Bioconversion of carbon dioxide and polluting gases into products with high added value

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INFORMAN

Que el trabajo titulado **Bioconversión de dióxido de carbono y gases contaminantes en productos de alto valor** ha sido realizado por **Kübra Arslan** en el Departamento de Química y que, como Directores del mismo, autorizan su presentación para optar al grado de **Doctor**.

Y para que así conste, expiden y firman la presente en La Coruña, a de de 2023.

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With Love

Kübra

Resumo

O uso de gases contaminantes o de efecto invernadoiro como o dióxido de carbono e o monóxido de carbono como substratos para producir produtos químicos é unha posibilidade para pechar os ciclos do carbono e establecer unha economía circular. A fermentación do gas de síntese é o proceso no que estes gases son convertidos en produtos químicos como etanol, ácido acético e butanol por bacterias acetóxenas.

O primeiro obxectivo desta tese foi mostrar a capacidade de produción de etanol duna bacteria acetóxenica, *Clostridium aceticum*. No primeiro paso determináronse individualmente os valores óptimos de pH para a produción de ácidos e disolventes e no segundo incrementouse a produción de etanol aplicando diferentes estratexias de regulación do pH. Tras este achado, investigouse a influencia das fontes de carbono co propósito de mellorar a produción de etanol. Tamén se mostrou o patrón de formación do produto desta cepa mentres crece co diferentes fontes de carbono, frutosa e CO.

En última instancia, a produción de acetona que non é un produto nativo de *Acetobacterium woodii* foi analizada en catro cepas de *A.woodii* diferentes, modificadas xenéticamente. Revelouse a viabilidade dun deseño de proceso de fermentación continuo con retención total de células.

Resumen

El uso de gases contaminantes o de efecto invernadero como el dióxido de carbono y el monóxido de carbono como sustratos para producir productos químicos es una posibilidad para cerrar los ciclos del carbono y establecer una economía circular. La fermentación de gas de síntesis es un proceso en el que estos gases se convierten en productos químicos como etanol, ácido acético y butanol por bacterias acetogénicas.

El primer objetivo de esta tesis fue mostrar la capacidad de producción de etanol de una bacteria acetógenica, *Clostridium aceticum*. En el primer paso, se determinaron los valores de pH óptimos individualmente para la producción de ácido y de disolvente y, en el segundo paso, se mejoró la producción de etanol mediante la aplicación de diferentes estrategias de regulación del pH. Tras este hallazgo, se investigó la influencia de las fuentes de carbono con el fin de mejorar la producción de etanol. También se mostró el patrón de formación de productos con esta cepa al crecer con diferentes fuentes de carbono, fructosa y CO.

Finalmente, la producción de acetona, que no es un producto nativo de *Acetobacterium woodii*, se analizó en cuatro cepas de *A.woodii* modificadas genéticamente. Se comprobó la viabilidad de un proceso de fermentación continua con retención celular completa.

Abstract

Using polluting or greenhouse gases such as carbon dioxide and carbon monoxide as substrates to produce bulk chemicals is one possibility to close carbon cycles and to establish a circular economy. Syngas fermentation is the process in which these gases are converted into chemicals like ethanol, acetic acid and butanol by acetogenic bacteria.

The first aim of this thesis was to show the ethanol production capability of an acetogen, *Clostridium aceticum*. In the first step optimum pH values for acid and solvent productions were determined individually and in the second step ethanol production was enhanced by applying different pH-regulating strategies. Following this finding, the influence of carbon sources was investigated with the purpose of enhancing ethanol production. The product formation pattern of this strain, while growing on different carbon sources, fructose and CO, was also showed. Ultimately, acetone production, which is not a native product of *Acetobacterium woodii*, was screened in four different metabolically engineered strains. Feasibility of a continuous fermentation process with full cell retention was demonstrated.

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1. INTRODUCTION

Global climate change and the depletion of fossil resources are the two biggest energy related problems of the 21st century. Increasing atmospheric CO_2 concentrations caused by combustion and extraction of fossil resources to produce chemicals, fuels, and energy are the common reasons behind these two problems (Appel et al., 2013). However, with the growing global energy demand more energy resources will likely to be consumed. The global community's common aims include reducing our reliance on fossil resources and so lowering high atmospheric CO_2 concentrations. In order to meet this aim, global communities set goals under agreements like Paris agreement to reduce atmospheric CO_2 release and limit global warming (United nations, 2015). This necessitates the replacement of fossil fuels with above-ground carbon and the construction of a circular economy (Köpke and Simpson, 2020; Liew et al., 2016).

At this point new alternative feedstocks are required, and these feedstocks must meet certain criteria, such as cost-effectiveness, when compared to traditional fossil fuel-based options in order to achieve a competitive production process. Lignocellulosic biomass, industrial, landfill, and residual waste streams are some of the potential streams proposed and studied during the last century (Kennes et al., 2016). Lignocellulosic materials are known to have a huge potential; however, they should be treated heavily to be able to make all fermentable sugars (mainly from cellulose and hemicellulose part of the lignocellulosic material) accessible, then the product yields and conversion efficiencies would be competitive, and the overall conversion process would be profitable (Jonsson and Martín, 2016; Kennes et al., 2016). Gasification is a process where lignocellulosic material is converted to syngas, a mixture of CO, CO_2, H_2, N_2 and some other gases and impurities at negligible concentrations under high temperature, and it appears as an alternative that allows to use all the lignocellulosic fractions, including lignin (Balan et al., 2013). Traditionally, syngas is further converted into products such as diesel, methanol or ethanol, among others, by using chemical

catalysts via the Fischer-Tropsch synthesis operated at high pressure and temperature (Griffin and Schultz, 2012). Bacterial syngas fermentation can also be used to convert syngas into alcohols and carboxylic acids by microbial catalysts.

Acetogenic bacteria are a group of chemolithoautotrophic microorganisms, capable to use CO and/or CO_2+H_2 under anaerobic conditions and convert these gases into organic acids, alcohols, and other compounds via the Wood-Ljungdahl pathway (WLP) (Schuchmann and Müller, 2014). The process is called gas fermentation or syngas fermentation and it is gaining importance due to its industrial potential and lower environmental impact (Redl et al., 2017). Acetogens are metabolically versatile organisms and in addition to the gas substrates they can utilize a very broad range of other substrates such as sugars, alcohols, and aldehydes (Müller, 2019). There are more than 100 cited species of acetogens (Drake et al., 2008) with various morphologies, and a wide range of optimum growth temperature and pH. Even though the main product of acetogens is acetate, some are capable of producing other valuable chemicals like ethanol, 2,3-butanediol, butanol, hexanol, butyrate and hexanoate (Bengelsdorf et al., 2013). Besides these naturally occurring products, it is possible to broaden the product spectrum to other platform chemicals by applying metabolic engineering approaches and discoveries of additional species with diverse fermentation capabilities generating promising new products from gas fermentation (Yang et al., 2021).

Syngas fermentation projects have advanced to commercial scale and they appear as other lignocellulosic biofuel production options as lignocellulosic biomass is not the only feedstock for syngas fermentation. Indeed, flue gases from several major industrial processes also contain syngas components; for instance the steel making industry is emitting 50% of the carbon utilized in the process as CO (Sun et al., 2019; Bengelsdorf et al., 2013). By utilizing dedicated energy crops, waste biomass, and industrial or municipal waste as raw materials for energy and chemical synthesis, we can encourage the repurposing and recycling of materials

that would otherwise go to waste. This approach can create a sustainable and environmentally friendly cycle of producing renewable, carbon-neutral energy and chemicals.

1.1 Wood-Ljungdahl Pathway (WLP) and Acetogenic Mixotrophy

Wood-Ljungdahl pathway (WLP) also termed reductive acetyl-CoA pathway is the only linear CO_2 fixation pathway that acetogenic bacteria employs to convert gaseous substrates into biocommodities (Drake et al., 2008). This pathway is used by acetogens for energy conservation, cell growth and for synthesizing acetyl-CoA from two molecules of CO_2 (Drake, 2008). The WLP consists of two separate branches (Figure 1) named as methyl branch and carbonyl branch and there are 15 enzymes closely associated with this pathway (Philips et al., 2017; Ragsdale, 2008). The methyl branch is utilized to reduce one molecule of CO_2 in a stepwise way to a methyl group which is then required to form acetyl-CoA. In the first step of the methyl branch, one molecule of CO_2 is reduced to formate catalyzed by formate dehydrogenase (Fdh). Depending on the organism, electrons required for this step are either obtained directly from H_2 or from a redox equivalent (Mock et al., 2015; Schuchmann and Müller, 2014). Then, formate is activated to formyl-tetrahydrofolate (THF) by a formyl-THF synthetase (Fhs) consuming one molecule of ATP. The reduction of formyl-THF to methyl-THF is catalyzed by an enzyme cascade consisting of the enzymes methenyl-THF cyclohydrolase (Mtc), methylene –THF dehydrogenase (Mtd), and methylene-THF reductase (Mtr). The methyl group is then transferred to a corrinoid iron-sulfur-containing protein (CoFeSP) and the methyl group, the carbonyl group, and the coenzyme A are fused by the enzyme complex COdh/Acs to form acetyl-CoA (Bengelsdorf et al. 2013; Ragsdale, 2008). In the carbonyl branch, the COdh/Acs complex catalyzes the reduction of another molecule of CO_2 to CO and then the formation of acetyl-CoA as well (Ragsdale, 2008). When acetogens grow on CO as sole carbon source, one molecule of CO is oxidized via CODH to CO_2 and CO_2 enters the pathway from the methyl branch while another molecule of CO enters the

carbonyl branch directly (Drake et al., 2008; Liew et al., 2016) (Fig 1). Acetyl-CoA serves as the key precursor for most further anabolic and catabolic processes.

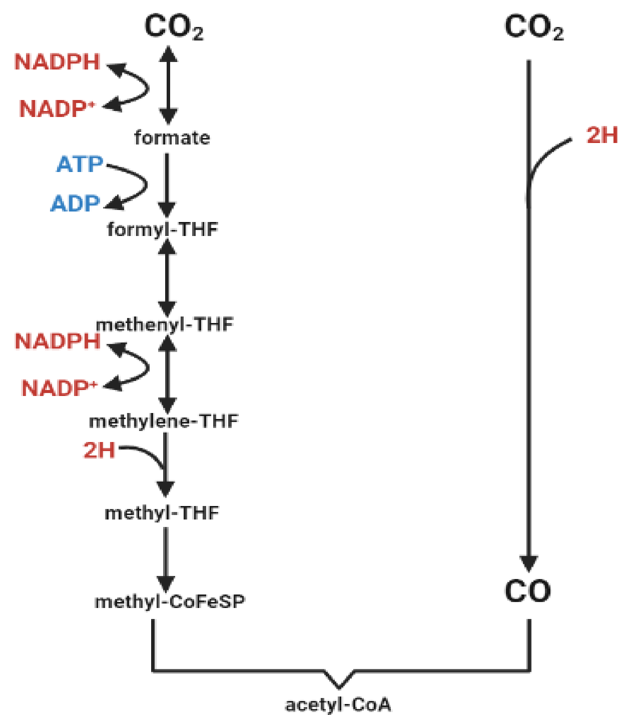

Figure 1. The Wood-Ljungdahl pathway in acetogens leading to acetyl-CoA which can be further converted into end-products

As mentioned before acetogens are also able to grow on several other carbon sources like sugars. This feature of acetogenic microorganisms is giving them a greater importance as coupling glycolysis and the WLP allows full sugar originated carbon fixing and thus higher product yields (Fast et al., 2015; Jones et al., 2016). Figure 2 presents the overall concept of WLP and glycolysis coupling in acetogens, also named as acetogenic mixotrophy (Fast et al., 2015). During heterotrophic growth, 2 molecules of CO_2, responsible for at least 33% of the sugar carbon, are released, while pyruvate is converted into acetyl-CoA catalayzed by the pyruvate:ferredoxin oxidoreductase (Pfor) (Jones et al., 2016)) . This CO_2 is lost in the absence of a CO_2 reassimilation mechanism together with the reducing equivalents in the form of H_2. This results in low substrate-carbon recovery into products and low product yields (Charubin and Papoutsakis, 2019). Acetogenic mixotrophy appears as an opportunity to

achieve better carbon recoveries by utilizing two molecules of CO_2 and electrons generated during glycolysis in the WLP and producing an additional acetyl-CoA.

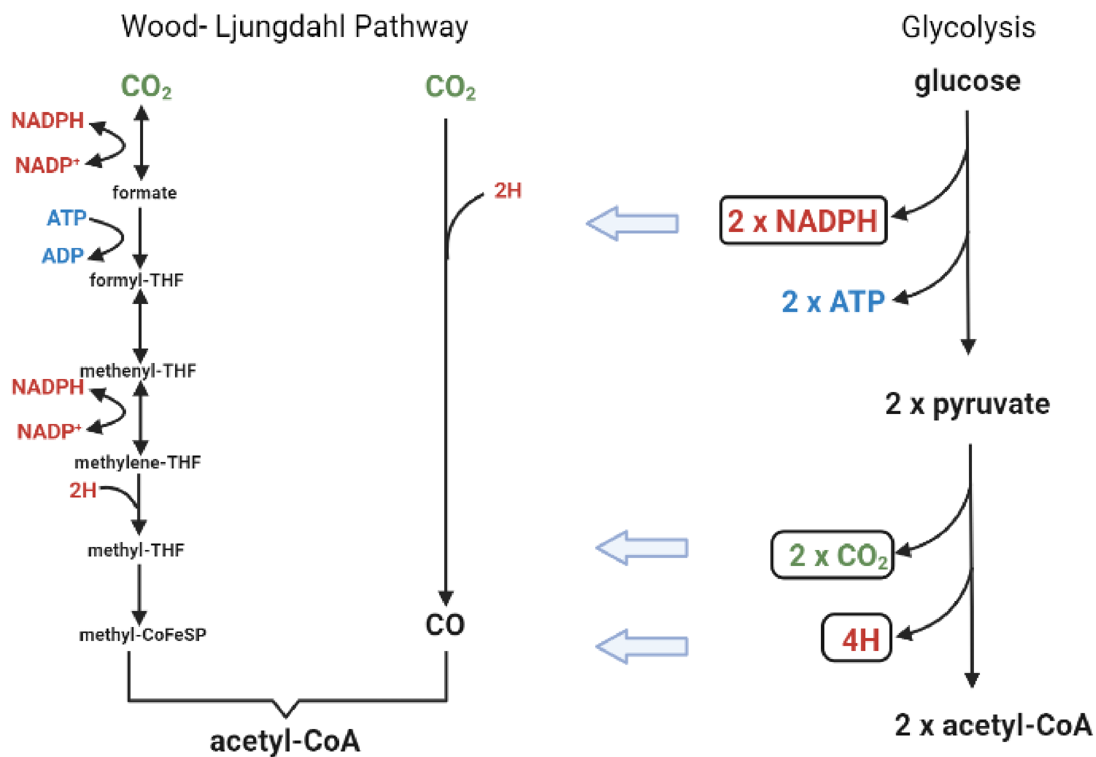

Figure 2. Overall concept of mixotrophy in acetogens; combination of WLP and glycolysis

Although the main product of many acetogenic bacteria is acetate and usually it is the sole end product, some other acids and alcohols can be formed as seen in Figure 3. For acetate formation, acetyl-CoA is first converted to acetyl phosphate then to acetate by the enzymes phosphotransacetylase (PTA) and acetate kinase (ACK) (Bengelsdorf et al., 2013). In the acetate kinase reaction, 1 mole of ATP is produced which is required for formate activation in the second step of the methyl branch of the WLP (Mock et al., 2015). Acetate production through the WLP gives zero ATP gain. It has been shown that acetogens use a chemiosmotic mechanism for additional ATP synthesis required for other metabolic activities like growth. Overview of energy conservation and additional ATP formation in *C. aceticum* is given in Figure 4 as an example.

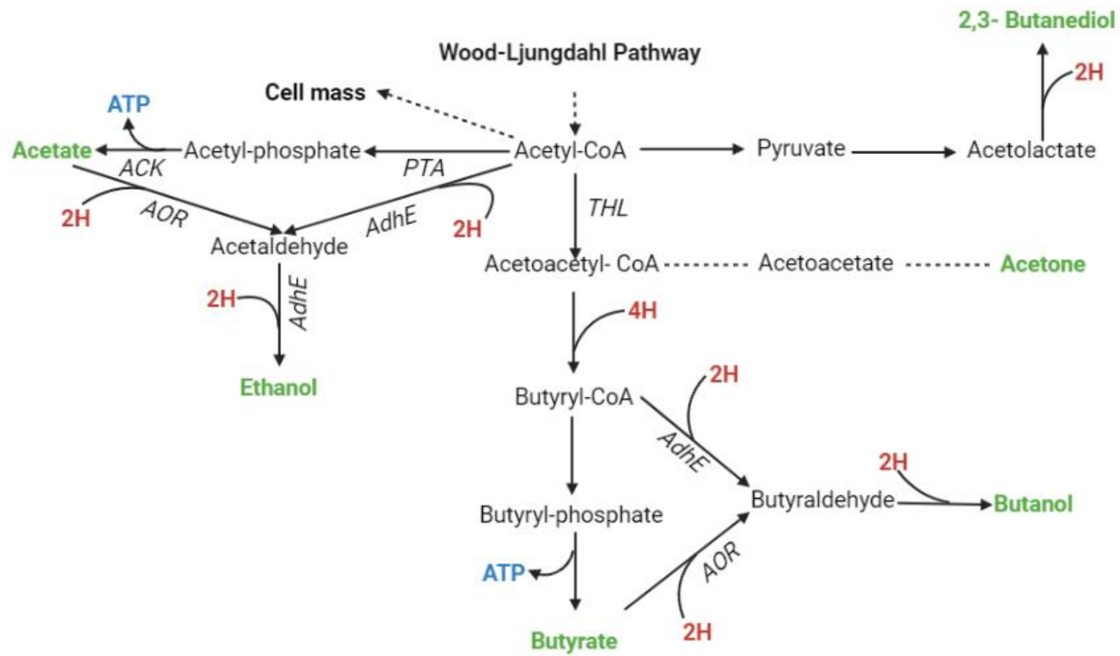

Figure 3. Potential native products of acetogens (Yang et al., 2021)

It was suggested that acetogens use respiratory enzymes that establishe a transmembrane electrochemical ion gradient across cell membrane by expelling ions in the form of H^+ or Na^+ from the cytoplasm using the free energy change of electron transport. This ion gradient drives additional ATP synthesis through a membrane-bound ATP synthase. Electron bifurcation (shown in purple color in Fig 4) generates the energy required for electron transport chain using hydrogen as reductant. So far, two respiratory enzymes were defined; ferredoxin: H oxidoreductase (Ech) and ferredoxin:NAD oxidoreductase (Rnf) and every acetogen sequenced so far is reported to have either one of these enzymes and the presence of these genes are mutually exclusive. Later on, cytochromes were found as indicative of proton-based energy conservation. In 2015, Poehlein (2015) and her team reported the genome sequence of *C. aceticum* and both *rnf* and cytochrome encoding genes were found. However, Wiechmann and Müller (2021) reported that *C.aceticum* has a Na^+ dependent respiratory chain with a Na^+ dependent Rnf and ATP synthase.

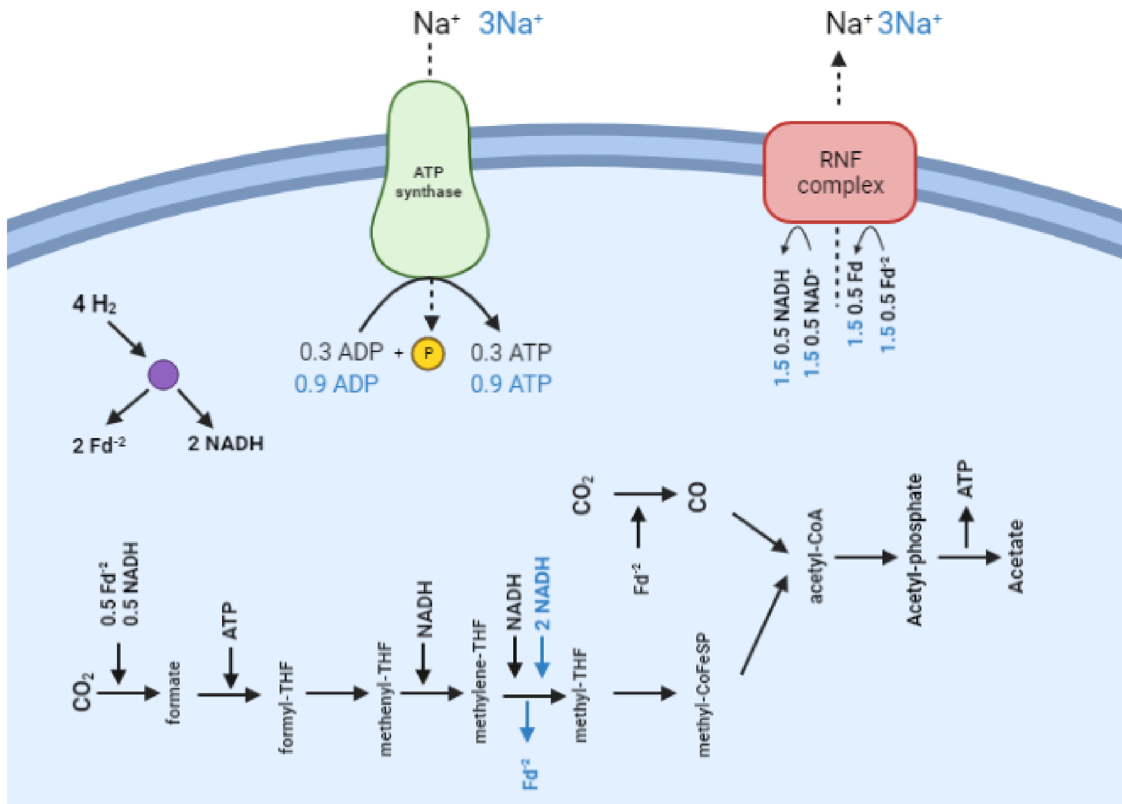

Figure 4. Overview of energy conservation during CO_2+H_2 fermentation in *C. aceticum* (Weichmann and Müller, 2021)

1.2 Potential Products of Acetogens

Ethanol and butanol are important alternative clean transportation fuels, ethanol can be blend with gasoline (10%) while butanol can be combusted directly and blended with gasoline at any concentration in existing automobile engines. Propionic acid, propanol, hexanoic acid, hexanol and isobutanol are other potential products (biochemical pathways are not presented here) reported to be produced via syngas fermentation (Hu et al., 2016; Köpke et al., 2011; Liu et al., 2014). Ethanol is one of the most available and most prominent biofuels in the market with high octane number and high oxygen content which allows complete combustion (Musatto et al., 2017; Sun et al., 2019). Several acetogenic bacteria are known to have the capability to produce ethanol naturally. Once acetyl-CoA is formed there are two possible routes to produce ethanol. In the first one acetyl-CoA is directly reduced to ethanol in sequential two step via acetaldehyde catalyzed by the enzyme aldehyde/alcohol

dehydrogenase (*AdhE*). In the second route ethanol is produced through acetate by the acetogens containing genes encoding aldehyde:ferredoxin oxidoreductase (AOR), a tungsten (W) dependent enzyme (Bengelsdorf et al., 2013; Roy and Adams, 2002). This way is thermodynamically more favorable due to the ATP production (Fig 3).

Common understanding holds that acid and solvent formation during syngas fermentation differs depending on the process conditions. Acid production is reported to be growth dependent which occurs at high growth rates and at optimum growth conditions. Optimum growth conditions are sufficient nutrient supply, optimal pH, optimal temperature, and no end product inhibition. Solvent production on the other hand occurs under unfavorable growth conditions like low temperature, nutrient limitation, and low pH. In addition, several acetogens are reported to reduce organic acids to respective solvents via the AOR route also at the expense of electron donor (reductant) like H_2 or CO. *Clostridium ljungdahlii* was the first acetogen reported to produce ethanol from syngas (Barik et al., 1988; Tanner et al., 1993). *Clostridium ragsdalei* reduced acetic, propionic, butyric, pentanoic and hexanoic acids to the corresponding alcohols in an optimized medium with low pH (Isom et al., 2015). Fernandez Naveira (2019) showed that *Clostridium carboxidivorans* is capable to convert acetic acid, butyric acid and hexanoic acid to ethanol, butanol and hexanol, respectively. This was possible under low pH conditions (around 5) and with the addition of trace metal tungsten. In *Clostridium autoethanogenum* enhanced ethanol productions were reached when bacteria was under stress conditions like low pH, limited yeast extract concentration, high gas pressure and high cysteine-HCI concentrations (Abubackar et al., 2012). New species that are capable to produce ethanol continue to be discovered.

Better understanding the acetogens at molecular and cellular biology levels enabled significant improvements in the application of genetic engineering tools in these microorganisms. These tools that have been applied before to several model microorganisms

like *E.coli* and yeasts, are used to expand the product range of acetogens beyond their natural products and also to improve the efficiency in producing their native products. One of the metabolic engineering approaches is to introduce genes encoding enzymes that are involved in the desired pathway from other bacteria to DNA of target acetogens (Yang et al., 2021). For instance, butanol and acetone synthesis pathway genes of *Clostridium acetobutylicum* were successfully expressed in *C. ljungdahlii* resulting in 0.148 g/L butanol production from synthesis gas mixture (Köpke et al., 2010) and 0.871 g/L acetone production from CO (Banerjee et al., 2014). In this thesis, two different acetogenic bacteria were studied, *C. aceticum* and *A. woodii*, which will briefly described hereafter.

1.3 *Clostridium aceticum*

C. aceticum was described many decades ago (Wieringa, 1939), as the first autotrophic acetogen capable of converting hydrogen and carbon dioxide to acetic acid and water in pure culture studies. However, no additional studies were reported due to the loss of the strain, until a test tube of spores of the original *C. aceticum* was found in Barker's cell culture collection (Braun et al., 1981). *C. aceticum* was described as an obligately anaerobic, spore-forming mesophilic bacteria capable to grow autotrophically on CO_2 plus H_2 and heterotrophically on sugars, organic acids, and alcohols (Braun et al., 1981). The main product of *C. aceticum* is acetate meaning that it is a homoacetogenic bacterium (Wieringa 1939; Braun et al., 1981). Autotrophic growth capability of *C. aceticum* on CO as sole substrate was first showed by Sim et al. (2007) and the main product was again acetic acid. Later on, Sim et al., (2008 a, b) conducted more studies in order to evaluate and optimize nutrient requirements of *C. aceticum* for acetic acid production from syngas and CO as sole substrate. No more studies were performed using this microorganism until Poehlein (2015) and her colleagues, sequenced completely the genome of *C.aceticum* and found that it contains a cytochrome proposing that *C. aceticum* might be a link between Rnf- and

cytochrome- containing authotrophic acetogens. Regarding these findings *C. aceticum* was considered to be an attractive organism with its energy metabolism offering new targets for metabolic engineering to improve ATP yield and enable the production of energetically challenging compounds (Poehlein et al., 2015). Several bioreactor operations were also conducted with this microorganism in order to elucidate Na^+ dependency of growth or other metabolic reactions (Mayer and Botz, 2017). As an unexpected result minor amounts of ethanol formation, with high concentrations of acetic acid as the major end metabolite, in *C. aceticum* was reported in one study when CO was used as sole carbon source at different partial pressures (Mayer et al., 2018; Riegler et al., 2019).

1.4 *Acetobacterium woodii*

A. woodii is also a homoacetogenic bacterium which was first discovered in 1977 in the black sediment of an Oyster pond in Massachusetts (US) (Balch et al., 1977). It is one of the most extensively studied acetogens today and serves as a model-organism (Braun and Gottschalk, 1981; Schuchmann and Müller, 2016, 2014; Wiechmann et al., 2020). Energy conservation of acetogens was first elucidated in *A. woodii* and it is classified as an Rnf and sodium dependent acetogen (Fritz and Müller, 2007; Müller et al., 2008; Schuchmann and Müller, 2014).
A. woodii is shown to utilize CO_2 and H_2 efficiently. However, unlike other acetogenic bacteria, CO cannot be used as the sole carbon and energy source by *A. woodii*, its utilization is reported to be possible only in the presence of CO_2 and H_2 or formate (Kantzow et al., 2015; Bertsch and Müller, 2015b; Takors et al., 2018; Bertsch et al., 2015). *A. woodii* is also known to use a wide range of organic substrates like formate, methanol, ethanol, lactate, 2,3-butanediol, glucose, fructose, and 1,2-propanediol and in addition *A. woodii* is capable to utilize CO_2 and organic substrates simultaneously (mixotrophy) (Hess et al., 2015; Kremp et al., 2018; Schuchmann et al., 2015).

As it was mentioned before *A. woodii* is a homoacetogenic bacterium meaning that its main product is acetate. Besides, it is also known to produce propionic acid and 1-propanol when growing on 1,2-propanediol, and ethanol is produced when acetaldehyde is used as the carbon source (Trifunovic et al., 2020; Schuchmann et al., 2015). Ethanol production was also reported under phosphate limited conditions and formate is shown to be the main product under sodium limited conditions (Müller, 2019).

Metabolic engineering tools have also been successfully used in *A. woodii* for the optimized/increased formation of natural products or to improve the product spectrum. Overexpression of different genes of the WLP resulted in increased native product, acetate, production in *A. woodii* with a maximum concentration of 51 g/L acetate in 3.8 process days (Straub et al., 2014). Another successful example is acetone production in *A. woodii* transformed with the plasmid expressing the acetone synthesis genes from *C. acetobutylicum*. In that study 26.4 mg/L.h maximum productivity was reached in a continuous stirred tank reactor from a mixture of CO_2 and H_2 gases (Hoffmeister et al., 2016).

1.5 Benefits and Limitations of Acetogenic Chemicals Production

Syngas has long been used as feedstock in industrial chemical synthesis and it is mainly produced from fossil resources such as coal or natural gas. Lignocellulosic wastes, municipal solid wastes, and organic industrial wastes can be used to generate syngas for a sustainable and circular production. This brings its own challenges; for example, these feedstocks are highly distributed, compositionally invariable and syngas produced from these sources is associated with contaminants (Clomburg et al., 2017; Köpke and Simpson, 2020). The Fisher-Tropsch (FT) process also converts syngas into chemicals such as ethanol and methanol by using chemical catalysts. The FT process requires high temperature and pressure. Besides, chemical catalysts require strict fixed H_2/CO ratio and can easily be poisoned by impurities. Syngas fermentation is advantageous as biological catalysts are flexible, independent from

gas ratio. It has better product selectivity, operates at low temperature and pressure and requires no gas clean up because gas impurities merely inhibit biological catalysts (Ahmed et al., 2006; Xu et al., 2011; Köpke and Simpson, 2020). Major barriers of gas fermentation as a commercial production system via acetogens include low productivity due to the gas-liquid mass transfer limitations, energy concerns, high production cost and successful scale up (Sun et al., 2019; Köpke and Simpson, 2020).

1.6 Important Process Parameters

Gas conversion by acetogenic bacteria is affected by the conditions inside and outside of the cell. Enzyme reactions and the rate of each reaction in the WLP are determined by the concentration and availability of the metabolites involved. Culture kinetics of growth as well depends on nutrients, carbon and energy sources taken from the medium. Fermentation process parameters should be optimized to create a favorable cell microenvironment for better cell growth and product formation. These conditions include pH, temperature, nutrient type and concentrations, gas composition, gas flow rate, mixing rate, mass transfer and end product concentration and the most relevant ones are described below.

1.6.1 Carbon sources and nutrient composition

The carbon source is reported to influence syngas fermentation processes, the yield, and the type of end product. This is mostly due to the differences between what substrates could provide (like byproducts or reducing equivalents, etc.) to the fermentation medium while metabolized by the microorganism. As it was mentioned in detail in section 1.1, during the WLP no net ATP is generated and reducing equivalents are needed to enhance ATP formation in acetogens. In addition to that reducing equivalents are electron donors for further reduced native products like ethanol and 2,3-butanediol as well. This apparently shows that the electron availability of carbon sources directly influences growth and product formation. Compared to CO_2 +H_2 and syngas mixtures, CO is reported to be a better substrate and

electron donor with respect to ATP supply, reduced product formation such as ethanol and 2,3-butanediol, and bacterial growth (Hermann et al., 2020). This was explained by excess NADH availability due to the low activity of the methyl branch of the WLP under CO consumption.

Acetogenic mixotrophy was suggested as a method to enhance energetical challenges of the WLP (Fast et al.,2015; Fast and Papoutsakis, 2012). Through mixotrophy, organic carbon is fixed via both the glycosis and WLP. It is hyphotised that the excess ATP generated by glycolysis could improve CO_2 fixation through the WLP to achieve maximum carbon yield (Jones et al., 2016; Fast et al., 2015). Additionally, mixotrophy seems to be a general trait of most acetogens which suggests the possibility of using mixotrophic applications to enhance gas fermentation processes. *C. ljungdahlii* and *C. autoethanogenum* were previously characterized in their mixotrophic behaviour (Jones et al., 2016).

The fermentation medium which is responsible for sustaining and maintaining the biocatalyst growth usually accounts for 60-70% of the production cost. Therefore, it is important to optimize nutrient requirements to create cost effective processes. An optimized medium typically should contain vitamins, minerals, amino acids, and trace metals. Vitamins and trace metals such as W, Se, Fe, and Co play critical roles as co-factors for metalloenzymes that are associated with the WLP (Sun et al, 2019). Nutrient limitation is also known to have some influence on the type and the yield of desired end product (Kundiyana et al., 2011; Gao et al., 2013).

1.6.2 pH

The end products of autotrophs are shown to depend highly on the pH of the medium (Abubackar et al., 2012; Kundiyana et al., 2011; Maddipati et al., 2011). As a result of substrate metabolism and the release of metabolic byproducts, pH of the medium changes during syngas fermentation. Therefore, a pH control strategy might have crucial importance

on the yield of the desired end product. In a study performed with no pH control, a pH drop in the culture media from 7.0 to 6.0 in 9 days of incubation was seen due to the acetate formation and only 8.2 g/L acetate could be produced in*A. woodii*. However, in the same study it was also shown that maintaining a neutral pH (7.0) with sodium hydroxide supplementation allowed32.4 g/L acetate production in 36 days of incubation (Demler and Botz, 2011).

During acid production medium pH falls below a thermodynamic threshold due to the accumulation of undissociated organic acids which in turn results in solvent production to take over converting the produced acids to alcohols (Ritcher et al., 2016). It has been shown for several *Clostridium* species pH values around 5.0-6.0 is favorable for acid formation associated with bacterial growth while operating at lower pH (4.5-5.0) solvent formation (conversion of acids to alcohols) is triggered (Abubackar et al., 2016; Martin et al., 2016). This phenomenon allowed researchers to apply different pH control strategies in order to reach high amounts of solvent productions in *C. autoethanogenum* and *C. carboxidivorans* (Abubackar et al., 2015, 2016, Fernández-Naveira et al., 2016).

Syngas fermentation has also been performed in separate stages to meet different operational requirements of cell growth and solvent production. Kundiyana et al. (2011) conducted a two-stage fermentation system by combining a growth reactor maintained at higher pH for acid production and a solvent production reactor at lower pH. In another study using the two-stage system with *C.ljungdahlii* 18 g/L acetate and 5.5 g/L ethanol was reached in growth reactor at pH 5.5 while 20.7 g/L ethanol was accumulated in the solvent production reactor which was kept between pH 4.5 to 4.8 (Ritcher et al., 2013).

1.6.3 Mass transfer

Gas-liquid mass transfer is a major challenge for syngas fermentation due to the sparing solubilities of CO and H_2 in water, and according to the Henry's law their solubility depends

on the partial pressure of the individual species (Phillips et al., 2017). As energy sources, CO and H_2 must be continuously provided to the liquid medium to sustain active fermentation. Many approaches have been suggested to improve gas-liquid mass tranfer. This includes using various reactor configurations like hollow fiber membrane reactor, bubble column reactor, gas-lift reactor, trickle bed reactor, monolithic biofilm reactor and others (Orgill et al., 2013; Rajagopalan et al., 2002; Munasinghe and Khanal, 2014; Shen et al., 2014). The continuous stirred tank reactors (CSTR) are the most commonly used reactors in laboratory and in industry due to the ease of operation. Type of impellers might influence the gas-liquid interaction by creating different stirring patterns. Effect of different types of impellers have been suggested as an alternative approach to enhance mass transfer in CSTR (Ungerman and Heindel, 2007). Product formation has also been reported to increase with increasing syngas flow rates and mixing rates (Atiyeh et al., 2016).

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2. OBJECTIVES

Industrial plants need to reduce their greenhouse gas emissions, for the transition to sustainable and carbon neutral future. Gas fermentation using acetogenic microorganism is an alternative to produce many important industrial chemicals from C1 carbon sources and has been suggested to achieve this purpose. These systems are mainly challenged by low productivity due to the gas-liquid mass transfer limitations and energetic constraints limiting the biotechnological potential of acetogenic microorganisms.

Overall aim of this thesis is to evaluate efficient operating parameters for gas fermentation to produce ethanol and acetone by the acetogenic bacteria *C. aceticum* and *A. woodii.* The results of this thesis were structured into four individual parts. Part 1 and 2 deal with the metabolism and energetic barriers of *C. aceticum.*

C. aceticum is thought to be an attractive host due to its energy metabolism. Acetate is the main product of this strain and ethanol production capability of *C. aceticum* is unknown. This thesis therefore aims to reveal optimum process parameters (mainly pH) for ethanol production in this strain. For this purpose, in a first step metabolite pattern of *C. aceticum* was screened at different pH values. This was achieved by not controlling the pH of the gas fermentation process which causes medium acidification as a result of acetic acid accumulation. It was hypothesized that medium acidification would allow to define optimum pH value that switches the acid production step to solvent production step in *C. aceticum*. After this goal was achieved and the limitations of the pH dependent solvent production in *C.* aceticum were determined*,* various approaches such as medium replacement and pH shifting strategies were applied in order to enrich ethanol concentrations in the fermentation medium. In the second step focus was laid on finding better carbon sources that would improve the process performance and ethanol production in *C. aceticum*. For this purpose, the influence of sole CO and sole fructose were evaluated with pH shifting.

In part 3, acetone production was studied in batch cultivation for four different genetically engineered *A. woodii* strains individually with the aim to find the best acetone producer among all. Within this part, acetone production in recombinant strains was observed to be dependent on the acetate concentration. The feasibility of a continuous fermentation process with full cell retention was shown in part 4, as a short chapter, where the aim was to keep the acetate concentration at an optimum level and improve acetone productivity. Additionally, focus was laid on the effect of agitation on metabolite formation and gas consumption.

3. MATERIALS AND METHODS

This section describes Materials and Methods common to all studies described in the section on “Results and Discussion”. More specific details of Materials and Methods of each specific individual study are provided in each chaper of the “Results and Discussion” section.

3.1 Microbial Culture

3.1.1 *Clostridium aceticum*

The native type strain of *C. aceticum* (DSM 1496) was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) in the form of freeze-dried pellets. Initial rehydration of the bacteria was carried out by following the procedure recommended by DSMZ. The medium composition used in all *C. aceticum* studies was given in Table 1. The bacteria were rehydrated with the specified medium under a stream of pure N_2 gas. The rehydrated microorganism was first transferred to serum bottles containing 20 mL of culture medium. The strain was then maintained by subculturing in 40 mL working volume in serum bottles. In the first three subcultures, fructose (10 g/L) was used as the carbon source. Afterward depending on the carbon source that will be used in the bioreactor study, inocula preparation was carried out in serum bottles, by using either the syngas mixture $CO:CO_2:H_2:N_2$ (30:5:15:50) or pure CO as carbon and energy source. For heterotrophic growth studies fructose was used as carbon source.

3.1.2 *Acetobacterium woodii*

Four different engineered strains were used in this study, namely, *A. woodii* [pJIR_actRBS], *A. woodii* [pMTL84151_act], *A. woodii* [pMJR_act], and *A. woodii* [pJIR_ac2t1]. They were kindly provided by Prof. Peter Dürre (Ulm University, Germany) in the form of freeze-dried pellets, as part of a collaborative European project. Details of the genetic modifications and other bacterial strains and plasmids used in genetic modifications are given in detail in the corresponding section (Chapter 4). The rehydration of the bacteria was carried out by following the same procedure as it was explained in the previous section (section 3.1.1).

Inocula for bioreactor studies were prepared heterotrophically using fructose as carbon source. The medium composition for engineered *A. woodii* strains was the same for all studies and was given in Table 2.

3.2 Bioreactor Studies

3.2.1 Preparation of inocula for bioreactor fermentation

The required medium was prepared, and 40 mL of medium was distributed into 100 mL glass serum bottles. Each bottle was flushed with aseptic pure N_2 for about 5 min. Afterwards, N_2 flushing was replaced with the syngas mixture or sole CO depending on the experiment and strain. The pH of each bottle was adjusted to the desired value with either a 2 M NaOH solution or a 2 M HCl solution. The bottles were then capped with butyl rubber septa and sealed tightly with aluminium crimp caps and were sterilized by means of autoclaving at 120°C for 20 min under 1 bar pressure. Temperature sensitive compounds like vitamins or precipitate forming compounds like $MgSO_4$ were added from anoxic sterile stock solutions to their final concentrations before inoculation. 10 % actively growing seed culture was used to inoculate the bottles. The inoculum bottles were then placed in an incubation chamber, maintained at constant temperature of 30 °C, with agitation at 150 rpm on an orbital shaker (Infors HT, Bottmingen, Switzerland). Details or differences in inocula preparation depending on the experiment is described in detail in each chapter.

Table 1. Medium composition of *C. aceticum*

Medium Composition		Trace Metal Solution		Vitamin Solution	
Chemical	Conc. (in 1 L)	Chemical	Conc. (in 1 L)	Chemical	Conc. (in 1 L)
Yeast Extract	3 g	Nitrilotriacetic acid	15 g	Biotin	0.025 gr
NH_4CI	0.20 g	$MgSO_4 \times 7H_2O$	30 g	Folic acid	0.025 gr
KH_2PO_4	1.76 g	$MnSO_4$ x H_2O	5 g	Pyridoxine - HCl	0.050 gr
K_2HPO_4	8.44 g	NaCl	10 g	Thiamine - HCl	0.050 gr
$MgSO_4 \times 7H_2O$	0.33 g	$FeSO_4$ x 7 H_2O	1 g	Riboflavin	0.050 gr
$NaHCO_3$	10 g	$CoSO_4$ x 7 H_2O	1.8 g	Nicotinic acid	0.050 gr
L-Cystein-HCl	0.30 g	$CaCl_2$ x 2 H_2O	1 g	D-Ca-pantothenate	0.050 gr
Na_2S x $9H_2O$	0.92 g	$ZnSO_4$ x 7 H_2O	1.8 g	Vitamin B12	0.025 gr
Rezasurin (stock sol. conc. 1 g/L)	1 mL	$KAl(SO_4)_2$ x 12 H_2O	0.2 g	α-Aminobenzoic acid	0.050 gr
Trace Metal Sol.	2 mL	$CuSO_4$ x 5 H_2O	0.1 g	Lipoic acid	0.025 gr
Vitamin Sol.	2 mL	H_3BO_3	0.1 g		
		Na_2MoO_4 x 2 H_2O	0.1 g		
		$NiCl_2$ x 6 H_2O	0.25 g		
		Na_2SeO_3 x 5 H_2O	3 mg		
		Na_2WO_4 x 2 H_2O	4 mg		

Table 2. Medium composition of *A. woodii*

Medium Composition		Trace Metal Solution		Vitamin Solution	
Chemical	Conc. (in 1 L)	Chemical	Conc. (in 1 L)	Chemical	Conc. (in 1 L)
Yeast Extract	2 g	Nitrilotriacetic acid	12.8 g	Biotin	0.025 gr
NH_4CI	0.20 g	$MnCl_2$ x $4H_2O$	0.1 g	Folic acid	0.025 gr
KH_2PO_4	1.76 g	NaCl	5 g	Pyridoxine - HCl	0.050 gr
K_2HPO_4	8.44 g	$FeCl_2$ x $4H_2O$	2.0 g	Thiamine - HCl	0.050 gr
$MgSO_4 \times 7H_2O$	0.33 g	$CoCl_2$ x $6H_2O$	0.2 g	Riboflavin	0.050 gr
$NaHCO_3$	10 g	$ZnCl_2$	70.0 mg	Nicotinic acid	0.050 gr
L-Cystein-HCl	0.30 g	$CuCl_2$ x $2H_2O$	2.0 mg	D-Ca-pantothenate	0.050 gr
Na_2S x $9H_2O$	0.30 g	H_3BO_3	6.0 mg	Vitamin B12	0.025 gr
Rezasurin (stock sol. conc. 1 g/L)	1 mL	Na_2MoO_4 x 2 H_2O	39.78 mg	α-Aminobenzoic acid	0.050 gr
Trace Metal Sol.	2 mL	$NiCl_2$ x 6 H_2O	24.0 mg	Lipoic acid	0.025 gr
Vitamin Sol.	2 mL				
Fructose	10 g				
Thiamphenicol (Antibiotic)	7.5 mg				

3.2.2 Bioreactor fermentations with continuous gas supply

The bioreactor experiments were carried out in 2L Eppendorf BIOFLO 120 stirred tank bioreactors (Eppendorf AG, Hamburg, Germany) with around 1.3 L working volume for all assays. A volume of 1.1 L medium was introduced in the bioreactor without vitamins, cysteine x HCl, Na_2S x $9H_2O$, fructose, and thiamphenicol and it was autoclaved at 120 °C for 20 min. The medium was then flushed for at least 2 h with aseptic pure nitrogen and then for 1 h with the feed gas in the case of autotrophic growth. After adjustment of the pH value, with 1 M HCl or 1 M NaOH, cysteine x HCl and Na_2S x $9H_2O$, vitamins, fructose and/or thiamphenicol was added from anoxic sterile stock solutions. The bioreactor experiments were initiated with the aseptic transfer of 120 mL culture medium containing the desired strain, which had reached late exponential growth phase, into the stirred tank reactor. The bioreactor was equipped with four baffles and a six blade Rushton turbine. Gas substrates were fed through a microsparger into the reactor at a flow rate of 10 mL/min. The gas flow rate was controlled and adjusted by means of a mass flow controller (Aalborg GFC 17, Müllheim, Germany). The pH value of the fermentation broth was monitored on-line with a pH (Mettler Toledo, Columbus, Ohio, USA) sensor and maintained at its corresponding value by using either 1 M HCl or 1 M NaOH solutions fed by means of peristaltic pumps during all experiments. All fermentation processes were run at a temperature of 30 °C, which was maintained constant by means of a water jacket or a heat blanket. Schematic setup of the fully controlled bioreactor system with the continuous gas supply were given in Figure 5.

3.2.3 Continuous fermentation with full cell retention

In case of continuous fermentation, a bottle filled with fresh sterile medium was conducted to the fermentation system by means of a peristaltic pump (Watson Marlow 101U/R, Marlow, United Kingdom). Various amounts of fresh medium were pumped into the bioreactor containing the respective *A. woodii* culture. An ultrafiltration module (Repligen, MiniKros

Sampler, Massachusetts, USA) was also included to the system to recover biomass and keep the metabolites at desired values.

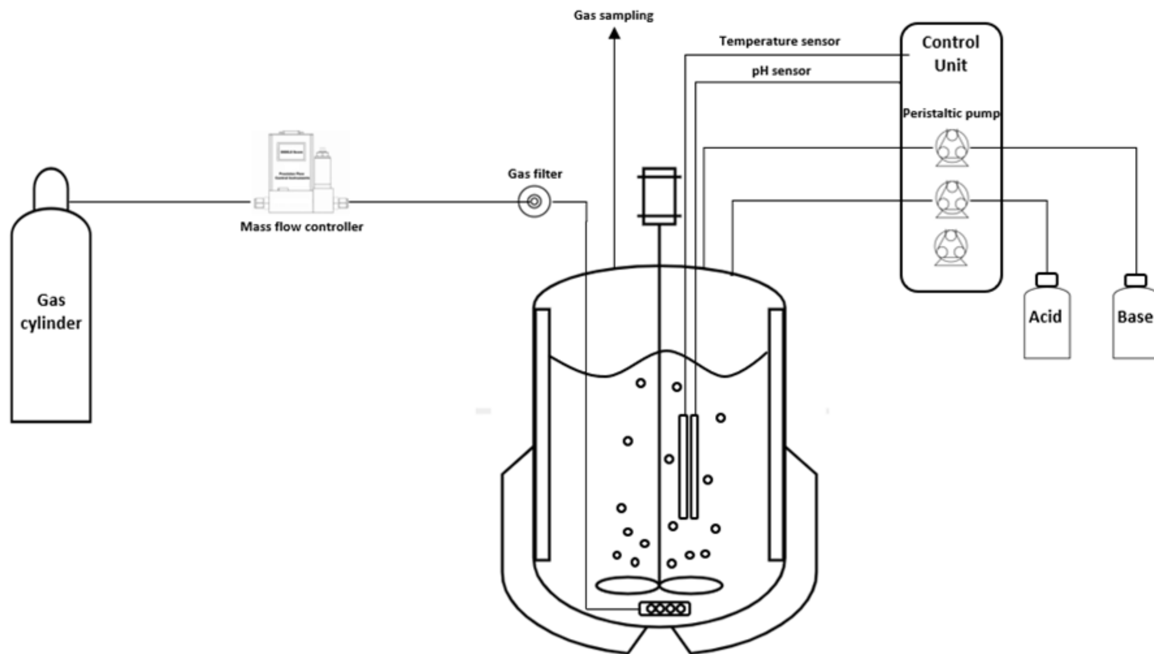

Figure 5. Schematic setup for the batch fermentation process with continuous gas supply

Schematic setup and the photo of the continuous fermentation process were given in Figure 6 Prior to use, the ultrafiltration module was autoclaved at 120 °C for 20 min and afterward it was flushed with aseptic pure N_2 for about 15 min. Media preparation in the bioreactor and inoculation was realized as it was explained in the previous section (Section 3.2.2). First, batch fermentation was performed until the strain reached the late exponential growth phase. Then, the fresh medium bottle providing 3 L of anaerobic medium, and the filtration module were connected to the system. Two identical external pumps regulated the flow of fresh medium feed and permeate, and the flow rates were kept equal in order to maintain the bioreactor working volume constant. The flow from the bioreactor to the filtration unit was controlled by an internal pump.

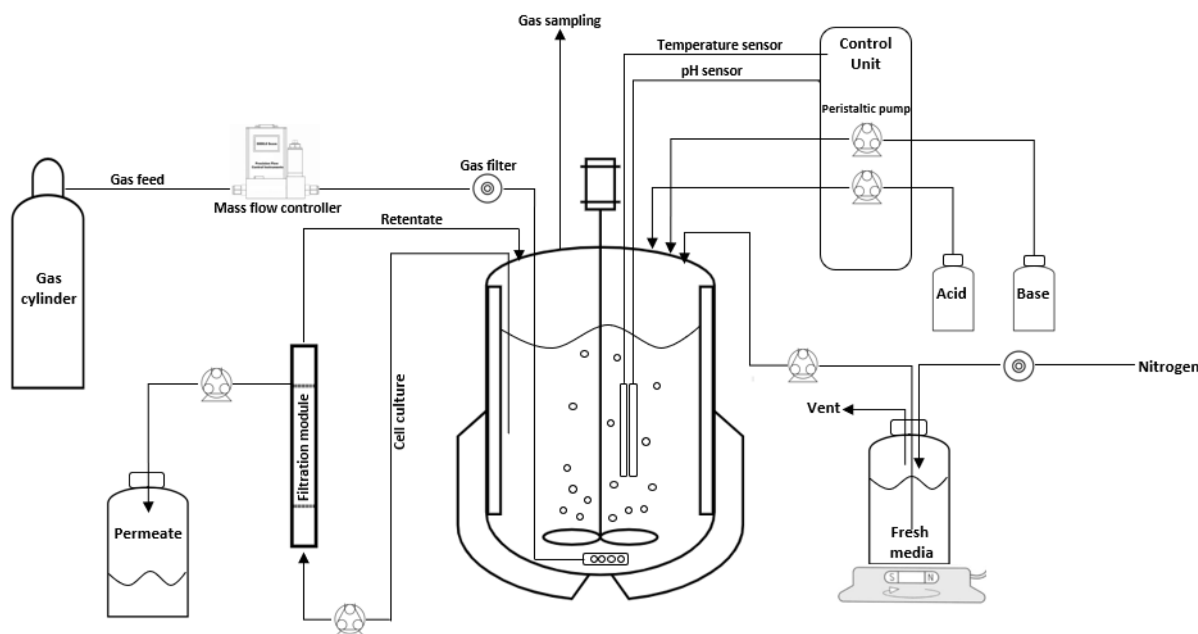

Figure 6. Schematic setup for the continuous fermentation process with cell retention

3.3 Analytical Methods

2 mL samples were withdrawn daily from the reactor in order to carry out analytical tests. The optical density, used to estimate biomass growth and concentration, was measured with a spectrophotometer (Hitachi, Model U-200, Pacisa& Giralt, Madrid, Spain) at a wavelength of 600 nm. Fructose, acetic acid, formic acid, acetone, isopropanol and ethanol concentrations were measured on a high performance liquid chromatograph-(HPLC) (HP1100, Agilent Co., USA) equipped with a diode array detector and a refractive index detector, operating at 50 °C. The possible presence of C4 and C6 acids and alcohols was checked as well. The HPLC samples were first centrifuged (ELMI Skyline Ltd CM 70M07) at 7000 rpm for 5 min and the supernatant was then filtered through a 0.22 µm filter (Labbox, Barcelona, Spain) before HPLC analyses. The mobile phase used for the HPLC analyses was a 5 mM H_2SO_4 solution, with a flow rate of 0.80 mL/min. 20 µL samples were injected in the Agilent Hi-Plex H Column (300 x 7.7 mm), which was kept at 45 °C. Carbon yields, C_M/C_S, were calculated as explained before (Maru et al., 2018). C_M refers the total produced acetic acid carbon and was calculated by multiplying the final acetic acid molar concentration by 2, while C_S refers the

total consumed substrate carbon and was calculated by multiplying the total consumed fructose molar concentration by 6.

4. RESULTS

4.1 Solventogenesis in *C aceticum* Producing High Concentrations of Ethanol from Syngas

Etanol is one of the bulk chemicals which is in the scope of syngas fermentation. Some operational approaches are known to be useful to enrich ethanol production and increase end ethanol concentration in acetogenic bacteria. *Clostridium aceticum* is reported to use CO, CO_2/H_2 efficiently and convert these gases into acetate. Very low amount of ethanol formation in this strain (a few mg/liter), with the main product acetate, was recently reported in the literature as an unexpected result under high CO partial pressure. However, full high ethanol production capability of the strain is still unknown. The scientific questions behind this study were; how process parameters influence solvent production and growth and if other approaches could be used to enrich ethanol production in this strain. It is known that low pH values stimulate solvent production in other acetogenic bacteria and a pH shifting approach could increase end ethanol concentration in the fermentation broth. It is also known that fresh nutrient feeding is a useful method for higher solvent productions. Our approach was to conduct a natural medium acidification experiment that could allow us to define the pH values optimum for growth and for solvent production in this strain. Later pH shifting and medium replacement approaches were studied in batch fermentation with continuous gas supply.

Citation – these data have been published as

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https://doi.org/10.1016/j.biortech.2019.121941

Abstract

The ethanol production capability of *Clostridium aceticum* was investigated and optimized, in order to evaluate the ability of that organism to produce high concentrations of fuel-ethanol. The results showed that *C. aceticum* can produce significant amounts of ethanol when a natural pH drop occurs in the fermentation broth as aconsequence of acetic acid production in a first stage. Applying different pH-regulating strategies allowed to optimize ethanol production, which proved to be more efficient in case of natural acidification due to acetic acid, reaching up to 5.6 g/L ethanol, compared to artificial pH adjustment through the addition of hydrogen chloride.

Playing with the pH value and the bioreactor operating conditions showed that, under specific conditions, *C. aceticum* is able to perform the reverse reaction as well and convert ethanol, produced at low pH, back to acetic acid, impeding, under those specific conditions, further accumulation of ethanol in the fermentation broth.

Keywords: acetogen; biofuel; bioethanol; Clostridia; syngas

Introduction

Environmental concerns and the expected increasing scarcity of fossil fuels in the near future, foster the search for new, renewable or environmentally friendly alternative energy sources. Ethanol is an important fuel that can be utilized as such in vehicle engines, or it can be blended with gasoline. (Bio)ethanol can be obtained from lignocellulosic biomass or organic wastes through different processes. Best known is the fermentation of carbohydrates, found in the cellulose and hemicellulose fractions of organic or lignocellulosic materials, to produce ethanol. Alternatively, the same feedstocks can be gasified to generate syngas, which can also be metabolized, by anaerobic bacteria, to yield ethanol. Depending on the gasification conditions, major components of syngas will be CO, CO_2 and H_2. Similarly, those same gases are also found in some industrial emissions, e.g. in steel producing industries. Gas fermentation offers a more sustainable and economical pathway for the production of fuels and chemicals by utilizing these readily available volatile pollutants and greenhouse gases such as CO_2 to obtain marketable products such as novel fuels (Liew et al., 2016; Abubackar et al., 2011).

Ethanol production is possible through C1-gas fermentation, as mentioned above, with autotrophic anaerobic bacteria as biocatalysts using the Wood-Ljungdahl pathway (Bengelsdorf et al., 2013). That pathway is used by acetogens to reduce CO directly and CO_2 in the presence of a reducing agent like H_2 into acetyl-CoA, the main intermediate of the pathway and the precursor for the further production of end metabolites (Ljungdahl, 1986; Wood, 1991; Ragsdale, 2004). The main product of the Wood- Ljungdahl pathway is acetic acid. However, a reduced number of acetogens like *Clostridium autoethanogenum* (Abubackar et al., 2015), *Clostridium ragsdalei* (Gao et al., 2013), *Clostridium ljungdahlii* (Köpke et al., 2011) and *Clostridium carboxidivorans* (Fernández-Naveira et al., 2016, 2017a) are reported to have the capability to produce other products, such as ethanol or even higher

fuel-alcohols such as butanol or hexanol. However, those solvent producing bacteria seem to be uncommon so far.

In autotrophic gas fermentation, C1 gases are converted first into acids, generally acetic acid, in a step called acidogenesis, and if the strain is one of the few able to produce solvents, e.g. ethanol, then the accumulated acids will be converted to alcohols in a second step called solventogenesis. Fermentation pH is one major parameter that determines the nature of end products and, in general, while higher pH values promote biomass growth and acid production in acetogenic bacteria, lower pH values would stimulate solvent production. A pH shifting strategy was suggested in *C. autoethanogenum* (Abubackar et al., 2016a), for the complete conversion of produced acetic acid and continuous production of ethanol. It was observed that maintaining the optimal growth pH at the beginning of the fermentation allows obtaining high biomass concentrations and high acid production. Later on, decreasing the pH to a lower value provides improved ethanol production and minimizes the accumulation of acids in the fermentation broth. The same phenomenon was observed in *C. carboxidivorans* (Fernández-Naveira et al., 2017b) and in anaerobic granular sludge as well (Chakraborty et al., 2019).

Gas fermentation is a novel alternative for the production of metabolites such as (bio)ethanol, but strains identified and reported for their ability to perform the solventogenic stage, i.e. ethanol rather than acetic acid accumulation, are scarce. A research gap needs therefore to be filled aiming at identifying and evaluating additional strains able to produce high amounts of ethanol through gas fermentation. This study focuses on an acetogenic strain, *Clostridium aceticum*, not reported previously to be able to produce ethanol as major end metabolite. The study aimed at identifying conditions that would allow to produce high concentrations of ethanol as end product in that organism. *C.aceticum* was first described many decades ago (Wieringa, 1939), as the first acetogen capable to convert hydrogen and carbon dioxide to acetic acid. Later on, no more studies were reported for several years as the strain was lost,

until a new isolate was obtained (Adamse, 1980). The original strain was found in Barker's cell culture collection and the complete microbiological description of the strain was published (Braun et al., 1981). Since then several studies have been done with *C. aceticum* to describe its metabolism for the production of acetic acid (Lux and Drake, 1992). Gas fermentation and the capability of *C. aceticum* to grow on CO and syngas was studied for the first time by Sim et al. (2007). The media for the conversion of CO to acetic acid was also optimized (Sim and Kamaruddin, 2008; Sim et al., 2008).

Recent research with this microorganism focused on the production of acetic acid and mostly on the energy conversation pathway and genome sequencing rather than optimizing any applied bioconversion process or bioreactor operation (Poehlein et al., 2015; Mayer and Weuster-Botz, 2017). Only some very recent research reported the detection of low amounts of ethanol in *C. aceticum*, as unexpected result, under different CO partial pressures, with high concentrations of acetic acid as the major end metabolite (Mayer et al., 2018; Riegler et al., 2019).

In the present study, the effect of bioreactor operating conditions and the fermentation pH, on the metabolism of *C. aceticum* and its ethanol production capability from syngas fermentation, was evaluated for the first time. Studies were performed in order to optimize ethanol production in that strain, identifying conditions that allow reaching high ethanol concentrations not reported before.

Materials & Methods

Microorganism and culture medium

The native type strain of *C. aceticum* (DSM 1496) was used in all the experiments. It was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) in the form of freeze-dried pellets and was rehydrated with the specified medium under a stream of pure N_2 gas. The rehydrated microorganism was first

transferred to serum bottles containing 20 mL working volume of culture medium. The strain was then maintained by subculturing in 40 mL working volume in serum bottles. In first three subcultures, fructose (10g/L) was used as the carbon source. Afterward, after complete exhaustion of fructose, autotrophic growth of the strain was carried out, in serum bottles, by using the syngas mixture $CO:CO_2:H_2:N_2$ (30:5:15:50) as carbon and energy source. The strain was subcultured on the gas mixture until the bioreactor set-up was ready for inoculation. Preparation of the serum bottles is detailed hereafter (section 3.2.2). The composition of the liquid culture medium used for growing *C. aceticum* is as follows (per liter distilled water): NH_4Cl, 0.20 g; yeast extract, 3 g; KH_2PO_4, 1.76; K_2HPO_4, 8.44 g; $MgSO_4 \times 7H_2O$, 0.39 g; $NaHCO_3$, 10 g; L-Cystein-HCl, 0.30 g; Na_2S x $9H_2O$, 0.92 g; rezasurin (stock sol. cons. 10 g/L), 1 mL; biotin, 40 µg; folic acid, 40 µg; pyridoxine - HCl, 200 µg; thiamine - HCl, 100 µg; riboflavin, 100 µg; nicotinic acid, 100 µg; D-Ca-pantothenate, 100 µg; vitamin B12, 10 µg; p-Aminobenzoic acid, 100 µg; lipoic acid, 100 µg; nitriloacetic acid, 30 mg; $MnSO_4$ x H_2O, 10 mg; NaCl, 20 mg; $FeSO_4$ x 7 H_2O, 2 mg; $CoSO_4$ x 7 H_2O, 3.6 mg; $CaCl_2$ x 2 H_2O, 2 mg; $ZnSO_4$ x 7 H_2O, 3.6 mg; $CuSO_4$ x 5 H_2O, 200 µg; $KAl(SO_4)_2$ x 12 H_2O, 400 µg; H_3BO_3, 200 µg; Na_2MoO_4 x 2 H_2O, 200 µg; $NiCl_2$ x 6 H_2O, 500 µg; Na_2SeO_3 x 5 H_2O, 6 µg; Na_2WO_4 x 2 H_2O 8 µg.

Serum bottle fermentation

The compounds given in section 3.2.1, except $MgSO_4$ and vitamins, were dissolved in 40 mL aqueous medium, which was introduced in 100 mL glass serum bottles. Each bottle was flushed with pure N_2 for at least 5 minutes. Afterwards, N_2 flushing was replaced by flushing with the syngas mixture. The pH of each bottle was adjusted to 8 with either a 2 M NaOH solution or a 2 M HCl solution, while keeping flushing syngas. The bottles were then capped with butyl rubber septa and sealed tightly with aluminum crimp caps. Sterilization of the bottles was done by autoclaving at 120 °C for 20 min. $MgSO_4$ and vitamins were added from

anoxic sterile stock solutions before the media were inoculated with 4 mL of seed microbial culture (10% of the working volume). The inoculated bottles were placed in an incubation chamber maintained at constant temperature of 30 °C and agitated at 150 RPM on an orbital shaker (Infors HT, Bottmingen, Switzerland).

Bioreactor experiments with continuous gas supply

Different experiments were performed in order to optimize solventogenesis and maximize the production of ethanol. The experiments were carried out either in a2L BIOFLO 110 bioreactor (New Brunswick Scientific, Edison, NJ, USA) with a working volume of 1.2 L for the first experiment or in a 2L Eppendorf BIOFLO 120 bioreactor (Eppendorf AG, Hamburg, Germany) with 1.2 L working volume for all subsequent assays. The preparation protocol for the liquid culture medium was the same for all studies. The medium was introduced in the vessel without vitamins, cysteine x HCl and Na_2S x $9H_2O$ and it was autoclaved at 120 °C for 20 min. After autoclaving, the medium was flushed with pure nitrogen for at least 2 hours. Afterwards, the culture broth was sparged for at least another 30 min using the syngas mixture ($CO:CO_2:H_2:N_2$ =30:5:15:50), before inoculation. A microsparger was used to feed the gas into the reactor at a rate of 10 mL/min and this rate was maintained constant during the whole experiment by using a mass flow controller (Aalborg GFC 17, Müllheim, Germany). All bioreactors were equipped with four baffles and six blade Rushton turbines and were operated with agitation at 250 RPM. The pH and redox potential were continuously monitored on-line with pH (Mettler Toledo, Columbus, Ohio, USA) and ORP (Mettler Toledo, Columbus, Ohio, USA) sensors. The initial pH of the fermentation medium was adjusted to 8.0 in all cases. Whenever required, it was also adjusted during the experiments by using either 1 M HCl or 1 M NaOH solutions, fed automatically to the fermenters. The temperature was kept constant at 30 °C by means of a water/heat jacket. Proper amounts of vitamins, cysteine x HCl and Na_2S x $9H_2O$ were added aseptically to the vessels from anoxic sterile stock solutions, prior to

inoculation. The inoculum (10% of the volume) was introduced aseptically into the reactor by using sterile syringes to initiate the batch fermentation. Independent bioreactor studies were conducted for the determination of the effect of different parameters, such as the pH value, on ethanol production in *C. aceticum*.

Analytical methods

Two milliliter liquid samples were withdrawn periodically from the bioreactors during the experiments to carry out analytical tests. The cell concentration was determined by using a spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain) at a wavelength of 600 nm. A high performance liquid chromatography (HPLC) system (HP1100, Agilent Co., USA) equipped with a diode array detector and a refractive index detector at 50 °C was used to determine the concentrations of acetic acid (HAc) and ethanol (EtOH). The possible presence of other metabolites, mainly C4 and C6 acids and alcohols, was checked as well. The samples were centrifuged (ELMI Skyline Ltd CM 70M07) at 7000 RPM for 5 min and the supernatant was filtered through a 0.22 μm PTFE syringe filter (Labbox, Barcelona, Spain) before HPLC analyses. A 0.005 M H_2SO_4 solution was used as mobile phase with a flow rate of 0.80 mL/min. Samples of 20 μL were injected into the Agilent Hi-Plex H Column (300 x 7.7 mm) at 45 °C. The concentrations of H_2 and CO were analyzed by using a gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD) and a15-m HP-PLOT Molecular Sieve 5A column (ID, 0.53 mm; film thickness, 50 μm). Helium was used as carrier gas and the temperatures of the injection port and the oven were 150 °C and 50 °C, respectively. The oven temperaturewas initially kept constant at 50 °C, for 5 min, and then raised by 20 °C/min for 2 min, to reach a final temperature of 90 °C. The detector temperature was maintained at 150 °C. Similarly, CO_2 was analyzed on an HP 5890 gas chromatograph, equipped with a TCD and Porapak Q 80/100 (inox) column (2 m × 1/8″). Helium was used as carrier gas and the injection, oven, and

detection temperatures were maintained at 90, 25 and 100 °C, respectively. The redox potential was monitored continuously using an Ag/AgCl reference electrode maintained inside the bioreactor and connected to a transmitter (M300, Mettler Toledo, Inc., USA).

Microbial analyses

The stability and purity of the culture was confirmed via 16S rDNA gene sequencing. The details of the procedure used are available elsewhere (Fernández-Naveira et al., 2017c).

Results and Discussion

pH stimulation of solventogenesis in C. aceticum

In all previous bioreactor studies, with *C. aceticum*, acetic acid was found to be the main C1-gas (CO, CO_2, syngas) fermentation product. Since it is known that fermentation pH is a major parameter affecting cell growth and the nature and concentration of end metabolites in gas fermentation (Abubackar et al., 2019; Fernández-Naveira et al., 2017a, 2019), possible stimulation of solvent production was studied here in *C. aceticum*, allowing the pH value to drop as a result of natural acidification in a STR (Stirred Tank Reactor) with continuous gas feeding.This experiment was started initially under optimum growth conditions (30ºC, pH 8) of the strain. The pH was kept constant at a value of 8.0 during the first 44 hours and a fast biomass growth reaching an optical density, measured at 600 nm (OD_{600nm}) of 1.8 and high acetic acid production were observed, reaching a concentration of 1.5 g/L acetic acid at that moment (Fig. 7). As a result of the continuous production of the acetic acid, stopping pH regulation will lead to a gradual pH drop through natural acidification. Therefore, in the early stage of the process, as a mean to evaluate the influence of the pH value on the production of metabolites, automatic pH regulation was turned off in order to let the pH decrease naturally with the simultaneous accumulation of acetic acid. The biomass concentration, pH and production of metabolites are shown in Figure 7a-c.

After pH regulation was turned off, the biomass concentration kept still increasing for a while and reached a maximum value of OD_{600nm} = 2.3, after 135 h bioreactor operation (Figure 7a). Herein, the acetic acid concentration reached 5.2 g/L once the pH had gradually decreased from 8 to 7.2 (Figure 7b, c). Afterward, the biomass concentration started to level off and then later decrease when reaching low pH values. In solventogenic clostridia, high and optimal pH conditions will generally favour both biomass growth and the accumulation of acids.
However, a short period of non-growth coupled acetic acid production was observed after the biomass concentration started to drop which caused a further decrease in pH, concomitant to the additional increase in acetic acid concentration. Ethanol first appeared when the pH value had dropped to 6.8.
Although the pH kept decreasing (pH 6.6), in order to avoid any possible inhibition, while maintaining conditions favorable for solventogenesis, it was adjusted to pH 6.8, after 210 h, and then kept constant until the end of the process. Although some trace amounts of ethanol were occasionally detected during the first days of the experiment, it is only when a low pH was reached that its production started increasing dramatically (Figure 7b, c). A low ethanol production was reported very recently in *C. aceticum* in a CO-fed batch process at high pH values (pH 8) simultaneous to acetic acid production (Mayer et al., 2018). The authors explained that even though the ethanol synthesis from acetyl-CoA is not useful since the pathway does not generate any ATP, the same pathway needs four reduction equivalents (NADH), and ethanol production might be used by the bacteria in order to regenerate the reducing equivalents in the case of accumulation.

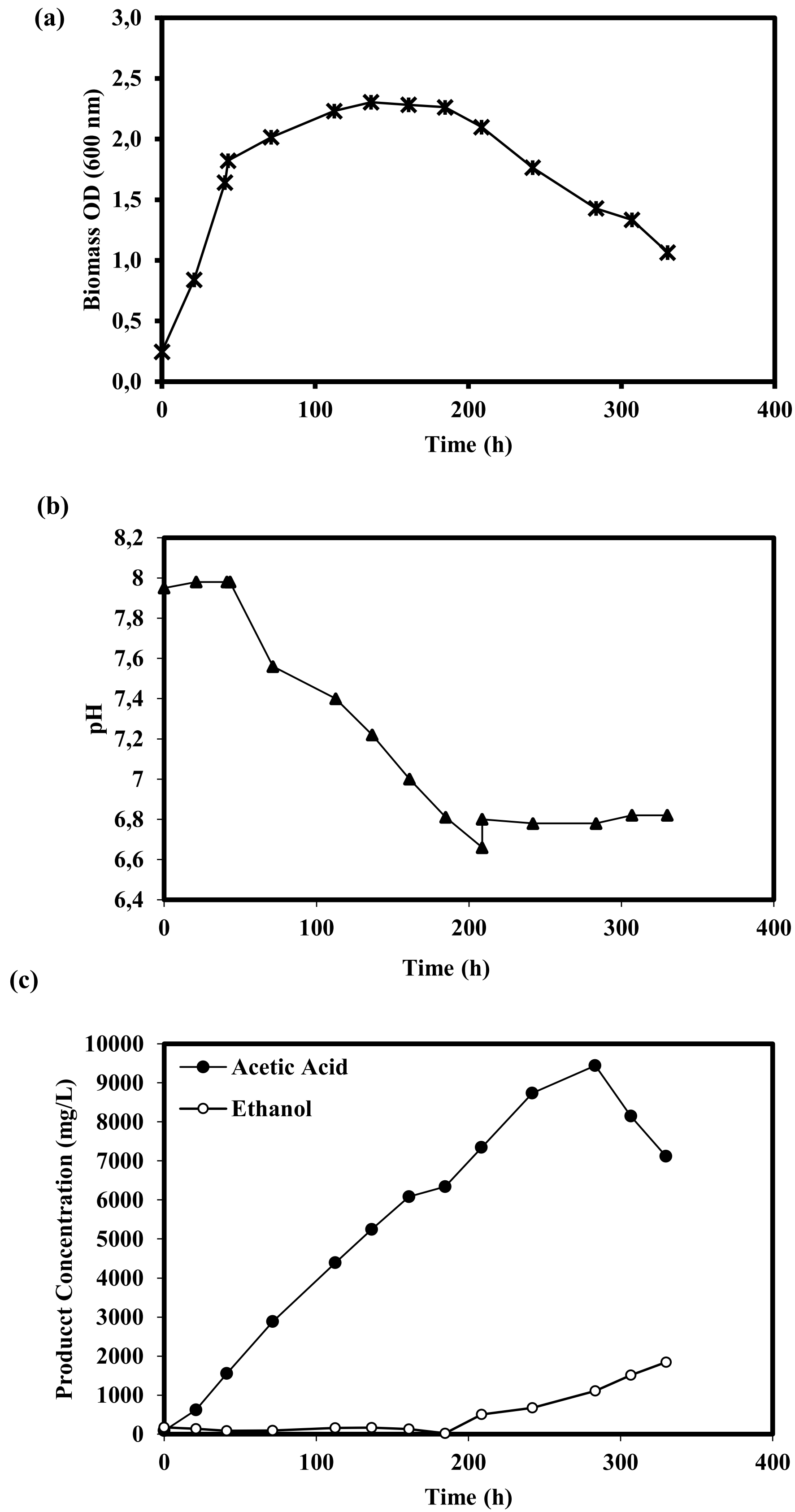

Figure 7. pH stimulated solventogenesis by natural acidification in C. aceticum. (a) biomass concentration (the OD value at 600 nm); (b) pH; (c) metabolite production

In the present study once, the low pH was reached, initially ethanol production occurred still simultaneously to acetic acid accumulation, until the acetic acid concentration reached a maximum value of 9.4 g/L. Thereafter, the acetic acid concentration decreased while the ethanol concentration kept increasing, which clearly confirms the conversion of acetic acid into ethanol in that strain. Since, in the Wood-Ljungdahl pathway, ethanol production is not an energy yielding process for the cell in acetogens, it is expected that the production of solvents does largely take place through the conversion of gases into acids, such as acetic acid, in a first stage, followed by the subsequent conversion of the acid into the corresponding alcohol. The reason behind bioconversion of acetic acid to ethanol is to overcome the stress conditions on the bacteria created by the accumulation of acetic acid in the fermentation media and the enzyme responsible for this conversion is a tungsten containing aldehyde:ferredoxin-oxidoreductase (AFOR) (Abubackar et al., 2015; 2018). Thus, once the low pH value was reached, although both acetic acid and ethanol production were observed initially, it is hypothesized that part of the acid produced was also partly and simultaneously converted to the corresponding C2-alcohol.

Ethanol was the only alcohol observed in this experiment, although some other clostridia, either in mixed or in pure cultures, have been described to be able to sometimes produce higher alcohols as well, such as butanol or even hexanol (Fernández-Naveira et al., 2019; Chakraborty et al., 2019). Acids with higher even carbon number were not found either (e.g., butyric acid, hexanoic acid). Since the main goal was to confirm, and later optimize, ethanol production in *C. aceticum*, the purity of the culture was regularly confirmed throughout the experiments. After 450 h bioreactor operation, some unexpected contaminant was detected, and the experiment was then stopped. The culture was otherwise confirmed to be pure until then.

Reproducibility of the present results is confirmed in the next assays. Besides, although solventogenesis is further optimized in the next experiments, it is worth highlighting that a high concentration of 1.8 g/L ethanol was already reached here, which is the highest value ever reported in that strain, demonstrating the potential of *C. aceticum* to produce significant amounts of ethanol as major product besides its more common metabolite, i.e. acetic acid. Although the experiment was stopped, at this stage reaching a still higher ethanol concentration was thus expected to be feasible.

Optimization of ethanol production through natural acidification and cyclic pH shift

The above described bioreactor study proves that *C. aceticum* has the ability to produce ethanol when a pH drop occurs in the fermentation medium, with concomitant acetic acid consumption. It did also show that solventogenesis is stimulated at a pH value below 7. This second experiment was aimed to increase ethanol production in *C. aceticum*, while decreasing the concentration of acetic acid accumulated, if possible. Minimizing the accumulation of any acid would be a major goal whenever the overall idea is to produce ethanol as target metabolite and as a possible alternative (bio)fuel. For this purpose, a cyclic pH shifting approach was applied, as it was previously shown to be an efficient strategy to reach higher ethanol concentrations in other solventogenic clostridia studied in STR with continuous gas supply (Abubackar et al., 2016a, 2016b). Indeed, it was confirmed experimentally, in *C. autoethanogenum*, that applying a high pH value near to optimal growth pH at the beginning of the syngas conversion would result in high biomass and acetic acid concentrations, as observed also in the above preliminary trial with *C. aceticum*. Then, if the pH drop strategy is optimized and followed by a new, second cycle, of high and low pH values, this might eventually provide high conversion of any produced acid, with the corresponding accumulation of increasing concentrations of alcohol in each subsequent pH shift cycle (Abubackar et al., 2016b). Therefore, in this experiment, shifting between high pH values and

low pH values, and *viceversa*, was performed in order to evaluate if a similar behavior would be observed in *C. aceticum* as in some other clostridia.

The experiment was also initiated under optimum growth conditions and the pH value was originally maintained at 8 through automatic pH control. Figure 8 shows the cell production, concentration of metabolites and pH evolution during the study. A similar and reproducible biomass growth pattern was observed as in the previous experiment. Automatic pH regulation was turned off before the maximum biomass concentration was reached and thus somewhat earlier than in the previous experiment to explore how this might affect the subsequent production of acetic acid, its maximum concentration, as well as biomass accumulation. pH regulation was stopped when reaching OD_{600nm} = 1.36, allowing then subsequent natural acidification. The acetic acid concentration was around 2 g/L at that point. Although pH started dropping, the biomass concentration kept increasing for some time until reaching a maximum value of OD_{600nm} = 2.4, which is very similar as in the previous experiment. The acetic acid concentration also increased and reached a maximum value of 9.4 g/L, which is also similar as in the first experiment, indicating that the somewhat earlier acidification did not affect the final biomass and total acid accumulation and highlighting the highly reproducible behavior of the bioconversion process. The production of acetic acid caused a slow decrease of the fermentation pH and ethanol first appeared after 335 h in the medium when the pH had dropped to 7.1. The pH value kept decreasing until it reached 6.98. However, it did not manage to further decrease and was then adjusted to 6.9, expected to be low enough for solventogenesis in that strain. The low pH was maintained for about 10 days and a maximum ethanol concentration of 5.6 g/L was achieved after about 710 h. The purity of the strain was checked at this stage and at the end of the experiment, confirming the absence of any possible contamination and the sole presence of *C. aceticum* throughout the whole experiment.

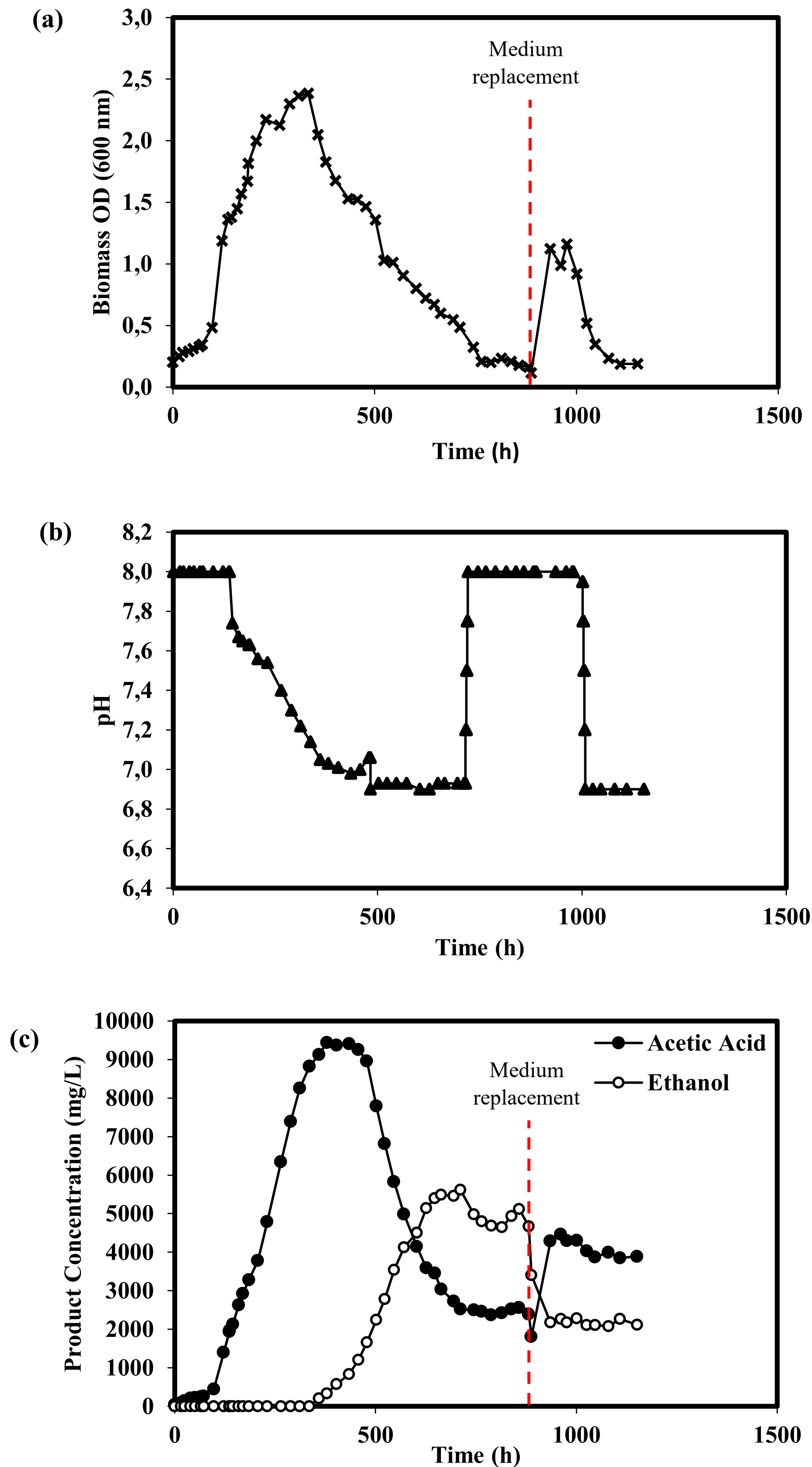

Figure 8. Optimization of ethanol production by applying natural acidification, pH shifting and medium replacement, respectively, in *C. aceticum*

(a) biomass concentration (the OD value at 600 nm); (b) pH; (c) metabolites production

Maintaining a low pH for several days resulted in severe biomass decay. The biomass concentration started to decrease when the pH value was under 7.1, and it dropped down to a minimum OD_{600nm} = 0.4 at the end of this period at pH 6.9. Since the accumulation of ethanol leveled off and hardly any acetic acid was converted anymore into the corresponding C2 alcohol, it was decided to apply the pH shift strategy, in order to check if a new round of acid production would be possible when increasing again the pH value, eventually followed by acid conversion to ethanol when allowing a new pH decrease later on. The pH value was readjusted to 8 by using a 1 M NaOH solution, at 715 h. However, contrary to what was observed in other clostridia, such as *C. autoethanogenum* (Abubackar et al., 2016b), acetic acid production did not take place. Instead, the concentrations of any metabolites remained stable and moreover the cell concentration kept decreasing down to OD_{600nm} = 0.164. Since the fermentation takes place with continuous gas feed while the liquid nutritive medium is maintained in batch mode, exhaustion of some key nutrient and/or accumulation of some inhibitory compound is not excluded. This was also observed in some other gas fermenting strain, such as *C. carboxidivorans* (Fernández-Naveira et al., 2016). It appeared that renewing part of the nutrient medium might then solve this issue and help restart microbial activity (Fernández-Naveira et al., 2016). Therefore, 400 mL of medium was removed from the bioreactor and replaced with fresh sterile medium. The pH was kept constant at 8 during this period. Right after medium replacement, the cell concentration immediately increased, exponentially, up to OD_{600nm} =1.12. Interestingly, while *C. aceticum* recovered its activity, with clear biomass growth, a significant decrease in ethanol concentration was observed with a concomitant increase in acetic acid concentration. This observation suggests that *C. aceticum* might be capable to use ethanol as a substrate, which to the best of our knowledge has not been reported before in that organism. The final concentrations of the metabolites at the end of this experiment were 3.8 g/L and 2.1 g/L for acetic acid and ethanol, respectively.

Effect of artificial acidification and pH shift on ethanol production

This new experiment was started under optimal growth conditions (pH 8, 30 ºC). During the first period of the experiment, the pH value was kept constant at 8 to promote growth and acetic acid production. As in the previous case, pH regulation was stopped once reasonable biomass growth and acetic acid accumulation had been observed. However, although in the previous experiments pH regulation had been stopped once about 2 g/L acetic acid had been produced, in the present case, the system was allowed to reach a higher concentration (double) of acetic acid before pH adjustment was stopped and its value started dropping. It was expected that this might allow an overall higher production of acetic acid and thus a higher subsequent accumulation of alcohol. About 4 g/L acetic acid was present in the medium, when automatic pH regulation was stopped. Nevertheless, although the pH did then indeed decrease, as a result of the additional production of acetic acid, it did not manage to drop below about pH 7.7 through natural acidification, even after several days (75 hours). It was considered that, in this experiment in which pH regulation had been maintained for a longer period by adding the required amount of NaOH (1 M), natural acidification below pH 7 would then not be possible as the additional production of acetic acid would not be enough to counteract the buffering capacity of the medium. For this reason, a 1 M HCl solution was used to artificially decrease the pH gradually to 6.9 in order to stimulate ethanol production in the first cycle of low pH. The artificial pH decrease was done stepwise, as shown in Figure 9; as our previous studies with different gas fermenting clostridia suggest that a sudden significant pH drop might negatively affect acetogens (Fernández-Naveira et al., 2017b). The accumulation of acetic acid in this case was quite slower than in the previous experiments. This could most probably be due to the artificial acidification strategy compared to natural acidification in the other experiments. Indeed, based on our own experience, artificial acidification has, in several cases, been shown to be less favourable or more inhibitory in the

switching process from acidogenesis to solventogenesis in clostridia. Ethanol production started 40 h after the pH was decreased to its minimum value and a concentration of 3.5 g/L ethanol was then reached in 185 h (Figure 9b). Simultaneously, the acetic acid concentration decreased from 6.6 g/L to 4 g/L, confirming the initial assimilation of gases to produce acetic acid, followed by the conversion of the acid into ethanol. The fermentation pH was then readjusted back to the initial optimum growth value for the second time in order to check if this would lead to a new increase in acetic acid concentration, which could then afterwards hopefully again be converted to additional ethanol. It would also allow to check if later stopping pH regulation combined to artificial acidification might affect the bioconversion process compared to the previous experiments. Interesting observation in this experiment is that in the second high pH period, while the acetic acid concentration doubled (from 4 g/L to 7.8 g/L), ethanol production decreased to half of its concentration (from 3.5 g/L to 1.7 g/L), as can be seen in Figure 3b. This result confirmed our former hypothesis that *C. aceticum* may have the ability to metabolize ethanol produced previously in the solventogenic phase and would be able to perform the reverse reaction converting ethanol back to acetic acid. The significant, fast, decrease in ethanol concentration in the two last experiments does not seem to be coincidence but instead suggests a possible metabolization of that alcohol in *C. aceticum*. The conversion of ethanol to acetic acid takes place at high pH (pH 8), under optimal growth conditions, but was not observed in acidified media. The high commercial interest of (bio)ethanol as a fuel, over the recent past, explains the higher interest in enzymes and mechanisms involved in ethanol production from C1 gases and acetic acid rather than the reverse reaction (Bertsch et al., 2016). Although the potential of *C. aceticum* to metabolize ethanol, which had, to the best of our knowledge, not been reported before, in *Acetobacterium woodii* it had been reported that 2 moles of ethanol would react with two moles of carbon dioxide to produce 3 moles of acetic acid, according to the following reaction: 2 C_2H_5OH + 2

$CO_2 \rightarrow 3\ CH_3COOH$ (Buschhom et al., 1989). The presence of bicarbonate in the culture medium as well as the presence of carbon dioxide, among others in the syngas mixture, supports the potential to perform this same reaction in *C. aceticum*. When considering the last stage of the last experiment, in which the concentration of acetic acid increased by about 3.8 g/L while the concentration of ethanol decreased by about 1.8 g/L, this would result in a consumption of 39 mM ethanol and a production of 63 mM acetic acid, which fits reasonably well to a ratio of 1.5 moles acetic acid produced per mol ethanol metabolized. If the goal is to produce ethanol as a renewable (bio)fuel, minimizing the concentration of bicarbonate might limit the possible reverse reaction of ethanol to acetic acid, while at low pH exclusive production of ethanol rather than any other metabolite was observed. Further optimization of the operating conditions would then certainly allow further improvement of the ethanol production efficiency in *C. aceticum*.

When the pH was again adjusted to a lower value for the second time, 780 h after start-up of the experiment, to check if it would stimulate solventogenesis again, an additional accumulation of 1.4 g/L ethanol was observed during this period, while acetic acid was indeed consumed. However, the ethanol consumption in the previous stage avoided any substantial overall increase in ethanol concentration in the fermentation broth and the final ethanol concentration obtained was then 3.2 g/L at the end of this experiment. In the last stage, since the previous pH shift was successful, the pH value was adjusted for the third time to a high value, however the acetic acid and ethanol concentrations remained almost stable in this case. As in the previous experiments, the sole presence of the inoculated strain was confirmed throughout this assay as well.

All three experiments are summarized and compared in Table 3. The maximum concentrations of metabolites are also given in that Table. In some other very recent study, although ethanol production was not the main focus, its production in *C. aceticum* from CO in

a stirred tank bioreactor was mentioned, reaching low maximum concentrations of a few hundreds of mg/L at high pH (pH 8) (Mayer et al., 2018; Riegler et al., 2019).

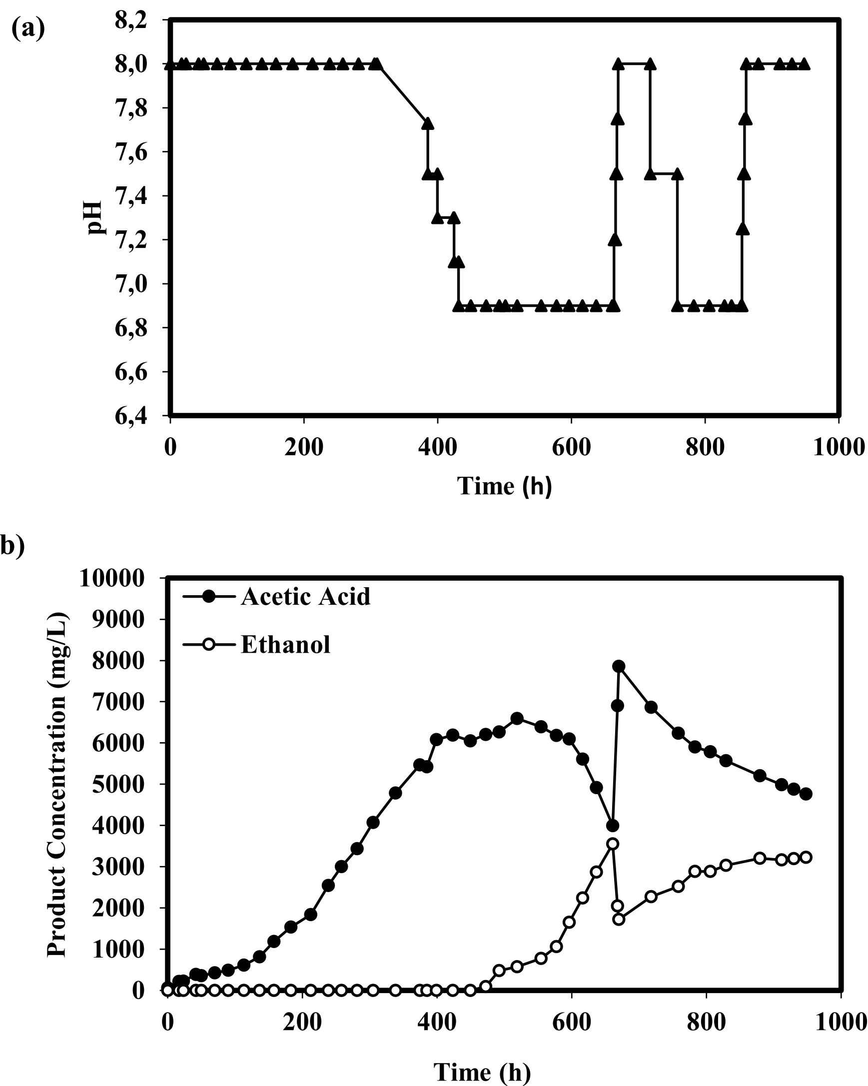

Figure 9. Optimization of ethanol production by applying artificial acidification and pH shifting, respectively, in *C. aceticum*

(a) pH; (b) metabolites production

The present optimization study shows that accurately adjusting the operating conditions allows to reach high ethanol concentrations of several g/L, not only from CO but also from syngas, and that the accumulation of that alcohol is possible when applying cycles of high and low pH; although under specific conditions, the reverse reaction may take place as well. In the

last experiment, the observed maximum concentration of acetic acid was lower than in the previous assays. The reason for this behavior would be the artificial acidification process, including also usage of high amounts of HCl and NaOH, and the higher stress conditions occurring therefore during the pH shift. This resulted also in a lower maximum ethanol concentration in case of artificial acidification compared to the natural pH drop (Table 1).

Table 3. Maximum acetic acid and ethanol production observed in different experiments

Experiment	Details	Maximum HAc Conc. (g/L)	Maximum EtOH Conc. (g/L)
1st	Natural acidification	9.4	1.8
2nd	Natural acidification + pH shifting + medium renewal	9.4	5.6
3rd	Artificial acidification + pH shifting	7.8	3.5

From all the above results it can clearly be observed that *C. aceticum* followed the typical acetogenic and solventogenic steps of the Wood-Ljungdahl pathway, metabolizing C1 gases to produce acetic acid, followed by its subsequent bioconversion to ethanol. Based on the present results and previously published research with other acetogens, it is interesting to observe that the pH drop rather than the pH value as such seems to stimulate solventogenesis. Indeed, in *C. aceticum* a pH value around 8 is near optimal for biomass growth and thus acetic acid production, while solventogenesis is already detected at a slightly acidic pH value of 6.9. Conversely, in strains such as *C. autoethanogenum* (Abubackar et al., 2012, 2016a), and *C. carboxidivorans* (Fernández-Naveira et al., 2017b), high efficient growth and acids production takes place at slightly acidic pH, around pH 6, rather than alkaline pH conditions

(i.e., pH 8 in *C. aceticum*), while efficient solventogenesis is observed after a pH drop down to values as low as 5 or even lower, to be compared to a suitable solventogenic pH around 6.8-6.9 in *C. aceticum*.

Production of ethanol and enrichment of solventogenic biomass inmixed culture by means of natural acidification was reported very recentlyand 11.1 g/L ethanol was obtained in an STR, using only CO asgaseous substrate (Chakraborty et al., 2019). Additionally, successfulassays were performed with C. autoethanogenum (Abubackar et al., 2016a) and C. carboxidivorans (Fernández-Naveira et al., 2019) to stimulate solventogenesis by applying a pH shifting strategy and by decreasingthe pH value through acid production. In such cases, 4.3 g/Land 5.9 g/L ethanol titers were observed in each one of these strains, respectively. Apart from the pH changing approaches, other strategies to enhanceethanol production in acetogens have also been reported, although applying pH drops is one of the most efficient alternatives. Abubackaret al. (2018) used the species C. carboxidivorans in a two‑stage continuoussystem composed of two STRs in series by maintaining the pHof the first reactor at 6 and of the second reactor at 5 in order to allowacetic acid production mainly in the first reactor and stimulate alcoholproduction in the second one. In a recent study with C. ragsdalei, it wasreported that ethanol production was enhanced up to 13.2 g/L with theaddition of poultry litter biochar as a nutrient supplement (Sun et al.,2018). In another study, a hollow fiber membrane biofilm reactor wasused for ethanol production from syngas (CO and H_2) with mixed cultureunder low pH (4.5) and a maximum concentration of 16.9 g/L ethanol was reached (Wang et al., 2018).

Conclusions

It was shown that *C. aceticum* can produce high ethanol concentrations from CO, CO_2, or syngas. Solventogenesis was initiated at near neutral pH values. High ethanol production and dominant solventogenesis in *C. aceticum* was detailed and optimized, reaching the maximum

ethanol concentration of 5.6 g/L when natural acidification was started in the early stage of the process. Another interesting finding was the possible ability of *C. aceticum* to convert ethanol, produced in the solventogenic phase, back to acetic acid, under specific conditions. Optimizing conditions to avoid the reverse reaction in cyclic pH shift would allow tofurther maximize ethanol production.

Acknowledgements

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4.2 Autotrophic (C1-gas) Versus Heterotrophic (Fructose) Accumulation of Acetic Acid and Ethanol in *Clostridium aceticum*

Ethanol formation in *Clostridium aceticum* was studied and it was observed that high ethanol concentration could be reached by applying certain pH regulation strategies. This study was performed by using a syngas mixture of $CO:CO_2:H_2:N_2$ (30:5:15:50). Syngas fermentation is known to suffer from poor product yields due to the low solubilities of gaseous substrates. Autotrophic CO_2 fixation requires and energy source and in syngas mixture this is H_2. However, CO can be a source of carbon and energy at the same time and it is known that compared to syngas mixture sole CO is a better carbon source for reduced products like ethanol. Addition to that organic carbon sources like fructose is known to provide more energy (ATP) and reducing power (NADH/NADPH). Therefore, it should be revealed how this different carbon sources would influence process performance and ethanol production with *Clostridium aceticum*. In this study our aim was to find an answer to the following question: what might be the limitations of sole CO or sole fructose usage for ethanol production in *C.aceticum* in a batch fermentation study while using a pH shifting approach.

Citation – these data have been published as

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Abstract

The influence of the carbon source on the metabolism and growth of *Clostridium aceticum* was investigated, supplyingeither CO or fructose as sole carbon source. Theacid and solvent production patterns were determined under either autotrophic or heterotrophic conditions, elucidating theeffect of pH on the bioconversion pattern of each carbon source.The highest biomass growth rate was observed with CO, under the organism´s optimal growth conditions, reaching 0.052 h^{-1} andan acetic acid concentrationof 18 $g \cdot L^{-1}$.The production of 4.4 $g \cdot L^{-1}$ethanol was also possible,after medium acidification,during CO bioconversion. Conversely, formic acid inhibition was observed during fructose fermentation under optimal growth conditions. In the latter experiments, it was not possible to stimulate solvent productionwhen growing *C. aceticum* on fructose, despite applying the same medium acidification strategy as with CO, showing the selective effect of the carbon source (autotrophic *vs* heterotrophic) on the metabolic pattern and solventogenesis.

Keywords: Carbon monoxide; Carbon dioxide; Solventogenesis; Syngas; Wood-Ljungdahl pathway

Introduction

Bioethanol has been considered a high potential clean transportation fuel alternative to gasoline, that can be produced from sustainable renewable resources. There are two major alternative production routes depending on the feedstock used for obtaining the alcohol. First generation bioethanol production is a commercially mature technology and food-based materials such as sugarcane, sugar beet, corn, and wheat are used as common feedstocks (Sun et al., 2019; de Medeiros et al., 2020; Kennes et al.,2016). However, usage of these food based materials in the production of fuels is criticized for creating food versus fuel competition, associated with increasing market prices of these foods and increasing global food insecurity, causing changes in land use, and deforestation (Kennes et al., 2016; Nanda et al., 2014).

On the other hand, second generation bioethanol production has the potential to minimize these impacts as lignocellulosic materials such as agricultural or municipal wastes, wood, straw, grasses, and crop residues are utilized as feedstocks (Groenestijn et al., 2013). Lignocellulosic biomass may contain up to 30-40% lignin in some cases and valorization of this fraction is an important goal to be reached in order to increase overall product yield and conversion efficiencies (Kennes et al., 2016; Safarian et al., 2020). A possible alternative that allows using all the lignocellulosic fractions, i.e., cellulose, hemicellulose, and lignin, is biomass gasification, in which the whole feedstock is converted into a gas mixture at high temperature. This gas mixture, known as syngas or synthetic gas, is composed of mainly CO, CO_2 and H_2 gases, with the possible presence of other gases, like CH_4 or nitrogen, and impurities, depending on the gasification conditions (Infantes et al., 2020).Besides the gasification of carbonaceous-rich materials, syngas components can also be found in flue gases of some heavy industrial processes (Sun et al., 2019). Carbon monoxide, CO, is also a highly dominant gas in some emissions of steel industries.

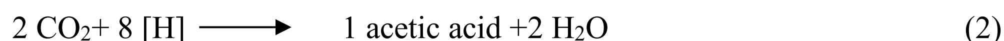
Acetogenic microorganisms are able to utilize CO and/or CO_2+$H_2$from syngas as sole carbon and/or energy sources and produce organic acids, alcohols and some other chemicals based on the Wood-Ljungdahl Pathway (WLP), under anaerobic conditions. Details of the WLP, including the development of the technology, reaction mechanisms and enzymology of the process, naturally occurring products, and recent accomplishments can be found in several review papers (Ragsdale 2008; Bengelsdorf et el., 2013; Drake et al., 2008; Schiel-Bengelsdorf and Dürre, 2012; Sun et al., 2019).

C. aceticum was the first reported autotrophic acetogen capable of converting carbon dioxide and hydrogen to acetic acid and water in pure culture studies (Wieringa, 1939). However, due to the loss of the strain, more studies were delayed until some spore preparation of the original *C. aceticum*species were found in a culture collection (Braun et al., 1981). Recently, several efforts have been made to studymore in details the ability of *C. aceticum* to grow autotrophically on CO and/or CO_2+H_2 in fully automated bioreactor systems (Mayer et al., 2018; Riegler et al., 2019; Arslan et al., 2019). The ethanol production capability of *C. aceticum* was also investigated and optimized very recently and it was shown that pH drops below 7.0 in the fermentation medium stimulate ethanol production in this strain. As much as 5.6 $g \cdot L^{-1}$ethanol production was recently reached with different pH regulating strategies from a syngas mixture of CO:CO_2:H_2:N_2 (30:5:15:50) (Arslan et al., 2019).

Beyond the characteristic feature of autotrophic growth on syngas components, most members of acetogens have the metabolic flexibility for utilizing a wide range of soluble substrates such as carbohydrates, among others (Fernández-Naveira et al., 2017a; Karekar et al., 2019; Weghoff et al., 2015; Buschhorn et al., 1989). Conversion of carbohydrates to acetic acid in acetogenic bacteria is called homoacetate fermentation and occurs in two steps. In the first step, sugars are oxidized to 2 moles of acetic acid and 2 moles of CO_2 by glycolysis. Following this step, both molecules of CO_2 are further converted to oneadditional molecule of

acetic acid through the WLP. In total, one mole of sugar is completely fermented to 3 moles of acetic acid according to reactions 1 and 2 (Schuchmann and Müller, 2016; Huang et al., 2012).

$$\text{Glucose} + 4\ \text{ADP} + 4\ P_i \longrightarrow 2\ \text{acetic acid} + 2\ CO_2 + 4\ \text{ATP} + 2\ H_2O + 8\ [H] \quad (1)$$



$$2\ CO_2 + 8\ [H] \longrightarrow 1\ \text{acetic acid} + 2\ H_2O \quad (2)$$

Theoretically, CO_2 reduction to one more mole of acetic acid in the WLP following glycolysis provides the highest known ATP gain and better mass yields in carbohydrate fermentationand only acetogens are natively capable of performing this conversion (Schuchmann& Müller, 2016; Fast et al., 2015). Therefore, it is important to understand the heterotrophic metabolism of acetogens in order tobetter understand their potential usein biotechnological applications. Comparison of autotrophic and heterotrophic metabolic patterns of acetogens and their influence onacetogenesis and solventogenesis has hardly been done (Fernández-Naveira et al., 2017a).

The present study was undertaken in order to compare the metabolic profile of *C. aceticum*o n two different carbon sources, either CO or fructose, as the heterotrophic metabolism of this strain on fructose and possible solventogenesis had not been reported before under controlled conditions, in automated bioreactor systems. Another objective of this research was to study the effect of pH control on the production of metabolites in *C. aceticum* growing on different carbon sources. Several different bioreactor studies, with and without pH control, were conducted with both carbon sources in order to elucidate how operational conditions, such as pH, affect the fermentation and bioconversion pattern in *C. aceticum*.

Materials and Methods

Microorganism and culture medium

C. aceticum (DSM 1496) was used in all the experiments and was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig,

Germany) in the form of freeze-dried pellets. Initial rehydration of the bacteria was carried out by following the procedure recommended by DSMZ.

The strain was maintained by subculturing in 40 mL working volume serum bottles. Preparation of the serum bottlesand growth of seed cultureswas done as described previously (Arslan et al., 2019). Autotrophic growth of the strainwas also carried out in serum bottles with 40 mL working volumeand using CO as carbon source, while fructose was used for heterotrophic growth. The composition of the liquid culture medium used in all studies with*C. aceticum*was as follows (per liter distilled water): NH_4Cl, 0.20 g; yeast extract, 3 g; KH_2PO_4, 1.76 g; K_2HPO_4, 8.44 g; $MgSO_4 \times 7H_2O$, 0.33 g; $NaHCO_3$, 10 g; L-Cysteine-HCl, 0.30 g; Na_2S x $9H_2O$, 0.92 g; rezasurin, 1 mL (from the stock solution of $1 g \cdot L^{-1}$); trace metal solution, 2 mL; vitamin solution, 2 mL.

Thecomposition of the trace metal solution was(per liter distilled water): Nitriloacetic acid, 15 g; $MgSO_4$ x 7 H_2O, 30 g; $MnSO_4$ x H_2O, 5g;NaCl, 10 g; $FeSO_4$ x 7 H_2O, 1 g; $CoSO_4$ x 7 H_2O, 1.8 g; $CaCl_2$ x 2 H_2O, 1 g; $ZnSO_4$ x 7 H_2O, 1.8 g; $CuSO_4$ x 5 H_2O, 0.1g; $KAl(SO_4)_2$ x 12 H_2O, 0.2g; H_3BO_3, 0.1 g; Na_2MoO_4 x 2 H_2O, 0.1g; $NiCl_2$ x 6 H_2O, 0.25g; Na_2SeO_3 x 5 H_2O, 3mg; Na_2WO_4 x 2 H_2O, 4mg.

The composition of the vitamin solution was (per liter distilled water): biotin, 0.025 g; folic acid, 0.025 g; pyridoxine-HCl, 0.050 g; thiamine-HCl, 0.050 g; riboflavin, 0.050 g; nicotinic acid, 0.050 g; D-Ca-pantothenate, 0.050 g; vitamin B12, 0.025 g; p-Aminobenzoic acid, 0.050 g; lipoic acid, 0.025 g.

Bioreactor operation

The fermentation studies were carried out in 2L EppendorfBIOFLO 120 bioreactors (Eppendorf AG, Hamburg, Germany) under anaerobic conditions, in batch for the liquid phase and with continuous gas supply when feeding a gas phase. The bioreactors were equipped with four baffles, a microsparger and a pH electrode (Mettler Toledo, Columbus,

Ohio, USA). The fermentation processes were performed at a temperature of 30 °C, which waskept constant by means of either a water jacket or a heating blanket. Mixing was realized with six blade Rushton turbines agitated at 250 rpm. In all studies the initial pH of the fermentation medium was adjusted to 8.0by using either 1 M HCl or 1 M NaOH solutions. The bioreactors filled with 1.1 L medium without vitamins, cysteinexHCl, Na_2Sx9H_2O and fructose were autoclavedat 120 °C for 20 min. A 50 mLsolution containing cysteinexHCl and Na_2Sx9H_2O and another 50 mL fructose (36 g) solution were prepared in separate bottles and autoclaved under the same conditions as well. After autoclaving, the medium was flushed with pure aseptic nitrogen for at least 2 hours. Following pH adjustment, cysteine x HCl, Na_2S x $9H_2O$, fructose and vitamins were addedaseptically to the vessels prior inoculation. For inoculation, 120 mL of late-exponential phaseadapted seed culture was used.

In autotrophic studies fructose was omitted from the medium and replacedwith pure CO, that was flushed through the medium for at least 30 minutes, immediately afternitrogen sparging through themicrosparger. The CO gas flow rate entering the reactor was adjusted to10 mL/min and it was maintained constant during the whole autotrophic experiments by means of a mass flow controller (Aalborg GFC 17, Müllheim, Germany).

Analytical methods

2 mL samples were withdrawn daily from the reactor in order to carry out analytical tests. The optical density, used to estimate biomass growth and concentration, was measuredwith aspectrophotometer (Hitachi, Model U-200, Pacisa& Giralt, Madrid, Spain) at a wavelength of 600 nm.

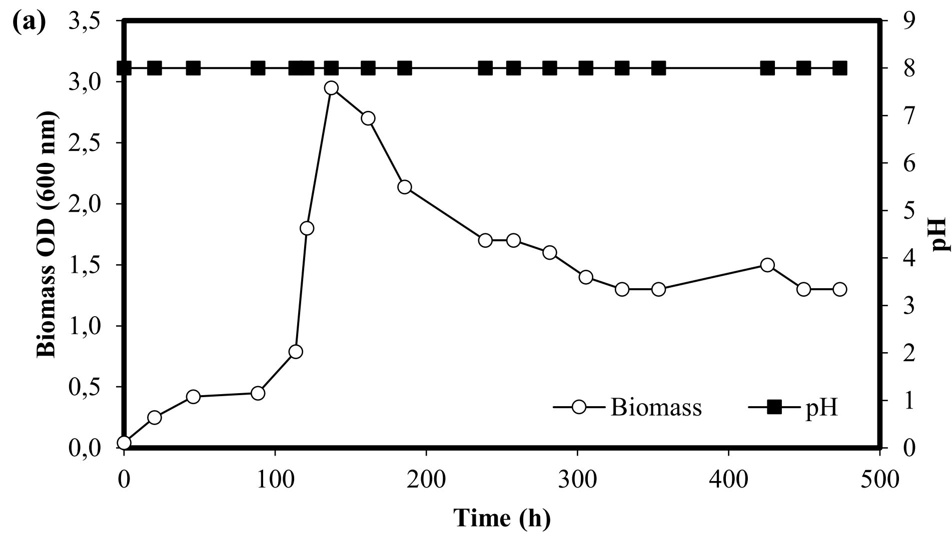
Fructose, acetic acid and ethanol concentrations were measured on a high performance liquid chromatograph (HPLC) (HP1100, Agilent Co., USA) equipped with a diode array detector and a refractive index detector, operating at 50 °C. The possible presence of C4 and C6 acids and alcohols was checked as well. The HPLC samples were first centrifuged (ELMI Skyline

Ltd CM 70M07) at 7000 rpm for 5 min and the supernatant was then filtered through a 0.22 μm filter (Labbox, Barcelona, Spain) before HPLC analyses. The mobile phase used for the HPLC analyses was a5 mM H_2SO_4solution, with a flow rate of 0.80 mL/min. 20 μL samples were injected in the Agilent Hi-Plex HColumn (300 x 7.7 mm) which was kept at 45 °C. Carbon yields, C_M/C_S, were calculated as explained before (Maru et al., 2018). C_M refers the total produced acetic acid carbon and was calculated by multiplying the final acetic acid molar concentration by 2, while C_S refers the total consumed substrate carbon and was calculated by multiplying the total consumed fructose molar concentration by 6.

Results and Discussion

CO fermentation with pH regulation and constant pH

In a first experiment, *C. aceticum* was inoculated in the fermentor with continuous CO gas feeding, and at constant pH = 8. This value was kept automatically stable during the whole fermentationprocess. Fig. 10 a and b show, respectively, the bacterial growth and the concentrations of products. A slow growth was first observed at the beginning of the process, which could have resulted from the common inhibitory effect of CO on the initially low biomass concentration. Then, a clear exponential growth phase took place, between about t = 100 h – 137 h, reaching a maximum OD value of 3 at t = 137 h, and with an exponential growth rate, μ_{exp} of 0.052 h^{-1} and a doubling time of 13.3 h. Afterwards, the biomass OD started to decrease and dropped below 1.5 at t = 300 h; and then it remained roughly constant, around 1.3, until the end of the process.



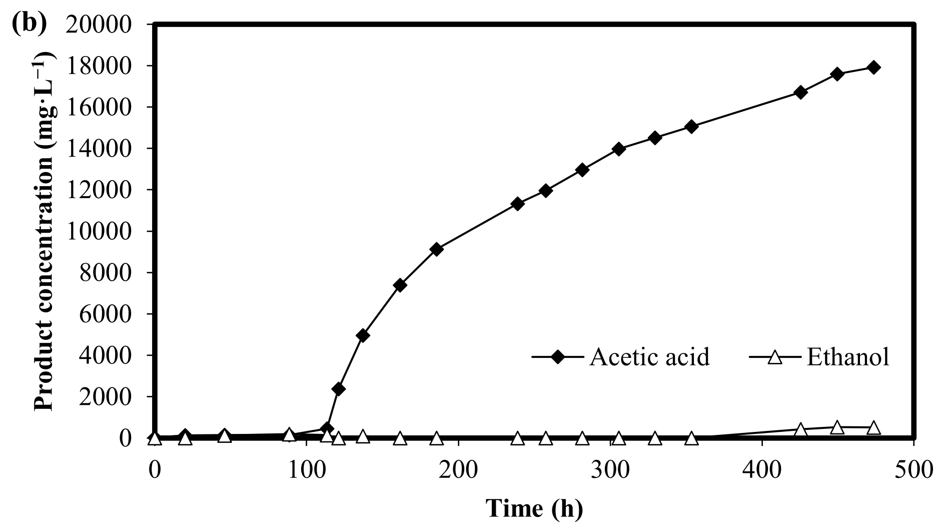


Figure 10. CO fermentation with pH regulation at 8

(a) biomass concentration (OD value at 600 nm) and pH; (b) metabolites production

The start of the exponential growth phase was concomitant with the first detection of acetic acid. The acetic acid concentration remained below 500 mg·L^{-1}during the first 100 h fermentation. The highest acetic acid production rate was observed during the exponential growth phase, reaching 0.26 g·L^{-1}·h^{-1}. This acetic acid production went on after the exponential growth phase ended, though with decreasing production rates, dropping gradually from 0.1 to 0.02 g·L^{-1}·h^{-1}at the end of the process, and reaching a final product concentration of 18 g·L^{-1}, after 475 h fermentation. Some ethanol production was observed as well, but only after almost 400 h, to finally reach around 500 mg·L^{-1} in the fermentation medium.

CO fermentation without pH regulation and with medium acidification

A subsequent CO conversion experiment was conducted in order to evaluate the effect of pH on metabolite production and on the biomass growth of *C. aceticum.* This experiment was started under optimal conditions, at pH = 8, but after inoculation, pH regulation was switched off and the pH value was allowed to drop freely, depending on the nature of the products and their concentrations. Fig. 11 a and b show the biomass growth, product formation, and pH fluctuations during the fermentation process. The initial bacterial growth was slow, similarly as in the previous experiment. Besides, a longer exponential growth phase was observed, which took place between t = 65h – 210 h of the fermentation processbut with a much lower growth rate, μ_{exp}, of 0.006 h^{-1} than in the previous case. This could certainly be related to the fast pH drop as a result of acetic acid production. Initially, slow production of acetic acid took place, before the exponential growth phase, and then a muchhigher acid production rate was observed, leading to a fast pH drop from 8 to 7.3, simultaneous to a sharp increase of the acetic acid concentration up to 2.1 g·L^{-1} (Fig. 11 a and b).

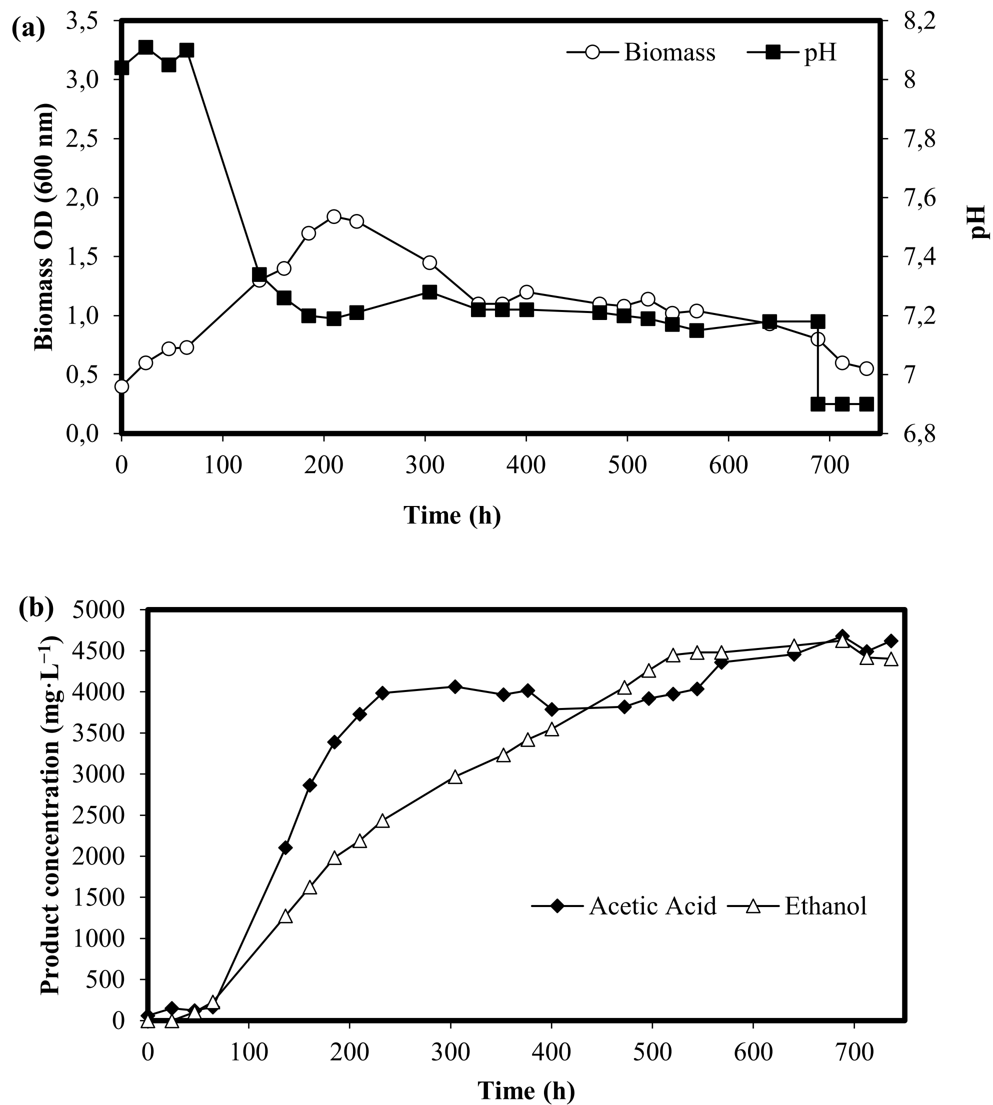


Figure 11. CO fermentation without pH regulation

(a) biomass concentration (OD value at 600 nm) and pH; (b) metabolites production

The optimum pH range of *C. aceticum* for cell growth was reported to be between 8 and 8.5 and it was also reported thatat pH values below 7 or above 10.5 generally no growth is observed (Wieringa 1940; Braun et al., 1981). In the present study, even though bacterial growth continued while the pHdecreased down to around 7.2, together with acetic acid production, the maximum OD reached a value of only 1.8, which is significantly lower than in the previous experiment at constant optimal pH. As expected, suppressing pH regulation ledto a drop of the fermentation pH and slowed down the bacterial growth,leading to a longer and

slower exponential growth phase with a significantly lowerfinal cell density. After the exponential growth phase, the biomass OD decreased to around 1.0and remained close to this value until the end of the process. Biomass decay may, among others, be attributed to the low pH value.

The acetic acid production rates were also lower compared to the previous study due to the lower bacterial growth and the highest production rate was observed during theexponential growth phase, corresponding to 0.03 $g \cdot L^{-1} \cdot h^{-1}$. The acetic acid concentration increased slowly and reached a stable value, around 4 $g \cdot L^{-1}$, at the end of the growth stage, causing a pH drop to 7.19. This acetic acid concentration is significantly lower than when maintaining the medium at constant, near optimal, pH. Ethanol first appeared in the fermentation medium at t = 46 h while the medium´s pH was still around 8, but it was detected in only minoramounts. Afterwardswith the pH value fastly decreasing from 8 to 7.3, a high ethanol production was observed, simultaneous to acetic acid accumulation and bacterial growth. The ethanol production rates were always lower than the acetic acid production rates during the exponential growth phase; however, the highest ethanol production rates, of around 0.015 $g \cdot L^{-1} \cdot h^{-1}$, were still observed during this stage as a result of the high active bacterial concentration. Ethanol continued accumulating in the fermentation medium after acetic acid had reached a stable, near constant, concentration. Later, some small decrease was detected, and when ethanol production leveled off, after 520 h, some very low increase in acetic acid concentration was still observed. Considering this production pattern, it is hypothesized that after the exponential growth phase non-growth coupled acetic acid formation continued with lower acid production rates, while part of the produced acid was simultaneously assimilated and converted to ethanol, resulting in both production and consumption of the acid. In a recent study with continuous syngas feeding ($CO/CO_2/H_2/N_2$), rather than pure CO, and with *C. aceticum*, lowering the pH also stimulated solvent production (Arslan et al., 2019).In that

study, high amounts of ethanol were only observed when the pH of the medium was decreased below neutral conditions, and a clear switch from acidogenesis to solventogenesis was also shown, slightly below pH 7.0, with a decrease in acetic acid concentration concominant to increases in ethanol concentrations. Hence, it was concluded that rather than a direct conversion of C_1 gases to alcohol, ethanol production took mainly place through the re-assimilation of acetic acid at low pH values. It was also reported before by other authors that rather than the direct reduction of acetyl-CoA to ethanol, conversion of acetic acid to ethanol through the aldehyde:ferredoxin oxidoreductase (AOR) (Fig.3) route is energetically more favorable in acetogens (Mock et al., 2015). According to these results, in order to analyze any further possibility of ethanol accumulation, after t = 690 h, the fermentation pH was decreased artificially to 6.9, by using 1M HCl, as a pH value slightly below neutral was recently reported as the most suitable average pH value for stimulation of solvent production in *C. aceticum* (Arslan et al., 2019). Nonetheless, lowering the fermentation pH to 6.9 only resulted in faster bacterial decay, while any possible improvement in ethanol production was not observed, which was most probably also a consequence of the low biomass concentration. At the end of the experiment, at t=690 h, the final concentrations of acetic acid and ethanol had reached4.7 $g \cdot L^{-1}$ and 4.6 $g \cdot L^{-1}$, respectively, highlighting the unfavorable effect of the applied pH conditions on acetogenesis and the solventogenic pattern leading to considerable ethanol accumulation.

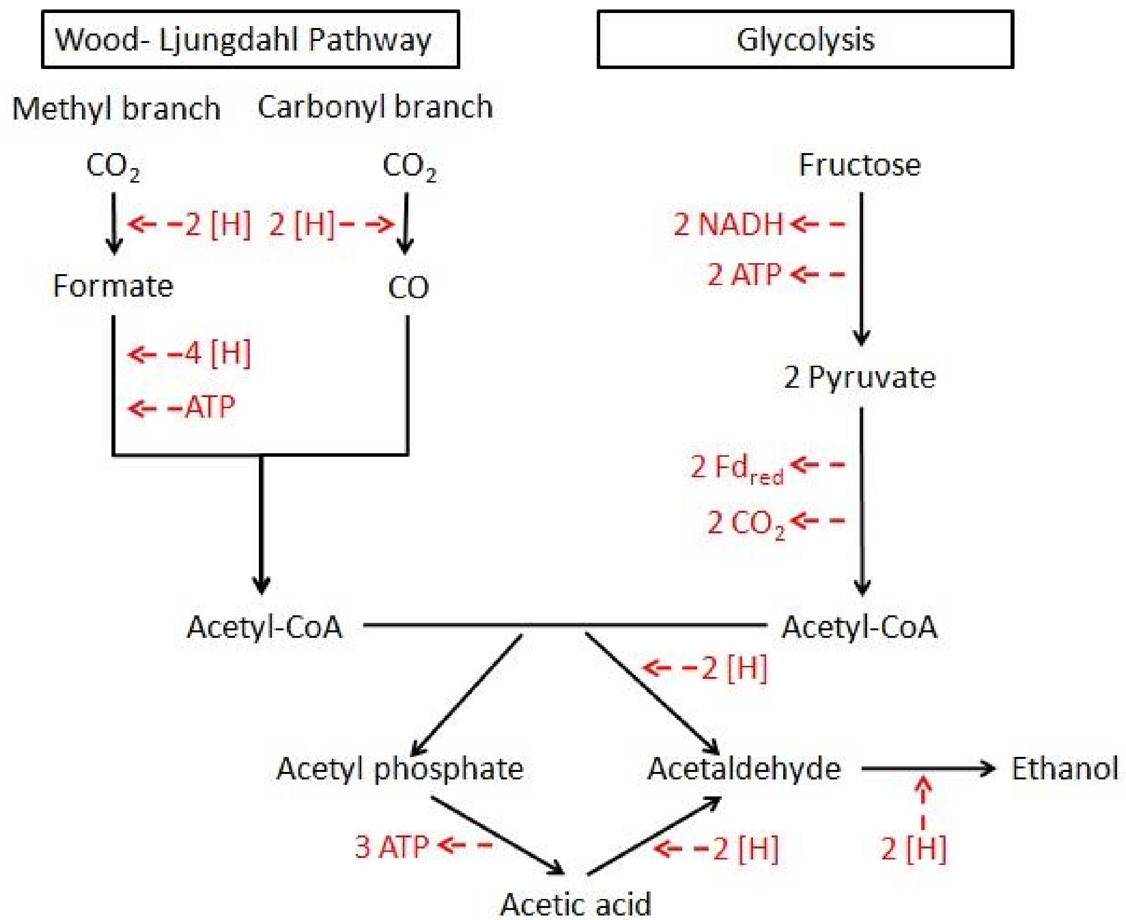


Figure 12. WLP and glycolysis in acetogens (modified from Bengelsdorf et al., 2013 and Schuchmann and Müller, 2016)

There are few acetogenic bacteria capable of growing on CO and convert this gas to acids and their corresponding alcohols (Köpke et al., 2011). Stimulation of the solventogenic phase and the shifting from acetate to ethanol production usuallyoccur under inhibitory or stress growth conditions, such as nutrient limitation (Saxena and Tanner, 2011) or below-optimal pH values (Abubackar et al., 2012; Fernández-Naveira et al., 2019). pH values below optimal generally stimulate solventogenesis, while they inhibit or slow down acetogenesis and biomass growth (Groenestijn et al., 2013). However, lower pH values are also causing fast bacterial decay and lower bacterial densities resulting in theslowing down of ethanol accumulationrates. In a previous study with a syngas mixture ($CO:CO_2:H_2:N_2$, 30:5:15:50), it was shown that *C.*

aceticum was capable of producing ethanol; however, the start of ethanol production was only possible at pH values slightly below 7 which is in turn inhibitory for bacterial growth. Pure CO was reported to be a better carbon and electron source compared to the CO_2+H_2 or syngas mixture, e.g., CO+CO_2+H_2. CO was found to allow higher bacterial growth rates and improved production of reduced metabolites like ethanol and 2,3-butanediol at higher amounts (Hermann et al.,2020; Mayer et al., 2017). In the present study, the growth of *C. aceticum*on CO was faster and the maximum biomass OD was higher compared to what was recently reported with syngas under optimal conditions (Arslan et al., 2019). In this second experiment, ethanol appeared in the fermentation medium already when the pH value was still 8, and the highest ethanol production rates were observed during the exponential growth phase, while pH was then fluctuating between 7.35-7.2. At the end of the exponential growth phase, at t=210 h, 50% percent of the total final ethanol concentrationshad already accumulated in the fermentation broth. However, it took another 480 h for the ethanol concentration to reach its maximum value of 4.6 $g \cdot L^{-1}$, which did not increase further as a result of the low bacterial concentration. In another study, the highest, maximum, ethanol concentration of 5.6 $g \cdot L^{-1}$, produced from syngas, after a process time of 710 h was reported with this strain by applying a pH shifting strategy which consisted of controlled pH shifts between high optimal pH values and low pH values, found to be optimal for solventogenesis (Arslan et al., 2019). It is interesting to highlight that, despite some slight differences, the behavior of *C. aceticum* observed here with CO is rather similar as with syngas (Arslan et al., 2019), though differences are observed between the autotrophic growth and the heterotrophic growth studied and described hereafter. It is also worth to highlight that in the few acetogenic bacteria known so far to produce alcohols from CO, CO_2 or syngas, solventogenesis is stimulated at low, acidic pH values, often around 5 or lower (Abubackar et al., 2016;

Fernández-Naveira et al., 2017b), while pH for solventogenesis in *C. aceticum* takes place around neutral pH values.

Fructose fermentation with pH regulation at constant pH

Acetogens are ableto fully metabolize and completely convertcarbon substrates to end products, often under either autotrophic or heterotrophic conditions. Mixotrophy and the simultaneous utilization of both organic and inorganic carbon sources is often also possible. Since *C. aceticum* was reported to grow efficientlyon fructose (Braun and Gottschalk, 1981), this sugar was chosen as carbon source for a newexperiment, carried out to assessthe behavior of *C. aceticum* in a stirred tank reactor (STR) at optimum growth pH of 8. Fig. 13 a and b show the heterotrophic growth profile and metabolites production. The experiment started with an initial fructose concentration of 27 $g{\cdot}L^{-1}$as this concentration was considered to be sufficient for bacterial growth and to assess the metabolites production profile. After reactor inoculation, the strain started growing and reached an OD of 1.5, after t = 90 h, with a growth rate, μ_{exp}, of 0.029 h^{-1}. This growth rate is slightly lower on fructose than on CO, while higher or similar growth rates are more usual when acetogenic bacteria grow on carbohydrates rather than on C_1 gases (Fernández-Naveira et al., 2017a, 2017b). After t = 90 h, a second, slower, growth phase was observed between t = 180 and 330 h. Finally, a maximum biomass OD of 2.2 was reached at t = 330 h, which later started slightly decreasing. A rather constant linear consumption of the carbon source, fructose, was observed and a similar pattern was found for the main product, acetic acid. The highest fructose consumption rate was found to be 0.06 $g{\cdot}L^{-1}{\cdot}h^{-1}$, during the first exponential growth phase, while highest acetic acid production rates were observed during second growth phase, after 180 h of the process, and reached 0.03 $g{\cdot}L^{-1}{\cdot}h^{-1}$.

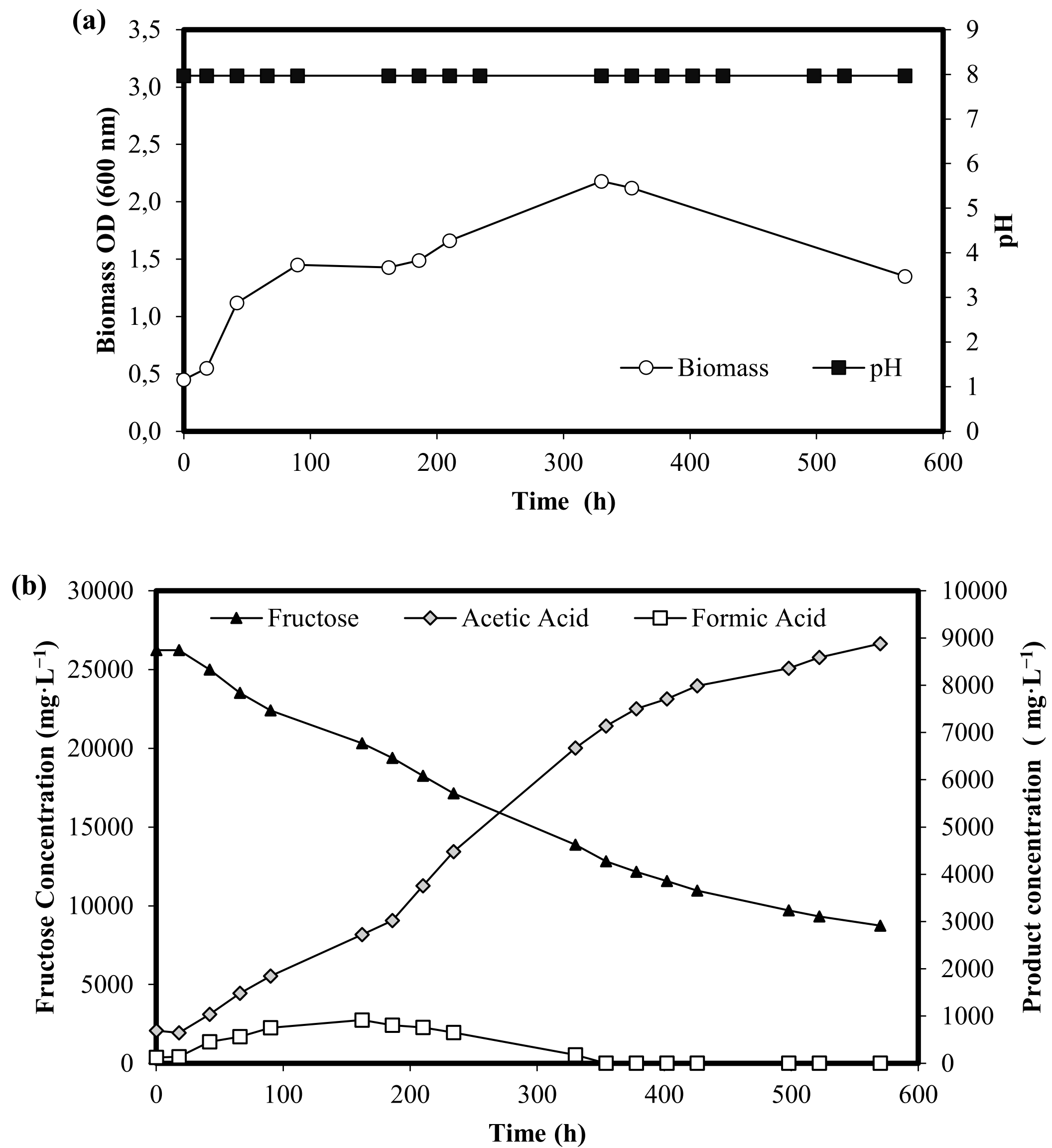


Figure 13. Fructose fermentation with pH regulation at 8

(a) biomass concentration (OD value at 600 nm) and pH; (b) metabolites production and fructose consumption

Formic acid was also transiently detected in the fermentation medium together with acetic acid, during the first 330 h of the process, increasing up to a maximum concentration of 0.9 $g \cdot L^{-1}$at t = 160 h. Afterward, itsconcentration decreased down to trace amounts within 170 h after reaching its maximum value. This same behavior was observed with *C. carboxidivorans*,

in which a transient production of formic acid was detected in that species when grown on glucose at a constant near optimal pH (Fernández-Naveira et al., 2017a), while this was not found in that species when grown on CO or syngas. The appearance of formic acid and acetic acid were basically simultaneous, both in *C. aceticum* grown on fructose (this study) and in *C. carboxidivorans* grown on glucose (Fernández-Naveira et al., 2017a). Thus, accumulation of formic acid, even transiently, is more common during heterotrophic growth on sugars than in autotrophic acetogenic growth. At the end of the 570 h fermentation process, the final remaining fructose concentration was 8.7 $g \cdot L^{-1}$ and acetic acid was the only fermentation product accumulating in the medium, with a final concentration around 8.8 $g \cdot L^{-1}$.

It was mentioned before that due to the low solubility of the syngas components in water, C_1-gas fermentation may suffer from low productivities as a consequence of poor cell growth. According to the previous studies carried out with different acetogenic microorganisms, growth on sugar based carbon, rather than syngas components, sometimes resulted in more dense cultures (Liu et al., 2015). It is also known that the doubling time for growth of *C. aceticum* with H_2 and CO_2 (20-25 h)is higher than with fructose (8h) (Braun et al., 1981). However, in this study growth rates and maximum bacterial density were lower compared to the experiment performed with CO under the same conditions. A possible explanation could be the accumulation of formic acid in the fermentation broth which was reported in a previous study with *C. carboxidivorans* grown on glucose as well. It was concluded that formic acid could lead to an inhibition effect, triggering acid crash in that species (Fernández-Naveira et al., 2017a). As can be seen in Fig 4 a and b, once the formic acid concentration reached 750 $mg \cdot L^{-1}$, biomass growth slowed down significantly and nearly stopped for a few hours. Later, when the formic acid concentration decreased below 800 $mg \cdot L^{-1}$, after t = 186 h, the biomass continued growing again. Given this pattern of biomass OD and metabolite formation it could be inferred that formic acid accumulation inhibited biomass growth in *C. aceticum* as well.

The autotrophic and heterotrophic metabolic pathways of acetogens are shown in Fig. 12. During glycolysis, fructose is converted to 2 moles of acetyl-CoA, 2 moles of CO_2, 2 moles ATP, and excess reducing equivalents (e.g. NADH, Fd_{red}). These reducing equivalents are used in the WLP to fix 2 moles of CO_2 into 1 more moleacetyl-CoA. Even though autotrophic and heteretrophic metabolisms of acetogens have been investigated and reported, the correlation of the pathways is species dependent (Liu et al.,2015; Maru et al., 2018). Fructose fermentation by *C. aceticum* resulted in formic acid accumulation in the culture medium, which is an indicator of WLP activity since formic acid is an intermediate product of the WLP's methyl branch (Fig.3). Theoretically, the amount of reducing equivalents synthesized by glycolysis is exactly the amount required to fix 2 moles of CO_2 to acetyl-CoA through the WLP. However, when the flux between glycolysis and WLP is not established, excess reducing equivalents are oxidized by hydrogenase activity which results in H_2 release. In the first step of the WLP's methyl branch, 2[H] are required for the conversion of CO_2 to formic acid, later 4 more [H] are used for complete conversion to acetyl-CoA. Formic acid accumulation in the fermentation medium is an evidence of the imbalance in fluxes of reducing equivalents between the two pathways. Fast formic acid accumulation in the fermentation medium was also reported before in *C. aceticum* when the WLP metabolism was purposely disturbed (Mayer and Weuster-Botz, 2017). In sugar fermentation by acetogenic bacteria, a possible WLP disruption could be explained by a phenomenon called carbon catabolite repression, a regulatory mechanism in which bacteria repress the consumption of a secondary carbon source in the presence of a preferred carbon source. This phenomenon is indicating that in the presence of fructose, WLP genes might be down regulated causing a disruption of CO_2 consumption by the WLP (Fast et al., 2015). Considering the above information, it could be concluded that, in the present study, the presence of fructose in the fermentation medium repressed the autotrophic metabolism in *C. aceticum*, resulting in

formic acid accumulation which created an inhibition of bacterial growth and prevented further fructose consumption and metabolite formation. The repressing effect from the presence of sugars on CO_2 assimilation through the WLP in homoacetogens was reported in *Blautiacoccoides*as well, showing that higher glucose concentrations are lowering the autotrophic ability of the bacteria (Liu et al., 2015). Maximum carbon yields C_M/C_S , or the ratio of carbon in produced metabolites to carbon in carbohydrate consumed, during the conversion of sugar are 67% at best due to the fact that one-third of all carbon in the sugar is lost to CO_2. Moreover, this ratio is actually even lower than 67% because of the carbon fixation in cell mass or the maintenance needs of the bacteria (Jones et al., 2016; Maru et al.,2018). Thus, it could be concluded that the observed C_M/C_S ratios higher than 67% can be considered as a successful mixotrophy. Final C_M/C_S derived in the present study was only 48% when considering acetic acid as the only product of the process. Even though formic acid formation is proving the occurrence of autotrophic metabolism in *C. aceticum*, it was strongly repressed by the presence of fructose. The CO_2 gas, that wasn´t reassimilated through the WLP was lost, resulting in poor carbon yields. Depending on the C_M/C_Svalues, poor mixotrophic behaviors were characterized before in some other acetogenic bacteria such as *Acetobacterium carbinolicum, Blautia product* and *Eubacterium limosum* growing on a preferred carbohydrate while some others like *Clostridium drakei, Clostridium magnum* and *Clostridium scatologenes* were shown to perform much better mixotrophy (Maru et al.,2018).

Fructose fermentation without pH regulationand with medium acidification

In order to determine the impact of pH on the metabolism of *C. aceticum*, depending on the carbon source, an additional experiment was performed with fructose, without pH adjustment. This experiment was designed and initiated as described in section 3.2. pH was adjusted to 8at the beginning and after inoculation, pH adjustment was stopped, so that its value could

fluctuate depending on the products and their concentrations. Fig 14 a and b show the growth of *C. aceticum* and the formation of fermentation products.

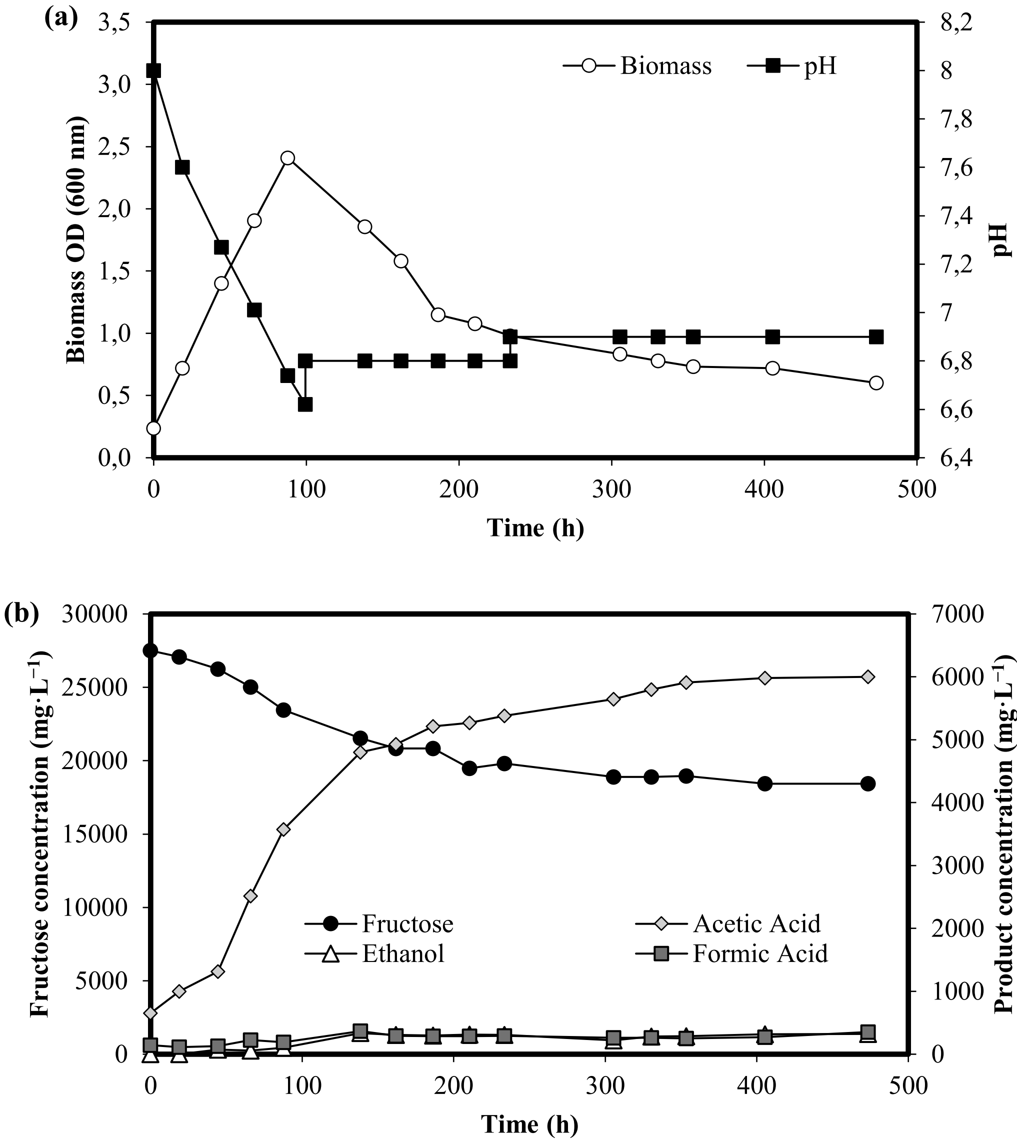


Figure 14. Fructose fermentation without pH regulation

(a) biomass concentration (OD value at 600 nm) and pH; (b) metabolites production

During the first 88 h, the biomass OD increased up to maximum value of 2.4, with a μ_{exp} of 0.017 h^{-1}. This growth coincided with acetic acid accumulation, up to 4 $g \cdot L^{-1}$, and a pH decrease from 8 to 6.7. The bacterial OD reached its maximum value when the pH dropped to approximately 6.7 at t=88 h. The pH value was then slightly adjusted and set at 6.8, between t

= 100 and 230 h, and later, it was further increased to 6.9 and controlled at these values in order to avoid any further pH drop which could cause a fast bacterial death and inhibition. Low amounts of ethanol in the fermentation medium first appeared after 50 hours when the pH of the medium was around 7.3. The ethanol concentration peaked at 330 mg·L^{-1}, at t= 140 h, when the pH value was 6.8 and no more substantial increase in ethanol production was observed for the rest of the process. Formic acid concentrations, between 100-300 mg·L^{-1}, were detected during the process and no evident inhibition effect was observed on biomass growth or metabolite production, that could be related to formic acid formation. About 400 h after fructose consumption and metabolites productions stabilized and at the end of the 475 h process time, the concentrations of fructose, acetic acid, ethanol and formic acid in the fermentation medium were 18.4 g·L^{-1}, 6 g·L^{-1}, 325 mg·L^{-1} and 350 mg·L^{-1}, respectively.

In comparison to the previous experiment, with fructose and pH maintained at 8, although some formic acid was observed in the fermentation medium, which could be interpreted as the occurrence of autotrophy (i.e., CO_2 coming from glycolysis), its concentration never reached inhibitory levels during the uncontrolled pH study. Bacterial growth was slower solely as a result of the pH drop, and while bacterial growth rates were lower than in the previous experiment, the maximum bacterial OD was observed to be marginally higher. The maximum acetic acid production and maximum fructose consumption rates were higher than in the previous study as well and were observed to reach 0.06 and 0.07 g·L^{-1}·h^{-1}, respectively, during the initial growth phase.

Once the pH was set at6.8, a fast bacterial death was observed, resulting in low final metabolite concentrations, and only 33% of the initial fructose concentration (27 g·L^{-1}) was consumed during the process. However, a better C_M/C_Sratio, of 60%, was observed, showing more efficient carbon fixation in the biomass and metabolites under uncontrolled pH conditions. It is suggested that the reason for the less efficient utilization of fructose in the

previous experiment was possibly the increased maintenance need caused by the inhibitory effect of formic acid accumulation.

As mentioned before, metabolite formation during heterotrophic growth of *C. aceticum*is dependent on the availability of sufficient amount of reducing equivalents. As can be seen in Fig 3, reduction of acetyl-CoA or acetic acid into ethanol requires 4 more [H]. pH drop clearly stimulated ethanol production when *C. aceticum* was growing on CO; however, the same effect was not observed in fructose fermentation. A possible explanation for this difference could be the lack of reducing equivalents. The same behavior was reported in *C. carboxidivorans*, which was stimulated to convert acids to alcohols by medium acidification when growing on a syngas mixture; however, this phenomenon was not so efficient when growing on glucose (Fernández-Naveira et al., 2017a). CO was also preferred by another acetogen, *C. ljungdahlii*, as a substrate and electron donor for the formation of reduced products such as ethanol since it provides excess NADH for the production of alcohols (Hermann et al., 2020). The summarized results of autotrophic and heterotrophic growth and production of metabolites are provided in Table 4.

Conclusions

The influence of the pH value and its regulation on the nature of metabolites produced in *C. aceticum*was shown to depend on the carbon sources. When fructose was used as sole substrate, the process suffered from formic acid accumulation, as shown in Table 1, and inhibition was observed as a consequence of reducing equivalents deficiency. A possible suggestion to overcome this problem might be H_2 enhanced mixotrophy in which H_2 is provided to the fermentation medium exogenously. Under the examined conditions, CO can be considered as better carbon source than fructose for the production of ethanol by *C. aceticum*.

Table 4. Summary of the results

	CO fermentation at pH 8	CO fermentation without pH control	Fructose fermentation at pH 8	Fructose fermentation without pH control
OD_{600} max.	3 (137 h)	1.8 (210 h)	2.2 (330 h)	2.4 (88 h)
μ_{exp}	0.052 h^{-1}	0.006 h^{-1}	0.029 h^{-1}	0.017 h^{-1}
Maximum acetic acid formation rate	0.26 $g{\cdot}L^{-1}{\cdot}h^{-1}$	0.03 $g{\cdot}L^{-1}{\cdot}h^{-1}$	0.03 $g{\cdot}L^{-1}{\cdot}h^{-1}$	0.06 $g{\cdot}L^{-1}{\cdot}h^{-1}$
Final acetic acid concentration	18 $g{\cdot}L^{-1}$	4.7 $g{\cdot}L^{-1}$	8.8 $g{\cdot}L^{-1}$	6 $g{\cdot}L^{-1}$
Final ethanol concentration	500 $mg{\cdot}L^{-1}$	4.6 $g{\cdot}L^{-1}$	-	325 $mg{\cdot}L^{-1}$
Maximum formic acid concentration	-	-	0.9 $g{\cdot}L^{-1}$	0.35 $g{\cdot}L^{-1}$
Final fructose concentration	-	-	8.7 $g{\cdot}L^{-1}$	18.4 $g{\cdot}L^{-1}$
C_M/C_S	-	-	48 %	60 %
Maximum fructose consumption rate	-	-	0.06 $g{\cdot}L^{-1}{\cdot}h^{-1}$	0.07 $g{\cdot}L^{-1}{\cdot}h^{-1}$

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4.3 Acetone and Isopropanol Production from Engineered *A. woodii*

It is required to broaden product range of the gas fermenting bacteria so other important bulk chemicals like acetone and isopropanol could also be produced from waste gas and syngas. Metabolic engineering approaches have been successfully used for the incorporation of synthetic pathways into acetogenic bacteria. Acetone is not natively produced in *A. woodii* and acetone production was achieved in this strain by introducing genes from *C. acetobutylicum* in it. However, some of *C. acetobutylicum*´s enzymes have drawbacks that make them unsuitable for synthesis in heterologous hosts.

For a better acetone production in *A. woodii* novel recombinant strains should be constructed and gas fermentation performance of these new constructed strains should be screened. To achieve better acetone production, two new acetone biosynthesis pathways were constructed by combining genes from *C. acetobutylicum* and *C. aceticum.* Batch fermentation in a stirred tank bioreactor with continuous gas supply using a defined media was used to test the capacity of newly generated strains to produce acetone. In this chapter, firstly strains were described then the results of batch fermentations with these strains were given.

First part of this study was published with the title "Engineering *Acetobacterium woodii* for the production of isopropanol and acetone from carbon dioxide and hydrogen" in Biotechnology Journal.

Citation – these data have partly been published as,

Arslan, K., Schoch, T., Höfele, F., Herrschaft, S., Oberlies, C., Bengelsdorf, F., Veiga, M. C., Dürre, P., and Kennes, C. (2022). Engineering *Acetobacterium woodii* for the production of isopropanol and acetone from carbon dioxide and hydrogen. Biotechnology Journal, September 2021, 1–12. https://doi.org/10.1002/biot.202100515.

Abstract

Four genetically engineered *A. woodii* strains were investigated for their ability to produce acetone from CO_2 and hydrogen. *C. acetobutylicum* and *C. aceticum* genes were combined to create the acetone production pathway.

Acetone production genes were expressed in all strains and all strains produced acetic acid, acetone, and, interestingly, isopropanol. The strain *A. woodii* [pJIR750 ac1t1] was found to be the most promising one as it produced the highest concentrations of all metabolites, acetic acid, acetone, and isopropanol, with ultimate concentrations of 438 mM, 7.6 mM, and 14.5 mM, respectively. Additionally, formation of isopropanol in *A. woodii* was shown for the first time.

Keywords: *Acetobacterium woodii*, acetogen, isopropanol, syngas, Wood-Ljungdahl pathway.

Introduction

The production of most commodity chemicals, including acetone, relies on fossil resources. Acetone is the simplest and most important ketone and it is a widely used industrial solvent as well as a platform chemical for the production of materials like polypropylene and acrylic glass (Howard, W.L. 2000; Liew et al., 2022). Acetone is also reported to have potential to be used as fuel additive that could reduce emissions and enhance the performance of existing fuels (Aguado-Deblas et al., 2020).

Most of the worldwide acetone supply is dependent on the cumene process, in which petrochemicals, benzene and propylene, are used in a highly energy consuming process and creates hazardous waste and emits greenhouse gases. In the meantime there is no any available green chemistry option for acetone production (Liew et al., 2022).

Biomanufacturing of acetone, dating back to the first half of the twentieth century, is considered as one of the first industrial chemical production by fermentation. In this process, acetone, butanol, and ethanol (ABE) are produced together through the Embden-Meyerhof-Parnas pathway (Dürre, P., 1998). *Clostridium beijerinckii, Clostridium saccharobutylicum, Clostridium saccharoperbutylacetonicum*, and *Clostridium acetobutylicum* are some solventogenic *Clostridium* species that are known to have the ability to covert sugar and starch feedstock to acetone, butanol and etanol (Jones and Woods, 1986). Microbial acetone production reached to big quantities to meet the high demand for smokeless powder during World War I and II (Dürre, P., 1998). However later ABE production was phased out with the development of petrochemical industry and high profitability of oil based acetone production. Biomanufacturing of bulk chemicals is gaining importance again as fermentation enables sustainable and cleaner production of bulk chemicals by using above ground renewable carbon sources.

Syngas fermentation using acetogenic bacteria appears as a promising alternative that can combine reduction of greenhouse gases with the production of bulk chemicals and fuels (Fernández-Naveira et al., 2017a). Syngas is a mixture of gases mainly contains of CO, CO_2 and H_2in different ratios and can be generated from biomass resources like lignocellulosic, municipal, and organic waste streams by gasification process (Redl et al., 2017). Besides, some waste gases from heavy industries like steel manufacturing also contains such gases, mainly CO, which can be utilized by acetogenic bacteria (Molitor et al., 2016). Acetogenic bacteria is fixing C1 gases mainly into acetyl-CoA by using the Wood-Ljungdahl pathway (WLP) which is then converted into metabolic end products and biomass. The WLP pathway is known as the most efficient CO_2 fixing pathway and historicaldevelopment and the underlying biochemistry of this pathway can be found in elsewhere (Drake et al., 2008; Ragsdale, 2008; Ragsdale and Pierce, 2008).

Acetone is not a native product of acetogens, low concentrations of acetone production was achieved only in several genetically engineered autotrophic acetogen hosts (Kato et al., 2021; Hoffmeister et al., 2016; Jones et al., 2016; Banerjee et al., 2014).

A.woodii is a model organism of acetogenic bacteria as it is one of the most studied acetogens. The genome of *A.woodii* was already sequenced and its energy conservation pathway is also elucidated (Schuchmann, and Müller, 2014; Poehlein et al., 2012).

Natively the main product of *A.woodii* is acetate however; acetone production in recombinant *A. woodii* strains using genes from *C. acetobutylicum*has been succeeded (Hoffmeister et al., 2016). In Hoffmeister's study, the clostridial acetone pathway was used for the formation of acetone in *A. woodii*. The acetone production operon (APO) containing the genes *thlA* (encoding thiolase A), *ctfA*/*ctfB* (encoding CoA transferase), and *adc* (encoding acetoacetate decarboxylase) from *C. acetobutylicum* were cloned under the control of the *thlA* promoter into vectors pJIR750 and pMTL84151 resulting in the plasmids pJIR750_act and

pMTL84151_act. In that study it was demonstrated that the strain *A. woodii* pMTL84151_act$_{thlA}$ is the best acetone producing recombinant in batch studies with continuous supply of H_{2+} CO_2 gas mixture. Furthermore, it was also shown that the acetate concentration within the bioreactor is critical for acetone formation in *A. woodii* due to the high Km value of the CoA transferase from *C.acetobutylicum.*

In the present study, several modifications were performed for further optimization of acetone production in *A. woodii* and for this purpose two new strains were constructed. For the plasmid pJIR750_ac1t1, the genes *adc* (CA_P0165) and *ctfA/ctfB* (CA_P0163/CA_P0164) from *C. acetobutylicum* including the corresponding ribosomal binding sites were used. For the construction of the second acetone production plasmid pJIR750_ac2t1, the *ctfA/ctfB* genes from *C. aceticum* (CACET_c04240 / CACET_ c04250) were used to replace the corresponding genes from *C. acetobutylicum*.

Autotrophic bioreactor experiments, with continuous CO_2+H_2 gas feed, were performed with four different recombinant strains to compare the growth and metabolite production of four *A. woodii* transformants.

Materials and Methods

Microorganisms, media and plasmid construction

Four different recombinant *A. woodii* strains ([pJIR750_ac1t1], [pMTL84151_act$_{thlA}$], [pJIR750_act$_{thlA}$], and [pJIR750_ac2t1]) used in this study were constructed in Professor Peter Dürre's laboratory. Details of the gene construction studies, and bacterial strains used in this study can be found in elsewhere (Arslan et al., 2022; Hoffmeister et al., 2016). Plasmids pJIR750_act$_{thlA}$ and pMTL84151_act$_{thlA}$ were constructed before by Hoffmeister et al., 2016, in addition to those, two newly constructed acetone production plasmids, pJIR750_ac1t1 and pJIR750_ac2t1, were used in this study. The plasmids and their relevant characteristics were giving in Table 6.

Table 5. Plasmids used in this study and their relevant characteristics

Plasmid	Relevant characteristics
pJIR750_act$_{thlA}$	pJIR750, PthlA, adc (CA_P0165), ctfA/ctfB (CA_P0163/CA_P0164), thlA (CAC2873) from *C.acetobutylicum*
pMTL84151_act$_{thlA}$	pMTL84151, PthlA, adc (CA_P0165), ctfA/ctfB (CA_P0163/CA_P0164), thlA (CAC2873) from *C.acetobutylicum*
pJIR750_ac1t1	pJIR750_act$_{thlA}$ including native ribosomal binding sites upstream of *adc* as well as ctfA
pJIR750_ac2t1	pJIR750, PthlA, ctfA/ctfB (CACET_c04240/CACET_c04250) from *C.aceticum*

Engineered strains were grown in modified DSMZ medium with the following composition (per liter distilled water): NH_4Cl, 0.20 g; yeast extract, 2 g; KH_2PO_4, 1.76 g; K_2HPO_4, 8.44 g; $MgSO_4 \times 7 H_2O$, 0.33 g; $NaHCO_3$, 10 g; L-Cysteine-HCl, 0.30 g; $Na_2S \times 9 H_2O$, 0.30 g; resazurin (stock solution concentration, 1 g L–1); 1 ml; vitamins solution, 2 ml; trace metals solution, 2 ml; and thiamphenicol, 7.5 mg.The composition of the vitamins solution was (per liter distilled water): biotin, 25 µg; folic acid, 25 µg; pyridoxine-HCl, 50 µg; thiamine-HCl, 50 µg; riboflavin, 50 µg; nicotinicacid, 50 µg; D-Ca-pantothenate, 50 µg; vitamin B12, 25 µg; p-amino benzoic acid, 50 µg and lipoic acid, 25 µg. The composition of the trace metals solution was (per liter distilled water): nitriloacetic acid, 12.8 g; $MnCl_2 \times 4 H_2O$, 0.1 g; NaCl, 5 g; $FeCl_2 \times 4 H_2O$, 2 g; $CoCl_2 \times 6 H_2O$, 0.2 g; $ZnCl_2$, 70 mg; $CuCl_2 \times 2 H_2O$, 2 mg; H_3BO_3, 6 mg; $Na_2MoO_4 \times 2 H_2O$, 36 mg, and $NiCl_2 \times 6 H_2O$, 24 mg.

Preparation of inocula for bioreactor fermentation

Recombinant strains were obtained in the form of freeze dried pellets. Rehydration of the bacteria was carried out under the pure N_2 gas stream by following the procedure recommended by DSMZ. Precultures were prepared heterotrophically with medium containin 10 gL^{-1} fructose. A total 100-ml glass serum bottles, with 40 ml working volume, were used to grow the inoculum. Serum bottle preparation procedure can be found in detail in material and method section 3.2.1. The pH of each bottle was adjusted to 7.5 with either a 2 M NaOH

solution or a 2 M HCl solution before autoclave sterilization. After sterilization $MgSO_4$, fructose, and vitamins were added from anoxic sterile stock solutions to their final concentrations. Thiamphenicol (prepared as a solution of 2:1 water:N-N,dimethylformamide) was used as antibiotic.Inoculated serum bottles were incubated at 30℃ and 150 RPM at least 48 hours before bioreactor cultivation.

Bioreactor cultivation with continuous gas supply

Engineered *A. woodii* strains were cultivated in the same medium as described in the section*Microorganisms, media and plasmid construction*with gas mixture (CO_2+H_2) as the only substrate. The experiments were carried out in 2-L Eppendorf BIOFLO 120 stirred tank bioreactors (Eppendorf AG, Hamburg, Germany) with around 1.3 L working volume for all assays, the details of the bioreactor preparation and the inoculation was given in material method section 3.2.2.

The bioreactor was equipped with four baffles and a six blade Rushton turbine. A gas mixture, consisting of 40% N_2, 25% CO_2, and 35% H_2, was fed through a microsparger into the reactor at a flowrate of 10 ml min^{-1}. That gas flow rate was maintained constant during the whole experiment by means of a mass flow controller (Aalborg GFC 17, Müllheim, Germany). A BIOFLO 120 condenser was connected to the bioreactors to avoid or minimize any possible gas losses, for example, acetone, using tap water at room temperature (below 20 °C). No gas losses were detected. The pH value of the fermentation broth was monitored on-line with a pH sensor (Mettler Toledo, Columbus, Ohio, USA) and maintained at 7.5 by using either 1 M HCl or 1 M NaOH solutions during all experiments. All fermentation processes were run at a temperature of 30°C, which was maintained constant by means of a water jacket or a heating blanket. The medium was continuously agitated at 250 rpm. Silicone oil was used as antifoam agent when required.

Analytical methods

2-ml liquid samples were withdrawn periodically from the bioreactors during the experiments to carry out analytical tests. The cell concentration was determined by using a spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain, or, Ultrospec 1100 Pro, Amersham Biosciences Europe GmbH, Freiburg, Germany, respectively) at a wavelength of 600 nm (OD_{600}). Fructose, acetic acid, acetone and isopropanol concentrations in bioreactor experiments were determined with a high performance liquid chromatograph (HPLC) HP-1100 (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector and a refractive index detector, at 50°C. The samples were centrifuged (ELMI Skyline Ltd CM 70M07, Riga, Latvia) at 7000 rpm for 5 min at room temperature and the supernatant was filtered through a 0.22-µm filter. Then, 20 µl of those samples were injected into the Agilent Hi-Plex HColumn (300 × 7.7 mm) (Agilent Technologies, Santa Clara, CA, USA) at 45°C, and a 5 mM H_2SO_4 solution was used as mobile phase with a flow rate of 0.80 ml min^{-1}.

Results and Discussion

Batch fermentation with continuous gas supply for the comparison of four engineered A.woodii

The bioconversion profile of the engineered strains, under autotrophic conditions, was studied in automated stirred tank reactors (STR), with constant pH regulation and with the same continuous gas supply for each strain. Fermentation processes were stopped once the concentration of all metabolites reached (near) stable values. In addition to the two newly constructed strains *A. woodii* [pJIR750_ac1t1] and *A. woodii* [pJIR750_ac2t1] their parent *A. woodii* [pJIR750_act_{thlA}] and strain *A. woodii* [pMTL84151_act_{thlA}] were included in these experiments, as this strain was found to be the best acetone producing engineered strain in the previous study (Hoffmeister et al., 2016). Figure 15 shows the growth and Figure 16 shows products formation profiles of the recombinant *A. woodii* strains.

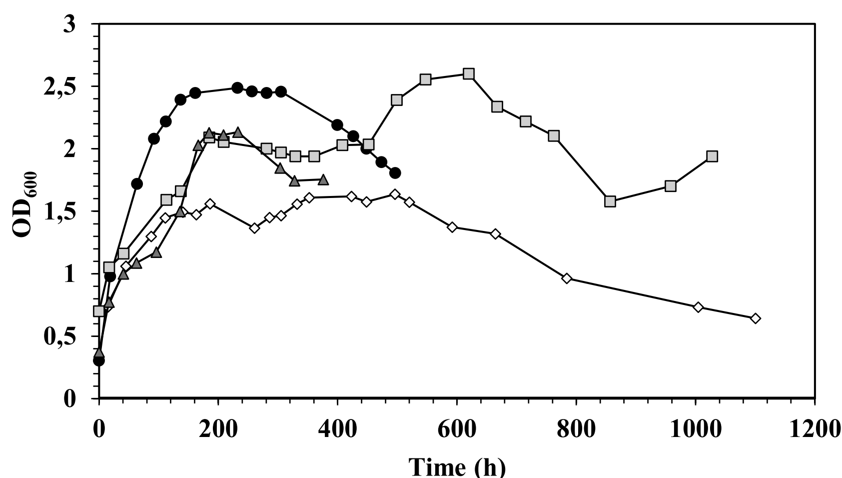

Figure 15. Optical density measured at 600 nm, *A. woodii* [pJIR750_ac1t1](circles, ●), *A. woodii* [pJIR750_ac2t1] (diamonds, ◆), *A. woodii* [pJIR750_ actthlA] (square, ■), and *A. woodii* [pMTL84151_ actthlA] (triangles, ▲).

Bacterial growth and acetic acid production in *A. woodii* [pJIR750_ac1t1] started right after inoculation without any lag phase. A high biomass OD_{600} of 2.4 was achieved 140 h after inoculation and it remained close to that same value up to t = 300 h, reaching a maximum OD_{600} of 2.54 during this period. Later, it decreased continuously, but slowly, until reaching an OD_{600} of 1.8 at the end of the experiment (t = 500 h), though the strain continued producing all three metabolites up to a few hours before stopping the bioreactor. Acetone production in that strain did not start upon inoculation but faced some delay of a few days and only started being detected 135 h after the reactor start-up, when the acetic acid concentration had already reached around 165–200 mM (10–12 g L–1). Surprisingly, also isopropanol appeared in the fermentation medium, about 70 h after acetone production had started and when the acetic acid concentration had already reached 280 mM. At the end of the experiment, the final concentrations of acetic acid, acetone, and isopropanol in the fermentation broth were 438 mM, 7.6 mM, and14.5 mM, respectively. This results in an acetone:acetic acid ratio of 0.017 and an acetone:isopropanol ratio of 0.52. The second recombinant strain, *A.woodii* [pJIR750_ac2t1], followed a similar biomass growth profile as the first one.

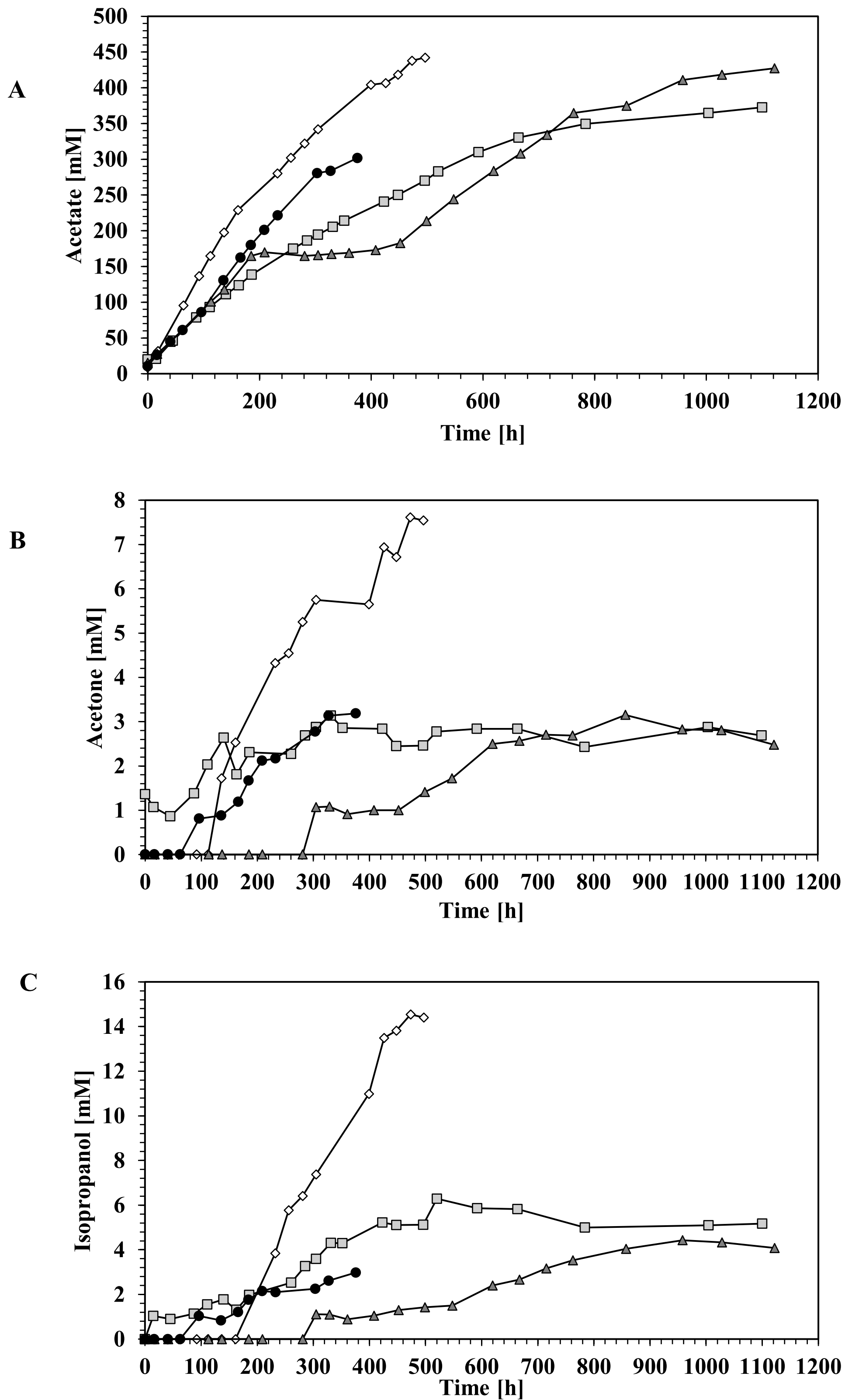

Figure 16. Product profile of four engineered strains, *A. woodii* [pJIR750_ac1t1] (circles, ●), *A. woodii* [pJIR750_ac2t1] (diamonds, ⬩), *A. woodii* [pJIR750_ actthlA] (square, ■), and *A. woodii* [pMTL84151_ actthlA] (triangles, ▲)

(A) acetate production; (B) acetone production; (C) isopropanol production

However, a maximum biomass OD_{600} of only around 1.5 was reached and no further increase was then seen after that. Contrary to all other three strains, in this case, acetone and isopropanol accumulation occurred at the same time as acetic acid production and they were all detected immediately upon inoculation. In this strain, compared to acetic acid, there was thus no delay in the production of acetone and isopropanol, contrary to what was observed in all the other three strains.

After about 500–600 h, slow and gradual biomass decay took place and, at the same time, accumulation of any metabolites did also slow down or even stop. The production of acetone did actually already reach steady state a few hours before the other metabolites, as it was the first one whose production stopped. After 1100 h, at the end of the experiment, basically no further acetic acid accumulation was observed, and its concentration remained nearly constant, while acetone and isopropanol production leveled off sooner. The highest acetone concentration was reached at t = 330 h, while isopropanol production continued to increase and reached its maximum concentration at t = 520 h. The maximum acetic acid, acetone, and isopropanol concentrations detected by HPLC were373 mM, 3.2 mM, and 6.3 mM, respectively. In this case, the acetone:acetic acid ratio was 0.008 and the acetone:isopropanol ratio was 0.49.Apparently, two distinctive growth phases and slower growth were observed in the biomass profile of the third recombinant strain, *A.woodii* [pJIR750_actthlA]. Each of the two growth phases appeared to be simultaneous to fast exponential productions of acetic acid, while a clear plateau of several days was observed in terms of growth as well as acetic acid production in between those two exponential phases. Acetone and isopropanol production both started near the beginning of that plateau and inactive growth phase. After reaching an OD_{600} value of 2, at t=185 h, the biomass concentration remained nearly constant until reaching t = 450 h. Even though acetone and isopropanol first appeared in the HPLC analysis

during this period, no considerable changes in the concentrations of any metabolites were observed. Later on, the second growth phase started, the biomass OD600 valuereached 2.6 at t = 620 h, and an additional increase in the formation of all metabolites, that is, acetic acid, acetone, isopropanol, took place. After t = 1122 h, near the end of the experiment, the concentrations of metabolites had reached nearly constant values, and a maximum acetic acid concentration of 427.3 mM was measured. Maximum accumulation of acetone and isopropanol was also observed, corresponding to3.2 mM and 4.4 mM, respectively. The acetone:acetic acid ratio was0.007 and the acetone:isopropanol ratio was 0.73.The biomass growth pattern of the fourth recombinant strain, *A.woodii* [pMTL84151_actthlA], was somehow similar to the third one.Two clear distinct growth phases were observed and even though a fastgrowth was observed on the first day of the process, once the biomass OD_{600} value was close to 1, growth reached a kind of short plateauand a decrease in the growth rate was observed between t = 40 and96 h. Later on, a second faster growth phase was observed, simultaneous with the initial production of acetone and isopropanol, and amaximum biomass OD_{600} value of 2.1 was reached, 166 h after inoculation. Here again, acetic acid accumulation was observed immediately after inoculation, while there was a delay in acetone and isopropanol production. Acetic acid accumulation was also slower than with the first recombinant strain and, after t = 375 h, acetic acid reached its maximum concentration of 302 mM in the fermentation medium. Acetone and isopropanol first appeared 100 h after inoculation, when the acetic acid concentration was 86mM. Unlike the previous recombinant strain, here acetone and isopropanol were produced simultaneously and at very similar rates. At the end of the process their final concentrations were 3.2 mM and 3 mM, respectively. In terms of ratios, this means an acetone:acetic acid ratio of 0.011, which is lower than in the previous strain, and an acetone:isopropanol ratio of 1.07.

After strain construction autotrophic uncontrolled batch experiments were performed (by Prof. Peter Dürre´s group, data not shown here) in serum bottles and acetone production of the two newly constructed strains was examined (Arslan et al., 2022).
This experiment revealed a better acetone production in recombinant *A. woodii* [pJIR750_ac2t1] when CoA transferase from *C. aceticum* was induced (Arslan et al., 2022). However, in bioreactor fermentations with continuous gas supply the strain carrying a construct with improved RBS performed best. High growth rates and high biomass concentrations are required to achieve high metabolite concentrations and process productivity. *A. woodii* [pJIR750_ac1t1] and *A. woodii* [pJIR750_act$_{thlA}$] showed the highest biomass concentrations amongst all transformants. However*, A. woodii* [pJIR750_act$_{thlA}$] reached its maximum biomass OD_{600} value 550 h after the beginning of the process, whereas *A. woodii* [pJIR750_ac1t1] reached its maximum biomass OD_{600} value at t = 160 h. The maximum biomass OD_{600} for *A. woodii* [pJIR750_ac1t1] was 2.54, which is higher than that reported recently for the genetically engineered acetone producing *A.woodii* [pMTL84151_act$_{thlA}$] autotrophically grown in a batch operated stirred-tank bioreactor (Hoffmeister et el., 2016). *A.woodii* is one of the most studied acetogens and it is known to have a high autotrophic acetic acid formation performance (Groher and Weuster-Botz, 2016). In the present study, acetic acid was the main product of all four studied engineered strains as well. The highest acetic acid concentrations were observed with *A. woodii* [pJIR750_ac1t1] and*A.woodii* [pJIR750_act$_{thlA}$], reaching 438 mM and 427.3 mM, respectively. The most important difference between these transformants was again the process duration. About 60% of the total produced acetic acid accumulated during the last 670 h of the process with *A.woodii* [pJIR750_act$_{thlA}$], while the same amount acetic acid was already produced in only 230 h after inoculation with *A. woodii* [pJIR750_ac1t1]. In batch fermentation of *A.woodii* [pJIR750_act$_{thlA}$] , three times higher acetic acid concentration

(1330 mM) was reported (Hoffmeister et al., 2016) in a shorter process time (360 h) and with a lower biomass accumulation (ODmax = 2.2), than what was observed with *A. woodii* [pJIR750_ac1t1] in the present study . A possible explanation for such difference between the productivities of the mutant strains could certainly be the operating process conditions. The poor aqueous solubility of the gas components is considered to be among the main reasons for low productivities of gas fermentation processes. In order to cope with this problem, increased agitation as well as higher gas flow rates and pressures were successfully applied in some studies and appeared to improve the gas fermentation process (Groher and Weuster-Botz, 2016; Kantzow and Weuster-Botz, 2016). However, this does also significantly increase operating costs, which may then become prohibitive at large scale. Slower agitation and low gas feeding rates applied in the present study reduce gas transfer and substrate availability to the biomass, resulting in reduced metabolic activities and lower production of metabolites. The gas flow rate was as much as 50 times lower in the present study (10 vs. 500 ml min^{-1}) compared to the previously reported one and the gas fed to the bioreactor in this case contained N_2 besides H_2 and CO_2 . Other gases than H_2, CO_2, or CO, for example, N_2, can actually also be found in syngas and in many industrial emissions.*A. woodii* [pJIR750_ac1t1] produced the highest concentration of acetone, reaching 442 mg L^{-1}. A total amount of 16.5 mM acetone production was reported before (Hoffmeister et al., 2016) with the recombinant strain *A. woodii* [pMTL84151_act_{thlA}] in batch gas fermentation and 52 mM acetone, so far the highest acetone accumulation in an engineered acetogen, from continuous gas fermentation with cell retention. Despite lower metabolic activities and lower acetic acid and acetone production in *A. woodii* [pJIR750_ac1t1], due to the lower substrate supply and consumption, a significant improvement can still be seen, considering the acetone to acetic acid ratio, observed in these recombinant strains. To the best of our knowledge, the acetone:acetic acid ratio of 0.017 reached in *A. woodii* [pJIR750_ac1t1], is the highest ever

reported ratio, which may therefore be considered to perform better in terms of acetone production.

Successful expression of the acetone production pathway genes in the acetogenic bacteria *C. ljungdahlii* and *A. woodii* was shown before (Hoffmeister et al., 2016; Banerjee et al., 2014). Here, acetone was produced autotrophically by the four recombinant strains under study. However, accumulation of isopropanol in the fermentation medium was, surprisingly, also detected in the bioreactor experiments of all recombinants while this was not the case in the experiments of Hoffmeister et al.,2016. The analytical system for batch fermentations used in that study, however, did not allow separation of acetone and isopropanol. Acetone and isopropanol peaks may easily overlap in chromatographic analyses, if not optimized, and could have been the case in that previous study. This might be the reason for the higher acetone concentration, due to additional undetected isopropanol formation, compared to the values reported here.

Isopropanol, also called isopropyl alcohol, is another important valuable organic molecule which is used in pharmaceutical, cosmetics, and painting. Isopropanol is also utilized as a gasoline and diesel additive and also disinfection is one of its most important applications (Chua et al., 2017). The market demand of isopropanol reaches 2 million tons per year, with growing demand as a result of the recent pandemic (Ávilaet al., 2021). Traditionally, isopropanol has been manufactured with two possible methods, that is, indirect hydration of propylene and direct hydration of propylene (Chua et al., 2017). Isopropanol can be produced through fermentation as well and *C. beijerinckii* is one of the major isopropanol-producing bacteria using sugars or lignocellulosic hydrolysate materials as carbon source (Ávilaet al., 2021; Liberato et al., 2019) It is also known that some gas fermenting acetogens, such as *C. ragsdalei*, are able to convert exogenous acetone into isopropanol (Cheng et al., 2019; Köpke et al., 2014; Ramachandriya et al., 2011) with the function of a single SADH. Even though *A.*

woodii was reported not to have such primary-SADH in its genome (Hoffmeister et al., 2016; Köpke et al., 2014) in the present study acetone conversion to isopropanol was observed in all recombinants. The highest maximum isopropanol concentration of 14.5 mM was observed in *A. woodii* [pJIR750_ac1t1], two times more than the maximum acetone concentration (7.6 mM) observed in this transformant. Studies describing the production of isopropanol in syngas-fermenting engineered acetogens are very scarce. Recently, isopropanol formation was confirmed in an engineered *C. ljungdahlii* (Philipps et al., 2019) and to the best of our knowledge the highest isopropanol production of 648 mg L^{-1}, which is 75% of what was observed in the present study (875 mg L^{-1}), from an engineered strain, was reported in *C.autoethanogenum* (Köpke et al., 2012). After submission of this manuscript, a report by Jia et al. (2021) was published, describing the production of up to 13.4 gL^{-1} by a recombinant *C. ljungdahlii*. Considering these results, it can be concluded that *A. woodii* might have an alternative acetone-isopropanol conversion pathway in its metabolism and it could be a better isopropanol producer. It was also attempted by Prof. Peter Dürre´s group (data not shown here) to identify the possible gene that might be encoding SADH responsible for isopropanol formation in *A. woodii* however the respective gene could not be identified (Arslan et al., 2022).

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4.4. Continuous Gas Fermentation with Cell Retention Using the Recombinant Strain *A. woodii* [pJIR750_ac1t1]

Abstract

Continuous fermentation with full cell retention was performed with the best acetone producing engineered *A. woodii* [pJIR750_ac1t1]. The objective was to maintain a consistent acetic acid level within bioreactor and increase biomass and acetone concentration. This set-up achieved the highest amount of isopropanol from anaerobic gas mixtures reported to date. Coupling the ultrafiltration module with the bioreactor system could further help increasing the biomass concentration compared to the batch process in a stirred tank bioreactor. The study also demonstrated that increasing agitation speeds resulted in improved mass transfer rates, enhanced biomass growth and, increased production of metabolites.

Keywords: *A. woodii*; acetone, isopropanol, cell retention, agitation speed

Citation- Data on continuous fermentation is unpublished yet

Introduction

Gas-liquid mass transfer limitations is the major bottleneck of syngas fermentation which causes low biomass growth and low product yield. Cell recycling, higher agitation speeds and high gas feeding rates are some approaches to increase density of biomass and productivity (Ritcher et al., 2013; Syahidah et al., 2008). In this section, it was aimed to increase acetone production in the best acetone producing engineered strain *A. woodii* [pJIR750_ac1t1]. In the previous section, batch fermentations with continuous gas supply revealed that the acetate concentration has a significant impact on acetone formation in recombinant *A. woodii* strains. Except for the strain *A. woodii* [pJIR750_ac2t1] (the low Km value CoA transferase from *C. aceticum* used), in all other three recombinant strains, acetone formation was only possible after acetate reached a certain concentration due to the high Km value of the CoA transferase from *C. acetobutylicum*. Additionally, it was also observed that the biomass growth started declining when the acetate concentration in the bioreactor exceeded 10 gL^{-1}. This work was conducted in order to suggest a bioreactor system where the acetate concentration within the bioreactor could be kept at an optimum value for biomass growth and also for acetone formation. To this end a continuous fermentation process was set-up in a 2 L stirred tank bioreactor. In order to increase the biomass concentration an ultrafiltration module was coupled to the bioreactor to provide full cell retention and recycling and continuous drainage of liquid medium (permeate). Three different hydraulic retention times were studied for the biomass and metabolite accumulation within the bioreactor under fully controlled fermentation conditions (30℃, pH:7.5, 10 mL min^{-1} gas flow rate, 250 RPM agitation).

Materials and Methods

Microorganisms and media

The best acetone producing engineered strain *A. woodii* [pJIR750_ac1t1] was used in this study. Details of media composition and gene constructions can be found in the Material & Method part of previous section (4.3).

Continuous fermentation with full cell retention

In case of continuous fermentation, two 2 L glass bottles were added to the bioreactor system with the help of external peristaltic pumps (Watson Marlow 101U/R, Marlow, United Kingdom). One of the bottles was used as feed bottle and 2 L of vitamin and antibiotic free medium was autoclaved in this bottle. Before connecting to the bioreactor system, the medium inside the feed bottle was flushed with pure nitrogen for at least 12 hours and then the headspace of the bottle was continuously flushed during the experiment as well. The other 2 L glass bottle was used to collect the effluent stream under continuous bioreactor operation. The peristaltic pumps and the gas tight tubes (Masterflex Norprene, Gelsenkirchen, Germany) that were used for the feeding and the effluent stream were equal and maintained at the same flow rate, in order to keep the reactor working volume constant at 1.5 L. The crossflow feed (5 mL min^{-1}) into the ultrafiltration module was controlled by an internal pump available on the control unit of the bioreactor system. An ultrafiltration module (Repligen, MiniKros Sampler, Massachusetts, USA) was connected to the system in order to achieve different acetate concentrations within the bioreactor and to recover biomass. Prior to use, the tubes of the ultrafiltration module were autoclaved and the module was flushed with sterile N_2 gas for at least 2 hours. Schematic setups for the batch and continuous fermentation processes were described in Material and Methods (Section 3.2.2 and 3.2.3).

First, the bioreactor was operated in batch fermentation mode, following the procedure described in the previous section (4.3), *Bioreactor cultivation with continuous gas supply*, until the modified *A. woodii* strain reached the late exponential growth phase. Then, the feed bottle, containing 2 L anaerobic and fructose-free medium (hydraulic retention time; 3–10.6 days), and the ultrafiltration module were connected to the system.

Product and biomass quantification

2-ml liquid samples were withdrawn periodically from the bioreactors during the experiments to carry out analytical tests. The cell concentration was determined by using a spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain, or, Ultrospec 1100 Pro, Amersham Biosciences Europe GmbH, Freiburg, Germany, respectively) at a wavelength of 600 nm (OD_{600}). Fructose, acetic acid, acetone and isopropanol concentrations in the bioreactor experiments were determined with a high performance liquid chromatograph (HPLC) HP-1100 (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector and a refractive index detector, at 50°C. The samples were centrifuged (ELMI Skyline Ltd CM 70M07, Riga, Latvia) at 7000 rpm for 5 min at room temperature and the supernatant was filtered through a 0.22-μm filter. Then, 20 μl of those samples were injected into the Agilent Hi-Plex H Column (300 × 7.7 mm) (Agilent Technologies, Santa Clara, CA, USA) at 45°C, and a 5 mM H_2SO_4 solution was used as mobile phase with a flow rate of 0.80 ml min^{-1}. A gas counter (Ritter, Miligascounter MGC-1 PMMA, Bochum, Germany) was connected to the exhaust gas tubes and the exhaust gas composition was analyzed periodically. Gas-phase H_2 concentrations were measured on an HP 6890 gas chromatograph (GC) equipped with a thermal conductivity detector (TCD). The GC was fitted with a 15 m HP-PLOT Molecular Sieve 5A column (ID: 0.53 mm, film thickness: 50 μm). The oven temperature was initially kept constant at 50 °C, for 5 min, and then raised by 20 °C min^{-1} for 2 min, to reach a final temperature of 90 °C. The temperature of the injection port and the detector were maintained constant at 150 °C. Helium was used as the carrier gas. Similarly, CO_2 was analyzed on an HP 5890 gas chromatograph, equipped with a TCD. The injection, oven and detection temperatures were maintained at 90, 25 and 100 °C, respectively.

Determination of the cell specific product formation rate

Estimation of the cell specific metabolite formation rates (q_p) were made based on the off-line measured metabolite and biomass OD values applying a central first-order difference approximation (Kantzow et al., 2015).

$$q_p = \frac{2}{Cx1 + Cx2} X \frac{\Delta Cp}{\Delta t} \quad (1)$$

where c_x is the biomass OD and c_p is the metabolite concentration.

Results and Discussion

Figure 17 shows the biomass OD, metabolite concentrations, and cell specific metabolite formation rate trends during the experiment. Figure 18 shows the inlet and outlet gas flow rates and the gas compositions depending on the areas determined from GC-TCD analyses. The experiment lasted for around 1400 h and the results were evaluated in five different periods. The results of those different periods are presented and discussed hereafter.

Period I (0-300 h): The fermentation was initially performed in batch operation mode with continuous gas supply (CO_2:H_2, 25:35, at 10 mL min^{-1}). After inoculation, the biomass OD_{600} increased from 0.45 to a maximum of 2.1 within the first 170 h and once the acetate concentration reached 10 gL^{-1}, the biomass OD_{600} started to decline slowly. These results were similar to the results observed previously in batch fermentation. Acetate formation started right after inoculation and reached 17 gL^{-1} after 300 h process time; thus still being the dominant metabolite. Acetone was first detected after the acetate concentration had increased over 8 g L^{-1}, while isopropanol was first detected after the acetate concentration exceeded 11.5 gL^{-1} and at an acetone concentration over 100 mgL^{-1} (Figure 17 B). The cell specific acetate formation rate showed a decreasing trend during this period, as expected. The outlet gas flow rate was always under 10 mL min $^{-1}$ during this period, and miminum decreases in the CO_2 and H_2 areas were observed at the beginning of the experiment (Figure 18, A and B) indicating that there wasn´t any limitation in gas supply.

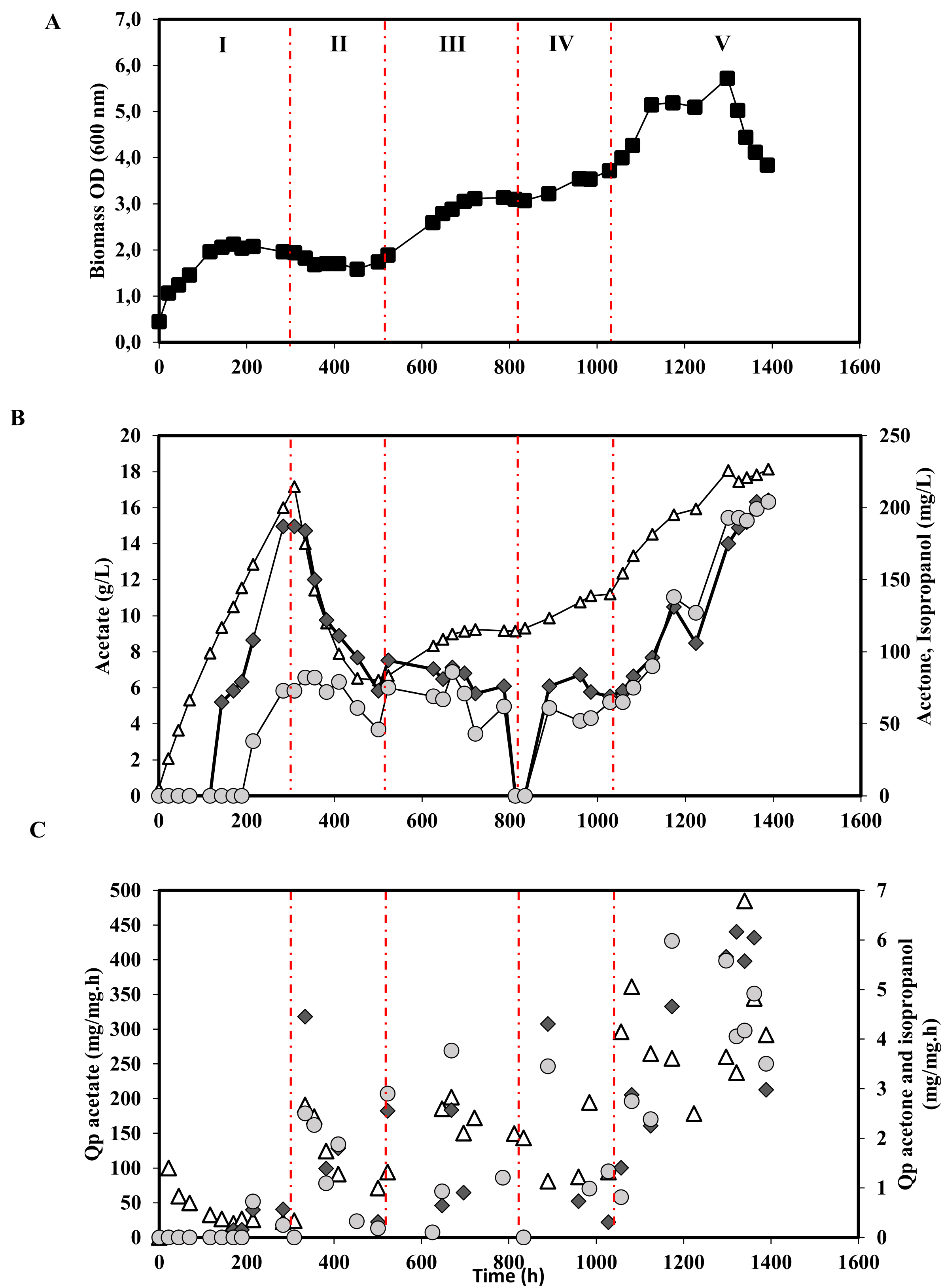

Figure 17. Continous gas fermentation with cell retention and recombinant *A. woodii* [pJIR750_ac1t1] (A) Biomass optical density measured at 600 nm; (B) Metabolites concentration trend; (C) Cell specific metabolite formation rate

Acetate (triangles, ▲), Acetone (diamonds, ⬥), Isopropanol (circles, ●).

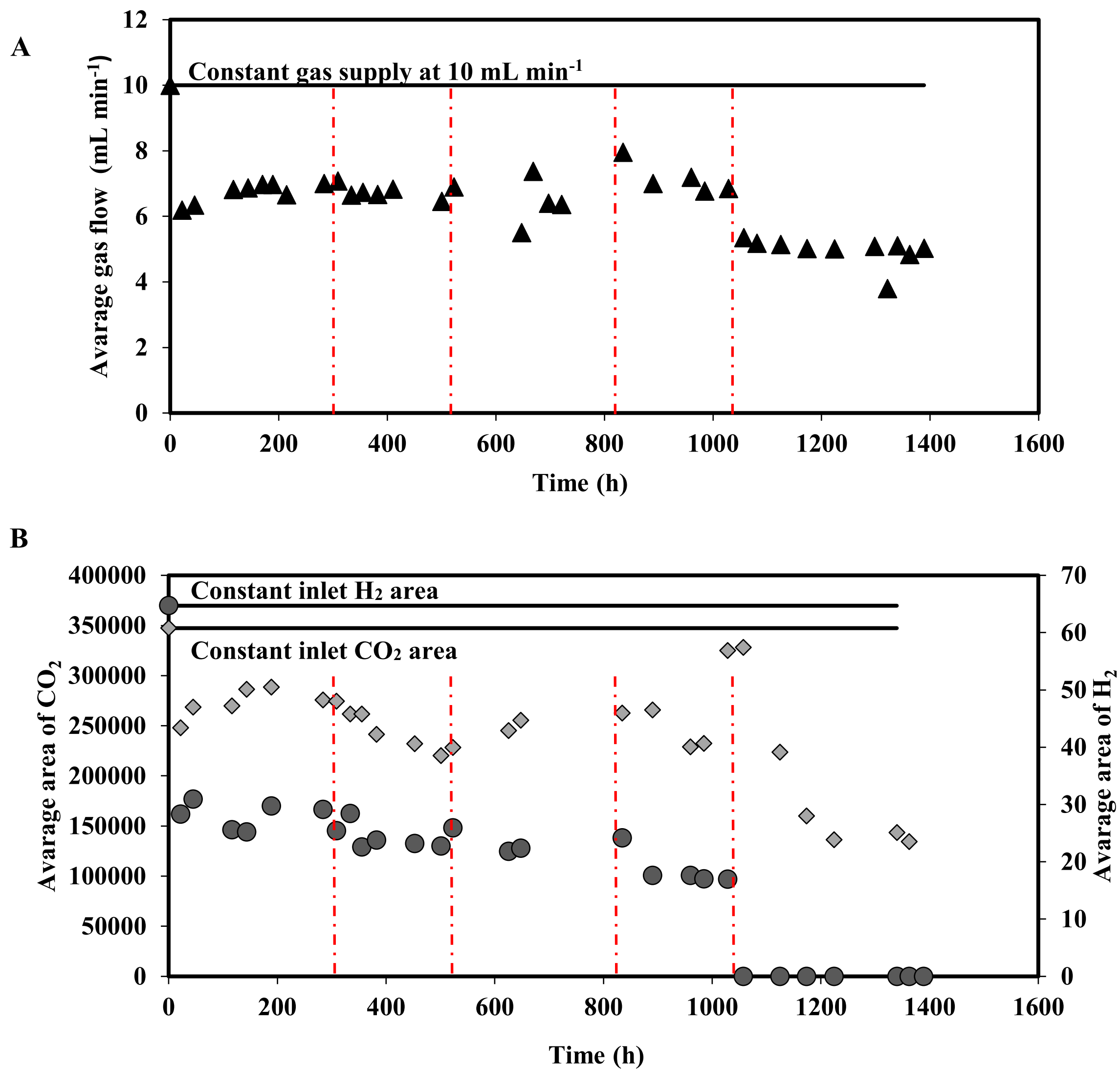

Figure 18. Continous gas fermentation with cell retention and recombinant *A. woodii* [pJIR750_ac1t1] (A) Outlet gas flow rate; (B) Areas of CO_2 (diamonds, ◆); Areas of H_2 (circles, ●)

Thus, the decreasing cell growth and cell specific acetate formation rates could be the result of inhibitory effects of the increasing acetate concentrations. After 300 h, as the biomass OD_{600} declined to 1.9, a 2 L glass feeding bottle and an ultrafiltration module were connected to the bioreactor system and continuous nutrient feeding was started, initiating what is considered period II.

Period II (300-500 h): In order to try to reduce the acetate concentration, continuous nutrient feeding was started with a flow rate of 0.34 mL min^{-1}, which corresponds to a hydraulic retention time (HRT) of 3 days. After switching to continuous operation, the biomass OD_{600}

decreased slowly to a steady state value of around 1.7. As a result of the dilution effect of the liquid feed, the concentrations of metabolite decreased as well to a steady state level of 6.5 gL^{-1} for acetate, 90 mgL^{-1} for acetone and 60 mgL^{-1} for isopropanol. Interestingly, isopropanol was much less affected by a drop of concentration than acetate and acetone. Cell specific metabolite formation rates were higher than during period I, showing that the continuous nutrient feed improved the accumulation of metabolites. However, there was no improvement in biomass concentration in the bioreactor even with the cell retention unit. CO_2 and H_2 areas in the outlet gas flow were lower than during the previous period indicating that the nutrient feed might improve the gas uptake by the bacteria. As expected, high dilution rates resulted in a lower stable acetate concentration of around 6.5 gL^{-1}.

Period III (500-800 h): In order to try to increase the concentration of metabolites and, eventually, biomass, the HRT was increased to 7 days by decreasing the nutrient flow rate to 0.15 mL min^{-1}. A slow steady increase was observed in terms of biomass OD_{600} reaching a value of 3. A similar trend was observed for the acetate concentration, reaching 9 g L^{-1}. However, it was not the case for the target metabolites, acetone and isopropanol. No important increase was observed for any of those two metabolites´ concentrations during this period. The cell specific metabolite formation rates did also not show any significant trend. Some improvements were only detected in q_p acetate values. At the end of this period, the acetate concentration stabilized around 9 gL^{-1}, while the concentrations of acetone and isopropanol both decreased dramatically at the end of this period. This seems to suggest that either increasing or decreasing the feed flow rates or the HRT may thus play a clear selection role on the nature of preferentially produced metabolites, though the exact mechanism behind this is not totally clear at this stage.

Period IV (800-1000 h): During this period, the HRT was adjusted to 10.6 days and decreasing dilution rates resulted in increases in the acetate concentration. Once the acetate

concentration reached 10 gL^{-1}, acetone and isopropanol were detected in the fermentation medium again at very low concentrations of 80 mgL^{-1} and around 50 mgL^{-1}, (maximum concentrations reached during this period) respectively. Lowering the feeding rate to 0.10 mL min^{-1} and dilution rate slightly improved the biomass concentration and H_2 consumption as well. At the end of this period, the biomass OD_{600} reached a high value of 3.7 and the acetate concentration stabilized at a concentration of 11 gL^{-1}.

Period V (1000-1400 h): After having observed that decreasing the liquid feed rate, increased more the concentration of acetate than of the other two target metabolites (i.e., acetone and isopropanol), whose concentrations remained rather stable, the effect of the gas supply was then tested in period V. In order to evaluate a possible limitation in gas-liquid mass transfer caused by the low solubility of the gaseous substrates, the agitation speed was increased from 250 RPM to 600 RPM during the last experimental period. Increased stirring rates improved all the reaction parameters, as expected. The highest biomass accumulation was observed, reaching an OD_{600} of 5.7, which is 2.7 times higher than the maximum biomass OD_{600} observed during the batch fermentation mode and 1.5 times higher than the best value found under the low agitation rate condition with continuous liquid feed. It should be mentioned that during all stages foam formation was observed and foaming was higher when cell densities were higher. Foam was controlled by antifoam (0.1 mL paraffin oil) injection. However, it was not possible to avoid biomass accumulation on the surface of the liquid medium and on the inside wall of the vessel. Initially, increased agitation enabled foam break and better mixing and this is possibly another reason for observing increasing biomass concentrations in the fermentation medium. Foam control is crucial for ensuring reactor stability and it is also important to determine the appropriate amount of antifoam to be added, in order to prevent cell death by excessive use of that agent which is known to be toxic at high concentration to most cells (Ritcher et al., 2013). One possible explanation for the observed decrease in

biomass concentration at the end of period V could be excess antifoam concentrations together with high acetic acid concentrations.

Gas consumption was also increased and H_2 was not found in the outlet gas flow showing that under these conditions H_2 became the limiting substrate. It is worth reminding that the solubility of H_2 in aqueous phase, at ambient temperature (e.g., 0.0015 gL^{-1} at 30 ºC), is about three orders of magnitude lower than for carbon dioxide (Fernández-Naveira et al., 2017). The highest cell specific metabolite formation rates were observed during this period as well. At the end of this period, acetate concentration reached 18 gL^{-1}, while acetone and isopropanol concentrations were 200 mgL^{-1}, each. It is interesting to mention that, besides the effect of the liquid dilution rate highlighted in the previous periods, in this period it can be observed and concluded that increasing the gas mass transfer (i.e., agitation speed) has a considerably more significant enhancing effect of the production of acetone and isopropanol than on acetate accumulation. Indeed, between period IV (low agitation rate) and period V (high agitation rate), the acetate concentration increased by about only 65% (from approximatly 11 gL^{-1} to 18 gL^{-1}), while both the acetone and isopropanol concentrations increased by close to as much as 340% and up to >200 mg/L in period V, at the end of the steady-states.

During this study it was observed that the lower liquid dilution rates were better for biomass growth and regarding gas consumptions it is also observed that at lower feeding rates outlet gas H_2 concentrations were also lower. It might be indicating that lower nutrient feeding rates, creating more favorable conditions for better H_2 solubility or higher dilution rates, result in gas/liquid mass transfer limitations, as also reported by Kantzow et al. (2015). Those authors suggested applying higher partial gas pressures at higher dilution rates for a better biomass growth and metabolite formation in *A.woodii*.

It can be concluded that the nutrient feeding at any rate improves cell specific metabolite formation rates compared to the batch operation mode. Hoffmeister et al. (2016) reported 10-

fold higher OD_{600} values in continuous gas fermentation and 20 times better acetone productivities compared to bottle fermentations. In their study, isopropanol was not identified nor analysed and thus data on that compound were not reported.

In this study, the highest q_q values for each metabolite (acetate, acetone, and isopropanol) were observed during the last period, when the agitation speed was increased from 250 RPM to 600 RPM. Higher agitation speeds are known to enhance the mass transfer coefficient, as was reported by Syahidah et al. (2008), who indicating that an increased agitation speed, from 350 RPM to 800 RPM, resulted in 3 times better mass transfer coefficient values and almost three times higher productions. During the first four periods of our study, both H_2 and CO_2 gases were found in the outlet gas at high levels, and for this reason it was concluded that there was no limitation in gas supply. In this study it was revealed very obviously that higher agitation speeds are improving biomass growth and metabolite formation by overcoming the mass transfer limitations. It is also important to take into consideration the high power inputs required for high agitation speeds and the foaming problems which is another major concern as it might keep reactants, products or biomass in foam and reduce the productivity of the process (Mohammadi et al., 2011).

Conclusions

Production of acetone and isopropanol from CO_2 is possible with engineered strains. According to the present findings, *A. woodii* [pJIR750_ac1t1] was shown to be the most promising engineered strain for an efficient acetone production, from a gas mixture of CO_2 and H_2, in terms of biomass growth and metabolites production. The present set-up also produced the highest amount of isopropanol from anaerobic gas mixtures reported so far.

It can also be concluded that the acetate concentration has a huge impact on both biomass growth and acetone formation in the recombinant *A.woodii* strains and the continuous nutrient feeding strategy is an efficient tool for keeping the metabolite concentrations at a certain

level. Coupling the ultrafiltration module with the bioreactor system could further help increasing the biomass concentration compared to the batch process in a stirred tank bioreactor. It is known that the efficiency of gas fermentation mainly suffers from low solubility of gas components which results in low growth rates and, consequently, low biomass concentrations. It was shown that increasing agitation speeds indeed improve mass transfer rates, biomass growth and the production of metabolites.

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5. CONCLUSIONS

The main achievements in this thesis were (i) to highlight the potential of *C. aceticum* as a promising host for ethanol formation from gaseous substrates, (ii) development of a pH control strategy to enhance ethanol production in *C. aceticum*, (iii) showing substrate flexibility, fructose metabolism and substrate preference of *C. aceticum* for ethanol production, (iv) the establishment of engineered *A. woodii* as a promising host for acetone and isopropanol production and (v) trial of a continuous fermentation system aiming acetone production enhancement.

- *C. aceticum* was challenged by medium acidification and it has been shown that solvent production in *C. aceticum* was triggered at around neutral pH values. It was clearly observed that *C. aceticum* followed the typical acetogenic and solventogenic steps of the Wood-Ljungdahl pathway, metabolizing C1 gases to produce acetic acid, followed by its subsequent bioconversion to ethanol most probably by the function of AOR enzyme.
- Based on the observations and previously published research with other acetogens, it was concluded that the pH drop rather than the pH value seems to stimulate solventogenesis. For *C. aceticum* a pH value around 8 is near optimal for biomass growth and thus acetic acid production, while solventogenesis was detected at a slightly acidic pH value of 6.9, which is a higher value than for other typical solventogenic species.
- It was also observed that medium replacement is a promising method to improve cell concentration and recover cell activity. Another interesting finding was the possible ability of *C. aceticum* to convert ethanol, produced in the solventogenic phase, back to acetic acid, under specific conditions. Optimizing conditions to avoid the reverse reaction in cyclic pH shift would allow to further maximize ethanol production.
- Ethanol formation was possible at low pH values but in such case the fermentation process suffered from fast biomass decay. Adjusting the operating conditions allowed to

reach high ethanol concentrations, not only from CO but also from syngas, and 5.6 g/L ethanol accumulation was possible when applying cycles of high and low pH and medium renewal.

- Organic carbon sources might enhance bacterial growth and so metabolite production by providing excess ATP. In order to determine substrate flexibility and the metabolic profile of *C. aceticum* on two different carbon sources, CO and fructose were used as sole carbon and energy sources.
- When fructose was used as sole substrate, the process suffered from formic acid accumulation, and inhibition was observed as a consequence of reducing equivalents deficiency. Formic acid formation was suggested as a sign of WLP activity, which means that *C. aceticum* was able to fix the CO_2 generated through glycolysis in the WLP. However, as a result of the poor flux establishment between glycolysis and WLP, excess reducing equivalents are oxidized by hydrogenase activity and H_2 was released. Lack of reducing equivalents (electrons) resulted in low bacterial growth and low metabolite formation. A possible suggestion to overcome this problem might be H_2 or CO enhanced mixotrophy in which H_2 or CO are provided to the fermentation medium exogenously as electron source.
- Final C_M/C_S derived in fructose fermentation was only 48% considering acetic acid as the only product of the process. Even though formic acid formation is proving the occurrence of autotrophic metabolism in *C. aceticum*, it was strongly repressed by the presence of fructose. The CO_2 gas, that was not reassimilated through the WLP was lost, resulting in poor carbon yields.
- The influence of the pH was found to be carbon source dependent. Acetic acid conversion to ethanol was clearly stimulated by pH drop when *C. aceticum* was growing on CO;

however, the same effect was not observed in fructose fermentation. CO was found to be a better C and energy source for ethanol production by *C. aceticum*.

- In the last section, the acetone production capability of genetically modified *A. woodii* strains was tested. Production of acetone and isopropanol from CO_2 and H_2 was possible with engineered strains. According to the findings, *A. woodii* [pJIR750_ac1t1] was shown to be the most promising engineered strain for an efficient acetone production from a gas mixture of CO_2 and H_2 in terms of biomass growth and metabolite production. 438 mM acetic acid, 14.5 mM isopropanol, and 7.6 mM acetone was produced in a batch cultivation with continuous gas feeding. Isopropanol appeared in the fermentation medium as an unexpected product and its concentration reached ~~to~~ the highest reported isopropanol value from anaerobic gas mixtures so far.
- Acetate concentration~~s~~ was found to have a huge impact on both biomass growth and acetone formation on recombinant *A. woodii*.
- Continuous nutrient feeding strategy was conducted in order to keep acetate concentrations at an optimum level for acetone or isopropanol production and it was shown to be an efficient tool for keeping the metabolite concentrations at a certain level. Coupling the ultrafiltration module with the bioreactor system also helped increasing the biomass concentration compared to the batch process in a stirred tank bioreactor.
- Nutrient feeding enhanced biomass accumulation and cell specific metabolite formation rates and lower dilution rates were found to be better for biomass growth.
- In order to observe the effect of agitation speed, agitation was increased from 250 RPM to 600 RPM, it revealed that higher agitation rates are improving biomass growth and metabolite formation, and improves much more acetone and isopropanol production than acetate production, but it is also important to take into consideration the high power inputs and foam formation problems at higher agitation speeds.

Appendix

Resumen de la tesis en castellano

El cambio climático global y el agotamiento de los recursos fósiles son los dos mayores problemas relacionados con la energía del siglo XXI. El aumento de las concentraciones atmosféricas de CO_2 causado por la combustión y extracción de recursos fósiles para producir productos químicos, combustibles y energía son las razones más comunes detrás de estos dos problemas. Sin embargo, con la creciente demanda energética mundial es probable que se consuman más recursos energéticos en el futuro. Los principales objetivos de la comunidad global incluyen reducir nuestra dependencia de los recursos fósiles y, por lo tanto, disminuir las altas concentraciones atmosféricas de CO_2. Para cumplir con este objetivo, estas comunidades establecen metas bajo acuerdos, como el Acuerdo de París, para reducir las emisiones de CO_2 atmosférico y limitar el calentamiento global. Esto implica la sustitución de los combustibles fósiles por carbono de orígen vegetal y el desarrollo de una economía circular.

En este punto, se requieren nuevas materias primas alternativas que cumplan ciertos criterios, como la rentabilidad en comparación con las opciones tradicionales basadas en fuentes fósiles, para lograr un proceso de producción competitivo. Durante el último siglo se han propuesto y estudiado diferentes materias primas sostenibles, cómo la biomasa lignocelulósica, residuos industriales, residuos de vertederos y otros tipos de residuos. Los materiales lignocelulósicos tienen un gran potencial, pero requieren un tratamiento exhaustivo para hacer accesibles todos los azúcares fermentables (principalmente de la celulosa y hemicelulosa del material lignocelulósico). De esta manera, los rendimientos de los productos y las eficiencias de conversión serían competitivos y el proceso de conversión en general sería rentable. La gasificación es un proceso en el que el material lignocelulósico se convierte en gas de síntesis, una mezcla de CO, CO_2, H_2, N_2 y otros gases e impurezas en concentraciones menores, a alta temperatura. Se presenta como una alternativa que permite utilizar todas las

fracciones lignocelulósicas, incluyendo la lignina. Tradicionalmente, el gas de síntesis se convierte en productos como diésel, metanol o etanol, entre otros, mediante el uso de catalizadores químicos y la síntesis de Fischer-Tropsch operada a alta presión y temperatura. La fermentación bacteriana del gas de síntesis también se puede utilizar para convertirlo en alcoholes y ácidos carboxílicos mediante catalizadores microbianos.

El gas de síntesis ha sido utilizado durante mucho tiempo como materia prima en la síntesis química industrial, y se produce principalmente a partir de recursos fósiles como el carbón o el gas natural. El uso de corrientes de desechos para la producción de gas de síntesis presenta sus propios desafíos; por ejemplo, estas materias primas están altamente distribuidas, tienen una composición invariable y el gas de síntesis producido a partir de estas fuentes está asociado con contaminantes. El proceso de Fischer-Tropsch requiere altas temperaturas y presiones. Además, los catalizadores químicos requieren una estricta relación fija de H_2/CO y pueden ser fácilmente envenenados por impurezas. La fermentación del gas de síntesis es ventajosa ya que los catalizadores biológicos son flexibles e independientes de la relación de gases. Tiene una mejor selectividad de productos, opera a baja temperatura y presión, y no requiere limpieza significativa del gas debido a que las impurezas en el gas apenas inhiben a los catalizadores biológicos. Las principales barreras de la fermentación del gas como sistema de producción comercial mediante bacterias acetogénicas incluyen la baja productividad debido a las limitaciones de transferencia de masa gas-líquido, preocupaciones energéticas, altos costos de producción y el escalado exitoso del proceso.

Sin embargo, los proyectos de fermentación del gas de síntesis han avanzado a escala comercial y se presentan como otras opciones de producción de biocombustibles lignocelulósicos, ya que la biomasa lignocelulósica no es la única materia prima para la fermentación del gas de síntesis. De hecho, los gases de escape de varios procesos industriales contienen componentes similares a los del gas de síntesis. Por ejemplo, la industria

siderúrgica emite aproximadamente un 50% del carbono utilizado en el proceso en forma de CO. Al utilizar cultivos energéticos dedicados, biomasa residual y residuos industriales o municipales como materias primas para la síntesis biológica o química, podemos fomentar la reutilización y el reciclaje de materiales que de otra manera se desperdiciarían. Este enfoque puede crear un ciclo sostenible y respetuoso con el medio ambiente para la producción de energía y productos químicos renovables y neutros en carbono.

Las bacterias acetogénicas son un grupo de microorganismos quimiolitoautótrofos capaces de utilizar CO y/o CO_2+H_2 en condiciones anaeróbias y convertir estos gases en ácidos orgánicos, alcoholes y otros compuestos a través de la vía metabólica de Wood-Ljungdahl (WLP). Las bacterias acetogénicas son organismos metabólicamente versátiles y, además de los sustratos gaseosos, pueden utilizar una amplia gama de otros sustratos como azúcares, alcoholes y aldehídos. Existen más de 100 especies de acetógenos citadas, con diversas morfologías y una amplia gama de temperaturas y pH óptimos para su crecimiento. La vía de Wood-Ljungdahl (WLP), también conocida como vía reductora del acetíl-CoA, es la única vía lineal de fijación de CO_2 que las bacterias acetogénicas emplean para convertir sustratos gaseosos en bioproductos. Esta vía es utilizada por las bacterias acetógenicas para la conservación de energía, el crecimiento celular y la síntesis de acetíl-CoA a partir de dos moléculas de CO_2. Como se mencionó anteriormente, estas bacterias también son capaces de crecer utilizando otras fuentes de carbono como azúcares. Esta característica les confiere una mayor importancia, ya que la combinación de la glicólisis y la WLP permite la fijación completa de carbono originado en azúcares y, por lo tanto, mayores rendimientos de productos.

Aunque el producto principal de las bacterias acetogénicas es el acetato, algunas son capaces de producir otros productos químicos valiosos como etanol, 2,3-butanodiol, butanol, hexanol, butirato y hexanoato. Además de estos productos que ocurren naturalmente, es posible

ampliar el espectro de productos a otras sustancias químicas mediante enfoques de ingeniería metabólica y el descubrimiento de especies adicionales con diversas capacidades de fermentación, lo que genera nuevos productos prometedores a partir de la fermentación del gas. El etanol y el butanol son importantes combustibles alternativos limpios para el transporte. El etanol puede mezclarse con gasolina (10%), mientras que el butanol puede ser utilizado directamente y mezclado con gasolina en cualquier proporción en los motores de automóviles existentes. El ácido propiónico, el propanol, el ácido hexanoico, el hexanol y el isobutanol son otros productos potenciales que se pueden producir mediante la fermentación del gas de síntesis. El etanol es uno de los biocombustibles más disponibles y prominentes en el mercado, con un alto número de octano y un alto contenido de oxígeno, que permite una combustión completa. Se sabe que varias bacterias acetogénicas tienen la capacidad de producir etanol de forma natural. Se cree comúnmente que la formación de ácidos y disolventes durante la fermentación del gas de síntesis depende de las condiciones del proceso. Se ha observado que la producción de ácido depende del crecimiento, lo cual ocurre a altas tasas de crecimiento y en condiciones óptimas de crecimiento. Las condiciones óptimas de crecimiento incluyen un suministro adecuado de nutrientes, un pH óptimo, una temperatura óptima y ninguna inhibición del producto final. Por otro lado, la producción de disolventes ocurre en condiciones desfavorables de crecimiento, como baja temperatura, limitación de nutrientes y bajo pH. Además, se informa que varias bacterias acetogénicas reducen los ácidos orgánicos a disolventes a través de la ruta AOR, también a expensas de un donante de electrones (agente reductor) como H_2 o CO.

El estudio y la mejor comprensión de las bacterias acetogénicas a nivel molecular y celular han permitido mejoras significativas en la aplicación de herramientas de ingeniería genética en estos microorganismos. Estas herramientas, que se han utilizado anteriormente en varios microorganismos modelo como *E. coli* y en levaduras, se utilizan para ampliar el rango de

productos de los acetógenos más allá de sus productos naturales y también para mejorar la eficiencia en la producción de sus productos nativos. *C. aceticum* fue descrito hace muchas décadas como el primer acetógeno autótrofo capaz de convertir hidrógeno y dióxido de carbono en ácido acético y agua en estudios de cultivo puro, y se consideró un organismo atractivo con su metabolismo energético que ofrece nuevos objetivos para la ingeniería metabólica para mejorar el rendimiento de ATP y permitir la producción de compuestos energéticamente desafiantes. *A. woodii* también es una bacteria homoacetogénica que fue descubierta por primera vez en 1977 en los sedimentos de un estanque de ostras en el estado de Massachusetts (EE.UU.). Es una de las bacterias acetogénicas más estudiadas en la actualidad y sirve como organismo modelo. La conversión de gas por parte de las bacterias acetogénicas se ve afectada por las condiciones dentro y fuera de la célula. Las reacciones enzimáticas y la velocidad de cada reacción en la WLP están determinadas por la concentración y disponibilidad de los metabolitos involucrados. La cinética de crecimiento del cultivo también depende de los nutrientes, el carbono y las fuentes de energía tomadas del medio. Los parámetros del proceso de fermentación deben optimizarse para crear un microambiente celular favorable para un mejor crecimiento celular y formación de productos. Estas condiciones incluyen pH, temperatura, tipo y concentraciones de nutrientes, composición del gas, velocidad de flujo de gas, velocidad de mezcla, transferencia de masa y concentración de productos finales.

Los sistemas de fermentación de gas de síntesis se ven principalmente desafiados por una baja productividad debido a las limitaciones de transferencia de masa gas-líquido y las limitaciones energéticas que limitan el potencial biotecnológico de los microorganismos acetogénicos. El objetivo general de esta tesis es evaluar parámetros de operación eficientes para la fermentación de gas y la producción de etanol y acetona utilizando las bacterias acetogénicas *C. aceticum* y *A. woodii*. Los resultados de esta tesis se estructuraron en cuatro

partes individuales. La Parte 1 y 2 tratan sobre el metabolismo y las barreras energéticas de *C. aceticum*. Se considera que *C. aceticum* es un huésped atractivo debido a su metabolismo energético. El acetato es el principal producto de esta cepa y se desconocía su capacidad de producción de etanol. Por lo tanto, esta tesis tiene como objetivo determinar los parámetros óptimos (principalmente el pH) para la producción de etanol en esta cepa. Con este fin, en un primer paso se analizó el patrón de metabolitos de *C. aceticum* a diferentes valores de pH. Esto se logró omitiendo la regulación del pH del proceso de fermentación de gas, lo que causa acidificación del medio como resultado de la acumulación natural de ácido acético. Se planteó la hipótesis de que la acidificación del medio permitiría alcanzar el valor óptimo de pH que cambiaría la etapa de producción de ácido a la etapa de producción de disolvente en *C. aceticum*. Después de lograr este objetivo y determinar las limitaciones de la producción de disolvente dependiendo del pH en *C. aceticum*, se aplicaron diversos enfoques como la renovación parcial del medio de fermentación y estrategias de cambio de pH para aumentar las concentraciones de etanol en el medio de cultivo. El segundo paso, se enfocó en encontrar mejores fuentes de carbono que mejorarían el rendimiento del proceso y la producción de etanol en *C. aceticum*. Con este fin, se evaluó la influencia del CO y la fructosa como únicas fuentes de carbono y mediante cambios de pH.

En la parte 3, se estudió la producción de acetona en cultivos por lotes para cuatro cepas de *A. woodii* modificadas genéticamente, con el objetivo de encontrar el mejor productor de acetona entre todas ellas. Dentro de esta parte, se observó que la producción de acetona en las cepas recombinantes dependía de la concentración de acetato. En la parte 4 se mostró la viabilidad de un proceso de fermentación continua con retención completa de células, con el objetivo de mantener la concentración de acetato en un nivel óptimo y mejorar la productividad de acetona. Además, se puso énfasis en el efecto de la agitación en la formación de metabolitos y el consumo de gas. A continuación se detallan los principales hallazgos de cada parte. El

etanol es uno de los productos químicos que se encuentra dentro del ámbito de metabolites formados en la fermentación del gas de sintésis. Se conocen algunos enfoques operativos que son útiles para mejorar la producción de etanol y aumentar su concentración final en las bacterias acetogénicas. Se ha descrito que *C. aceticum* utiliza CO y CO_2/H_2 de manera eficiente y convierte estos gases en acetato. Recientemente, se informó en la literature científica que esta cepa produce una cantidad muy baja de etanol (unos pocos mg/litro), con el acetato como producto principal, como resultado inesperado bajo una alta presión parcial de CO. Sin embargo, antes de estas tesis, todavía se desconocía por completo la capacidad de producción de etanol, a altas concentraciones, de la cepa. Las preguntas científicas detrás de este estudio fueron: cómo influyen los parámetros del proceso en la producción de disolventes y el crecimiento, y si se podrían utilizar otros enfoques para mejorar la producción de etanol en esta cepa. Se sabe que valores de pH bajos estimulan la producción de disolventes en otras bacterias acetogénicas, y una de las hipótesis de partida era que un cambio de pH podría aumentar la concentración final de etanol en el medio de fermentación. También se sabe que la alimentación de nutrientes frescos es un método útil para obtener una mayor producción de disolventes. Nuestro enfoque fue llevar a cabo un experimento de acidificación natural del medio que nos permitiera definir los valores de pH óptimos para el crecimiento y la producción de disolventes en esta cepa. Posteriormente, se estudiaron aspectos cómo el cambio de pH y la renovación parcial del medio en la fermentación por lotes con suministro continuo de gas. Los principales hallazgos de este estudio son:

• Se desafió a *C. aceticum* mediante la acidificación del medio y se demostró que la producción de disolventes en *C. aceticum* se desencadenó alrededor de valores de pH neutros. Se observó claramente que *C. aceticum* seguía los pasos típicos de procesos acetogénicos y solventogénicos de la vía de Wood-Ljungdahl, metabolizando los gases C1 para producir

ácido acético, seguido de su posterior bioconversión a etanol, probablemente mediante la actividad de la enzima AOR.

• Basándonos en las observaciones y en investigaciones previamente publicadas con otras bacterias acetogénicas, se concluyó que la caída del pH, más que el valor de pH en sí, parece estimular la solventogénesis. Para *C. aceticum*, un valor de pH alrededor de 8 es óptimo para el crecimiento de la biomasa y, por lo tanto, para la producción de ácido acético, mientras que la solventogénesis se detectó a un valor de pH ligeramente ácido, alrededor de 6,9, que es un valor más alto que el de otras especies solventogénicas típicas.

• También se observó que la renovación del medio de fermentación es un método prometedor para mejorar la concentración celular y recuperar la actividad celular. Otro hallazgo interesante fue la capacidad de *C. aceticum* para convertir el etanol, producido en la fase solventogénica, de vuelta a ácido acético bajo condiciones específicas. La optimización de las condiciones para evitar la reacción inversa en el cambio cíclico de pH permitiría maximizar aún más la producción de etanol.

• La formación de etanol fue posible a valores de pH bajos, pero en este caso, el proceso de fermentación sufrió una rápida degradación de la biomasa. Ajustar las condiciones operativas permitió alcanzar altas concentraciones de etanol, no sólo a partir de CO sino también de gas de sintésis, y fue posible acumular 5,6 g/L de etanol al aplicar ciclos de pH alto y bajo y renovación del medio.

En el primer paso se estudió la formación de etanol en *C. aceticum* y se observó que se podía alcanzar una alta concentración de etanol aplicando ciertas estrategias de regulación del pH. Este estudio se realizó utilizando una mezcla de gas de sintésis con $CO:CO_2:H_2:N_2$ (30:5:15:50). La fermentación de gas de sintésis se conoce por tener bajos rendimientos de productos debido a las bajas solubilidades de los sustratos gaseosos. La fijación autotrófica de CO_2 requiere una fuente de energía, y en la mezcla de gas de sintésis, esta fuente es el H_2. Sin

embargo, el CO puede ser una fuente de carbono y energía al mismo tiempo, y se sabe que, en comparación con la mezcla de gas de síntesis, el CO puro es una mejor fuente de carbono para productos reducidos como el etanol. Además, se sabe que fuentes de carbono orgánicas como la fructosa proporcionan más energía (ATP) y poder reductor (NADH/NADPH). Por lo tanto, era necesario determinar cómo estas diferentes fuentes de carbono influirían en el rendimiento del proceso y en la producción de etanol con *C. aceticum*. En el segundo estudio, nuestro objetivo era encontrar respuesta a la siguiente pregunta: ¿cuáles podrían ser las limitaciones del uso exclusivo de CO o fructosa para la producción de etanol en *C. aceticum* en un estudio de fermentación por lotes utilizando un enfoque de cambio de pH? Los principales hallazgos de esta parte son:

• Las fuentes de carbono orgánicas pueden mejorar el crecimiento bacteriano y la producción de metabolitos al proporcionar un exceso de ATP. Con el fin de determinar la flexibilidad de los sustratos y el perfil metabólico de *C. aceticum* con dos fuentes de carbono diferentes, se utilizó CO y fructosa como únicas fuentes de carbono y energía.

• Cuando se utilizó fructosa como único sustrato, el proceso llevó a la acumulación de ácido fórmico, y se observó inhibición como consecuencia de la deficiencia de equivalentes reductores. La formación de ácido fórmico se sugirió como indicativo de la actividad de la WLP, lo que significa que *C. aceticum* era capaz de fijar el CO_2 generado a través de la glucólisis en la WLP. Sin embargo, debido a la deficiente establecimiento del flujo entre la glucólisis y la WLP, los equivalentes reductores en exceso se oxidaron mediante la actividad de la hidrogenasa y se liberó H_2. La falta de equivalentes reductores (electrones) resultó en un bajo crecimiento bacteriano y baja formación de metabolitos. Una posible sugerencia para superar este problema podría ser la mixotrofia mejorada con H_2 o CO, en la que el H_2 o el CO se proporcionan al medio de fermentación exógenamente como fuente de electrones.

• La CM/CS final derivada de la fermentación de fructosa fue solo del 48% considerando el ácido acético como el único producto del proceso. Aunque la formación de ácido fórmico demuestra la ocurrencia de metabolismo autotrófico en *C. aceticum*, este fue fuertemente reprimido por la presencia de fructosa. El gas CO_2, que no fue reassimilado a través de la WLP, se perdió, lo que resultó en bajos rendimientos de carbono.

• Se encontró que la influencia del pH dependía de la fuente de carbono. La conversión de ácido acético a etanol fue claramente estimulada por la disminución del pH cuando *C. aceticum* crecía con CO; sin embargo, no se observó el mismo efecto en la fermentación de fructosa. El CO se encontró que era una mejor fuente de carbono y energía para la producción de etanol por *C. aceticum*.

Es necesario ampliar el rango de productos producidos por bacterias en la fermentación de gases para que otros productos químicos importantes, como la acetona y el isopropanol, puedan también ser producidos a partir de gases residuales y gas de sintésis. Los enfoques de ingeniería metabólica se han utilizado con éxito para la incorporación de nuevas vías sintéticas en bacterias acetogénicas. La acetona no se produce de forma natural en *A. woodii* y la producción de acetona se logró en esta cepa mediante la introducción de genes de *C. acetobutylicum* en ella. Sin embargo, algunas de las enzimas de *C. acetobutylicum* tienen inconvenientes que las hacen inadecuadas para la síntesis en huéspedes heterólogos.

Para lograr una mejor producción de acetona en *A. woodii*, se deben construir nuevas cepas recombinantes y se debe evaluar el rendimiento de fermentación de gas de estas nuevas cepas modificadas. Para lograr una mejor producción de acetona, se construyeron dos nuevas vías de biosíntesis de acetona combinando genes de *C. acetobutylicum* y *C. aceticum*. Se utilizó una fermentación por lotes en un biorreactor con agitación y con suministro continuo de gas utilizando un medio de cultivo definido para comprobar la capacidad de las cepas recién generadas para producir acetona. En el tercer capítulo, se describieron primero las cepas y

luego se presentaron los resultados de las fermentaciones por lotes con estas cepas. El principal hallazgo de esta sección fue:

• La producción de acetona e isopropanol a partir de CO_2 y H_2 fue posible con cepas modificadas. Según los hallazgos, *A. woodii* [pJIR750_ac1t1] se mostró como la cepa modificada, más prometedora, para una producción eficiente de acetona a partir de una mezcla de gases, CO_2 y H_2, en términos de crecimiento de biomasa y producción de metabolitos. Se produjeron 438 mM de ácido acético, 14,5 mM de isopropanol y 7,6 mM de acetona en un cultivo por lotes con alimentación continua de gas. El isopropanol apareció en el medio de fermentación como un producto inesperado y su concentración alcanzó el valor más alto reportado hasta ahora para el isopropanol a partir de mezclas de gases en condiciones anaeróbias.

• Se encontró que las concentraciones de acetato tienen un gran impacto tanto en el crecimiento de la biomasa como en la formación de acetona en cepas de *A. woodii* recombinantes.

En la cuarta y última sección, se buscó aumentar la producción de acetona en la mejor cepa modificada de *A. woodii* para la producción de acetona, *A. woodii* [pJIR750_ac1t1]. En la sección anterior, los cultivos por lotes con suministro continuo de gas revelaron que la concentración de acetato tiene un impacto significativo en la formación de acetona en las cepas recombinantes de *A. woodii*. Excepto en la cepa *A. woodii* [pJIR750_ac2t1] (que utiliza la transferasa CoA de *C. aceticum* con un valor de Km bajo), en las otras tres cepas recombinantes, la formación de acetona solo fue posible cuando el acetato alcanzó una cierta concentración debido al alto valor de Km de la transferasa CoA de *C. acetobutylicum*. Además, también se observó que el crecimiento de la biomasa comienza a disminuir cuando la concentración de acetato en el biorreactor supera los 10 g/L. Este trabajo se realizó con el objetivo de proponer un sistema de biorreactor donde la concentración de acetato dentro del

biorreactor se pueda mantener en un valor óptimo para el crecimiento de la biomasa y la formación de acetona. Para ello, se estableció un proceso de fermentación continua en un biorreactor, con agitación, de 2 L. Para aumentar la concentración de biomasa, se acopló un módulo de ultrafiltración al biorreactor para proporcionar retención completa de células y drenaje continuo del medio líquido (permeado). Se estudiaron tres diferentes tiempos de retención hidráulica para la acumulación de biomasa y metabolitos dentro del biorreactor bajo condiciones de fermentación totalmente controladas (30℃, pH 7.5, velocidad de flujo de gas de 10 mL/min, agitación a 250 RPM). Los principales hallazgos de la última sección son:

• Se llevó a cabo una estrategia de alimentación continua de nutrientes con el fin de mantener las concentraciones de acetato en un nivel óptimo para la producción de acetona o isopropanol, y se demostró que es una herramienta eficiente para mantener las concentraciones de metabolitos en un nivel determinado. El acoplamiento del módulo de ultrafiltración con el biorreactor también ayudó a aumentar la concentración de biomasa en comparación con el proceso por lotes en un biorreactor agitado.

• La alimentación de nutrientes mejoró la acumulación de biomasa y las tasas de formación de metabolitos específicos de las células, y se encontró que tasas de dilución más bajas eran mejores para el crecimiento de la biomasa.

• Para observar el efecto de la velocidad de agitación, se aumentó la agitación de 250 RPM a 600 RPM, lo cual reveló que mayores velocidades de agitación mejoran el crecimiento de la biomasa y la formación de metabolitos, y mejoran mucho más la producción de acetona e isopropanol que la producción de acetato, pero también es importante tener en cuenta los altos requerimientos de energía y los problemas de formación de espuma a velocidades de agitación más altas.

List of publications

Arslan, K., Bayar, B., Nalakath Abubackar, H., Veiga, M. C., & Kennes, C. (2019). Solventogenesis in *Clostridium aceticum* producing high concentrations of ethanol from syngas. *Bioresource Technology, 292 (August).* https://doi.org/10.1016/j.biortech.2019.121941

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Conferences – oral presentation

Arslan, K., Bayar, B., Abubackar, H. N., Veiga, M. C., & Kennes, C. 2019. Solventogenesis in *Clostridium aceticum* producing high ethanol concentrations from CO_2, CO and syngas. 8th International Conference Biotechniques for Air Pollution Control & Bioenergy, Galway, Ireland, August 28-30.