Universidade do Minho
Escola de Engenharia

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Syngas Fermentation Using Pressurized Systems

July 2021

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Syngas Fermentation Using Pressurized Systems

Doctoral Thesis
Ph.D. in Chemical and Biological Engineering

Work developed under supervision of
Professor Doctor Maria Madalena dos Santos Alves
Professor Doctor Diana Zita Sousa
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DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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"I never lose, I win or I learn." – said Nelson Mandela. I feel this is the best way to describe this very important, challenging and defining journey. It is difficult to put into words the feelings that overwhelm me when I think of the acknowledgments I must give after all this journey. But being deserved and owed, I will do my best to honor the people I am grateful to.

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho

SYNGAS FERMENTATION USING PRESSURIZED SYSTEMS

Summary

One of the major drawbacks of syngas fermentation is the limited gas-liquid mass transfer that generally limits productivities. Most research has been focusing in increasing the k_La (volumetric gas-liquid mass transfer coefficient), investigating different reactor typologies and gas dispersion devices. However, the driving force for mass transfer can also be increased, for instance, by operating at increased pressure. This thesis aims to explore the effect of increased pressure in syngas fermentation to improve the gas solubility, evaluating the effect on the biocatalysts and on the process itself. The starting point of this work was to use an adapted culture (from a syngas converting reactor) as inoculum. This culture was able to mainly produce methane and acetate from syngas. Methane was not produced directly from CO, but via the conversion of acetate and H_2 by the bacteria present in the mixed culture (*Acetobacterium* and *Sporomusa* species). Later on, this culture was subsequentially transferred with syngas, originating an enriched culture mainly composed by *Acetobacterium* and *Methanospirillum* species that, besides acetate and methane, was able to produce propionate from syngas. From that highly enriched culture a new strain of *Acetobacterium wieringae*, strain JM, was isolated and characterized. This highly enriched culture was tested in an axial agitation reactor (AAR) with different initial syngas (CO, H_2 and CO_2 (60:30:10 %, v/v)) pressures, from 100 kPa to 600 kPa. No substrate inhibition was observed, even at the highest pressure and an increase of 45 % in titres (of acetate and propionate) were obtained. Moreover, the increase of pressure resulted in a shift in the metabolic pathways from acetate towards propionate, which is an uncommon product of syngas fermentation. The effect of pressure on the conversion of syngas by anaerobic mixed sludge was then studied using two different reactor typologies, AAR and GLR (gas-lift reactor). Initial syngas pressures of 100 kPa, 300 kPa and 500 kPa were tested. Overall, the GLR showed better performance in terms of CO consumption rates and product titres. The main product obtained was methane and the results showed, for the first time, that methanogenic activity was not inhibited with initial syngas pressures up to 500 kPa, achieving methane yields of 75 % for the GLR, and 92 % for the AAR. At 300 kPa and 500 kPa, volatile fatty acids were also produced, namely acetate, proprionate and n-butyrate. Propionate was the most abundant acid produced, reaching 4.4 mM at 300 kPa and 4.8 mM at 500 kPa in the AAR.

Overall, the use of moderate pressures in syngas fermentation was shown beneficial, as it resulted in better productivities and titres without having significant detrimental effects on cell growth. The increase of pressure also did not inhibit methanogenesis. Moreover, higher pressures seem to induce the production of different chemicals, broadening the product spectrum of syngas fermentation. In this way, these findings could be the basis of new developments in the industrialization of syngas fermentation to produce platform chemicals and/or for biomethanation processes.

Keywords: gas solubility, methane, moderate pressures, reactors, syngas fermentation, VFA.

FERMENTAÇÃO DE GÁS DE SÍNTESE USANDO SISTEMAS PRESSURIZADOS

SUMÁRIO

Um dos principais desafios da fermentação do gás de síntese é a limitação na transferência de massa gás-líquido que geralmente limita a produtividade. A maioria das pesquisas têm-se concentrado no aumento do k_La (coeficiente de transferência de massa gás-líquido volumétrico), investigando diferentes tipologias de reatores e dispositivos de dispersão de gás. No entanto, a força motriz para a transferência de massa também pode ser aumentada, por exemplo, operando sistemas pressurizados. Esta tese tem como objetivo explorar o efeito do aumento da pressão na fermentação de gás de síntese para melhorar a solubilidade do gás, avaliando o efeito nos biocatalisadores e no próprio processo. O ponto de partida deste trabalho foi a utilização de uma cultura adaptada (de um reator de conversão de gás de síntese) como inóculo. Essa cultura foi capaz de produzir principalmente metano e acetato a partir do gás de síntese. O metano não foi produzido diretamente do CO, mas através da conversão de acetato e H_2 pelas bactérias presentes na cultura mista (espécies dos géneros *Acetobacterium* e *Sporomusa*). Essa cultura foi consecutivamente transferida usando gás de síntese como substrato, originando uma cultura enriquecida composta principalmente por espécies de *Acetobacterium* e de *Methanospirillum* que, além de acetato e metano, foi capaz de produzir propionato a partir de gás de síntese. A partir dessa cultura altamente enriquecida, uma nova strain de *Acetobacterium wieringae*, strain JM, foi isolada e caracterizada. Esta cultura altamente enriquecida foi testada num reator de agitação axial (AAR) com diferentes pressões de gás de síntese (CO, H_2 e CO_2 (60:30:10%, v/v)), de 100 kPa a 600 kPa. Não foi observada qualquer inibição pelo substrato, mesmo na pressão mais elevada e obteve-se um aumento de 45 % na produção final de acetato e propionato. Além disso, o aumento da pressão originou um desvio metabólico de acetato para propionato, que é um produto incomum da fermentação do gás de síntese. O efeito da pressão na conversão de gás de síntese utilizando biomassa anaeróbia foi também estudado usando duas tipologias de reatores diferentes, AAR e GLR (reator gas-lift). Foram testadas as seguintes pressões iniciais de gás de síntese: 100 kPa, 300 kPa e 500 kPa. O GLR apresentou melhor desempenho em termos de taxas de consumo de CO e de concentração final de produtos. O principal produto obtido foi o metano e os resultados mostraram, pela primeira vez, que a atividade metalogénica não foi inibida com pressões iniciais de syngas de até 500 kPa, atingindo rendimentos de metano de 75 % para o GLR e 92 % para o AAR. A 300 kPa e 500 kPa, também foram produzidos ácidos gordos voláteis, nomeadamente, acetato, propionato e n-butirato. O propionato foi o ácido mais abundante produzido, atingindo 4,4 mM a 300 kPa e 4,8 mM a 500 kPa no AAR.

No geral, o uso de pressões moderadas na fermentação do gás de síntese mostrou-se benéfico, pois resultou em melhores produtividades e concentrações finais de produto sem ter efeitos prejudiciais significativos no crescimento celular. O aumento da pressão também não inibiu a metanogénese. Além disso, pressões mais altas parecem induzir a produção de diferentes produtos químicos, ampliando o espectro de produtos da fermentação do gás de síntese. Desta forma, estas descobertas podem ser a base de novos desenvolvimentos na industrialização da fermentação do gás de síntese para a produção de produtos químicos e/ou para processos de biometanação.

Palavras-chave: fermentação de gás de síntese. metano, pressões moderadas, reactores, solunilidade de gás, VFA.

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LIST OF SYMBOLS AND ABBREVIATIONS

ACS (acs)	acetyl co-A synthase
ACK	acetate kinase
ADH (adh)	alcohol dehydrogenase
ATP	adenosine triphosphate
AOR (aor)	aldehyde:ferredoxin oxidoreductase
BCR	bubble column reactor
CoFeSP	corrinoid iron-sulphur-containing protein
CODH	carbon monoxide dehydrogenase
CooS	CODH catalytic subunit
CSTR	stirred-tank reactor
dDDH	digital DNA-DNA hybridization
DNA	deoxyribonucleic acid
Ech	Energy converting hydrogenase
EU	European Union
Fd2	reduced ferrodoxin
FDH (fdh)	formate dehydrogenase
FdhH	formate dehydrogenase subunit H
FT	Fischer-Tropsch
GHG	greenhouse gases
GLR	gas lift reactor
HDCR	hydrogen dependent carbon dioxide reductase
HFM	hollow fiber membrane
k_La	volumetric gas-liquid mass transfer coefficient
NADH	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
Nfn	ferrodoxin:NADP+ oxidorreductase
RNA	ribonucleic acid
Rnf	sodium-motive ferredoxin: NAD oxidoreductase
rRNA	ribosomal RNA

THF	tetrahydrofolate
tRNA	transfer RNA
WL	Wood-Ljundgdahl
WLP	Wood-Ljundgdahl pathway

OUTPUTS

The overall work presented in this PhD thesis gave origin to the following publications:

PAPERS PUBLISHED TO PEER REVIEWED JOURNALS

Arantes AL, Moreira, JPC, Diender M, Stams AJM, Alves MM, Alves JI, Sousa DZ. Enrichment of anaerobic syngas-converting communities and isolation of a novel carboxydotrophic *Acetobacterium wieringae* strain JM. Frontiers in Microbiology, 11:58, 2020, (Q1/Q1) doi: 10.3389/fmicb.2020.00058

Arantes AL, Alves JI, Stams AJM, Alves MM, Sousa DZ. Enrichment of syngas-converting communities from a multi-orifice baffled bioreactor. Microbial Biotechnology, 11(4), 639-646, 2018. (Q1/Q1) doi: 10.1111/1751-7915.12864

PAPERS IN PREPARATION FOR SUBMISSION TO PEER REVIEWED JOURNALS

Arantes AL, Alves JI, Sousa DZ, Alves MM, Effect of moderate pressures on syngas biomethanation by anaerobic mixed cultures

Arantes AL, Alves JI, Sousa DZ, Alves MM, Production of propionate by an enriched syngas-converting anaerobic culture at moderately elevated pressure.

ABSTRACTS AND POSTERS IN CONFERENCES

Arantes AL, Moreira J, Diender M, Stams AJM, Alves MM, Alves JI and Sousa DZ. *Acetobacterium* sp. strain JM, a biotechnological platform organism for syngas conversion. MICROBIOTEC19, December 5-7, Coimbra, Portugal, 2019.

Alves JI, Lopes M, **Arantes AL**, Belo I, Sousa DZ and Alves MM. Pressurized syngas bioconversion: physiological and microbial characterization. MICROBIOTEC17, December 7-9, Porto, Portugal, 2017.

Arantes AL, Lopes M, Alves JI, Pereira MA, Alves MM. Boosting hydrogenotrophic methanogenesis in pressurized bioreactors. AD 15th IWA World Conference on Anaerobic Digestion, October 17 - 20, Beijing, China, 2017.

Alves JI, Lopes M, **Arantes AL**, Sousa DZ and Alves MM. Pressurized syngas bioconversion: physiological and microbial characterization. 3rd International Conference on Biogas Microbiology (ICBM-3), May 1-3, Wageningen, The Netherlands, 2017.

Arantes AL, Alves JI, Lopes M, Alves MM and Sousa DZ. Microbial diversity of anaerobic syngasconverting enrichments from a multi-orifice baffled bioreactor (MOBB). Proceedings of the 3rd International Conference on Biogas Microbiology (ICBM-3), Wageningen, The Netherlands, 1st – 3rd May, 2017.

Arantes AL, Lopes M, Alves JI, Sousa DZ and Alves MM. Pressurized syngas bioconversion: turning gaseous residues into valuable products. International Workshop on Environment and Alternative Energy 2017, The European Space Research and Technology Centre (ESTEC), May 30 – June 2, Noordwijk, The Netherlands, 2017.

Alves JI, **Arantes AL**, Lopes M, Belo I, Sousa DZ and Alves MM. Perspectives on syngas fermentation. CEB Annual Meeting 2017, July 6, Braga, Portugal, 2017. ISBN: 978-989-974788-3.

"Those who have never been wrong have never tried anything new."

Albert Einstein

Chapter 1.

Context, Aim and Thesis Outline

1.1. CONTEXT AND THE MAIN RESEARCH QUESTIONS

Depletion of natural resources and intensification of industrialization are consequences of the exponential growth of world population in the last decades. Demand for water, energy, fertilizers, raw materials accompany this growth with a consequent unsustainable overexploitation of natural ecosystems (Asimakopoulos *et al.*, 2019; Teixeira *et al.*, 2018). The excessive use of fossil-based chemicals and fuels continues to contribute to the problem of greenhouse effect that results in one of the major environmental issues, global warming. Therefore, there is an increasing necessity to use renewable and alternative resources towards a biobased economy. Synthesis gas is a promising feedstock for the environmentally friendly production of commodity chemicals and biofuels (Abubackar *et al.*, 2019; Asimakopoulos *et al.*, 2018; Bengelsdorf *et al.*, 2018; Yasin *et al.*, 2019a). This gas mixture (mainly composed by CO, H_2 and CO_2) is produced from the gasification of various types of waste materials including hardly biodegradable feedstocks. Furthermore, a huge amount of waste gases, containing CO and CO_2, are produced daily by several industries *e.g.* steel manufacturing, petroleum refining and others (Asimakopoulos *et al.*, 2018; De Tissera *et al.*, 2017; Molitor *et al.*, 2016; Yasin *et al.*, 2019a). Syngas fermentation is based on the ability of the biocatalysts (microorganisms belonging to different physiological groups) to convert syngas constituents in alcohols and carboxylic acids (Munasinghe and Khanal, 2010a; Phillips *et al.*, 2017; Sun *et al.*, 2019). Efforts for the commercial application of this technology have been made in the last few years by some companies, LanzaTech®, INEOS Bio and Coskata Inc. Lanzatech® has an operating commercial facility of fermentation of CO rich gases from steel mills to ethanol, in China and is building a new commercial facility in Belgium with the partnership of AcellorMittal (Asimakopoulos *et al.*, 2019; Redl *et al.*, 2017; Stoll *et al.*, 2020). However, syngas fermentation technology still faces some challenges that require further investigation, such as low productivities caused by poor gas-liquid mass transfer (Asimakopoulos *et al.*, 2018; Liew *et al.*, 2016; Sun *et al.*, 2019; Yasin *et al.*, 2019b). One way to improve the problem of gas-liquid mass transfer is the increase of the partial pressure of syngas constituents (Bengelsdorf *et al.*, 2018; Stoll *et al.*, 2020). Utilization of pressurized systems for syngas bioconversion, can be a solution, once it has the potential to increase the dissolution of gaseous substrates, promoting gas-liquid mass transfer. However, the utilization of pressurized systems raises some questions that are addressed in this thesis:

- How reactor configuration affects pressurized syngas fermentation?
- What is the effect of increasing headspace pressures in syngas fermentation?
- How the increase of syngas pressure influences the metabolic pathways of the biocatalysts/microorganisms?

1.2. AIM

The aim of this thesis is to explore syngas fermentation processes using different reactor technologies at moderate pressures in the range of 100 - 600 kPa. Within this main goal, the research is oriented to study the effect of pressure on the growth and physiology of carboxydotrophic microorganisms with a focus in the production of added value chemicals. This will contribute to the knowledge on syngas fermentation at moderate pressure and point to alternative processes and technologies that can ultimately be commercialized in the medium to long term.

1.3. THESIS OUTLINE

In **Chapter 1** the context and aim of this thesis are presented. A state of the art review on syngas fermentation is done in **Chapter 2**, focusing on the microbiology, biochemistry and process technology. In **Chapter 3** a mixed anaerobic culture, highly adapted to syngas was studied, to identify the key players in CO conversion. The culture was mainly composed of *Acetobacterium* and *Sporomusa* species (bacterial community) and *Methanobacterium* and *Methanospirillum* species (archeal community). *Sporomusa* species showed to be responsible for CO conversion, producing H_2 and acetate, acting as conveyers of direct substrates to the methanogens in culture. In **Chapter 4** this mixed culture was further enriched with syngas resulting in the isolation of a new carboxydotrophic microorganism (*Acetobacterium wieringae* strain JM). The effect of pressure (from 100 kPa to 600 kPa) in the syngas-converting enriched culture was studied in **Chapter 5**, using an axial agitation reactor (AAR). The main products formed by this culture were acetate and propionate. In **Chapter 6** a mixed culture approach was used to perform syngas fermentation at moderate pressures (at 100 kPa, 300 kPa and 500 kPa), in two different reactor configurations: a gas-lift reactor (GLR) and an AAR, with the goal to assess the effect of pressure in the community and in product formation. Methane was the main product in both typologies, for all the tested syngas pressures. At higher pressures (300 kPa and 500 kPa) acetate, propionate and n-butyrate were also produced (mainly in the AAR). The main conclusions of this thesis are summarized in **Chapter 7**, where also ideas for future research are suggested.

Chapter 2.

The 3B's of Syngas Fermentation – Biocatalysts, Bioreactors and Biocommodities

In this chapter the existing literature on syngas fermentation is reviewed. Diversity of carboxydotrophic microorganisms and their pathways, and fundamental aspects on process bottlenecks' and reactor designs, are explained. Current developments and future perspectives on syngas fermentation are also presented.

2.1. General Introduction

The increasing world population, associated to an overuse of fossil-based chemicals and fuels, have conducted our society to unprecedented levels of concern in terms of climate change and threats on sustainability. A more circular economy is required to comply with the EU, that targeted that 55 % of energy needs to come from for Renewable Energy Sources by 2030 (European Comission, 2020). Therefore, there is an urgent need for new technologies that convert greenhouse gases (GHG) to renewable fuels and chemicals, moving from a fossil-based towards a bio-based economy. Several technologies have been proposed that enable this transition, as the use of agricultural resources for fuel production (fermentation of sugars, refining of plant oils), recovery of methane through anaerobic digestion of a diversity of organic wastes and, more recently, gasification of lignocellulosic biomass combined with gas fermentation. Nearly any form of organic matter can be transformed, through gasification, into a gas mixture, commonly named syngas, composed of carbon monoxide (CO), hydrogen (H_2) and carbon dioxide (CO_2). Fermentation of these gaseous streams, derived from gasification of poorly biodegradable biomass is a recent approach to produce alternative fuels and commodity chemicals, such as biomethane, fatty acids or alcohols, Figure 2. 1 contributing at the same time to the reduction of GHG (Bredwell *et al.*, 1999; Daniell *et al.*, 2015; Guiot *et al.*, 2011a; Molitor *et al.*, 2016). Additionaly, tons of CO are also released from heavy industry (*e.g.* it is estimated that steelmaking industry produces around 1.4×10^9 megatons of CO worldwide, per year (Bengelsdorf *et al.*, 2018)) and they can also be fermented with this process, saving GHG emissions.

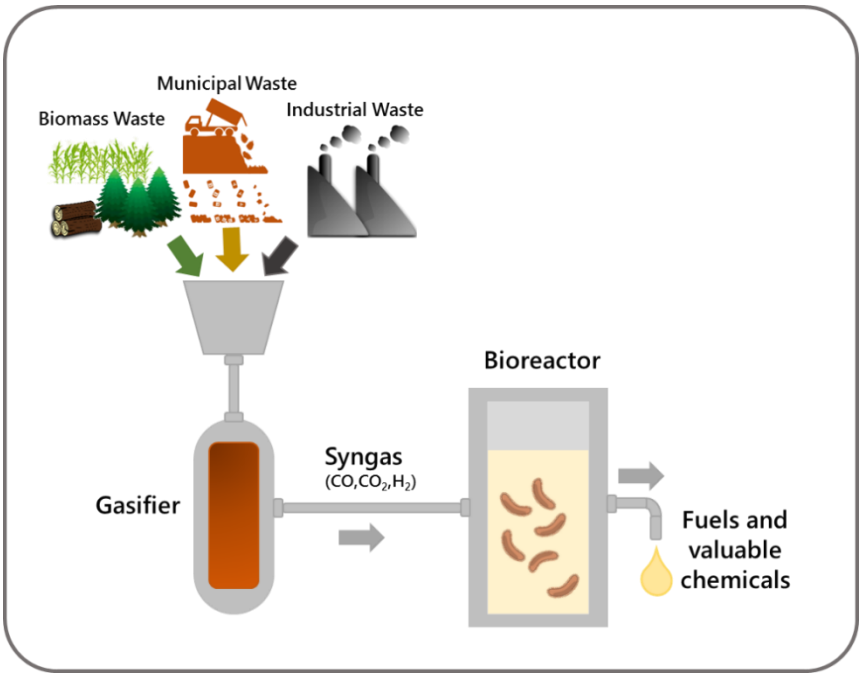

Figure 2. 1 Schematic representation of the gasification process and further syngas fermentation into valuable fuels and chemicals.

Lignocellulosic biomass is the most abundant natural feedstock for the production of chemicals with new sustainable technologies (Kim and Lee, 2016; Redl *et al.*, 2017). This type of biomass is already used to produce biofuels at full-scale, for instance ethanol, through biochemical pathways. However, the economy of such process is still challenged by some constrains including: need of pre-treatments to alter the structure of biomass and expose cellulose and hemicellulose for enzymatic hydrolysis, high enzyme costs, generation of soluble compounds (furan derivatives, and various phenolic compounds) that inhibit fermantation, inneficient conversion of pentose (C5) sugars. Additionally, most sources of biomass (such as straw and wood) contains around 10 % to 25 % of lignin, which cannot be converted by microorganisms to ethanol (Daniell *et al.*, 2012). One way to circumvent these issues, and increase biomass-specific product yields, might be to gasify biomass feedstocks and to use the produced synthesis gas to generate ethanol and other valuable products by gas fermentation technologies, in hybrid thermochemical-biochemical approaches (Henstra*et al.*, 2007(b); Liew *et al.*, 2016; Mohammadi *et al.*, 2011a; Munasinghe and Khanal, 2011).

Gasification is a thermochemical conversion process, performed at high temperatures (500 °C to 900 °C) with a controlled input of oxygen (Abubackar *et al.*, 2011; Daniell *et al.*, 2012; Molino *et al.*, 2016; Tirado-Acevedo *et al.*, 2010). Traditionally, coal, shale oil, tar sands and heavy and hardly biodegradable waste are used as feedstocks in the gasification processes and the resulting syngas is

mainly used for the production of methane, methanol, formaldehyde and acetic acid, via chemical catalysis. More recently, gasification technology has evolved for the utilization of other materials, such as forestry and agricultural biomass, organic waste and some industrial residues. Table 2-1 shows the typical composition of produced syngas according to different feedstocks. Syngas derived from coal gasification is the most common type of syngas, though gasification of lignocellulosic biomass has been widely studied in the last years once it has a great potential and is a renewable source of energy.

Table 2-1 – Some examples of the typical composition of synthesis gas originated by different feedstocks (adapted from Sipma *et al.* 2006 and Tirado-Acevedo *et al.* 2010).

	Composition (vol %)					
Origin	**CO**	**CO_2**	**H_2**	**N_2**	**CH_4**	**Others**
Coal gasification	59.4	10.0	29.4	0.6	0.0	0.6
Partial oxidation of heavy fuel oil	47.0	4.3	46.0	1.4	0.3	1.0
Water gas	30.0	3.4	31.7	13.1	12.2	9.6
Pine Wood	16.1	13.6	16.6	37.6	2.7	13.4
Gramineae	14.7	16.5	4.4	56.8	4.2	3.4
Wood scrap / waste paper	9.2	16.1	6.1	63.2	2.8	2.6
Dairy industry biomass	8.7	15.7	18.6	56.0	0.6	0.4
Cocoa shells	8.0	16.0	9.0	61.5	2.3	3.2

2.2. OPPORTUNITIES FOR SYNGAS FERMENTATION

Synthesis gas can be converted to fuels and chemicals through different processes: *e.g.* Fischer-Tropsch (FT) process, using inorganic or metal-based catalysts, or through syngas fermentation, using microbes (Henstra*et al.*, 2007(b); Mohammadi *et al.*, 2011a; Sun *et al.*, 2019). Metal based catalytic conversion (FT) is well understood and has been employed at commercial scale since 1930 (Molitor *et al.*, 2016). This process occurs at high temperatures (150 - 350 °C) and elevated pressure (30×10^5 Pa) and consists on passing syngas over heterogeneous metal catalysts (iron, cobalt and ruthenium). At these conditions, the CO and H_2 are thermo-chemically converted into a mixture of liquid hydrocarbons

(Liew *et al.*, 2016; Molitor *et al.*, 2016). Despite the high productivity of hydrocarbons in the FT process, there are several common problems: poisoning of the catalysts by low concentrations of syngas contaminants (*e.g.* sulphides), the requirement for fixed ratio of CO and H_2, among others. Biological syngas conversion (syngas fermentation) is an emerging alternative for the production of fuels/chemicals. This process has several strengths, such as (Daniell *et al.*, 2012; Liew *et al.*, 2016; Molitor *et al.*, 2016; Munasinghe and Khanal, 2011; Yasin *et al.*, 2015):

i) independence of the H_2:CO ratio;
ii) high affinity to CO by the microbial catalysts, and lower susceptibility to syngas contaminants (*e.g.* sulfides);
iii) bioreactor operation at mild temperatures;
iv) lower operational costs.

2.3. MICROBIOLOGY AND BIOCHEMISTRY OF SYNGAS FERMENTATION

2.3.1. Microbiology of syngas fermentation

A number of anaerobic microorganisms can grow with CO and/or H2 as electron donors (Table 2-2). These microorganisms are suitable cell factories for the conversion of CO and H2+CO2 to added-value chemicals such as methane (by methanogens), fatty acids (acetate, formate, butyrate) (by acetogens) or/and alcohols (ethanol, butanol, hexanol) (by acetogens) (Bredwell et al., 1999; Diender et al., 2015; Guiot et al., 2011a; Henstra et al., 2007(b); Liu et al., 2014; Sun et al., 2019). CO can also be converted to H2 by hydrogenogens, able to perform the water-gas shift reaction. The main reactions with syngas components during anaerobic syngas fermentation are summarized on Table 2-3.

Most carboxydotrophic hydrogenogens, recently described, are thermophilic organisms. *Carboxydothermus hydrogenoformans*, *Moorella stamsii* and *Desulfotomaculum nigrificans* are good examples of microorganisms able to perform this type of metabolism, which results in the formation of H_2 and CO_2 from CO (Alves *et al.*, 2013 (b); Diender *et al.*, 2015; Henstra*et al.*, 2007(b); Parshina *et al.*, 2005; Svetlitchnyi *et al.*, 2001; Visser *et al.*, 2014). The ability to grow at the expense of hydrogenogenic CO oxidation was also shown for organisms from the archaeal domain, such as members of the genus *Thermococcus, Thermofilum* and *Archeoglobus* (Henstra *et al.*, 2007(a); Kochetkova *et al.*, 2020, 2011; Sokolova *et al.*, 2004)

Acetogenic bacteria, such as *Clostridium carboxidivorans*, *C. ljungdahlii*, *C. autoethanogenum* and *Butyribacterium methylotrophicum*, can convert CO into short-chain fatty-acids (mainly acetate) and alcohols (ethanol, butanol, hexanol, 2,3-butanediol) (Diender *et al.*, 2015; Henstra*et al.*, 2007(a); Jang *et al.*, 2012; Köpke *et al.*, 2011, 2010; Munasinghe and Khanal, 2011; Sun *et al.*, 2019). The biotechnological interest on acetogens from *Clostridium* genus has been increasing in the last years, since these organisms are able to produce butanol and/or 2,3-butanediol using a CO-containing industrial waste gas or syngas as the sole energy and carbon source. Butanol is a good alternative fuel because its energy content is 30 % higher than ethanol, and 2,3-butanediol is a high-value chemical, since it is a precursor in the manufacture of a variety of chemical products (Daniell *et al.*, 2012; Tirado-Acevedo *et al.*, 2010).

Methane production as a direct product from CO conversion has been reported for some species of mesophilic and thermophilic methanogens, such as *Methanosarcina barkeri*, *Methanosarcina acetivorans* and *Methanothermobacter thermoautotrophicus* (Henstra*et al.*, 2007(b); Rother and Metcalf, 2004). Methanogens are the best-studied archaea able to grow with CO as the sole energy source. *M. acetivorans* is the most well studied mesophilic methanogen with respect to the use of CO as growth substrate. Hydrogenotrophic methanogens can also utilize H_2 and CO_2, initially present in syngas, to produce methane. Moreover, CO can be converted into acetate and H_2 by acetogenic or other bacteria, and further used by acetoclastic and hydrogenotrophic methanogens for methane production, which is likely the case when anaerobic sludge, a complex microbial community, is used as inoculum (Sun *et al.*, 2019; Wang *et al.*, 2018).

Several sulphate reducing prokaryotes are also able to utilize CO as energy source, despite typically being inhibited by its presence. Usually they oxidize CO to CO_2 and the derived reducing equivalents are used for sulphate reduction and/or are released in the form of H_2 (Oelgeschläger and Rother, 2008a). Some sulphate reducing prokaryotes, such as *Desulfofundulus thermobenzoicus* subsp. *thermosyntrophicus* or *D. kuznetsovii*, besides the H_2 production, also produce acetate from CO (Parshina *et al.*, 2005). Species from the *Desulfovibrio, Desulfotomaculum* and *Desulfofundulus* genera are the most studied carboxydothrophic sulfate reducing organisms (Parshina *et al.*, 2010). *Desulfotomaculum nigrificans* is a very interesting example of a sulfate reducing bacterium that can grow in the presence of 100 % CO, both in the presence and in the absence of sulfate (Parshina *et al.*, 2005; Visser *et al.*, 2014).

Most of the published studies so far describe syngas/CO conversion by pure cultures. Yet, the use of mixed open- or defined co-cultures to convert syngas is also promising because it allows different routes

and combination of pathways that can result in the production of a broader range of products (Alves *et al.*, 2013 (a); Diender *et al.*, 2016b; Henstra*et al.*, 2007(b); Jang *et al.*, 2012; Köpke *et al.*, 2010; Richter *et al.*, 2016). One of the big challenges, though, is product selectivity. As demonstrated in several different works (Alves *et al.*, 2013 (a); Guiot *et al.*, 2011a; Sancho Navarro *et al.*, 2016; Sipma *et al.*, 2004, 2003) most of the mixed open-cultures result in methane and acetate production and knowledge on defined cultures is still emerging. Besides product selectivity, syngas conversion rates need to be considered as well when prospecting biotechnological applications.

Table 2-2 –Microorganisms capable of degrading CO. (nr, stands for not reported).

Species	T_{opt} (ºC)	pH_{opt}	Products	References
Bacteria				
Mesophilic				
Acetobacterium woodii	30	6.8	Acetate	Sharak Genthner and Bryant, 1987
Acetobacterium wieringae strain JM	30	7	Acetate	Arantes *et al.*, 2020
Butyribacterium methylotrophicum	37	6	Acetate, ethanol, butyrate, butanol	Lynd *et al.*, 1982; Grethlein *et al.*, 1991; Heiskanen *et al.*, 2007
Citrobacter sp Y19	30–40	5.5–7.5	H_2	Jung *et al.*, 1999; *Jung et al.*, 2002
Clostridium autoethanogenum	37	5.8–6.0	Acetate, ethanol	Abrini *et al.*, 1994
Clostridium carboxidivorans	38	6.2	Acetate, ethanol, butyrate, butanol	Liou *et al.*, 2005
Clostridium ljungdahlii	37	6	Acetate, ethanol	Tanner *et al.*, 1993
Eubacterium limosum	38–39	7.0–7.2	Acetate	Sharak Genthner and Bryant, 1987
Peptostreptococcus productus	37	7	Acetate	Lorowitz *et al.*, 1984
Pleomorphomonas carboxyditropha	30	6.5– 7.3	Acetate, H_2	Esquivel-Elizondo *et al.*, 2018
Oxobacter pfennigii	36–38	7.3	Acetate, n-butyrate	Krumholz *et al.*, 1985
Rubrivivax gelatinosa	34	6.7–6.9	H_2	Uffen 1976; Maness *et al.*,2005
Rhodopseudomonas palustris	30	nr	H_2	Jung *et al.*, 1999
Rhodospirillum rubrum	30	6.8	H_2	Kerby *et al.* 1995
Thermophilic				
Caldanaerobacter subterraneus subsp. *Pacificus*	70	6.8–7.1	H_2	Sokolova *et al.*, 2001; Fardeau *et al.*, 2004

Carboxydocella sporoproducens	60	6.8	H_2	Slepova *et al.*, 2006
Carboxydocella thermoautotrophica	58	7	H_2	Sokolova *et al.*, 2002
Carboxydothermus hydrogenoformans	70–72	6.8–7.0	H_2	Svetlitchnyi *et al.*, 2001
Carboxydothermus islandicus	65	55–6.0	H_2	Novikov *et al.*, 2011
Carboxydothermus pertinax	65	6.0–6.5	H_2	Yoneda *et al.*, 2012
Carboxydothermus siderophilus	65	6.5–7.2	H_2	Slepova *et al.*, 2009
Desulfofundulus kuznetsovii	60	7	Acetate, H_2S	Parshina *et al.*, 2005
Desulfofundulus thermobenzoicus subsp. *Thermosyntrophicus*	55	7	Acetate, H_2S	Parshina *et al.*, 2005
Desulfotomaculum nigrificans	55	7	H_2, H_2S	Parshina *et al.*, 2005; Visser *et al.*, 2014
Dietyoglomus carboxydivorans	75	6.5–7.5	H_2	Kochctkova *et al.*, 2011
Moorella stamsii	65	7.5	H_2	Alves *et al.*, 2013(b)
Moorella thermoacetica	55	6.5–6.8	Acetate	Daniel SL *et al.*, 1990
Moorella thermoautotrophica	58	6.1	Acetate	Savage *et al.*,1987
Moorella strain AMP	60–65	6.9	H_2	Jiang *et al.*, 2009
Natranaerofaba carboxydovora	48-50	9.5-9.7	Acetate/Formate	Sorokin *et al.*, 2020
Thermincola carboxydiphila	55	8	H_2	Sokolova *et al.*, 2005
Thermincola ferriacetica	57–60	7.0–7.2	H_2	Zavarzina *et al.*, 2007
Thermoanaerobacter kivui	60	7.5	Acetate, H_2	Weghoff and Müller, 2016
Thermincola potens	55	nr	H_2	Byrne-Bailey *et al.*, 2010
Thermococcus onnurineus	80	8.5	H_2	Bae *et al.*, 2006
Thermolithobacter carboxydivorans	70	7	H_2	Sokolova *et al.*, 2007
Thermosinus carboxydivorans	60	6.8–7.0	H_2	Sokolova *et al.*, 2004
Archaea				
Mesophilic				
Methanosarcina barkeri	37	7.4	CH_4	O'Brien *et al.*, 1984
Methanosarcina acetivorans strain C2A	37	7	Acetate, formate, CH_4	Rother *et al.*, 2004
Thermophilic				

Methanothermobacter thermoautotrophicus	65	7.4	CH_4	Daniels *et al.*, 1977
Thermococcus strain AM4	82	6.8	H_2	Sokolova *et al.*, 2004
Archaeoglobus fulgidus	83	6.4	Acetate, formate, H_2S	Klenk *et al.*, 1997

Table 2-3 – Microbial reactions in CO/syngas anaerobic conversion (adapted from Mazumder *et al.* 1985, Sipma *et al.* 2006, Diender *et al.* 2015 and Sun *et al.* 2019).

Products		$\Delta G^{0'}$ (25 °C) kJ reaction^{-1} (pH 7.00)
From CO		
formate	$CO + H_2O \rightarrow CO_2 + H_2$	- 16
acetate	$4\ CO + 2\ H_2O \rightarrow CH_3COO^- + H^+ + 2\ CO_2$	- 174
butyrate	$10\ CO + 4\ H_2O \rightarrow CH_3(CH_2)_2COO^- + H^+ + 6\ CO_2$	- 440
hexanoate	$16\ CO + 6\ H_2O \rightarrow C_5H_{11}COOH + 10\ CO_2$	-663
ethanol	$6\ CO + 3\ H_2O \rightarrow CH_3CH_2OH + 4\ CO_2$	- 222
n-butanol	$12\ CO + 5\ H_2O \rightarrow CH_3(CH_2)_3OH + 8\ CO_2$	- 480
hexanol	$18\ CO + 3\ H_2O \rightarrow C_6H_{13}OH + 12\ CO_2$	-759
hydrogen	$CO + H_2O \rightarrow CO_2 + H_2$	- 20
methane	$4\ CO + 2\ H_2O \rightarrow 3\ CO_2 + CH_4$	- 211
From H_2/CO		
acetate	$2\ CO + 2\ H_2 \rightarrow CH_3COO^- + H^+$	- 134
butyrate	$4\ CO + 6\ H_2 \rightarrow CH_3(CH_2)_2COO^- + H^+ + 2\ H_2O$	- 240
methanol	$CO + 2\ H_2 \rightarrow CH_3OH$	- 39
ethanol	$2\ CO + 4\ H_2 \rightarrow CH_3CH_2OH + H_2O$	- 288
n-butanol	$4\ CO + 8\ H_2 \rightarrow CH_3(CH_2)_3OH + 3\ H_2O$	- 324
methane	$CO + 3\ H_2 \rightarrow CH_4 + H_2O$	- 151

From H_2/CO_2		
acetate	$2\ HCO_3^- + 4\ H_2 + H^+ \rightarrow CH_3COO^- + 4\ H_2O$	- 104
butyrate	$10\ H_2 + 4\ CO_2 \rightarrow C_3H_7COOH + 6\ H_2O$	-220
hexanoate	$16\ H_2 + 6\ CO_2 \rightarrow C_5H_{11}COOH + 10\ H_2O$	- 341
n- butanol	$12\ H_2 + 4\ CO_2 \rightarrow C_4H_9OH + 7\ H_2O$	- 245
hexanol	$18\ H_2 + 6\ CO_2 \rightarrow C_6H_{13}OH + 11\ H_2O$	-397
methane	$HCO_3^- + 4\ H_2 + H^+ \rightarrow CH_4 + 3\ H_2O$	- 135
From acetate		
methane	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	- 31

2.3.2. Biochemistry of syngas fermentation

CO-fermenting microorganisms use the Wood-Ljungdahl pathway (WLP), also known as reductive acetyl-CoA pathway (Figure 2. 2) to reduce CO_2 to acetyl-CoA. The carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) is a key enzyme complex in this pathway (Diender *et al.*, 2015; Latif *et al.*, 2014; Liew *et al.*, 2016, 2013) The WLP is suggested to be the most ancient CO_2 fixation pathway on earth, and it was first characterized by Wood and Ljungdahl in 1966 in *Clostridium thermoaceticum*, nowadays classified as *Moorella thermoacetica* (Latif *et al.*, 2014; Ljungdahl and Wood, 1969; Wood, 1991). The mechanisms and enzymes involved in the WLP are extensively reviewed elsewhere (Drake *et al.*, 2006; Latif *et al.*, 2014; Ragsdale and Pierce, 2008; Robb and Techtmann, 2018; Wood, 1991). In a brief way, the WLP is composed by two branches: a methyl and a carbonyl branch. Firstly, the CO_2 is reduced to formate in the methyl branch. After, the formate will be activated by condensation with tetrahydrofolate (THF), consuming one molecule of adenosine triphosphate (ATP), to originate formyl-THF. After several reactions, the formyl-THF will be reduced to methyl-THF. In the end of the methyl branch, this methyl group will be transferred to a corrinoid iron-sulphur-containing protein (CoFeSP) and then merged to a molecule of CO, originated from the carbonyl branch, to form acetyl-CoA, through the CODH/ACS complex. In the case of autotrophic growth with CO, the CO_2 required for the methyl branch is formed by the CODH-catalysed water-gas shift reaction. Similarly, when grown autotrophically on CO_2,

the CO is formed from CO_2 by CODH in the carbonyl branch (Liew *et al.*, 2016). Acetyl-CoA can be further converted to acetate (yielding one ATP by substrate level phosphorylation) or reduced to ethanol, butanol or other byproducts (Bengelsdorf *et al.*, 2013; Liew *et al.*, 2013; Munasinghe and Khanal, 2011).

Despite being considered the most efficient CO_2 fixation pathway known (Fast and Papoutsakis, 2012), the WLP is ATP neutral, once one ATP is invested (conversion of formate to formyl-THF) and one ATP is formed (from acetyl-CoA being converted to acetate). For this reason, acetogens have a RnF (sodium-motive ferredoxin:NAD oxidoreductase) or a Ech (energy-converting, ion-translocating hydrogenase) complex to create a sodium or proton motive force to conserve net energy. The Rnf and the Ech complexes are known as the respiratory enzymes present in acetogens, therefore acetogens can be bioenergetically classified as "RnF-" or "Ech-acetogens" (Schwarz and Müller, 2020). In Moorella thermoacetica genome, an Ech complex is coded, suggesting cation export through this enzyme. Based on the presence of this Ech complex, an energy metabolism for growth on H_2/CO_2 has been proposed (Diender *et al.*, 2015). Rnf complexes have been identified, in many acetogens, as the cation extruding mechanism, forming a sodium or proton gradient. It is predicted that this membrane-bound electron transfer complex, reduces NAD^+ simultaneously to the translocation of Na^+/H^+, using reduced ferredoxin (Fd^{2-}) generated from CO oxidation, carbohydrate utilization and/or hydrogenase reactions. The ion gradient generated is collected by H^+ - or Na^+ - ATP synthase to generate ATP (Diender *et al.*, 2015; Drake *et al.*, 2008; Müller *et al.*, 2008). The coupling to a Rnf complex in *Acetobacterium woodii* and *Clostridium ljungdahlii* was suggested (Biegel *et al.*, 2009; Li *et al.*, 2008; Müller e*t al.*, 2008*). A. woodii* genome sequencing, revealed that Rnf complex, probably, is the only active ion-pumping enzyme during autotrophic growth (Poehlein *et al.*, 2012).

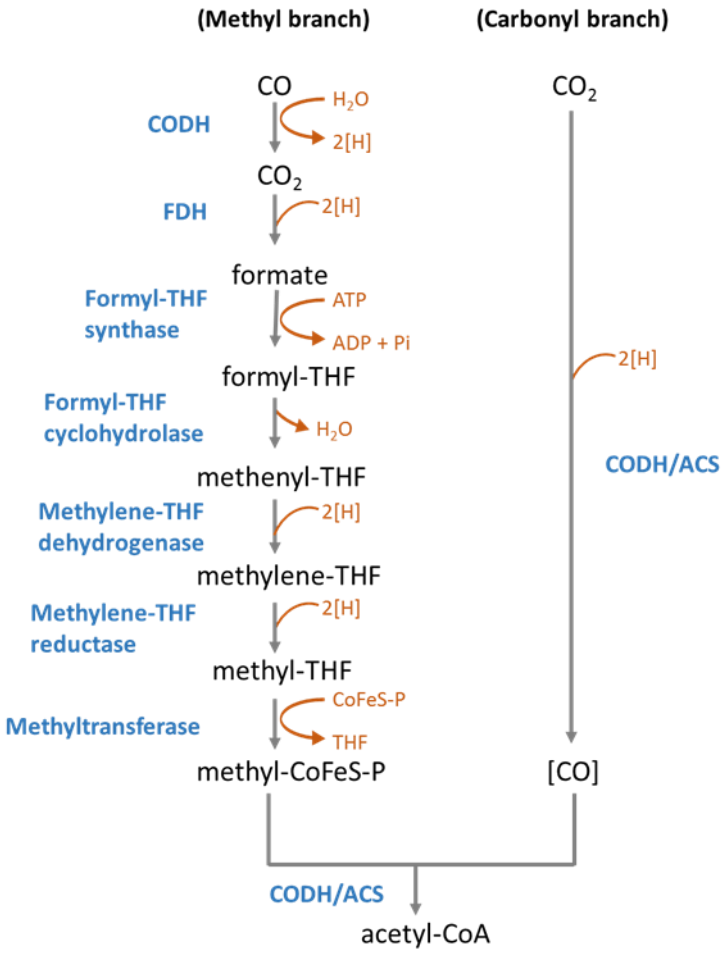

Figure 2. 2 Schematic representation of the Wood-Ljungdahl Pathway. ACS, acetyl-CoA synthase; CODH, carbon monoxide dehydrogenase; CoFeSP, corrinoid iron sulfur protein; FDH, formate dehydrogenase; THF, tetrahydrofolate. (adapted from Liew *et al.* 2013).

2.4. COMMERCIALIZATION AND BIOREACTOR DESIGN

Fermentation of biomass derived syngas still involves practical challenges, though its commercial interests as a platform for biofuels production has grown in the last years (Liew *et al.*, 2013; Molitor *et al.*, 2016; Stoll *et al.*, 2020; Sun *et al.*, 2019). A description of patents in the field of syngas fermentation focusing on reactor design, process development/control and on microorganisms and gene manipulation is provided by Sun *et al.* (2019). Currently, industrial application of this technology focuses on ethanol production (Dürre and Eikmanns, 2015; Sun *et al.*, 2019).

Some companies that initially invested in this technology unfortunately did not advance, such as INEOS Bio (USA) and Coskata inc (USA). In both cases their facilities closed shortly after the first year. Coskata inc technology was eventually bought, in 2016, by Synata Bio (Illinois, USA), founded in 2015, that

posteriorly acquired Abengoa ethanol plants. At the moment, LanzaTech® (Illinois, USA), is actively engaged in the development and commercialization of syngas fermentation technology. LanzaTech® has one gas to fuel demonstration plant in Beijing (China) with a capacity of 378 541 L of ethanol per year, and a waste gas to fuel demonstration plant in Taiwan with a capacity of 36.5 tons of ethanol per year. Currently, LanzaTech®, is building two commercial scale ethanol facilities using waste gases from steel industry, one in China with Shougang (China's largest steel company) and one in Belgium with the world's largest steel manufacturer, ArcelorMittal, with a total capacity of 47 000 tons of ethanol per year. Furthermore, LanzaTech® is now building, in California, the first plant with synthesis gas produced from agricultural and forestry waste in cooperation with Aemetis (Stoll *et al.*, 2020). Other companies are now giving their first steps into research of syngas conversion, although they are entirely catalytic processes: Covestro with the project Carbon4PUR, which focuses on turning CO/CO_2 industrial streams into intermediates for polyurethane plastics; and a joint research project between Evonik and Siemens to produce valuable specialty chemicals from carbon dioxide and green electricity.

Even though many studies were performed in the last few years on syngas fermentation, still some bottlenecks limit the process, as for example the gas-to-liquid mass transfer (Bredwell *et al.*, 1999; Munasinghe and Khanal, 2010a; Yasin *et al.*, 2019a, 2015). The volumetric gas-liquid mass transfer coefficient (k_La) can be used as a reliable parameter to compare mass transfer rates in different reactors and depends on the hydrodynamic conditions in a reactor. The volumetric mass transfer rate is calculated by the multiplication of the k_La by the mass transfer driving force (C* - C), in which C* stands for liquid-phase concentration in equilibrium with the gas phase and C stands for the actual liquid-phase concentration of the transferred gas (Bredwell *et al.*, 1999; Munasinghe and Khanal, 2012). CO and H_2 have considerably low solubility in water, 0.022 g/kg and 0.0014 g/kg, respectively. The low gas-liquid mass transfer of gaseous substrates reduces the microorganism's access to the substrate and consequently reduces the process productivity (Bailey and Ollis, 1986). Thus, it becomes necessary to comprehend the factors that affect k_La, aiming the optimization of operational conditions in bioreactors and to develop alternatives that promote the enhancement of mass transfer driving force (Bredwell *et al.*, 1999; Klasson *et al.*, 1992).

Stirred-tank reactors (CSTR) have been widely used for synthesis gas fermentation at laboratory scale (Klasson *et al.*, 1992; Liew *et al.*, 2016; Munasinghe and Khanal, 2011; Yasin *et al.*, 2019b). This bioreactor design offers excellent mixing, since gaseous substrates are continuously fed and sheared by baffle impellers into smaller bubbles, enhancing mass transfer between the substrate and the microbes. Furthermore, fine bubbles have a slower rising velocity which results in a longer gas retention in

aqueous medium, enhancing mass transfer rates (Liew *et al.*, 2013; Munasinghe and Khanal, 2011). A way to improve mass transfer in this type of reactors is the use of a microbubble sparger, that reduces the size of the bubbles, increasing the contact of microorganisms with the substrate; smaller bubble diameter also increases the flux (Bredwell *et al.*, 1999; Munasinghe and Khanal, 2011). However, this design is not economically feasible for commercial scale due to the high power per unit volume required by the stirrers (Bredwell *et al.*, 1999; Munasinghe and Khanal, 2011; Yasin *et al.*, 2019b). Other bioreactors designs, as pneumatically agitated reactors and cell immobilized reactors have also been studied (Chen *et al.*, 2015; Datar *et al.*, 2004; Ebrahimi *et al.*, 2005; Kreutzer *et al.*, 2005; Yasin *et al.*, 2019b).

Bubble column reactors (BCR), which are the most commonly used pneumatically agitated reactors, are advantageous when compared with CSTR once mixing is achieved by sparging (pneumatically mixing generated by the bubbles, sparged at the bottom, rise through the liquid) without any mechanical agitation, thereby making these reactors a good alternative for practical applications (Abubackar *et al.*, 2011; Liew *et al.*, 2013; Munasinghe and Khanal, 2011). One modified version of the BCR, is the gas lift reactor (GLR) where the vessel is divided into a riser (from which gases are bubbled) and downcomer zones, which can improve the homogeneity in the reactor (Yasin *et al.*, 2019b). BCR or similar configurations are promising candidates for scale-up of syngas fermentation, being, for instance, the ones used by LanzaTech® (Yasin *et al.*, 2019b).

Cell immobilized reactors are an alternative that helps to maintain higher cell densities in the reactor (Yasin *et al.*, 2019b). In monolith biofilm reactors, microbes grow on a biofilm, and are operated under atmospheric pressures, which is an advantage for commercial purposes (Munasinghe and Khanal, 2011). Trickle-bed reactor (another type of cell immobilized reactors) have shown to be more efficient (higher productivities and excellent gas conversion rates) than the ones mentioned before (CSTR and bubble column) (Devarapalli *et al.*, 2016; Klasson *et al.*, 1992; Orgill *et al.*, 2013). This design consists in a packed bed continuous reactor in which the liquid culture flows down through the packing media, resulting in an easier uptake of the dissolved gases by the fixed biofilm. Gaseous substrate is carried either co-currently or counter-currently to the liquid flow and no mechanical agitation is needed (Liew *et al.*, 2013; Munasinghe and Khanal, 2011; Yasin *et al.*, 2019b).

Recent studies have also been focusing on membrane-based systems. In hollow fiber membrane reactors (HFM), the gaseous substrate diffuses through the walls of membranes without forming bubbles and is consumed by the biofilm that is adhered directly in the membrane. This guarantees a higher dissolution of gases in the aqueous fermenting broth and the formation of a biofilm in the

membranes, increasing the cell density (Lee *et al.*, 2012). This innovative approach showed many advantages in what concerns achieving a higher yield and reaction rate (Munasinghe and Khanal, 2011). Different examples of reactor configurations for the biological conversion of synthesis gas are summarized on Table 2-4.

Table 2-4 – Different reactor configurations used in bioconversion of syngas/CO. (n.d. – not determined)

Reactor typology	Substrate	Biocatalyst	Operation condition	Main Products	Productivities	Other kinetic parameters	References
Stirred tank reactor	syngas	*Rhodospirillum rubrum* and *Methanobacterium formicicum*	Continuous	CH_4	methane:1.6 mmol h^{-1}	n.d.	Klasson *et al.*, 1991
Stirred tank reactor	syngas	*Clostridium ljungdahlii*	Continuous	ethanol	n.d.	n.d.	Klasson *et al.*, 1991
Stirred tank reactor	syngas	*Rhodopirillum rubrum*	Continuous	H_2	n.d.	n.d.	Younesi *et al.*, 2008
Stirred tank reactor	syngas	*Clostridium carboxidivorans* strain P7	Continuous	acetate, butyrate, hexanoate	n.d.	n.d.	Abubackar *et al.*, 2018

Bubble column reactor	syngas	*Clostridium ragsdalei*	Continuous	ethanol	n.d.	n.d.	Datar *et al.*, 2004
Trickle-bed reactor	syngas	*Butyribacterium methylotrophicum*	Continuous (variable)	n.d.	n.d.	n.d.	Chatterjee *et al.*, 1996
Trickle-bed reactor	syngas	*Rhodospirillum rubrum*	Continuous	n.d.	n.d.	n.d.	Cowger *et al.*, 1992
Trickle-bed reactor	syngas	*Clostridum ragsdalei*	Semi-continuous	ethanol, acetate	ethanol: 0.38 mmol L^{-1} h^{-1} ; acetate: 0.68 mmol L^{-1} h^{-1}	*Growth rate*: 0.09 h^{-1}	Devarapalli, 2016
Gas-lift reactor	syngas	Mixed anaerobic biomass (animal feces)	Batch	acetate, ethanol, butanol	n.d.	n.d.	Park *et al.*, 2013

Gas-lift reactor	CO	*Carboxydothermus hydrogenoformans*	Semi-continuous	H_2	n.d.	*CO conversion activity:* 0.05 mol L^{-1} reactor day^{-1}; *Growth rate:* 0.1 h^{-1}	Haddad *et al*, 2014
Packed bed reactor	CO	Mixed anaerobic biomass	Semi-continuous	n-caproate, n-heptylate, n-caprylate	n-caproate: 0.28 mmol L^{-1} day^{-1}; n-heptylate: 0.442 mmol L^{-1} day^{-1}; n-caprylate: 0.11 mmol L^{-1} day^{-1}	*CO conversion rate:* 3.3 mmol h^{-1}	He *et al.*, 2018
Monolithic biofilm reactor	syngas	*Clostridium carboxidivorans* strain P7	Batch and Continuous	acetate, ethanol	ethanol: 2.35 g_e L^{-1} day^{-1}; acetate: 1.45 g L^{-1} day^{-1}	n.d.	Shen *et al.*, 2014a
Membrane based reactor	CO	Mixed anaerobic biomass	Continuous	CH_4	methane: 943 mL L^{-1} d^{-1}	*Carboxydotrophic activity:* 5.2 $mmol_{CO}$ L^{-1} g_{VS}^{-1} d^{-1}	Luo *et al.,* 2013

Hollow fiber membrane reactor	syngas	*Clostridium carboxidivorans* strain P7	Batch and Continuous	ethanol	ethanol: 3.44 g L^{-1} day^{-1}	*Consumption rates:* CO: 600 $mmol L^{-1}$ day^{-1}; H_2: 200 $mmol_{H2} L^{-1}$ day^{-1}	Shen *et al.*, 2014b
Hollow fiber membrane reactor	syngas	Mixed anaerobic biomass	Batch and Continuous	acetate, butyrate, caproate	acetate: 0.6 g L^{-1} d^{-1}; butyrate: 0.2 g L^{-1} d^{-1}; caproate: 0.14 g L^{-1} d^{-1}	n.d.	Wang *et al.*, 2018
Hollow fiber membrane reactor	CO	*Clostridium autoethanogenum*	Batch	acetate, ethanol	ethanol: 0.049 g L^{-1} h^{-1}	*Growth rate:* 0.19 h^{-1}	Jang *et al.*, 2018
Reverse membrane reactor	syngas	Mixed anaerobic biomass	Batch	CH_4	n.d.	n.d.	Youngsukkasem *et al.*, 2015
Reverse membrane reactor	syngas	Mixed anaerobic biomass	Continuous	CH_4	methane: 186 mL L^{-1} day^{-1}	*Conversion rates:* CO: 15.2 mmol L^{-1} day^{-1}; H_2: 7.0 mmol L^{-1} day^{-1}	Westman *et al.*, 2016

2.5. CONCLUSION AND PERSPECTIVES

Syngas fermentation is an attractive and sustainable alternative technology to obtain fuels and commodity chemicals. In the past years, research has been done, with the isolation of new syngas fermenting organisms, the development of bioreactors for syngas microbial conversion and engineering microbial strains. Research is still necessary to study the biocatalysts (microorganisms) and their metabolic routes, aiming the production of specific compounds at competitive titres, yields and productivities. The isolation of novel syngas- and/or CO-degrading bacteria can provide new, better biocatalysts for syngas fermentation processes. To accomplish this purpose, efficient bioreactors should promote high mass transfer rates and high cell densities. Moreover, optimization of the existing reactor designs and the use of new and innovative designs should also continue to be studied, so that more industrial installations of this process succeed and proliferate, in a way that syngas fermentation could really be widely applied to contribute to a sustainable bioeconomy.

Chapter 3.

ENRICHMENT OF SYNGAS-CONVERTING COMMUNITIES FROM A MULTI-ORIFICE BAFFLED BIOREACTOR

The substitution of natural gas by renewable biomethane is an interesting option to reduce global carbon footprint. Syngas fermentation has potential in this context, as a diverse range of low-biodegradable materials can be used. In this study, anaerobic sludge acclimatized to syngas in a multi-orifice baffled bioreactor (MOBB) was used to start enrichments with CO. The main goals were to identify the key players in CO conversion and evaluate potential interspecies metabolic interactions conferring robustness to the process. Anaerobic sludge incubated with 70 kPa CO produced methane and acetate. When the antibiotics vancomycin and/or erythromycin were added no methane was produced, indicating that direct methanogenesis from CO did not occur. *Acetobacterium* and *Sporomusa* were the predominant bacterial species in CO-converting enrichments, together with methanogens from the genera *Methanobacterium* and *Methanospirillum*. Subsequently, a highly enriched culture mainly composed of a *Sporomusa* sp. was obtained that could convert up to 170 kPa CO to hydrogen and acetate. These results attest the role of *Sporomusa* species in the enrichment as primary CO utilizers and show their importance for methane production as conveyers of hydrogen to methanogens present in the culture.

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3.1. INTRODUCTION

Biomass can be biologically converted to fuels and chemicals, but poorly biodegradable biomass (*e.g.* straw, wood) requires costly chemical or enzymatic hydrolysis prior to microbial fermentation. Gasification of biomass, and subsequent syngas fermentation, is an alternative that can maximize carbon recovery compared to conventional fermentations. Syngas is mainly composed of CO, H_2 and CO_2, and these compounds can be used by anaerobic microorganisms to produce value-added chemicals. In the absence of external electron acceptors, CO can be converted by hydrogenogenic, acetogenic and methanogenic microorganisms. The hydrogenogenic conversion of CO results in the formation of H_2 and CO_2, and is typically performed by thermophilic carboxydotrophs, such as *Carboxydothermus hydrogenoformans*, *Moorella stamsii* and *Desulfotomaculum nigrificans*. Carboxydotrophic mesophiles, such as *Clostridium carboxidivorans*, *C. ljungdahlii*, *C. autoethanogenum, Acetobacterium woodii*, *Sporomusa ovata*, and *Butyribacterium methylotrophicum* have the capability of converting CO into short-chain fatty-acids (mainly acetate) and alcohols (ethanol, butanol, 2,3-butanediol). In general, CO is a poor substrate for methanogens. Methane production from CO has been reported only for some species of *Methanosarcina* and *Methanothermobacter*. For this reason, the utilization of mixed cultures (sludges) for the production of methane from syngas may be advantageous as normally these systems are more robust and less susceptible to inhibition. Although methane production directly from CO is possible, hydrogenotrophic and acetoclastic methanogens can co-exist with carboxydotrophic hydrogenogenic and acetogenic bacteria and use the bacterial products (H_2 and acetate) to ultimately produce methane. An additional advantage of using anaerobic sludge for syngas conversion is the possibility of implementing low cost, open-fermentation systems. Methane is a direct substitute of natural gas and infrastructure to distribute methane to industry and households is existing.

Bioreactor technology for open-fermentation of syngas is also developing, with special attention to the requisites of high gas-liquid mass transfer rates and cell retention times. Several reactor types have been studied for syngas open-fermentations, including gas-lift reactors, reverse membrane bioreactors (Westman *et al.*, 2016; Youngsukkasem *et al.*, 2015), and multi-orifice baffled bioreactor (MOBB) (Pereira, 2014) . The MOBB described by Pereira (2014) showed methane production rates of about 1.5-2 times higher than reported for the other systems. In this study, we analysed the microbial communities in the MOBB sludge and performed enrichment and microbial diversity studies to get

more insight into key players and metabolic networks involved in CO biomethanation. The main aim was to verify whether methane production from CO occurs predominantly via direct methanogenesis, or indirectly via bacterial-archaeal associations. Identifying key microbial interactions could explain and justify the higher robustness of mixed-cultures for syngas conversion to methane.

3.2. MATERIALS AND METHODS

3.2.1. Source of inoculum and media composition

Granular sludge obtained from a 10-L MOBB fed with syngas mixture (60 % CO, 30 % H_2 and 10 % CO_2 (v/v)) (Pereira, 2014) was used as inoculum for batch incubations with CO. Cultures were prepared in 120-mL serum bottles containing 30 mL of bicarbonate-buffered mineral salt medium and sealed with butyl rubber stoppers and aluminium crimp caps. Medium was prepared as described by Stams *et al.* (1993). Carbon monoxide was used as the sole energy and carbon source and added to the bottles' headspace to the desired final partial pressure using a syringe. All the assays were carried out at initial total pressure of 170 kPa, for which a mixture of N_2/CO_2 (80:20 (v/v)) was added to the headspace when necessary. For assays with CO partial pressures higher than 130 kPa a phosphate-buffered mineral salt medium was used and prepared as described by Alves *et al.* 2013(b). Medium was reduced prior to inoculation with 0.8 mM (final concentration) sodium sulphide ($Na_2S \times H_2O$, ×=7-9). Cultures were monitored by measuring CO depletion and methane, hydrogen and acetate production.

3.2.2. Batch assays

An overview of the experimental set-up implemented in this study is illustrated in Figure 3.1. To test the direct methanogenesis from CO, methanogenic sludge was incubated with the antibiotics vancomycin (v) and erythromycin (e) (bacterial growth inhibitors): CO-$v_{50}e_{100}$ (50 μmol L^{-1} vancomycin + 100 μmol L^{-1} erythromycin) and CO-$v_{100}e_{100}$ (100 μmol L^{-1} of each antibiotic). Incubations without antibiotics were also performed. Bottles' headspace contained 40 % CO (pCO = 68 kPa) as substrate and cultures were incubated at 37 °C. None of the incubations with antibiotics could use CO or produce methane. Incubations without antibiotics were successively transferred (10 % (v/v)) to new bicarbonate-buffered medium and fed with CO for two years with 40 % CO, resulting in an enrichment series designated as CO(x) (where x stands for number of successive transfers). Since an early enrichment stage, the presence of sporulating cells in the CO cultures was evident (microscopic observation). An aliquot of culture CO(4) was pasteurized and used as inoculum to start the enrichment series CO-P(x) (where P

stands for pasteurization; x stands for number of successive transfers). Pasteurization procedure consisted in heating up the culture to 80 °C for 20 min. Culture CO-P(1) was transferred four times to fresh medium, and at this time a second pasteurization was performed (culture CO-P(4)). Pasteurization temperature was now increased to 95 °C (for 20 minutes). In transfers immediately after each pasteurization, yeast extract and ethanol were used to stimulate growth, being removed in posterior transfers. CO was the sole carbon and energy source added (40 %; pCO = 68. kPa) in the next transfers (CO-P(5) and CO-P(6)). Then, the CO partial pressure in the headspace was raised gradually from 40 % (pCO = 68 kPa) to 100 % CO (pCO = 170 kPa) (CO-P(7) to CO-P(12)).

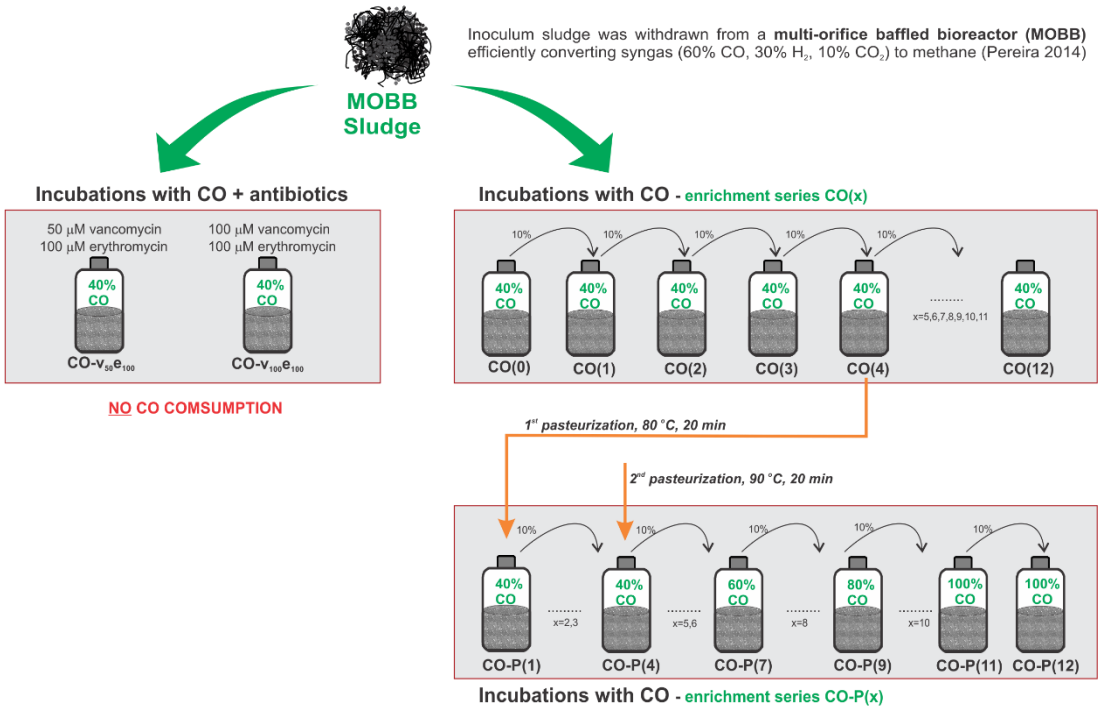

Figure 3. 1 - Experimental set-up and identification of enrichment cultures promoted in this study. In the enrichment series CO(x) and CO-P(x), (x) stands for the number of transfers over 2 years period.

3.2.3. Analytical methods

Gas samples were analysed by gas chromatography with a Bruker Scion 456-GC (Billerica, MA, USA) with a thermal conductivity detector and equipped with two columns: a BR-QPLOT column (30 m length, 0.53 mm internal diameter; film thickness, 20 µm) and a Molsieve packed column (13X 80/100, 2 m length, 2.1 mm internal diameter). The Molsieve column was used to measure CO, H_2 and CH_4, and argon was used as carrier gas at a flow rate of 30 mL min^{-1}; temperatures in the injector, column and detector were 100, 35 and 130 °C, respectively. Volatile fatty acids (VFA), such as acetate,

were determined by high performance liquid chromatography using an HPLC (Jasco, Tokyo, Japan) with a Phenomenex - Rezex ROA – organic Acid H+ (8 %) column (300×7.8 mm). The mobile phase used was sulfuric acid (0.005 N) at a flow rate of 0.6 mL min^{-1}. Column temperature was set at 60 °C. Detection of VFA was made sequentially with an UV detector at 210 nm.

3.2.4. DNA extraction and amplification

5 mL of well-homogenized MOBB sludge, CO(x) and CO-P(x) cultures were stored at -20 °C. DNA extraction from these samples was performed using the FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH, USA) accordingly to the manufacturer's protocol. The microbial 16S rRNA genes were amplified by PCR using a Taq DNA polymerase kit (Invitrogen, Carlsbad, CA, USA). PCR programs and reactions mixtures used were as described elsewhere (Sousa *et al.*, 2007) and all primers set were synthesized by Invitrogen (Carlsbad, CA, USA). For 16S rRNA gene amplification for denaturing gradient gel electrophoresis (DGGE), primers set 1401r/968-GCf was used for bacteria and 515-GCr/A109(T)f for archaea (Sousa *et al.*, 2007). The yield and size of PCR products were assessed by electrophoresis in 1 % agarose gel (wt/vol), using a 1 Kb extended DNA ladder (ThermoScientific, Waltham, MA 02451, USA) and a green safe staining.

3.2.5. DGGE analysis

DGGE analysis was performed using a DCode system (Bio-Rad, Hercules, CA, USA). For the purpose, gels of 8 % (wt/vol) polyacrylamide (37.5:1 acrylamide/bis-acrylamide) were used with a denaturing gradient of 30 to 50 % for Archaea and 30 to 60 % for Bacteria, with 100 % of denaturant corresponding to 7 M urea and 40 % (v/v) formamide. Electrophoresis ran in a 0.5 TAE buffer at 60 °C for 16 hours at 85 V. Posteriorly gels were stained with silver nitrate (Sanguinetti *et al.*, 1994) and scanned in an Epson Perfection V750 PRO (Epson, Long Beach, CA 90806, USA).

3.2.6. 16S rRNA gene sequencing

Cloning and Sanger sequencing were performed using the methodologies previously described by Sousa *et al.* (2007). Similarity searches were performed using the NCBI blast search program within the GenBank database (http://www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1990). Illumina Miseq platform sequencing was done at the Research and Testing Laboratory - RTLGenomics (Lubbock, TX, USA). The MiSeq method used was the Illumina two-step using universal primers for Bacteria and Archaea, 515f and 806r developed by Caporaso *et al.* (2011). After sequencing the data were processed using the data analysis pipeline from RTL, which consists in two major steps, the denoising

and chimera detection step and the microbial diversity analysis step, as in described in the company procedures.

3.2.7. Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in this study have been deposited in the European Nucleotide Archive (ENA) under the accession numbers LT671598-LT671603 (Sanger sequencing) and ERS1422865-ERS1422866 (Illumina MiSeq platform; these sequences can be viewed by following URL http://www.ebi.ac.uk/ena/data/view/PRJEB16760).

3.3. Results and discussion

Multi-orifice baffled (bio)reactors (MOBB) are recognized for their excellent performance in gas-liquid mass transfer and therefore suited for the conversion of gaseous substrates (Ni *et al.*, 2003). In a previous work, Pereira (2014) described the application of a MOBB to continuous syngas conversion to methane using open anaerobic mixed cultures (sludge). Although methane was efficiently produced from CO, the microorganisms involved in this conversion and possible metabolic interactions were not disclosed. In this study, we used a combination of enrichment studies and microbial diversity analyses to identify key microbial players and microbial interactions occurring in the MOBB sludge.

3.3.1. CO to methane conversion by MOBB enrichments is dependent on bacteria-archaea interactions

Incubation of MOBB sludge with 40 % CO as sole carbon and energy source resulted in the production of methane and acetate. In Figure 3.2, substrate consumption and product formation is shown for a stable enrichment culture obtained after 12 successive transfers of the MOBB sludge on CO (CO(12)): 68.9 mmol L^{-1} CO resulted in the formation of 10.4 mmol L^{-1} methane and 7.9 mmol L^{-1} acetate.

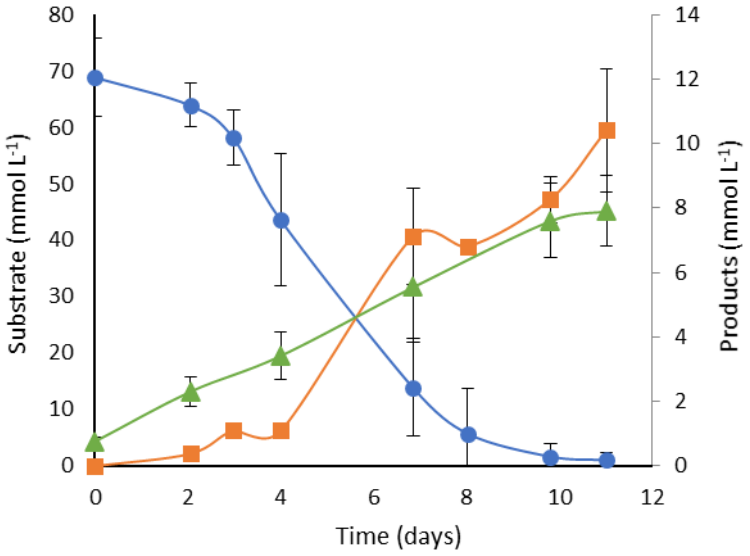

Figure 3. 2 - Substrate consumption and product formation by stable enrichment CO-degrading cultures: CO(12). Symbols: (●) carbon monoxide, (▲) acetate and (■) methane.

When the MOBB sludge was incubated in the presence of the antibiotics vancomycin and erythromycin (CO-$v_{50}e_{100}$ and O-CO-$v_{100}e_{100}$)) no CO conversion or methane production were observed over a long incubation period (over 3 months) (data not shown). Vancomycin and erythromycin are bacterial inhibitors and the fact that no CO conversion was observed in their presence indicates that CO is not directly metabolised by carboxydotrophic methanogens. Carbon monoxide is inhibitory for most methanogens, and only a few species can actually grow on this substrate: *Methanothermobacter thermoautotrophicus* (Daniels *et al.*, 1977), *Methanothermobacter marburguensis* (Diender *et al.*, 2016a), *Methanosarcina barkeri* (Bott *et al.*, 1986; O'Brien *et al.*, 1984), and *Methanosarcina acetivorans* (Rother and Metcalf, 2004). Both thermophilic *Methanothermobacter* species grow significantly slower on CO than on H_2/CO_2 (Daniels *et al.*, 1977; Diender *et al.*, 2016a). *M. acetivorans* can withstand higher CO partial pressures than the hydrogenotrophic methanogens but its methanogenic metabolism shifts towards formation of acetate and formate at increased CO pressures (Rother and Metcalf, 2004)

Analysis of the archaeal communities in the inoculum and initial CO enrichment cultures by cloning and sequencing (Sanger) revealed the presence of hydrogenotrophic methanogens closely related to *Methanobacterium* and *Methanospirillum* species (Figure 3. 3). However, microorganisms related to known carboxydotrophic methanogens were not detected, which again supports the hypothesis that methane is not directly produced from CO in these cultures. *Methanobacterium* and *Methanospirillum*

species are capable to use H_2/CO_2 to produce methane (Whitman *et al.*, 2006). Although H_2 could not be detected in CO enrichments (Figure 3. 2), microbial H_2 production from CO is possible (water-gas shift reaction, $CO + H_2O \rightarrow H_2 + CO_2$) (Diender *et al.*, 2015). The two predominant bacteria identified in the enrichments were related to *Acetobacterium* and *Sporomusa* species (Figure 3. 3), which normally have a homoacetogenic metabolism when growing on CO (Balk *et al.*, 2010; Diender *et al.*, 2015; Sharak Genthner and Bryant, 1987). However, interspecies H_2 transfer during growth of *Acetobacterium woodii* with different methanogens on sugars has been previously reported (Winter and Wolfe, 1980), indicating that a similar mechanism could take place during CO fermentation. Acetoclastic methanogens were not detected in the enrichment cultures (Figure 3. 3), and acetate accumulation was observed (Figure 3. 2). *Methanosarcina* and *Methanosaeta* species are commonly present in anaerobic sludges (Karakashev *et al.*, 2005; Pan *et al.*, 2016; Sancho Navarro *et al.*, 2016) and the reason for their absence in the MOBB sludge is not clear. It might be that these microorganisms suffer from the shear stress caused by the oscillations in the MOBB. In addition, effects of CO toxicity towards aceticlastic methanogens cannot be excluded.

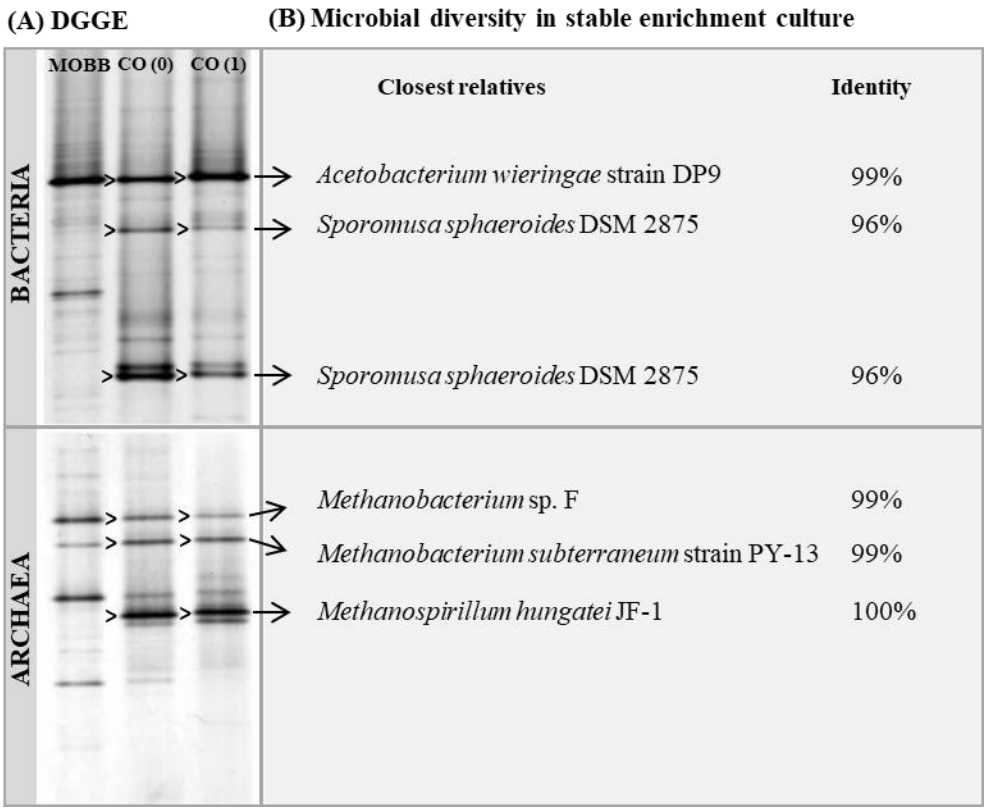

Figure 3. 3 - Microbial diversity in CO-converting anaerobic enrichment (enrichment series CO(x)): (A) bacterial and archaeal DGGE profiles, and (B) closely relative microorganisms (based on 16S rRNA gene idendity) of predominant clones obtained from the enrichment cultures. MOBB – inoculum sludge withdrawn from a MOBB fed with syngas; CO(0) and CO(1) – enrichment cultures incubated with CO as sole carbon and energy source, where (x) stands for number of successive transfers.

3.3.2. A novel *Sporomusa* sp. is present in MOBB sludge that can convert CO to H_2

From the DGGE profiles of cultures CO(0) and CO(1) it is evident that bacterial communities were dominated by *Acetobacterium* and *Sporomusa* species (Figure 3. 3). Carbon monoxide metabolism is well studied in *Acetobacterium woodii*. This organism was initially described to grow homoacetogenically on CO as a sole energy source (Sharak Genthner and Bryant, 1987), but it has been recently found that it can use CO only in co-fermentation with H_2 or formate (Bertsch and Müller, 2015). *Acetobacterium* population in the CO enrichments is most closely related to *A. wieringae* (99 % 16S rRNA gene identity). Recently, Sancho Navarro *et al.* (2016) reported the presence of bacterial species closely related to *Acetobacterium wieringae* after incubation of anaerobic sludge at high CO concentrations. While only *A. woodii* is reported as carboxydotrophic organism, *A. wieringae* genome contains the gene clusters for both carbonyl and methyl branches of the Wood-Ljungdahl pathway, identical to what is found in *A.*

woodii (Poehlein *et al.*, 2016; Sharak Genthner and Bryant, 1987). *Acetobacterium* population became more predominant during the enrichment process (Figure S. 1), and based on MiSeq results it represented about 82 % of the community in enrichment culture CO(12) (Table 3. 1A).

Table 3. 1 - Microbial diversity of enrichments (A) CO(12) and (B) CO-P(8).

(A)

	Closest relatives	**Amount (%)** [a]	**Coverage (%)** [c]	**Identity (%)** [c]
BACTERIA	***Acetobacterium* sp.** (*Acetobacterium* sp. enrichment culture isolate DGGE gel band K1-IRE21-Sa 16S ribosomal RNA gene, partial sequence) [c]	82	100	100
ARCHAEA	***Methanospirillum* sp.** (*Methanospirillum hungatei* strain JF-1 16S ribosomal RNA gene, complete sequence) [c]	7	99	100

(B)

	Closest relatives	**Amount (%)** [a]	**Coverage (%)** [c]	**Identity (%)** [c]
BACTERIA	***Sporomusa* sp.** (*Sporomusa ovata* strain DSM 2662 16S ribosomal RNA gene, partial sequence) [c]	97	100	96
	Caloramator quimbayensis (*Caloramator quimbayensis* strain USBA A 16S ribosomal RNA gene, partial sequence) [c]	2	98	97

(a) Percentage calculated based on a total number of counts of 28146.

(b) Percentage calculated based on a total number of counts of 41718.

(c) Results of sequence analysis on NCBI-BLAST.

The presence of closely related organisms to *Sporomusa* species in the initial enrichments was rather striking. Carbon monoxide metabolism in *Sporomusa* species is poorly studied, although CO conversion to acetate has been reported for *S. termitida*, *S. ovata* and *Sporomusa* strain An4 (Balk *et al.*, 2010; Breznak *et al.*, 1988). *Sporomusa* strains are known to form spores (Möller *et al.*, 1984) and it was clear from microscopic examination that spores were present in early CO-enrichment cultures (data not shown). With the aim of enriching/isolating the *Sporomusa* species in the CO enrichments we proceeded with the pasteurization of culture CO(4) and, later on, a second pasteurization was done with culture CO-P(4) (see experimental set-up in Figure 3. 1). Culture CO-P(12) was highly enriched in

Sporomusa (97 % of the total microorganisms) (Table 3. 1B) and was able to grow under a 100 % CO headspace. Culture CO-P(12) produced H_2 from CO (Figure 3. 4), which considering the composition of the initial CO enrichment cultures, could benefit growth of both *Acetobacterium* species and hydrogenotrophic methanogens. H_2 production by *Sporomusa* strains from CO was not shown before, but these bacteria have an important role in CO conversion in the mixed culture. A remarkable observation is that the *Sporomusa* species in CO-P(12) can grow with 170 kPa CO. Growth inhibition of *S. termitida* was observed for CO partial pressures higher than 40 kPa (Breznak *et al.*, 1988). Further research is needed to compare the enriched *Sporomusa* strain with other *Sporomusa* type strains.

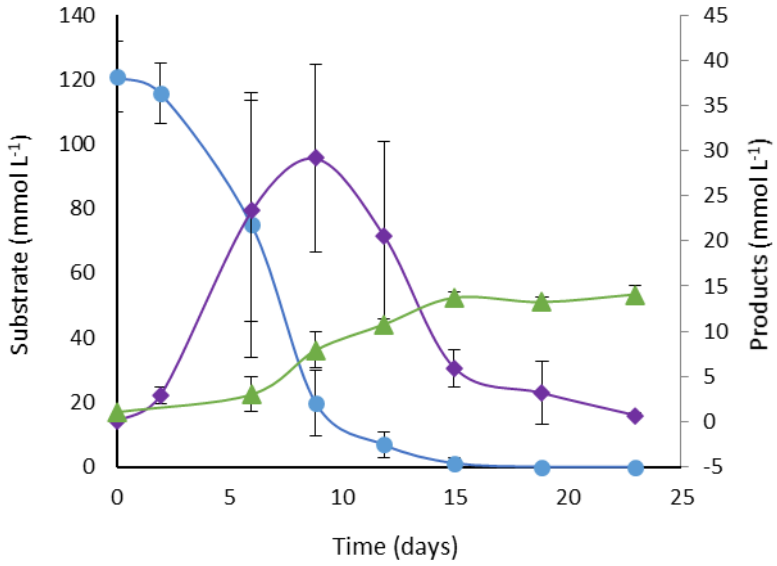

Figure 3. 4 - Substrate consumption and product formation by stable enrichment CO-degrading cultures after pasteurisation: CO-P(8). Symbols: (●) carbon monoxide, (▲) acetate and (◆) hydrogen.

3.4. Conclusion

In the studied syngas adapted culture (from the MOBB reactor) *Acetobacterium* and *Sporomusa* species were the predominant bacterial species, cohabiting with hydrogenotrophic methanogens belonging to the *Methanobacterium* and *Methanospirillum* genera. The results allowed to conclude that methane production from CO in the MOBB was likely resulting from the combined activity of carboxydotrophic hydrogenogenic bacteria and hydrogenotrophic methanogens. Furthermore, in this mixed culture, methanogens have shown an adaptation and higher tolerance for CO concentrations. The *Sporomusa* species found did also show higher tolerance to increased CO concentrations that, for instance, *S.*

termitida (which is known as a carboxydrotroph). In this way, this work allowed to get a deeper insight on CO tolerance on microorganisms. Moreover, it supports the importance and versatility of using mixed cultures in syngas/CO fermentation in order to obtain a broader range of products, as methane for instance.

Chapter 4.

ENRICHMENT OF ANAEROBIC SYNGAS-CONVERTING COMMUNITIES AND ISOLATION OF A NOVEL CARBOXYDOTROPHIC *ACETOBACTERIUM WIERINGAE* STRAIN JM

Syngas is a substrate for the anaerobic bioproduction of fuels and valuable chemicals. In this study, anaerobic sludge was used for microbial enrichments with synthetic syngas and acetate as main substrates. The objectives of this study were to identify microbial networks (in enrichment cultures) for the conversion of syngas to added-value products, and to isolate robust, non-fastidious carboxydotrophs. Enrichment cultures produced methane and propionate, this last one an unusual product from syngas fermentation. A bacterium closely related to *Acetobacterium wieringae* was identified as most prevalent (87 % relative abundance) in the enrichments. *Methanospirillum* sp. and propionate-producing bacteria clustering within the genera *Anaerotignum* and *Pelobacter* were also found. Further on, strain JM, was isolated and was found to be 99 % identical (16S rRNA gene) to *A. wieringae* DSM 1911^T. Digital DNA-DNA hybridization (dDDH) value between the genomes of strain JM and *A. wieringae* was 77.1 %, indicating that strain JM is a new strain of *A. wieringae*. Strain JM can grow on carbon monoxide (100 % CO, total pressure 170 kPa) without yeast extract or formate, producing mainly acetate. Remarkably, conversion of CO by strain JM showed shorter lag phase than in cultures of *A. wieringae* DSM 1911^T, and about 4 times higher amount of CO was consumed in 7 days. Genome analysis suggests that strain JM uses the Wood-Ljungdahl pathway for the conversion of one carbon compounds (CO, formate, CO_2/H_2). Genes encoding bifurcational enzyme complexes with similarity to the bifurcational formate

dehydrogenase (Fdh) of *Clostridium autoethanogenum* are present, and possibly relate to the higher tolerance to CO of strain JM compared to other *Acetobacterium* species. *A. wieringae* DSM 1911^T grew on CO in medium containing 1 mM formate.

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https://doi.org/10.3389/fmicb.2020.00058

4.1. INTRODUCTION

In the frame of a circular bio-economy, it is essential to develop technologies for the sustainable conversion of waste materials to fuels and chemicals. Solutions combining the gasification of low-biodegradable wastes, such as lignocellulosic materials, plastic-based wastes or municipal solid waste, with the biological conversion of the generated syngas have been subject of growing interest and show excellent perspectives (Bengelsdorf *et al.*, 2018; Yasin *et al.*, 2019b). Some microbes can grow on carbon monoxide (CO) and/or CO_2/H_2, which are the main components in syngas. Acetogenic organisms are used in commercial syngas fermentation, such as the LanzaTech® process, to produce ethanol from CO-rich streams (De Tissera *et al.*, 2017; Dürre and Eikmanns, 2015; Molitor *et al.*, 2016; Redl *et al.*, 2017). Carboxydotrophic acetogens are phylogenetically diverse and have been isolated from a variety of habitats including soil, sediments, intestinal tracts of animals and humans (Diender *et al.*, 2015). Acetogens utilize the Wood-Ljungdahl pathway (WL pathway), also known as reductive acetyl-CoA pathway, to conserve energy for growth and perform CO_2 fixation (Ragsdale and Pierce, 2008). The most studied acetogenic bacteria include *Acetobacterium woodii*, *Clostridium ljungdahlii*, *C. autoethanogenum*, *C. carboxidivorans*, *Eubacterium limosum*, *Moorella thermoacetica*, and *M. thermoautotrophica* (Bengelsdorf *et al.*, 2018; Müller, 2019). With C1-compounds, some acetogens mainly produce acetate, while others also produce alcohols, such as butanol and hexanol (Abubackar *et al.*, 2018, 2016; Bengelsdorf *et al.*, 2018; Diender *et al.*, 2015; Phillips *et al.*, 2015).

In this work, anaerobic sludge, previously acclimatized to syngas in a continuous reactor (Pereira, 2014), was used to start the enrichment of microorganisms capable of converting CO/syngas. Analysis of microbial communities in enrichment cultures allowed the identification of a predominant acetogen closely related to *Acetobacterium wieringae*, together with bacteria clustering within *Anaerotignum* and *Pelobacter* genera. A novel carboxydotrophic acetogen, *Acetobacterium wieringae* strain JM, was isolated. Growth of strain JM on CO was compared with that of *A. wieringae* DSM 1911^T and *A. woodii* DSM 1030^T.

4.2. MATERIAL AND METHODS

4.2.1. Media and Microorganisms

The basal medium for the cultivation of the microbial cultures contained the following (l^{-1}): $Na_2HPO_4 \cdot 2H_2O$, 0.53 g; KH_2PO_4, 0.41 g; NH_4Cl, 0.3 g; $CaCl_2 \cdot 2H_20$, 0.11 g; $MgCl_2 \cdot 6H_20$, 0.10 g; NaCl, 0.3 g; $NaHCO_3$, 4.0 g; and $Na_2S \cdot 7\text{-}9H_20$, 0.48 g (as well as acid and alkaline trace elements (each, 1 mL/liter) and vitamins (0.2 mL/liter) prepared as described by Stams *et al.* (1993)). For incubations with 100 % CO, phosphate buffer medium was used and prepared as described previously by Alves *et al.,* 2013(b). The headspace of the bottles was pressurized to 170 kPa with 100 % (v/v) CO, syngas mixture (CO, H_2 and CO_2 (60:30:10 %, v/v)) or H_2-free syngas (CO, N_2 and CO_2 (60:30:10 %, v/v)). The final pH of the media was 7.0 - 7.2. Medium was autoclaved and before inoculation supplemented with vitamins and reduced with 0.8 mM sodium sulfide ($Na_2S \cdot 7\text{-}9H_2O$) (Stams *et al.*, 1993).

Anaerobic granular sludge from a multi-orifice baffled bioreactor (MOBB) (temperature: 35-37 °C; pH: 5.8 - 6.7) fed with a syngas mixture (60 % CO, 30 % H_2 and 10 % CO_2 (v/v)) (Pereira, 2014) was used as inoculum for enrichment. *Acetobacterium wieringae* (DSM 1911^T) and *A. woodii* (DSM 1030^T) were purchased from DSMZ (German Collection of Microorganisms and Cell Culture, Braunschweig, Germany).

4.2.2. Enrichment cultures and isolation of strain JM

Enrichment cultures were coded as culture JM(x), where x represents the number of successive transfers (in a total of 18 transfers). Enrichments were started by inoculation of anaerobic sludge (5 %, v/v) in anaerobic basal medium (described above). First incubations were done with 170 kPa of syngas (CO, H_2 and CO_2 (60:30:10 %, v/v)); acetate (20 mM) was added to the medium as a trial to promote solventogenic metabolism and divert acetogenesis; no yeast extract or formate were supplemented. Cultivation of enrichments was done under non-shaking conditions at 37 °C and pH 7.0.

Growth of the highly enriched culture JM(16) was tested using a syngas mixture 60 % CO, 30 % H_2 and 10 % CO_2 (v/v)) (total pressure 170 kPa) with or without acetate (20 mM). The microbial communities of cultures JM(7) and JM(16) were by 16S rRNA gene analysis (Illumina® MiSeq sequencing and cloning and sequencing, respectively).

Culture JM(16) was used for the isolation of *Acetobacterium* sp. strain JM (the most dominant bacterium in that enrichment). Strain JM was further enriched by using dilution technique (up to 10^{-10}), using medium described above and supplemented with 1 mM of formate and under a headspace of 60 % CO and 40 % N_2 (v/v) (total pressure 170 kPa). The resulting culture was inoculated in roll tubes with

1.5 % low melting point agarose (using the same medium and headspace composition) and incubated at 37 °C. Colonies were picked and inoculated in fresh liquid phosphate-buffered basal medium supplemented with 1 mM of formate and 0.1 g/l of yeast extract and under a headspace of 60 % CO and 40 % N_2 (v/v) (total pressure 170 kPa) and incubated at 37 °C statically. Purity was checked by phase contrast microscopy using a Leica DM2000 microscope (Leica, Microsystems, Weltzar, Germany) and by direct sequencing of the 16S rRNA gene (GATC Biotech, Konstanz, Germany).

4.2.3. Characterization of strain JM

The optimum and range of temperature for growth, and ability of growth with different soluble (final concentration of 20 mM) and gaseous (total pressure 170 kPa) substrates were tested. Substrates tested included: D-fructose, D-glucose, sucrose, xylose, lactate, formate, glycerol, ethanol, methanol, pyruvate, fumarate, citrate, glycine, malate, mannitol, galactose, melibiose, glutamate, galactitol, sorbitol, lactose, maltose, serine, H_2/CO_2 (80:20 % (v/v)), CO (100 % (v/v)), CO (50 % (v/v)), CO (50 % (v/v)) plus acetate, and mixture of CO + H_2/CO_2 (Syngas: 60 % CO, 30 % H_2 and 10 % CO_2 (v/v)). Substrate tests were done at the optimum temperature (30 °C) and shaken at 130 rpm. Additionally, comparison tests of strain JM and type strains *A. wieringae* DSM 1911^T and *A. woodii* DSM 1030^T were also done at 30 °C and at 130 rpm shaking using CO (50 %, 170 kPa); medium was supplemented with 20 mM acetate and 1 mM formate. In these experiments CO was refilled as it was consumed.

4.2.4. DNA isolation, PCR, sequencing and phylogenetic analysis

20 mL of enrichment cultures JM(7) and JM(16) were used for DNA extraction using the FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH, USA), according to the manufacturer's instructions. Bacterial and archaeal 16S rRNA gene fragments were amplified by PCR, using respectively the primer sets 27F/1492R (Nübel *et al.*, 1996) and A109F/1386R (Gagliano *et al.*, 2015). PCR programs and reaction mixtures used were as described elsewhere (Sousa *et al.*, 2007). The PCR products were purified and cloned in Escherichia coli XL-blue competent cells (Agilent Technologies, Santa Clara, CA, USA) as previously described by Sousa *et al.* (2007). Plasmid amplification and Sanger sequencing was done by GATC Biotech (Konstanz, Germany). For bacterial isolates, colony PCR was performed using the same primer set and programme described above, and PCR products were sent to GATC Biotech (Konstanz, Germany) for sequencing. 16S rRNA gene sequences were assembled with DNA Baser software version 4.36.0 (Heracle BioSoft S.R.L, http://www.dnabaser.com) and further compared with the GenBank database (Altschul *et al.*, 1990) using the NCBI BLAST search tool. Illumina Miseq

platform sequencing was performed at the Research and Testing Laboratory – RTL Genomics (Lubbock, TX, USA). The MiSeq method used was the Illumina two-step using universal primers for bacteria and archaea, 515f and 806r developed by Caporaso *et al.* (2011). After sequencing, the data were processed using the data analysis pipeline from RTL, which consists in two major steps, the denoising and chimera detection step and the microbial diversity analysis step, as described in the company procedures.

The 16S rRNA gene sequence of strain JM was submitted to the European Nucleotide Database (ENA) and is available under the accession number LR655884. All the other 16S rRNA gene sequences obtained were submitted to ENA, under the following accession numbers: clones sequences (Sanger sequencing) – from LR657299 to LR657303; sequences from Illumina MiSeq platform – project PRJEB33623.

4.2.5. Genome sequencing, assembling and annotation

DNA was extracted from 50 mL of a grown culture of strain JM using MasterPureTM Gram positive DNA purification Kit (Epicenter, Madison, WI, USA). DNA quality was checked by electrophoresis in a 0.8 % (w/v) agarose gel, using a mass standard (lambda phage DNA) and a size marker (Hind III digested lambda phage DNA). The genome of strain JM was sequenced using Illumina HiSeq X Ten platform (Illumina Ic., San Diego, CA, USA) at Novogene (Beijing, China). Genome was assembled using a pipeline comprising: Ray (Boisvert *et al.*, 2012) to generate an initial assembly, followed by Opera (Gao *et al.*, 2011) for genome scaffolding, and CAP3 (Huang and Madan, 1999) for assembling optimization. For Ray assembler, the optimal kmer size was calculated with KmerGenie (Chikhi and Medvedev, 2014). Automated annotation was performed using the RAST annotation server (Aziz *et al.*, 2008), followed by manual curation. Digital DNA-DNA hybridisation value (dDDH) of strain JM and *A. wieringae* DSM 1911^{T} were obtained using the Genome-to-Genome Distance Calculator 2.1 (GGDC; https://ggdc.dsmz.de) (Meier-Kolthoff *et al.*, 2013; 2014).

The Whole Genome Shotgun project of *Acetobacterium* sp. strain JM has been deposited at DDBJ/ENA/GenBank under the accession VSLA00000000.

4.2.6. Analytical techniques

Organic acids and alcohols were analysed via high pressure liquid chromatography (HPLC) equipped with a MetaCarb 67H column (Agilent Technologies, Santa Clara, CA, USA). The column was operated at a temperature of 45 °C with a flow rate of 0.8 mL min^{-1}. Detection was done via a RI and UV

detector. 0.01 N H_2SO_4 was used as eluent. Samples of 1.0 mL were taken and immediately centrifuged at 13000 g. Subsequently, vials for HPLC analysis were prepared with the supernatant and 30 mM of arabinose solution with the ratio of 8:2 (v/v). Gas analysis was done by gas chromatography (GC). Gas samples of 0.2 mL were taken using a 1 mL syringe and analysed in a Compact GC 4.0 (Global Analyser Solutions, Breda, The Netherlands). CO, CH_4 and H_2 were measured using a molsieve 5A column operated at 100 °C coupled to a Carboxen 1010 pre-column. CO_2 was measured using a Rt-Q-BOND column operated at 80 °C. Detection was done via a thermal conductivity detector.

4.3. Results

4.3.1. Physiological and microbial characterization of enrichment culture JM

Incubation and several transfers of anaerobic sludge with syngas and acetate as substrates, resulted in an enriched culture (culture JM), producing methane and propionate. Substrate consumption and product formation by culture JM(7) are shown in Figure 4. 1: syngas (43 mmol L^{-1} of CO and 20 mmol L^{-1} of H_2) and acetate (17 mM) were completely converted and resulted in 16 mmol L^{-1} of methane and 2.4 mM of propionate (Figure 4. 1 A). In subsequent transfers, acetate consumption by the enrichment cultures stopped as shown for culture JM(16) (Figure 4. 1 B). When only syngas was added to the culture as substrate, acetogenic activity could be observed (Figure 4. 1 C)

The microbial diversity of the enriched culture JM(7) consisted for about 50 % of bacteria affiliated with the genus *Acetobacterium*, while the most abundant methanogen was closely related to *Methanospirilum hungatei* (24 %) (Table 4. 1 A). In culture JM(16), an organism closely related to *Acetobacterium wieringae* DSM 1911^T (99 % of 16S rRNA gene identity) was highly prevalent (87 %) (Table 4. 1 B). A small fraction of organisms (3 %) was related to known propionate producers, namely *Anaerotignum neopropionicum* strain DSM 3847^T (former *Clostridium neopropionicum*) (97 % of 16S rRNA gene identity) and *Pelobacter propionicus* DSM 2379^T (92 % of 16S rRNA gene identity). From the archaeal domain, *Methanospirillum hungatei* was most dominant (94 % of the archaeal clones) (Table 4. 1 B).

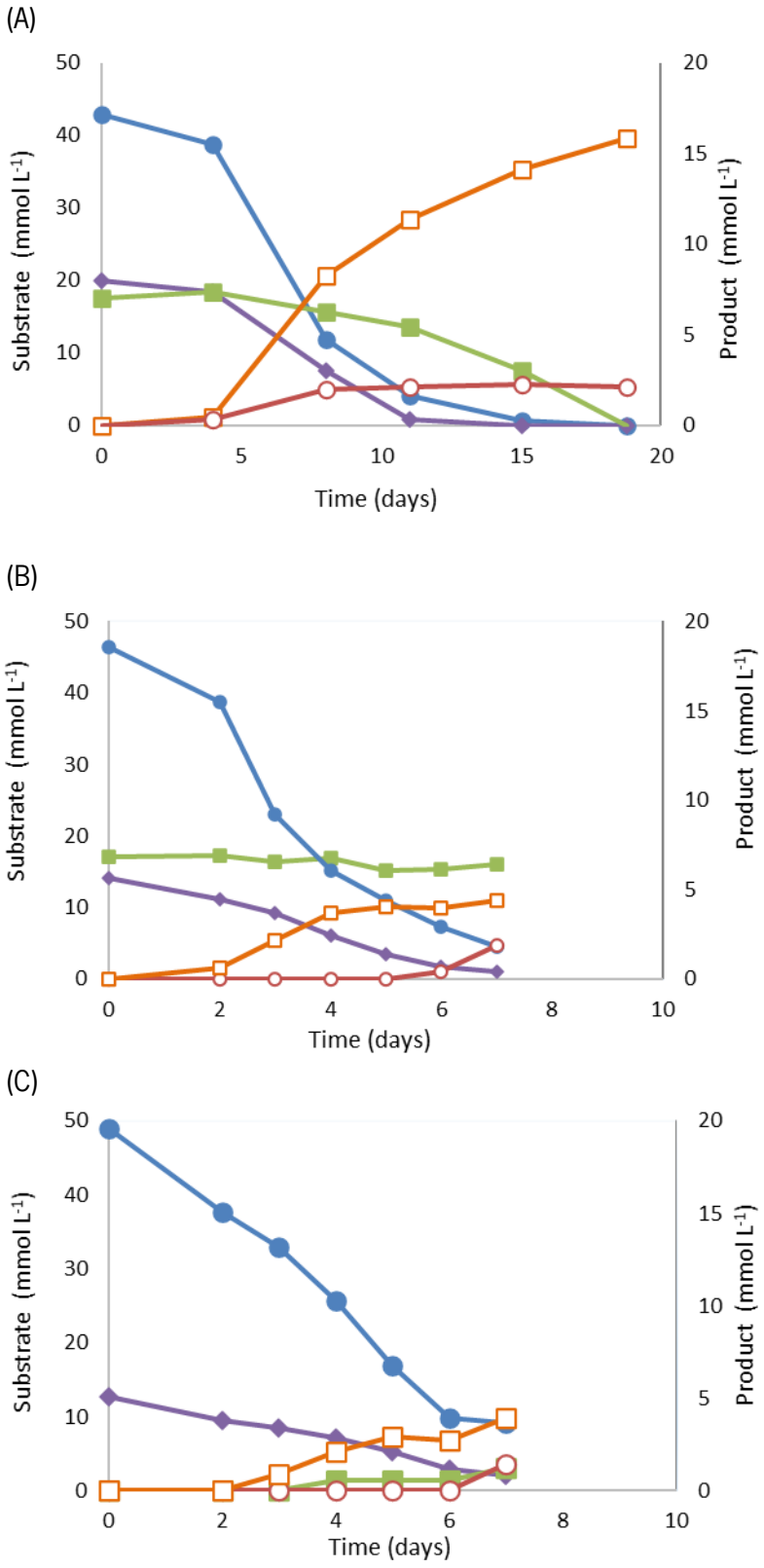


Figure 4. 1 - Substrate consumption and product formation by stable enrichment JM cultures with different substrates: (A) Syngas and acetate (JM(7)), (B) Syngas and acetate (JM(16)) and (C) Syngas (JM(16)). Symbols: (●) carbon monoxide, (◆) hydrogen, (■) acetate, (□) methane, and (○) propionate.

Table 4. 1 - Microbial community analysis of cultures JM(7) and JM(16).

(A) Microbial community analysis of culture JM(7) – Illumina MiSeq

	Closest relatives	Number (%) [a]	Query Coverage (%)	Identity (%)
BACTERIA	***Acetobacterium* sp.** (*Acetobacterium* sp. strain SVCO-15 16S ribosomal RNA gene, partial sequence) [b]	50	100	99
BACTERIA	***Desulfovibrio* sp.** (*Desulfovibrio* sp. S10 gene for 16S ribosomal RNA, partial sequence) [b]	8	100	100
ARCHAEA	***Methanospirillum* sp.** (*Methanospirillum hungatei* strain JF-1 16S ribosomal RNA gene, complete sequence) [b]	24	93	99

(B) Microbial community analysis of culture JM(16) – Cloning and Sanger Sequencing

	Closest relatives	Relative abundance (%) [c]	Query Coverage (%)	Identity (%)
BACTERIA	***Acetobacterium wieringae*** (*Acetobacterium wieringae* strain DP9 16S ribosomal RNA gene, partial sequence) [d]	87	98	99
BACTERIA	***Anaerotignum neopropionicum*** (*Anaerotignum neopropionicum* strain DSM 3847, 16S ribossomal RNA,partial sequence) [d]	2	94	97
BACTERIA	***Pelobacter propionicus*** (*Pelobacter propionicus* strain DSM 2379 16S ribossomal RNA, partial sequence) [d]	1	94	92
ARCHAEA	***Methanospririllum hungatei*** (*Methanospirillum hungatei* JF-1, complete genome) [d]	94	95	99
ARCHAEA	***Methanothrix soehngenii*** (*Methanothrix soehngenii* GP-6, complete genome) [d]	4	94	99

(a) Percentage calculated based on the number of sequence counts obtained for the total community by Illumina sequencing, 27817.
(b) Results of sequence alignment by using BLAST towards the NCBI nucleotide database of partial 16S rRNA gene sequences (approximately 291 bp; results obtained from amplicon Illumina sequencing).
(c) Percentage calculated based on the total number of clones obtained for each domain: 96 clones for Bacteria and 96 clones for Archaea.
(d) Results of sequence alignment by using BLAST towards the NCBI nucleotide database of partial 16S rRNA gene sequences (approximately 1000 bp; results obtained from cloning and sequencing).

4.3.2. Isolation and physiological characterization of *Acetobacterium* sp. strain JM

Isolation of strain JM was done by 10-fold dilution series (up to 10^{-10}) of culture JM(16), using CO as sole carbon and energy source. After several rounds of dilution series in liquid and solid media, a pure culture (strain JM) was obtained. The 16S rRNA gene sequence was 99 % identical to that of *Acetobacterium wieringae* DSM 1911^T. Digital DNA-DNA hybridization (dDDH) between strain JM and *Acetobacterium wieringae* DSM 1911^T was 77.1 %, which is above the 70 % cut-off value generally recommended for species differentiation (Meier-Kolthoff *et al.*, 2013). These results indicate that strain JM is a novel *A. wieringae* strain.

Strain JM is a rod-shaped bacterium with an optimal temperature for growth at 30 °C (growth between 20-37 °C). Strain JM can utilize and grow on CO, without the need of supplementation with yeast extract or formate. Growth on syngas (60 % CO, 30 % H_2 and 10 % CO_2, 170 kPa), CO (50 % CO and 50 % N_2, 170 kPa), CO (50 % CO and 50 % N_2, 170 kPa) plus acetate, and CO (100 %, 170 kPa) yielded acetate and CO_2 (Figure 4. 2). Growth on syngas (Figure 4. 2 A) led to the production of higher amounts of acetate (25.3 ± 0.8 mM) and lower CO_2 accumulation (22.9 ± 0.9 mM) than growth on 50 % CO (13.7 ± 0.1 mM acetate, 56.1 ± 2.9 mM CO_2) (Figure 4. 2 B). When acetate was added as co-substrate (Figure 4. 2 C), lower acetate concentrations were reached (11.5 ± 0.9 mM acetate), though no different fermentation products were detected. On the other hand, growth of strain JM with 100 % CO in the headspace (55.6 ± 0.8 mmol L^{-1}), yielded ethanol (1.8 ± 0.2 mM) in addition to acetate and CO_2 (Figure 4. 2 D).

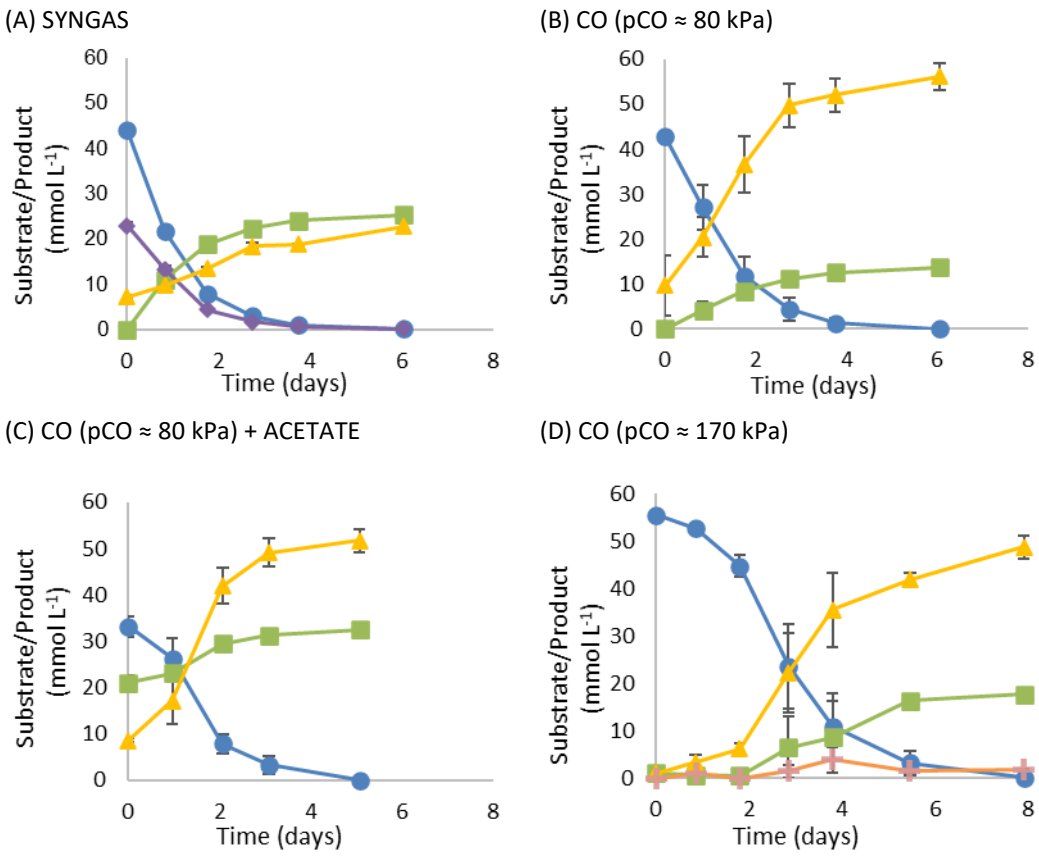
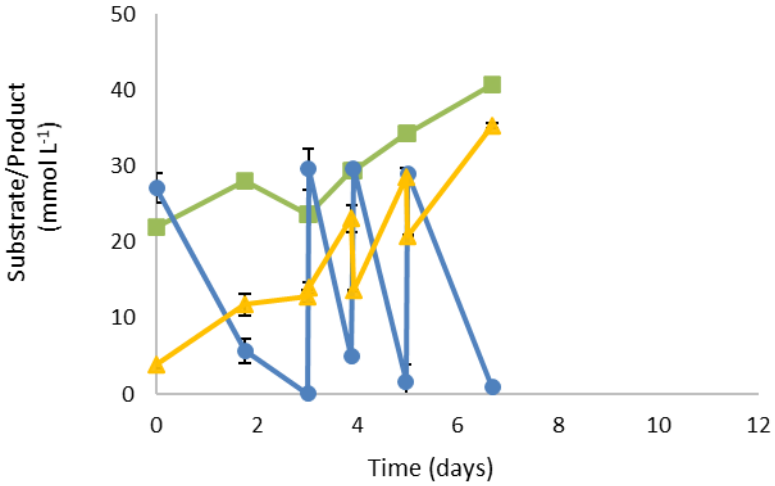


Figure 4. 2 - Batch growth of strain JM with different substrate combinations: (A) syngas, (B) 50 % CO, (C) 50 % CO and acetate (20 mM), (D) 100 % CO. Symbols: (●) carbon monoxide, (■) acetate, (◆) hydrogen, (▲) carbon dioxide, (+) ethanol. All cultures grown using basal medium, without supplementation with yeast extract or formate.

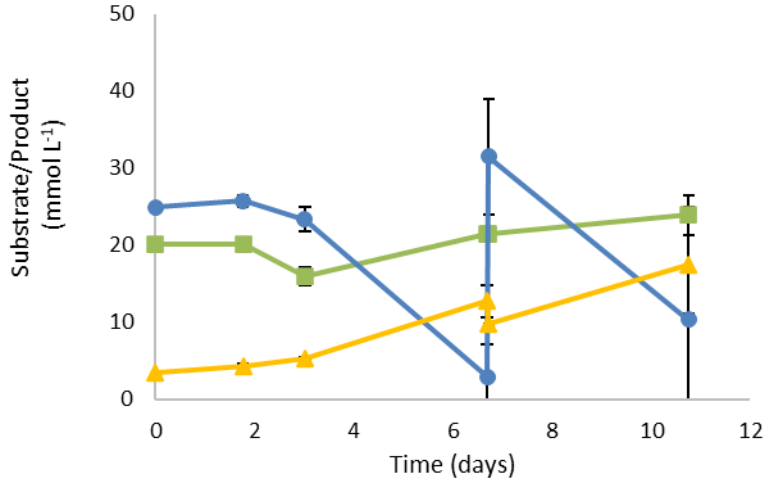
The following substrates were tested and utilized by strain JM: H_2/CO_2, CO, H_2/CO_2 + CO, D-fructose, D-glucose, sucrose, xylose, lactate, formate, glycerol, ethanol, methanol, pyruvate, fumarate, citrate, glycine, malate, mannitol, galactose, melibiose, glutamate, galactitol and sorbitol. Substrates tested that could not be utilized were lactose, maltose and serine.

Parallel growth experiments with CO-acetate as substrates (supplemented with 1 mM formate) were performed for strain JM, (Figure 4. 3 A) and its closest relatives *A. wieringae* DSM 1911^T (Figure 4. 3 B) while *A. woodii* was able to convert 78.5 mmol L^{-1} of CO in 7 days on CO-acetate (Figure 4. 3 C).

(A)



(B)



(C)

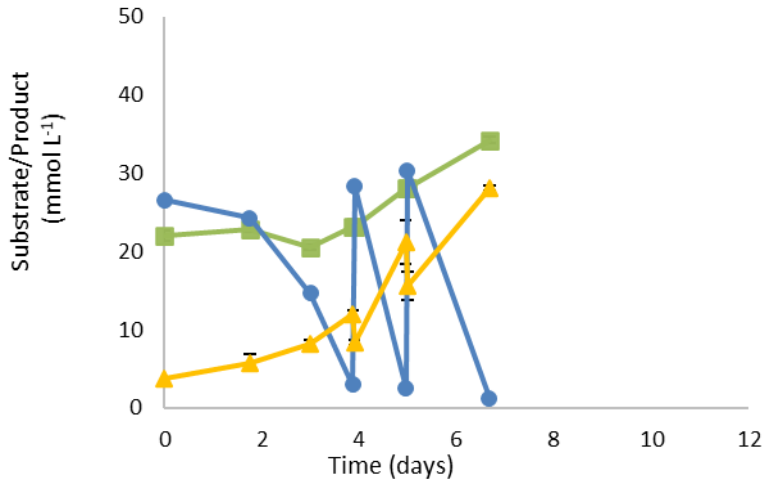


Figure 4. 3 - Gas fed-batch growth with (1) CO-acetate of (A) strain JM (B) A. wieringae DSM 1911^{T} and (C) A. woodii DSM 1030^{T}. Symbols: (●) carbon monoxide, (▲) carbon dioxide, (■) acetate. All cultures grown in basal medium supplemented with 1mM of formate (without yeast extract).

4.3.3. Genome analysis

Genome assembly of strain JM produced 44 contigs with an N50 size of 195,031 bp. The draft genome sequence consists of 3,61 Mbp and a G+C content of 44.3 mol%. The genome has 3240 protein-coding genes, 46 tRNA genes and 12 rRNA genes. All enzymes of the WL pathway are encoded for in the genome of strain JM (Figure 4. 4), supporting its ability to grow on H_2/CO_2 and/or CO. One formate dehydrogenase (Fdh) (TYC86388) was annotated in the genome, showing similarity to the formate dehydrogenase subunit H (FdhH) of the hydrogen-dependent carbon dioxide reductase (HDCR) complex found in *A. woodii* (Bertsch and Müller, 2015). The genes of the HDCR associated hydrogenase were not found in the vicinity of this Fdh. As the Fdh was located at the end of a contig it is possible that associated hydrogenase subunits were missed. Genes of the rest of the methyl-branch of the WLP are located adjacent to each other, including formyl-THF ligase (TYC83982-83), a bifunctional 5,10-methylenetetrahydrofolate dehydrogenase/5,10-methenyltetrahydrofolate cyclohydrolase (TYC83959-60) and a methylene-THF reductase (TYC83962-63). Two carbon monoxide dehydrogenases (codh) encoding genes (TYC86630, TYC87911-12) were identified. TYC87911-12 is located in close vicinity to a gene sequence encoding for an acetyl-CoA synthase (acs) complex (TYC87909-87910) and thus likely serves a dual function: CO-oxidation and acetyl-CoA formation. TYC 86630 appears to have a CODH catalytic subunit (CooS) motive and is next to an iron-sulfur cluster domain protein, suggesting it encodes for a monofunctional CODH. Several genes in the genome (*e.g.* TYC85757-59, TYC86583-84) show similarity to bifurcating complexes such as the NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase (Nfn) complex, or the bifurcating Fdh/[Fe-Fe] hydrogenase complex (Wang *et al.*, 2013). Additionally, two blocks of genes encode for a Ferredoxin:NAD^+ oxidoreductase (Rnf) complex (TYC 88316-21, TYC84275-84280), typically involved in the build-up of a cation gradient.

Genes encoding for acetate and ethanol formation pathways are present. This includes an acetate kinase (ack) (TYC88392) and several alcohol/acetaldehyde dehydrogenase genes (Figure 4.4).

Additionally, the genome contains two acetaldehyde:ferredoxin oxidoreductase genes (TYC88292, TYC84206), of which the latter is located next to a gene coding for an alcohol dehydrogenase. Pyruvate:ferredoxin oxidoreductase (TYC86008) is present for the formation of pyruvate from acetyl-CoA, allowing for assimilation metabolism.

General propionate formation pathways (*e.g.* methylmalonyl-pathway), are not annotated or not complete in strain JM. Nevertheless, pathways for conversion of propanoyl-CoA to propionate are present, so indirect formation of propionate from *e.g.* amino acid metabolism is potentially possible.

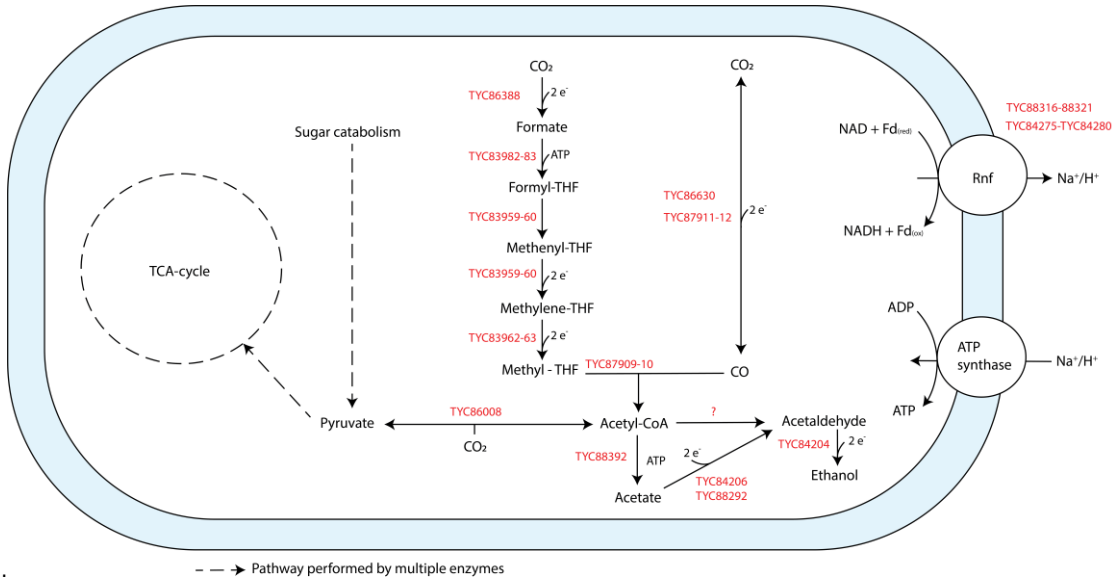


Figure 4. 4 - Schematic representation of the physiology of strain JM when grown on CO. Genes found in the genome that are annotated to perform specific reactions are indicated in red. Reactions are not displayed stoichiometrically.

4.4. Discussion

A novel carboxydotrophic *Acetobacterium wieringae* (strain JM) was isolated from a syngas-converting enrichment culture, producing mainly acetate and small amounts of ethanol from CO. The fact that *Acetobacterium* species were the most predominant bacteria in the enrichment cultures (Table 4. 1) and acetate one of the main product detected in the enrichments, suggests that this bacterium was the main CO-utilizer in the enrichment cultures. Microorganisms closely related to *Anaerotignum neopropionicum* (2 % of total sequences) and *Pelobacter propionicus* (1 % of total sequences) were present in the enrichment cultures JM(16) (Table 4. 1) and were likely responsible for propionate production (Figure 4. 1C). *A. neopropionicum* and *P. propionicus* are known for their capability to convert ethanol to propionate (Schink *et al.*, 1987; Tholozan *et al.*, 1992; Ueki *et al.*, 2017). These results suggest that a synergistic interaction between the *Acetobacterium* species and propionate-forming bacteria was taking place in the enrichments, where *Acetobacterium* is consuming CO to produce acetate and ethanol is further used by close relatives to *A. neopropionicum* and *P. propionicus* to form propionate. Such interactions can be relevant for the overall fitness of microbial communities as they influence thermodynamics of the system. Diender *et al.* (2019) have recently shown a similar synergistic relation in synthetic co-cultures of *Clostridium autoethanogenum* and *Clostridium kluyveri*. In that study, it was shown that the presence of the ethanol-consuming bacterium *C. kluyveri* induced a higher degree of solventogenesis by the carboxydotrophic organism (compared with monocultures of *C.*

autoethanogenum). In the present work, we could derive possibly the same type of interaction by natural enrichment of anaerobic sludge, which points out to a possible significance of this process in natural ecosystems too. Methanogens persisted in the enrichments, despite the reported toxicity of CO towards methanogens (Klasson *et al.*, 1991); species closely related to *Methanospirillum hungatei* JF-1 and *Methanothrix soehngenii* GP6 were present in the enriched cultures (Table 4. 1). There are a few methanogens capable of metabolizing CO to methane, belonging to *Methanobrevibacter*, *Methanosarcina*, *Methanothermobacter* genera (Diender *et al.*, 2015). However, *Methanospirillum* is only reported to produce methane from H_2/CO_2 or formate (Iino *et al.*, 2010), indicating that these microorganisms might be responsible for methane production, using H_2 and not CO. We previously tested CO utilization by *Methanospirilum hungatei* JF-1 (DSM 864) but no growth was observed (unpublished data).

Strain JM can grow on CO alone (without supplementation of yeast extract, formate or H_2/CO_2) (Figure 4. 2). The type strain of *A. wieringae* (DSM 1911^T) was described by Braun and Gottschalk (1982), but its capability to use CO has not been tested before. Here we show that *A. wieringae* type strain can also grow on CO in the presence of formate. The related *A. woodii* can also grow on CO, but only with H_2/CO_2 or formate as a co-substrate (Bertsch and Müller, 2015). In *A. woodii*, a hydrogen-dependent carbon dioxide reductase (HDCR) complex has been found responsible for the production of formate from CO_2, coupling CO_2 reduction directly to H_2 oxidation (Bertsch and Müller, 2015). A similar HDCR complex is present in the genome of *A. wieringae* (OFV70223- OFV70228). Fe-Fe hydrogenases in the HDCR complex were thought to be sensitive to high CO concentrations (Bertsch and Müller, 2015), which could explain the need for formate when *A. woodii* was grown on CO. However, later it was shown that CO inhibition of the HDCR is fully reversible (Ceccaldi *et al.*, 2017). Also, the thermophilic *Thermoanaeroacter kivui* employs a similar HDCR complex and, after prolonged adaptation to CO, was able to grow on 100 % CO without formate (Weghoff and Müller, 2016). This suggests that hydrogenases in HDCR complex can adapt to CO. Strain JM was isolated from a long-term enrichment growing on syngas, and this could have resulted in a better adaptation to CO. The genome of strain JM encodes for a formate dehydrogenase with high similarity to the HDCR of *A. woodii*, but the associated hydrogenases were not found. The fdh gene of strain JM was located at the end of a contig, and therefore we cannot exclude the possibility of missing part of the sequence of the HDCR. It thus remains unclear if strain JM employs a HDCR, but formate formation does not seem to be a limiting step in its metabolism. Besides adaptation of hydrogenases to CO, a link between the abundance of the monofunctional CODH CooS and the bifunctional CODH/ACS and the efficiency in CO utilization was

proposed (Weghoff and Müller, 2016). The genome of strain JM encodes for both, a bifunctional CODH/ACS complex (TYC87911-12), and an apparent monofunctional CODH (TYC86630). However, as the genomes of both *A. woodii* and *A. wieringae* also appear to carry genes of mono- as well as bi-functional CODH this appears not to make a difference here.

Comparison of CO conversion by strain JM and by the type strains of *A. wieringae* and *A. woodii* shows that strain JM can convert a higher amount of CO during the 7 days of incubation (up to 4- and 2.5-fold higher, respectively) (Figure 4. 3), which again could result from metabolic adaptation to CO during enrichment and isolation of strain JM. Adaptation to CO has been previously shown to play a role to increase the growth rate of *A. woodii* up to 3-fold compared to non-adapted cultures (with maximum 75 % CO and 100 mM formate) (Bertsch and Müller, 2015). Duplication times in mesophilic carboxydotrophs range from 4 to 14 hours, with the lowest being achieved by *Clostridium* species (namely *C. ljungdahlii*, *C. autoethanogenum* and *C. ragsdalei*); *A. woodii* has reported (or calculated) duplication times between 5.5 and 13 hours (Diender *et al.*, 2015).

Strain JM encodes for both the carbonyl and methyl branches of the WL pathway as found in *A. woodii* (Poehlein *et al.*, 2016; Sharak Genthner and Bryant, 1987). Additionally, the genome analysis of *A. wieringae* strain JM revealed the presence of two CODH encoding sequences, explaining its CO utilizing properties. Strain JM could grow in the presence of different initial partial pressures of CO or CO/H_2, producing acetate and CO_2; to note, though, that incubation of strain JM with 100 % CO (170 kPa) led to the production of ethanol as well (Figure 4. 2 D). Both, aldehyde:ferredoxin oxidoreductase (aor) (TYC84204, TYC88292) and alcohol dehydrogenase (adh) (TYC84204) encoding genes are present in the genome of strain JM, and are potentially linked to ethanol production by this strain. Conversion of carboxylic acids to alcohols via the AOR-ADH pathway has been previously observed in several carboxydotrophs (Perez *et al.*, 2013; Simon *et al.*, 1987), and further genetic evidence for the pathway reported by Basen *et al.* (2014). The presence of the AOR may contribute for the efficient growth of strain JM on CO, as redox equivalents can be shuttled into ethanol, without interfering with energy conservation (Köpke *et al.*, 2010). Earlier reports on *A. wieringae* type strain show ethanol formation from fructose and H_2/CO_2 conversion (Buschhorn *et al.*, 1989; Groher and Weuster-Botz, 2016) and on *A. woodii* from glucose fermentation (Buschhorn *et al.*, 1989), though not with CO. The same authors also reported that *A. woodii* and *A. wieringae* could use ethanol as substrate as well (Buschhorn *et al.*, 1989), which is also the case for strain JM. This can also explain ethanol consumption by strain JM in later phase of CO fermentation (Figure 4. 2 D).

4.5. Conclusions

Enrichment cultures mainly composed of strain JM and close relatives to *Anaerotignum neopropionicum* and *Pelobacter propionicus* were able to produce propionate from syngas, which is an uncommon product from syngas fermentation. A novel carboxydotrophic *Acetobacterium wieringae* strain JM was isolated from the syngas enriched culture. Strain JM could efficiently convert CO to acetate (and CO_2) and small amounts of ethanol. This is the first report of an *A. wieringae* strain able to use CO, and proof that type strain (DSM 1911^T) can also utilize CO, but only in the presence of formate. It is also the first report of isolation of an *Acetobacterium* species from a CO-fed enrichment.

Chapter 5.

PRODUCTION OF PROPIONATE BY AN ENRICHED SYNGAS-CONVERTING ANAEROBIC CULTURE AT MODERATELY ELEVATED PRESSURE

Syngas fermentation is gaining momentum as a suitable technology to produce biobased chemicals. Because microbial conversions occur in the liquid phase, the low solubilities of its constituents (CO, H_2, CO_2) is still a major limitation of the process. One way to increase gas solubility is to operate bioreactors at higher headspace gas pressure. Whereas an increase in gas solubility will enhance or damage the process performance is a relevant research question. For example, higher concentrations of dissolved CO could be toxic for microorganisms. Furthermore, it is not possible to dissociate increased pressure from increased gas concentrations, therefore it is also relevant to infer about the limiting capacity of the microorganisms, in terms of gas concentration uptake. In this work, the kinetic and metabolic performance of a highly enriched culture growing on syngas, mainly composed of *Acetobacterium wieringae* strain JM, was assessed in pressurized bioreactors working at initial pressure of 100, 300, 400 and 600 kPa of syngas. The CO consumption rates were similar up to 400 kPa, suffering a 2-fold decrease at 600 kPa. With the increase of pressure, a shift towards the production of ethanol and propionate was observed, though acetate was still the main product for all the pressures tested. The enriched culture was also able to grow well with mixtures of CO/H_2 (60:30:10 % (v/v)) and CO (60:40 % (v/v)), showing propionate production in both cases at 300 kPa, suggesting that propionate production is associated with CO consumption and increase of pressure. When comparing to previous studies with this culture in serum bottles at 170 kPa, propionate production was increased in 23 % in these pressurized bioreactors.

5.1. INTRODUCTION

The effects of intensive fossil-fuel consumption are causing a severe climate transition and measures to mitigate this problem should be considered. A growing relevance in the production of fuels and platform chemicals using biobased technologies has been emerging in the last years (Abubackar *et al.*, 2019). Syngas fermentation is a suitable technology for the production of chemicals and fuels. In this process, syngas – a mixture of CO, H_2 and CO_2 generated by gasification of carbon-containing materials – is used as electron and carbon sources by microbes and transformed into aded-value products CO/CO_2 rich gases are also a waste product of several industrial processes (Daniell *et al.*, 2015; De Tissera *et al.*, 2017; Molitor *et al.*, 2016). In syngas fermentation, carboxydotrophic acetogens are usually the elected biocatalyst to capture carbon, which they do via the Wood-Ljungdahl pathway, using CO as electron donor to generate adenosine triphosphate (ATP) (Bengelsdorf *et al.*, 2018; Diender *et al.*, 2015; Liew *et al.*, 2016; Yasin *et al.*, 2019a). The mechanisms and enzymes involved in the pathway are published and discussed in several reviews (Bengelsdorf *et al.*, 2018; Diender *et al.*, 2015; Latif *et al.*, 2014; Liew *et al.*, 2016, 2013; Sun *et al.*, 2019). Some of the acetogens able to convert carbon monoxide are *Moorella thermoacetica*, *M. thermoautotrophica*, *Eubacterium limonusum*, *Sporomusa ovata*, *Clostridium autoethanogenum*, *C. carboxidivorans*, *C. ragsdalei*, *C. ljungdahlii*, and *Acetobacterium woodii* (Bengelsdorf *et al.*, 2018; Müller, 2019; Yasin *et al.*, 2019a). Acetogenic carboxydotrophs can produce a range of relevant products (acetate, ethanol, butanol, 2,3-butanediol, etc) from syngas, and have been investigated for their application in commercial syngas fermentation (Köpke *et al.*, 2011). For example, LanzaTech® company is successfully implementing commercial syngas fermentation, using a highly efficient proprietary strain of *C. autoethanogenum* to produce ethanol (Abubackar *et al.*, 2019; De Tissera *et al.*, 2017; Molitor *et al.*, 2016).

Acetogens produce mainly acetate and ethanol from syngas fermentation. Odd-chain carboxylates, such as propionate, are not produced by these organisms alone (or have not been yet shown). Mixed cultures could have an advantage in this matter once some studies have already reported the biological production of propionate from C1 compounds (Arantes *et al.*, 2020; Sancho Navarro *et al.*, 2016).

Propionate has a high market demand with numerous industrial applications, *e.g.* as antimicrobial (Huang *et al.*, 2011; Rivero *et al.*, 2013) and anti-inflammatory (Loaiza-Ambuludi *et al.*, 2013; Turan-Zitouni *et al.*, 2015) agents, as food and feed preservatives (Del Nobile *et al.*, 2016; Sabra *et al.*, 2013), and as a building block to produce herbicides (Degenhardt *et al.*, 2011). Currently, at industrial level,

propionate production occurs via chemical synthesis from petroleum-based feedstock, being timely and appropriate to replace this process for a more sustainable one (Eş *et al.*, 2017), as for example through syngas fermentation.

One of the main obstacles of syngas fermentation is the gas-liquid mass transfer limitations (Bengelsdorf *et al.*, 2018; Bredwell *et al.*, 1999; Mohammadi *et al.*, 2011a; Stoll *et al.*, 2020; Yasin *et al.*, 2015). One way to improve mass transfer is the increase of partial headspace pressure of the gas, that will increase the gas solubility according to Henry's Law ($p_g = H_g \cdot C^*$, where (p_g) partial pressure; (H_g) Henry's law constant; (C) gas concentration in the liquid phase that is in equilibrium with the gas phase) (Bengelsdorf *et al.*, 2018; De Tissera *et al.*, 2017; Klasson *et al.*, 1991; Stoll *et al.*, 2020). Besides enhancing mass transfer and therefore increase productivities, pressure can influence the range of products obtained from syngas fermentations.

In the previous chapter (Chapter 4), a highly enriched culture able to produce propionate from syngas was obtained. The culture was mainly composed of an acetogenic bacteria, a new strain of *Acetobacterium wieringae,* able to efficiently convert syngas, that was posteriorly isolated, *A. wieringae* strain JM (Arantes *et al.*, 2020). Here in this chapter we intended to study the effect of moderate pressures in propionate production with this enriched culture and the effect in the microbial community when higher syngas pressures (higher CO partial pressures) were tested in bioreactors.

5.2. Material and Methods

5.2.1. Media and inoculum

The inoculum was a highly enriched syngas-degrading culture obtained in a previous work (Arantes *et al.*, 2020). Since then, the enrichment culture was kept active by successive transfers on syngas with 60 % CO (v/v), 30 % H_2 (v/v) and 10 % CO_2 (v/v) (over two years, transferred in average with monthly intervals). The basal medium for the cultivation of the microbial cultures contained the following (l^{-1}): $Na_2HPO_4 \cdot 2H_2O$, 0.53 g; KH_2PO_4, 0.41 g; NH_4Cl, 0.3 g; $CaCl_2 \cdot 2H_20$, 0.11 g; $MgCl_2 \cdot 6H_20$, 0.10 g; NaCl, 0.3 g; $NaHCO_3$, 4.0 g; and $Na_2S \cdot 9H_20$, 0.48 g [as well as acid and alkaline trace elements (each, 1 mL/liter) and vitamins (0.2 mL/liter) prepared as described by Stams *et al.* (1993)]. To prepare the inoculum for the reactor experiments, the culture was inoculated in bottles with headspace pressurized to 170 kPa with a syngas mixture with CO and CO_2 (60:40 %, v/v) or with CO, H_2 and CO_2 (60:30:10 %, v/v), depending on which of the reactors the inoculum will be used. The final pH of the media was 7.0–7.2. Before inoculation, medium was autoclaved and, after cooling, supplemented with vitamins and

reduced with 0.8 mM sodium sulfide ($Na_2S \times H_2O$; ×=9-12). Cultures were grown at optimal temperature, 30 °C with shaking at 100 rpm.

5.2.2. Experimental set-up of the pressurized axial agitation reactors

Syngas bioconversion was carried out in stainless steel cylindrical pressurized reactors with a total volume of 1 L. The reactors and pipes connections were all constructed in stainless steel and completely sealed to maintain strict anaerobic conditions. The reactors were installed in a shaker bath (that allows an axial agitation and heating). Gas sampling was performed through a stainless-steel chamber coupled to a pipe connected to the reactor's headspace, with a SampleLockTM syringe (Hamilton, Reno, NV, USA). Figure 5. 1 depicts a scheme of the reactor system used in all experiments. In a first approach, assays were performed with $CO/H_2/CO_2$ (60:30:10 %, v/v) to assess the effect of pressure and to infer which would be the best conditions for growth, conversion rates and productivities. On a second phase, comparison assays with different syngas mixtures (one set with $CO/H_2/CO_2$ (60:30:10 %, v/v) and other set without H_2 (only CO/CO_2 mixture (60:40 %, v/v)), at the optimal chosen conditions, were performed. These last experiments were done in triplicates. All syngas batch fermentations were carried out in the 1L-reactors with 400 mL of anaerobic medium. The reactors were sterilized *in situ* (100 °C, 20 min) and afterwards filled with autoclaved medium (310 mL). Salts and vitamins solution (20 mL) and bicarbonate and reducing solution (20 mL) were added from sterile stock solutions prepared as previously described. The reactors were inoculated with 40 mL of a well -grown enrichment culture (OD ≈ 0.4)). After inoculation, the reactors were sealed, and the headspace was first flushed with N_2 and then pressurized with syngas mixtures (100 kPa to 600 kPa) until the desired initial total pressure. Reactors were kept at 30 °C and 100 rpm, until the total consumption of CO or CO and H_2 in the gas phase.

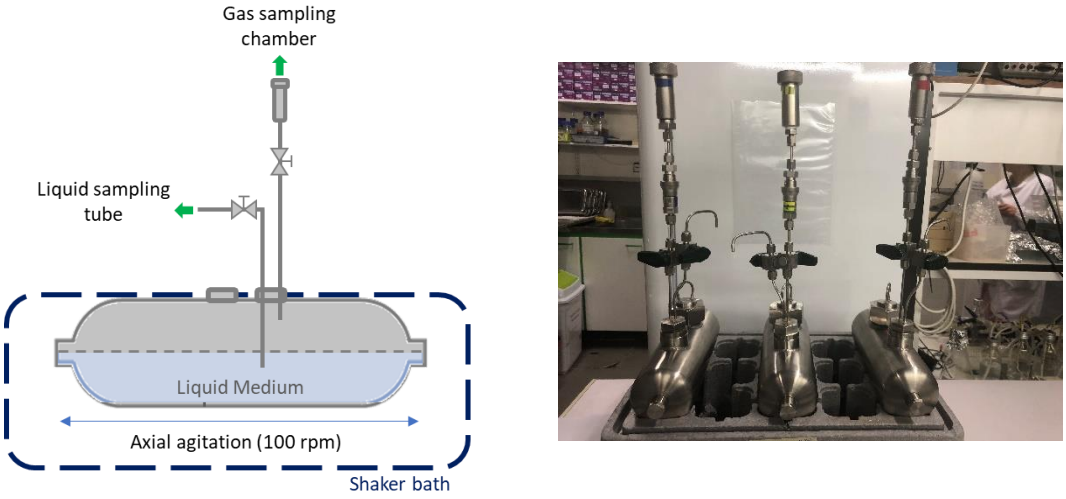


Figure 5. 1 - Schematic representation of the pressurized axial agitation reactor.

5.2.3. Analytical techniques

Growth was monitored by measuring the optical density at 600 nm on a UV–visible spectrophotometer (Hach-Lange, Loveland, CO, USA). Organic acids and alcohols were analysed via high pressure liquid chromatography (HPLC) equipped with a Phenomenex Rezex ROA – organic acid H+ (8 %) column (300 x 7.8 mm) and an ultraviolet/visible detector ((UV-2070 Plus, JASCO, Tokyo, Japan) at 210 nm). The column was operated at 60 °C, at a flow rate of 0.6 mL min^{-1}. The mobile phase used was a 2.5 mM H_2SO_4 solution. The column was operated at a temperature of 45 °C with a flow rate of 0.8 mL min^{-1}. Detection was done via a RI and UV detector. Gas analysis was done by gas chromatography (GC). Gas samples of 0.5 mL were taken using a 1 mL syringe and analysed in a Bruker Scion 456-GC (Bruker, Billerica, MA, USA) with a thermal conductivity detector and equipped with two columns: a BR-QPLOT column (30 m length, 0.53 mm internal diameter; film thickness, 20 µm) and a Molsieve packed column (13 9 80/100, 2 m length, 2.1 mm internal diameter). CO and H_2 were measured in a Molsieve column and argon was used as carrier gas at a flow rate of 30 mL min^{-1}; CO_2 was measured using a BR-QPLOT column (30 m length, 0.53 mm internal diameter; film thickness, 20 µm) and helium was used as carrier gas at a flow rate of 4 mL min^{-1}. Temperatures in the injector, column oven and detector were 100 °C (Molsieve) and 80 °C (BR-QPLOT), 35 °C and 130 °C respectively.

5.2.4. DNA Isolation and 16S rRNA gene sequencing

In the end of operation of each reactor, at 100 and 300 kPa, with both syngas mixtures ($CO/H_2/CO_2$ and CO/CO_2), sixty milliliters of the culture were used for DNA extraction using the FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH, USA), according to the manufacturer's instructions. Illumina MiSeq platform sequencing was performed at the research and testing laboratory - RTL Genomics (Lubbock, TX, USA). The MiSeq method used was the Illumina two-step using universal primers for bacteria and archaea, 515f and 806r developed by Caporaso *et al.* (2011). (Caporaso *et al.*, 2011). After sequencing, the data were processed using the data analysis pipeline from RTL, which consists in two major steps, the denoising and chimera detection step and the microbial diversity analysis step, as described in the company procedures. The 16S DNA gene sequences of the cultures were submitted to the European Nucleotide Database (ENA) at EMBL-EBI under the project accession number PRJEB42521.

5.3. Results

The syngas enriched culture was grown in batch pressurized reactors with syngas ($CO/H_2/CO_2$ (60:30:10 % v/v)) at increasing initial pressures from 100 kPa to 600 kPa (Figure 5. 2). Growth was observed for all the pressures tested, though the increase in pressure resulted in a delay in growth, with a lag phase of 4 and > 10 days at 400 kPa and at 600 kPa, respectively (Figure 5. 2C). At 100 kPa, CO and H_2 were consumed in 3 days, and with the raise of pressure, the degradation time was also higher, reaching 14 days at 600 kPa (Figure 5. 2 A and B). The pattern of CO and H_2 consumption was similar for each pressure, showing no preference for one or the other electron donor (Figure 5. 2 A and B), neither inhibition. The CO consumption rate had a similar behavior in all pressures until 400 kPa (between 1.2 and 1.5 mmol CO L^{-1} h^{-1}), suffering a decrease at 600 kPa (0.72mmol CO L^{-1} h^{-1}) (Table 5. 1). At higher pressures (300 kPa, 400 kPa and 600 kPa), there was a slight drop in pH from 7.5 to 6.5. The main product from syngas fermentation, for all the pressures tested, was acetate (Table 5. 1), and this acid was the only product formed at 100 kPa.

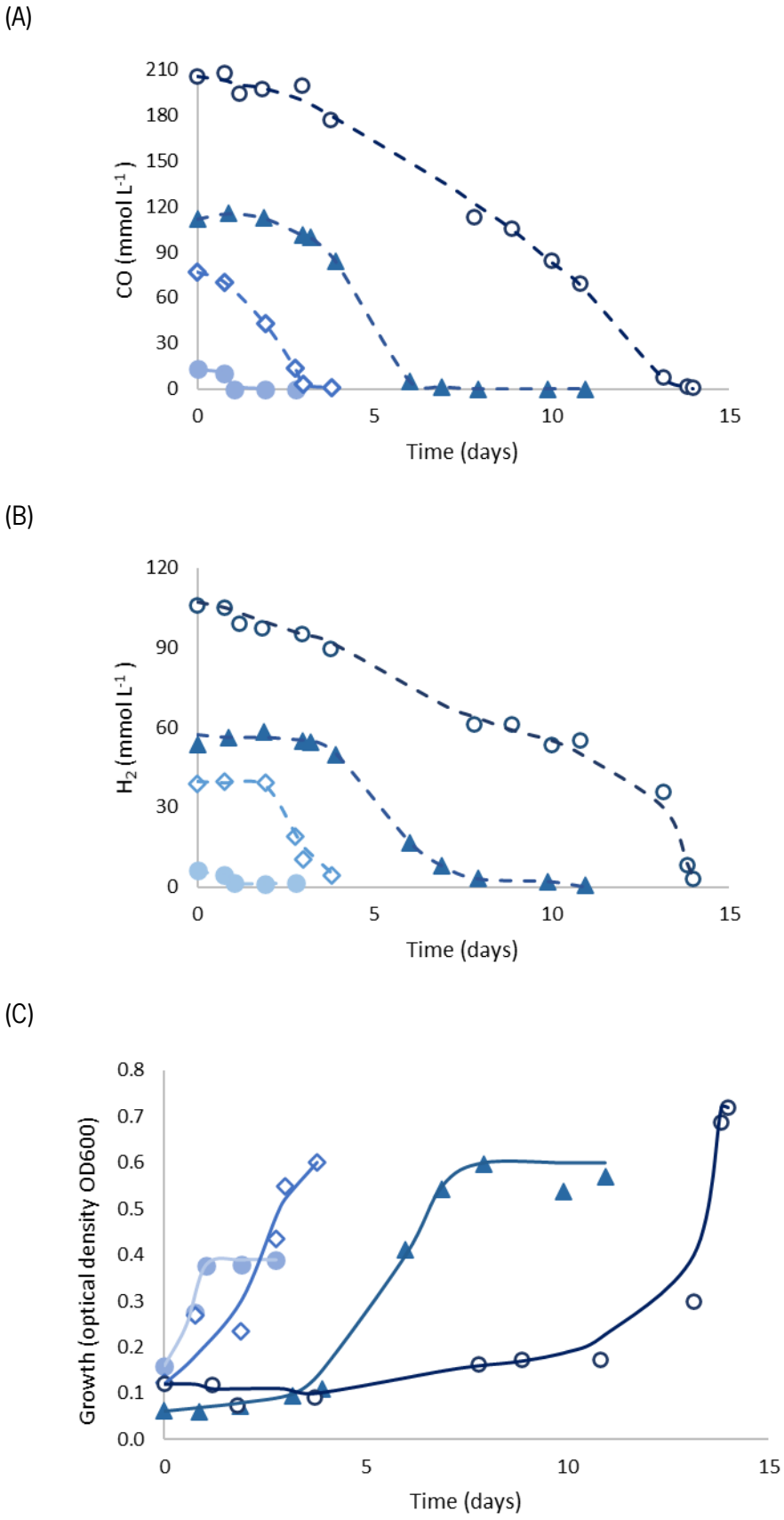


Figure 5. 2 - Syngas consumption (CO (A) and H_2 (B)) and growth (C) of the enriched culture at different syngas pressures: (●) 100 kPa; (◇) 300 kPa; (▲) 400 kPa; (○) 600 kPa.

Product titres increased with the increase in syngas initial pressure: acetate from 5.2 mM at 100 kPa to 10.5 mM at 600 kPa; propionate (not produced at 100 kPa) from 6.4 mM at 300 kPa to 10.4 mM at 600 kPa; and ethanol from 1.4 mM at 300 kPa to 2.9 mM at 600 kPa (Table 5. 1). Additionally, the increase of pressure also led to an increase of cell density from 0.065 g/L at 100 kPa to 0.120 g/L at 600 kPa.

Table 5. 1 - Soluble products and CO consumption rate at the different syngas pressures tested.

Syngas total pressure (kPa)	CO utilization (mmol L^{-1} day^{1}) (a)	Cell concentration (at the end) (g/L)	Acetate (mM)	Propionate (mM)	Ethanol (mM)
100	12.5	0.065	5.2	0	0
300	20.3	0.100	9.2	6.4	1.4
400	14.1	0.099	8.5	6.9	2.4
600	14.7	0.120	10.5	10.4	2.9

(a) Calculated with the total amount of CO consumed by the end of the incubation time and calculated with reference to the volume of liquid medium

Acetate productivity was higher at 100 kPa (0.2 mmol L^{-1} h^{-1}), decreasing slightly with the increase of pressure, though for the three other pressures tested (300, 400 and 600 kPa) acetate productivity was identical (0.1 mmol L^{-1} h^{-1}) (Table 5. 2). In the case of propionate and ethanol, the increase of pressure led to a small decrease in productivity (for propionate at 300 kPa a productivity of 0.12 mmol L^{-1} h^{-1} was obtained dropping to 0.08 mmol L^{-1} h^{-1} at 600 kPa; for ethanol at 300 kPa a productivity of 0.03 mmol L^{-1} h^{-1} was obtained dropping to 0.02 mmol L^{-1} h^{-1} at 600 kPa) (Table 5. 2). Apart from 100 kPa, where only acetate was formed, for all the other pressures tested, propionate yield was higher than acetate and ethanol. Therefore, the best condition for propionate production was 300 kPa initial syngas pressure where both productivity (0.12 mmol L^{-1} h^{-1}) and yield (45 %) were highest (Table 5. 2).

Table 5. 2 - Productivities and yields at the different syngas pressures tested.

Syngas total pressure (kPa)	Acetate productivity (mmol L^{-1} h^{-1})	Acetate yield (%)	Propionate productivity (mmol L^{-1} h^{-1})	Propionate yield (%)	Ethanol productivity (mmol L^{-1} h^{-1})	Ethanol yield (%)
100	0.20	100	-	-	-	-
300	0.11	37.5	0.12	45.0	0.03	8.3
400	0.11	23.1	0.07	32.4	0.03	9.7
600	0.10	15.1	0.08	26.0	0.02	6.2

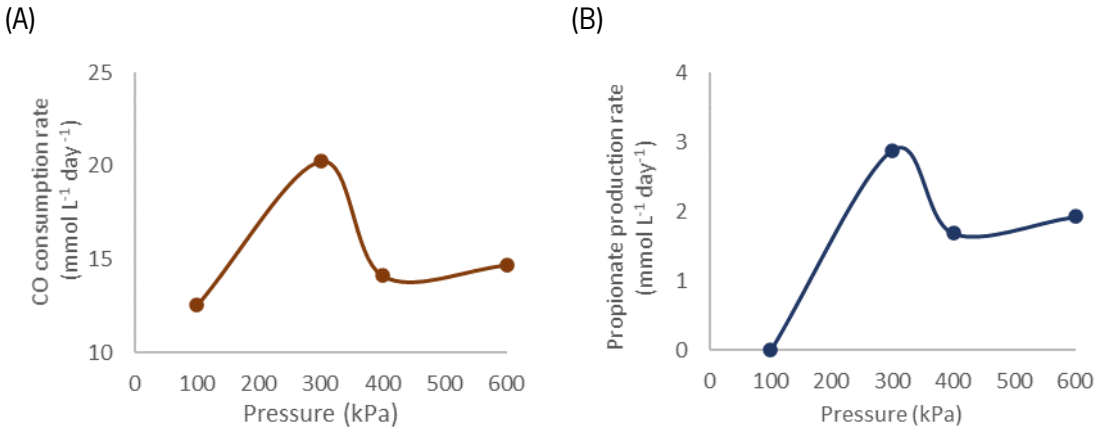


Figure 5. 3 - CO consumption (A) and propionate productivities (B) at different initial syngas pressures (initial screening).

Further studies were done, using the best compromise between CO consumption rate (Figure 5. 3) and better propionate productivity and yield (Table 5. 2), that happened at 300 kPa. Studies were done using two different syngas mixtures, with the same CO partial pressure but with and without H_2 (mixture 1: 60 % CO, 30 % H_2, 10 % CO_2 (v/v); mixture 2: 60% CO and 40 % CO_2, (v/v)) with initial total pressures of 100 kPa (as reference) and 300 kPa (Figure 5. 4). Growth with CO as the only carbon and energy source was studied to infer if the shift in the metabolic pathways would also happen in the absence of H_2 within the syngas mixture. For 100 kPa, complete conversion of CO (mixture 1) and CO/H_2 (mixture 2) was achieved in 2 days (Figure 5. 4). At 300 kPa, total conversion of substrates was slower, though it was faster with CO/H_2 (8 days) than with only CO (11 days) (Figure 5. 4).

The growth rate of the culture was the same for CO/H_2 and CO at each pressure (100 kPa or 300 kPa), suggesting a similar behavior with both substrates, though the increase of pressure induced a decrease in the culture growth rate (CO/H_2:0.094 day^{-1}, CO: 0.192 day^{-1} (100 kPa) and CO/H_2 0.070 day^{-1}, CO:

0.055 day^{-1} (300 kPa)) (Table 5. 3) Nevertheless, the increase in initial syngas pressure was beneficial in terms of carbon recovery, with an increase of 13 % for CO and 17 % for CO/H_2. As expected, the increase in initial syngas pressure continued to induce a shift in the metabolic pathways of the culture, deviating the metabolism for propionate production, reaching higher propionate yields (45.2 % for CO/H_2 and 23.1 % for CO) than acetate (38.6 % for CO/H_2 and 7.4 % for CO) (Table 5. 3). Better productivities were obtained in the presence of H_2 in the gas mixture instead when CO was used as only carbon and energy source.

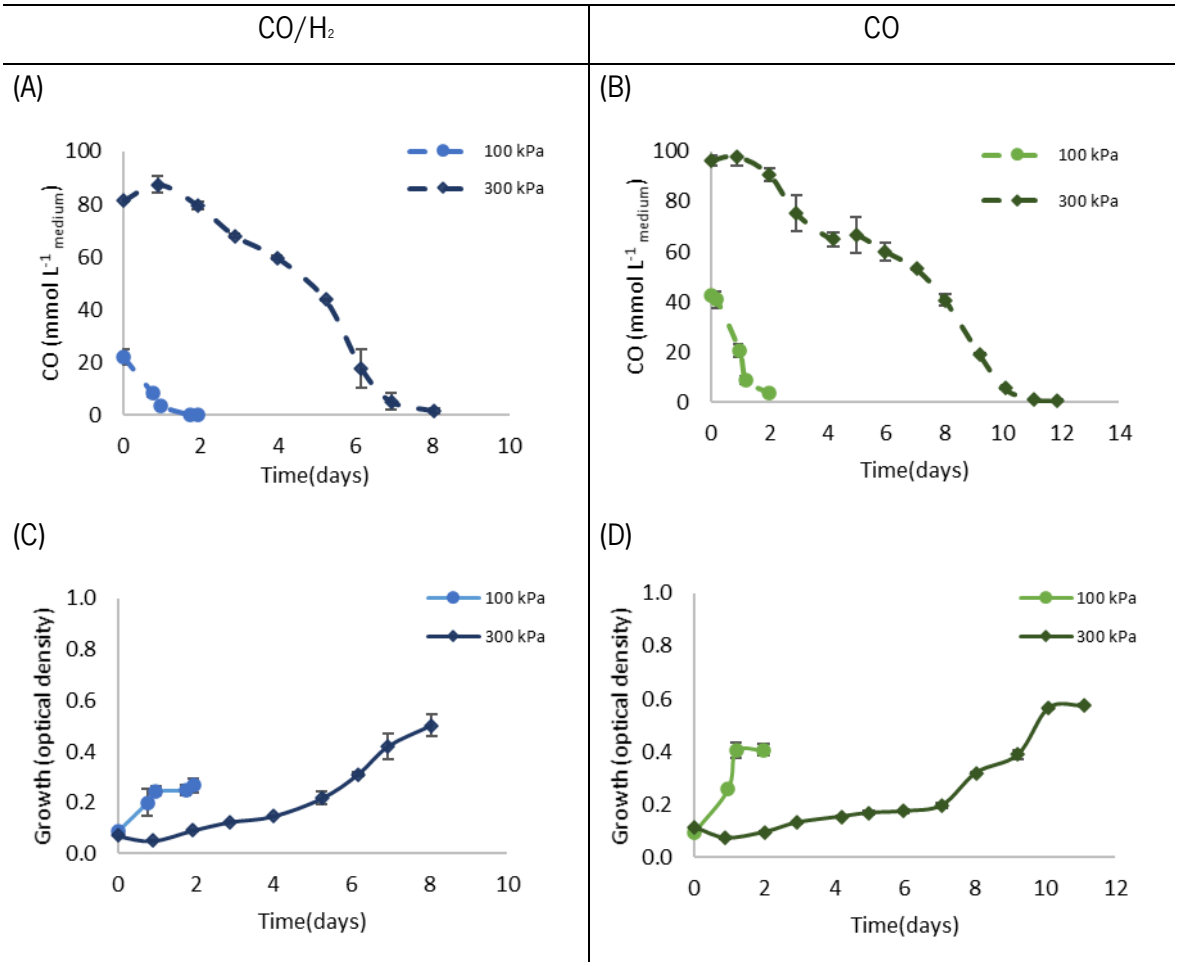


Figure 5. 4 - Syngas consumption by the enriched culture CO/H_2 (A) and CO (B). (C) and (D) show the growth (OD_{600}) of the enriched culture for both substrates at different pressures: (●,●) 100 kPa and (◆,◆) 300 kPa. Average values of triplicates. Bars represent standard deviation.

Table 5. 3 - Soluble products, growth rate and carbon recovery with $CO/H_2/CO_2$ and CO/CO_2 at 100 and 300 kPa initial pressures.

Pressure (kPa)		Growth rate (day^{-1})	CO utilization ($mmol\ L^{-1}\ day^{-1}$)	Carbon Recovery (%)	Acetate (mM)	Acetate yield (%)	Propionate (mM)	Propionate yield (%)
100	$CO/H_2/CO_2$	0.094 ±0.012	12.3 ± 2.2	83	6.75± 0.27	73.2	0	0
	CO/CO_2	0.192±0.024	21.7 ±1.0	72	8.37 ±0.12	57.6	0	0
300	$CO/H_2/CO_2$	0.070 ± 0.012	12.3 ± 2.9	100	10.66 ± 0.53	38.6	6.76 ± 0.47	45.2
	CO/CO_2	0.055 ±0.007	9.3 ±1.3	85	2.34 ± 0.09	7.4	3.27 ± 0.05	23.1

(a) Calculated with the total amount of CO consumed by the end of the incubation time and calculated with reference to the volume of liquid medium

Microbial community analysis was performed at the end of the operation of each reactor, for all the tested conditions, at 100 and 300 kPa initial pressures (Table 5. 4).

Table 5. 4 - Microbial community analysis of the enriched culture at the end of each pressured and condition tested.

	Closest relatives	**Amount (%)** [a]	**Query Coverage (%)**	**Identity (%)**
CO 100 kPa	***Acetobacterium wieringae*** (*Acetobacterium wieringae* partial 16S rRNA gene) [b]	**84**	100	100
	Anaerotignum neopropionicum (*Anaerotignum neopropionicum* strain DSM 3847 16S ribosomal RNA, partial sequence) [b]	11.5	100	100
	Anaerotignum lactatifermentans (*Anaerotignum lactatifermentans* strain ClaCZ174 16S ribosomal RNA gene, partial sequence) [b]	8	100	96.7
CO 300 kPa	***Acetobacterium wieringae*** (*Acetobacterium wieringae* partial 16S rRNA gene) [b]	**88**	100	100
	Anaerotignum neopropionicum (*Anaerotignum neopropionicum* strain DSM 3847 16S ribosomal RNA, partial sequence) [b]	11	100	100
CO/H_2 100 kPa	***Acetobacterium wieringae*** (*Acetobacterium wieringae* partial 16S rRNA gene) [b]	**100**	100	100
CO/H_2 300 kPa	***Acetobacterium wieringae*** (*Acetobacterium wieringae* partial 16S rRNA gene) [b]	**81.5**	100	100
	Anaerotignum neopropionicum (*Anaerotignum neopropionicum* strain DSM 3847 16S ribosomal RNA, partial sequence) [b]	16	100	100
	Anaerotignum lactatifermentans (*Anaerotignum lactatifermentans* strain ClaCZ174 16S ribosomal RNA gene, partial sequence) [b]	2	100	96.7

(a) Percentage calculated based on the number of sequence counts obtained for the total community by Illumina sequencing, 27817.

(b) Results of sequence alignment by using BLAST towards the NCBI nucleotide database of partial 16S DNA gene sequences (approximately 291 bp; results obtained from amplicon Illumina sequencing).

The inoculum culture was highly enriched in *A. wieringae* (87 % bacterial community). *A. wieringae* was predominant also after reactor incubations, for all the initial syngas pressures tested. However, for higher pressures, relative abundance of sequences of *Anaerotignum propionicum* increased (Table 5. 4).

This is a propionate producing bacterium, and at these higher partial pressures we did observe propionate production. When grown with only CO it was not possible to observe a notorious change in the community. On the other hand, when grown in the presence of H_2 (CO/H_2), at 100 kPa we had a community only composed of *A. wieringae* strain JM, thought when the system pressure was increased to 300 kPa, where we also observed a higher propionate production, the presence of *A. neopropionicum* related organisms increased.

5.4. Discussion

In autotrophic fermentations the low solubility of the gases (*e.g.* CO and H_2) usually results in low productivities. Reactor operation at higher headspace gas pressure favors the solubility of gaseous compounds (Van Hecke *et al.*, 2019). With this work it was intended to evaluate the effect of pressure, increasing the gases solubility. Increasing the pressure represents also increasing the concentration of the gases, and therefore this work also allowed us to test the substrate uptake rate at different concentrations.

In Chapter 4, an enriched culture was grown with syngas and was able to produce 17 mmol L^{-1} of acetate, 16 mmol L^{-1} of methane (L refers to liquid medium) and 2.4 mmol L^{-1} of propionate. The culture was mainly composed of *A. wieringae* strain JM and *Methanospirillum hungatei*, though small amounts of a propionigenic bacteria were also found – *Anaerotignum neopropionicum* (Arantes *et al.*, 2020). With continued transfers on syngas (for 1 year), methanogens were eliminated from the culture. The culture was capable of handling total pressures up to 600 kPa (CO partial pressure (pCO) = 360 kPa; H_2 partial pressure (pH_2) = 180 kPa) without being completely inhibited by syngas components (Table 5. 2). In syngas fermentation, CO is considered to be the most probable source of inhibition, however CO_2 and H_2 can likely cause inhibition of cell growth as well (Mohammadi *et al.*, 2014). CO_2 can cause a drop of pH due to the formation of carbonic acid (Amos, 2004) and increase of H_2 partial pressure can cause an alteration of the electron flow in the microorganisms biological pathway, inhibiting, *e.g.*, acetogenesis (Nie *et al.*, 2008). In this case a slight drop in pH from 7.5 to 6.5 was observed at higher pressures (300 kPa, 400 kPa and 600 kPa). Some studies have shown growth inhibition with increasing CO partial pressures: *Clostridium ljungdahlii* growth has shown inhibition with CO partial

pressures above 80 kPa (Mohammadi *et al.*, 2014); and a co-culture of *Peptostreptococcus productus* and *Methanothrix* sp. showed some inhibition at CO partial pressures of 101.6 kPa, ending up by having no substrate consumption at 196.8 kPa of CO partial pressure (Ko *et al.*, 1989). Contrarily, in this enriched culture, cell concentration increased with the increase of pressure, as expected in theory once there is more substrate (Table 5. 1). Results in literature are somehow contradictory. Husrt and Lewis (2010) reported an increase in cell concentration of *C. carboxidivorans* with the increase of pressure (up to a maximum pCO of 200 kPa), while Oswald *et al.* showed a decrease in biomass of *C. ljungdhalii* with increasing H_2/CO_2 pressures (no significant growth at 400 kPa (total pressure)). These studies indicate that growth inhibition at elevated pressures might not be linked to the inhibitory effect of CO alone. However, considering our results, this effect might be different for each microorganism, suggesting that the carboxydotrophic microorganism present in our culture should be well adapted to syngas components. This can also be observed in the average CO utilization rate, where the increase of pressure showed to have a beneficial effect, achieving the best result at 300 kPa (Table 5. 1). Nevertheless, the increase of pressure also led to the appearance a lag phase, at 400 kPa and 600 kPa, that can be seen as an adaptation time of the culture to pressure and/or concentration of substrate (Figure 5. 2 C), which is something that was also reported by other authors (Kantzow and Weuster-Botz, 2016; Vega *et al.*, 1989). It is noteworthy that the increase in the initial syngas pressures also led to a shift in the product spectrum from only acetate to additionally propionate and ethanol (Table 5. 1), being important to stand out that those two products were not produced at 100 kPa. Other studies have shown the same effect: growth of *C. ljungdhalii* at increasing H_2 pressures shown a shift in the product spectrum towards formic acid (Oswald *et al.*, 2018); increase of H_2/CO_2 pressures in *Acetobacterium woodii* showed a reduction of acetate production while formate production increased (Kantzow and Weuster-Botz, 2016); in a mixed anerobic sludge fed with CO and ethanol, carboxylic acids formation was detected for higher CO partial pressures (Esquivel-Elizondo *et al.*, 2017). Production of propionate from syngas is uncommon, though it has been reported in the growth of mixed anaerobic sludge grown with syngas and ethanol (Esquivel-Elizondo *et al.*, 2017) and also in our previous work with this syngas enriched culture (Arantes *et al.*, 2020). According to Esquivel-Elizondo *et al.*, a maximum of 12 mmol L^{-1} of propionate, among other acids, were produced when using CO and ethanol as substrates (Esquivel-Elizondo *et al.*, 2017). In this study, our highly enriched culture was able to produce a maximum of 10 mmol L^{-1} of propionate solely from syngas. In our previous work it was hypothesized that the *Acetobacterium* present in the enriched culture was consuming the CO to produce acetate and ethanol, and that the ethanol was further used by *Anaerotignum neopropionicum*

species to produce propionate (Arantes *et al.*, 2020), and these new results still endorse this hypothesis. To produce ethanol from acetate four electrons are required. Therefore it is feasible to say that as growth slows down, and not as much ATP is needed, the excess electrons, that are no longer required for growth, might be channeled to convert acetate to acetaldehyde and posteriorly to ethanol (Hurst and Lewis, 2010). The increase of pressure has also shown an increase in propionate titres (Table 5. 1), up to 10.4 mmol L^{-1} showing additionally an increase when comparing to the results in Chapter 4, where only 2.4 mmol L^{-1} of propionate were produced. Furthermore, when pressure was raised (≥ 300 kPa) higher propionate yields than acetate, were always achieved (Table 5. 2). In this way, with our results, it is possible to say that the increase of pressure resulted in higher cell growth and higher propionate yields.

When comparing growth with and without H_2 in the syngas mixture, the first highlight is that conversion of substrates at 300 kPa was 1.5 times faster with H_2 than without (Figure 5. 4 A and B). In addition, propionate productivities were also higher in the presence of H_2. Indeed, when H_2 is also supplied as electron donor, more acetate can be produced, than from CO alone, according to equations 1 and 2 (Eq. 1 -production of acetate from CO and Eq. 2 - production of acetate from CO_2/H_2): The increased propionate productivities, in the presence of H_2, might be due to a similar behavior as it happens for acetate.

$$4\,CO + 2\,H_2O \rightarrow CH_3COOH + 2\,CO_2 \quad \text{(Eq. 1)}$$

$$2\,CO_2 + 4\,H_2 \rightarrow CH_3COOH + 2\,H_2O \quad \text{(Eq. 2)}$$

On the other hand, higher growth rates were observed with CO as substrate (Figure 5. 4 C and D). This could be due to the H_2 inhibitory effect mentioned above and also observed in earlier studies (Oswald *et al.*, 2018).

The shift of the product spectrum was again observed, once at 100 kPa only acetate was produced, for both gas mixtures, while at 300 kPa propionate was also produced, likely because a higher presence of *A. neopropionicum* was noticed. *A. neopropionicum* is known for its capability to convert ethanol to propionate (Tholozan *et al.*, 1992; Ueki *et al.*, 2017), which supports a synergistic interaction between *A. wieringae* strain JM and *A. neopropionicum* in this enriched culture: strain JM should be consuming CO to produce acetate and ethanol, while ethanol would be further used by *A. neopropionicum* to produce propionate, as suggested before. Since with 100 kPa *A. neopropionicum* was barely present (Table 5. 4), we suggest that the increase of pressure induced a shift of the metabolism towards the production of ethanol (that despite of not being detectable in HPLC analysis, was most likely being

produced), which makes *A. neopropionicum* thrive at higher pressures. The fact that propionate was produced with or without the presence of H_2 might indicate that propionate production should be related with CO consumption. Though ethanol production was no longer observed, the hypothesis mentioned above still stands, but probably ethanol conversion to acetate was faster in this case, thus, it was not detectable. The increase of pressure was also shown to be beneficial in what concerns carbon recovery (Table 5. 3), for both substrates tested.

5.5. Conclusion

In this work, the increase of syngas pressure has shown to be beneficial in the growth of a highly enriched syngas-degrading culture, mainly composed of *A. wieringae* strain JM and *A. neopropionicium*. The culture was able to handle pressures up to 600 kPa (pCO = 360 kPa; pH_2 =180 kPa) without being inhibited, although suffering from a lag phase of about 4 days for 400 kPa and 10 days for 600 kPa. No substrate inhibition was observed.

The increase of pressure led to higher titres up to 10.5 mM of acetate and 10.4 mM of propionate and induced a shift in the metabolic pathway towards propionate production leading to propionate yields of 26 %, well above the ones obtained for acetate (15 %). When compared to our previous work, where the enriched culture grew in serum bottles at 170 kPa, propionate titre increased 23 %. Additionally, the culture was able to grow in the presence of only CO/CO_2, generally presenting the same behavior as with $CO/H_2/CO_2$. However, at higher pressures, acetate and propionate titres were 79 % and 52 % higher, respectively, in the presence of H_2. Lastly, the microbial community was not severely affected by the increase of pressure, once *A. wieringae* strain JM was able to prevail in percentages higher than 80 %, though *A. neopropionicum* thrived at increasing syngas pressures (slightly increasing their presence), most probably due to the shift of the metabolism of strain JM towards ethanol production. In this way, with this work it is possible to state that the increase of pressure (up to an optimal condition) can significantly improve syngas fermentation, allowing to broader the product spectrum towards more valuable compounds. Therefore, it is highly relevant to continue to investigate the effect of increased pressures in gas fermentation processes.

Chapter 6.

EFFECT OF MODERATE PRESSURES ON SYNGAS BIOMETHANATION BY ANAEROBIC MIXED CULTURES

Tons of carbon monoxide are released every day in several industrial processes including metal manufacturing, electricity supply, mining, or oil and gas extraction. Syngas fermentation is attracting the attention of biotechnologists as a way of recycling these CO-rich streams and produce renewable fuels and chemicals. Yet, one of the most critical bottlenecks in syngas fermentation are the gas-liquid mass transfer limitations of syngas components. Several efforts were made to overcome this problem, *e.g.* testing alternative reactor configurations with innovative gas dispersion technologies. In this chapter, two types of pressurized reactor were tested with the goal to enhance gas-liquid mass transfer: an axial agitation reactor (AAR) and a gas-lift reactor (GLR). Syngas (60 % CO, 30 % H_2,10 % CO_2) was supplied to reactors inoculated with granular sludge at three different initial pressures, 100 kPa (pCO ≈ 60 kPa), 300 kPa (pCO ≈ 180 kPa) and 500 kPa (pCO ≈ 300 kPa). The main fermentation product in most of the runs and in the two types of reactors tested was methane, though at pCO ≥ 180 kPa, small amounts of propionate, acetate and n-butyrate were also produced. At an initial pCO of 180 kPa methane yields were between 65 % and 90 % in both systems. Even at the highest pCO tested (500 kPa), methanogenesis was not completely inhibited, with methane yields of 61 % (GLR) and 92 % (AAR). Microbial analysis also showed that methanogens were present in all the biomass samples.

6.1. INTRODUCTION

Our society has been facing unprecedented levels of concern in terms of climate change and threats on sustainability. The European Union is committed to an ambitious climate policy. Under the Green Deal it aims to become the first continent that removes as many CO_2 emissions as it produces by 2050. It is a new growth strategy that aims to transform the EU into a fair and prosperous society, with a modern, resource-efficient and competitive economy where there are no net emissions of greenhouse gases in 2050 and where economic growth is decoupled from resource use (European Commission, 2019). To meet these ambitious goals, there is a growing need for new technologies that aim the reduction of greenhouse gases and the production of new renewable fuels.

Gasification of carbonaceous materials generates a stable gaseous mixture - synthesis gas (or syngas) - mainly composed by carbon monoxide (CO), hydrogen (H_2) and carbon dioxide (CO_2). Although syngas has been traditionally produced from coal, renewable carbonaceous feedstocks (*e.g.* wood, agricultural and forestry residues, dedicated energy crop and municipal solid waste) can also be gasified (D. Ramachandriya *et al.*, 2016; Mohammadi *et al.*, 2011b). Additionally, steelmaking industry produces worldwide around 1.4×10^9 megatons of CO that is combusted to CO_2 before emission to the atmosphere (Bengelsdorf *et al.*, 2018). Both, syngas especially waste- and biomass-derived syngas and CO-rich flue gases are an important, not yet sufficiently exploited substrate for gas fermentation bioprocesses (Asimakopoulos *et al.*, 2019). Syngas biocatalysis is a developing field, and presently only one functioning company, LanzaTech®, recycles CO-rich waste streams at full commercial scale to produce ethanol. Despite the several advantages of the biological route compared to chemical catalysis, *e.g.* the independence of a specific H_2:CO ratio and the lower requirements for gas pre-treatment steps (Liew *et al.*, 2016; Munasinghe and Khanal, 2011; Yasin *et al.*, 2015), this process still has some limitations. Some of the most critical limitations are the low solubility of CO and H_2 and the low gas-liquid mass transfer rates of syngas components leading to reduced availability of the gases as substrate to the microorganisms and low conversion rates (Bengelsdorf *et al.*, 2018; Bredwell *et al.*, 1999; Mohammadi *et al.*, 2011b; Verma *et al.*, 2016; Yasin *et al.*, 2015). Most of the syngas fermentation studies so far were done in continuous stirred tank reactor (CSTR) (Asimakopoulos *et al.*, 2018; Bengelsdorf *et al.*, 2018). The typical approach to enhance gas-to-liquid mass transfer in CSTRs is increasing the stirring rate or the gas flow rate, resulting in an increment of the mass transfer coefficient (k_La). The high-energy consumption, resulting from reactor stirring, limits the economic feasibility of the process, and high gas flow rates reduce the syngas conversion efficiency (Asimakopoulos *et al.*, 2018; Bredwell *et al.*, 1999; Ungerman and Heindel, 2007; Yasin *et al.*, 2015).

In addition, high stirring rates could induce shear stress to microorganisms, microbial biofilms or granules. Besides CSTR, alternative reactor configurations, designed for improved gas-liquid mass transfer, have been tested for syngas fermentation, such as: column diffuser (Munasinghe and Khanal, 2010b), gas-lift reactor (Guiot *et al.*, 2011b), monolithic biofilm reactor (Shen *et al.*, 2014a), hollow fiber membrane reactor (Shen *et al.*, 2014b; Yasin *et al.*, 2014) and trickle bed reactor (Devarapalli *et al.*, 2016). Another strategy to enhance gas-liquid transfer is by increasing the driving force by increasing the partial pressure of syngas components (CO and/or H_2), which will increase the gas solubility according to Henry´s law ($p_g = H_g \cdot C^*$) ((p_g) partial pressure; (H_g) Henry's law constant; (C) gas concentration in the liquid phase that is in equilibrium with the gas phase) (Bengelsdorf *et al.*, 2018; De Tissera *et al.*, 2017; Klasson *et al.*, 1991). The partial pressure and the k_La are related according to the equation: $\frac{dN_S^G}{V_L dt} = \frac{K_L a}{H_g}(P_S^G - P_S^L)$ that defines the gas flux from the gas phase to the liquid phase (N_S^G) number of moles of substrate transferred from the gas phase; (V_L) volume of the liquid phase; (t) is time; (K_L), overall mass transfer coefficient; (a) gas-liquid interfacial area per unit volume; (P_S^G) partial pressure of the substrate in the bulk gas phase; and (P_S^L) partial pressure of the substrate in the liquid phase (Klasson *et al.*, 1991).

The typical syngas fermentation products are acetate and ethanol, once acetogenic bacteria are the predominant microbial group capable of syngas conversion (Bengelsdorf *et al.*, 2018; De Tissera *et al.*, 2017; Grimalt-Alemany *et al.*, 2018). However, other products can be obtained, such as methane, formate, butyrate, or higher alcohols, i.e. butanol and 2,3-butanediol (Diender *et al.*, 2015; Grimalt-Alemany *et al.*, 2018; Köpke *et al.*, 2010; Munasinghe and Khanal, 2011). For instance, n-butyrate and propionate have great potential to provide significant additional revenue streams, once n-butyrate could be converted into other industrial chemicals or fuels and propionate is involved in a wide range of industrial applications (Bertleff, 2000; Marshall *et al.*, 2013; Phillips *et al.*, 2015; Tirado-Acevedo *et al.*, 2010). Concurrently, biomethane is an interesting renewable fuel that can be directly incorporated in the existing natural gas grid (Asimakopoulos *et al.*, 2019; Grimalt-Alemany *et al.*, 2018). Syngas fermentation to biomethane is performed by a microbial community, which avoid the need of sterilization costs (Asimakopoulos *et al.*, 2019). A complex microbial community may increase adaptation capacity and resilience of the system and may result in the formation of products that cannot be produced by pure cultures (Diender *et al.*, 2016b).

This study focusses on the effect of total initial syngas pressure (100 kPa, 300 kPa and 500 kPa) on CO and H_2 consumption, and biomethane and liquid metabolites production by anaerobic mixed cultures (granular sludge) in two types of pressurized reactors: an axial agitation reactor (AAR) and a gas-lift reactor (GLR). Additionally, the effect of syngas pressure on the microbial community composition was assessed.

6.2. MATERIALS AND METHODS

6.2.1. Inoculum and substrate

Anaerobic granular sludge (0.084 g VS g^{-1} biomass ± 0.001 g VS g^{-1} biomass), collected from the wastewater treatment plant of a brewery industry, was used as inoculum for AAR and GLR. Synthetic syngas (60 % CO, 30 % H_2 and 10 % CO_2, v/v) was fed to the reactors as sole carbon and energy source.

6.2.2. Anaerobic medium composition

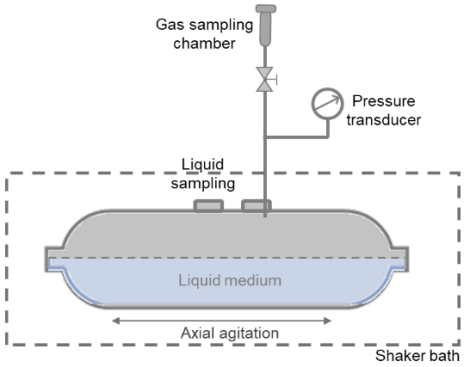
A phosphate-buffered mineral salt medium (20 mM, pH 7.0), containing (per liter) NaH_2PO_4 1.02 g; Na_2HPO_4 1.63 g and resazurin 1 g, were used in all experiments. Salt solution composed by (per liter) $CaCl_2 \cdot 2H_20$ 0.11 g; $MgCl_2 \cdot 6H_20$ 0.10 g; NH_4Cl 0.3 g; NaCl 0.3 g, together with 0.2 mL of vitamin stock solution were added to the medium. Vitamin solution was prepared as described by Stams *et al.* (1993). Before inoculation, medium was reduced with sodium sulfide 1 mM (final concentration).

6.2.3. Experimental set-up of the pressurized bioreactors

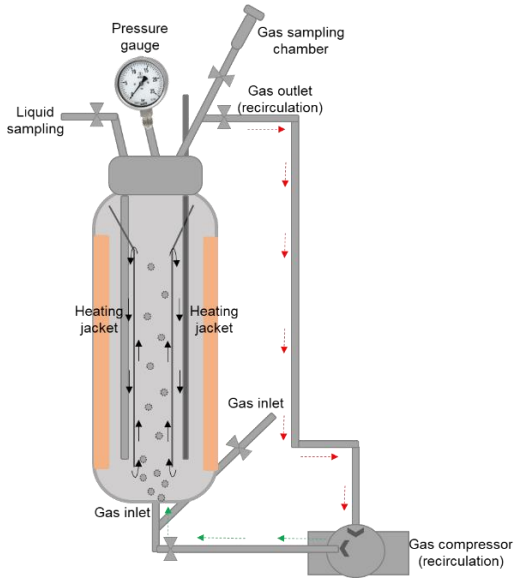
Syngas bioconversion was carried out in two different types of reactors: a cylindrical pressurized reactor (named axial agitation reactor - AAR) with a total volume of 1 L (Figure 6. 1 A) and a gas-lift pressurized reactor (GLR) with a total volume of 3.5 L (Figure 6. 1B). Reactors, pipes and connections were all constructed in stainless steel and completely sealed to maintain strict anaerobic conditions. AAR was installed in a shaker bath (for axial agitation and heating) and was equipped with a pressure transducer (HD 9220, Delta OHM, Caselle di Selvazzano, Italy) to monitor internal pressure. As for the GLR, pressure was monitored with a pressure gauge and temperature was set using a built-in heating jacket and controlled with a temperature probe located inside the reactor; initially the gas was sparged at the bottom of the reactor and then was continuously recirculated and sparged, using a gas compressor system.

For both reactors, gas sampling was performed through a stainless-steel chamber, coupled to a pipe connected to the reactor's headspace, with a SampleLock™ syringe (Hamilton, Reno, NV).

(A)



(B)



(A1)

(B1)

Figure 6. 1 - Schematic representation of the pressurized reactors used on syngas bioconversion studies: (A) Axial agitation reactor (AAR) and (B) Gas-lift reactor (GLR). A1 and B1 are live pictures of the respective reactors.

6.2.4. Batch syngas bioconversion in the pressurized bioreactors

Batch experiments were carried out in the reactors with 400 mL of anaerobic medium and 600 mL of headspace, for the AAR, and with 2.6 L of anaerobic medium and 900 mL of headspace, for the gas-lift reactor. Reactors were inoculated with anaerobic granular sludge (4 g·VS), so that the initial mmol CO/ initial gVS ratio was from ≈ 5 (100 kPa) to ≈ 21 (500 kPa) and was comparable for both systems. After inoculation, the reactors were sealed, and the headspace was first flushed with N_2. The inoculum was

then acclimatized at 37 °C (in the reactor vessel), for approximately 24 hours, to consume the residual substrate. Afterwards, the headspace was flushed with N_2 to remove any gaseous products and, then, pressurized with syngas until the desired initial total pressure (100 kPa, 300 kPa or 500 kPa). Reactors were operated at 37 °C and at 100 rpm (in the case of the AAR), until the total consumption of syngas components (CO and H_2) in the gas phase.

6.2.5. RNA isolation and 16S rRNA gene sequencing

Sludge samples (biomass at the end of reactor operation for each condition tested) were collected, preserved with RNA*later* (Sigma-Aldrich, St. Louis, MO, USA) and stored at -20 °C. Total RNA was extracted from approximately 500 µL of sample using a FastRNA Pro™ Soil-Direct Kit (MP Biomedicals, Solon, OH, USA), in accordance with the manufacturer's instructions. To remove genomic DNA, samples were submitted to a treatment with DNase I (Thermo Fisher Scientific, Waltham, MA, USA). The treated RNA samples were used in a reverse transcription reaction for cDNA synthesis, by using a reverse transcriptase SuperScript III (Thermo Fisher Scientific, Waltham, MA, USA) with the primer Uni1492-r (5'-CGG CTA CCT TGT TAC GAC-3') (Nübel *et al.* 1996). The resulting cDNA samples, representative of the inoculum and the final biomass of each condition tested on the reactor, were sent to sequence using an Illumina MiSeq platform (Research and Testing Laboratory-RTL, TX, USA) following the procedure described elsewhere (Salvador *et al.*, 2019). The Illumina MiSeq method was performed using a two-step method with universal set of primers for Bacteria and Archaea, 515f and 806r (Caporaso *et al.* 2011). The data were later processed using the data analysis pipeline from RTL, which consists in the denoising and chimera detection step and the microbial diversity analysis step, as described by the company procedures.

6.2.6. Nucleotide sequence accession numbers

The nucleotide sequences obtained by Illumina MiSeq were deposited in the European Nucleotide Archive (ENA) under the accession numbers ERS1465383 to ERS1465389 (for the AAR) and ERS3780203 to ERS3780205 (for the GLR). Sequences can be viewed at URL http://www.ebi.ac.uk/ena/data/view/PRJEB18485 (AAR) and /PRJEB34608 (GLR). Similarity searches were performed using the NCBI blast search program within the GenBank database (http://www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1990).

6.2.7. Analytical methods

Gaseous compounds (CH_4, CO and H_2) were analyzed by gas chromatography (GC) (Bruker Scion 456-GC) (Billerica, MA, USA) equipped with a thermal conductivity detector (TCD) and a Molsieve packed column (13 × 80/100, 2 m length, 2.1 mm internal diameter). Argon was used as carrier gas at a flow rate of 30 mL·min^{-1}. The temperatures of column, injector and detector were set at 35 °C, 100 °C and 130 °C, respectively. Volatile fatty acids (VFA's) and alcohols were quantified in culture supernatants by high-performance liquid chromatography (HPLC) (Jasco, Tokyo, Japan) equipped with a UV detector (210 nm), a RI detector and a Phenomenex-Rezex ROA-organic acid H+ (8 %) column (300 × 7.8 mm) at 60 °C. Sulphuric acid (0.005 N) was used as mobile phase at a flow rate of 0.6 mL·min^{-1}. Samples were lyophilized to perform quantification of polyhydroxyalkanoates (PHA) according to Duarte, 2018. MCFA and LCFA (C8 up to C18) were measured by gas chromatography (GC Varian 3800, Agilent, Santa Clara, CA, USA) after esterification with propanol and extraction with dichloromethane, as described by Neves *et al.* 2009. Mass spectrometry data were generated by the Mass Spectrometry Unit (UniMS), ITQB/iBET, Oeiras, Portugal.

6.3. Results

Figure 6. 2 shows the consumption of CO (1) and H_2 (2). For all the initial syngas pressures, GLR performance was better, in the sense that all the syngas constituents were converted faster than in the AAR. Increase of initial syngas pressure resulted in longer conversion times in the AAR, but in the GLR the pressure effect was only noted for the highest pressure tested (500 kPa, corresponding to a pCO of 300 kPa) (Figure 6. 2 C).

Additionally, it is possible to observe that the consumption pattern achieved a plateau, after approximately 30 % of CO (Figure 6. 2 C(1)) and H_2 (Figure 6. 2 C(2)) consumption, for the AAR system. This lag phase persisted around 4 days, and only after, the consumption of substrates seems to resume the regular rate.

H_2 consumption rate was generally slower than CO consumption rate for all the conditions tested, except at 100 kPa for the GLR, where the H_2 consumption rate was 2-fold higher than the CO consumption rate (Table 6. 1). The CO consumption rate increased with the initial CO pressure up to 300 kPa. Overall, CO and H_2 consumption rates in GLR are 2 to 8-fold higher than in the AAR. The main product of syngas fermentation by the granular sludge, in both reactors and all the conditions tested, was methane. For the AAR, a 100 % CH_4 yield was obtained for all pressures dropping slightly to 92 % at

500 kPa, and for the GLR, at 100 kPa a 100 % CH_4 yield was obtained, though with higher pressures (300 kPa and 500 kPa), CH_4 yields dropped to 78 % and 75 % respectively. (Table 6. 1). The CH_4 production rate in the AAR, was similar for all the conditions tested (between 0.08 mmol CH_4 h^{-1} and 0.11 mmol CH_4 h^{-1}), though in the GLR the CH_4 production rate decreased from 0.29 mmol CH_4 h^{-1} (100kPa) to 0.17 mmol CH_4 h^{-1} (500kPa).

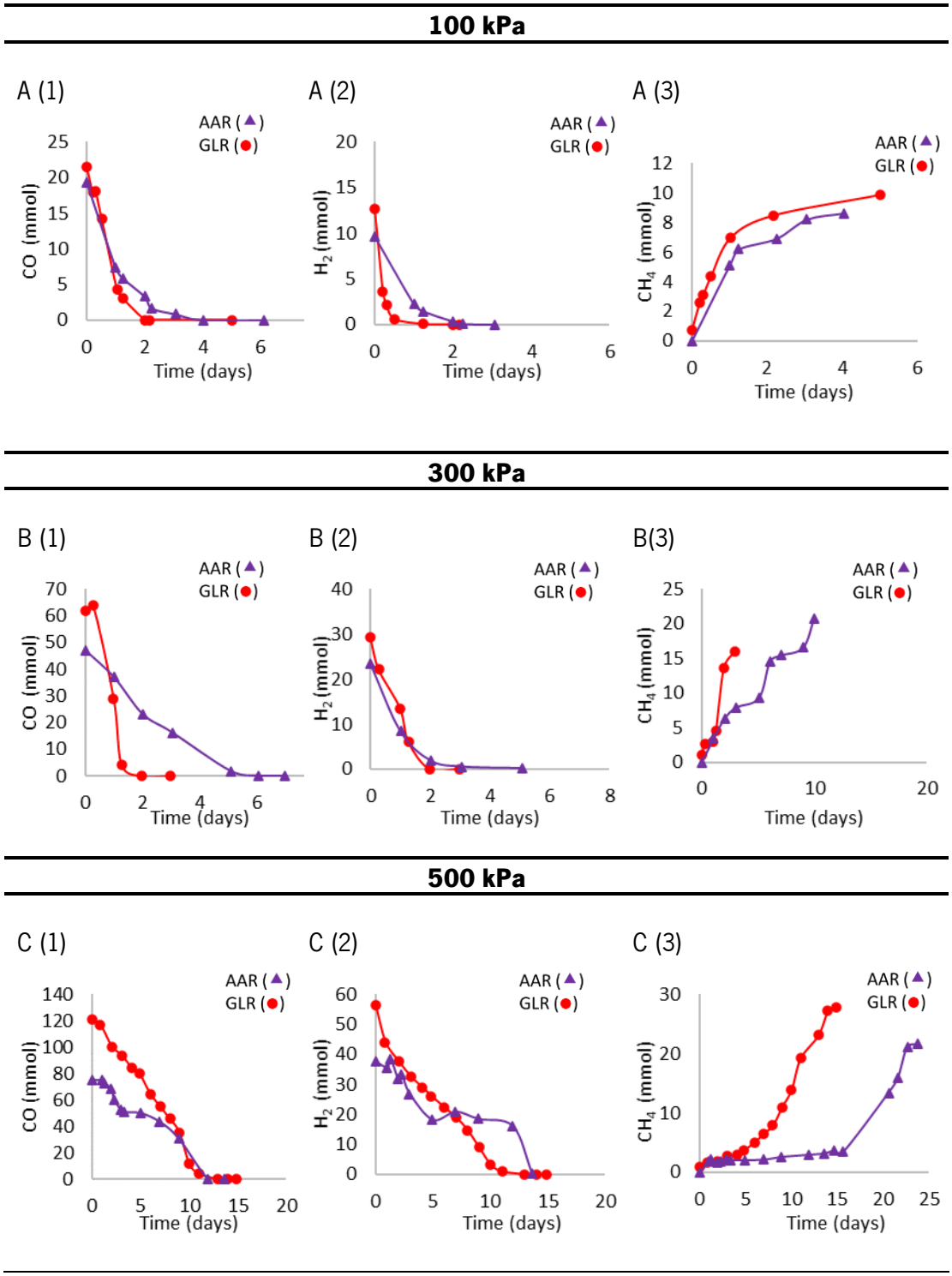


Figure 6. 2 - Substrate consumption (CO (1) and H_2 (2)) and CH_4 production (3) by anaerobic sludge at different pressures ((A) 100 kPa, (B) 300 kPa and (C) 500 kPa) in two different pressurized reactor typologies: (●) Gas-lift reactor (GLR); (▲) Axial agitation reactor (AAR). Note: Assays were stopped when substrate reached zero.

Volatile fatty acids (VFA's) and alcohols were not produced at 100 kPa in both reactor types (Table 6. 1). For higher pressures, small amounts of VFA's were produced, namely acetate, propionate and n-butyrate. Propionate was the main VFA produced, reaching higher titres in the AAR (4.4 mM at 300 kPa and 4.8 mM at 500 kPa) (Table 6. 1). At 500 kPa the VFA analysis, for both reactor typologies, revealed some unknown peaks (Figure 6. 3) that did not fit any of the standards available in the laboratory. In order to try to identify those compounds other types of analysis were done, such as LCFA analysis and PHAs analysis, though the results allowed to infer that no LCFA or PHA were identified. The same samples were also analysed with LC-MS chromatography. Based on the mass spectrum of LC-MS analysis we inferred that heptanoic acid could be produced suggesting that chain elongation might be occurring at higher pressures (500 kPa). However, this hypothesis was not confirmed due to the bad quality of the peaks separation in the LC-MS analysis.

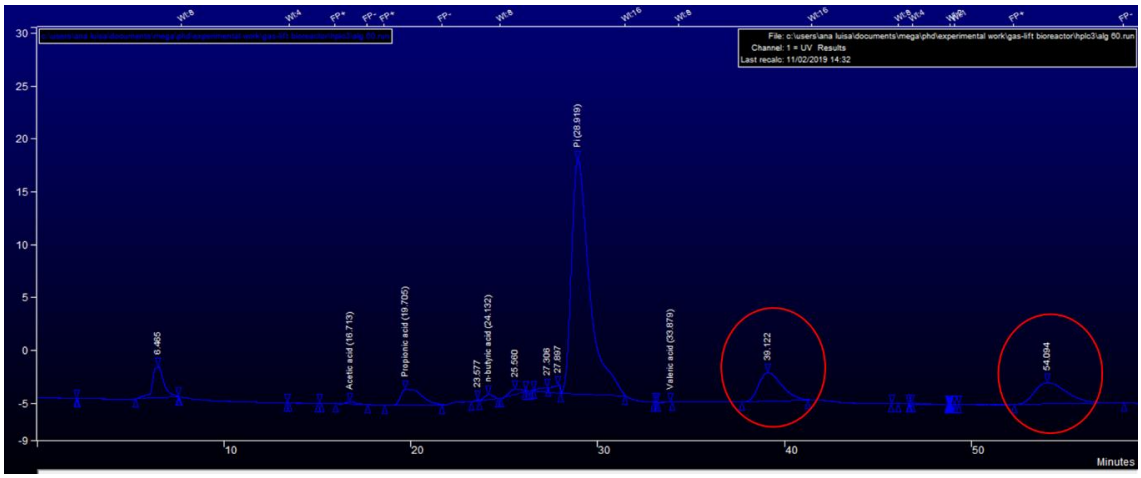


Figure 6. 3 - Example of a chromatogram from HPLC analysis, referent to samples taken in the end of operation at 500 kPa in the GLR. Red circles show the unidentified peaks observed.

In order to access the effect of syngas pressure in the microbial community, the microbial diversity was studied by sequencing the 16S rRNA gene pool at the end of operation of each pressure condition tested (Table 6. 2).

Table 6. 1 - Comparison of batch syngas fermentation in a pressurized axial agitation reactor (AAR) and a pressurized gas-lift reactor (GLR) with anaerobic sludge at different starting total pressures. (n.p. – not present)

Total pressure (kPa)	**Reactor typology**	**CO consumption rate** (mmol h^{-1})	**H_2 consumption rate** (mmol h^{-1})	**CH_4 yield** (%)	**CH_4 production rate** (mmol h^{-1})	**CH_4** (mmol)	**Acetate** (mM)	**Propionate** (mM)	**n-Butyrate** (mM)
100 (pCO ≈ 60)	AAR	0.33	0.19	100	0.11	7.7	n.p.	n.p.	n.p.
	GLR	0.7	1.52	100	0.29	9.1	n.p.	n.p.	n.p.
300 (pCO ≈ 180)	AAR	0.37	0.45	100	0.08	17.8	n.p.	4.4	0.5
	GLR	2.4	0.70	78	0.09	14.9	0.8	n.p.	1.1.
500 (pCO ≈ 300)	AAR	0.21	0.13	92	0.10	19.6	n.p.	4.8	1.2
	GLR	0.44	0.19	61	0.11	26	0.5	1	n.p

Table 6. 2 - Microbial composition until the order level (relative abundance ≥ 1 %) of samples collect at the end of operation of AAR and GLR, using syngas as substrate at different pressures (100, 300 and 500 kPa). Variation in colour intensity reflects the relative abundance of microbial groups, from light colour, less abundant, to dark colour, more abundant.

ARCHAEA				**Relative abundance (%)**			
				AAR		**GLR**	
Taxonomic classification				**Total community**	**Archaeal community**	**Total community**	**Archaeal community**
Total pressure syngas	**Phylum**	**Class**	**Order**				
100 kPa	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	15,0	51,0	22,0	56,0
	Euryarchaeota	*Methanomicrobia*	*Methanomicrobiales*	9,0	29,0	15,0	38,0
	Euryarchaeota	*Methanobacteria*	*Methanomicrobiales*	5,0	18,0	2,0	5,0
300 kPa	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	1,0	5,0	12,0	46,0
	Euryarchaeota	*Methanomicrobia*	*Methanomicrobiales*	5,0	21,0	9,0	32,0
	Euryarchaeota	*Methanobacteria*	*Methanomicrobiales*	19,0	73,0	5,0	19,0
500 kPa	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	15,0	51,0	21,0	52,0
	Euryarchaeota	*Methanomicrobia*	*Methanomicrobiales*	9,0	29,0	13,0	33,0
	Euryarchaeota	*Methanobacteria*	*Methanomicrobiales*	5,0	18,0	5,0	12,0

BATERIA				**Relative abundance (%)**			
				AAR		**GLR**	
Taxonomic classification				**Total community**	**Bacterial community**	**Total community**	**Bacterial community**
Total pressure syngas	**Phylum**	**Class**	**Order**				
100 kPa	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	20,0	29,0	16,0	27,0
	Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	1,0	1,0	9,0	14,0
	Proteobacteria	*Deltaproteobacteria*	Unclassified	7,0	10,0	1,0	1,0
	Chloroflexi	*Anaerolineae*	*Anaerolineales*	6,0	8,0	1,0	2,0
	Synergistetes	*Synergistia*	*Synergistales*	1,0	2,0	1,0	1,0
	Unclassified	Unclassified	Unclassified	30,0	43,0	24,0	40,0
300 kPa	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	22,0	30,0	21,0	28,0
	Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	1,0	1,0	15,0	21,0

	Proteobacteria	*Deltaproteobacteria*	Unclassified	11,0	15,0	1,0	1,0
	Chloroflexi	*Anaerolineae*	*Anaerolineales*	6,0	8,0	2,0	3,0
	Synergistetes	*Synergistia*	*Synergistales*	4,0	6,0	1,0	1,0
	Bacteroidetes	*Bacteroidia*	*Bacteroidales*	1,0	2,0	1,0	1,0
	Firmicutes	*Clostridia*	*Clostridiales*	1,0	2,0	1,0	1,0
	Thermotogae	Unclassified	Unclassified	2,0	2,0	1,0	1,0
	Unclassified	Unclassified	Unclassified	33,0	45,0	17,0	23,0
500 kPa	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	11,0	23,0	27,0	46,0
	Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	1,0	1,0	8,0	13,0
	Proteobacteria	*Deltaproteobacteria*	Unclassified	8,0	18,0	1,0	2,0
	Chloroflexi	*Anaerolineae*	*Anaerolineales*	1,0	2,0	2,0	4,0
	Synergistetes	*Synergistia*	*Synergistales*	6,0	14,0	1,0	1,0
	Firmicutes	*Clostridia*	*Clostridiales*	3,0	6,0	1,0	2,0
	Thermotogae	Unclassified	Unclassified	4,0	9,0	1,0	1,0
	Thermotogae	*Thermotogae*	*Kosmotogales*	1,0	1,0	1,0	3,0
	Unclassified	Unclassified	Unclassified	9,0	19,0	8,0	14,0

Regarding the archaeal community composition, *Methanomicrobiales*, *Methanobacterales* and *Methanosarcinales* were the three orders that prevailed in the pressurized systems tested (AAR and GLR). At 500 kPa of total pressure, the archaeal community of the AAR system is dominated by the *Methanobacterium* genus (79 % of Archaea); as for the GLR system most of the archaeal community belongs to the *Methanobacterium* (12 % of Archaea) and the *Methanosaeta* genera (52 % of Archaea) (Table S. 1).

In both systems, for any of the pressures tested, the prevailing groups in the bacterial community were the *Proteobacteria* (around 30 % to 70 % of Bacteria) and the *Chloroflexi* (2 % to 8 % of Bacteria). For the highest total pressure tested, 500 kPa, despite presenting different relative abundances, the dominant groups were: *Proteobacteria* (of which 20 % to 35 % belong to *Deltaproteobacteria* class), *Chloroflexi* (the majority belonging to the *Anaerolineales* order), *Firmicutes* (that were mainly from *Clostridia* class) and *Thermotogae* (predominantly members of *Mesotoga* genus) (Table 6. 2, Table S. 1). The exception lays on the *Synergistetes* phylum, that represents 14 % of the bacterial community in the AAR, while at the same pressure, this phylum was not identified in the GLR.

6.4. Discussion

Gas-liquid mass transfer limitations are still one of the major bottlenecks of syngas fermentation, due to the low solubility of syngas main constituents (CO and H_2). Thus, this issue is one of the biggest hurdles for the scale-up and application of this technology. Mass transfer limitations can be decreased by improving the volumetric gas-liquid mass transfer coefficient (k_La), which is usually related with reactor characteristics. One other way to improve the gas-liquid mass transfer is to increase the gas partial pressure of the system which is the driving force for their transference to the liquid phase (Bengelsdorf *et al.*, 2018; De Tissera *et al.*, 2017; Grimalt-Alemany *et al.*, 2018; Yasin *et al.*, 2019b). In this work two reactor typologies were studied, in the same conditions (of pressure, temperature and ratio of initial CO to VS (CO mmol/ gVs) with the goal to evaluate the effect of the reactor configuration in syngas (mainly CO and H_2) conversion and the effect of pressure in the conversion and in the behavior of the microbial community. When comparing the two different reactor typologies, the GLR shows a better

performance than the AAR, in terms of conversion of syngas components (Figure 6. 2). These results are in line with the data obtained by Munasinghe and Khanal, where it was shown that the gas-lift reactor, combined with a bubble diffuser, had the highest mass transfer coefficient (k_La) for CO (Munasinghe and Khanal, 2010b). Due to its morphology, the GLR enhances the mass transfer between the gas and the liquid phase: the gas is injected at the bottom of the reactor with a sparger and ascends through an inner channel for gas-liquid upflow, the riser, and the downflow happens through a different channel (Figure 6. 1(B), creating circulation channels that enhance the system mixing, allowing constant agitation without moving parts (Haddad *et al.*, 2014; Merchuk and Garcia Camacho, 2010). Additionally, in GLR, microorganisms are not exposed to shear stress, as for example in mechanically agitated reactors, which can be detrimental for growth (Merchuk and Garcia Camacho, 2010).

With the exception of 500 kPa in the GLR, for all the other conditions, the main syngas fermentation product was always CH_4.This result is somehow contradictory with the literature that states that typically hydrogenogenesis (production of H_2) and acetogenesis (production of acetate) are more common pathways in CO/syngas conversion, while methanogenesis is highly sensitive to CO (Ferry, 2010; Guiot *et al.*, 2011b; Klasson *et al.*, 1991; Oelgeschläger *et al.*, 2004). Despite no significant changes being observed in CH_4 production rate in the AAR, high CH_4 yields were always obtained, even at the highest pressure, 500 kPa (pCO ≈ 300 kPa), a 92 % CH_4 yield was reached, which is opposing with many studies that state that usually methanogenesis is partially or completely inhibited with CO partial pressures between 11 kPa and 100 kPa: Esquivel-Elizondo *et al.* stated that methanogenesis was partially inhibited with pCO above 11.2 kPa (Esquivel-Elizondo *et al.*, 2017); complete inhibition of CH_4 production at CO partial pressure of 50.7 kPa was reported by Jing *et al.*(Jing *et al.*, 2017); and Sancho-Navarro *et al.* reported total block of methanogenic activity at 101 kPa of CO partial pressure (Sancho Navarro *et al.*, 2016), but suggested that methane production with high pCO (≥ 101 kPa) is

possible, but only after sufficient acclimation to CO over time (Sancho Navarro *et al.*, 2016). In terms of consumption rates, the results were more satisfactory for the GLR, once the CO consumption rate increased with the increase of pCO and only slightly decreased at 500 kPa (pCO ≈ 300 kPa). Though, lower CH_4 yields were obtained with the increase of pressure. Even though that this effect was not observed in the AAR, it was still likely to occur, once it is reported in several other works, that the increase of pCO usually results in a decrease on the methanogenic activity (Asimakopoulos *et al.*, 2019; Esquivel-Elizondo *et al.*, 2017; Sancho Navarro *et al.*, 2016). Nevertheless, at 500 kPa (pCO ≈ 300 kPa) the CH_4 yield was still upheld at 61 % in the GLR, considering that for 100 kPa (pCO ≈ 60 kPa) and 300 kPa (pCO ≈ 180 kPa) a 100 % and 78 % CH_4 yield, respectively, were obtained (Table 6. 1). Though, in both systems the sludge was directly inoculated in the reactor with syngas without previously acclimation to CO, in all cases, without any lag phase observation, and still higher methane yields than usually reported were achieved. Therefore, this work reveals, for the first time, methanogenic activity at CO partial pressures higher than 101 kPa with relevant CH_4 yields. Additionally, all the CO was totally consumed for all the pressures tested. The results obtained with microbial community analysis (Table 6. 2 and Table S. 1) also support that methanogenesis was not inhibited by the high partial pressures of syngas components, namely by the CO, once we still had a predominance of methanogens within the culture, even with the highest pressure tested (500 kPa; pCO ≈ 300 kPa). Furthermore, the GLR system seems to favor a higher stability of the microbial culture (both for Archaea and Bacteria), when submitted to increased pressures. This was an expected result, once the GLR shows higher CO conversion rates, meaning that it will remove, at a faster rate, the potential initial inhibitory/toxic effect of CO to both archaeal and bacterial communities. Moreover, Sancho Navarro *et al.* stated that the sensitivity of methanogens to CO varies depending on the characteristic of the sludge (granular or disaggregated) (Sancho Navarro *et al.*, 2016). Therefore, one should not rule out the hypothesis that the longevity of methanogens at higher CO partial pressures, in the GLR, can also be due to its

configuration, once it is known to be less aggressive to the microorganisms (Merchuk and Garcia Camacho, 2010). The bacterial community was more affected in the AAR system than in the GLR, being notorious a drop in the bacteria relative abundance, suggesting some inhibition caused by the increase of pressure and therefore a specialization of the culture, supporting the higher CH_4 yields and consequently the lower VFA's production in the AAR. Overall, the global analysis of the microbial community reveled an adaptation to the moderate pressures tested, suggesting a specialization of the community for the substrate (Table S. 1). Accordingly to the microbial analysis, methane production from CO was done via acetate as an intermediate metabolite, as previously observed by other authors (O'Brien *et al.*, 1984; Sancho Navarro *et al.*, 2016; Sipma *et al.*, 2003), once there was a high prevalence of *Methanosaeta* species, especially in the GLR (Table S. 1), and a total absence of carboxydothrophic methanogens. This was expected as direct conversion of CO to CH_4 is uncommon (Sancho Navarro *et al.*, 2016; Sipma *et al.*, 2003). Moreover, this is coherent as well with the higher CO affinity reported by the carbon monoxide dehydrogenase (CODH) enzyme in carboxydothrophic acetogens (Oelgeschläger and Rother, 2008b), suggesting that in this particular case, this higher affinity allows the acetogens to thrive removing the CO at a faster rate, producing acetate, that will be available for methanogenic growth without CO inhibition.

The increase of the initial pressure of syngas triggered a change in the metabolic pathways in syngas conversion by anaerobic granular sludge, leading to the production of VFA's (Table 6. 1), coinciding as well with the partial inhibition of methanogenesis. It has been previously shown that CO partial pressure can induce changes in metabolic routes of syngas-fermenting microorganisms. Hurst and Lewis reported an increase in ethanol with a reduction in acetic acid production by *C. carboxidivorans* strain $P7^T$ with an increase of CO partial pressure from 35 kPa to 200 kPa (Hurst and Lewis, 2010). Moreover, Sancho Navarro *et al.* described a decrease in CH_4 production and the formation of intermediate metabolites (H_2, acetate and propionate), by disaggregated anaerobic sludge, at CO partial

pressures above 50 kPa (Sancho Navarro *et al.*, 2016). In this work, for the AAR, the production of propionate and butyrate was observed at 180 kPa and 300 kPa of CO partial pressure, and for the GLR, at 300 kPa and 500 kPa small amounts of VFA's were measured, namely acetate, propionate and n-butyrate. This result is of great importance, since both propionate and n-butyrate are important industrial platform chemicals, with a wide range of applications that have been showing a continuously growing market demand (Tirado-Acevedo *et al.*, 2010; Ueki *et al.*, 2014). Thus, the increased syngas pressure might be an alternative option to direct the biochemical pathways of microbial community towards the specific products formation, namely propionate and/or butyrate. The potential of anaerobic granular sludge for biomethanation from syngas is also noteworthy.

6.5. Conclusion

This work has shown that the increase of CO partial pressure can have a beneficial effect on syngas fermentation to methane. This is the first report with pCO above 101 kPa, without showing relatively high or complete inhibition of methanogenesis. The microbial analysis showed an adaptation to moderate pressures of syngas and a specialization of the microbial community. Microbial mixed cultures seem to be a stable and robust biocatalyst for the biomethanation of syngas, being able to withstand higher CO partial pressures while still producing methane. Even at the highest pressure (500 kPa, pCO ≈ 300 kPa) CH_4 yields of 92 % in the AAR and 61 % in the GLR were obtained at the end of the assay, when no more CO or H_2 were detected. When comparing both reactor configurations, the GLR showed a better performance, in terms of consumption rates, VFA's production and microbial stability, than the AAR, though the AAR revealed better CH_4 yields in all the conditions. These results are revolutionary on what was so far thought about inhibition of methanogenesis with high CO concentrations and opens perspectives for implementation of sustainable and environmentally friendly biomethanation processes from syngas. Moreover, with this work it was also possible to reinforce the

hypothesis that the increase of pressure can trigger a change in syngas fermentation metabolic pathways, which can be groundbreaking in the industrialization of syngas fermentation.

Chapter 7.

General Conclusions and Suggestions for Future Work

7.1. GENERAL CONCLUSIONS

One of the major hindrances of gas fermentation is the limited gas-liquid mass transfer rate that generally limits productivities, which can be an obstacle in terms of economic feasibility, slowing down the industrialization of this technology. Increasing the system pressure, increases the gases solubility, thus the driving force for mass transfer. Some studies have already shown that higher pressures could improve the microbial growth rates and product formation (Oswald *et al.*,2018; Kantzow and Webster-Botz, 2016; Esquivel_Elizondo *et al.*, 2017), though the effect of pressure in syngas fermentation is not yet widely studied. The work presented in this thesis aimed at further understand how pressure affects the biocatalysts and the product formation spectrum in syngas fermentation, contributing to the development of this technology. The main conclusions achieved were:

i. Increase of syngas partial pressure (up to 600 kPa), had a general beneficial effect on the process. In all assays, with highly enriched cultures and mixed cultures, respectively, the increase of pressure, generally resulted in faster growth rates and higher titres

ii. Methanogenesis in anaerobic sludges was not inhibited with initial CO partial pressures of 60 kPa, 180 kPa or 300 kPa. Microbial mixed cultures are more stable and robust biocatalyst for the production of methane from syngas at higher pressures.

iii. A gas lift pressurized bioreactor was more efficient for the biomethanation of syngas that an axially agitated pressurized bioreactor.

iv. A shift in the metabolic pathways was observed with the increase of pressure both with mixed- and highly enriched-cultures. At higher pressures, a shift from acetate towards production of other VFAs, namely butyrate and propionate, was observed.

v. *Acetobacterium weringae* strain JM was isolated and shown to be more efficient than its closest relatives *A. wieringae* type strain and *A. woodii*.

7.2. SUGGESTIONS FOR FUTURE WORK

This thesis shows that use of moderate pressures can be a step forward to the industrialization of syngas fermentation. Therefore, further studies on the topic should be encouraged. In this way the following topics are suggested:

i. The study of moderately high-pressure systems working in a continuous mode, which can improve productivities, thus being more appropriate for industrial application.

ii. Once gas-liquid mass transfer was improved by the increase of absolute pressure of the systems, resulting in *e.g.* better productivities and better consumption rates, the same experimental approach should be applied to other carboxydrotrophic microorganisms with high biotechnological interest, such as *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, or even synthetic co-cultures with high potential for syngas fermentation, *e.g. Clostridium autoethanogenum* and *Clostridium kluyveri.*

iii. The new isolated *A. wieringae* strain JM, should be studied as a novel and highly efficient acetogen for syngas fermentation, using genetic engineering tools, to induce the production of non-native chemicals based on C1 gases and/or to improve productivities.

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SUPPLEMENTARY MATERIAL

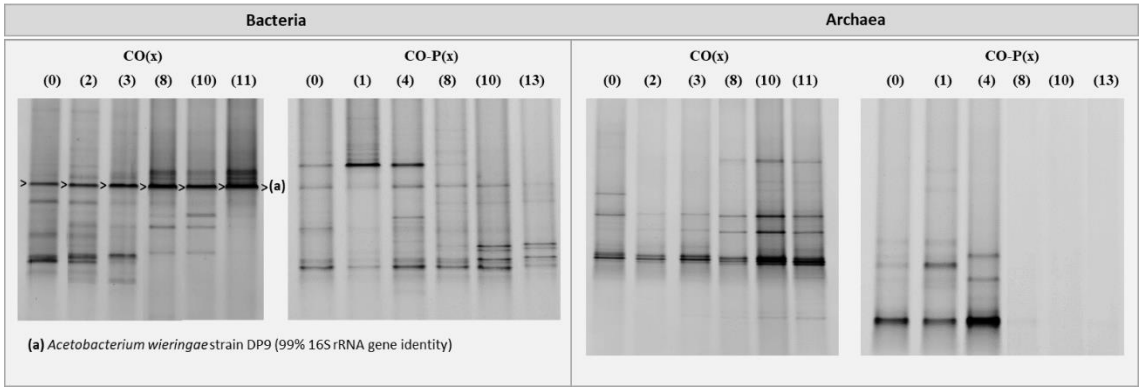

Figure S. 1 - Bacterial and archaeal DGGE profiles of the enrichments CO(x) and CO-P(x), where (x) corresponds to number of successive transfers (nomenclature in Figure 3. 1).

Table S. 1 - Microbial composition until the genus level of inoculum and reactor samples AAR and GLR, using syngas as substrate at different pressures (100, 300 and 500 kPa). Variation in colour intensity reflects the relative abundance of microbial groups, from light colour, less abundant, to dark colour, more abundant.

						Relative abundance in the microbial community (%)		
	Taxonomic classification					120 kPa (pCO≈ 72 kPa)	300 kPa (pCO≈ 180 kPa)	520 kPa (pCO≈ 312 kPa)
	Phylum	Class	Order	Family	Genus			
Archaea	**Total *Archaea***					**39.4**	**26.7**	**40.7**
	Euryarchaeota	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*	1.8	5.0	4.8
	Euryarchaeota	*Methanomicrobia*	*Methanomicrobiales*	*Methanoregulaceae*	*Methanolinea*	0.1	0.0	0.1
	Euryarchaeota	*Methanomicrobia*	*Methanomicrobiales*	*Methanospirillaceae*	*Methanospirillum*	0.0	0.1	0.1
	Euryarchaeota	*Methanomicrobia*	*Methanomicrobiales*	*Unclassified*	*Unclassified*	14.8	8.5	13.2
	Euryarchaeota	*Methanomicrobia*	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*	22.3	12.3	21.0
	Euryarchaeota	*Thermoplasmata*	Unclassified	Unclassified	Unclassified	0.0	0.0	0.1
	Crenarchaeota	Unclassified	Unclassified	Unclassified	Unclassified	0.0	0.0	0.3
	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified	0.3	0.7	1.1
Bacteria	**Total *Bacteria***					**60.2**	**72.5**	**58.3**
	Acidobacteria	*Acidobacteriia*	*Acidobacteriales*	*Unclassified*	*Unclassified*	0.1	0.1	0.0
	Actinobacteria	*Thermoleophilia*	*Solirubrobacterales*	*Solirubrobacteraceae*	*Solirubrobacter*	0.0	0.0	0.1
	Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*	0.1	0.0	0.0
	Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Parabacteroides*	0.0	0.1	0.1
	Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Petrimonas*	0.0	0.0	0.2
	Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*Anaerolinea*	0.9	1.6	1.5
	Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*Bellilinea*	0.1	0.1	0.1

	Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*Levilinea*	0.0	0.0	0.1
	Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*Longilinea*	0.1	0.4	0.5
	Chloroflexi	*Anaerolineae*	*Anaerolineales*	Unclassified	Unclassified	0.0	0.1	0.3
	Chloroflexi	*Caldilineae*	*Caldilineales*	Unclassified	Unclassified	0.0	0.2	0.2
	Chloroflexi	*Chloroflexia*	*Chloroflexales*	Unclassified	Unclassified	0.0	0.0	0.2
	Chloroflexi	Unclassified	Unclassified	Unclassified	Unclassified	0.1	0.2	0.1
	Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	0.0	0.1	0.5
	Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Tyzzerella*	0.0	0.3	0.2
	Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	Unclassified	0.0	0.0	0.1
	Firmicutes	*Clostridia*	Unclassified	Unclassified	Unclassified	0.0	0.1	0.1
	Firmicutes	*Negativicutes*	*Selenomonadales*	*Veillonellaceae*	*Centipeda*	0.0	0.1	0.0
	Lentisphaerae	*Lentisphaeria*	*Victivallales*	*Victivallaceae*	*Victivallis*	0.0	0.0	0.1
	Nitrospirae	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	Unclassified	0.1	0.3	0.2
	Planctomycetes	*Phycisphaerae*	Unclassified	Unclassified	Unclassified	0.4	0.4	0.1
	Planctomycetes	*Planctomycetia*	*Planctomycetales*	Unclassified	Unclassified	0.0	0.1	0.0
	Planctomycetes	*Planctomycetia*	Unclassified	Unclassified	Unclassified	0.5	0.2	0.4
	Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Paracoccus*	0.0	0.0	0.1
	Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Ralstonia*	0.0	0.1	0.0
	Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*	0.0	0.0	0.1
	Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Curvibacter*	0.0	0.0	0.1
	Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	Unclassified	*Aquabacterium*	0.0	0.0	0.4
	Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Propionivibrio*	0.0	0.0	0.1
	Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Thauera*	0.0	0.0	0.3
	Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfomicrobiaceae*	*Desulfomicrobium*	0.1	0.5	0.4
	Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	0.0	0.0	0.0
	Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Desulfuromonadaceae*	*Desulfuromonas*	0.0	0.0	0.0
	Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacteraceae*	*Geobacter*	8.4	15.0	7.5
	Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Pelobacteraceae*	*Pelobacter*	0.0	0.0	0.1

	Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	Unclassified	Unclassified	0.1	0.1	0.2
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Desulfomonile*	0.2	0.2	0.1
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Smithella*	0.1	0.1	0.1
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Syntrophus*	0.3	0.6	0.7
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	Unclassified	0.0	0.1	0.0
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophobacteraceae*	*Syntrophobacter*	15.3	18.7	25.3
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophorhabdaceae*	*Syntrophorhabdus*	0.0	0.1	0.1
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	Unclassified	Unclassified	0.3	0.8	0.5
	Proteobacteria	*Deltaproteobacteria*	Unclassified	Unclassified	Unclassified	0.2	0.4	1.1
	Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Arcobacter*	0.0	0.1	0.1
	Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Sulfuricurvum*	0.0	0.3	0.1
	Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Alteromonadaceae*	*Alishewanella*	0.0	0.0	0.1
	Proteobacteria	*Gammaproteobacteria*	*Chromatiales*	Unclassified	Unclassified	0.0	0.0	0.1
	Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	0.0	0.0	0.6
	Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Paraperlucidibaca*	0.0	0.1	0.1
	Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	0.0	0.1	0.0
	Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	Unclassified	Unclassified	0.0	0.0	0.1
	Proteobacteria	Unclassified	Unclassified	Unclassified	Unclassified	7.3	12.1	3.8
	Spirochaetes	*Spirochaetia*	Unclassified	Unclassified	Unclassified	0.0	0.2	0.1
	Spirochaetes	Unclassified	Unclassified	Unclassified	Unclassified	0.5	0.6	0.9
	Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*Synergistes*	0.0	0.0	0.1
	Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	Unclassified	0.1	0.0	0.1

	Synergistetes	*Synergistia*	*Synergistales*	Unclassified	Unclassified	0.0	0.1	0.0
	Thermotogae	*Thermotogae*	*Kosmotogales*	*Kosmotogaceae*	*Mesotoga*	0.2	0.3	1.5
	Thermotogae	*Thermotogae*	*Thermotogales*	Unclassified	Unclassified	0.0	0.0	0.4
	Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*	Unclassified	Unclassified	0.2	0.5	0.3
	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified	24.2	16.9	8.0
No Hit	No Hit	No Hit	No Hit	No Hit	No Hit	0.2	0.3	0.7

						Relative abundance in the microbial community (%)		
	Taxonomic classification (AAR)					**120 kPa (pCO≈ 72 kPa)**	**300 kPa (pCO≈ 180 kPa)**	**520 kPa (pCO≈ 312 kPa)**
	Phylum	Class	Order	Family	Genus			
	Total *Archaea*					**30.2**	**26.0**	**53.9**
Archaea	*Euryarchaeota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*	5.4	19.0	42.8
	Euryarchaeota	*Methanomicrobia*	*Methanomicrobiales*	*Methanoregulaceae*	*Methanolinea*	2.8	2.3	9.9
	Euryarchaeota	*Methanomicrobia*	*Methanomicrobiales*	*Methanospirillaceae*	*Methanospirillum*	6.0	3.1	0.8
	Euryarchaeota	*Methanomicrobia*	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*	15.4	1.3	0.1
	Euryarchaeota	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanosarcina*	0.0	0.0	0.1
	Euryarchaeota	*Thermoplasmata*	Unclassified	Unclassified	Unclassified	0.3	0.1	0.1
	Crenarchaeota	Unclassified	Unclassified	Unclassified	Unclassified	0.2	0.1	0.1
	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified	0.1	0.1	0.1
	Total *Bacteria*					**68.2**	**72.9**	**45.5**
Bacteria	*Acidobacteria*	*Acidobacteriia*	*Acidobacteriales*	Unclassified	Unclassified	0.1	0.1	0.0
	Actinobacteria	*Actinobacteria*	*Corynebacteriales*	*Corynebacteriaceae*	*Corynebacterium*	0.0	0.0	0.1
	Actinobacteria	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Micrococcus*	0.0	0.0	0.1
	Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*	0.1	1.1	0.2
	Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Marinilabiliaceae*	*Anaerophaga*	0.0	0.3	0.0

Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Petrimonas*	0.0	0.1	0.0
Bacteroidetes	*Cytophagia*	*Cytophagales*	*Cytophagaceae*	*Cytophaga*	0.1	0.0	0.0
Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*Anaerolinea*	3.9	1.0	0.4
Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*Bellilinea*	0.1	0.3	0.0
Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*Levilinea*	0.1	0.4	0.0
Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*Longilinea*	1.0	3.4	0.0
Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*Pelolinea*	0.0	0.0	0.1
Chloroflexi	*Anaerolineae*	*Anaerolineales*	Unclassified	Unclassified	0.5	0.5	0.4
Chloroflexi	*Chloroflexia*	*Chloroflexales*	Unclassified	Unclassified	0.0	0.0	0.1
Chloroflexi	*Dehalococcoidia*	*Dehalococcoidales*	*Dehalococcoidaceae*	*Dehalococcoides*	0.1	0.0	0.0
Chloroflexi	Unclassified	Unclassified	Unclassified	Unclassified	0.0	0.1	0.1
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Caloramator*	0.0	0.1	0.7
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	0.1	0.4	1.7
Firmicutes	*Clostridia*	*Clostridiales*	*Eubacteriaceae*	*Acetobacterium*	0.1	0.2	0.0
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Tyzzerella*	0.0	0.5	0.0
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Acetivibrio*	0.0	0.0	0.1
Firmicutes	*Clostridia*	*Clostridiales*	Unclassified	*Proteocatella*	0.0	0.0	0.2
Firmicutes	*Clostridia*	*Clostridiales*	Unclassified	Unclassified	0.0	0.2	0.1
Firmicutes	*Clostridia*	Unclassified	Unclassified	Unclassified	0.2	0.2	0.4
Firmicutes	*Negativicutes*	*Selenomonadales*	*Veillonellaceae*	*Centipeda*	0.0	0.2	0.2
Firmicutes	Unclassified	Unclassified	Unclassified	Unclassified	0.1	0.0	0.1
Lentisphaerae	*Lentisphaeria*	*Victivallales*	*Victivallaceae*	*Victivallis*	0.1	0.1	0.2
Nitrospirae	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	Unclassified	0.0	0.1	0.0
Planctomycetes	*Phycisphaerae*	Unclassified	Unclassified	Unclassified	0.1	0.0	0.0
Planctomycetes	*Planctomycetia*	Unclassified	Unclassified	Unclassified	0.8	0.1	0.0
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Burkholderia*	0.0	0.0	0.1
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Ralstonia*	0.0	0.1	0.0
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Pelomonas*	0.0	0.0	0.1

	Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Propionivibrio*	0.1	0.0	0.0
	Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfobulbus*	0.2	0.1	0.2
	Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	Unclassified	Unclassified	0.2	0.1	0.0
	Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	0.1	0.1	0.0
	Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacteraceae*	*Geobacter*	0.4	0.2	0.0
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Desulfomonile*	0.5	0.1	0.0
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Syntrophus*	0.7	0.3	0.0
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	Unclassified	0.1	0.0	0.0
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophobacteraceae*	*Syntrophobacter*	17.0	10.0	10.0
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophorhabdaceae*	*Syntrophorhabdus*	0.2	0.0	0.1
	Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	Unclassified	Unclassified	1.4	0.4	0.5
	Proteobacteria	*Deltaproteobacteria*	Unclassified	Unclassified	Unclassified	7.0	10.8	8.3
	Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Stenotrophomonas*	0.0	0.0	0.6
	Proteobacteria	Unclassified	Unclassified	Unclassified	Unclassified	2.0	1.2	0.6
	Spirochaetes	*Spirochaetia*	Unclassified	Unclassified	Unclassified	0.1	0.1	0.0
	Spirochaetes	Unclassified	Unclassified	Unclassified	Unclassified	0.1	0.0	0.0
	Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*Aminivibrio*	0.7	1.2	0.1
	Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*Aminobacterium*	0.2	1.2	0.5
	Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*Aminomonas*	0.0	0.0	0.2
	Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*Lactivibrio*	0.0	0.5	5.0

	Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*Synergistes*	0.0	0.2	0.5
	Synergistetes	*Synergistia*	*Synergistales*	Unclassified	Unclassified	0.2	1.3	0.0
	Synergistetes	Unclassified	Unclassified	Unclassified	Unclassified	0.1	0.8	0.1
	Thermotogae	*Thermotogae*	*Kosmotogales*	*Kosmotogaceae*	*Mesotoga*	0.2	0.6	0.4
	Thermotogae	*Thermotogae*	*Thermotogales*	Unclassified	Unclassified	0.0	1.7	4.3
	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified	29.5	32.9	8.7
	No Hit	No Hit	No Hit	No Hit	No Hit	0.8	0.4	0.4