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Microbial production of propionate from carbon monoxide/syngas and glycerol

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SUMÁRIO

O monóxido de carbono (CO) é um gás poluente produzido por vários setores industriais (por exemplo, energia e indústria química). É também um componente importante, habitualmente o mais abundante do gás síntese (syngas), que é gerado a partir da gaseificação de materiais contendo carbono. O glicerol é um inevitável subproduto gerado em grandes quantidades durante processos industriais de produção de bioetanol e de biodiesel. Syngas/CO e glicerol podem ser usados por alguns microrganismos anaeróbios para produzir combustíveis ou produtos químicos de elevado valor económico. O objetivo deste trabalho foi construir um consórcio microbiano capaz de converter CO/Syngas e/ou glycerol em propionato. O propionato é um composto de elevado valor com inúmeras aplicações industriais: aplicações variadas na indústria farmacêutica, agente antifúngico na indústria alimentar e funciona também como um percursor para a construção química para produzir plásticos e herbicidas. Um enriquecimento mesofílico (37 ºC) capaz de produzir propionato foi obtido a partir biomassa anaeróbia (biomassa granular) através de transferências sucessivas em CO (≈1.0 bar de pressão parcial) mais acetato (20 mM). A diversidade microbiana desta cultura foi estudada por análise do gene 16S rRNA, sendo identificados como predominantes os microrganismos pertencentes ao gênero *Acetobacterium*. Microrganismos relacionados com *Clostridium propionicum* e *C. neopropionicum* (identidade do gene 16S rRNA> 98%) e com *Pelobacter* sp. (93% de identidade do gene 16S rRNA) também foram abundantes. Uma nova estirpe, designada strain JM, 99% de identidade do gene 16S rRNA *Acetobacterium* sp. foi isolada de um enriquecimento microbiano usando técnicas de diluição em série, em meio líquido e sólido, e usando CO como única fonte de carbono e energia. Este organismo consegue converter CO em acetato e / ou etanol, e é também capaz de converter glicerol em 1,3-propanodiol. Foi construído um consórcio sintético (co-cultura) composto por Acetobacterium sp. estirpe JM e por *Clostridium neopropionicum*, que tem a capacidade de produzir propionato a partir de CO (0,1 mol de propionato/ mol de CO convertido). Com o objetivo de obter informações sobre a fisiologia e bioquímica da conversão biológica de CO a propionato e as potenciais interações microbianas entre os dois microrganismos no consórcio, foi aplicada a técnica de proteómica.

PALAVRAS-CHAVE: SYNGAS, MONÓXIDO DE CARBONO, PROPIONATO, *ACETOBACTERIUM* SP., *CLOSTRIDIUM NEOPROPIONICUM*.

ABSTRACT

Carbon monoxide (CO) is a polluting gas produced by several industrial sectors (*e.g.* energy, chemical industry). It is also a major component of syngas generated from the gasification of carbon-containing materials. Glycerol is an inevitable byproduct generated in large amounts during the production processes of both bioethanol and biodiesel industries. Syngas/CO and glycerol can be used by some anaerobes to produce fuels or valuable chemicals. The aim of this work was to construct a microbial consortium capable of converting CO/ Syngas and/or glycerol to propionate. Propionate is a value-added compound with numerous industrial applications, *e.g.* as an antifungal agent in foods and feeds, and as a building block to produce plastics and herbicides. A mesophilic (37 ºC) propionate-producing enrichment was obtained from anaerobic sludge by successive transfers on CO (≈1.0 bar of partial pressure) plus acetate (20 mM). Microbial diversity of this culture was studied by 16S rRNA gene analysis, and microorganisms belonging to *Acetobacterium* genus were identified as predominant. Microorganisms related to *Clostridium propionicum* and *C. neopropionicum* (16S rRNA gene identity > 98%) and to *Pelobacter* species (93% identity) were also abundant. A new *Acetobacterium* sp. strain JM was isolated from the enrichment culture using dilution series technique and solid medium using CO as sole carbon and energy source. This organism was able to convert CO to acetate and/or ethanol, and able to convert glycerol into 1,3-propanediol. A synthetic consortium (co-culture) composed of *Acetobacterium* sp. strain JM and *Clostridium neopropionicum* was constructed and was capable of producing propionate from CO (0.1 mol propionate/mol CO converted). A proteomic analysis was performed to get insights into the physiology of CO conversion to propionate and microbial interactions between the two microorganisms in the consortium.

KEYWORDS: SYNGAS, CARBON MONOXIDE, PROPIONATE, *ACETOBACTERIUM* SP., *CLOSTRIDIUM NEOPROPIONICUM*

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1. STATE OF THE ART

1.1 Introduction

In the frame of a sustainable and bio-based economy, it is essential to go beyond the unsustainable use of oil and other fossil resources and develop alternative and/or complementing technologies with the ability to produce fuels and chemicals from waste matter.

At the current rate of fossil resources consumption, it is estimated the exhaustion of the global reserves of petroleum within 50 years [5]. This, in addition to the resulting noxious environmental impacts from the atmospheric CO_2 accumulation caused by the burning of fossil fuels, the development and establishment of sustainable and affordable fuels are urgently required. In response to this need, many countries have legislated mandates and introduced policies in order to promote research and development (R&D) and set up technologies that allow the production of fuels and chemicals with associated low cost and low fossil carbon emission. For instance, the European Union (EU) Directive 2009/28/EC of the European Parliament and of the Council states that, until 2020, all state members have to derive 10 % of all transportation fuel from renewable sources [6].

Regarding the scheme of a biobased economy, bioethanol production is the most well-established alternative to fossil fuel resources. This way, bioethanol production know-how can be used as a model to obtain other valuable compounds, using direct or indirect fermentation of renewable sources of feedstock (sugar, starch, or lignocellulosic materials), or from catalytic conversion of producer gas. Yeasts or bacteria are the catalytic converters, through direct fermentation of feedstocks such as sugar-based crops (e.g. cane, beet, and sorghum) or starch-based crops (e.g. corn, barley, wheat, and potatoes) into alcohols [7, 8]. This process is known as first-generation technology [8].

An alternative, known as second-generation technology, is the utilization of lignocellulosic feedstocks, as agricultural wastes, wood, straw, grasses and crop residues. Through these feedstocks composed by three main components (cellulose, hemicelluloses, and lignin) [9], it is possible to obtain fermentable sugars after acid or enzymatic pre-treatment [10, 11]. The available sugars can be then fermented to yield a wide range of

compounds [12, 13]. However, most biomass sources like straw and wood contain a large proportion of materials which microorganisms cannot convert. Another approach might be the gasification technology where organic biomass is converted into a mixture of gases called produced synthesis gas or syngas. Syngas can be subsequently converted to valuable products. This can be achieved either by using a chemical process (Fischer-Tropsch Synthesis, FTS) [14], either by means of anaerobic fermentation [15].Syngas, formed by gasification or steam reforming of coal, natural gas or biomass, is a key intermediate in synthetic fuels production [16]. Thus, syngas can be obtained from both fossil and renewable resources, thereby allowing a gradual transition to a more sustainable energy and chemical production.

The use of syngas obtained from by-products or waste streams from biomass feedstocks fermentation, aiming the yield of high-value products throught anaerobic gas fermentation, relies on the concept of biorefinery [17], which is analogous to the production of multiple fuels and products from a single feedstock in petroleum refineries. Therefore, by the use of both byproduct streams and a small amount of the feedstock to obtain higher value chemicals along with the biofuels, the biomass feedstock can be fully monetized, increasing its derived value.

One promising route to the scene of coupling processes in a biorefinery is the utilization of glycerol as a substrate to produce biochemicals and biofuels. Glycerol is an inevitable byproduct generated in large amounts during the production processes of both bioethanol [18] and biodiesel [19]. The tremendous growth of these industries has generated a glycerol surplus that has led to a dramatic decrease in crude glycerol prices over the past years [20, 21]. In addition to the large amounts of glycerol produced by bioethanol and biodiesel industries, industries using animal fats and vegetable oils also generate waste streams containing high levels of glycerol [22, 23]. Thereby, the development of efficient and cost-effective processes for the conversion of crude glycerol into higher value products is a urgent need for the development and implementation of biorefineries, and a opportunity for the creation of new and complementary economies to other industries.

Many strategies based on chemical and biological reaction have been pursued in the conversion of glycerol into more valuable products. Several disadvantages of chemical catalysis as low product specificity, use of high pressure and/or temperatures, inability to use crude glycerol with high levels of contaminants, etc. can be transcended with biological conversion, while offering the chance to synthesize a wide range of products and

functionalities [24]. The low cost of glycerol is a very competitive alternative to sugars, as raw sources, in the production of chemical products through microbial fermentation. Given the highly reduced nature of carbon atoms in glycerol, fuels and reduced chemicals can be produced from it at higher yields than those obtained from common sugars such as glucose or xylose [25]. However, the utilization of glycerol under fermentative conditions requires microorganisms that can metabolize such a reduced carbon source in the absence of external electron acceptors. The degree of carbon reduction, κ [26] (a measure of available electrons per unit of carbon), of glycerol (κ = 4.67) is greater than that of cell mass (κ = 4.3 for $CH_{1.9}O_{0.5}N_{0.2}$ biomass [26]), in contrast to traditional carbon sources such as glucose (κ = 4) and xylose (k = 4), which means that the formation of cell mass from glycerol will result in the generation of reducing equivalents, namely, electrons [26]. The capability of a microorganism to maintain the overall redox balance, and thus possess the cellular machinery to ferment glycerol as carbon source, is directly linked to the production of a product more reduced than glycerol such as succinic acid (Figure 1), and it is evident that the conversion of glycerol into the metabolic intermediates phosphoenolpyruvate (PEP) and pyruvate (PYR) generates the double of reducing equivalents produced during the catabolism of lignocellulosic sugars such as glucose or xylose (Figure 1). In the fermentative reduction of glycerol, redox balance should remain stable through the terminal transfer of electrons to internally generated organic compounds [27] and, as such, anaerobic fermentation provides a means of maximizing the production of reduced chemicals and fuels [25]. Furthermore, anaerobic fermentation also provides lower operating and capital costs than aerobic fermentation [2] (Figure 1).

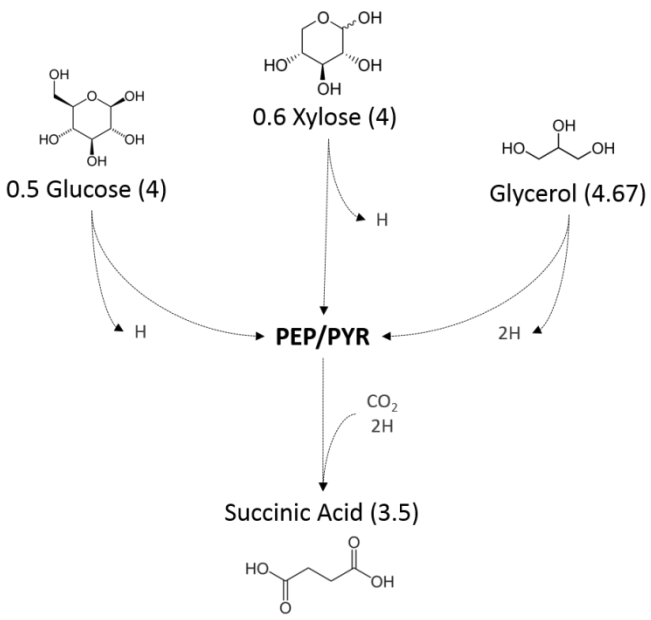


Figure 1. Succinic acid production from glucose, xylose, and glycerol in bacteria. Broken lines represent multiple reaction steps. H, reducing equivalents (H = NADH/NADPH/$FADH_2$). The degree of reduction per carbon, κ, is indicated in parenthesis and was estimated as described elsewhere [26].

By this way, it becomes more evident the need and opportunity of exploiting anaerobic fermentation of syngas and glycerol. Both compounds are enormous potential substrates which can be used singly or in combination for the synthesis of several interesting products, using as means of catalysis pure or defined mixed cultures of anaerobic microorganisms.

1.2 Syngas fermentation

Synthesis gas (syngas) fermentation offers an opportunity to utilize resources from a wide range of non-biodegradable materials, such as coal, biomass, municipal solid waste or other recalcitrant materials for the production of fuels and valuable chemicals. Syngas, a gas mixture of mostly CO and H_2, can be produced by gasification of solid fuels (coal, petroleum coke, oil shale, and biomass); by catalytic reforming of natural gas; or by partial oxidation of heavy oils (tar-sand oil). Syngas composition varies with the type of resource used, its moisture content, and the gasification process [28]. Gasification is a process based on the thermochemical conversion of carbonaceous materials, in the presence of a controlled amount of oxidant (air/O_2), to gas mixture mainly composed of CO, H_2, CH_4, CO_2, and N_2 [29]. The composition of the produced gas can be narrowed to mainly CO and H_2 through

optimization of the gasification operation, thus promoting the downstream syngas fermentation owing to the higher composition of the main compounds (CO and H_2) [30]. As mentioned before, the resulting syngas can be further converted to biofuels and to a variety of chemicals through the Fischer-Tropsch (FT) cycle. However, syngas fermentation has several advantages over the metal catalyst-based approaches despite being characterized by a slower reaction rate. Such advantages include 1) lower temperatures and pressures resulting in a significant energy and cost savings [31]; 2) near complete conversion efficiencies due to the irreversible nature of biological reactions which avoids thermodynamic equilibrium relationships [32, 33]; 3) higher conversion specificity, provided by enzymatic activity, leading to higher yields which simplify further downstream processing and reduces the accumulation of toxic by-products [34]; 4) wider operation range of CO:H_2 ratios in the feed gas, producing the same set of products, unlike chemical catalysis which requires a defined gas composition to yield desired product ratios [35]; 5) higher tolerance to sulfur [36] and the adapting ability to contaminants [37]. Additionally, after exposure to oxygen, anaerobic biomass generally dies, thereby the process does not create any health hazard and generates less environmental pollution [38].

Through bioconversion, anaerobic microorganisms can be used to produce valuable metabolites like organic acids and alcohols. These products include acetic, propionic, butyric, formic, and lactic acids as well as methanol, ethanol, propanol, and butanol [39, 40].

1.2.1 Biochemical pathways of syngas fermentation

In order to produce a variety of valuable metabolites from syngas, anaerobic microorganisms usually use the Wood-Ljungdahl pathway, also known as reductive acetyl-CoA pathway [41]. It was first characterized by Wood and Ljungdahl in 1966 when they presented a scheme for acetate synthesis from CO_2 by the anaerobe *Clostridium thermoaceticum*, now classified as *Moorella thermoacetica*, which became a model acetogen [42, 43].

The Wood-Ljungdahl pathway (Figure 2) can be found in a wide range of microorganisms such as homoacetogenic bacteria and methanogenic archaea [44]. It is constituted by the carbonyl (Western) and methyl (Eastern) branches. The Western branch is an exclusive route to anaerobes for the generation of CO from CO_2 or for the direct uptake of CO from the extracellular environment, serving as the carbonyl group for the acetyl-CoA synthesis [45],

while the Eastern branch comprises several reductive steps to produce the methyl group acetyl-CoA through reduction of CO_2.

CO can enter in the pathway through the two routes: into the Western branch being directly used by the enzymatic carbon monoxide dehydrogenase/ acetyl-CoA synthase (CODH/ACS) complex; and into the Eastern branch after conversion to CO_2 through an oxidation process called biological water gas shift reaction, catalyzed by a carbon monoxide dehydrogenase (CODH), having the resultant released energy of this reaction being captured into a reduced ferredoxin [46, 47]. The CO_2 input by the Eastern branch depends if CO fits as both carbon and energy source, or if hydrogen is present and then it can be utilized in a hydrogenase reaction. However, it should be noted that this energy-yielding process is thermodynamically more favorable from CO than from H_2 [48] and that hydrogenases are reversibly inhibited by CO [49-51].

In the Eastern branch (Figure 2), CO_2 is reduced to formate by formate dehydrogenase (FDH), followed up by the attachment of formate to tetrahydrofolate (THF) by 10-formyl-THF synthase [41]. Then, the attached resulting molecule (formyl-TH)) undergoes several reductive steps catalyzed by the enzymes methylene-THF cyclohydrolase (MTC), methylene-THF dehydrogenase (MTD) and methylene-THF reductase (MTRS). The methyl group from methyl-THF is then transferred by methyltransferase (MTR) to the cobalt center of the corrinoid/iron-sulfur protein (CFeSP) [52], then serving as the methyl group of Acetyl-CoA.

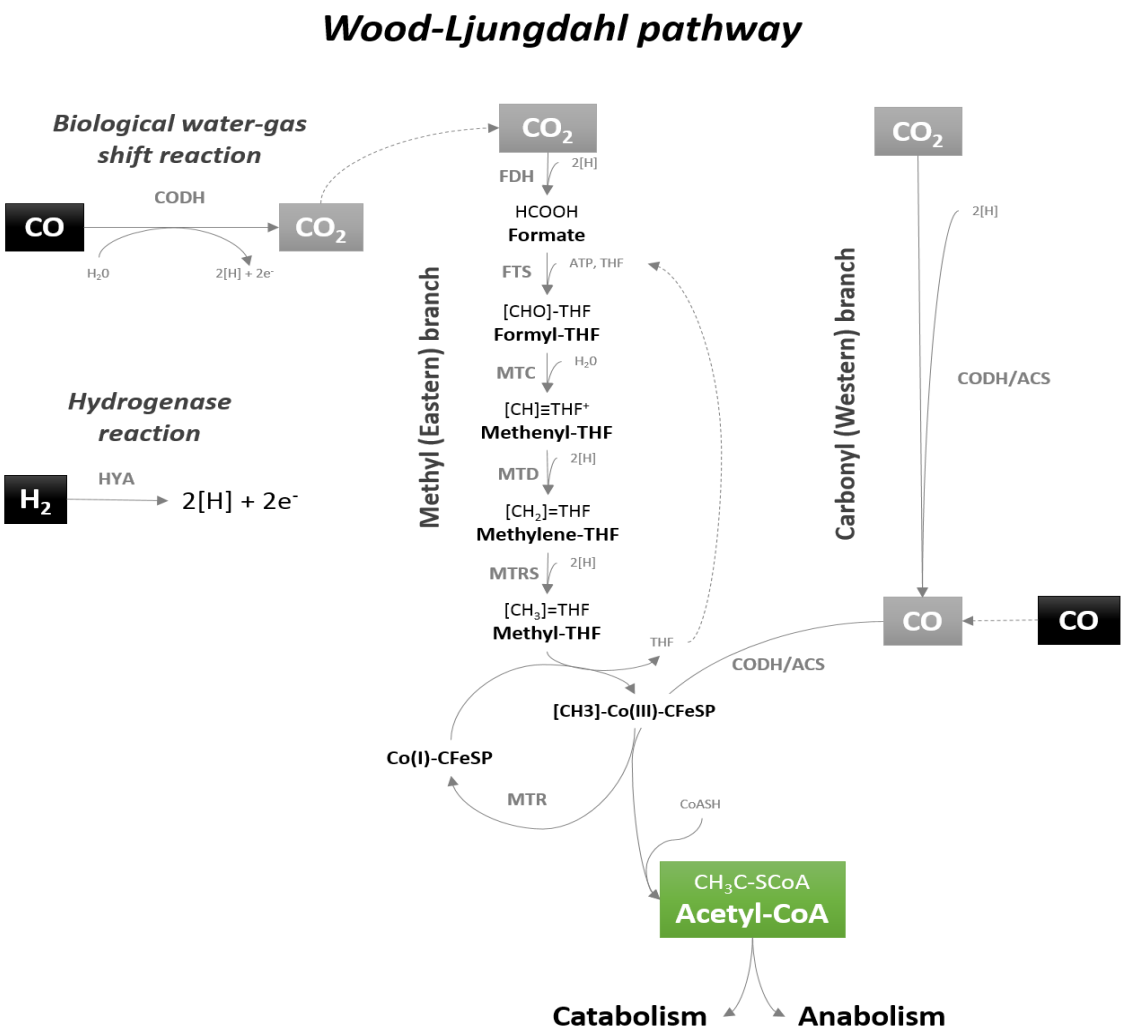


Figure 2. Simplified scheme of Wood-Ljungdahl pathway. Abbreviations: THF – Tetrahydrofolate; CFeSP – Corrinoid iron sulfur protein; CODH – CO dehydrogenase; CODH/ACS – CO dehydrogenase/ acetyl-CoA synthase; FDH – Formate dehydrogenase; FTS – Formyl-THF synthase; MTC – methenyl-THF cyclohydrolase; MTD – methylene-THF dehydrogenase; MTRS – methylene-THF reductase; MTR – methyltransferase; HYA – hydrogenase. Adapted from M. Kopke et al., 2011.[4].

In the Western branch (Figure 2), CO serves as the carbonyl group for acetyl-CoA, in a reaction catalyzed by one of the main enzymes in the Wood-Ljungdahl pathway, CO dehydrogenase [53]. This Ni-CODH can be monofunctional [54] or bifunctional CODH [55]. Monofunctional CODH is responsible for the oxidation of CO to CO_2 in the Eastern branch, while the bifunctional CODH converts CO_2 to CO, which serves as the carbonyl group of acetyl-CoA, and also catalyzes the formation of acetyl-CoA along with acetyl-CoA synthase (ACS) [56]. The next step to the attachment of CO in the bifunctional CODH (CO migrates to the Ni_p site of ACS forming organometallic intermediate, Ni-CO [57]) involves the transfer of the methyl group from the methylated CFeS protein to the CODH/ACS complex, leading to the formation of a methyl-Ni complex [58, 59]. In the next step, condensation of methyl and carbonyl groups

occurs at the Ni_p site, forming an acetylmetal, followed up by the final thiolysis step of the acetylmetal by CoA together with ACS, leading to the formation of acetyl-CoA [60, 61].

Acetyl-CoA can be further used in catabolic or anabolic pathways. The formation of acetyl-CoA from $CO/H_2/CO_2$ has a negative energy balance. Products that can be formed from $CO/H_2/CO_2$ are thus limited to those that allow sufficient conservation of metabolic energy unless an additional energy substrate is provided [62].

1.2.2 Microbiology and products of syngas fermentation

During the last twenty years, new isolates and some known anaerobic microorganisms were reported by their capability to grow on syngas. Microorganisms with this ability are predominantly mesophilic or thermophilic. The optimal operating temperature for the mesophilic microorganism is between (37 and 40) °C, while for thermophilic microbes, is between (55 and 80) °C [62]. Examples of mesophilic microorganisms widely studied in syngas fermentation, are *Acetobacterium woodii, Butyribacterium methylotrophicum, Clostridium carboxidivorans* and *C. ljungdahlii* [15, 63]. Mesophilic organisms have been shown to mainly produce organic compounds from syngas, such as acetate, butyrate, ethanol, butanol, 2,3-butanediol, etc. The use of thermophiles on syngas fermentation is still in an early stage, where *Carboxydothermus hydrogenoformans*, *Carboxydocella sporoproducens, Desulfotomaculum nigrificans, Moorella thermoacetica* and *Moorella thermoautotrophica* were found to grow on CO [39, 64, 65].

Regarding the syngas fermenting microorganisms, they can be classified by their fermentation resulting products. For instance, acetate producers are widely referred as acetogens [66]. The genus *Acetobacterium* and *Clostridium* own the best characterized and researched acetogens [1]. However, the best definition of an acetogen is described as the ability of using the acetyl-CoA pathway 1) as a mechanism for the reductive synthesis of acetyl-CoA from CO_2; 2) as a terminal electron-accepting, energy-conserving process; 3) and as a mechanism for CO_2 fixation upon cell carbon synthesis [66, 67]. In addition to acetate, acetogens can also produce other compounds such as ethanol (*Clostridium ljungdahlii*, *C. autoethanogemum*, *Alkalibaculum bacchi*), butanol (*C. carboxidivorans*, *Butyribacterium methylotrophicum*), butyrate (*C. drakei*, *C. scatologenes*) and 2,3-butanediol *(C. ljungdahlii*, *C. autoethanogenum*) [68].

The metabolic diversity in acetogens relies on coupling the Wood-Ljungdahl with other pathways. For instance, a key central molecule for anabolism is pyruvate which is predominantly generated from glycolysis along heterotrophic growth. However, it can also be synthesized under autotrophic growth by pyruvate ferredoxin oxidoreductase (PFOR), as reported on *Clostridium thermoaceticum* [69]. PFOR is a pyruvate synthase that catalyzes the conversion of acetyl-CoA to pyruvate, the first step in the incomplete reductive tricarboxylic acid cycle (TCA) (Figure 3). Despite its presence in anabolic pathways, pyruvate is also a precursor to other products such as lactic acid or 2,3-butanediol [70, 71].

Many autotrophic anaerobes use the TCA cycle in the reductive direction to transfer the acetyl group of acetyl-CoA into cell mass and to generate metabolic intermediates. This reductive TCA cycle (Figure 3) is also present in the metabolism of green sulphur bacteria [72] and *Epsilonproteobacteria* [73]. In acetogens and other anaerobes, the reverse TCA cycle is

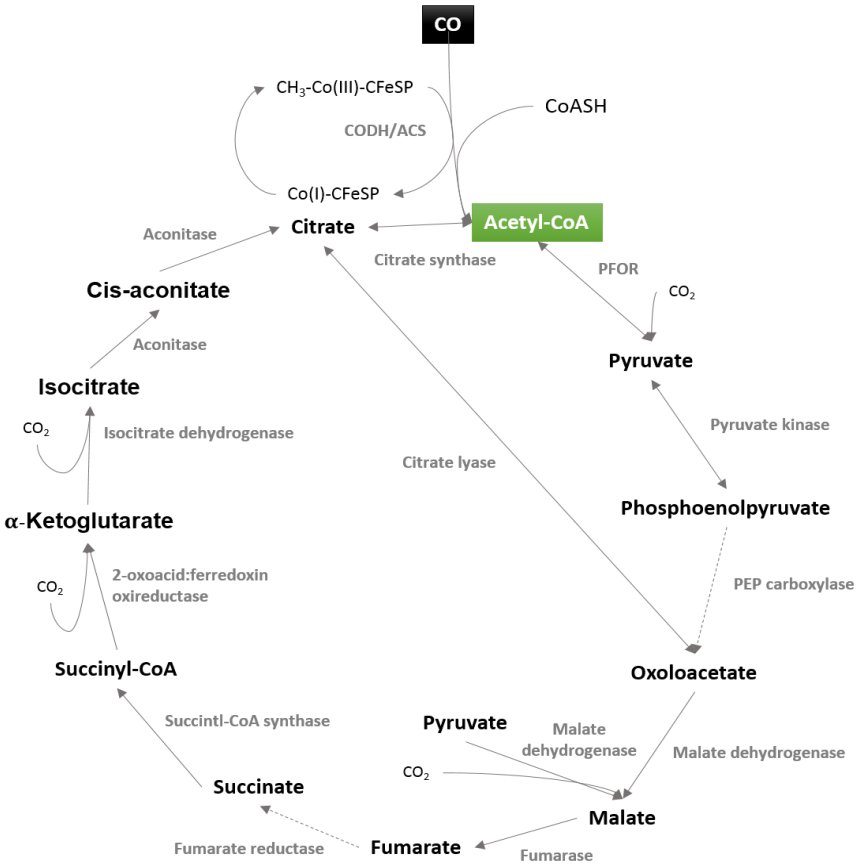


Figure 3. Incomplete TCA cycle allowing conversion of acetyl-CoA to cellular intermediates. Dashed arrows represent enzymes that are not identified in the *M. thermoacetica* genome. Adapted from SWR Rasdale, 2008 [3].

incomplete (Figure 3) and is used in both oxidative and reductive directions in order to generate metabolic intermediates for amino acid synthesis [74, 75].

In anaerobic mixed cultures, methanogens are usually present due to their dominant hydrogenotrophic characteristics: 1) they have a lower threshold for H_2 than acetogens [76]; 2) the energy yield in the conversion of CO_2 and H_2 to methane (CH_4) is greater than in acetate conversion [77, 78]. In these mixed environments where methanogens are present, acetogens can grow due to their ability to use a wide variety of carbon sources as well as electron donors and acceptors. A typical acetogen can use most of the substrates shown in Figure 4.

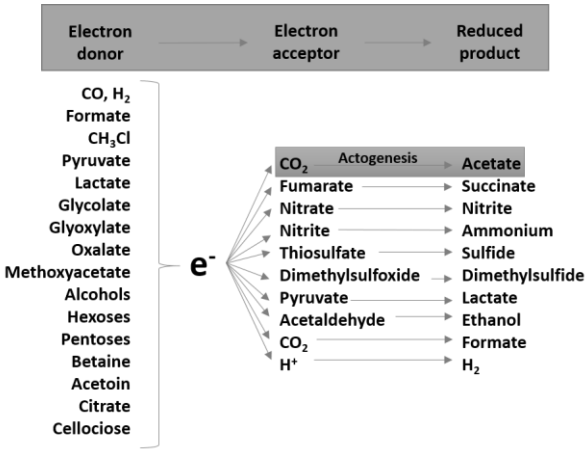
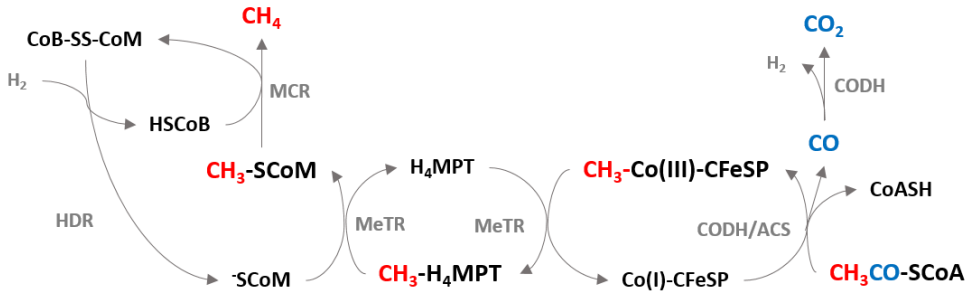


Figure 4. Redox couples that can be used by acetogens. Adapted from Drake, 2008[1].

Furthermore, methanogenic sulfate reducers can degrade the acetate produced by acetogens [79]. The pathway of acetate catabolism by methanogens is shown in Figure 5, where acetate is converted to acetyl-CoA by the enzymes acetate kinase and



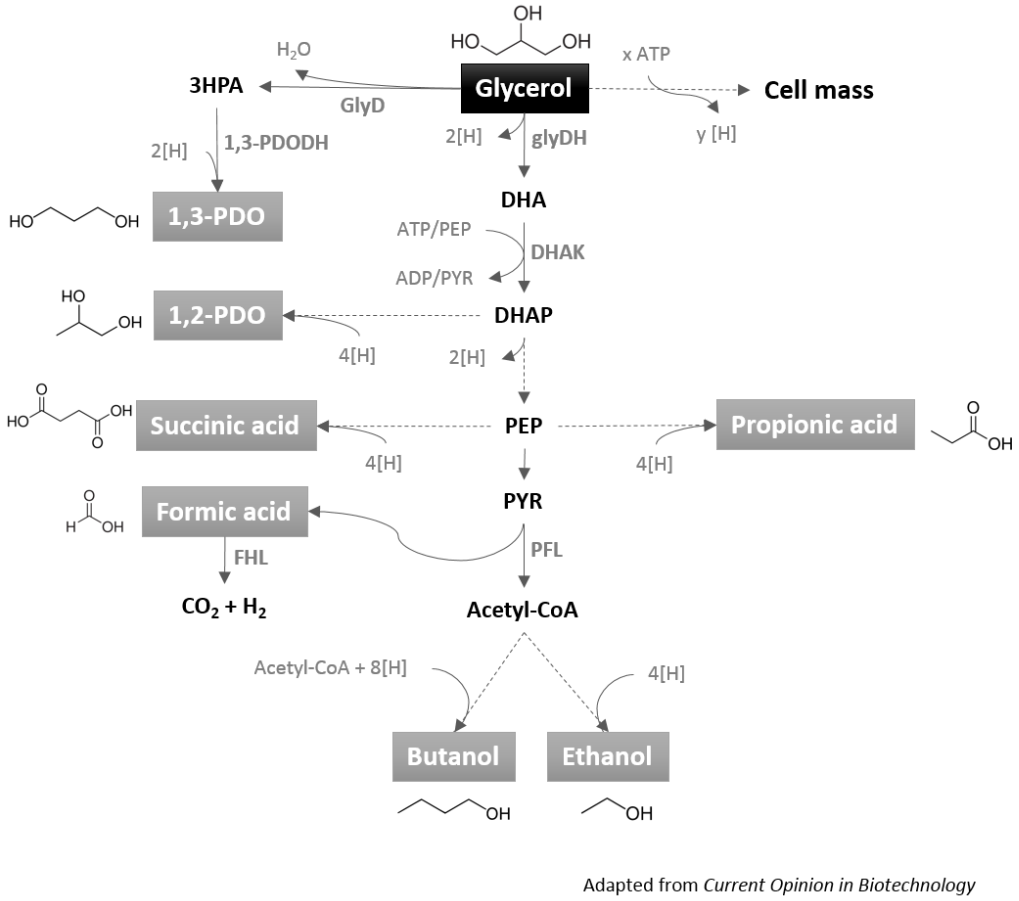
$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$$

Figure 5. Acetoclastic methanogenesis: coupling methanogenesis to the Wood-Ljungdahl pathway (reverse acetogenesis). MCR – methyl-SCoM resuctase; HDR – heterodisulfide reductase; CODH – CO dehydrogenase; CODH/ACS – CO dehydrogenase/ acetyl-CoA synthase; MeTR – methyltransferase. Adapted from Ragsdale, 2008 [3].

phosphotransacetylase. Later in a final stage, the methyl and carboxyl groups from acetate are respectively converted to methane and CO_2.

1.3 Pathways, products, and microbiology of glycerol fermentation

The low cost, abundance, and the significant reduction degree of glycerol make this compound a promising substrate for its bioconversion to high-value compounds through microbial fermentation. Although there are many reported microorganisms with the ability to metabolize glycerol in the presence of external electron acceptors (respiratory metabolism) [80, 81], few can perform it in the absence of electron acceptors (fermentatively). In this process, bacteria from *Enterobacteriaceae* family, such as *Citrobacter freundii* and *Klebsiella pneumoniae*, are the most studied microorganisms concerning to fermentative metabolism of glycerol. In these organisms, dissimilation of glycerol is closely linked to their ability to synthesize the reduced product 1,3-propanediol (1,3-PDO) [82]. This phenomenon is caused by a dismutation process involving two pathways (Figure 6) [80]. In *Klebsiella, Citrobacter, Clostridium* and *Enterobacter* genus, glycerol is metabolized both oxidatively and reductively [83]. In the oxidative pathway, glycerol is dehydrogenated by a NAD^+-dependent enzyme glycerol dehydrogenase (glyDH) to dihydroxyacetone (DHA), which is phosphorylated by PEP- and ATP-dependent DHA kinases (DHAK) and then funneled to glycolysis. In the reducing pathway, glycerol is dehydrated by the coenzyme B_{12}-dependent glycerol dehydrogenase and related diol dehydratases to form 3-hydroxyproprionaldehyde (3-HPA), which is then reduced to the major fermentation product, 1,3-PDO by the NADH-dependent enzyme 1,3-PDO dehydrogenase (1,3-PDODH), generating NAD^+ (Figure 6).



Adapted from *Current Opinion in Biotechnology*

Figure 6 – Anaerobic fermentation of glycerol for the production of fuels and reduced chemicals. The high degree of reduced carbon atoms in glycerol results in pathways to each product representing a redox-balanced or redox-consuming conversion. In each case, the theoretical maximum yield is higher than that obtained from common sugars, such as glucose or xylose. Broken lines represent multiple reaction steps. Abbreviations: DHA, dihydroxyacetone; DHAK, DHA kinase; DHAP, DHA phosphate; FHL, formate hydrogen lyase; GlyD, glycerol dehydratase; glyDH, glycerol dehydrogenase; PEP, phosphoenolpyruvate; PFL, pyruvate formate lyase; PYR, pyruvate; 1,3-PDO, 1,3-propanediol; 1,3-PDODH, 1,3-PDO dehydrogenase; 3HPA, 3-hydroxypropionaldehyde; 2[H] = NADH/NADPH/FADH2. Adapted from Yazdani, 2007 [2].

The final 1,3-PDO product is highly specific in glycerol fermentation and cannot be obtained from any other anaerobic conversion [84, 85]. Only eight taxa of the *Enterobacteriaceae* (out of 1123 strains from 128 taxa tested) are reported to grow fermentatively on glycerol and all produce 1,3-PDO and possess both glyDH type I and 1,3-PDODH [82]. These pathways provide the basis of the current model for the fermentative metabolism of glycerol in microorganisms. The highly reduced state of carbon in glycerol implies the need for an active 1,3-PDO pathway, whose conversion generates reducing equivalents (Figure 6). As the conversion of glycerol into 1,3-PDO results in the net consumption of reducing equivalents, this pathway provides a means to achieve redox balance in the absence of electron acceptors. *Enterobacteriaceae* species, such as *Citrobacter*

freundii and *Klebsiella pneumoniae*, the glycerol fermentation produces 1,3-PDO and acetic acid as the main fermentation products [84]. In its turn, Clostridial strains produce 1,3-PDO along with a variety of another fermentation products such as butyric acid, *n*-butanol, acetic acid, and lactate depending on the strain and conditions [86-91]. Thus, the yield of other products rather than 1,3-PDO using these glycerol-fermenting organisms has also been reported: butanol was found to be the major product of glycerol fermentation by *Clostridium pasteurianum* [90]; ethanol and formate were shown to be the two main products of glycerol fermentation by a *Klebsiella planticola* strain [92]; the co-production of ethanol and hydrogen from glycerol-containing wastes was possible using an *Enterobacter aerogens* mutant [93].

Another product that can be obtained throw glycerol fermentation with higher yields than its recovery through sugar fermentation is the 1,2-propanediol (1,2-PDO) [94]. Just a few microorganisms like *Thermoanaerobacterium thermosaccharolyticum* have been shown to naturally produce 1,2-PDO in large amounts [95]. In addition, when considering 1,2-PDO production from glycerol, its typical fermentative pathways require the conversion of glycerol to dihydroxyacetone phosphate (DHAP) through glycolytic pathways, in contrast with 1,3-PDO pathways which take two steps to produce 1,3-PDO from glycerol (Figure 6).

A small number of reports also document the anaerobic conversion of glycerol to succinic acid. Succinic acid production from glycerol represents a redox balanced pathway [2], which should facilitate the readiness at which high yield of succinic acid can be produced. The anaerobic fermentative conversion of glycerol to succinic acid has been reported in *Anaerobiospirillum succiniciproducens*[96], *Actinobacillus succinogenes* [97], and *Escherichia coli* [98].

The production of propionic acid has also been studied with anaerobes from the *Propionibacterium* genera, which have been shown a positive response in the conversion of glycerol to propionic acid under fermentative conditions [99, 100]. In fact, *Propionibacterium acidipropionici* yields higher amounts of propionic acid using glycerol as carbon source rather than glucose [100].

1.4 Microbial Propionic Acid Production

Propionic acid (propionate) is a compound that has a high market demand for numerous industrial applications, e.g. as antimicrobial [101, 102] and anti-inflammatory [103, 104] agents, as food and feed preservatives [105, 106], and as a building block to produce herbicides [107]. At the industrial level, propionate production occurs via chemical synthesis from petroleum-based feedstock [108]. Bio-based technologies have been attempting propionate production from renewable biomass [109], or cheap industrial waste streams like glycerol using *Propionibacterium*, the most commonly used bacteria in propionic acid production [110]. However, genetically engineered *E. coli* and cultures have also reported activity on propionate production from glycerol [111, 112]. Additionally, other propionogenic bacteria such as *Clostridium neopropionicum*, *Pelobacter propionicus,* and *Desulfobulbus propionicus* have been shown the ability of produce propionate from other substrates like alcohols and organic acids such as ethanol, propanol, 2,3-butanediol, lactate, and acetate [113-116].

The microbial propionic acid production, as a primary fermentation product, occurs via pathways that use 1,2-PDO as an intermediate, the acryloyl-CoA pathway and methylmalonyl-CoA or succinate pathways [117] (Figure 7).

In 1,2-PDO associated pathways (Figure 7A), PDO can be generated from deoxy sugars catabolism via lactate or also from the glycolytic intermediate glycerine phosphate (DHAP) [118], which is also an intermediate in glycerol degradation pathways (Figure 6). The consequent catabolism of PDO to propionate yields one ATP molecule and one reduced cofactor [117]. *Salmonella typhimurium* and *Roseburia inulinivorans* are known microorganisms capable of performing these PDO associated pathways [119, 120].

Through the acrylate pathway (Figure 7B), a variety of substrates such as lactate, serine, alanine, and ethanol can be catabolized to propionate and acetate [117]. Considering lactate conversion to propionate, this pathway has a neutral ATP balance along with NADH consumption [117]. *Clostridium propionicum, Clostridium neopropionicum,* and *Megasphaera elsdenii* are microorganisms capable of fermentatively produce propionate using the acrylate pathway [121-123].

The succinate pathway (Figure 7C) relies on the catabolism of pyruvate using the dicarboxylic branch of TCA cycle to generate succinate. This pathway is usually linked with an

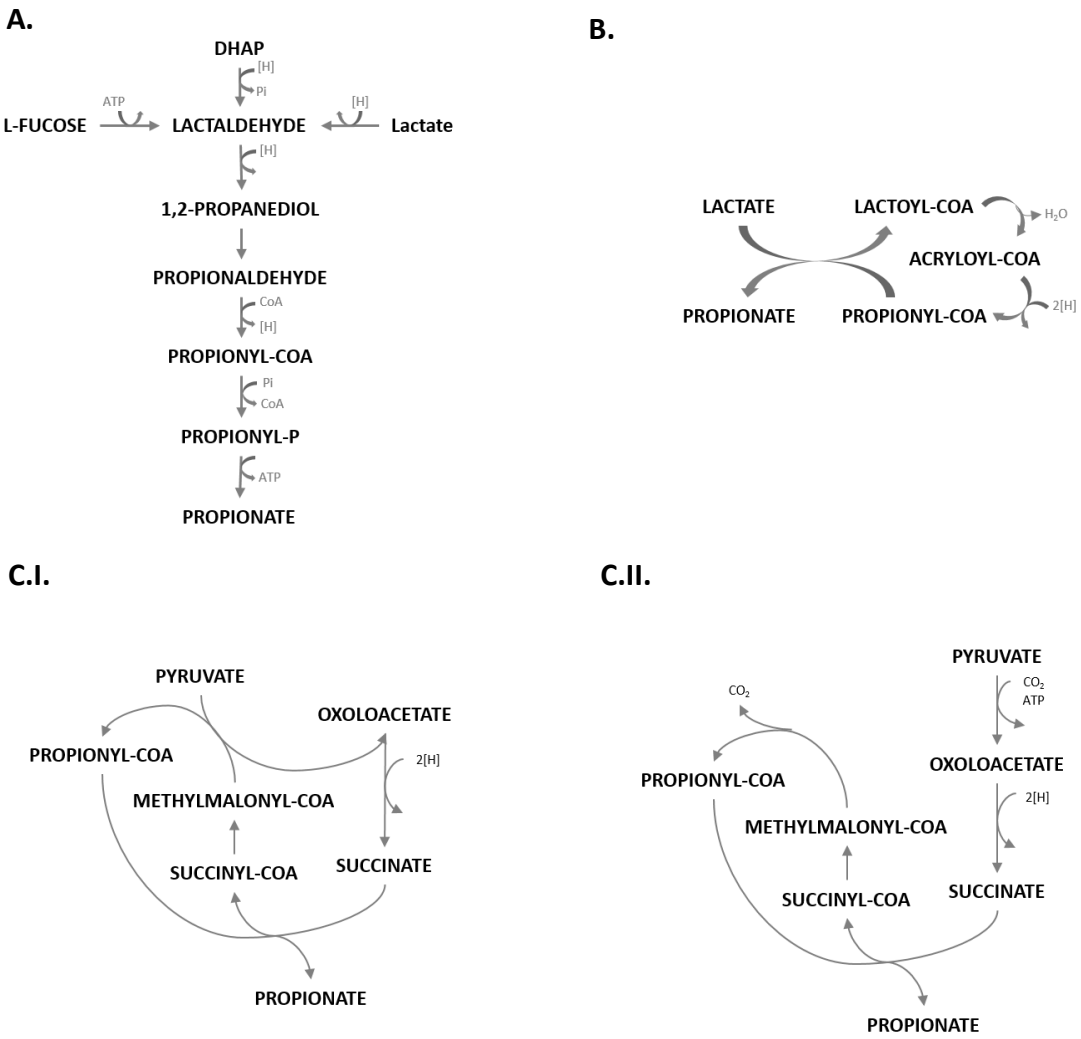


Figure 7. Fermentation pathways for propionate biosynthesis. A.) 1,2-Propanediol pathway. B.) Acrylate Pathway. C.) Succinate pathway configurations: I.) Wood-Werkman cycle, methylmalonyl-CoA:pyruvate transcarboxylase; II.) Sodium pumping pathway, methylmalonyl-CoA decarboxylase.

anaerobic electron transport chain consisting of NADH dehydrogenase and fumarate reductase to compensate the required ATP molecule that is required to fix carbon dioxide and pyruvate or phosphoenolpyruvate into oxaloacetate [117]. To improve the energy yields of this succinate producing pathway, some microorganisms have developed a mixed acid fermentation strategy by further decarboxylation of succinate to propionate, using the sodium pumping methylmalonyl-CoA decarboxylase (Figure 7CII) or the methylmalonyl-CoA: pyruvate transcarboxylase (Wood-Werkman cycle) (Figure 7CI)[117]. *Veillonella parvula* is an example of using the sodium pumping pathway for energy conservation along with propionate and acetate production from malate [124]. For its turn, the Wood-Werkman cycle is predominantly found in *Propionibacterium* species like *P. acidipropionici* [125] and *P. freudenreichii* [126]. In opposite to the sodium pumping pathway, the Wood-Werkman cycle

replaces the decarboxylation step with the methylmalonyl-CoA: pyruvate transcarboxylase which transfers a carboxyl group from methylmalonyl-CoA to pyruvate to yield propionyl-CoA without ATP being required, providing this way the conservation of an entire ATP molecule [117]. This way, the Wood-Werkman cycle represents the best pathway to produce propionate in considering the metabolic goal of energy maximization.

The microbial production of propionate has a defined yield and productivity goals by techno-economic analyses. Until now, the explored bioprocesses have been failing to economically compete with chemical processes due to limitations like the high complexity of substrates, end-product inhibition, and required downstream processes for recovery and purification [127].

Approaches to overcome these limitations are usually based on the use of metabolically-engineered mutants of *propionibacteria*. Through advanced genetic engineering, mutants can be generated with increased productivity and yield in terms of propionate production, as well as enhanced resistance to final product inhibition, significant viability during long fermentation time and adaptation to different culture environments [108]. Some studies have successfully reported the modification of *P. shermanii*, *P. jensenii* and *P. acididiprpionici* aiming to decrease by-products, improve acid tolerance, and increase metabolic flux towards propionate production. The genetic strategies that have been used are genome editing, overexpression, and overexpression of heterologous enzymes. For instance: 1) *P. shermanii CoAT* overexpression strain have increased yield and productivity respectively by 10% and 46%, after aiming the increase of metabolic flux towards propionate production [128]; 2) *P. jensenii paxB* or *Idh* knock out and ppc overexpression aiming the by-products decrement have resulted in a maximum of 30% improvement in titre and 24% improvement productivity [129]; 3) *P. acididiprpionici otsA* overexpression strain have increased propionic acid yield by 11% after improvement on acid tolerance [130].

Although numerous studies have focussed on optimizing the fermentation process in order to improve production, these studies focus on monocultures of natural or engineered propionate producers have not been used on an industrial scale. Attention should be placed on cheap and sustainable feedstock's based bioprocesses which can economically compete with chemical processes. Co-culture techniques might be an interesting approach to couple the conversion pathways of syngas with propionate fermentation. By co-culture cultivation, different cell populations are cultured together with a certain degree of interactions [131]

which might stimulate the production of specified cells, which cannot easily occur in a monoculture system [105]. Co-culture approaches for propionate production have already been tested with the cultivation of propionic acid producing bacteria in the presence of lactic acid bacteria, namely *Propionibacterium shermanii* and *Lactobacillus acidophilus*, which resulted in an increase in propionate yield when compared with its production using *P. shermanii* as a monoculture [132]. In this system, the produced lactate by *L. acidophilus* serves as a substrate that can be used by *P. shermanii* to produce propionate as well as acetate.

2. AIMS OF THE WORK

The main objective of this master thesis, entitled “Microbial production of organic chemicals from either syngas or glycerol, or combined syngas and glycerol”, was to develop efficient anaerobic bioprocesses capable of convert industrial excesses such as syngas and/or glycerol to high-value products, such as succinic acid and/or propionic acid, among others.

From previous work, it was obtained an enrichment culture that produces propionic acid in trace amounts from syngas. In order to contribute to the success of this thesis, the specific objectives were the following:

1) To study the microbial syngas and/or glycerol conversion to organic acids by the obtained enrichment culture, pure cultures, and co-cultures.

2) To identify relevant microorganisms and to study important metabolic pathways involved in syngas and/or glycerol bioconversion to propionic acid.

This plan constituted a step forward to open new opportunities for biological processes as an alternative for the production of biofuels or other added-value chemicals via syngas and/or glycerol conversion route.

All the experimental work was done in both institutions (Laboratory of Microbiology of the University of Wageningen (WUR), Wageningen, The Netherlands, and Centre of Biological Engineering of the University of Minho, Braga, Portugal (CEB-UMinho)), accordingly to the Gantt Diagram presented in this document.

3. MATERIALS AND METHODS

3.1 Medium composition, source of inoculum and microorganisms

The basal medium for the cultivation of the microbial cultures contained the following (grams per liter): KH_2PO_4, 0.93; K_2HPO_4, 1.07; Na_2HPO_4 . $2H_2O$, 0.53; NH_4Cl, 0.3; NaCl, 0.3; $MgCl_2$. $6H_2O$, 0.1; and resazurin, 0.0005; $NaHCO_3$, 4.0; and Na_2S . $9H_2O$, 0.48 (as well as acid and alkaline trace elements [each, 1 ml/liter] and vitamins [0.2 ml/liter], from the stock solutions prepared as described by Stams et al. [133]. The medium was boiled and cooled on ice under N_2 flow and then filled into bottles in portions of under 50 % of the total bottle volume. The bottles were immediately capped with butyl rubber stoppers and aluminum caps. The headspace was filled with the desired gas or gas mixture (e.g., CO, H_2, CO_2, N_2) to a final pressure of 170 kPa. Bottles were autoclaved immediately after preparation. The medium was further supplemented in 1 % v/v with a filter sterile calcium/vitamin solution prepared through the 1:10 dilution of an 11 g/l $CaCl_2$. $2H_2O$ solution and through the 1:100 dilution of a vitamin solution containing (grams per liter): biotin, 0.02; nicotinamide, 0.2; p-aminobenzoic acid, 0.1; thiamine, 0.2; pantothenic acid, 0.1; pyridoxamine, 0.5; cyanocobalamine, 0.1; and riboflavin, 0.1. Other supplements/ substrates such as yeast extract, formate, acetate, ethanol, and glycerol were also added from sterile stock solutions. Just before inoculation, the medium was reduced adding from a sterile solution for a final concentration of 4.0 g/l $NaHCO_3$ and 0.48 g/l Na_2S . $9H_2O$. The final pH of the medium was 7.0 - 7.2. Depending on the experiment, cultivation was done non-shaking or 130 rpm shaking at (30 or 37) ºC.

Methanogenic anaerobic granular sludge from a multi-orifice baffled bioreactor (MOBB), fed with syngas mixture (60 % CO, 30 % H_2 and 10 % CO_2 (v/v)) (Pereira 2014) was used as inoculum for enrichment series; the microorganisms used in this work were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures) strain collection (Braunschweig, Germany): *Acetobacterium wieringae* (DSM 1911), *Acetobacterium woodii* (DSM 1030), *Pelobacter propionicus* (DSM 2379) and *Clostridium neopropionicum* (DSM 3847).

3.2 Enrichment and isolation

A syngas-enriched culture was obtained from a granular sludge from a 10-L MOBB fed with syngas mixture (60 % CO, 30 % H_2 and 10 % CO_2 (v/v)), that was efficiently converting syngas into methane (Pereira 2014). Acetate and syngas were the substrates used to build a series of enrichments, coded as Ace-Syn(x), where x represents the number of successive transfers. During the enrichment process, the culture Ace-Syn(7) was used for the isolation of *Acetobacterium* sp. strain JM. Using carbon monoxide as the sole energy and carbon source, several dilution series of the Ace-Syn enriched culture were done using the basal medium without $NaHCO_3$, supplemented with 1 mM of formate under the headspace composition of 60 % CO and 40 % N_2 (v/v). The resulting culture was further purified using 1.5 % low melting point agarose in sealed roll-tubes with basal medium supplemented with 1 mM of formate and 0.5 g/l of yeast extract with the headspace composition of 50% CO, 30% N_2, and 20% CO_2 (v/v). Colonies were picked and inoculated in fresh liquid basal medium without NaHCO3 supplemented with 1 mM of formate and 0.1 g/l of yeast extract under the headspace composition of 60 % CO and 40 % N_2 (v/v).

Growth was promoted at 37 ºC and non-shaking conditions. The purity of the cultures was checked by microscopic examination and direct sequencing of the 16S rRNA (GATC Biotech, Germany). Isolation of *Acetobacterium* sp. strain JM was also accomplished using ethanol as substrate (20 mM).

3.3 Defined co-cultures experiments

To build the synthetic co-cultures, different pure cultures of microorganism were used: *Acetobacterium* sp. strain JM, *Acetobacterium wieringae* (DSM 1911), *Pelobacter propionicus* (DSM 2379) and *Clostridium neopropionicum* (DSM 3847). Initial co-culture experiments were performed in 117 ml bottles with 50 ml liquid phase. Pre-cultures of *Acetobacterium* species (*A. wieringae* sp. strain JM and *A. wieringae* DSM 1911) were incubated with basal medium at 30 ºC, 130 rpm shaking, and in the presence of 1 mM formate and 0.1 g/l yeast extract as supplements, under a headspace of 50 % CO, 30 % N_2 and 20 % CO_2 (v/v) as substrate. Pre-cultures of *P. propionicus* and *C. neopropionicum* were grown at 30 ºC, non-shaking, in the presence of 20 mM ethanol as substrate, with the addition of 1 mM formate and 0.1 g/l yeast

extract under a headspace of 80 % N_2 and 20 % CO_2 (v/v). After clear detection of growth in all pure cultures experiments, 25 ml of each culture was inoculated into the other culture, building the following co-cultures: *Acetobaterium* sp. strain JM and *P. propionicus; Acetobaterium* sp. strain JM and *C. neopropionicum; A. wieringae* DSM 1911 and *P. propionicus; A. wieringae* DSM 1911 and *C. neopropionicum*. Immediately after inoculation, the headspace of the bottles was re-defined to a 50 % CO, 30 % N_2 and 20 % CO_2 (v/v) composition. The bottles were further incubated at 130 rpm shaking and 30 ºC (or non-shaking during the initial lag phase of growth). Along the experiments, before CO depletion, bottles had been re-pressurized to a final CO composition of 50 % (v/v).

3.4 Analytical techniques

Organic acids and alcohols were measured by high-performance liquid chromatography (HPLC) with Agilent HiPlex-H, 300 x 7.7 mm column operated at a temperature of 45 °C with a flow rate of 0.9 ml/min. 0.01 N H_2SO_4 was used as eluent. The used detectors were a refractive index (RI) detector and a UV light detector set at 210 nm. Samples of (0.5 or 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 was used as internal standard with the ratio of 8:2.

High-performance liquid chromatography is a powerful tool in analysis. It is an improved form of column chromatography: a solvent is forced through a column under high pressures of up to 400 bar, which makes the process much faster than the traditional elution through a column under gravity force [134]. HPLC also allows using a smaller particle size surface of the column packing material, which gives a bigger surface area of interactions between the stationary and mobile phases [135]. This allows a much better separation of the components of the mixture. Another improvement over column chromatography concerns about the detection methods which are highly automated and extremely sensitive. The time for a particular compound to travel through the column to the detector from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound[136]. Different compounds have different retention times due to different specific interactions with the stationary phase [137]. The detection can happen through several

different ways, a common method is the UV absorption [138]. Many organic compounds absorb UV light in various wavelengths, the amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time [134]. The output is recorded as a series of peaks, each one representing a compound in the mixture passing through the detector and absorbing UV light [134]. The retention times could be used to identify the compounds present in the sample, and the area under each peak could be used to quantification of the compounds since the area is proportional to the amount of the compound that has passed the detector [134].

Gas composition analysis was done by gas chromatography (GC). Gas samples of 0.2 ml were taken with a 1 ml syringe and analyzed in a Compact GC 4.0 (Global Analyser Solutions, The Netherlands). CO, CH_4, and H_2 were measured using a Molsieve 5A column, coupled to a Carboxen 1010 pre-column. The standard GC setting for measuring was: 300 kPa, valve (injection) oven 60 ºC, column oven temperature 100 ºC, TCD temperature 110 ºC, filament temperature 175 ºC. CO_2 was measured using a Rt-Q-BOND column operated at pressure 80 kPa, valve (injection) oven 60 ºC, column oven temperature 60 ºC, TCD temperature 110 ºC, filament temperature 175 ºC.

Gas chromatography (GC) is a group of analytical separation techniques which analyses volatile substances at the gas phase. In GC, the components present in a sample are dissolved in a solvent and vaporized in order to separate the compounds by distributing the sample between stationary and mobile phases [139]. The mobile phase consists of a chemically inert gas that is capable to carry the compounds present in the sample through the heated column [140]. Commonly used gases include nitrogen, helium, argon, and carbon dioxide. The choice of carrier gas is usually dependent upon the type of detector which is used. The stationary phase is either a solid absorbant or a liquid on an inert support [141]. The output has the same principle of HPLC, thus the identification and quantification are done through retention times and areas of the peaks, respectively.

3.5 Molecular techniques

3.5.1 DNA isolation, PCR, sequencing, and phylogenetic analysis

DNA from the Ace-Syn enrichment culture (Ace-Syn(5)) was extracted from liquid culture samples using the FastDNA SPIN kit for soil (MP Biomedicals, Ohio, 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 [142] and A109F/1386R [143], and the following programme: initial denaturation at 94 °C for 10 min, and 30 cycles of denaturation at 94 ºC for 30 s, primer annealing at 52 ºC for 40 s and elongation at 72 ºC for 1 min 30 s. The final elongation was at 72 ºC and lasted 7 min. The PCR products were purified and cloned in *Escherichia coli* XL-blue competent cells (Agilent) by using pGEM-T easy vector (Promega). Positive clones were selected by blue/white screening in plates containing 100 mg ampicillin ml^{-1} and further incubated overnight in LB/ampicillin liquid medium at 37 ºC and 250 rpm shaking. Plasmid amplification and Sanger sequencing were done by GATC Biotech (Konstanz, Germany).

For the obtained 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 Sanger sequencing.

All 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 [144] using the NCBI BLAST search tool (http://www.ncbi.nlm.nih.gov/blast/).

3.5.2 Genome sequencing, assembling, and annotation

For genome sequencing, DNA from a pure culture of *Acetobacterium* sp. strain JM was extracted using MasterPure™ Gram-positive DNA purification Kit (Epicenter, Madison, WI) from 50 ml culture grown at 30 ºC, 130 rpm shaking, and with 1 mM formate, 0.1 g/l yeast extract and the headspace composition of 50 % CO, 30 % N_2, 20 % CO_2 (v/v). The quality of the extracted DNA 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).

For genome assembling and annotation, the pipeline was comprised of: Ray [145] to generate an initial assembly, followed by Opera [146] for genome scaffolding, and CAP3 [147]

for assembling optimization. For Ray assembler, the optimal kmer size was calculated with KmerGenie [148].

3.6 Proteomics

Proteomics is a growing field of molecular biology and concerns to the large-scale of proteins. Proteomics provides a global view of the protein complement of biological systems and, in combination with other omics technologies, has a key role in helping uncover the mechanisms of cellular processes, being, therefore, a useful tool for the study microbial physiology, metabolism, and ecology.

3.6.1 Sample preparation for proteomics

Triplicate co-cultures of *Acetobacterium* sp. strain JM and *C. neopropionicum* were grown in 500 ml sealed bottles with 200 ml of liquid phase constituted by basal medium plus 1 mM formate, 0.1 g/l yeast extract, 20 mM acetate, under 50% CO, 30% N_2, and 20% CO_2 (v/v) of gas phase, at 30 ºC and 130 rpm shaking (after 3 days non-shaking). CO had been refilled along the experiment. Cultures were sampled at three different stages of growth: 4^{th}, 11^{th}, and 15^{th} day of incubation. Before cell harvesting by centrifugation, cultures were quickly cooled down on ice to decrease cell activity. Cell pellets were resuspended in 0.5 ml SDS-lysis buffer (100 mM Tris/HCl, pH 7.5; 4 % SDS (w/v)) plus 50 µl 1 mM PMSF (phenylmethylsulfonyl fluoride), sonicated 6 times (30 s pulse, 30 s rest) on ice. Cell debris was removed by centrifugation (13000 g, 10 min). Final protein concentration in samples obtained for LC-MS/MS analysis were assessed using Pierce BCA Protein Assay Kit (Life Technologies, Thermo Fisher Scientific), following the manufacturer instructions. Samples were subjected to protein separation using 12 % Mini-PROTEAN® TGX™ Precast Protein Gels, 12-well, 20 µl (Bio-Rad), loading 60 µg proteins per well. Reduction of cysteine disulfide bridges was performed by adding 50 mM NH_4HCO_3 plus 10 mM dithiothreitol, pH 8, and overnight incubation at room temperature. Reforming of disulfide bridges was prohibited via alkylation of reduced cysteines by adding 1 M Tris pH 8 plus 20 mM iodoacetamide pH 8, and subsequent incubation at room temperature in the dark with gentle shaking for one hour. After gel cutting, protein digestion was performed by adding a 5 ng/µl trypsin solution prepared in 1.5 mM ammonium bicarbonate, and subsequent overnight incubation at room temperature.

3.6.2 LC-MS/MS data acquisition

Peptides from the protein samples obtained from the three sets of biological triplicates were analyzed in duplicate using C18 reversed-phase liquid chromatography with online tandem mass spectrometry (LC-MS/MS). Measurements were performed using a nanoflow ultra-high-pressure liquid chromatograph (nano-Advance; Bruker Daltonics) coupled online to an orthogonal quadrupole time-of-flight mass spectrometer (maXis 4G ETD, otofControl v3.4 build 14; Bruker Daltonics) via an axial desolvation vacuum assisted electrospray ionization source (Captive sprayer; Bruker Daltonics). Five microliters of tryptic digest were loaded onto the trapping column (Acclaim PepMap 100, 75µm x 2 cm, nanoViper, 3µm 100Å C18 particles; Thermo Scientific) using 0.1 % FA at a flow rate of 9000 nl/min for 3 minutes at room temperature. Next, peptides were separated on a C18 reversed phase 15 cm length x 75 µm internal diameter analytical column (Acclaim PepMap RSLC, 75µm x 15 cm, nanoViper, 2µm 100Å C18 particles; Thermo Scientific) at 40 °C using a linear gradient of 3-35 % ACN 0.1 % FA in 120 minutes at a flow rate of 600 nl/min. The mass spectrometer was operated in positive ion mode and was tuned for optimal ion transmission in the range of m/z 300-1400. Electrospray ionization conditions were 3 l/min 180 °C N_2 drying gas, 1400 V capillary voltage and 0.4 Bar N_2 for gas phase supercharging (nanobooster) using acetonitrile as dopant. Parameters for optimal ion transmission were funnel RF: 400 Vpp, multipole RF: 400 Vpp, quadrupole ion energy: 5.0 eV, quadrupole low mass: 300 m/z, collision cell energy: 9.0 eV, collision cell RF: 3500 Vpp, ion cooler transfer time: 64 µs, ion cooler RF: 250 Vpp, pre-pule storage: 22 µs. Data-dependent acquisition of MS/MS spectra (AutoMSn) was performed using a 3 second duty cycle at 2 Hz acquisition rate for full MS spectra and a variable number of MS/MS experiments at precursor intensity scaled spectra rate (3Hz MS/MS spectra rate at 2000 counts, 20Hz MS/MS spectra rate @ 100.000 counts). Precursor ions within the range of 400-1400 m/z with charge state z = 2+ or higher (preferred charge state range of z = 2+ to z = 4+) were selected for MS/MS analysis with active exclusion enabled (excluded after 1 spectrum, released after 0.5 min, reconsidered precursor if current intensity/previous intensity >= 4, smart exclusion disabled). Spectra were saved as line spectra only and were calculated from profile spectra as the sum of intensities across a mass spectral peak (5 counts absolute threshold, peak summation width 7 points).

4. Results and Discussion

4.1 Physiological and microbial characterization of Ace-Syn enrichment culture

4.1.1 Starting point: Ace-Syn enrichment growth curve

The anaerobic mixed culture obtained as a result of enrichment series on syngas and acetate used as substrate have shown the ability to convert the syngas components CO and H_2 to CO_2, methane, acetate, and propionate (Figure 8 (a)). In the presence of acetate along with syngas, the enrichment produced CO_2, methane, and propionate but there was no acetate production (Figure 8 (b)). Syngas is reported to be consumed by acetogenic bacteria due to their ability to use the Wood-Ljungdahl pathway to grow in one-carbon substrates, such as carbon monoxide, carbon dioxide, or formate [149]. Acetate is a usual product of fermentation in acetogens [150], while acetate utilization is reported to occur in methanogenic pathways [151]. Propionate formation from syngas is highly uncommon to occur but it has already been reported occur as a by-product in an anaerobic sludge from a reactor treating wastewater [152].

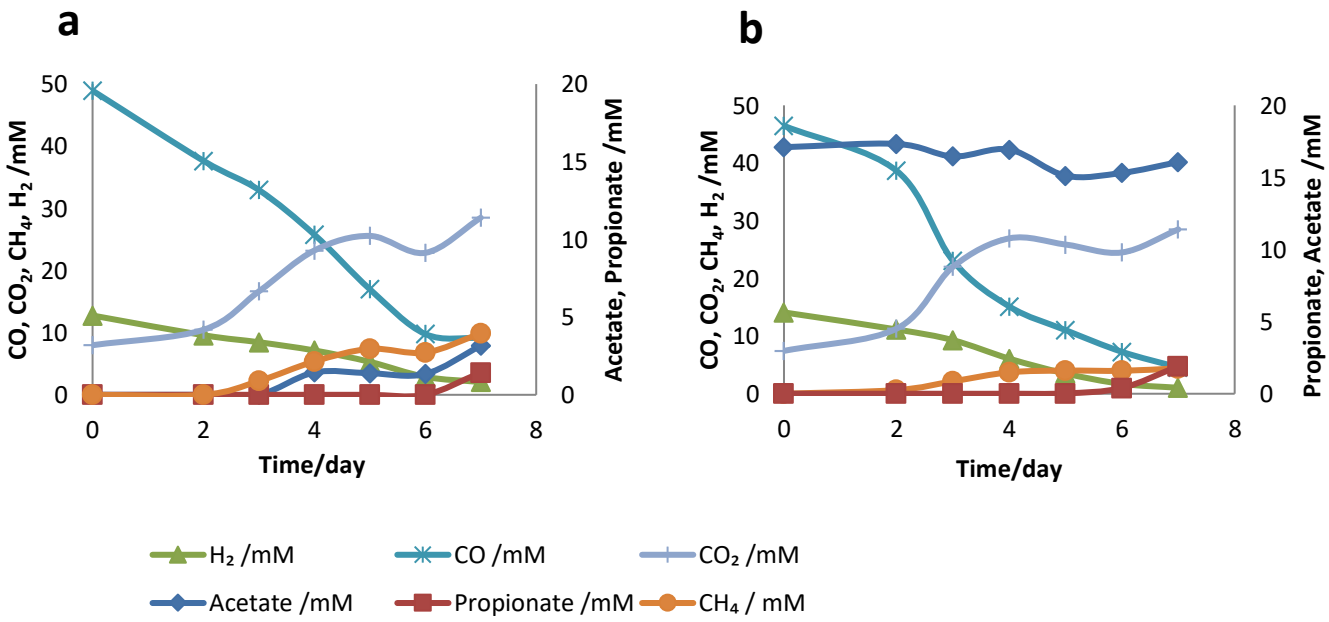


Figure 8. Batch growth of Ace-Syn enrichment culture under (a) syngas and (b) acetate plus syngas.

4.1.2 *Behaviour of Ace-Syn enrichment culture under different substrate combinations*

In order to figure out the critical intervener(s) and possible booster(s) for propionate production in this culture, different substrate combinations were tested, and its production profiles are shown in Figure 9. These results indicate that for this mixed culture, the growth on carbon monoxide as sole carbon and energy source is possible and leads to higher levels of propionate production along with lower methane and acetate production than growth on syngas. This way, the use of formate is not crucial to step into the methyl branch of the Wood-Ljungdahl pathway as compensation for a possible bottleneck created in the formate-formation step, as it happens in *Acetobacterium woodii* when cells are grown with CO [149]. Although, coupling formate with CO increases the acetogenesis but not the propionate production. The presence of acetate (20 mM) increases propionate production compared with the same conditions without acetate, but also increases the methane production, which indicates that methanogenic organisms in the culture use acetate as substrate. Glycerol shows a big positive effect on propionate production when coupled with syngas, syngas and acetate,

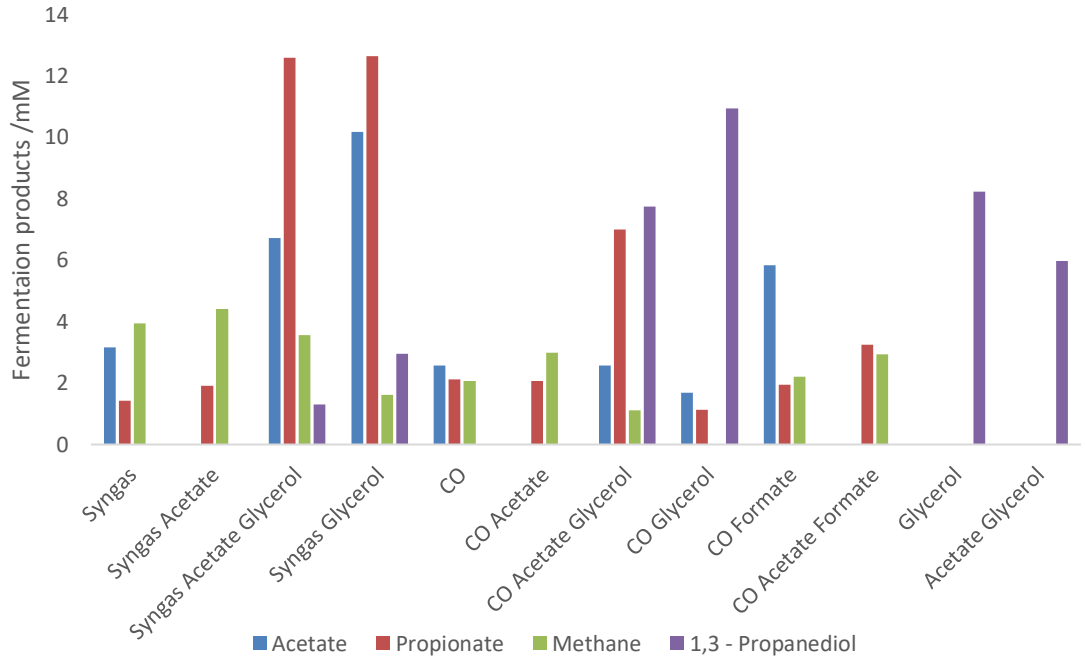


Figure 9. Product production per batch experiment for different substrate combinations. Headspace composition of bottles with syngas was 60% CO, 30% H_2, 10% CO_2 (v/v). Bottles with CO had the headspace composition of 60% CO, 40% N_2 (v/v). Bottles without gas substrate had were flushed with 100% N_2 (v/v). Liquid substrates (acetate, formate, glycerol) had the initial concentration of 20 mM. Experiments in sole acetate and formate are not shown because growth was not accomplished.

or with CO and acetate which reflects the potential of using glycerol as co-substrate to produce propionate. Along with propionate, 1,3-propanediol (1,3-PDO) is also a fermentation product. This reflects the ability of this enriched culture to ferment glycerol through both 1,3-PDO and 1,2-PDO-ethanol models [153]. While 1,3-PDO model provides an easy way to achieve redox balance through the oxidation of NADH released by the formation of cell mass [153], the 1,2-PDO-ethanol model provides the redox balance through the formation of 1,2-PDO, and also provides ATP generation through ethanol production [154], however in some microorganisms the use of this pathway is associated with low pH and CO_2 presence [155], which explains a bigger production ratio of propionate/1,3-PDO when using syngas rather than CO. In order to figure out the importance of CO for propionate production in this culture, growth in acetate and formate was attempted but not possible, while growth on sole glycerol and glycerol with acetate led to the production of 1,3-PDO, which shows that in this culture, propionate production is strictly connected with CO conversion (Figure 9).

4.1.3 Microbial characterization of Ace-Syn enrichment

Attempting to correlate the activity of the Ace-Syn enriched culture with the responsible microorganisms, microbial community analysis was performed by 16S rRNA sequencing. Resulting sequences were aligned with GenBank database [144] using the NCBI BLAST search tool. Results in Figure 10 are represented per taxonomic genus for bacterial and archaeal domains. The main bacterial genus present in the Ace-Syn culture is *Acetobacterium* which allows the association of CO conversion to acetogens. The high rated blast hits for *Acetobacterium* species were *A. wieringae* DSM 1911 (99 % 16S rRNA gene identity), *A. malicum* DSM 4132 (98 % 16S rRNA gene identity), and *A. woodii* DSM 130 (97 % 16S rRNA gene identity). *A. wieringae* and *A. malicum* are not reported to grow on CO [156, 157], while *A. woodii* can grow on CO in combination with H_2/CO_2 or formate as a co-substrate, but not

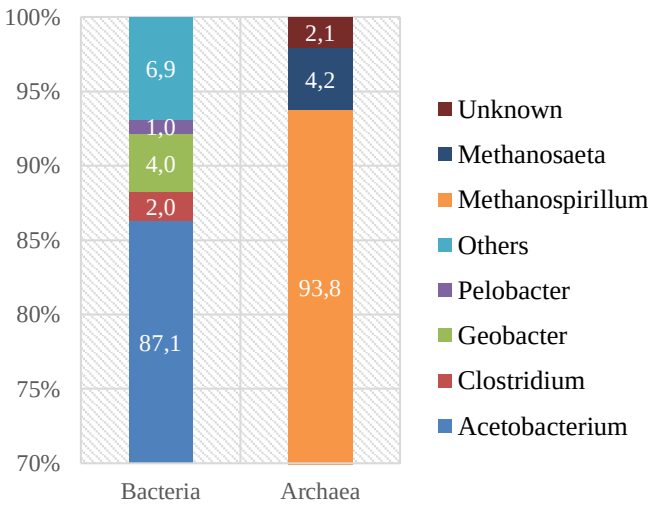


Figure 10. Microbial community analysis of the Ace-Syn enriched culture. Bacterial and archaeal analysis were performed for the same biological sample.

on CO as sole carbon and energy source [149]. Propionate production is also not reported to occur using *Acetobacterium* species. Moreover, some bacterial species present in the culture from the genus *Pelobacter* and *Clostridium* have reported activity on propionate production, namely *P. propionicus* DSM 2379 (92 % 16S rRNA gene identity), *C. propionicum* JCM 1430 (99 % 16S rRNA gene identity), *C. propionicum* strain X2 (99 % 16S rRNA gene identity), and *C. neopropionicum* DSM 3847 (97 % 16S rRNA gene identity). *P. propionicus* produces propionate from 2,3-butanediol, acetoin ethanol, lactate and mixtures of acetate and propanol or butanol [158]. In *C. propionicum*, propionic acid fermentation occurs with alanine, lactate, pyruvate, acrylate, serine, or threonine [159]. By its turn, *C. neopropionicum* can ferment ethanol,

threonine, alanine, cysteine, serine, and glucose to propionate [122]. The propionate producing activity from C_2 compounds of these microorganisms coupled with homoacetogenic activity might be crucial for the propionate production from syngas/CO by this Ace-Syn enriched culture. Similar propionate formation from C_2 units was found in freshwater isolates and other physiologically-related bacteria described [160, 161], while propionic acid formation from H_2/CO_2 was also shown to occur in mixed anaerobic populations [162].

The archaeal community analysis in this culture showed the dominance of *Methanospirillum* genus, namely *M. hungatei* JF-1 (99 % 16S rRNA gene identity), as well as the minority presence of *Methanosaeta* genus, namely *M. concilli* GP6 (99 % gene identity). *M. hungatei* is a methanogen that usually grows in an atmosphere with H_2/CO_2 and it can use formate or acetate as a carbon source [163]. *M. concilli* can grow on acetate as sole energy source, and on acetate or CO_2 as carbon source [164]. Therefore, these microorganisms might be responsible for the methane production in this Ace-Syn enrichment.

4.2 Isolation of *Acetobacterium* sp. strain JM and its physiological characterization

From the Ace-Syn enrichment culture, an acetogenic bacterium was isolated (*Acetobacterium* sp. strain JM) through dilution series with carbon monoxide and 1mM of formate as carbon and energy sources. After four dilution series, where transfers were performed from the most diluted bottle with visible growth (1^{-4}, 1^{-4} ,1^{-5}, and 1^{-6}), short rods were the only shape of bacteria identified by phase contrast microscopy. After purification by growth in solid medium and consequent colony picking, pure liquid cultures growing in carbon monoxide were obtained. 16S rRNA gene sequencing was further performed and results show 99 % of 16S rRNA gene identity to *Acetobacterium wieringae* DSM 1911. Same isolation results were obtained through a different approach using ethanol as substrate.

These results represent the first report of the *Acetobacterium wieringae* as a carboxidotrophic bacterium. The use of ethanol as sole carbon and energy source was already reported to be possible for *A. wieringae* [165] and it is confirmed again in the present work.

The growth of the isolated *Acetobacterium* sp. strain JM on CO-acetate and CO-acetate-glycerol was also performed (Figure 11). Headspace composition was set to 50 % CO, 30 % N_2, and 20 % CO_2 (v/v) before inoculation (total pressure 170 kPa). CO was refilled along the growth experiments; the drop on CO_2 concentration along the growth plots results from the process of refilling the bottles with CO, where bottles were depressurized to 100 kPa and subsequently pressurized with to a final total pressure of 170 kPa. Despite the presence of other carbon sources such as acetate and glycerol, CO was continuously consumed in both experiments. In contrast to the conditions used with the Ace-Syn enrichment, under 30 ºC and 130 rpm shaking, *Acetobacterium* sp. strain JM becomes highly active on CO conversion at the third day of incubation, being able to keep the same CO conversion rate for several days, causing four CO depletions in 7 days which results in a total of 5 mmol of CO conversion in each experiment. On CO-acetate *Acetobacterium* sp. strain JM yields acetate and CO_2 upon carbon monoxide conversion, while with CO-acetate-glycerol acetate, ethanol, 1,3-PDO, and CO_2 are the products of carbon monoxide and glycerol conversion. Glycerol is consumed simultaneously with CO till the first CO depletion but after that glycerol concentration keeps stable at a low value while CO conversion keeps highly active, which shows a preference of *Acetobacterium* sp. strain JM for CO rather than glycerol. At this phase where glycerol

consumption is stopped, ethanol appears as a product of CO and/or acetate conversion (Figure 11 (b)). This indicates that ethanol production in *Acetobacterium* sp. strain JM is linked with the Wood-Ljungdahl pathway and it occurs via acetyl-CoA and acetaldehyde, or via acetate and acetaldehyde routes [166]. Since acetate concentration decreases when ethanol is produced, ethanol production might occur via an acetate highly energy-consuming reduction to its corresponding aldehyde, representing an endergonic reaction [167]. Although some microorganisms like *P. furiosus* can catalyze this reaction using reduced ferredoxin as an electron donor [168]. With the use of glycerol, 1,3-PDO appears as a fermentation product.

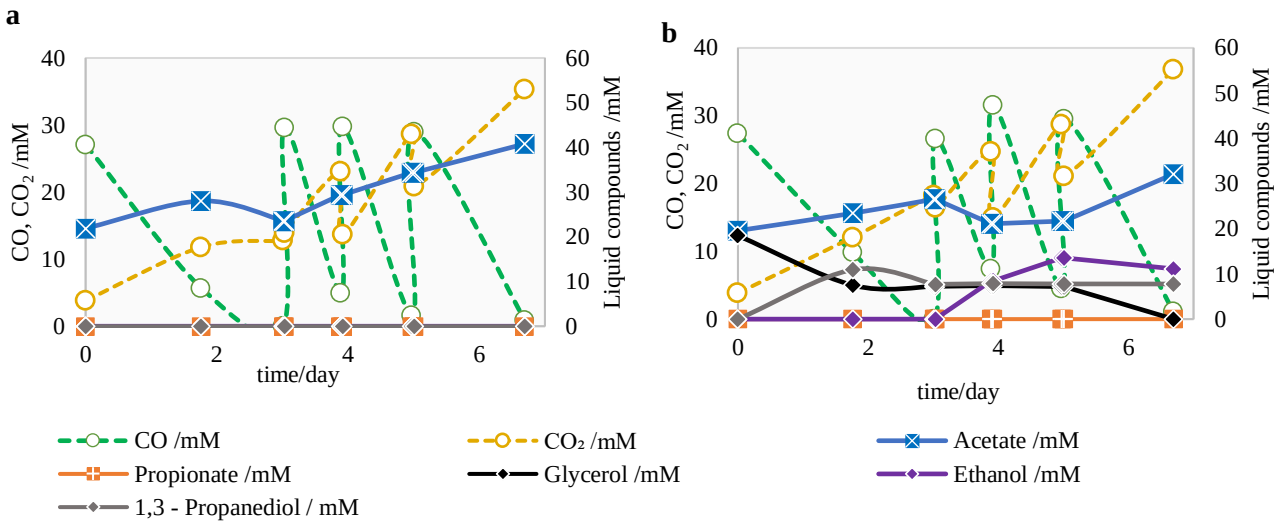


Figure 11. Semi-batch growth of *Acetobacterium* sp. strain JM under two different substrate combinations. (a) Growth in CO and acetate **(b)** Growth in CO, acetate and Glycerol.

In order to compare *Acetobacterium* sp. strain JM physiologically with *Acetobacterium* relatives, *A. wieringae* DSM 1911 and *A. woodii* DSM 1030 were grown in CO-acetate and CO-acetate-glycerol (Figure 12). These strains were not capable of converting CO at the same rate of the isolated *Acetobacterium* sp. strain JM: *A. wieringae* DSM 1911 did not reach any substrate depletion on CO-acetate-glycerol and consumed 2.15 mmol of CO in 11 days on CO-acetate (Figure 12 (a1) and (b1)), while *A. woodii* DSM 1030 was able to convert 2.47 mM of CO in 11 days on CO-acetate-glycerol, and 3.93 mM of CO in 7 days on CO-acetate (Figure 12 (a2) and (b2)). The resulting products in these strains were acetate, CO_2, and 1,3-PDO (when using glycerol), and no ethanol production was observed. These results show that the isolated *Acetobacterium* sp. strain JM has physiological differences from its closest relative, *A.*

wieringae DSM 1911, which can be explained by genomic differences or by an adaptation to CO-acetate environments during the previous enrichment process.

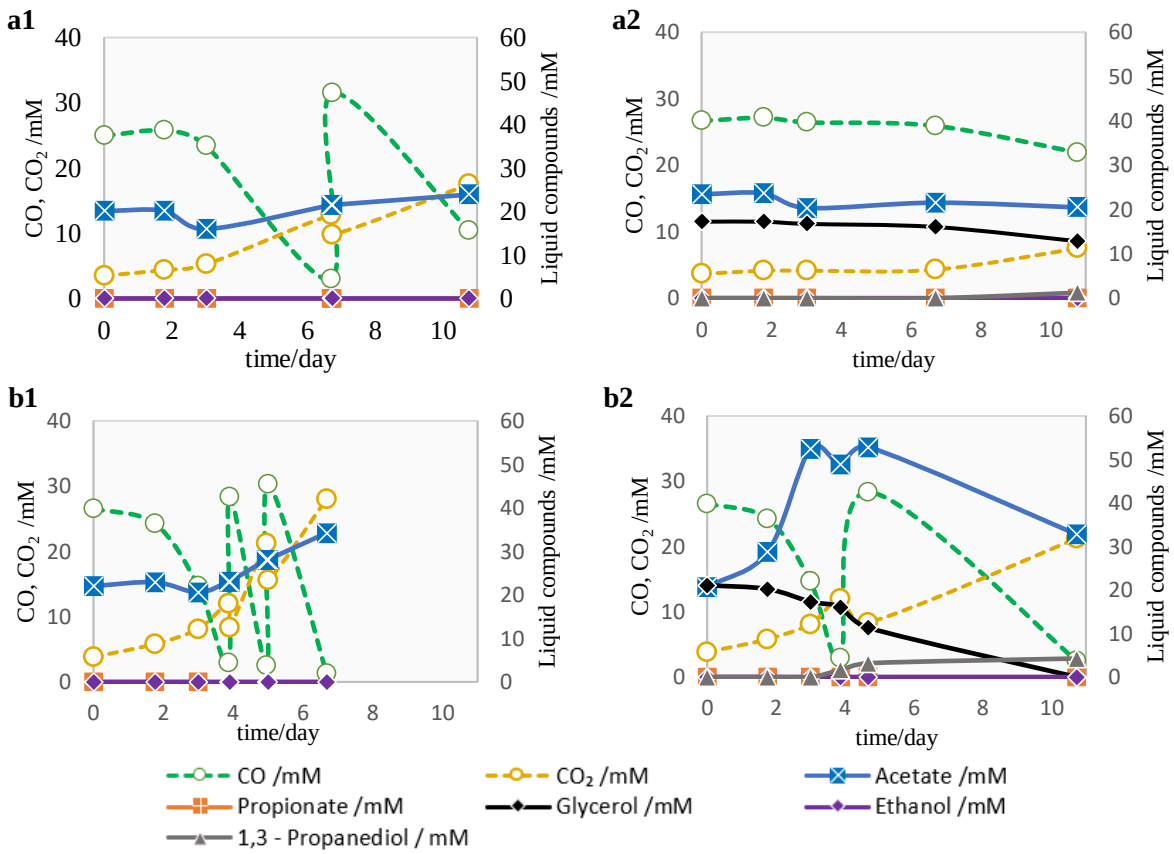


Figure 12. Semi-batch growth of (a) *A. wieringae* DSM 1911 and (b) *A. woodii* DSM 1030 under two different substrate combinations. (1) CO and acetate (**2**) CO, acetate and glycerol.

4.3 Defined co-cultures of CO consumers with propionate producers

4.3.1 *Defined co-cultures building*

Knowing that ethanol can, in specific conditions, be produced from CO by *Acetobacterium* sp. strain JM, synthetic co-cultures of this acetogen and *A. wieringae* DSM 1911 (*A. wieringae*^{T}) with *Pebolacter propionicus* or *Clostridium neopropionicum* were built to attempt the production of propionate from carbon monoxide. Their growth is represented in Figure 13. The last two microorganisms have the ability to produce propionate from ethanol, *P. propionicus* uses the succinate-methylmalonyl CoA pathway [114], while *C. neopropionicum* uses the acrylate pathway [113].

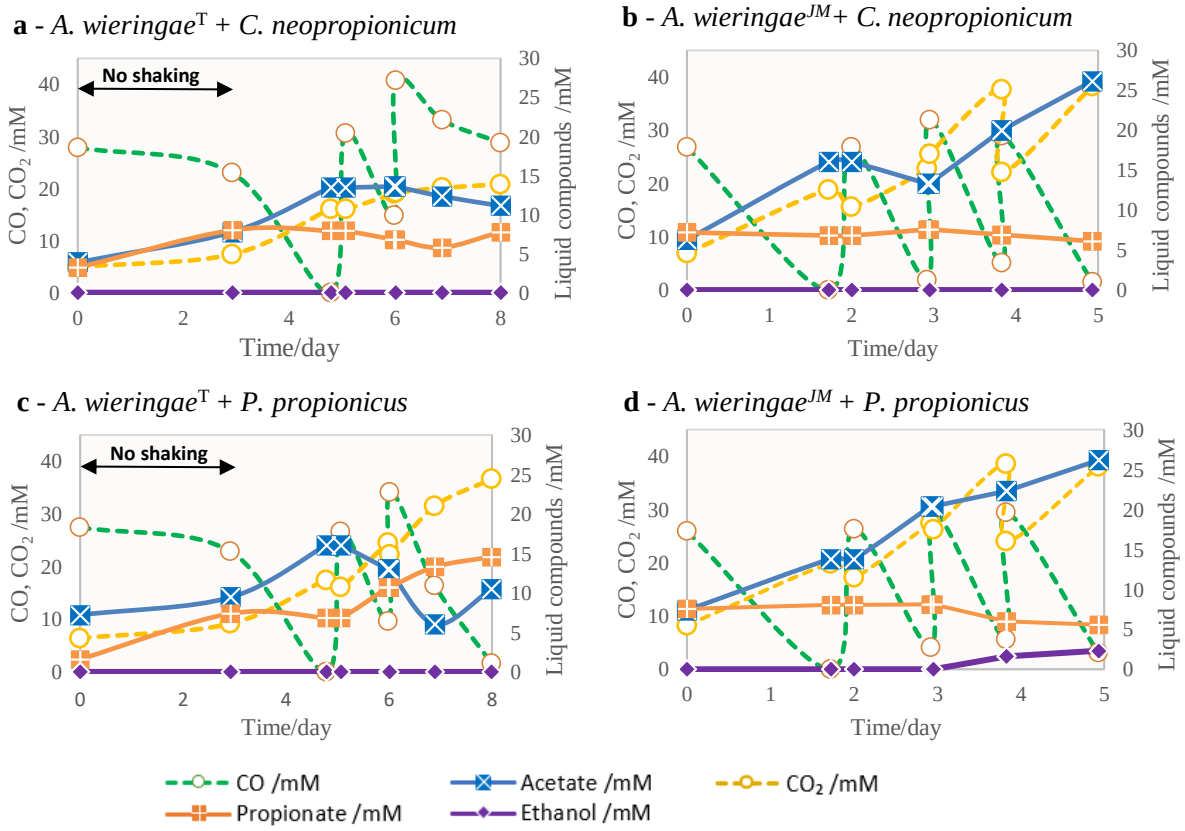


Figure 13. Semi-batch growth of defined co-cultures (a) *A. wieringae*^{T} with *C. neopropionicum*. **(b)** *Acetobacterium* sp. *strain JM* with *C. neopropionicum*. **(c)** *A. wieringae*^{T} with *P. propionicus*. **(d)** *Acetobacterium* sp. *strain JM* with *P. propionicus*.

Considering the yield of propionate in these defined co-cultures, propionate production from CO was accomplished using *A. wieringae*^{T} with either *C. neopropionicum* or *P. propionicus* yielding respectively 4.6 mM and 13.0 mM of propionate. For the other side, co-cultures containing the isolated *Acetobacterium* sp. strain JM did not show a positive

propionate production yield, acetate was the major product of CO conversion. However, these parallel experiences using *A. wieringae*^{T} or *Acetobacterium* sp. strain JM had two setup differences that might explain these results: three days of incubation without shaking for *A. wieringae*^{T} co-cultures and higher propionate concentration at the beginning of the experience which resulted from the monoculture growth of *P. propionicus* or *C. neopropionicum* before mixing into co-cultures.

4.3.2 *Growth of stable defined co-cultures on CO-Acetate and CO-Acetate-Glycerol*

The built defined co-cultures were submitted to stability pressure by inoculating them to fresh medium and their growth on CO-acetate and CO-acetate-glycerol was tested for propionate production (Figure 14 and Figure 15). Cultures were exposed to agitation throughout the incubation time. The resultant growth curves were very different from the ones immediately after mixing the two pure cultures for co-culture construction. Co-cultures containing *A.wieringae*^{T} did not produce propionate and the activity on CO conversion was

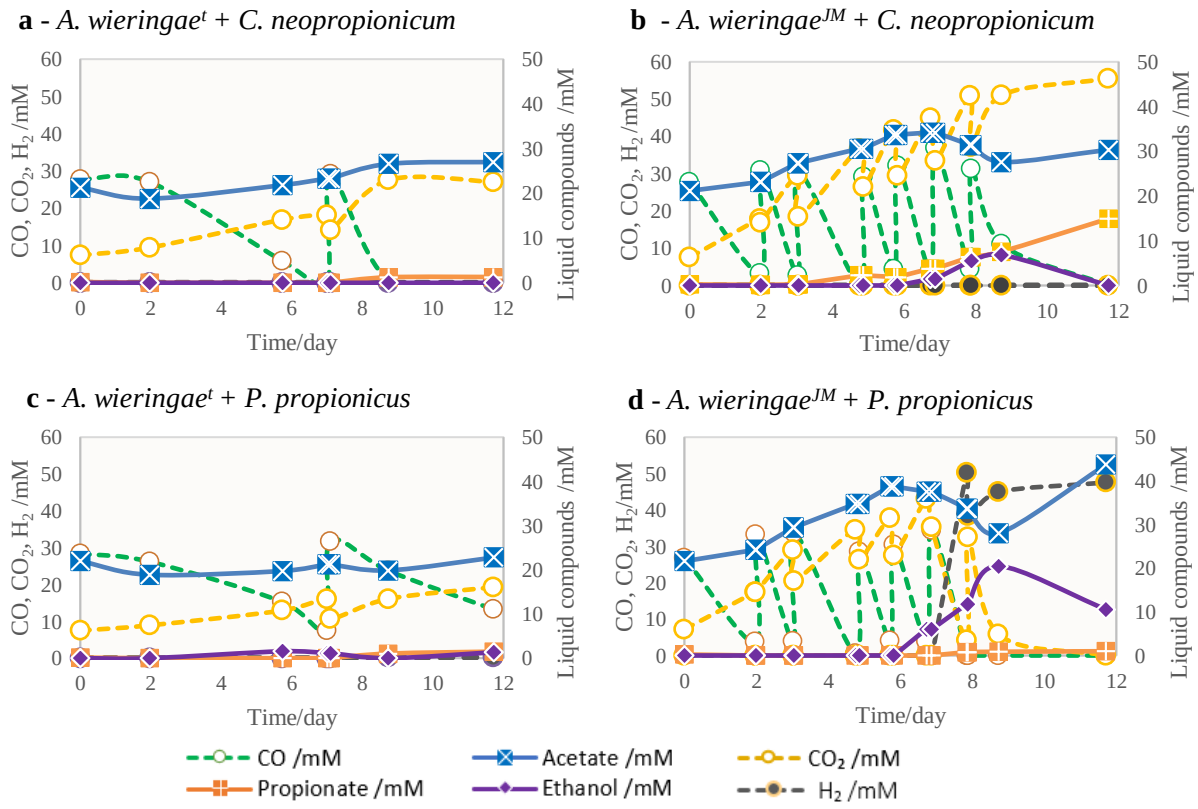


Figure 14. Semi-batch growth of stable defined co-cultures on CO-Acetate (a) *A. wieringae*^{T} with *C. neopropionicum*. **(b)** *Acetobacterium* sp. *strain JM* with *C. neopropionicum*. **(c)** *A. wieringae*^{T} with *P. propionicus*. **(d)** *Acetobacterium* sp. *strain JM* with *P. propionicus*.

low (Figure 14 (a) and (c)) which confirms that this *A. wieringae*^{T} does not grow well on CO-acetate environments, as shown in Figure 12 (a1).

On the other hand, cultures containing *Acetobacterium* sp. strain JM were highly active on CO conversion on a CO-acetate environment. In the co-culture constituted by *Acetobacterium* sp. strain JM and *P. propionicus*, ethanol and acetate were the main products of CO conversion while propionate concentration was lower than 1 mM (Figure 14 (d)), which means that the experience conditions did not allow *P. propionicus* to survive or to be metabolic active since its preferential substrate, ethanol, have been accumulated.

Propionate was the major CO conversion product in the co-culture defined by *Acetobacterium* sp. strain JM and *C. neopropionicum* reaching a final concentration of 14.9 mM (Figure 14 (b)). During the growth of this culture, acetate starts as the main product of CO conversion, but its concentration drops once ethanol starts to accumulate. At this point, the metabolic activity of *C. neopropionicum* might be exponentially increased, leading to the propionate production.

Concerning to the experiments on a CO-acetate-glycerol environment, co-cultures constituted with *A. wieringae*^{T} did not grow, which confirms that this strain does not grow on CO-acetate-glycerol environments (Figure 12 (a2)). For their turn, co-cultures containing *Acetobacterium* sp. strain JM did grow on CO-acetate-glycerol (Figure 15), consuming CO and glycerol at a high rate during the first days of growth. Glycerol was not depleted, being CO the preferred carbon source for *Acetobacterium* sp. strain JM during the entire experiment. Glycerol fermentation to 1,3-PDO occurred in both co-cultures. The co-culture constituted by

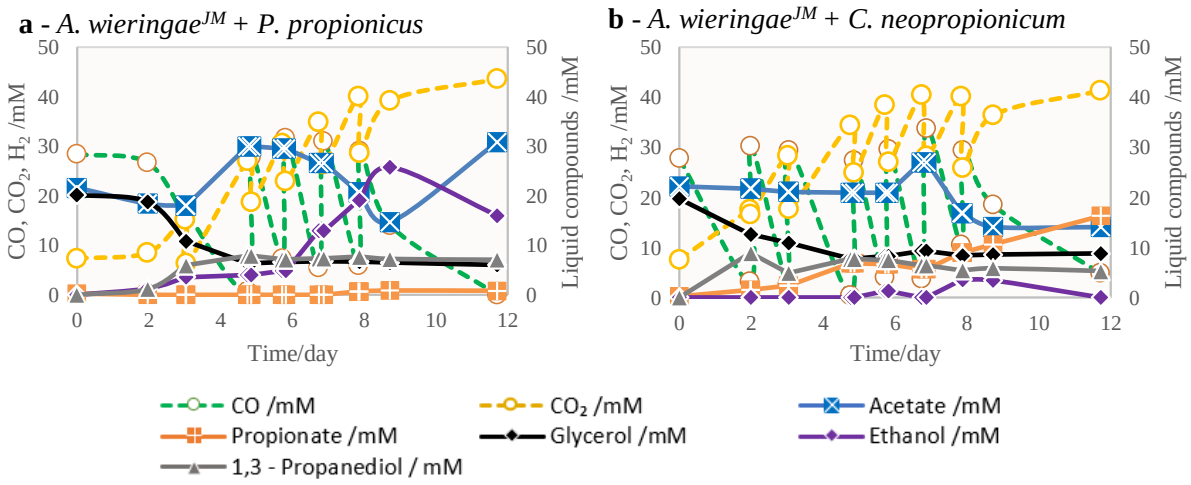


Figure 15. Semi-batch growth of stable defined co-cultures on CO-Acetate-Glycerol (a) *Acetobacterium* sp. *strain JM* with *P. propionicus* **(b)** *Acetobacterium* sp. *strain JM* with *C. neopropionicum*.

Acetobacterium sp. strain JM and *P. propionicus* did not produce propionate (Figure 15 (a)) but the production of ethanol was higher than in the condition represented in Figure 14 (d) which might be associated with the presence of glycerol as an additional substrate. The co-culture constituted by *Acetobacterium* sp. strain JM and *C. neopropionicum* once again showed propionate production reaching a final concentration of 16.3 mM (Figure 15 (b)).

4.3.3 *Physiological characterization of Acetobacterium* sp. strain JM *in co-culture with C. neopropionicum*

The co-culture with best activity and stability on propionate production from carbon monoxide was constituted by *Acetobacterium* sp. strain JM and *C. neopropionicum.* The growth of this co-culture was performed in a CO-acetate environment for proteomic analysis and its behavior is presented in Figure 16 a). Due to the use of bottles with bigger gas phase than the bottles used before, shaking of the culture at 130 rpm was started at the third day of growth, allowing *Acetobacterium* sp. strain JM to overcome its lag phase and become active on CO conversion, avoiding a possible noxious effect on *C. neopropionicum* caused by an accumulation of CO into the liquid phase due to agitation.

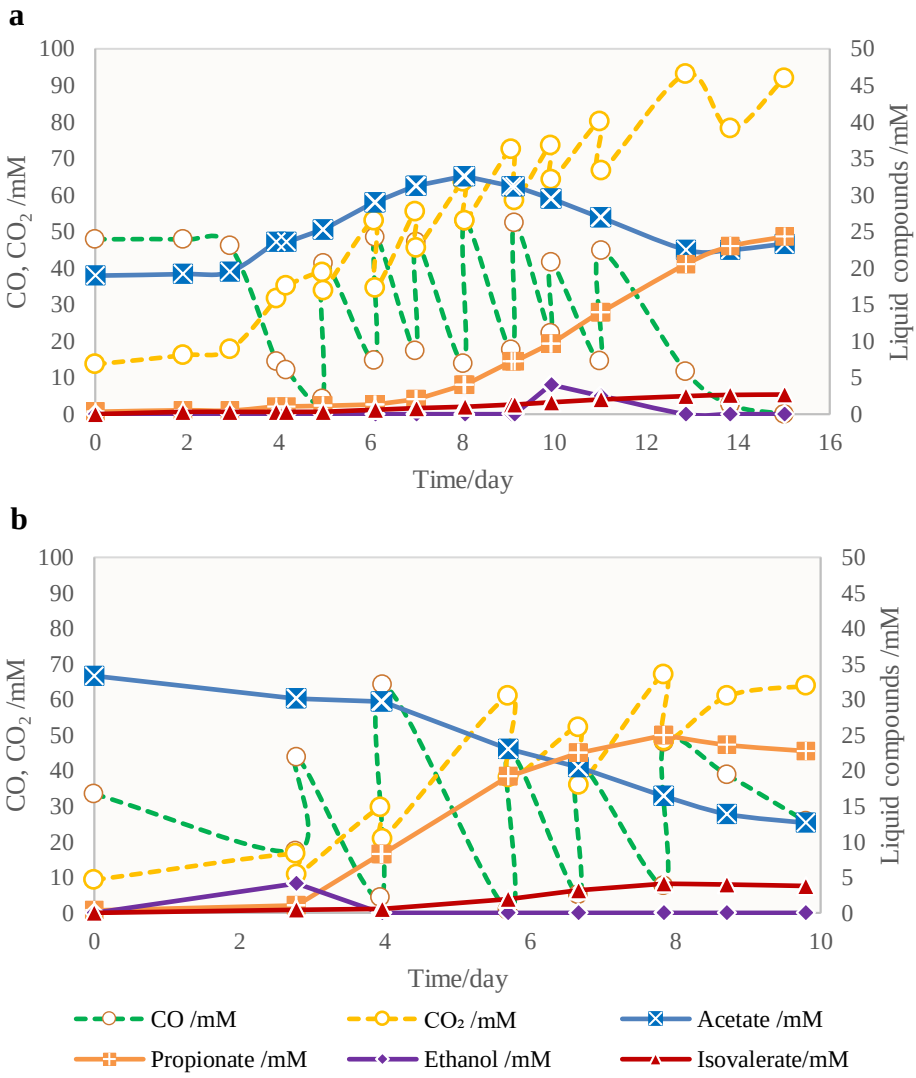


Figure 16. Semi-batch growth of *Acetobacterium* sp. *strain JM* in co-culture with *C. neopropionicum*. (a) Initial acetate concentration of 20 mM. **(b)** Initial acetate concentration of 30 mM.

CO has been refilled throughout the experiment and the total amount of CO converted in 15 days of growth was 54.7 mmol. Till the 8th day of growth, this co-culture produced mainly acetate, reaching the concentration of 32 mM. Knowing that acetate concentration at the beginning of the experiment was 20 mM, a 12 mM yield of acetate was obtained during this acetogenic phase, while propionate emerged as a by-product reaching the concentration of 4 mM at the 8th day. However, during the following days, acetate started to be degraded and propionate production increased exponentially, reaching a final concentration of 24 mM after 15 days of growth, having CO being continuously converted.

This data indicates that *Acetobacterium* sp. strain JM converts CO to ethanol/acetate and acetate to ethanol, while *C. neoprionicum* produces propionate from ethanol. The presence of ethanol is only detected at a low concentration at the 10th and 11th days (Figure 16 (a)). Since ethanol is the only substrate available for *C. neopropionicum*, it might be consumed at the same rate that it is being produced.

Isovalerate was also formed as a by-product at a final concentration of 3 mM. It might be a product of *C. neoprionicum* metabolism since *Clostridium spp.* have reported activity on isovalerate production in contrast with *Acetobacterium spp.* [169].

Figure 16 (b) represents the growth of an experiment with the co-culture *Acetobacterium* sp. strain JM and *C. neopropionicum* where 30 mM of acetate were used at the beginning of the experiment, instead of the usual 20 mM. This condition led *Acetobacterium* sp. strain JM to instantly produce ethanol from CO and acetate, skipping the acetogenic phase shown in Figure 16 (a), which resulted in a high propionate production rate from the 3rd day of incubation.

This confirms that the standard growth of this co-culture of propionate production from CO, represented in Figure 16 (a) is divided into two phases, based on the metabolic activity of *Acetobacterium* sp. strain JM: the acetogenic growth, and the non-acetogenic growth. The switch point between these two metabolic behaviors depends on the acetate concentration of (30-40) mM. Figure 17 represents a scheme that illustrates this metabolic switch. Acetate switch has been best reported in Escherichia coli, it refers to the transition from acetate production to acetate utilization, occurring when carbon sources such as D-glucose or L-serine are depleted in the acetate-producing environment and cells begin to scavenge for the previously produced and excreted acetate [170]. However, the acetate switch reported here is not driven by substrate depletion.

During the phase 1, the acetogenic growth, *Acetobacterium* sp. strain JM converts CO to acetate gaining one ATP per each acetate molecule formed. Although when acetate concentration reaches 30 mM, acetate formation might be no longer favorable and for that reason *Acetobacterium* sp. strain JM initiates the non-acetogenic growth by driving CO conversion to ethanol and by converting acetate to ethanol using CO as a reductant. The conversion of organic acids to their respective alcohols using CO as a reductant was already reported to be possible [168, 171]. In these reports CODH gene was inserted in order to allow the use of CO as a reductant in the conversion of organic acids to alcohols, since *Acetobacterium* sp. strain JM has the CODH gene naturally in its genome because performs CO conversion without being genetically engineered, it might also use this mechanism to convert acetate to ethanol. After ethanol starts to be produced during phase 2, *C. neopropionicum* can convert the ethanol to propionate (Figure 17).

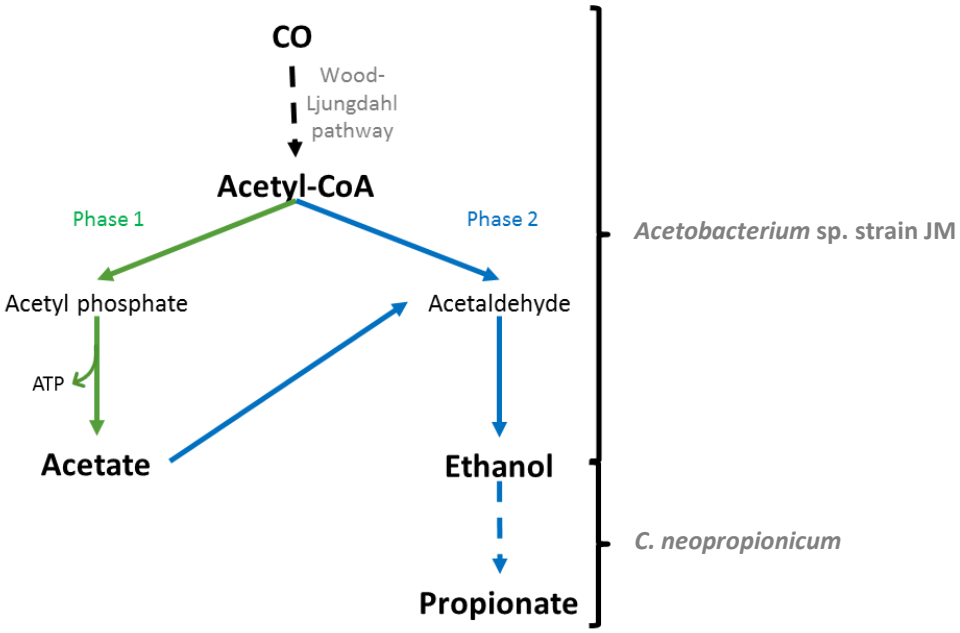


Figure 17. Representation of the metabolic switch during the growth of the co-culture *Acetobacterium* sp. strain JM and *C. neopropionicum*

4.4 Proteomic analysis and pathways of propionate production from CO by *Acetobacterium* sp. strain JM and *C. neoproprionicum*

Using quantitative mass-spectrometry, significant changes in protein abundance at the 4th, 11th and 15th days of the growth of the defined co-culture *Acetobacterium* sp. strain JM and *C. neopropionicum* were detected. The comparison statistical tests were performed in groups of two to detect metabolic differences among the acetogenic phase (4th day) and the solventogenic (ethanol)/ propionogenic phases (11th and 15th days). A total of 1616 proteins were detected and quantified, 989 were identified as proteins belonging to *Acetobacterium* sp. strain JM, and 517 to *C. neopropionicum*.

To identify the proteins, genome sequences were accessed. Since *Acetobacterium* sp. strain JM was isolated in this work, genome sequencing, assembling and annotation was performed, resulting in a final size of 3.668.938 bp with 33 scaffolds.

Figure 18 represents a 2-sample t-test to assess differential expression between the 4th and 11th days of growth. The most statically significant differences were from proteins with big sample variability. This Figure 18 also shows that *Acetobacterium* sp. strain JM was expressing much more proteins than *C. neopropionicum* at the 4th day of growth, confirming the acetogenic behavior of the co-culture at the beginning of the experiment. On the 11th day of growth, proteins belonging to *C. neopropionicum* were being highly expressed, confirming

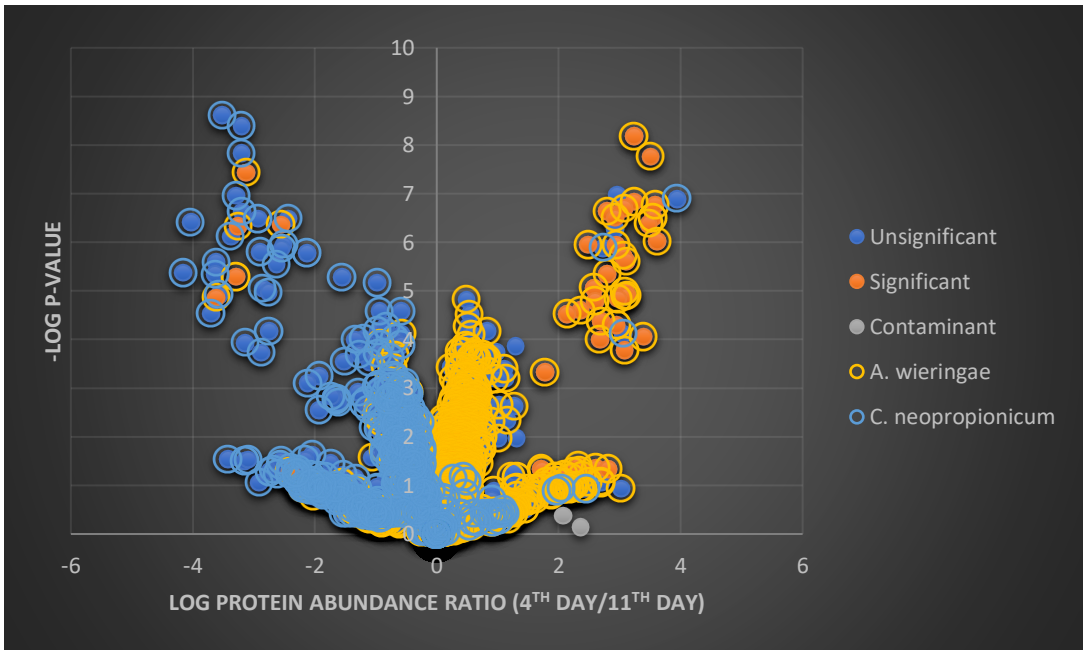


Figure 18. Volcano plot showing the estimated fold changes between the 4th and 11th day of growth (x-axis) versus the -log10 p-values (y-axis) for each protein.

the solventogenic (ethanol) / propionogenic behavior of the co-culture since *C. neopropionicum* converts ethanol to propionate.

Figure 19 shows the differential expression between the 4th and 15th days of growth. A lot of significant differences were identified for proteins with big sample variability but also with small sample variability. On the 15th day of growth, proteins with significant differences belonging to *C. neopropionicum* were highly expressed, confirming the solventogenic (ethanol) / propionogenic behavior of the co-culture at the end of the experiment, matching with the high propionate concentration obtained.

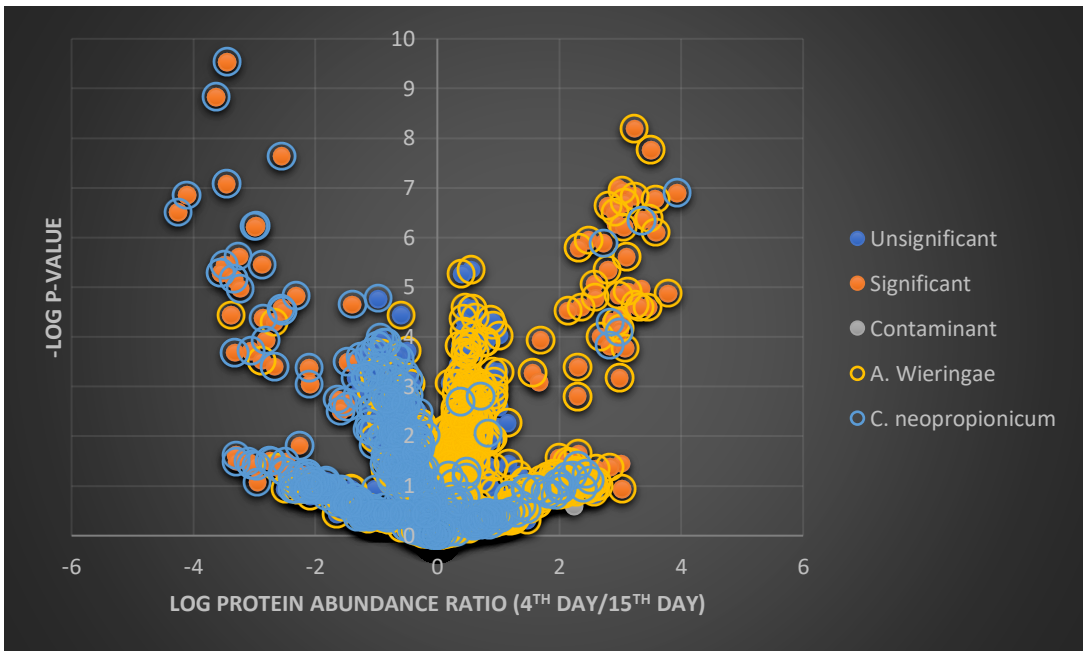


Figure 19. Volcano plot showing the estimated fold changes between the 4th and 15th day of growth (x-axis) versus the -log10 p-values (y-axis) for each protein.

Figure 20 lists the relevant identified proteins involved in the pathways which drives CO to propionate in the defined co-culture *Acetobacterium* sp. strain JM and *C. neopropionicum*. The presence of proteins such as carbon monoxide dehydrogenase, carbon monoxide dehydrogenase/acetyl-CoA synthase complex, formate dehydrogenase, and methylenetetrahydrofolate reductase are involved in the Wood-Ljungahl pathway, driving CO to acetyl-CoA. The presence of acetate kinase and alcohol dehydrogenases confirms that *Acetobacterium* sp. strain JM is involved in acetate conversion to ethanol as discussed above. Belonging to *C. neopropionicum*, the presence of alcohol dehydrogenases confirms the consumption of ethanol as a substrate by this microorganism, and the presence acryloyl-CoA reductase, D,L-lactate dehydrogenases, acetate kinase, and acetate CoA-transferase indicates

that the acrylate pathway proposed by Tholozan J. L. et al. [113], is used by *C. neopropionicum* in this co-culture to produce propionate.

Acetobacterium sp. **strain JM**	**EC number**	***C. neopropionicum***	**EC number**
Acetate Kinase	2.7.2.1	Pyruvate synthase	1.2.7.1
Acetyl-coA carboxylase	6.4.1.2	Phosphate acetyltransferase	2.3.1.8
Acetyltransferase (GNAT)	-	L-lactate dehydrogenase	1.1.1.27
Alcohol dehydrogenase 2	1.1.1.1	D-lactate dehydrogenase	1.1.1.28
Aldehyde oxidoreductase	1.2.99.7	Aldehyde-alcohol dehydrogenase	-
Aldehyde-alcohol dehydrogenase	-	Alcohol dehydrogenase 2	1.1.1.1
Carbon monoxide dehydrogenase	1.2.7.4	Alcohol dehydrogenase	1.1.1.4
Carbon monoxide dehydrogenase/acetyl-CoA synthase	2.3.1.1.169	Acyl-CoA dehydrogenase	1.3.99.-
Formate dehydrogenase	1.2.1.2	Acryloyl-CoA reductase	-
Formate-tetrahydrofolate ligase	6.3.4.3	Acetate kinase	2.7.2.1
Methylenetetrahydrofolate reductase	1.5.1.20	Acetate CoA-transferase	2.8.3.8
Periplasmic [Fe] hydrogenase	1.12.7.2	3-isopropylmalate dehydratase	4.2.1.33

Figure 20. Relevant identified proteins belonging to the microbes *Acetobacterium* sp. strain JM and *C. neopropionicum* and corresponding EC numbers.

The overall CO fermentation reaction of this co-culture (eq. 1) is based on the carbon monoxide conversion to ethanol by *Acetobacterium* sp. strain JM (eq. 3) after acetogenic growth (eq.2) and further ethanol conversion to propionate by *C. neopropionicum* (eq. 4).

$$CO \xrightarrow{A.wieringae^{JM}} C_2H_5OH \xrightarrow{C.neopropionicum} C_3H_5O_2 \qquad \textbf{Equation (1)}$$

$$4\,CO + 2\,H_2O \xrightarrow{\Delta G^0 = -174\,kJ} CH_3COO^- + H^+ + 2\,CO_2 \qquad \textbf{Equation (2)}$$

$$6\,CO + 3\,H_2O \xrightarrow{\Delta G^0 = -224\,kJ} C_2H_5OH + 4\,CO_2 \qquad \textbf{Equation (3)}$$

$$3\,C_2H_5OH + 2\,HCO_3^- \xrightarrow{\Delta G^0 = -124\,kJ} 2\,C_3H_4O_2^- + CH_3COO^- + H^+ + 3H_3O \qquad \textbf{Equation (4)}$$

The proposed pathway that drives propionate production from carbon monoxide is represented in Figure 21. It couples the Wood-Ljungdahl pathway (Figure 2) with the acrylate pathway (Figure 7 (b)).

The Wood-Ljungdahl pathway was based on the acetogenic carbon monoxide conversion by *Acetobacterium woodii* [172] and adapted to ethanol production based on the solventogenic growth on CO *by Clostridium ljungdahlii* [172]. It's proposed here that the

isolated *Acetobacterium* sp. strain JM and *Clostridium ljungdahlii* are physiologically equivalent in what it concerns to solventogenic growth on CO. The enzyme aldehyde oxidoreductase is known for using ferredoxin to reduce acetate to acetaldehyde and was shown to be expressed in CO-grown *Clostridium ljungdahlii* [173], and is also shown here to be expressed by *Acetobacterium* sp. strain JM on CO growth (Figure 20). The expression of this enzyme together with an acetaldehyde/alcohol dehydrogenase complex (Figure 20) confirms that during the solventogenic phase, *Acetobacterium* sp. strain JM forms ethanol via acetaldehyde from directly acetyl-CoA after CO assimilation and indirectly via acetate reduction (Figure 21). The final ethanol formation step from acetaldehyde is catalyzed by the enzyme alcohol dehydrogenase 2 (Figure 20), utilizing additional reducing equivalents such as NADH or NADPH [174].

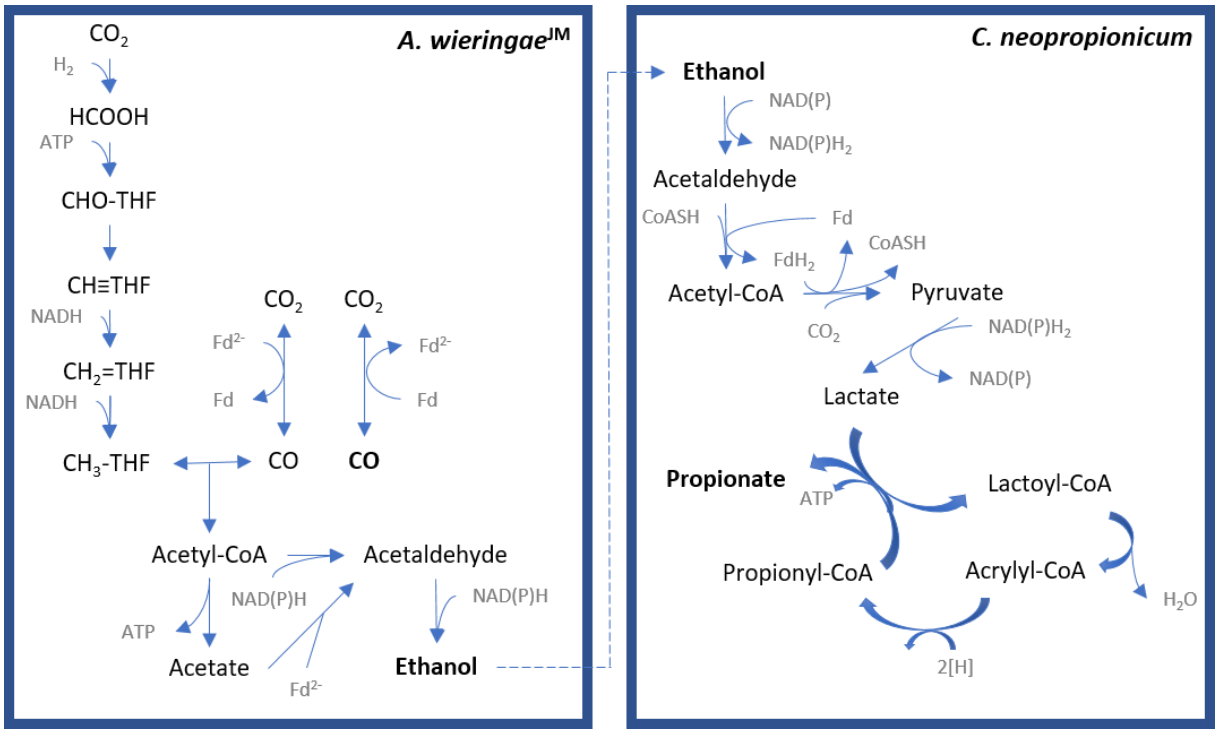


Figure 21. Proposed pathway of propionate production from carbon monoxide.

In the acrylate pathway, as previously described by Tholozan J. L. et al. [113], *C. neopropionicum* shows the presence of two alcohol dehydrogenases (Figure 20), one of them a NADP-dependant highly specific ethanol dehydrogenase which catalyzes the oxidation of ethanol to acetaldehyde. In its turn, acetaldehyde yields acetyl-CoA, pyruvate and lactate (Figure 21). This last step is catalyzed by a D-, or L- lactate dehydrogenase (Figure 20) which was already reported to be involved in propionate formation in *C. neopropionicum* [113] and

also in *Peptostreptococcus elsdenii* [175]. Although Tholozan J. L. et al. suggests a propionyl-CoA dehydrogenase to catalyze the formation of propionyl-CoA from acrylyl-CoA, we report here the presence of an acyl-CoA dehydrogenase (Figure 20) as presented by Paputsakis E. T. and Meyer C. L. [176].

5. CONCLUSIONS

This thesis had as main goal the development of an efficient anaerobic bioprocess capable of converting industrial excesses such as syngas and/or glycerol to high value products such as propionic acid, which was successfully accomplished through the first report of propionate production from carbon monoxide by a co-culture defined by the isolated *Acetobacterium* sp. strain JM and *C. neopropionicum*. Glycerol was also shown to be converted by this co-culture, boosting propionate production and yielding 1,3-propanediol as a co-product, which is also a high value product. Further studies need to be performed on a bioreactor scale, testing different headspace compositions and pressures as well as glycerol conversion.

In this thesis was proved for the first time the growth capability of a *Acetobacterium* species on sole carbon monoxide as a carbon and energy sources. It is then necessary to find out if *Acetobacterium* sp. strain JM's formate dehydrogenase is inhibited by CO, as reported for *Acetobacterium woodii*, where CO conversion was possible only in a co-fermentation regime with formate, using levels of CO lower than 50 kPa [149]. However, it is reported here than even on *A. woodii* experiments, acetogenic growth on sole CO as carbon and energy sources was possible using CO pressures above 50 kPa. This way, it is proposed that with the defined medium used here, cells increase their metabolic activity in such a way that CO is converted at a high rate, keeping a low CO concentration in the medium and avoiding the CO inhibitory effect on formate dehydrogenase.

Acetobacterium sp. strain JM has shown acetogenic and solventogenic behaviors, being able to switch its CO conversion activity from producing acetate to producing ethanol. Moreover *Acetobacterium* sp. strain JM was also able to oxidize acetate, which represents a new feature for an *Acetobacterium* species.

The presence of isovalerate as a by-product of fermentation might represent a starting point to perform chain elongation from carbon monoxide.

To conclude, this work constitutes a step forward on opening new opportunities for biological processes as an alternative for the production of valuable chemicals via syngas/CO and glycerol conversion route.

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