were performed before starting the isolation process in solid media. The purpose of the large number of sample transfers was to serially dilute the organic matter present in the original sample and to facilitate enrichment of autotrophic bacteria.

4.6.2.2 Isolation on pure cultures

After the enrichment, 0.2 mL aliquots were inoculated in solid Reinforced Clostridia Medium (RCM, BD, New Jersey, USA). All preparation steps were done in an anaerobic chamber. RCM agar plates were incubated inside the anaerobic chamber at ambient temperature (approximately 23 ºC). Once grown, several colonies having different morphologies visible at naked eye, were selected, and reinoculated in freshly prepared agar plates. Re-inoculation steps were repeated for at least 10 times from single colonies to ensure pure culture isolation.

Once pure cultures were obtained, the ability of the isolated bacteria to grow in a syngas fermentation broth was tested using batch conditions. Two to three colonies of the same isolate were selected from an agar plate and inoculated into 25 mL glass anaerobic tubes with 6 mL liquid modified ATCC 1754 PETC medium. Conditions for medium preparation, inoculation and incubation have been described elsewhere (Ramió-Pujol et al., 2015c). Headspace was flushed with synthetic syngas (32% CO - 32% H_2 - 28% N_2 - 8% CO_2) with an overpressure of 100 kPa.

4.6.2.3 *Fermentation experiments*

Fermentation batch tests were conducted to characterize growth and production of new isolates in order to get a better grasp of the kinetics and production of the new isolates. Incubation experiments were conducted in triplicates following the exact procedure explained in materials and method section of the present thesis. Headspace of each tube was flushed with syngas every 24 hours for 1 minute to ensure replenishment of gas substrates during the experiment. Cultures were incubated at 25 ºC and 100 rpm. Three independent culture tubes were sampled at 0 h, 70 h, 141 h, 213 h, 309 h, 356 h, and at the end of the experiment, 404 h.

4.6.2.4 *Morphological determination and gram stain*

Cell morphology was observed under a phase-contrast microscope (Zeiss, Axioskop). The presence of spores was determined after a routine Malachite green staining (Gerhardt, 1994). Gram staining was done as previously described (Gram and Friedlaender, 1984).

4.6.2.5 *Nucleic acids extraction from the isolates and PCR conditions*

DNA was isolated from active growing cells using the Chelex chelating resin extraction method following the manufacturer's instructions (Chelex® 100, Bio-Rad). 16S rRNA genes were amplified by PCR in a GeneAmp PCR system 2700 (Applied Biosystems, Thermo Fisher Scientific Inc., Massachusetts, USA) with the primer pair 27F/1492R (Lane, 1991). PCR reactions contained: 1X PCR buffer, 2.5 mM $MgCl_2$, 800 µM total dNTPs, 0.4 µM of each primer, 10 ng of the DNA extracts, and 0.25 units of Taq polymerase (Qiagen, Germany), in a total volume of 50 µL. The PCR amplification was carried out with an initial denaturation step at 94 ºC for 5 min and followed by 35 cycles of denaturation (94 ºC, 30 seconds), annealing (55 ºC, 45 s) and extension (72 ºC, 90 s). Final extension was done for 7 min at 72 ºC. PCR products were analyzed by

electrophoresis on 1.5% agarose gels and visualized after staining with GelRed™ (Biotium Inc.).

4.6.2.6 *Sequencing and phylogenetic analysis*

Sequences of 16S rRNA genes were obtained and used for identification of isolates. Prior to sequencing, the PCR products were purified with 1 U of Exonuclease I (Fermentas, Sankt Leon-Rot, Germany) and 0.5 U of Thermosensitive Alkaline Phosphatase (FastAP TM; Fermentas) at 37 ºC for 1 h, followed by an inactivation step at 85 ºC for 15 min. Purified PCR products were sequenced using forward and reverse primers located at different positions within the 16 rRNA gene sequence in order to obtain the complete sequence from both directions. Used primers were the above mentioned, 27F and 1492R, and the internal primers 357F, 787F, 1114F, and 907R. Big Dye Terminator kit v3.1 cycle-sequencing kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA) was used for sequence reactions. Sequences were obtained in an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Almost complete 16S rRNA gene sequences were reconstructed for each isolate using Bioedit (Hall, 1999). Finally, good quality 1371 bp sequences could be obtained and were compared to public databases using a BLAST search at the NCBI. Closely related sequences were retrieved from GenBank database and aligned using BioEdit. A phylogenetic tree was reconstructed using MEGA v.6 software (Tamura et al., 2013). Neighbour joining and maximum likelihood methods were used to reconstruct phylogenies using Bootstrap replication (1000 iterations).

4.6.3 Results and Discussion

4.6.3.1 *Isolates*

A total of 13, 11, 17, 13, and 23 different colonies were selected from DMP, TL, SM, RB, and CW enrichments, respectively. However, some of the colonies did not show good growth as pure cultures on agar plates and were lost during isolation. We ended up with

49 different isolates, which were inoculated in liquid media and tested for syngas fermentation. After several dilutions, 2 isolates showed their ability for growing with syngas as the sole carbon and reducing equivalents source. The obtained isolates were named as I19 and I53 and were obtained from SP and CW samples, respectively.

Phase-contrast microscopy revealed that I19 and I53 cells were morphologically similar, rods of approximately 0.5 µm width and 2.0 µm length. Both isolates stained positive in a Gram stain.

Growth experiments using syngas showed that the two strains differed on the lag phase, the specific growth rate (µ), and the maximum optical density (Figure 4.6.1). Isolate I19 had a longer lag phase compared to I53, although the growth rate of the latter was significantly higher, 0.036 ± 0.004 h^{-1} compared to 0.021 ± 0.003 h^{-1} ($p<0.05$, T3-Dunnet test, $n\geq11$).

During the kinetic experiment, I19 and I53 were able to convert syngas into products. Initially, mainly acetate was produced during the exponential phase, up to concentrations of 120.2 and 66.0 mmol C/L for I19 and I53, respectively. I19 exhibited a significant production of alcohols at early stages of growth. The percentage of alcohols ranged from 20 to 50% (Figure 4.6.2), with a maximum ethanol concentration of 110.5 mmol C/L. Butanol was only produced at trace amounts (3.6 mmol C/L). Low extracellular pH has been reported to be a crucial factor triggering solventogenesis (Jones and Woods, 1986). I53 did not present a clear shift from acidogenesis to solventogenesis probably because acid production was not high enough to cause a decrease in pH to values lower than 5.0. The maximum concentrations of ethanol and butanol observed for I53 were 1.26 and 1.29 mmol C/L, respectively. This led us to think that, despite having the ability to produce alcohols, I53 is a poor alcohol producer compared to I19.

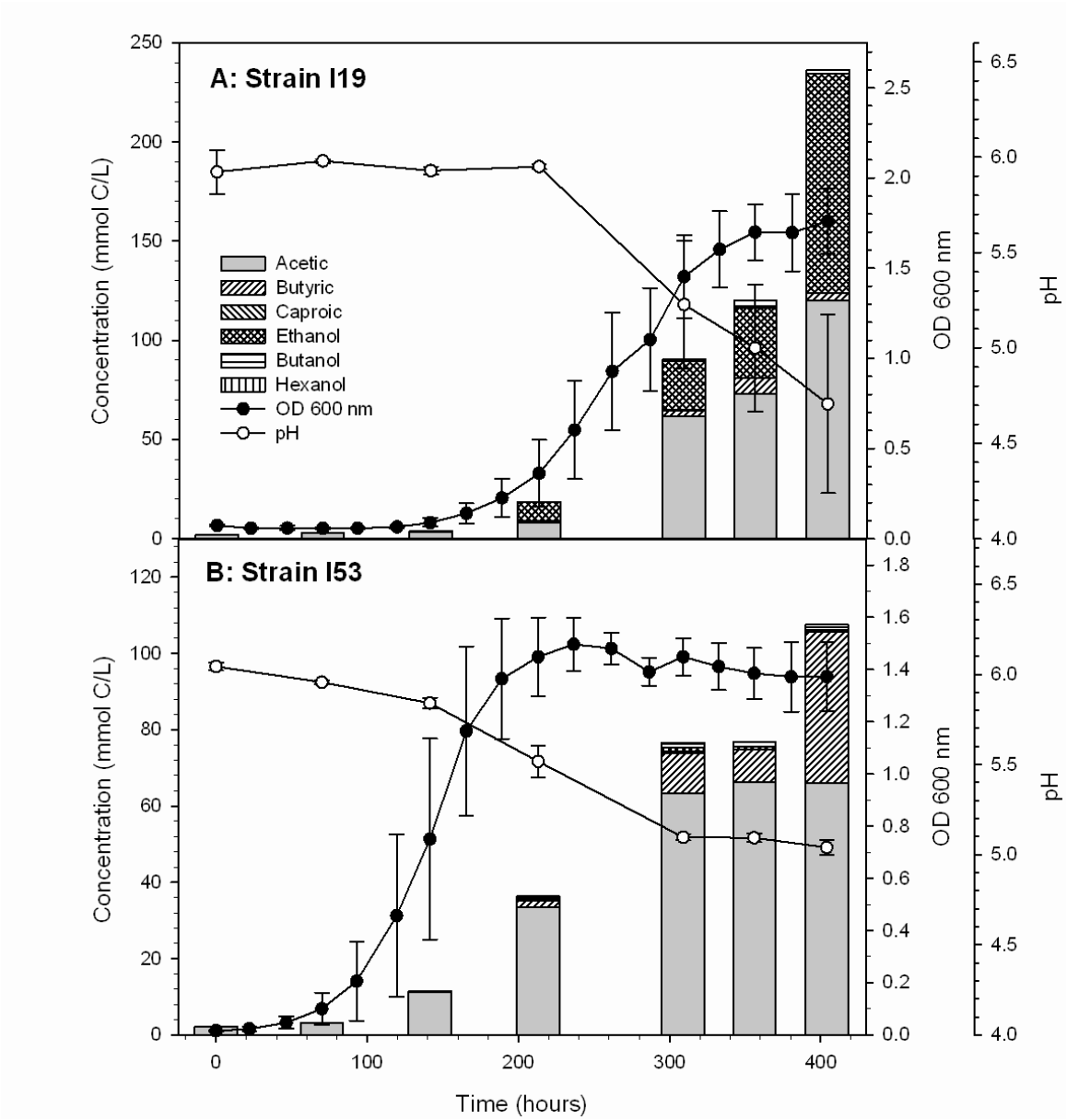

Figure 4.6.1 Optical density (black dots) and pH (light grey squares) evolution every 24 hours, and concentration of organic acids (acetate, butyrate, and caproate) and alcohols (ethanol, butanol, and hexanol) during kinetic experiments at seven different times (0, 70, 141, 213, 309, 356, and 404 hours) produced by strain I19 (**A**) and strain I53 (**B**).

Both of the isolates presented C2 compounds as the main products derived from syngas fermentation. However, strain I53 showed a significant increase in the concentration of C4, mainly butyric acid (30.8 mmol C/L). Traces of C6, mainly caproic acid, were also observed.

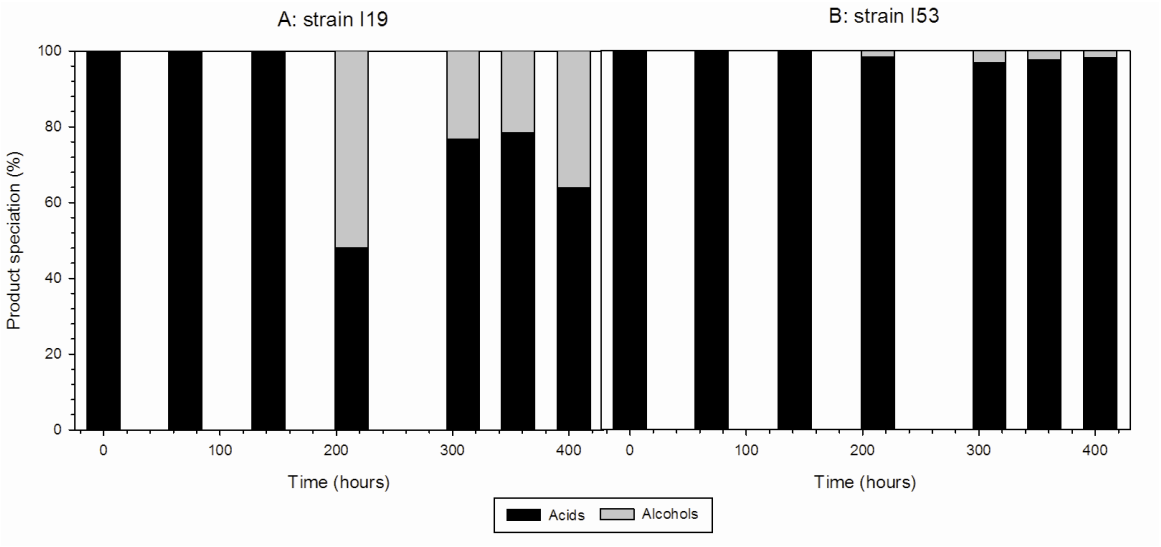

Figure 4.6.2 Percentage of the products in organic acids (black) and alcohols (light grey) form during the kinetic experiment of strain I19 (**A**) and strain I53 (**B**).

4.6.3.2 Phylogenetic analysis of strain I19 and strain I53

I19 and I53 exhibited an almost identical 16S rRNA gene sequence. Phylogenetic analysis based on comparisons with existing sequences on data bases indicated a close relationship to *Butyribacterium methylotrophicum* (100% sequence similarity) and

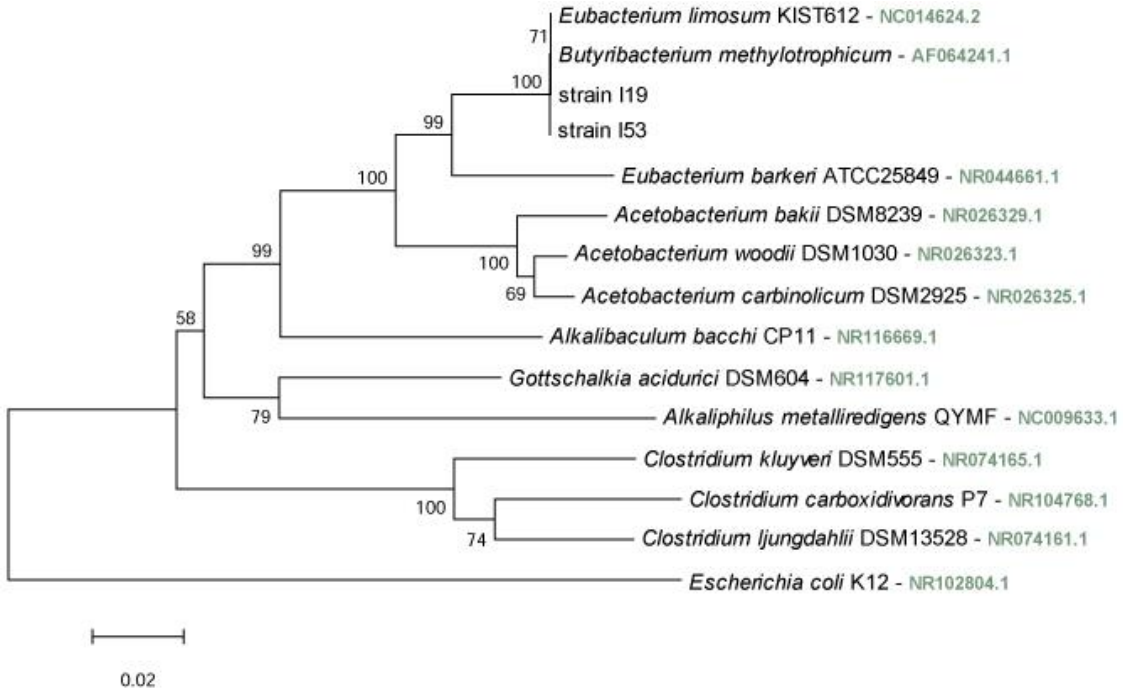

Figure 4.6.3 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1371 bp) showing the relationship of isolates I19 and I53, with other related species of the class *Clostridia*. Bootstrap values greater than 50% are given at the nodes.

Eubacterium limosum KIST612 (99.9% similarity) (Figure 4.6.3), and fall within the *Clostridia*. Despite having an almost identical 16S rRNA gene sequence, *B. methylotrophicum* and *E. limosum* are clustered into two distinct phylogenetic groups, the *Clostridiaceae* and the *Eubacteriaceae*, respectively.

In order to substantiate if strains I19 and I53 represent different and novel species, a comparison among the closest known relatives of both strains was made (Table 4.6.1). As being described for *B. methylotrophicum* and *E. limosum* (Table 4.6.1), I19 and I53 are strict anaerobic bacteria and show a positive gram stain. However, I19 and I53 showed a rather smaller cell size when compared to *B. methylotrophicum* and *E. limosum*, although differences in cell size may be the result of adapting cell physiology to growth conditions and must be taken cautiously as a species determinant difference.

Table 4.6.1 Some key features among *E. limosum*, *B. methylotrophicum*, strain I19, and strain I53.

	Eubacterium limosum	***Butyribacterium methylotrophicum***	**Strain I19**	**Strain I53**
Isolation medium	Basal medium + 5% rumen fluid	Mineral medium + methanol + acetate	Mineral PETC medium + syngas	Mineral PETC medium + syngas
Morphology	Cylindrical cells with rounded ends	Often displayed rudimentary branching	Cylindrical cells with rounded ends	Cylindrical cells with rounded ends
Size	1.2 µm width 3.3 µm length	0.8 ± 0.2 µm width 2.7 ± 0.5 µm length	0.54 ± 0.05 µm width 2.10 ± 0.26 µm length	0.54 ± 0.05 µm width 1.82 ± 0.41 µm length
Spore	Not detected	Retractile endospore like structures	Not detected	Not detected
Gram staining	Positive	Positive	Positive	Positive
Oxygen tolerance	Oxygen was an inhibitor of growth	Oxygen was an inhibitor of growth	Oxygen was an inhibitor of growth	Oxygen was an inhibitor of growth
Inorganic carbon sources	Methanol, CO, CO_2	CO, CO_2	CO, CO_2	CO, CO_2
Syngas fermentation products	Acetic acid, butyric acid, ethanol, and butanol	Acetic acid, butyric acid, lactic acid, ethanol, and butanol	Acetic acid, butyric acid, ethanol, and butanol	Acetic acid, butyric acid, ethanol, and butanol
Reference	(Chang et al., 2001; Genthner and Bryant, 1982; Song and Cho, 2015)	(Heiskanen et al., 2007; Shen et al., 1999; Zeikus et al., 1980)	This chapter	This chapter

I19 presented significant differences in the product spectrum compared to strains I53, *B. methylotrophicum* or *E. limosum*. The I19 production of ethanol in the present experiment reached concentrations up to 200 mmol C/L, which is more than the maximum concentration of ethanol reached in syngas fermentation by *C. carboxidivorans* P7, 141.0 mmol C/L (Phillips et al., 2015). However, it is still far below ethanol production by *C. ljungdahlii* PETC, which in our lab has reached concentrations up to 600 mmol C/L (unpublished results). Therefore, this new isolate has the potential to be considered in ethanol production in industrial fermentation of syngas and future work should be aimed at assessing its real production potential in syngas fermentation with continuous syngas supply.

DISCUSSION

The determination of key parameters for bio-alcohol production using model carboxydotrophic bacteria is necessary to increase the efficiency of CO_2+H_2 or CO reduction. The knowledge that the scientific community has about carboxydotrophic bacteria metabolism is still limited and additional efforts are required to fully understand the process before this can be implemented in the industry. In this dissertation we have contributed to this effort by studying some key aspects that may be important in future research in the field.

5.1 The importance of the electron flow during inorganic carbon reduction

Inorganic carbon reduction to acetyl-CoA requires reducing power. The production of reduced NAD (NADH in this text) and ferredoxin (Fd^{2-}) is restricted to the oxidation of molecular H_2 and CO. Reduced Fd is oxidized by membrane-bound enzymes that couple exergonic electron transfer to an acceptor with the translocation of ions across the membrane, thus establishing an electrochemical ion gradient across the membrane (Schuchmann and Müller, 2014). In clostridia, the ferredoxin:NAD reductase (Rnf) complex catalyzes electron transfer from Fd^{2-} to NAD while protons are pumped out the cell (Figure 5.1). This reaction compensates for the disequilibrium in Fd^{2-}/NADH derived from autotrophic growth at the expenses of CO or H_2, contents of syngas and is used as a proton-motive force energy generating mechanism (Biegel et al., 2011). Flow of energy is linked to the pools of Fd^{2-} and NAD, which need to be constantly regenerated to carry out other cell needs. However, when the availability of reducing power is not compromised, the higher the imbalance between demand and supply of Fd^{2-} the more H^+ can be translocated by the Rnf complex and the more ATP can be synthesized. In the Methyl branch of the WLP, ATP is consumed to convert formate into formyl-THF in the first step of carbon activation (Ragsdale and Pierce, 2008). This negative energy balance is compensated by the synthesis of metabolites, such as acids, that allow ATP production by substrate-level phosphorylation compensating the initial energy expenditure.

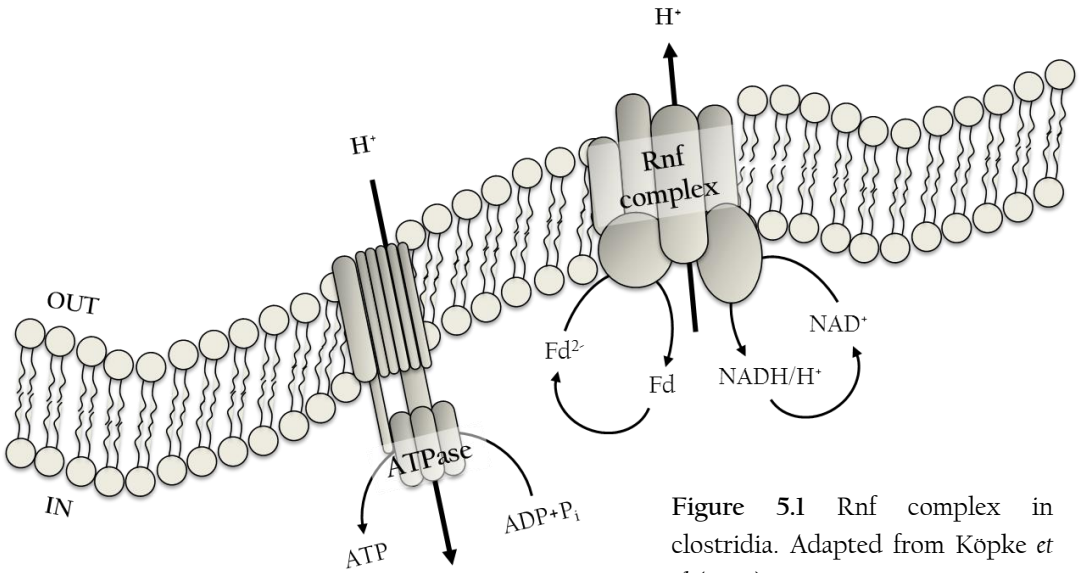

Figure 5.1 Rnf complex in clostridia. Adapted from Köpke *et al* (2010).

Ethanol production in clostridia can be the result of a reduction of *de novo* synthesis by the reduction of acetyl-CoA, or the result of a reassimilation of previously excreted acetate and its conversion to acetaldehyde via acetyl-CoA, or aldehyde:ferredoxin oxidoreductase (AOR) (Bertsch and Müller, 2015; Jones and Woods, 1986) (Figure 5.2). In two of these situations, either *de novo* synthesis or the reassimilation of acetate via acetyl-CoA, ethanol is produced as a two-step reducing reaction catalyzed by the bifunctional ADHE and the ADH, both at the expenses of NADH. Correpondingly, ethanol production via acetyl-CoA reduction does not give a positive energy balance in the form of ATP. On the other side, AOR is capable of catalyzing the reversible reduction

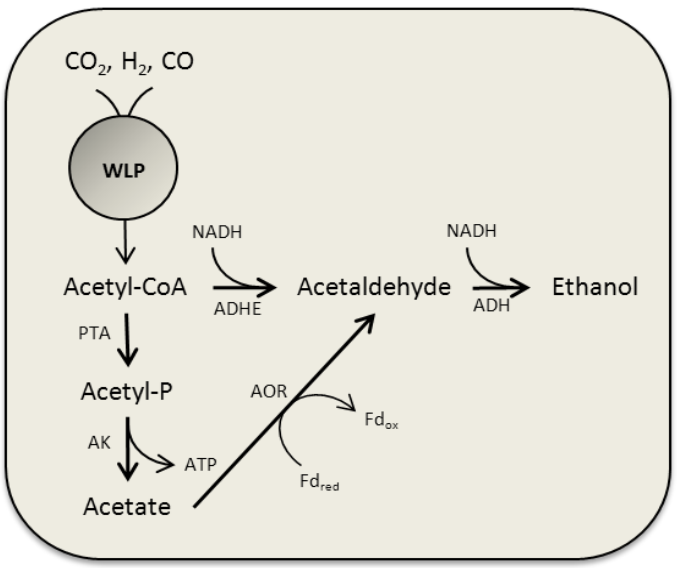

Figure 5.2 Ethanol formation from acetyl-CoA. Acetyl-CoA is synthesized via the Wood-Ljungdahl pathway (WLP) and can be reduced to ethanol either by means of acetaldehyde dehydrogenase (ADHE) or by means of aldehyde:ferredoxin oxidoreductase (AOR). Adapted from Bertsch and Müller (2015).

of an acid to the corresponding aldehyde (White et al., 1989) with the consumption of Fd^{2-}. For this reaction to occur in clostridia, acetate must be formed and an ATP equivalent can be obtained via substrate-level phosphorylation due to acetate kinase action. Theoretically, the reduction of acetyl-CoA to ethanol via acetate synthesis by AOR assimilation yields 1 ATP, and requires Fd^{2-} and NADH equivalents. Consequently, net energy gain compared to direct synthesis from acetyl-CoA (two NADH equivalents required, no ATP formation) can be obtained using this route.

Due to energy and reducing power requirements during growth, and the fact that energy generating systems (proton motive force) may be impacted by the decrease in pH during growth, it has been for long considered that acidogenesis and solventogenesis occur at two separate growth phases (Figure 5.3). However, in our experiments culturing carboxydotrophs in autotrophic conditions, we have often observed that transit from acidogenesis to solventogenesis is gradual and acids and alcohols are concomitantly produced at different rates during growth. This has also been observed in other studies by different authors (Bruant et al., 2010; Köpke et al., 2010; Kundiyana et al., 2011b; Phillips et al., 2015). This is likely linked to the gain of ATP during alcohol production through the AOR route, which allows the regeneration of NADH and Fd pools.

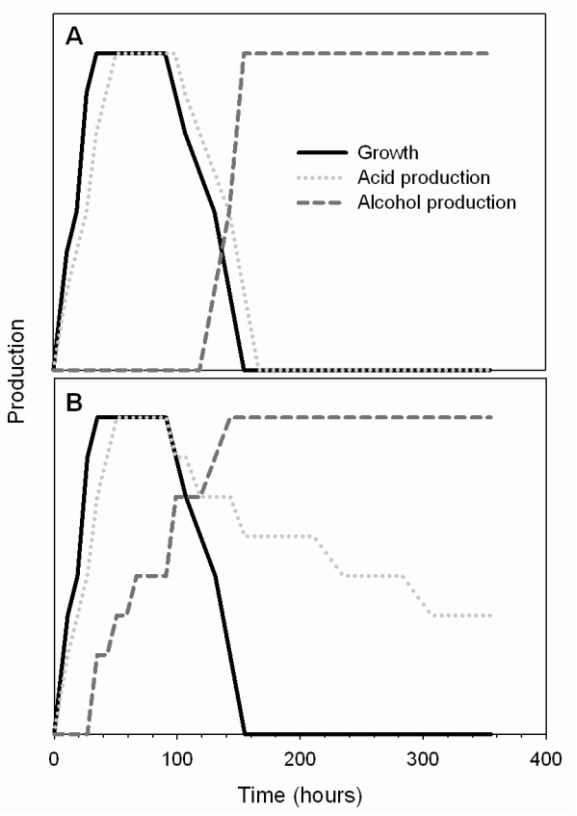

Figure 5.3 Theory (A) and what we have observed (B) about when the acidogenesis (orange line) and solventogenesis (green line) take place related to the growth (brown line) of any carboxydotrophic bacteria.

Recently, Bertsch *et al.* reported that the energy requirements of acetogenic bacteria in WLP differ depending on the carbon source used (Bertsch and Müller,

2015). Reduction of CO_2 to acetate with hydrogen as electron donor requires 2 NADH and 2 Fd^{2-} (Figure 5.4A). On the other hand, when CO is the electron donor, 2 NADH equivalents are required (Figure 5.4B). However, the use of CO as electron donor is concomitant to the production of CO_2 (Eq. (2)):

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

Reducing equivalents from CO are obtained through the activity of the ACS/CODH enzyme complex activity. In contrast, an electron bifurcating hydrogenase (HydABC) is used to obtain electrons from H_2. However, these two activities are unlikely to occur simultaneously at high rates due to most hydrogenases known are strongly inhibited by low concentrations of CO. So far no CO-tolerant hydrogenase has been found in acetogens. In pure extracts of *A. woodii* hydrogenase, the protein activity was reduced to less than 50% even when trace levels of CO (7 nM) were present in the solution

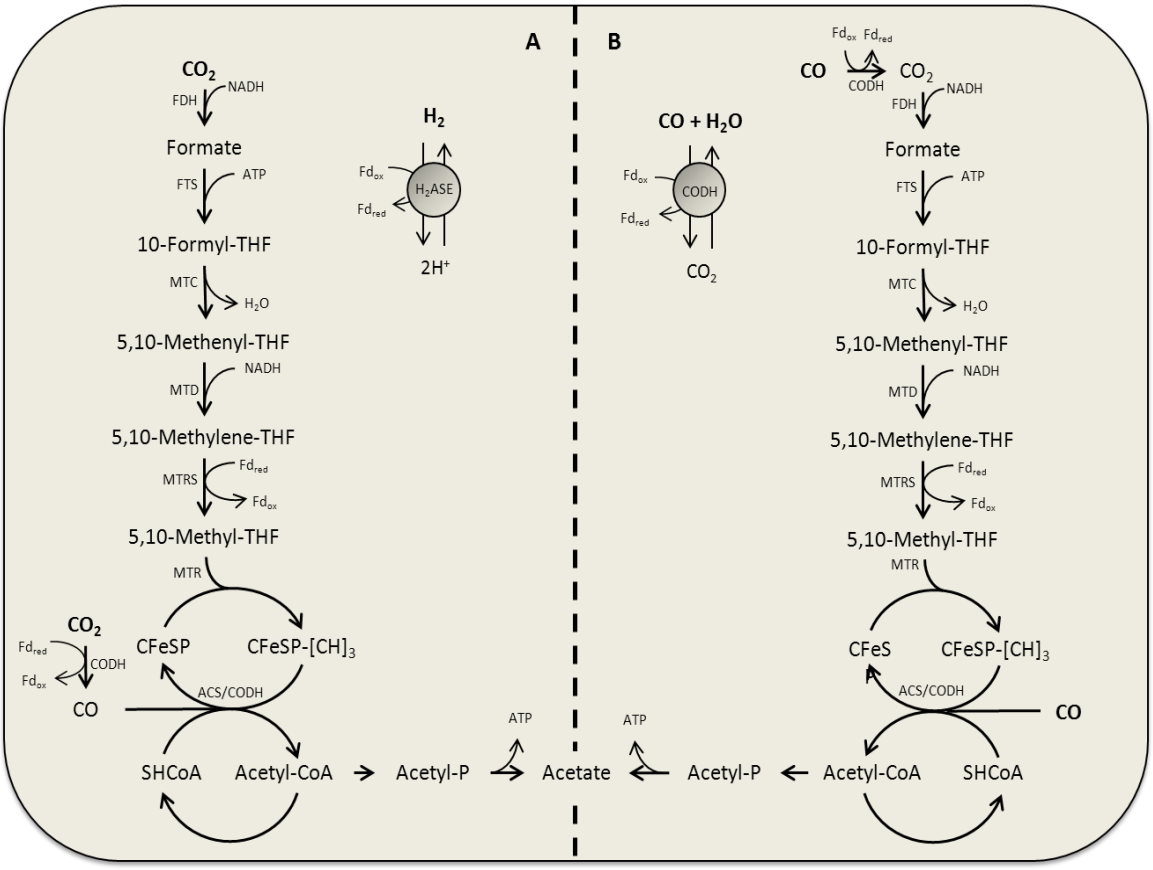

Figure 5.4 Bioenergetics of acetate formation from H_2 + CO_2 (**A**) or CO (**B**) in carboxydotrophic clostridia.

(Ragsdale and Ljungdahl, 1984). This was also demonstrated in batch cultures of *C. ljungdahlii*, H_2 did not start to be used before CO had been almost completely consumed (60 h) (Najafpour and Younesi, 2006). Although this cannot be generalized to all carboxydotrophic strains due to the lack of experimental evidences, the analysis of the gas consumption during the autotrophic growth of P7 in our laboratory revealed a preference for the use of CO when CO and H_2 were simultaneously present in the media (Ramió-Pujol et al., 2015b).

In a situation in which hydrogenases are inhibited by the presence of CO, the external supply of partially reduced compounds could have positive effect on energy conservation. In chapter 4.1, we assessed the impact of formate as a partially reduced carbon source with the potential to diminish the hydrogen/CO demand in the WLP. Formate was chosen since formate synthesis is recognized as one of the reducing equivalents sinks that may diminish the growth capacity of cells (Daniell et al., 2012). Besides, formate can be synthesized from CO_2 in a BES (Srikanth et al., 2014), which could be coupled to the fermentation. Results showed that growth rate and productivity at low formate concentrations was enhanced, especially at pH 5.0 and 6.0 for *C. ljungdahlii* PETC. This is of interest from the biotechnological point of view since the ability of carboxydotrophic bacteria to use other sources of reducing equivalents.

Other medium component that may act as potential sources of external reducing equivalents is YE. This component is regularly used as a nitrogen source in microbiology media formulations and contains a large variety of organics, which may decrease carbon fixation needs. Based on our results (chapter 4.4), the addition of YE does not seem to benefit the growth of carboxydotrophic clostridia during syngas fermentation beyond a slight decrease of the cultures' lag phase. Additions of complex organics may have other functions in carboxydotrophic bacteria metabolism, such as being sources of complex vitamins and co-factors. However, according to our results this is not the case for *C. carboxidivorans* P7.

5.2 Key variables to enhance alcohol production

Production of solvents by classical ABE fermentation of carbohydrates has been extensively described in literature (Lütke-Eversloh and Bahl, 2011). Bacterial growth is usually divided in two phases: first, exponential growth and acidogenesis; and second, stationary phase with the re-assimilation of acids and concomitant formation of alcohols. In classical ABE fermentation the shift from acidogenesis to solventogenesis occurs at pH values ranging from 4.5 to 5.0 (Jones and Woods, 1986). This has been assumed to also hold true for syngas fermenting carboxydotrophic bacteria (Gaddy et al., 2007; Grethlein et al., 1990; Phillips et al., 1993). However, according to our results, this may not be the case in many experimental situations.

The onset of solvent production is normally associated with the accumulation of acids as end products and linked to a pH drop. Thus, we studied the impact of the initial pH values on solvent production in chapter 4.2 using a series of batch cultivation of *C. carboxidivorans* P7 (Ganigué et al., 2015). Our results showed that pH played a significant role on product speciation; ethanol was favored over acetic acid at lower initial pH values. Conversely, Kundiyana *et al.* also studied the pH effects over "C. ragsdalei" production concluding that incubations at pH lower than 6.0 was not associated with increased ethanol concentrations (Kundiyana et al., 2011b). This contradiction may reveal species-specific differences among bacteria and support the idea that pH is not the only variable that governs the transit to alcohol production. Under acidic conditions, a higher proportion of acids will be present in its undissociated form. The accumulation of higher concentration of undissociated acids has also been reported as a parameter favoring solvent production as a means to detoxify accumulated acids (Jones and Woods, 1986).

Extremely high acid production rates can lead to a sudden accumulation of organic acids in the medium, which can not be transformed into alcohols. This drastic situation, known as acid crash, results in the absence of a transition to the solventogenesis phase,

which invariably reduces substrate consumption (Maddox et al., 2000; Mohammadi et al., 2014). In chapter 4.3 we show that the incubation of *C. carboxidivorans* P7 at 37 ºC led to an accumulation of undissociated acids (36.4 mM), reaching critical concentrations in which acid crash had been reported for *C. ljungdahlii* (Mohammadi et al., 2014). As observed in our study, a decrease on the metabolic rate of the strain due to incubation at suboptimal temperatures resulted in a significant mitigation of the acid crash negative effects (Ramió-Pujol et al., 2015b), thus allowing the culture to follow its normal growth phases and alcohol production. Kundiyana *et al.* also reported that decreasing temperature had a significantly positive effects on ethanol production using "C. ragsdalei" (Kundiyana et al., 2011b), which were also observed in our study with P7. Most likely, temperature positive effects on growth and productivity arise from the increase of gas solubility. However, additional hypotheses need to be considered. At lower temperatures, phospholipid composition of the membrane is changed to adapt functionality to variations in viscosity of the medium. Adaptations to temperature include decreasing fatty acids chain length, and changing the presence of unsaturated fatty acid chains (Lepage et al., 1987; Sinensky, 1974). It is realistic to consider that toxic effects of either acids and alcohols to the cell may be decreased at lower temperatures due to changes in membrane viscosity and permeability. However, resistance to ethanol and butanol for carboxydotrophic clostridia had not been previously investigated.

In chapter 4.5, the inhibitory effect of ethanol and butanol on *C. ljungdahlii* PETC, *C. carboxidivorans* P7, and *B. methylotrophicum* DSM3468 was analyzed. These three carboxydotrophic clostridia showed kinetic inhibition parameters in line with the threshold toxicity of some other ethanol and butanol producers. Toxic effects were not observed at concentrations lower than 15 g/L for ethanol, and between 12 and 15 g/L for butanol. Therefore, since the maximum production of ethanol and butanol during the growth phase in carboxydotrophic clostridia from syngas fermentation is still far below the reported values for toxicity, we considered alcohol inhibition is not an issue to be

addressed in the short term in industrial process development with carboxydotrophic clostridia.

5.3 The importance of the culture inoculum

Scientists are deeply interested in culturing variables because they not only impact cell growth, but also affect product yield and speciation. Conversely, not much attention has been paid to inoculum development and maintenance. Although this was not one of the aims of the present PhD, experimental results derived from this thesis (mainly *C. carboxidivorans* P7 although also for *C. ljungdahlii* PETC) showed that the state of the inoculum may be a determinant factor on the outcomes of syngas fermentation experiments.

Acid crash events are characterized by relatively poor growth and an almost absence of solventogenesis. As shown earlier, temperature exerts a significant effect of the advent of an acid crash event in *C. carboxidivorans* P7 cultures. However, in some experiments performed with the same strain at 25 ºC (Figure 5.5), we observed a poor alcohol production, at similar titers to those observed when acid crash events occurred. But, high culture densities were obtained in the same cultures (1.0 au), indicating that not all conditions for the definition of an acid crash event were observed. As far as we controlled the experimental conditions, differences among experiments were only related to the inoculum state and preparation, here named as Inocculum 1 (I1), 2, and 3 (I2 and I3, respectively).

Changes in the growth rates were recorded for the three inoccula when incubated at the same temperature. The growth rates for I2 (0.044 h^{-1}) and I3 (0.035 h^{-1}) were slightly lower than that of I1 (0.052 h^{-1}), thereby indicating changes in the metabolic rate. Moreover, the concentration of undissociated acids for I2 and I3 were slightly higher than those found for I1, being 26.8, 33.8, and 20.9 mM, respectively. Although in some cultures undissociated acid concentration was above the critical acid crash point

according to previous authors (Mohammadi et al., 2014; Ramió-Pujol et al., 2015b), no growth inhibition was observed. We hypothesized that inoculum cultures may have an effect on the observed results since all other operational variables and media composition were constant. Supporting this hypothesis, Kawagoshi *et al.* proved that pH and temperature of the inoculum could affect the production of value-added compounds and hydrogen production in anaerobic hydrogen production (Kawagoshi et al., 2005).

However, other differences in the inoculum are also able to affect growth or production during fermentation experiments. One such example is the age of the inoculum. To assess the potential effect of the inoculum age on the fermentation outcome, a simple experiment was performed using strains P7 (ES1 and ES3) and PETC (ES2 and ES4) inocula, with extended stationary phase times (Figure 5.6). The inoculum used for the kinetic tests ES3 and ES4 had been cultured for an extra day than those used in ES1 and ES2. Younger inocula attained much higher optical densities than older cultures (1.0 au vs 0.4 au), supporting the hypothesis that the state of the inoculum determines the outcome of the fermentation experiments.

The state of the inoculum is therefore an important aspect that may alter growth kinetics and alcohol productivity. Although this may not be an issue at large scale fermentation, the effects reported at lab-scale experimentation may end up with some conclusions caused by the divergence in the inoculum. Once these effects adopted at large scale fermentation could not work and be the cause of tremendous costs. According to our experience, most of these problemes could be avoided by working with continuous cultures where constant cell growth is supported by a continuous replenishment of substrates, and no accumulation of potentially toxic products occurs.

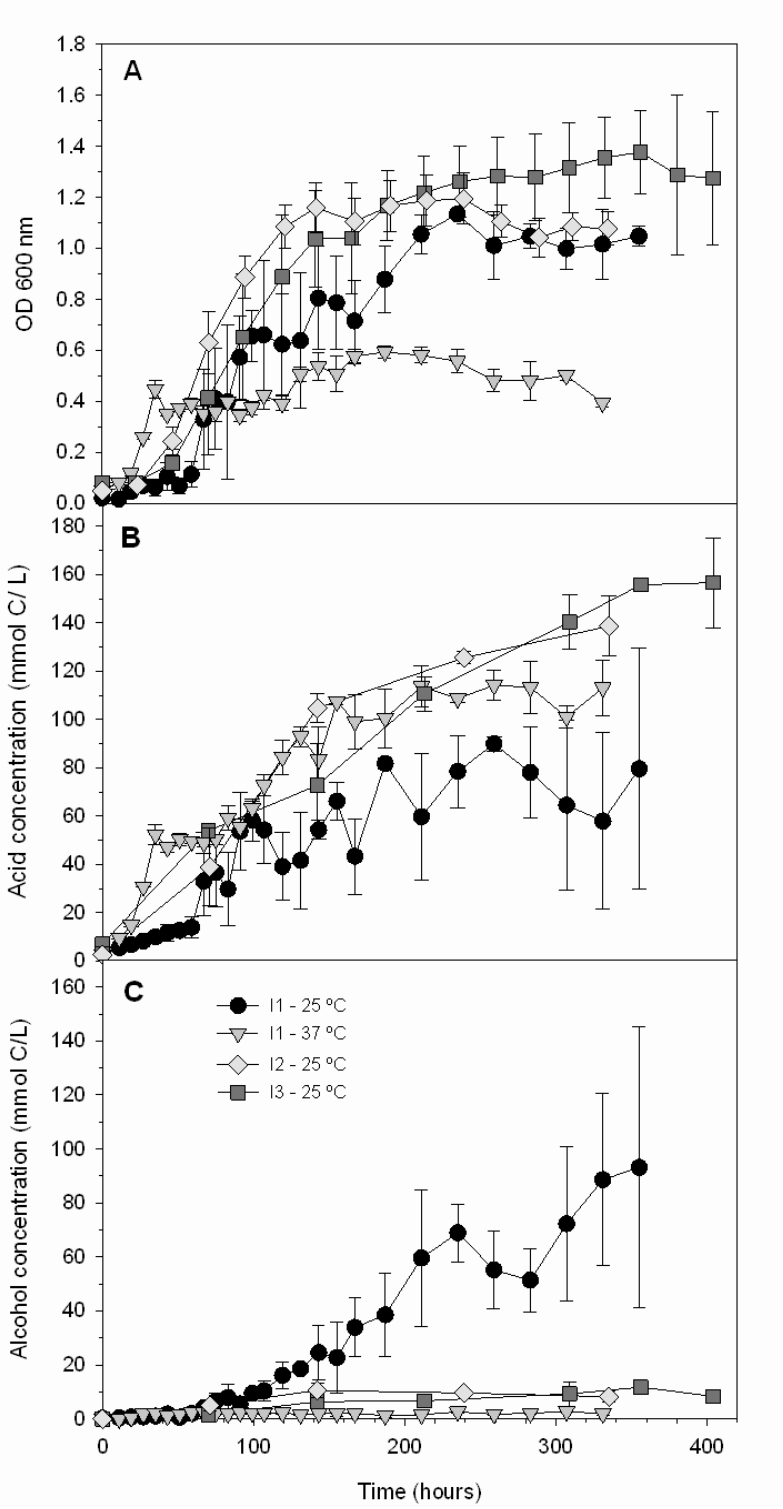

Figure 5.5 Kinetic experiments of *C. carboxidivorans* P7 with the same inoculum I1 incubated at 25 ºC (black dots) and 37 ºC (light grey inverse triangles), and with different inoculum incubated at 25 ºC: I2 (light grey rhombus) and I3 (dark grey square). Growth curves (**A**), acid concentration (**B**), and alcohol concentration (**C**) are shown in this graph. Mean values and standard errors of three replicates are shown.

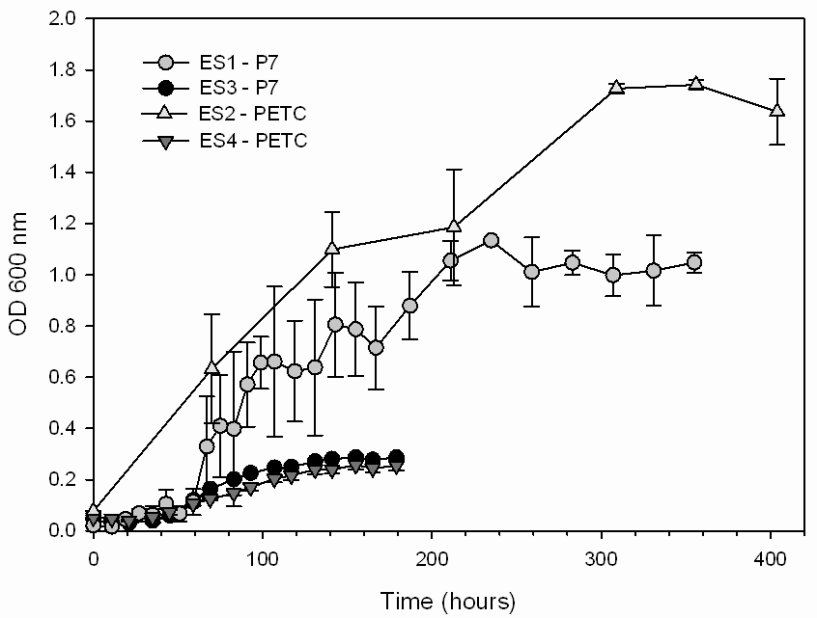

Figure 5.6 Growth curves of *C. carboxidivorans* P7 (dots) and *C. ljungdahlii* PETC (triangle) with an inoculum without (ES1 and ES2) and with (ES3 and ES4) extended stationary phase time.

Although all experiments in the present thesis were conducted using lab-scale reactors, most results are directed to central aspects of cell physiology. Thus, the results obtained provide new insights into key physiological aspects of carboxydotrophic bacteria during syngas fermentation which could help to improve the process at industrial-scale level.

5.4 Outlook and future perspectives

Research outputs directed to enhance culture conditions and on-line control of fermentation have caused significant increases in alcohol production from syngas using naturally occurring bacterial strains. However, improvements in this area have somehow come to a limit and, therefore, other perspectives need to be explored in order to increase the interest in syngas fermentation by carboxydotrophs technology. A future direction for research in this area is metabolic engineering, as this may provide new biological platforms for the efficient transformation of inorganic carbon to specific target compounds (Liew et al., 2013). The modification of metabolic routes in microbial hosts will expand the spectrum of compounds that can be synthesized by microorganisms.

However, a good understanding of the regulatory circuits and molecular mechanisms for the solvent production in carboxydotrophic bacteria is still needed. In this sense, the application of recent technological advances, such as genome sequencing, computer aided design and modeling, and genome-scale metabolic modeling, will ease the process.

Another experimental field in which carboxydotrophs may be a keystone for significant improvements in fuel production is bio-electrochemical fermentation and bio-electrosynthesis. Recent studies have reported microbial electrosynthesis of added-value compounds by mixed cultures, including acetogenic bacteria, exclusively fed with electricity as the sole electron donor and CO_2 as the only carbon source (Batlle-Vilanova et al., 2016; Ganigué et al., 2015; Marshall et al., 2012; Nevin et al., 2010). The conversion of electrical energy to covalent chemical bonds may be one of the best storage and distribution options to fully exploit the potential of the production of energy from renewable technologies.

CONCLUSIONS

The main conclusions of this thesis are:

1. *C. ljundahlii* PETC showed better growth rate and productivity at low formate concentrations and at pH 5.0 and 6.0. These results show the ability of PETC to use formate as a substrate diminishing the energy demand for growth and enhancing strain productivity, which opens up potential to upgrade carboxydotrophic fermentation process with external formate supply.

2. Experiments proved a successful production by mixed cultures of longer organic compounds (C4 or C6) than P7 pure cultures, with butyrate as the main product.

3. Incubation at sub-optimal temperature (i.e. 25 ºC) diminished acid crash risk in *C. carboxidivorans* P7 minimizing the negative effects resulting from the accumulation of organic acids. It also enhanced alcohol production. Molecules with longer carbon chains accumulated at lower temperatures. These effects are probably due to the decrease in the metabolic rate of cells and the increase in gas solubility.

4. Yeast extract was proven unnecessary for improving syngas fermentation by *C. carboxidivorans* P7. This opens up the possibility to reduce media supplementation in large scale syngas fermentation, lowering production costs.

5. Key model organisms for syngas fermentation, *C. carboxidivorans* P7, *C. ljungdahlii* PETC, and *B. methylotrophicum* DSM3468 have a moderate to high resistance to ethanol and butanol. Alcohol toxicity was on the range of 10 – 15 g/L and was not considered an issue for development of industrial production processes, since maximum production are still far below the inhibitory threshold for the tested strains.

6. Strain I19 and I53 were isolatd from organic-rich environments. On the basis of 16S rRNA comparisons, isolates should be classified either as *Butyribacterium* or *Eubacterium* spp. despite displaying significant differences on product spectrum compared to type strain.

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