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Mixotrophic fermentation for butanol production

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob orientação científica da Doutora Ana Lopez Contreras, Cientista e Coordenadora de Projetos no Food and Biobased Reasearch Center na Universidade de Wageningen, Holanda, e co-orientada pela Doutora Ana Maria Rebelo Barreto Xavier, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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palavras-chave Fermentação mixotrófica, *Clostridium beijerickii* NCIMB 8052, butanol, Wood-Ljungdahl pathway, engenharia genética

resumo

Os organismos heterotróficos têm a capacidade de metabolizar carbono orgânico para gerar produtos de fermentação indispensáveis para a sociedade atual. Numa economia ainda dominada pela industria química à base de recursos fósseis, a urgência em otimizar e viabilizar os processos fermentativos é cada vez mais significativa. Em fermentações onde os açucares são utilizados como fonte principal de carbono, sabe-se que cerca de um terço do carbono proveniente do açúcar é perdido na forma de CO_2. Este fenómeno deve-se a uma reação de descarboxilação, durante a via glicolítica Embden-Meyerhof-Parnas (EMP), responsável por converter o piruvato em acetil-CoA. Numa tentativa de colmatar estas perdas de carbono, o presente trabalho revê uma via alternativa para recapturar o CO_2 desenvolvido usando o metabolismo de fixação de CO_2 Wood-Ljungdahl (WLP), num processo chamado fermentação mixotrófica anaeróbia, não-fotossintética (ANP). O mixotrofismo ANP, definido como a utilização simultânea de substratos orgânicos (como açucares) e inorgânicos (como CO_2) por um único organismo, evita as perdas de carbono, aumentando os rendimentos de produção e reduzindo as emissões de CO_2 durante as fermentações.

O objetivo deste trabalho foi o de tentar aumentar a produtividade de biobutanol em fermentações anaeróbias Acetona-Butanol-Etanol (ABE) realizadas pela bactéria *Clostridium beijerickii* NCIMB 8052. Para isso delineou-se uma estratégia de engenharia genética para ativar o metabolismo ANP mixotrófico na estirpe em causa. Através de um conjunto de diferentes fermentações experimentais e de diferentes análises bioinformáticas, concluiu-se que *C. beijerickii* NCIMB 8052 não é capaz de realizar o metabolismo mixotrófico ANP de forma natural e que isso se deve à ausência, no seu genoma, de um grupo de genes considerados essenciais para o funcionamento do metabolismo de WLP. Usaram-se várias técnicas de clonagem na tentativa de inserir os respetivos genes, via plasmídeo, em *C. beijerickii* NCIMB 8052, mas não foram obtidos os resultados esperados. Comprovou-se que nenhum dos genes de interesse foi clonado com sucesso.

keywords

Mixotrophic fermentation, *Clostridium beijerickii* NCIMB 8052, butanol, Wood-Ljungdahl pathway, genetic engeneering,

abstract

The current economy is still dominated by the fossil-based chemical industry that represents a nefarious contribution to the environment. To avoid the permanence of this industry, the necessity to optimize fermentations to cost-competitive processes started to arise. It is known that heterotrophic organisms can transform organic carbon into fermentation products with great economic interest. However, for most fermentations where sugars are used as carbon source, over one-third of the sugar carbon is lost to CO_2. The CO_2 evolves from the Embden-Meyerhof-Parnas (EMP) glycolysis decarboxylation reaction that converts pyruvate into acetyl-CoA. To overcome this carbon loss, one route to recapture evolved CO_2 using the Wood-Ljungdahl carbon fixation pathway (WLP), in a process called anaerobic, non-photosynthetic (ANP) mixotrophy, was reviewed in the present work. The ANP mixotrophy is defined as the concurrent utilization of organic (for example, sugars) and inorganic (for example, CO_2) substrates in a single organism. Comparing with the EMP glycolysis, this metabolism allows higher productivities and lower CO_2 emissions during fermentations.

With the purpose of increasing the biobutanol productivity in anaerobic ABE fermentations performed by *Clostridium beijerickii* NCIMB 8052, a genetic engineering strategy was designed to enable the ANP mixotrophic metabolism in this strain. Through a set of different fermentations and bioinformatic researches, it was concluded that *Clostridium beijerickii* NCIMB 8052 is not naturally capable of performing the ANP mixotrophic metabolism due to a group of genes, considered as essential for the WLP, that were found to be missing in this strain. Several cloning techniques were used to insert and overexpress, via plasmid, these genes into *Clostridium beijerickii* NCIMB 8052. At the end, none of the genes were successfully transformed.

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Abbreviations

ABE – Acetone-Butanol-Ethanol;

ABFA – Advanced Biofuels Association;

ACFA – Asian Clean Fuels Association;

AOR – Aldehyde Ferredoxin Oxidoreductase;

BLAST – Basic Local Alignment Search Tool;

CCR – Carbon Catabolic Repression;

CPEC – Circular Polymerase Extension Cloning;

DNA – Deoxyribonucleic Acid;

DW – Dry Weight;

EBTP – European Biofuels Technology Platform;

EMP – Embden-Meyerhof-Parnas;

EU – European Union;

FFV – Flexible Fuel Vehicles;

HF – High Fidelity;

MCS – Multiple Cloning Site;

NOG – Non-Oxidative Glycolysis;

OD – Optical Density;

OPEX – Operation expenditure/expenses;

PCR – Polymerase Chain Reaction;

SOC – Super Optimal Broth;

TAE – Tris-Acetate-EDTA;

Tm – Melting temperature;

UV – Ultra-violet;

WLP – Wood-Ljungdahl pathway;

Introduction

The today's society energy system is deeply dependent on the fossil resources. They are so engrained that they started to constitute one of the main building blocks of the economic world. Therefore, with the purpose of minimize their prices, intensive researching to optimize the technologies that convert these resources into fuels and commodity chemicals are being made for many years. Coupling these very efficient technologies to the 2007-2010 financial crisis, that raised up the necessity to recover the global economies and political instability in the Middle East, the crude oil prices suffered a considerable decrease to US$100 per barrel (Liew et al., 2013). Such low prices constitute one of the biggest barriers for the production of cost-competitive alternative fuels. However, at the current rate of consumption, the global reserves of petroleum are expected to be exhausted within 35 years, resulting in an increasing trend of the fossil fuels price along the next years. Apart of this, these fuels also represent a considerable environmental impact, since their burning lead to the accumulation of atmospheric CO_2 that directs the environment to the nefarious climate changes that global warming represents. The use of these resources is then unsustainable and under these circumstances a very controversial public discussion began to emerge focusing on the question of how long the petroleum reserves will last and for how much time the environment will resist to the inadvertent use of them (Shafiee & Topal, 2009).

In response to this problem and to face the increasing demand of energy, especially from developing countries, environmentally sustainable fuels are urgently required. For instance, the European Union (EU) has mandated member countries to a target of deriving 10% of all transportation fuels from renewable sources, in a cost-effective way, by 2020, as well as a target of a 20% share of energy from renewable sources in overall community energy consumption. Even more, the European Council of March 2008 mentioned that is essential to develop and fulfil effective sustainability criteria for biofuels and ensure the commercial availability of second-generation biofuels (The European Parliament and the Council of the European Union, 2009). In fact, the dangerous CO_2 output, derived from fossil fuels burning, can be counterbalanced by using renewable biomass for biofuel production. Using biomass, we can guarantee that the amount of CO_2 that is released, by the burning of these fuels, will be affordable for plants and bacteria to recapture. Lignocellulosic biomass such as agricultural, industrial, and forestry residues, as well as dedicated crops, are renewable and

abundant resources with great potential for a low-cost and uniquely sustained bioconversion. However, this kind of feedstock needs to be pre-treated to become fermentable sugars, as C-5 and/or C-6 sugars, by biological or chemical processes. In nature, a variety of microorganisms including bacteria and fungi have the ability to degrade lignocellulosic biomass. Moreover, lignocellulosic biomass is also able to be converted, by chemical processes (encompassing drying, size reduction, pyrolysis, fractionation and leaching) in synthesis gas (syngas) that can be also used by microorganism such as acetogens, carboxytrophs and methanogens to produce biofuels. Yet, one of the primary factors affecting economic viability of next generation biofuels is the cost of the feedstock pre-treatment, which can exceed 50% of total operating expenditure (OPEX) (Fast et al., 2015; Morvan & Fonty, 1996). The best way to minimize high feedstock costs is by maximizing feedstock conversion to the product of interest. Focusing on increase the fermentation efficiency seems to be an important solution.

The ability to achieve high mass yields from carbohydrate fermentations can be considerably improved. Actually, one third of hexose carbons consumed by the microorganisms are lost as CO_2 in the heterotrophic metabolism, during classical Embden-Meyerhof-Parnas (EMP) glycolysis. This CO_2 is formed by the decarboxylation of pyruvate to form acetyl-CoA. In practice, it means that the theoretical maximum mass yields for biofuels range from 33% for butanol and 51% for ethanol (Rude & Schirmer, 2009). For this reason, researchers are attempting to find a new biological approach to eliminate CO_2 losses and direct it to the formation of more acetyl-CoA, the main building block to produce biofuels (Tracy et al., 2012). Mixotrophic microorganisms seem to be perfect candidates to solve this problem. These organisms are able to use a mix of different sources of carbon and perform both heterotrophic and autotrophic metabolisms. So, through mixotrophic fermentation, the CO_2 evolved during EMP glycolysis can be reassimilated into biomass and fermentation products by a carbon dioxide fixation metabolic pathway. Only the bacteria that use the Wood-Ljungdahl carbon fixation pathway (WLP), termed acetogens, are natively capable of performing the stoichiometric conversion of 1 mol of C-6 sugars into 3 mol of acetyl-CoA. Glycolysis and WLP are then complementary pathways in which two molecules of CO_2 and eight electrons generated from glycolysis can be fully utilized by the WLP to produce an additional acetyl-CoA. This co-utilization of the two pathways simultaneously represents an

increase of 50% of the acetyl-CoA yield, when compared with the standard EMP glycolysis (Fast et al., 2015).

In the present work, a bibliographic review about the ANP mixotrophic metabolism was elaborated, focusing on butanol production, as well as an experimental analysis of the capability of *Clostridium beijerickii* NCIMB 8052 to produce butanol.

1.Background Information

1.1 Biofuels

1.1.1 What are biofuels?

In today's society, gasoline is the predominant fuel to power petrol engine automobiles. Gasoline is a C_4-C_{12} hydrocarbon mixture produced by cracking crude oil. One barrel of crude oil (159 L) can be refined to produce 72L of gasoline. It is known that nowadays there are approximately 600 million passenger cars moving around the world, representing a daily consuming amount of 3520 million liters of gasoline. Considering the tremendous environmental and socioeconomic impacts that this dependence on fossil fuels represents, we need urgently to develop renewable sources of gasoline substitutes that fit the existing liquid fuel supply systems (Guo et al., 2015; Shafiee & Topal, 2009).

To limit greenhouse gas emissions and improving air quality, some energy alternatives were created. Biofuels, hydrogen, natural gas and syngas (synthesis gas) are considered the most reliable strategies to fight this problem. Among all these alternatives, biofuels seem to be the most promising, as they are considered most environmental friendly, they are suitable for the use in the power petrol engines, that are already very engrained in the society, and they are safe to transport (Nigam & Singh, 2011).

Biofuels are considered as liquid, gas and solid fuels that are produced from biomass. They can be made from agricultural crops (first generation biofuels) or from agricultural, industrial and municipal wastes (second generation biofuels). The second-generation biofuels have the particular advantage of reutilizing some compounds that are present in the wastes and that represent a considerably environmental impact when released directly into the environment. They are also a good alternative to avoid ethical and social discussions related with the use of crops, dedicated to food production, as raw-material for biofuels. There is a diverse variety of technologies to produce biofuels such as fermentation of sugar substrates, catalytic conversion of ethanol to mixed hydrocarbon, hydrolysis of cellulose, transesterification of natural oils and fats to biodiesel, hydrocracking of natural oils and fats, pyrolysis and gasification of various biological materials. Biofuels are CO_2 neutral fuels, as the amount of CO_2 that is released from their combustion is exactly the same that was initially absorbed by the biomass used to produce them. Therefore, the CO_2 released is able of being completely

absorbed again by plants and bacteria, representing a minimum environmental impact. At the end, they can be used in conventional fuels and vehicles engines, either totally or partially in a blend (Demirbas & Balat, 2006; Faaij, 2006).

The most commercialized biofuels in the recent years have been ethanol, manufactured from corn starch in the United States and from sugarcane in Brazil and biodiesel produced primarily from rapeseed oil in Germany and France. However, other biofuels with great potential can be produced, for instance butanol (Demirbas & Balat, 2006).

Renewable resources are more evenly distributed than fossil and nuclear resources. Additionally, the energy flows from renewable resources are more than three orders of magnitude higher than current global energy use. So, the use of these renewable resources to produce fuels is a promising way to preserve our environment without compromising the use of our technologies. However, the prices of the biofuel accessibility and production are still considerably high when compared with the fossil fuels. Considering the crude oil prices and the refining costs, gasoline can be produced for 0,14 €/Kg (Clifford, 2016a) while ethanol production costs vary between 0,11 – 0,41 €/Kg (Clifford, 2016b) and butanol production costs between 0,76 – 1,27 €/Kg (Clifford, 2016c). To achieve competitiveness and efficiency there are a lot of improvements that should be considered, mainly the availability and long distance supply of the biomass, the transportation system between the biofuel production facilities and the clients and the production efficiency. All these topics should be targets of investigation to guarantee a faster transition to clean energies and limit, as much as possible, the environmental impact that the use of fossil fuels is causing every day (Solomon, 2010).

1.1.2 Advantages of butanol as biofuel

Recently, another player entered in the game and that is the biologically produced butanol (biobutanol). It is a four carbon primary alcohol, with the molecular formula C_4H_9OH (MW 74,12 g mol^{-1}). This alcohol is a colorless liquid and it has a very distinct odor. There are four isomers of butanol, isobutanol, sec-butanol, tert-butanol and n-butanol, the latter having fuel interesting characteristics. In this bibliography, the term “butanol” refers to n-butanol unless otherwise stated. The alcohol is produced by acetone-ethanol-butanol (ABE) fermentations and it has superior fuel properties when compared with ethanol (Szulczyk, 2010).

On the one hand, butanol is a longer chain hydrocarbon (four carbons) than ethanol (two carbons), a characteristic that makes this compound resemble gasoline more closely than ethanol, allowing butanol to be blended with gasoline at any concentration. On the order

hand, ethanol presents some limitations concerning the blending properties. It has a blending range between 15%, when is used in standard car engines without any engine modifications, up to 85%, considering flexible fuel vehicles (FFV). The combustion of butanol, as a sole fuel or blended with gasoline, does not require any modification on the existing car engines (Ramey, 2007).

Table 1 – Comparison between gasoline, pure ethanol and pure butanol with focus on the fuels properties. The parameters analysed on the table are considered the most relevant from a fuels industry point of view. Table adapted from (Szulczyk, 2010);

	Units	***Gasoline***	***Pure ethanol***	***Pure butanol***
Oxygen Content	%	Close to 0	36	22
Octane Number	%	85 to 94	112,5 to 114	87
Reid Vapor Pressure	Bar	0,480 to 1,034	0,159	0,023
Higher Heating Value*	MJ L^{-1}	34,8	23,6	-
Lower Heating Value **	MJ L^{-1}	31,2 to 32,4	21,1 to 21,3	27,8

* Heat energy release during the combustion, including the water vaporization; ** Heat energy release during the combustion, excluding the water vaporization;

Considering the comparison made in table 1, it is possible to emphasize some of the benefits of butanol. Starting with the oxygen content, this characteristic defines the level of combustion that the fuel can reach. Fuels with more oxygen are able to achieve a more complete combustion, reducing the amount of carbon monoxide emissions. Looking at table 1, gasoline has almost 0% of oxygen, what implicate that it must be blended with additives, richer in oxygen, in order to reduce carbon monoxide emissions. Ethanol or butanol are great options for this purpose (Gallagher et al., 2003; Reynolds, 2000). The octane number is considered as a standard measure of the performance of an engine. The higher this number, the higher is the compression that the fuel should be exposed to detonate. Thus, the higher the octane number, the easier is the prevention of premature ignition that can lead to engine damages. In this aspect, ethanol is the best option as it has the highest-octane number. Either butanol or ethanol can be blended with cheaper low-octane fuels to increase their octane number (ACFA NEWS, 2008; Szulczyk, 2010). The Reid vapor pressure is considered as the minimum vapor pressure needed to start a cold engine. At the beginning, the fuel should

allow an easy vaporization of some of its compounds, that are compressed and ignited. When the engine gets warmer the other compounds in the fuel will not have difficulties to vaporize too. As shown at table 1, butanol has a lower Reid vapor pressure when compared with ethanol and gasoline, meaning that it turns more difficult the ignition in a cold engine. However, considering the legislations applied by the Environmental Protection Agency, who demands a maximum vapor pressure for fuels, butanol could have an interesting contribution. As the easy vaporization of fuels is responsible for higher pollution levels, because sun's ultra-violet (UV) radiation converts these volatile gases into ground ozone pollution (Environmental Protection Agency, 2008), if the petroleum producers blend butanol with low-cost fuels, they can decrease their high vapor pressure. For the case of ethanol, it must be blended with more expensive fuels with lower vapor pressure. The lower vapor pressure of butanol is also an advantage because it makes butanol safer to handle (Dürre, 2007; Reynolds, 2000; Wu et al.,2007). Considering the next property in the table 1, the heating values, it expresses the energy content of each fuel. This energy is the heat released by the fuels combustion that is used by the engine to produce movement. Once the engines are not able to utilize the energy from vaporized water, we should consider the lower heating values. Therefore, butanol has 86 % of gasoline energy while ethanol has just 65 %. It means that butanol has more acceleration and mileage per liter of fuel comparing with ethanol (Van Gerpen et al.,2004).

In addition to all these butanol properties referred above, there are others of special interest from an industrial point of view. Butanol is not hygroscopic. This characteristic allows it to be blended with gasoline at the refinery, much before the storage and distribution, since the mixture butanol-gasoline does not separate in the presence of water. On the contrary, ethanol is a hygroscopic compound and the mixture ethanol-gasoline can be easily separated if it enters in contact with water at any stage of the storage and distribution processes. For that reason, it is mandatory that the ethanol stays separated from the gasoline in different tanks until they both reach the gas stations. This butanol property also prevents groundwater contamination in case of spills and if there is water inside the pipelines it will not be a problem either. For ethanol, the same cannot be considered. If there is water somewhere along the pipeline, the ethanol gets contaminated and the mixture gasoline-ethanol is compromised. Furthermore, ethanol is corrosive to the pipes joints and dissolves some impurities, increasing the possibility of buildups in the pipe (Dürre, 2007; Ramey, 2004; Szulczyk, 2010).

1.2 Butanol fermentation

1.2.1 Clostridia

Clostridia are one of the oldest prokaryotes known until now. They appeared approximately 2.7 billion years ago, before "the great oxidation event". It is known that they could be the evolutionary predecessors of the aerobic Firmicutes, such as bacilli (which contain the genus *Bacillus*), that derived from Clostridia before the big oxidation event (Paredes et al., 2005). Clostridia are anaerobic, gram-positive, non-sulphur-reducing rods that can form heat-stable endospores with the capability to survive to long exposures to oxygen/air (Dürre, 2014; Paredes et al.,2005). This prokaryote can be found as living cells or dormant spores in soils and animal intestines. Despite some Clostridia represent one of the most known pathogens (*Clostridium tetani*, *Clostridium perfringens*, *Clostridium botulinum* and *Clostridium difficile),* they also include other species like solvent producers (*Clostridium acetobutylicum* and *Clostridium beijerinckii*), cellulolytic species (*Clostridium phytofermentans* and *Clostridium thermocellum*) and species with major importance to the human and animal health and physiology, anaerobic degradation of simple and complex carbohydrates, acidogenesis, the carbon-cycle on earth and bioremediation of complex organic chemicals (Mitchell, 1998; Paredes et al., 2005; Tracy et al., 2012). Solventogenic clostridia can naturally produce, for instance, butanol, acetone, ethanol or isopropanol from a large variety of substrates, including monosaccharides, in the form of pentoses or hexoses, to polysaccharides and inorganic gases, such as CO, CO_2 and H_2 (Jones & Woods, 1986). They are also capable to perform mixotrophic fermentations in which they utilize both substrates, organic sugars and inorganic gases, to perform a heterotrophic and autotrophic complementary reaction, allowing the stoichiometric conversion of sugars into acetyl-CoA (Fast et al., 2015). The present work will focus on the solvent producing clostridia and in the ABE fermentations from which butanol is produced.

1.2.2 ABE fermentation

For many years, a lot of bacterial genera are used in industry to perform fermentations in order to obtain a large variety of bioproducts. The first historical report about the use of fermentations remit to 5000 B.C., where the fermentative production of ethanol was used for brewing beer in Mesopotamia (Liew et al., 2013). The ABE fermentations appeared several millennia later. Pasteur discovered bacterial butanol production from his landmark anaerobic

cultivation in 1861. This discovery made the ABE fermentations prosper in the early 20th century (Jones & Woods, 1986). During the World War I, Chaim Weizmann started to use starchy materials as substrate to perform ABE fermentations, using *Clostridium acetobutylicum* to produce acetone (used for cordites, an important propellant to the ballistic industry) (Dürre, 2007). ABE fermentations became one of the biggest biotechnological processes (after the ethanol fermentation) ever performed. However, the low demand of acetone after the end of the war caused the closure of all the plants. In 1945, the ABE fermentations became strong again, with butanol as a product of great interest for the automobile industry and the U.S. as producer of two thirds of industrially used butanol. Nevertheless, once again, in the 1960s, the process started to lose competitiveness, due to the increase of feedstock costs and advancement of the petrochemical industry, leading to the second big crash of the ABE fermentations (Lee et al., 2008). Recently, the ABE fermentations started to resurge due to the increasing demand of biofuels, such as ethanol and butanol, mainly in China, USA, United Kingdom (UK), Brazil, France and Austria (Dürre, 2007). This demand is motivated by the devastating pollution values reached from the inadvertent burning of fossil fuels, which tend to be even more severe due to the increasing mobility, especially in countries such as China and India. It is predicted that the worldwide energy consumption will grow by 57 % until 2030 (Shafiee & Topal, 2009). Furthermore, the governments started to impose laws mandating that fuels should increasingly derive from renewable resources, in a cost-effective way. Investments are being directed for the research and development of sustainable criteria for biofuels as well as commercial availability of them, mainly the second-generation biofuels (The European Parliament and the Council of the European Union, 2009). For instance, there are initiatives such as the EBTP (European Biofuels Technology Platform), ABFA (Advanced Biofuels Association), BAA (Biofuels Association of Australia) and ACFA (Asian Clean Fuels Association) that bring together a large number of companies and research centers with the objective of working together for a sustainable future, through the development of cost-competitive world-class biofuels value chains, healthy biofuels industries and accelerating the sustainable deployment of biofuels in the daily life. Now is probably the best time to bring again the ABE fermentations on the table and start thinking about how we can improve it in order to decrease the prices and fulfil the governmental demands.

To perform ABE fermentations two solventogenic strains of clostridia, *Clostridium acetobutylicum* and *Clostridium beijerickii,* are commonly used. The fermentation process is conducted under anaerobic conditions and in a sterile environment. To produce butanol, a large variety of raw materials can be used, such as molasses, whey permeates, corn and even seaweed biomass (Ezeji et., 2004; Ezeji et al., 2007a; Ezeji et al., 2007b; Jones & Woods, 1986; Van der Wal et al., 2013). ABE fermentations performed by clostridia are usually divided in two different phases. The first phase is designated as acidogenesis (acid production) and the second one as solventogenesis (solvent production) (Johnson, Peterson, & Fred, 1931). The first phase is characterized by exponential cell growth and expression of acids forming pathways in which acetate, butyrate, hydrogen and carbon dioxide are produced as major products (Andersch et al., 1983). By the excretion of these acids to the extracellular medium a decrease of the external pH occurs. The accumulation of these acids in the external medium is considered as an inducer of the expression of enzymes involved in the solventogenesis. Then, at a critical point of the external pH, the solventogenesis starts and the acids formed during the acidogenesis are reassimilated by the cells and used as co-substrates for the production of acetone, butanol and ethanol (Fond et al., 1985; Kell et al., 1981). This transition between acidogenesis and solventogenesis is the result of a dramatic change in gene expression patterns (Dürre, 2007) and is an adaptive response of the cells to the low pH of the medium (Bahl et al., 1982). During the solventogenic metabolism the cells do not grow anymore, reaching stationary phase, the acid production ends and the carbon and reducing equivalents are directed to solvent formation. At this stage a slightly increase of the external pH is noticed, as the acids are being consumed by the solventogenic metabolism (Terracciano & Kashket, 1986). When the solvents reach a concentration high enough to destabilize the cells membrane structure by affecting the membrane fluidity, the cellular metabolism ceases and the end of the fermentation is achieved (Vollherbstschneck et al., 1984).

Considering a batch fermentation, depending on the conditions, it should take 2-6 days to be completed. Normally, fed-batch and continuous fermentations are not feasible for solvent production because of the solvent toxicity in higher concentrations and the disturbances that these kinds of fermentations can have in the biphasic metabolism (Lee et al., 2008).

The buffering capacity of the medium should be target of major consideration during the fermentation process, because, if the pH decreases below 4.5, the solventogenesis will be

brief and unproductive (Bryant & Blaschek, 1988). Other practice that could have great influence in the solvent production is the use of excess of carbon under nitrogen limitation (Madihah et al., 2001) as well as the use of iron supplementation, as the conversion of pyruvate to acetyl-CoA involves a ferredoxin oxidoreductase iron-sulphur protein (Kim et al., 1988).

It is known that the solventogenic metabolism is deeply related with the sporulation. The transcription factor responsible for the sporulation in solvent clostridia, Spo0A, is also responsible for the solvent production as it is responsible for the activation of transcription of acetoacetate decarboxylase (adc), alcohol dehydrogenase (adhE) and CoA transferase (ctfAB) genes (Sullivan & Bennett, 2006). Harris et al., 2002, demonstrated that Spo0A deletion mutants are very deficient in solvent production, while in mutants with overexpressed Spo0A, an overexpression of solventogenic genes occurred as well. However, the strain failed to produce more solvents due to an accelerated sporulation process.

1.2.3 Metabolism

1.2.3.1 Wood-Ljungdahl Pathway

Acetogenic bacteria are known to use the Wood-Ljungdhal pathway as their main mechanism for energy conservation, synthesis of acetyl-CoA and cell carbon. This pathway confers them the ability to anaerobically convert CO_2 and CO into acetyl-CoA. There are studies that suggest that these organisms may have been the first autotrophs using inorganic compounds, like CO and H_2, as an energy source and CO_2 as an electron acceptor, approximately 1 billion years before the O_2 appeared (Brock, 1989). The WLP is described as a two branches pathway which converge to a common product, the acetyl-CoA (Figure 1). The Eastern, or methyl branch, in which one molecule of CO_2 undergoes reduction by six electrons to a methyl group and the western branch, or carbonyl branch, that involves the reduction of other CO_2 molecule to carbon monoxide and condensation of the bound methyl group with CO and coenzyme A (CoA) to make acetyl-CoA. Acetyl-CoA is then either incorporated into cell carbon or converted to acetylphosphate, whose phosphoryl group is transferred to ADP to generate ATP and acetate, the main growth product of acetogenic bacteria (Drake et al., 1997; Ljungdahl, 1986; Shafiee & Topal, 2009).

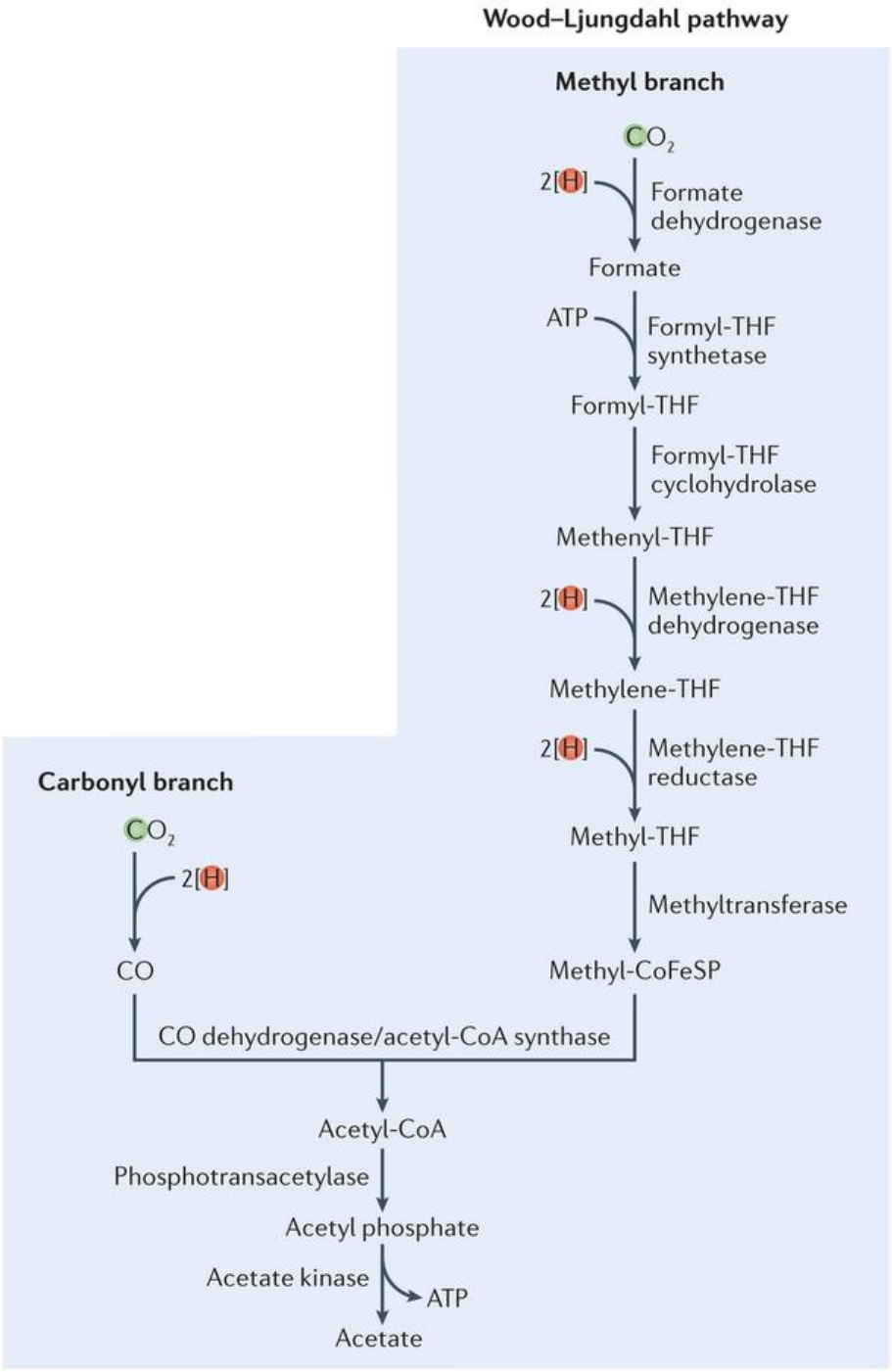


Figure 1 – Wood-Ljungdahl pathway illustration, with the western branch represented on the left and the eastern branch on the right. THF stems from tetrahydrofolate and CoFeSP from corrinoid/iron-sulphur protein. Figure adapted from (Fast et al., 2015);

The eastern branch of the WLP starts with the reduction of CO_2 to formate. This first reaction is catalyzed by formate dehydrogenase in which $NADP^+$/NADPH were shown essential to the CO_2/HCOOH half-reaction (Fu et al., 2010; Qureshi et al., 2008; Ragsdale,

1997). Once the formate is formed, in a reaction catalyzed by formyl-H_4folate synthetase, it undergoes an ATP-dependent condensation with H_4folate, forming 10-formyl-H_4folate. The next two steps are catalyzed by a bifunctional enzyme that contains a cyclohydrolase activity, that converts 10-formyl-H_4folate to 5,10-methenyl-H_4folate, and a dehydrogenase activity for the reduction of 5,10-methenyl-H_4folate to 5,10-methylene-H_4folate, in a NAD(P)H-dependent reaction. There are some organism, such as *Clostridium formicoaceticum* and *Acectobacterium woodii*, that are considered as an exception, concerning this two steps, as they pocess two monofunctional enzymes to catalyze these reactions instead of one byfuctional enzyme (Ragsdale, 1997). The next step is the reduction of 5,10-methylene-H_4folate to 5-CH_3-H_4folate. The enzyme that catalyzes this step is the 5,10-methylene-H_4folate reductase. At the end of the branch, an enzyme called methyltetrahydrofolate: corrinoid/iron-sulfur protein methyltransferase (MeTr) catalyzes the tranfer of the N^5 methyl group from (6S)-CH_3-H_4folate to the cobalt center of a corrinoid iron-sulfur protein (CFeSP).

The western branch, in the contrary of the eastern, involves just one enzyme, the byfunctional protein carbon monoxide dehydrogenase/ Acetyl-CoA synthase (CODH/ACS). CODH is responsible for the first reaction of this branch and it catalyzes the conversion of CO_2 to CO (Ragsdale, 1997; Ragsdale, 2008). When the organisms are grown on CO, CODH generates CO_2, which is then converted to formate in the eastern branch of the pathway and CO is incorporated directly as the carbonyl group of acetyl-CoA. In the next step the ACS catalyzes the condensation of CO, CoA, and the methyl group of a methylated corrinoid-sulfur protein (CfeSP) to generate Acetyl-CoA. It is the step where western branch meets the eastern.

At the end of the WLP, the acetyl-CoA molecule can be converted into acetate to produce one mole of ATP by substrate-level phosphorilation in the acetate kinase reaction. However, this mole of ATP is consumed in the eastern branch in the formyl- H_4folate synthetase reaction. Therefore, the net ATP at the end of the WLP is zero and the organisms still need energy to grow chemolithoautotrophically. To face this lack of energy, the WLP is coupled to a energy conservation complex (figure 2). With respect to the energy conservation mechanisms, the acetogens can be divided into two groups, the Na^+ and the H^+ organisms. The Na^+-dependente acetogens, such as *Acetobacterium woodii*, take use of membrane-bound corrinoids and couple the WLP to primary and electrogenic translocation of Na^+. The H^+-dependente acetogens, such as *Moorella thermoacetica* (formerly *Clostridium*

thermoaceticum), contain cytochromes and a membre-bound H^+ motive electron transport chain (Müller, 2003). H_2 supply is considered the driving force behind the production of reducing equivalents, since this molecule is the "spark" that initiates all WLP-associated energy conservation reactions. The energy conservation complex starts with H_2 oxidation by an electron bifurcating [FeFe]-hydrogenase, resulting in equal amounts of reduced ferredoxin and NADH. The reduced ferredoxin is then oxidized at the Rnf complex, in which the electrons are transferred to NAD^+, yielding NADH. During this process, the high-energy electrons from reduced ferredoxin are used by the Rnf complex to drive sodium/hydrogen cations translocation across the membranes (Hess et al., 2013). The established sodium/hydrogen gradient is used by a Na^+/H^+-dependent F_1F_0-ATPase to generate ATP. It is noteworthy that the same architecture and mechanisms apply to Na^+ and H^+ F_1F_0ATPases (Müller, 2003). NADH is used for the reduction of methenyl-H_4folate, via methylene-H_4folate, to methyl-H_4folate in the methyl branch of WLP. Additional reduced ferredoxin is required for CO_2 reduction to enzyme-bound CO, at the carbonyl branch. This reduced ferredoxin is thought to stem from the exergonic reduction of methylene-H_4folate to methyl-H_4folate, either by electron bifurcation or by coupling to the Rnf complex. As the ATP formed by the acetate kinase activity (acetate formation) is used to activate the formate to formyl-H_4folate, the sodium/hydrogen cation gradient is the only source of ATP formation. (Henstra et al., 2007; Liew et al., 2013; Müller, 2003; Ragsdale & Pierce, 2008; Tracy et al., 2012). NAD(P)H is another co-factor needed to perform the CO_2 reduction reaction. This necessity of NAD(P)H was shown in *Moorella thermoacetica* (previously known as Clostridium thermoaceticum). Therefore, to obtain NAD(P)H, an electron bifurcating NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase (NfnAB) is used to reduce two molecules of $NADP^+$, using NADH and reduced ferredoxin. Using the transference of electrons between reduced co-factores, *Moorella thermoacetica* enables the simultaneous utilization of glycolysis and WLP (Huang et al., 2012).

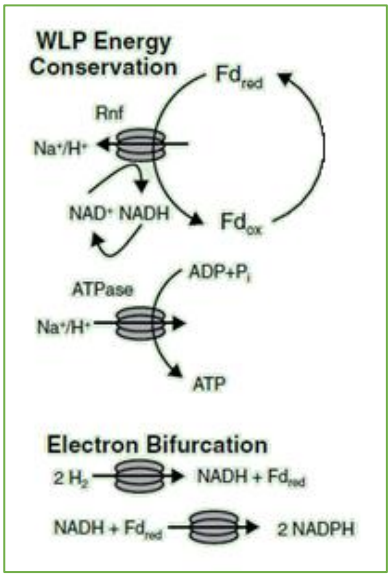


Figure 2 – WLP energy conservation complex with the Rnf complex, the ATPase and the electron bifurcation system. Figure adapted from (Fast et al., 2015);

To be sure about the relevant influence of the Rnf complex in the energy conservation mechanism of the acetogens, Heise et al., 1989, and Trembley et al., 2012, performed two different experiments to confirm if the Na^+-dependent and H^+-dependent Rnf complexes, respectively, are essential or not for the metabolism. Heise et al., 1989, studied the effects of removing all the sources of Na^+ from the medium, on the Na^+-dependent Rnf complex of *Acetobacterium woodii*. They concluded that: (i) in the absence of Na^+ a shift of the acetate-fructose ratio occurs from 2.7 to 2.1; (ii) the growth on H_2/CO_2 or on methanol/CO_2 was strictly dependent on the presence of Na^+; and (iii) the reduction of methylene-H_4folate to methyl-H_4folate is an Na^+-requiring reaction. Trembley et al., 2012, performed a knockout of the H^+-dependent Rnf complex of *C. ljungdahlii*, by a single-crossover integration that removed genes for two subunits of the Rnf complex, and analysed its effects on autotrophic and heterotrophic metabolism. They conclude that: (i) the absence of the H^+-dependent Rnf complex led to a complete inhibition of autotrophic growth; and (ii) the heterotrophic metabolism is also dependent on the H^+-dependent Rnf complex activity, as the knock out mutants could not grow at the same rate than the wild type that possessed the intact Rnf complex.

The WLP is the autotrophic metabolism addressed to perform the mixotrophic fermentation.

1.2.3.2 EMP Glycolysis

The Embden-Meyerhof-Parnas glycolysis is the most common sequence of reactions for the conversion of glucose-6-phosphate into pyruvate in all domains of life (Figure 3). This pathway allows the metabolic use of glucose to generate ATP, NADH, and several biosynthetic precursors such as 3-phosphoglycerate or pyruvate. This metabolic pathway is performed either by anaerobes, leading to several fermentation pathways, or by aerobes through the conversion of pyruvate to acetyl-CoA and the connection with the tricarboxylic acids cycle (TCA). The entire pathway occurs in the cytoplasm and it consists in ten enzymatic steps (Berg et al., 2002; Cohen, 2014; Taillefer & Sparling, 2016).

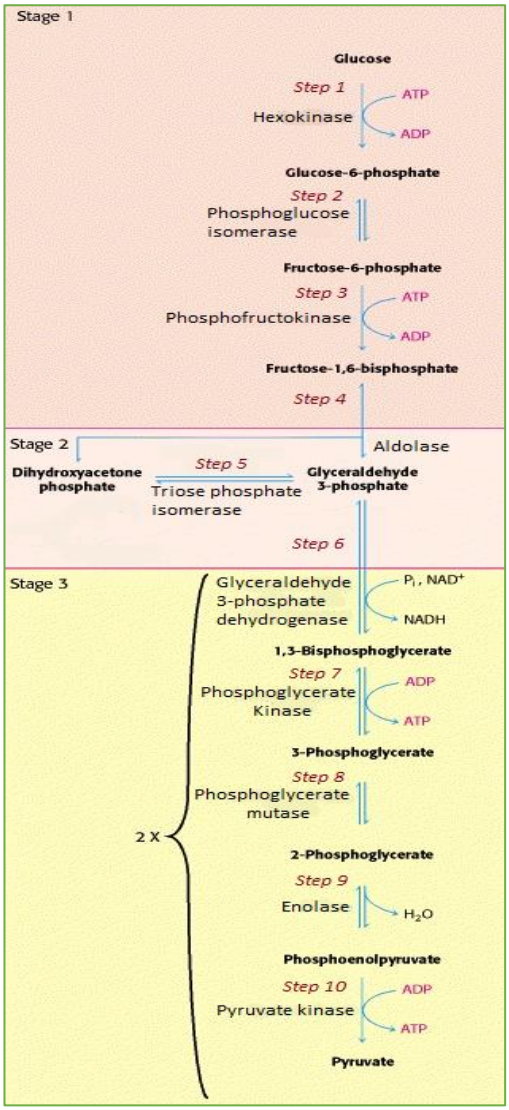


Figure 3 – EMP glycolysis illustration including the three different stages, (1) glucose is trapped and destabilized, (2) two interconvertible three-carbon molecules are generated by cleavage of six-carbon fructose and (3) ATP is generated. The enzymes involved and the steps that are inherent to each enzyme are also illustrated. Figure adapted from (Berg et al., 2002)

The first step in EMP glycolysis is the conversion of D-glucose into glucose-6-phosphate. The enzyme that catalizes this reaction is hexokinase and it performs the transfer of a phosphate group derived from ATP, to the glucose molecule. As a result, at this point in glycolysis, one molecule of ATP has been consumed. The second step involves an isomerization reaction, catalyzed by glucose phosphate isomerase, in which glucose-6-phosphate is rearranged into fructose-6-phosphate. In the third step, fructose-6-phosphate is converted to fructose-1,6-biphosphate. The enzyme that catalyzes this reaction is the phosphofructokinase and similar to the reaction that occurs in step 1, a second molecule of ATP provides the phosphate group that is added on to the fructose-6-phosphate. The fourth step is characterized by the split of fructose-1,6-biphosphate into two sugars that are isomers of each other. Aldolase is the enzyme that catalyzes this cleavage to yield glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Gliceraldehyde-3-phosphate is the only molecule that continues in the glycolytic pathway. Therefore, in the fifth step, the dihydroxyacetone phosphate is reorganized into glyceraldehyde-6-phosphate by the enzyme triphosphate isomerase. Now, we have two molecules of glyceraldehyde-3-phosphate proceding in the further glycolysis steps, meaning that all the products that are formed in the next steps are formed in twice the amount. The sixth step consists in two main reactions, the oxidation of glyceraldehyde-3-phosphate by the coenzyme nicotinamide adenine dinucleotide (NAD) and the phosphorylation of the resulting molecule. This reaction is catalyzed by glyceraldehyde-3-phosphate dehydrogenase and it yields 1,3-bisphosphoglycerate, NADH and hydrogen atoms. In the seventh step phosphoglycerate kinase transfers a phosphate group from 1,3-bisphosphoglycerate to ADP to form ATP and 3-phosphoglycerate. Since there are two molecules of 1,3-bisphosphoglycerate, this synthesis of ATP compensates the first two molecules of ATP consumed, resulting in a net of 0 ATP. The eighth step involves a simple rearrangement of the position of the phosphate group in the 3-phosphoglycerate molecule, making it 2-phosphoglycerate. The enzyme that catalyses this reaction is called phosphoglycerate mustase. The nineth step leads to the formation of phosphoenolpyruvic acid. This molecule results from the enolase work, by removing one water group from the 2-phosphoglycerate. In the final step the enzyme pyruvate kinase transfers a phosphate group from phosphoenolpyruvate to ADP resulting in pyruvic acid and ATP. Again, since there are two molecules of phosphoenolpyruvate, this step generates 2 ATP molecules. At the end of this pathway we have a net of +2 ATP. Once synthetized,

pyruvic acid can be converted into acetyl-CoA in a reaction called pyruvate decarboxylation. This reaction is catalysed by the enzyme pyruvate dehydrogenase. As the name of the reaction indicates, there is a release of CO_2. Since we have two molecules of pyruvic acid at the end of glycolysis, this reaction will produce two molecules of acetyl-CoA and two molecules of CO_2 (Cohen, 2014; Hatti-Kaul, 2016).

The EMP glycolysis constitutes the heterotrophic pathway that was addressed to perform the mixotophic fermentation.

1.2.3.3 Mixotrophy

Clostridia are able to perform both, heterotrophic and autotrophic metabolisms. However, these two pathways represent some constraints for the production of fermentation products. On the one hand, the heterotrophic metabolism in which clostridia cannot fix carbon and uses organic carbon for growth, leads to a loss of CO_2 during the Embden-Meyerhof-Parnas (EMP) glycolysis because of the decarboxylation of pyruvate to form acetyl-CoA. This loss of CO_2 results in a considerable drop of the maximum mass yield capacity. However, a positive ATP net is obtained at the end of this pathway (+2 ATP) (Fast & Papoutsakis, 2012; Fast et al., 2015; Jones et al., 2016). On the other hand, the autotrophic pathway can produce complex organic compounds through the fixation of CO_2 and H_2, as sole carbon and energy sources, respectively. But, for instance, the WLP, that is responsible for the fixation of CO_2 in acetogenic clostridia, besides the formation of acetyl-CoA, it is ATP consuming, making the cell growth limited. To create and maintain biomass, organisms that take use of the WLP must convert acetyl-coA into acetate, a process that generates ATP trough substrate-level phosphorylation, but deviates the carbon flux to a different metabolic direction than the one that allows butanol formation (Fast et al., 2015; Fontaine et al., 1942; Jones et al., 2016). Moreover, in order to generate ATP for the CO_2 fixation the organisms with this pathway can also utilize Rnf complexes and electron bifurcations. Even so, these ATP generating methods are unwanted for a fermentation process once they increase the cost and complexity of the bioprocess (Henstra et al., 2007; Liew et al., 2013; Müller, 2003; Ragsdale & Pierce, 2008; Tracy et al., 2012).

A very interesting and promising alternative to face the constraints associated with the heterotrophic and autotrophic metabolisms is the mixotrophic metabolism (Figure 4). In the present work, the anaerobic, non-photosynthetic (ANP) mixotrophic metabolism, a pathway that stoichiometrically converts sugars into acetyl-CoA, was the one exploited. Mixotrophy

is defined as the simultaneous utilization of organic compounds (like sugars) and inorganic compounds (like CO_2, CO and H_2) as substrate for growth and metabolism (Fast & Papoutsakis, 2012). As EMP glycolysis generates one molecule of CO_2 per molecule of acetyl-CoA and generates an excess of reducing equivalents, namely NAD(P)H + H^+, using the mixothrophic metabolism the cells can now direct these surplus compounds to enhance other metabolism with these compounds requirement. Taking in account the type of compounds released during the glycolysis, there is no better metabolism to couple with this than the WLP pathway. This carbon fixation pathway is particularly well-suited for mixotrophy because it exhibits a low ATP requirement, comparing with other carbon fixation pathways (Fast & Papoutsakis, 2012), and it requires the exact amount of NAD(P)H, generated through glycolysis, to fix two molecules of CO_2 into acetyl-CoA. Overall, glycolysis yields 2 mol of acetyl-CoA, 2 mol of ATP, 2 mol of CO_2 and 8 H^+/reducing equivalents from 1 mol of glucose. Without a mechanism for CO_2 reassimilation, the CO_2 is lost and a big part of the reducing equivalent pool is oxidized by hydrogenase activity to H_2. By the co-utilization of both metabolisms, the cells are able to produce 3 moles of acetyl-CoA using the surplus of the glycolysis, instead of producing 2 moles of acetyl-CoA trough glycolysis.

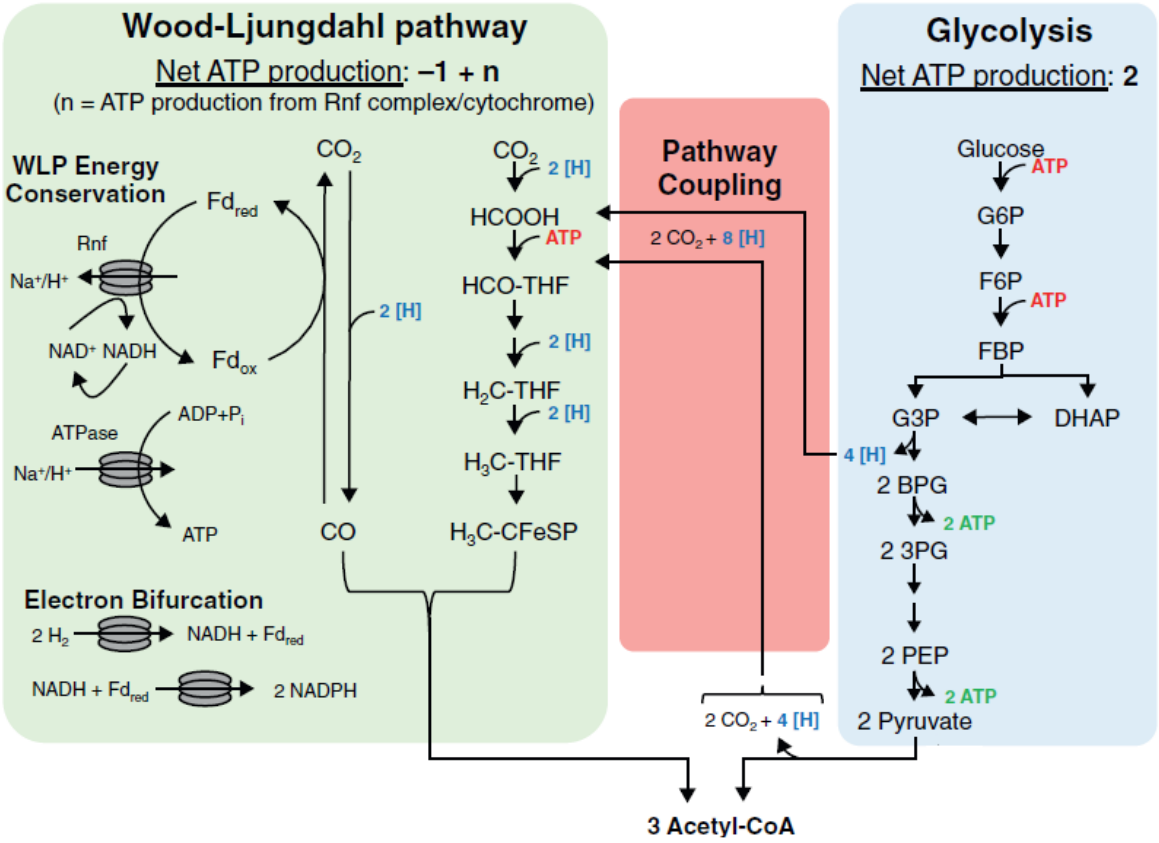


Figure 4 – Scheme for mixotrophic metabolic pathway and energy conservation in acetogenic clostridia, representing the pathway coupling between EMP glycolysis and WLP. Figure adapted from (Fast et al., 2015);

The degree of reduction of the desired product has a great influence on the amount of CO_2 reassimilated by the mixotrophic metabolism. For more reduced products, lesser amount of CO_2 can be fixed, because the reducing equivalents (like NAD(P)H + H^+) are directed towards product formation rather than CO_2 fixation. Through an H_2-enhanced mixotrophy (addition of H_2 to the fermentation medium), it is possible to overcome this reducing equivalents deficiency, as the H_2 molecules can be used to produce NAD(P)H by a hydrogenase activity. Since H_2 is an expensive compound, it is possible to use syngas to provide necessary reducing power and carbon. Syngas is an abbreviation for synthesis gas, which is a mixture comprising of carbon monoxide, carbon dioxide and hydrogen (Jones et al., 2016).

As ANP mixotrophic metabolism allows the production of great amounts of acetyl-CoA with the benefit of a positive net production of ATP, this metabolism is the best suited for biofuel production.

1.2.3.4 Fermentative alcohol production

After acetyl-CoA formation, the metabolic pathway must continue to generate the desired biofuels. Since the butanol production is the main goal of this work, it will focus on this metabolic pathway. The fermentative alcohol production is the metabolic approach most acceptable to explain butanol and ethanol production (Figure 5). While ethanol is produced in a two-step reduction, the synthesis of butanol requires a longer chain of reactions. Acetyl-CoA needs to be condensed to a second acetyl-CoA molecule by the activity of the thiolase enzyme, thereby forming acetoacetyl-CoA. Then, acetoacetyl-CoA is reduced to 3-hydroxyl-CoA, which in turn is dehydrated to form crotonyl-CoA that is reduced again to butyryl-CoA. Now, the butyryl-CoA can be converted into butanol by a bifunctional aldehyde/alcohol dehydrogenase (AdhE), with no ATP production. Another way to obtain butanol consists of the conversion of butyryl-CoA to butyrate, a step that generates ATP by substrate level phosphorylation, and the subsequent butyrate oxidation to butyryaldehyde by the activity of an aldehyde ferredoxin oxidoreductase (AOR) enzyme. Butyrylaldehyde is then reduced to butanol by an alcohol dehydrogenase. The butanol production via AOR activity has been suggested as an important step to fulfil the ATP requirements in acetogenic alcohol fermentation (Fast et al., 2015; Kopke et al., 2010).

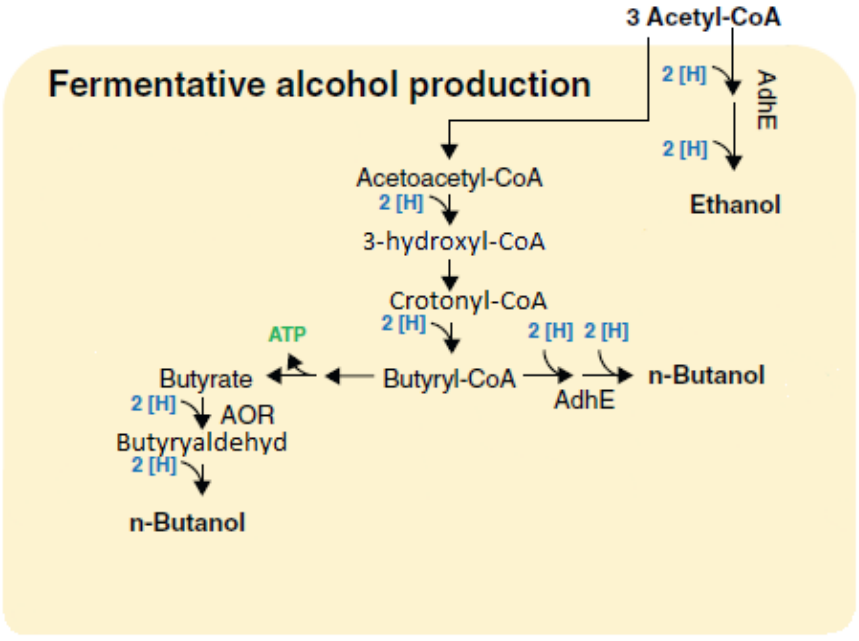


Figure 5 - Fermentative alcohol production metabolism after acetyl-CoA formation by a mixotrophic metabolic pathway, in acetogenic bacteria. Figure adapted from (Fast et al., 2015);

1.2.4 Butanol toxicity

The ratio of ABE in typical batch fermentation using *C. acetobutylicum* ATCC 824 is 3:6:1, with 20 g/L being the maximum ABE concentration. The same accounts for *C. beijerinckii* NCIMB 8052, with butanol being produced two times more than acetone and six times more than ethanol (Qureshi & Blaschek, 2001a). Therefore, butanol is considered as the only produced solvent that becomes toxic to the cells. Moreover, the lipophilic character of butanol makes it more toxic to the cells than the other two products. The production of butanol during the fermentation results in a negative effect for the cell membranes, which become more and more fluid. This increasing in the membrane fluidity leads to the disruption of the phospholipids components, that consequently causes the destabilization of membrane-associated functions such as transport processes, glucose uptake and membrane-bound ATPase activity (Bowles & Ellefson, 1985). It is known that clostridial metabolism ceases at a concentration of approximately 20 g/L of solvents (Woods, 1995) and a concentration of 0.1-0.15 M of butanol causes a 50 % inhibition of both cell growth and sugar uptake (Moreira et al., 1981). Shafiee & Topal, 2009, concluded that an addition of 7-13 g/L of butanol, to the fermentation medium, causes a 50% inhibition of growth, while for the other two products, ethanol and acetone, a concentration of 40 g/L is needed to generate the same effect.

There are some approaches being made to minimize butanol toxicity. The development of genetically modified strains, able to support bigger concentrations of butanol, is one of them. For instance, Qureshi & Blaschek, 2001b, developed a genetically modified strain by random mutagenesis, called *C. beijerinckii* BA101, derived from *C. beijerincki* NCIMB 8052, that has the advantage of resisting higher solvent concentrations. This strain is able to produce up to 33 g/L of total solvents with a ratio of ABE in the order of 3:16:1, which is considerably high when compared with the 20 g/L of total solvents produced by the wild strains, with an ABE ratio in the order of 3:6:1 (Formanek et al., 1997). Another approach is the integration of an *in situ* solvent recovery process with the fermentation. If the butanol is being retired from the fermentation medium while the fermentation is being processed, it allows the maintenance of a basal butanol concentration, supportable for the culture. It is expected that, by coupling the development of new genetically enhanced strains with an efficient butanol recovery process, it is possible to overcome the butanol toxicity problem (Qureshi & Blaschek, 2001a).

1.2.5 Butanol recovery techniques

A crucial problem in the butanol production is the product recovery. The most traditional technique to separate and obtain butanol is by distillation. However, there are different processes which are also of remarkable interest, such as pervaporation, adsorption, liquid-liquid extraction, gas-stripping and reverse osmosis. The distillation process has fallen into disuse due to the low concentrations of butanol in the fermentation broths, thereby requiring higher operation costs for product recovery (Dürre, 1998). Therefore, the use of different separation techniques to perform an *in situ* recovery of butanol has been a target of intensive research.

Pervaporation is a membrane-based process that allows selective removal of volatile compounds from the fermentation broth. This membrane is usually solid and it is in contact with the fermentation broth. The volatile liquids or solvents are then able to diffuse through this membrane, as a vapor, that is recovered posteriorly by condensation. Previous experiments already proved that the integration of this recovery system with the fermentation process is able to enhance the butanol productivity. Qureshi & Blaschek, 1999, experiment, with *C. beijerinckii* BA101, in which an integrated batch-pervaporation process was made, a two-fold increase in the total solvent concentration was achieved (from 24.2 g/L in the simple batch fermentation, to 51.5 g/L in batch-pervaporation fermentation). The recovery process conditions did not affect the growth of the culture and it required just one tenth of the energy used in the conventional distillation. The recovered solution, however, contained in addition to butanol some acetone and ethanol, what required an extra distillation process. The same process was also applied to a fed-batch reactor, in which a productivity of 165.1 g/L of total solvents was achieved (Qureshi & Blaschek, 2000a). A continuous-pervaporation fermentation, with *C. acetobutylicum*, achieved a productivity of 2.34 g/L/h (Izák, et al., 2008).

Adsorption is a simple technique that can be used to remove butanol from the fermentation broth energy efficiently. A diverse variety of materials can be used as adsorbents to butanol recovery, such as silicalite, activated charcoal, bone charcoal, active carbon, amberlite, polyvinylpyridine, among others. This kind of materials allow the formation of a film of alcohols on their surfaces when they enter in contact with the fermentation broth. After the adsorption of the alcohols they are desorbed by heat treatment, or displacers, to give concentrated butanol solutions as final products. Activate carbon and bone charcoal were

shown to be the most capable adsorbents to butanol recovery, reaching 252 and 206 mg of butanol per g of the adsorbent, respectively (Qureshi et al., 2005).

Liquid-liquid extraction is based on utilizing two immiscible phases in which butanol diffuses from the aqueous phase (fermentation broth) to the organic phase, whose composition allows an easier solubilisation of butanol. Butanol is then selectively concentrated in the organic phase. The problem of this recovery process is the toxicity that the extractant can have on the culture. By using a membrane that allows a surface for butanol exchange between the two immiscible phases, it is possible to avoid the toxicity of the extractant and emulsion formation. Using this kind of recovery system integrated in a continuous fermentation with *C. acetobutylicum*, a solvent productivity of 3.08 g/L/h was obtained utilizing n-decanol as extractant (Eckert & Schiigerl, 1987).

Gas stripping is a simple and efficient butanol recovery technique, that relies on bubbling gas through the fermentation broth. The gas sustains some of the solvents in the medium which are recovered when it reaches a condenser. After the condenser, this gas can be recycled back to the fermentor and the process continues until all the sugar in the fermentation broth is consumed. In this way, it is possible to obtain high sugar consumptions, a reduction in butanol inhibition and it allows the use of concentrated sugar solutions to perform the fermentation (Maddox et al., 1995; Qureshi & Blaschek, 2000b). Ezeji et al., 2003, applied gas stripping technique to a batch fermentation with *C. beijerinckii* BA101 and they obtained 75.9 g/L of total solvent production. Ezeji et al., 2004, did the same experiment in a fed-batch fermentation and a productivity of 1.16 g/L/h was achieved. When they integrate the same process with a continuous fermentation, the productivity reached the 0.91 g/L/h of total solvents.

Reverse osmosis is another recovery technique that relies on membranes. This technique requires the removal of suspended vegetative organisms, for instance, using a hollow-fiber ultrafilter, before the reverse osmosis is carried out. Then, reverse osmosis starts by rejecting solvents but allowing water to pass through the membrane. The products are concentrated in the membrane and the volume of liquid to be distillated is dramatically reduced. It is reported that polyamide membranes exhibit rejection rates as high as 98%, and the optimum rejection of butanol in the fermentation liquor occurs at recoveries of 20-45% (Garcia III et al., 1986).

From an economic point of view, reverse osmosis is the most preferable recovery method, but it has the disadvantage of membrane clogging and fouling. In turn, liquid-liquid

extraction has a higher capacity and selectivity but it is expensive to utilize. Basically, all the recovery methods have their advantages and disadvantages, that is why they need to be optimized for a cost-competitive biofuel production (Dürre, 1998). It is important to understand that these recovery methods are deeply dependent on the fermentation performance and strain characteristics. If the strain is able to produce butanol with just trace quantities of ethanol and acetone, it will significantly simplify the recovery process. The same accounts if the strain is more tolerable to the solvents. In this case, a higher butanol concentration can be achieved and it also facilitates the recovery process. In conclusion, to maximum optimize the fermentation, the overall process must be considered, since the strain development to the downstream processes (Dürre, 1998).

1.2.6 Fermentation techniques

The bioindustry has been choosing to work with batch reactors, as they are simple to operate and they have a reduced risk of contamination. A disadvantage of batch fermentations is the low productivity due to the lag phase, product inhibition and all the time that they need to be stopped for cleaning, sterilization and filling purposes. For these reasons, the continuous culture reactors and the fed-batch reactors started to be an alternative option. On the one hand, the continuous culture reactors can eliminate the problems with the lag phase and preparation time, however, a single-stage continuous fermentation is not feasible because of the complexity of butanol production in clostridia. On the other hand, the fed-batch reactors can be useful to overcome the problems with the substrate inhibition and increase cell growth (Li et al., 2011). Different approaches like immobilized cell reactors (Qureshi & Blaschek, 2000b; Qureshi & Blaschek, 2001a) and cell recycle reactors (Pierrot et al., 1986) are also been applied to butanol production, with the purpose of increasing the productivity.

1.3 Why choose this type of fermentation process?

1.3.1 Advantages of the mixotrophic fermentation for butanol production

In a broad point of view, comparing the mixotrophic metabolism with other well-known and utilized metabolisms to perform fermentations, it is easy to highlight some interesting advantages. Looking at some of the requirements for acetyl-CoA production, such as net ATP and CO_2 evolution or consumption, we can see considerable differences between glycolysis, WLP, non-oxidative glycolysis (NOG) and mixotrophy metabolisms (Table 2). NOG is a synthetic metabolic pathway, design with the purpose of eliminate the carbon loss during the classical EMP glycolysis. This is a non-oxidative, cyclic pathway that allows the production of stoichiometric amounts of C2 metabolites from hexose, pentose and triose phosphates without carbon loss (Bogorad et al., 2013). Taking into account that an ideal fuel production should maximize acetyl-CoA formation, minimize CO_2 evolution and be able to generate enough ATP for the creation and maintenance of biomass, mixotrophic metabolism is certainly the closest to these desired characteristics. While there are pathways involving CO_2 production (glycolysis) and ATP consumption (WLP and NOG), the mixotrophic pathway is the only one that produces large amounts of acetyl-CoA (3 mol per mol of hexose consumed), does not produce CO_2 and has a positive net ATP. Moreover, to fulfill the ATP requirements, NOG and WLP metabolisms need to produce acetate or, regarding the WLP, utilize the energy conserving membrane reactions (Fast et al., 2015).

Table 2 - Analysis of four different metabolic pathways for fuel production. Glycolysis refers to EMP glycolysis, WLP refers to Wood– Ljungdahl pathway of carbon fixation, NOG refers to non-oxidative glycolysis and mixotrophic to the ANP mixotrophy. Table adapted from (Fast et al., 2015);

	Feedstock	Net ATP	CO_2 evolved	Acetyl-CoA
Glycolysis	Hexose	2	2	2
WLP	$CO_2 + H_2$	Less than 1	−2	1
Mixotrophic	Hexose	1	0	3
NOG	Hexose	−1	0	3

Previous studies were already made (Fast & Papoutsakis, 2012; Fast et al., 2015) in order to create a stoichiometric reliable model to allow the comparison between the natural carbon fixation pathways, for biofuel and biochemistry production, under autotrophic, heterotrophic

and mixotrophic conditions (Table 3). To perform this kind of analyses it is first necessary to develop the stoichiometric model for each pathway. The heterotrophic pathway, for which just glucose was added as substrate, was designed according to the EMP glycolysis model. The autotrophic pathway, for which the substrate was given as three different mixes of CO_2 and H_2, was based on the WLP. For the ANP mixotrophic pathway two different types of substrate were considered, one consisting of glucose and CO_2 and the second consisting of glucose, CO_2 and H_2. The stoichiometric models were built towards acetyl-CoA production and considering NAD(P)H as reducing equivalent.

Table 3 - Biofuel and biochemical production, under autotrophic, heterotrophic and mixotrophic conditions based on a stoichiometric model for acetogenic bacterial metabolism. Table adapted from (Fast et al., 2015);

Stoichiometric calculations to determine the maximum yield of *n*-butanol using glycolysis, the WLP, and ANP mixotrophic fermentation

Substrate or metabolite	Heterotrophic	Autotrophic			Limited mixotrophic	Supplemented mixotrophic
	Hexose	Gas (0.4 ATP)	Gas (0.7 ATP)	Gas (1 ATP)	Hexose + gas evolved	Hexose + gas evolved + H_2
ATP cons.	N/A	0.4	0.7	1	0.4	0.4
Substrate						
CO_2	200.0	−320.7	−282.0	−249.7	193.0	−11.2
H_2	21.0	−700.0	−700.0	−700.0	0.0	−600.0
Hexose	−100.0	0.0	0.0	0.0	−100.0	−100.0
Metabolite						
Cell	30.0	8.0	7.0	6.2	30.2	35.3
ATP	80.0	0.0	0.0	0.0	77.2	0.0
Acetate	0.0	128.3	70.5	25.2	0.0	4.5
n-BuOH	92.5	14.0	33.5	48.7	94.2	141.7
Output						
Carbon effic.	61.7%	17.5%	47.5%	78.0%	76.0%	94.5%
n-BuOH mass yield	38.1%	7.4%	20.0%	32.8%	38.8%	58.3%
Hydrogen effic.[a]	N/A	2.0%	4.8%	7.0%	N/A	8.2%

[a] Hydrogen efficiency is calculated as the ratio of butanol produced per mole hydrogen for autotrophic cases (mol butanol/mol H_2) and as additional moles butanol produced per mole hydrogen for the supplemented mixotrophic case ((mol $butanol_{supp\ mixo}$ − mol $butanol_{hetero}$)/mol H_2).

According to the estimations suggested in table 3, H_2-supplemented mixotrophic fermentations have the greatest potential for the production of butanol and also achieve the highest biomass concentration. As mentioned before, if the intended product has a high degree of reduction, as it is the case for butanol, a higher amount of reducing equivalents is needed. In the mixotrophic metabolism, the reducing equivalents are produced by the hydrogenase reaction in the presence of hydrogen. In the absence of hydrogen, no additional reducing equivalents are produced and they end up being wasted during product formation, becoming unavailable for CO_2 fixation. This is the reason why the limited mixotrophic fermentation exhibits results so similar to the heterotrophic fermentation. The autotrophic fermentation proved to be the less efficient one, with a large amount the carbon being directed to the acetate formation to compensate the low net ATP production.

In the H_2-enhanced mixotrophic fermentation, all carbons from hexose can be converted theoretically into butanol. It results in a 53% increase over what is possible in EMP glycolysis alone. Considering that the sugar costs represent approximately 50% of the OPEX for biofuel production, the 53% increase in mass yield decreases the total OPEX by more than 17% (Fast et al., 2015; Gnansounou & Dauriat, 2010). Considering that the OPEX for butanol production ranges between 0.82 – 1.37 €/Kg (0.82 €/Kg in plants that are annexed to already made facilities, in which butanol is obtained by membrane recovery techniques and 1.37 €/Kg in plants dedicated just to butanol production, using wheat straw as substrate and distillation as butanol recovery technique) (Clifford, 2016c), with the H_2-enhanced mixotrophic fermentation it is possible to decrease these costs to a range of 0.62 – 1.05 €/Kg.

Table 4 - Comparison between chemoorganotrophic growth and product formation of *Acetobacterium woodii*, under an N_2-atmosphere and mixotrophic growth under N_2/CO_2-atmosphere. Table adapted from (Braun & Gottschalk, 1981);

Chemoorganotrophic growth (under N2-atmosphere)			
Substrate	**Final OD (600nm)**	**Substrate consumed (nmol/culture)**	**Product formation / Acetate (mmol/culture)**
D-Fructose	0,80	0,50	1,30
D-Glucose	0,95	0,50	1,14
L-Lactate	0,47	0,96	1,20

Mixotrophic growth (under H2/CO2 -atmosphere)				
Substrate	**Final OD (600nm)**	**Substrate consumed (nmol/culture)**		**Product formation / Acetate (mmol/culture)**
		Organic compound	**H2 + CO2**	
D-Fructose	1,30	0,50	7,37	2,32
D-Glucose	1,30	0,51	13,30	3,04
L-Lactate	0,87	0,93	12,00	2,96

Braun & Gottschalk, 1981, work is a great example of the advantages of mixotrophic fermentations, especially for the growth and product formation of acetogenic bacteria. In this work, the acetogenic organism *Acetobacterium woodii* was used to study the product formation and growth capacity under chemoorganotrophic metabolism, using three different substrates (D-fructose, D-glucose and L-lactate), and under mixotrophic metabolism using the same substrates in the presence of H_2 + CO_2 (Table 4). The acetate formed was used as indicator for product formation and it can be compared with alcohol formation, as both derive

from acetyl-CoA. Looking in table 3, it is apparent that the final optical density of the culture was considerably higher, as well as the amount of acetate, during mixotrophic conditions when compared to heterotrophic conditions. As no diauxic growth was observed, it can mean that the organic and inorganic substrates were consumed simultaneously.

The CO2 that is formed during glycolysis should be totally fixed by the WLP in order to maximally optimize the mixotrophic conversion of substrate into products. Nagarajan et al., 2013, work with *C.ljungdahlii* used the stoichiometric metabolic model that is mentioned in this chapter, to predict the amount of CO_2 that is generated during the glycolysis and the amount of CO_2 that is captured during the CO_2 fixation metabolism. Considering an experimentally determined 1.9 mmol/gDW/h uptake of fructose, the stoichiometric model was used to predict the CO_2 flow. Accordingly to the model, 3.8 mmol/gDW/h of CO_2 are released during fermentation and a maximum CO_2 uptake rate of 10 mmol/gDW/h was determined. Therefore, all the CO_2 formed during the heterotrophic metabolism is completely used by the autotrophic one, allowing an efficient mixotrophic metabolism with feasible growth rates. However, it is just a prediction based on the stoichiometric model and it was not experimentally proven.

1.3.2 Viability of the mixotrophic metabolism in acetogenic bacteria

There are some concerns related with the viability of the mixotrophic metabolism that should be reviewed:

1) Carbon catabolic repression (CCR), could be a problem once we are dealing with two different substrates. CCR is defined as a regulatory mechanism in which, in the presence of different substrates, the organisms trend to preferentially consume the one that is easier to metabolize than the other secondary sources of carbon. This mechanism results from a downregulation of the genes involved in the consumption of the carbon sources considered secondary (Stulke & Hillen, 1999). CCR started to be noticed between different organic substrates, such as different sugars. Grimmler et al., 2010, study, with *Clostridium acetobutylicum*, demonstrated a CCR in a medium containing D-glucose and D-xylose. D-glucose was the preferred carbon source, that was exclusively utilized until its complete consumption from the medium. After that, a short lag phase occurred (characteristic for diauxic growth) and the D-xylose started to be consumed. For the ANP

mixotrophic metabolism, the co-utilization of carbohydrates and gas may also stand in conflict to each other resulting in a different regulation of the genes involved (Tan et al., 2013). According to previous works, the regulation of the WLP genes depend on the species of bacteria and culture conditions. To study CCR in acetogens, Loubière et al., 1992, performed an experiment where the acetogenic bacteria *Eubacterium limosum* was exposed to an equimolar carbon mixture of glucose and methanol (50 mM methanol and 8.3 mM glucose). During the first hours, solely glucose was consumed, with growth rates identical to normal growth on glucose. After the decreasing of glucose concentration to 6 mM, the culture started a short period of mixotrophy with the glucose and methanol being consumed simultaneously and the growth rate reaching is highest level. When the glucose was completely consumed, the culture underwent a lag phase for 12 h after which the growth restarted with the consumption of the rest of methanol, although at lower rates than the consumption of glucose. Despite of the CCR mechanisms observed in many acetogenic species (Braun & Gottschalk, 1981; Liu et al., 2015), accompanied by the downregulation of WLP genes in the presence of sugars, there are other examples that demonstrate the ability to utilize both sugars and gas, simultaneously. At Jones et al., 2016, experiment, *Clostridium ljungdahlii* was grown on C^{12}-fructose and C^{13}-labelled syngas mixture. Based on the carbon analysis of the metabolites produced, it was possible to quantify how much product derived from the sugars and how much from the syngas. Since the beginning of the fermentation (t=24h), the majority (between 70 and 80 %) of the acetate formed derived from the labelled syngas rather than fructose and no alterations in the expression or translation of the WLP genes were noticed when compared to the autotrophic control. The same experiment was done with *Clostridium autoethanogenum* and a high degree of C^{13}-labelled acetate was found as well (51-58%), supporting the idea that for both strains, glycolysis and the WLP operate simultaneously without CCR. Experiments with different organisms (*Clostridium aceticum* and *Acetobacterium woodii*) affirm the absence of CCR mechanisms in acetogenic bacteria when grown under mixotrophic conditions (Braun & Gottschalk, 1981). Because there are studies that either confirm the existence of CCR, under mixotrophic conditions, either affirm its absence, this phenomenon must be considered as a possible concern;

2) The balances between the CO_2 uptake rate of the WLP in comparison with the amount of CO_2 produced during glycolysis. If the kinetics of these two pathways are incompatible, there will be a release of CO_2 that could be utilized to enhance the production of the product of interest. It was already showed that if we follow the stoichiometric model presented by Fast & Papoutsakis, 2012, and Fast et al., 2015, considering an experimentally determined 1.9 mmol/gDW/h uptake of fructose, all CO_2 produced by glycolysis is eventually utilized by the WLP. However, this is just a prediction based on the stoichiometric model and it was not experimentally proven, reason why we still should consider it a possible concern;

3) The amount of ATP and reducing equivalents that can be produced during the mixotrophic metabolism, which are essential for the biosynthesis of fermentation products other than acetate (Fast et al., 2015). As already stated, the ATP and reducing equivalents required to produce fermentation products are essential to obtain good yields. It was also stated that, the higher the degree of reduction of the desired product is, the more reducing equivalents are required. Therefore, the medium conditions have a decisive role in the energy conservation complex. For instance, butanol is a highly reduced product that requires sufficient reducing equivalents to be formed, namely a 2:1 NAD(P)H to acetyl-CoA ratio. To provide this necessity, H_2 can be added to the medium in order to generate higher amounts of reducing equivalents via the hydrogenase-catalyzed reaction;

2. Objectives

The present experiment has a main objective from which secondary objectives derived according to the different discoveries that came out during the experimental work. This was the first time that this kind of experiment was made with *Clostridium beijerinckii* NCIMB 8052 and obstacles that were unknown in the beginning started to arise along the experiment, leading to new secondary objectives mandatory to achieve the primary one.

Primary objective: Enable the ANP mixotrophic metabolism in *Clostridium beijerinckii* NCIMB 8052 with the purpose of increasing the butanol productivity;

Secondary objectives:

1. Evaluation of the natural capability of *C. beijerinckii* NCIMB 8052 to perform the ANP mixotrophic metabolism;

2. Gathering of butanol production values, as well as for other relevant fermentation products, to construct a productivity database, concerning the native strain (*C. beijerinckii* NCIMB 8052), to further comparison with a possible genetic modified strain;

3. Characterization of the genes and enzymes involved in the WLP using the *Clostridium autoethanogenum* DSM 10061 genome;

4. BLASTn researches between the WLP genes of *C. autoethanogenum* DSM 10061 and the *C. beijerinckii* NCIMB 8052 genome, to understand if all the WLP genes have homologous pairs in *C. beijerinckii* genome;

5. Selection of relevant genes to transform into *C. beijerinckii* NCIMB 8052 and enable the WLP;

6. Selection of a suitable promoter and terminator genes for overexpression of the genes of interest in *C. beijerinckii* NCIMB 8052;

7. Primers design for amplification of all the genes considered;

8. Isolation of all the genes of interest;

9. Construction of plasmids containing the genes of interest and all the genetic support necessary to express them;

10. Transformation of the constructed plasmids into *C. beijerinckii* NCIMB 8052;

11. Evaluation of the plasmids overexpression effects in the butanol productivity of *C. beijerinckii* NCIMB 8052, by comparison with the productivity database constructed in point 2;

3.Materials and Methods

The experiment was divided in 4 main steps that followed the first month of theoretical and practical background apprenticeship:

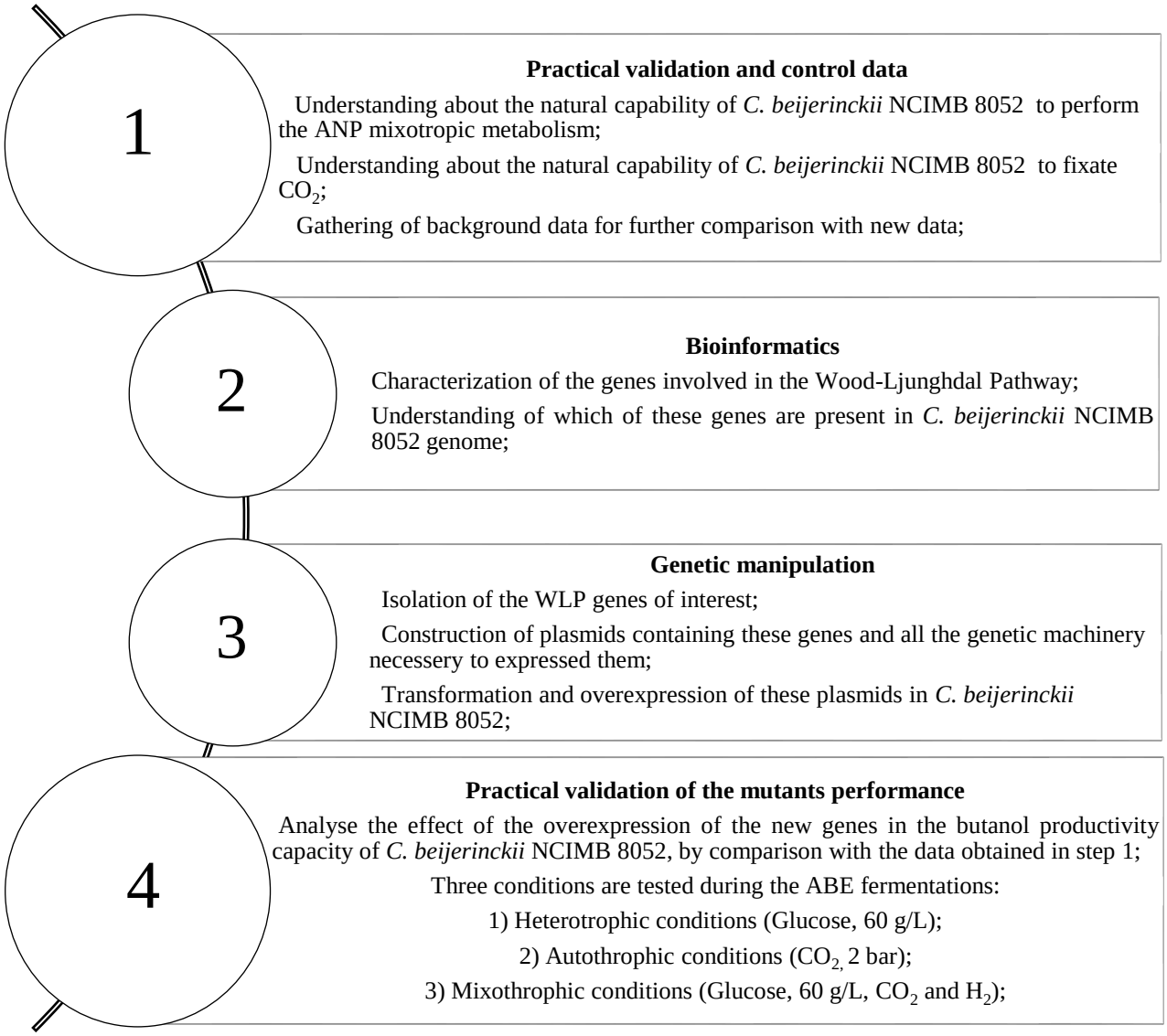


Figure 6 – Schematic illustration of the overall experimental structure traced for the present study;

Step 1 – Practical validation and control data;

It was already known that *C. beijerinckii* NCIMB 8052 was not able to perform the Wood-Ljunghdal pathway. However, it was uncertain if other carbon dioxide fixation pathways were present. To evaluate the capability of *C. beijerinckii* NCIMB 8052 to fixate carbon dioxide and to practically validate its incapability to perform the ANP mixotrophic metabolism, fermentations at different conditions were performed and monitored. Different parameters were assessed along the fermentations, namely the pH, the OD (600nm) and the concentration of substrates and products considered as relevant for an ABE fermentation monitoring. This step allowed the construction of a control database for further comparison with new data.

Strain: *Clostridium beijerinckii* NCIMB 8052 were stored as spore suspension in 15% glycerol solution at -20ºC. Spore suspensions were heat shocked 1 min at 95ºC, prior to inoculation in liquid medium.

Media and growth conditions: For fermentation assays, 1 mL of the heat shocked spore solution of *Clostridium beijerinckii* NCIMB 8052 were grown overnight in 50 mL liquid CM2 medium (Appendices 8.1). Fermentations were performed in 50 mL working volume of CM2 medium in pressure resistant Schott bottles of 250 mL. All the mediums, including the one for growing *Clostridium beijerinckii* NCIMB 8052, were sterilized by autoclavation and made anaerobic by flushing with nitrogen gas for 30 min. All the clostridial culture fermentations were performed at 37ºC and anaerobically. Different conditions were tested in each fermentation, namely: i) heterotrophic conditions, the CM2 medium was supplemented with glucose, 60g/L; ii) autotrophic conditions, the medium was just supplemented with 2 bar of CO_2 or with a mixture H_2/CO_2; iii) mixotrophic conditions, the medium was supplemented with glucose together with CO_2 or a CO_2/H_2 mixture. Fermentations supplemented with hydrogen were always performed inside an anaerobic chamber (Sheldon Manufacturing, Oregon USA; gas mixture consisting of 15% CO_2, 5% H_2 and 80% N_2). All the fermentation conditions were tested with and without shaking (50 and 0rpm, respectively), except the conditions with H_2 that were always performed without shaking. The fermentations were run for 6 days.

Sampling: At different times along the fermentations, samples of 1.5 mL were taken from the fermentation broth. The first samples were always collected at the same time as the inoculation and six hours afterwards. After the first day of fermentation one sample per day was taken. The pH and the optical density at 600nm were measured right after each sampling. For further substrate and products quantification, the samples were centrifuged for 5 min at 14000 rpm and the respective supernatants collected and stored at -20ºC. The previous centrifugation is advisable prior HPLC analysis, to avoid the clogging of the column. At the end of the 6 days of fermentation all the stored samples were analysed by HPLC (Shodex KC-811 column, refractive index detector, 0.003M H_2SO_4 as mobile phase, 10% IPA as seal wash solution, mili-Q water as needle wash solution and 250 mM valeric acid in 1M H_2SO_4 as internal standard solution) to quantify substrates and products considered as relevant for a proper monitoring of an ABE fermentation. The glucose consumption, as well as the production of acetic acid, butyric acid, propionic acid, acetone, butanol and ethanol, were quantified. For fermentations supplemented with both glucose and CO_2, or just with CO_2, measurements of the pressure inside the fermentation bottles were also made together with the sampling.

Step 2 - Bioinformatics;

Once confirmed the absence of a carbon dioxide fixation pathway in *C. beijerinckii* NCIMB 8052, it was mandatory to evaluate if all the genes responsible for the WLP were present in its genome. First, it was necessary to identify all the genes that are possibly involved in the WLP. For that purpose, *Clostridium autoethanogenum* DSM 10061, a clostridial strain known to perform the WLP, was the model organism used to find out which genes are responsible for this metabolism. Based on the articles Liew et al., 2016 and Brown et al., 2014, and after bioinformatic searches on KEGG and MaGe platforms, a table with all the essential genes for the WLP was constructed. A BLASTn research was then made, between the considered genes and the *C. beijerinckii* NCIMB 8052 genome. Based on the BLASTn research results, a group of genes was selected as promising to enable the WLP in *C. beijerinckii* NCIMB 8052.

Bioinformatics: The WLP genes were firstly identified using the KEGG platform. It contains all the *C. autoethanogenum* DSM 10061 carbon metabolism genes and enzymes, identified

and associated with the respective metabolic steps. To confirm if the previously selected genes were enough to support the entire WLP, the articles Liew et al., 2016 and Brown et al., 2014 were accessed. These publications describe the *C. autoethanogenum* DSM 10061 genome fraction that encodes the WLP and they were useful to validate and complete the group of genes previously catalogued. The GeneBank database and the MaGe platform, which contain the entire *C. autoethanogenum* DSM 10061 genome, were then used to obtain the FASTA sequences for all the selected genes. Using the BLASTn tool from the NCIMB website, local alignments between the FASTA sequences and the *C. beijerinckii* NCIMB 8052 genome were performed. When the BLATSn alignments presented statistical significance ($E < e^{-10}$ and positive percentage above 50%), the respective genes were considered to have a homologous correspondent in *C. beijerinckii* NCIMB 8052 genome, while the other ones were considered as missing genes.

Step 3 – Genetic manipulation;

According to the previous chapter, there are some WLP genes missing from *C. beijerinckii* NCIMB 8052 genome. These genes were accepted as promising for the WLP activation. Therefore, the next step encompassed the isolation, insertion into a plasmid and transformation of these genes into *C. beijerinckii* NCIMB 8052. A promoter and terminator had to be selected to allow the proper overexpression of the genes of interest once inside the cells. As the genes must be expressed within *C. beijerinckii* NCIMB 8052, the promoter and terminator must belong to its genome and allow the constitutive transcription of the genes of interest at a relative constant level, regardless of the cell environmental conditions. For this reason, the promoter and terminator from the C. *beijerinckii* NCIMB 8052 thiolase gene (Cbei_0411) were selected as the most suitable.

Primers design: Primers were needed to amplify all the genes of interest, including the promoter and terminator. The SerialCloner 2.6.1 was the program used to design the primers. Prior to the primers design, the pEC500E was chosen as the vector in which the genes of interest were going to be inserted. Then, the primers were designed with 20bp complementary regions to the genomic sequences where the primers must attach and additional 20bp overhangs were added to create homologous sequences between PCR products and between the promoter/terminator with the vector. The 20bp overhangs were designed in such a way that just allowed the linkage between genes in the desired order. Within the overhangs,

specific restriction sites were added to allow the replacing of the different genes, once they are linked to each other and to the vector. The primers designed are listed in table 5.

Table 5 - Description of all the designed primers. The name of the primers describes the sequence from the left to the right. The first part corresponds to the primers overhangs, with the name of the sequence to which the overhangs must attach, after it comes the name of the restriction enzyme that cuts in the specific site added between the overhangs and the primers (represented in small letters) and, at the end, the name of the gene to which the primers should anneal, followed by the letter F or R depending if they are forward or reverse primers. The P1 and P2 correspond to the pEC500E region 1 and 2, respectively.

Name	Sequence	Lenght [nt]	DNA target
P1_SphI_thlP_F	CGACGTTGTAAAACGACGGCgcatgcTAATAAAAGGTATAATTTAG	46	thlP
CAETHG_1608-12_XmaI_thlP_R	AGCTATTTTATATCCCATcccgggGTTTGACCTCCTAAAATTTTATAG	48	thlP
thlP_XmaI_CAETHG_1608-12_F	AAAATTTTAGGAGGTCAAACcccgggATGGGATATAAAATAGCTGTAGC	49	CAETHG_1608-12
thlT_NotI_CAETHG_1608-12_R	TCTTAATTTATAgcggccgcCTACATTATTGGATCCATCTTTAATG	46	CAETHG_1608-12
CAETHG_1608-12_NotI_thlT_F	TGTAGgcggccgcTATAAATTAAGATTTAAAAAGG	35	thlT
P2_XhoI_thlT_R	GCGGATAACAATTTCACACActcagaATACATGCATTTCTATTTTCTTC	49	thlT
thlP_XmaI_CAETHG_1620-21_F	AAAATTTTAGGAGGTCAAACcccgggATGGAAGAAAAAGCAAAATC	46	CAETHG_1620-21
thlT_NotI_CAETHG_1620-21_R	TCTTAATTTATAgcggccgcTTAGATACCTAATTTTTTACGTTTTTC	47	CAETHG_1620-21
thlP_XmaI_CAETHG_3005_F	AAAATTTTAGGAGGTCAAACcccgggATGTCAAATAACAAAATTTG	46	CAETHG_3005
thlT_NotI_CAETHG_3005_R	TCTTAATTTATAgcggccgcTTAATTAAGACCTAAGCTTTTCCTTC	46	CAETHG_3005
thlP_XmaI_CAETHG_3899_F	AAAATTTTAGGAGGTCAAACcccgggATGAGTCAAACTACACTAG	45	CAETHG_3899
thlT_NotI_CAETHG_3899_R	TCTTAATTTATAgcggccgcTTATAACCCCAAACCCTCTCTTTTAC	46	CAETHG_3899

The primers were designed in order to construct a plasmid with the following characteristics:

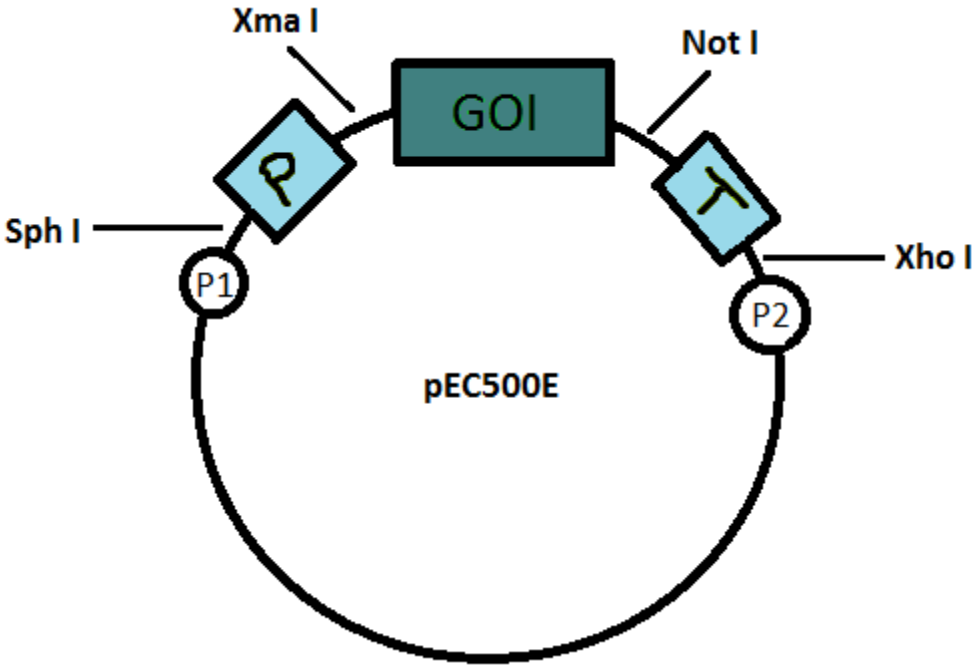


Figure 7 – Illustration of how the pEC500E plasmid looks, after the insertion of the genes of interest for which the primers were designed. The P1 and P2 regions correspond to the pEC500E ends, formed after the pEC500E digestion with the enzymes SphI and XhoI and to which the promoter and terminator sequences should ligate, respectively. The P represents the promoter sequence, the T the terminator sequence and the GOI is the abbreviation for genes of interest;

Once this plasmid is constructed, by making a restriction digestion with XmaI and NotI, it is possible to remove the gene of interest (GOI) from the plasmid and easily replace it for a different gene of interest. The same applies to the promoter and terminator that can also be replaced, but in these cases using different restriction enzymes.

Genome extraction: The *C. autoethanogenum* DSM 10061 genome, from where the selected genes of interest derive from, and the C. *beijerinckii* NCIMB 8052 genome, where the thiolase gene promoter and terminator are present, had to be fully extracted from the cells. C. *beijerinckii* NCIMB 8052 heat shocked spore solution were grown overnight in liquid CM2 medium. *C. autoethanogenum* DSM 10061, also heat shocked from a stored spore suspension in 15% glycerol solution at -20ºC, were grown for 3 days in DSM 10061 liquid medium (Appendices 8.1). The cells were then lysed by incubation at 99ºC during 10 minutes and the respective genomes were extracted using the Genelute Bacterial Genomic DNA Kit (SIGMA-AlDRICH), within which the protocol for Gram-Positive bacteria was followed.

PCR for amplification of the genes of interest: All the genes of interest were initially amplified by PCR using the Q5 High-Fidelity DNA polymerase master mix, the respective primers and the *C. autoethanogenum* DSM 10061 genome as DNA template. Three different PCRs were made considering the difference between the annealing temperatures of the primers and the elongation times needed to the DNA polymerase to amplify the desired sequences in all their extensions.

Touchdown PCR for the promoter and terminator amplification: To increase the specificity of the PCR reaction and avoid non-specific sequences to contaminate our PCR product solutions, a Touchdown PCR was done to amplify the promoter and terminator. A Q5 High-Fidelity DNA polymerase master mix was used together with the primers and the C. *beijerinckii* NCIMB 8052 genome as DNA template. The earliest steps of the Touchdown PCR had high annealing temperatures (56ºC) that decreased in increments for every subsequent set of cycles (-1ºC / cycle). The annealing temperature decreased during 10 cycles (reaching 46ºC) and maintained at this value during 25 additional cycles. The high annealing temperatures at the beginning of the PCR reaction allows the least-tolerant annealing to non-specific regions of the genome. Thus, the first sequence amplified will most likely be the sequence of interest. These sequences will then be further amplified during subsequent rounds of lower temperatures, excluding the competition with non-specific sequences to which the primers may bind at lower temperatures. The specificity increases during the reactions at higher temperatures and the efficiency increases along the cycles by lowering the annealing temperatures.

PCR products purification: An aliquot of the PCR products was initially loaded onto an electrophoresis gel (1% Agarose) and migrated at 80V for 60 min, to confirm that all PCR products corresponded to the desired fragments. Once confirmed, each PCR product was again loaded and run in separated electrophoresis gels to avoid cross contamination by different fragments and allow a clean gel extraction of the same. For gel extraction, the clearer and migrated the band of the desired product is, easier is the isolation of that specific region from the gel. To obtain an improved separation of the PCR products, the agarose content of the gel must be less than 1% (0.8%) for bigger PCR products and more than 1% for smaller ones (1.2%). The voltage and migration time are as well very important for the gel extraction, high voltages can burn the gel and affect the DNA quality and too long running's lead to the solubilization of the DNA in the Tris-acetate-EDTA (TAE) buffer.

When the electrophoresis finished, the regions of the gels that contained the fragments of interest were cut out over a UV lamp as quick as possible to avoid DNA mutations due to UV exposure. The Gene JET Gel Extraction Kit (Thermo Fisher Scientific) was then used to extract and purify the DNA from the pieces of gel cut previously. Mili-Q water was used as an alternative to the elution buffer that came with the kit. At the end, the concentration of DNA in the gel purified solutions was measured using a NanoDrop Lite Spectrophotometer (Thermo Scientific). To concentrate the purified solutions, a CentriVap Micro IR Vacuum Centrifugal Concentrator (Cole-Parmer) was used for 30min, at 60ºC with infrared radiation (IR).

Cloning attempt 1

Restriction ligation: Restriction enzymes can generate a wide variety of ends in the DNA they digest. Certain enzymes generate single-stranded overhangs, called sticky or cohesive ends, that can anneal to other compatible ends and become ligated in a sticky-end ligation. When the same restriction enzyme is used to cut two different DNA fragments, it generates ends that are compatible with each other and allows the linkage between the different DNA fragments. To apply this technique in the construction of a vector containing the promoter, gene of interest and terminator in this precise order, firstly the vector pEC500E needed to be digested to become a linear DNA sequence with sticky ends to which the promoter and terminator can be linked. The pEC500E was digested, at the multiple cloning site (MCS), by the restriction enzymes SphI and XhoI (the use of two different restriction enzymes avoid the self-ligation of the vector after digestion). The digestion was made in CutSmart buffer, at 37ºC during 60 min, after which the restriction enzymes were inactivated at 65ºC during 20 min. To remove from the solution the 14bp fragments that were cut out from the plasmid, the Gene JET Gel Extraction Kit, that purifies DNA fragments from 25bp to 20kb in size, was used to retain the digested plasmid and discard the 14bp fragments. The promoter gene was then digested, at the same conditions than the pEC500E, but by the restriction enzymes SphI (to allow the linkage between the promoter and the pEC500E) and XmaI (for the linkage between the promoter and the gene of interest). The restriction enzymes were as well inactivated at 65ºC for 20 min. The terminator digestion was the next step. For this case, the reaction was made in NEBuffer 3.1, using the enzyme NotI (that allows the linkage between the terminator and the gene of interest) and the enzyme XhoI (for the linkage between terminator and pEC500E), at 37ºC for 60 min, followed by the enzymatic inactivation at 65ºC

for 20 min. The genes of interest were the only DNA fragments that still needed to be digested. It was decided that, as a first approach, there were some genes, from all the genes selected to enable the WLP, more promising than the others. The clusters CAETHG_1608-1611 and CAETHG_1620-21 were the most promising group of genes and they were selected for this first cloning attempt. Thus, these two clusters were digested with the restriction enzymes XmaI (for the linkage between the clusters and the promoter) and NotI (for the linkage between cluster and terminator). The digestion reaction was made in NEBuffer 2.1, at 37ºC for 90 min, followed by the restriction enzymes inactivation at 65ºC for 20min. After all the genes were digested, the concentration of DNA in each solution was measured using a NanoDrop Lite Spectrophotometer. With all the DNA fragments properly digested, the ligation reaction took place. Two ligation solutions were prepared. The first one contained, in a ratio 1:3 (vector:inserts), the digested plasmid, digested promoter, digested CAETHG_1608-1612 cluster, digested terminator, the T4 DNA ligase and the T4 buffer. The second solution contained the same constituents than the first one, except the CAETHG_1608-1611 cluster that was replaced by the CAETHG_1620-1621. After the ligation solutions were prepared they were slightly homogenized in a vortex and the reaction occurred at room temperature for 40 min.

Selection plates: The vector pEC500E contains intrinsic genes that confer resistance to antibiotics. Ampicillin and Erythromycin are the antibiotics to which the cells that contained this vector are resistant to. Considering this, a culture medium supplemented with one of these antibiotics just allows the growing of cells containing the pEC500E. These mediums are of great interest to select colonies after the transformation of them with the constructed plasmids. A stock solution of ampicillin (10mg/ml) was prepared and sterilized by filtration on a 0.2µm filter. The antibiotic stock solution was then diluted at 1:100 into a 35g/L LB Agar medium and petri dishes of 25mL of the resulting solution were prepared. The LB Agar medium was previously autoclaved, to become sterilized, and it must cool down to temperatures below 60 ºC before adding the antibiotics. Every step was made closed to the flame to avoid contaminations. The plates were left slightly open inside a foam hood, for 30 min, to let the agar solidified. Once the agar solidified the plates were stored at 4ºC.

Transformation in *E. coli*: At the end of the ligation reaction, the products must be transformed into competent *E. coli* for amplification of the constructed plasmids. For this reason, the ligation products were firstly transformed into Z-competent *E. coli* XL-1 Blue.

These *E. coli* cells were stored as vegetative cells in 15% glycerol solution at -80ºC. 4µL of each ligation solution was mixed with 50µL of thawed Z-competent *E. coli* XL-1 Blue and the solution was incubated on ice for 30 min. A heat shock was not required. To regenerate the cells from transformation and to allow the expression of the antibiotic resistance conferred by the pEC500E, 200µL of SOC (Super Optimal Broth, Thermo Scientific) medium was added to the transformation solutions and they were incubated at 37ºC for 60 min and 250 rpm. In the meantime, two selection plates were pre-heated at 37ºC. At the end of the regeneration process the *E. coli* solutions were incubated into the pre-heated selection plates and left overnight at 37ºC. The inoculation steps were all made near the flame to assure the most sterilized environment possible.

Colony PCR: After the overnight incubation, visible colonies appeared in both the plates. Along the *E. coli* growth, the plasmids suffered successive replications and reached a concentration feasible to work with. The colony PCR is a high-throughput method for determining the presence or absence of insert DNA in plasmid constructs. To perform this method, five colonies from each plate were picked and resuspended in 10µL of Mili-Q water. These individual transformants were then lysed with a short heating step, 10min at 99ºC, to release the plasmid DNA from the cells. This plasmid DNA served as template for a PCR reaction in which a specific group of primers were used to amplify the insert DNA and determine if the plasmids contained the DNA fragment of interest and if this fragment was in the desired order. Two pairs of primers were used, one with the promoter forward and genes of interest reverse primers (for the CAETHG_1620-1621 and CAETHG_1608-1611 clusters) and other group with the genes of interest forward primers and terminator reverse primer. The PCR reaction was made using a DreamTaq Green PCR master mix (Thermo Fisher Scientific). When the PCR finished, the PCR products were directly loaded onto an electrophoresis gel (1% Agarose) and migrated for 60min at 80V to check for the presence of the desired plasmid.

Plasmids isolation: Once confirmed that the colony PCR products were the expected ones, the colonies that were previously picked for the colony PCR were now inoculated in 10mL of a 25g/L LB liquid medium, with 100µg/mL of ampicillin. The cultures were grown overnight at 37ºC and 250rpm. The cells rapidly multiplied during the night as well as the plasmids that they contained. To avoid damage the plasmids and facilitate their isolation from the culture broth, is advisable that the cultures do not have more than 18h. Thus, in the next

day, the plasmids needed to be isolated from the cell cultures. 6mL of each cell culture were centrifuged at 14000rpm for 5 min and the resultant pellets were used in a GeneJET Plasmid Miniprep Kit (Thermo Scientific) that isolates the plasmids from the remaining pellet constituents. In the elution step of this kit it was used Mili-Q water instead of the elution buffer that came with it. The DNA content of the plasmids solutions was measured in a NanoDrop Lite Spectrophotometer.

Restriction confirmation: The colony PCR made previously had the purpose of indicating which colonies from the petri dishes had perhaps the desired DNA fragment. The assertive confirmation for the plasmids identity was made after plasmid isolation by restriction digest. The plasmids were digested by different restriction enzymes to produce multiple plasmid fragments in known numbers and sizes. Five different digestions were made for each plasmid, one with the restriction enzyme XcmI, one with HindIII, one with NdeI and the last two with a mixture of the previous enzymes, namely XcmI with HindIII and HindIII with NdeI. All the digestions were made in NEBuffer 2.1 for 60min at 37ºC. After the digestion, all the restriction enzymes were inactivated at 65ºC for 20min. The number and size of the resulting plasmid fragments were analysed by electrophoresis in a 1% Agarose gel run for 70min at 80V.

Cloning attempt 2

Gibson Assembly: The NEBuilder® HiFi DNA Assembly kit was now used as a new approach to construct the desired plasmids. This kit contains a solution of different enzymes that work together in the same buffer to allow for seamless assembly of multiple DNA fragments, regardless of fragment length or end compatibility. In the same way as the Gibson assembly method, the reaction starts with an exonuclease that creates single-stranded 3' overhangs to facilitate the annealing of fragments that share complementarity at one end (the overlap region). A DNA polymerase will then fill the gaps within each annealed fragment and a DNA ligase seals the nicks in the assembled DNA. The result is a double-stranded fully sealed DNA molecule that can be directly transformed into *E. coli.* For this attempt the cluster CAETHG_1608-1611 was used because of it promising predictable capacity of enable the WLP. In this technique the promoter, terminator and cluster PCR products did not needed to be digested, although, for the pEC500E, it was mandatory to open its circular DNA structure and form a linear one with two open ends. The pEC500E was digested in the exactly same

way than before, in the restriction ligation method, and as well purified from the 14bp fragments that derived from its digestion. Because four different DNA fragments were used for the assembly and considering that the cluster PCR product was almost the same size than the digested pEC500E (6242 and 6260bp, respectively), a ratio of 1:1:3:3 (vector : cluster : promoter : terminator) was advisable according to the kit instructions. The amount of each DNA fragment needed to stablish this ratio was calculated based on a desired concentration of vector of 0,2 pmols. The DNA concentration in each PCR product solution and in the digested pEC500E solution, was previously measured with the NanoDrop Lite Spectrophotometer. Once calculated the needed amounts of each solution, all the DNA fragments were mixed together with the HiFi assembly master mix and Mili-Q water. The assembly reaction occurred in a block heater at 50ºC for 60min.

Transformation in *E. coli*: While the assembly reaction was occurring, one selection plate with LB Agar and ampicillin was pre-heated at 37ºC. Once the Gibson assembly finished, 50µL of NEB 5-alpha Chemical Competent *E. coli*, previously stored at -80ºC as vegetative cells in 15% glycerol solution, were thawed on ice for transformation. 2µL of the assembled products were added to the *E. coli* solution and gently mixed by pipetting. The mixture was placed on ice for 30min, followed by a heat shock at 42ºC for 30 seconds, after which it was again transferred to ice for more 2 min. To regenerate the cells, 950µL of room temperature SOC media was added to the solution that was placed at 37ºC for 60min with vigorous shaking at 250rpm. 100µL of the regenerated cells were at the end spread onto the pre-heated selection plate and incubated overnight at 37ºC.

Colony PCR: In the day after the plate incubation, there were colonies spread all over the plate. Ten of the colonies were picked from the plate and resuspended in 10µL of Mili-Q water, where they were lysed by a short heating step for 10min at 99ºC. For the colony PCR, to discover if the plasmids contained the inserts and to check if the genes were assembled in the proper way, two pairs of primers were used, one with the promoter forward and cluster reverse primers and other with the cluster forward primer and terminator reverse primer. The PCR reaction was then made using a DreamTaq Green PCR master mix, the DNA from the lysed cells as template DNA and the primers. When the PCR finished, to evaluate if the inserts were amplified, the PCR products were directly loaded onto an electrophoresis gel (1% Agarose) and migrated for 60min at 80V.

Cloning attempt 3

New primers: The failure in the previous attempts of constructing the desired plasmids lead to a re-evaluation of the promoter and terminator structure and primers design. Looking in detail with a more critical perspective to the C. *beijerinckii* NCIMB 8052 thiolase gene, it was concluded that both promoter and terminator should contain a bigger sequence of DNA than the one chose before. By increasing their size, it was expected that cloning would be easier and feasible. Table 6 shows the sequences of the new primers.

Table 6 – Description of the new promoter and terminator primers;

Name	Sequence	Lenght [nt]	DNA target
P1_thlP_F	CGACGTTGTAAAACGACGGCgcatgcAGCCTGTACTTGTGTTGAGAGACG	50	thlP
thlP_R	gtttgacctcctaaaattttATAGATTATTTTATTAACTTTTG	43	thlP
thlT_F	gcggccgcTATAAATTAAGATTTAAAAAGGTTACTATG	38	thlT
P2_thlT_R	gcggataacaatttcacacaTTATTGAATATAGAAATTAATCC	43	thlT

Touchdown PCR for the promoter and terminator amplification: To amplify the new promoter and terminator the most precise as possible, a touchdown PCR was made using the Q5 High-Fidelity DNA polymerase master mix together with the new primers and the C. *beijerinckii* NCIMB 8052 genome as DNA template. The PCR program was stablished to decrease the annealing temperature in 1ºC / cycle during the first 17 cycles and stabilize the temperature at the minimum in the last 13 cycles. When the PCR finished, an aliquot of the products was loaded onto an electrophoresis gel (1.2% Agarose) and they run at 90V for 30 min to confirm if the PCR products size corresponded to the expected ones.

PCR products purification: When confirmed that the PCR products had the correct size, the remainder PCR product solutions were entirely loaded again in a new electrophoresis gel (1.2% Agarose) and migrated at 80V for 60min. After the electrophoresis, the gel regions that contained the bands of interest were cut out over a UV lamp as fast as possible. The Gene JET Gel Extraction Kit was then used to extract and purify the DNA from the pieces of gel. Mili-Q water was used as an alternative to the elution buffer that came with the kit. At the end, the concentration of DNA in the gel purified solutions was measured using the NanoDrop Lite Spectrophotometer.

Gibson Assembly: The NEBuilder® HiFi DNA Assembly kit was again used in this new attempt. It was decided that, despite of the promising capacity of the cluster CAETHG_1608-1611 to enable the WLP, it was too big for direct cloning and probably his size could be one of the reasons why the previous attempts did not work. For this reason, the cluster CAETHG_1620-1621 and the gene CAETHG_3005 were chosen for cloning in this new attempt. The pEC500E was digested and purified following the same procedures mentioned in the cloning attempt 2. A ratio of 1:1:2:2 (vector : gene of interest : promoter : terminator) was used and the needed amount of each of these solutions was determined considering that the digested pEC500E concentration should be between 50-100ng. The DNA fragments were then mixed together with the HiFi assembly master mix and Mili-Q water. Two different assembly solutions were prepared, one containing the cluster CAETHG_1620-1621 and other the gene CAETHG_3005. The assembly reactions were done in a block heater at 50ºC for 60min.

Transformation in *E. coli*: While the assembly reaction was occurring, two selection plates were placed at 37ºC to be warm for the inoculation step. The NEB 5-alpha Chemical Competent *E. coli* that came with the NEBuilder® HiFi DNA Assembly kit were finished, so the Z-competent *E. coli* XL-1 Blue were used for transformation. After the thawing of the cell solutions on ice (50µL of cells per tube), 4µL of the assembly reaction products were added. Two different transformation reactions were performed, one with the assembly products containing the cluster CAETHG_1620-1621 and the other containing the gene CAETHG_3005. Once the solutions were gently mixed by pipetting, they were left on ice for 30min for the transformation reaction to occur. When the 30min passed, 200 µL of LB liquid media were added to each solution and they were transferred to an incubator at 37ºC and 250rpm for 60min. At the end of the regeneration process, each solution was inoculated in the pre-heated selection plates and they were left overnight at 37ºC.

Colony PCR: After the overnight growth of the transformed cells, it was visible that they formed around 20-30 colonies in each plate. 8 colonies, from each plate, were picked and resuspended in 10µL of Mili-Q water. A short heating step was made, 10min at 99ºC, to lysate the cells and released the plasmids that were inside them. For this colony PCR, it was used different pairs of primers, one pair containing the new promoter forward and reverse primers and the other the new terminator forward and reverse primers. The PCR solutions were then made using a DreamTaq Green PCR master mix, the template DNA from the lysed

cells and the primers. Once finished the PCR, all the products were loaded onto an electrophoresis gel (1% Agarose) and migrated for 60min at 80V. No band appeared for any of the analysed colonies, suggesting that the desired plasmid was not present. Although, considering that the colony PCR is not such a trustworthy method, two colonies of each plate were chosen to further restriction confirmation.

Plasmids isolation: The colonies were then inoculated in 10mL of a 25g/L LB liquid medium with 100µg/mL of ampicillin and left overnight at 37ºC and 250rpm. In the next day, 6mL of each cell culture were centrifuged at 14000rpm for 5 min and the resultant pellets were used in a GeneJET Plasmid Miniprep Kit to isolate the plasmids from the remaining pellet. As well as before, it was used Mili-Q water instead of the elution buffer that come with the kit. All the plasmids solutions were measured in a NanoDrop Lite Spectrophotometer to quantify their amount of DNA.

Restriction confirmation: To confirm if the plasmids corresponded to the desired ones, they were digested by the restriction enzymes SphI HF (High Fidelity) and NotI, in NEBuffer 3.1 for 90min at 37ºC. It was decided that the inactivation step was not necessary for this situation, once it was just for identification and the digested plasmids were not going to be used for further steps. After the digestion finished, the solutions were loaded in a 1% Agarose electrophoresis gel and migrated during 60min at 80V.

Cloning attempt 4

Fusion PCR: A different approach than before was selected for a new attempt. The fusion PCR is thought to be a simple and easy method to produce fused DNA fragments without the need of restriction enzymes or DNA ligation. Two DNA fragments that have overlap sequences between each other are able to anneal one to the other after the denaturation step in a PCR reaction. This step opens the double-stranded structure of the DNA and allows the contact between the overlap sequences of both DNA fragments. If the PCR solution contains the primers that anneal to the ends of this attached sequence, the DNA polymerase can run over the single-stranded regions and turn the entire sequence into a double-stranded DNA structure. The resulting sequence corresponds to the fusion between the two DNA fragments used in the beginning. To perform this technique it was decided that, in addition to the genes used in the cloning attempt 3, it should be utilized also the gene CAETHG_3899. In a first fusion PCR reaction, the promoter was fused to the genes of interest. For this purpose, three

PCR reactions were made, one for each gene of interest, in which the promoter was added together with the primers (promoter forward and gene of interest reverse) and the Q5 High-Fidelity DNA polymerase master mix. It was used 5ng of each gene of interest in a ratio 1:1 with the promoter. For a fusion PCR, the annealing temperature should be the lowest one between the annealing temperature of the promoter forward primer, the gene of interest reverse primer and the annealing temperature of the overlap sequences between the promoter and gene of interest. Once constructed the fused DNA fragment promoter-gene of interest, the PCR solutions were loaded onto an electrophoresis gel (1% Agarose) for 60min at 80V. Then, the gel regions that contained these fused DNA fragments were cut out over a UV lamp and the DNA was purified from the gel using the Gene JET Gel Extraction Kit. The concentration of DNA in the purified DNA fragments was measured using a NanoDrop Lite Spectrophotometer. With the promoter attached to the genes of interest, now it was necessary to fuse these new DNA fragments to the terminator. A new fusion PCR was made, identical to the previous one, with the exception that, for this case, it was used the fused DNA fragments together with the terminator, what lead to a different annealing temperature, and a different pair of primers (promoter forward and terminator reverse). The PCR products were equally loaded and extracted from an electrophoresis gel using the Gene JET Gel Extraction Kit. The DNA concentration in each PCR product was measured in a NanoDrop Lite Spectrophotometer. At the end, the fused fragments promoter-CAETHG_3005-terminator and promoter-CAETHG_3899-terminator were obtained. It did not work for the cluster CAETHG_1620-1621, however.

Circular Polymerase Extension Cloning: This method uses the polymerase extension mechanism to join overlapping DNA fragments into a double-stranded circular form, such as a plasmid. Firstly, the linear double-stranded insert(s) and vector are heat-denatured resulting in single strands that anneal with their overlapping ends and extend using each other as a template to form a double-stranded circular plasmid. There is no need for primers in this method, because the DNA polymerase uses the annealed overhangs between DNA fragments as “primers” from which the hybridized insert and vector extend, using each other as a template, until they complete a full circle and reach their own 5’-ends. Therefore, this method was used to join the fused DNA fragments, previously obtained, with the pEC500E. First, the pEC500E was digested and purified exactly in the same way than before. Then, two PCR solutions were prepared, one containing the fused DNA fragment promoter-CAETHG_3005-

terminator and other the promoter-CAETHG_3899-terminator. To each PCR solution it was added the digested pEC500E and the Q5 High-Fidelity DNA polymerase master mix. It was used 50-100ng of the digested pEC500E in a ratio 1:1 with the fused DNA fragments. After the PCR, the concentration of DNA in the solutions was measured using a NanoDrop Lite Spectrophotometer and the products were loaded onto an electrophoresis gel (1% Agarose) and migrated at 80V for 40min.

Transformation in *E. coli*: The PCR products from the circular polymerase extension cloning were transformed into Z-Competent *E.coli* XL-1 Blue, following the same procedure than before.

Cloning attempt 5

Circular Polymerase Extension Cloning: The CPEC method was repeated and it was decided that the concentration of inserts in the PCR solutions should be bigger than before. In this attempt, 50-100ng of the digested pEC500E was used, although, the ratio vector:insert was changed to 1:2. After the PCR, the concentration of DNA in the solutions was measured using a NanoDrop Lite Spectrophotometer and the products were loaded onto an electrophoresis gel (1% Agarose) and migrated at 80V for 40min.

Transformation in *E. coli*: The PCR products from the circular polymerase extension cloning were transformed into Z-Competent *E.coli* XL-1 Blue, following the same procedure than before. After two days of incubation, at 37ºC, both plates contained around 20 colonies.

Colony PCR: 16 colonies from each plate were chosen to perform a colony PCR. They were resuspended in 10µL of Mili-Q water and lysed with a heat shock of 99ºC for 10min. It was used just one pair of primers for this colony PCR, the promoter forward and terminator reverse primers. Using these primers, it was expected that the entire insert was amplified. The PCR solutions were made mixing the template DNA, extracted from the colonies, the primers and the DreamTaq Green PCR master mix. The PCR products were loaded onto an electrophoresis gel (1% Agarose) and migrated at 85V for 35min. The expected bands, with the insert size, did not appeared.

Plasmids isolation: Despite of the lack of results in the colony PCR, three of the analysed colonies were inoculated in 10mL of a 25g/L LB liquid medium with 100µg/mL of ampicillin and left overnight at 37ºC and 250rpm. The same procedures than before were used to extract

the plasmids from the liquid cultures. All the plasmids solutions were measured using a NanoDrop Lite Spectrophotometer to quantify their concentrations in DNA.

Restriction confirmation: To determine the plasmids identity, they were digested by the restriction enzymes SphI HF and NotI HF, in a CutSmart buffer. The digestion reaction was made at 37ºC for 60min. No enzymatic inactivation step was needed and the digested plasmids were directly loaded onto an electrophoresis gel (1% Agarose) and run for 40min at 80V.

Cloning attempt 6

Restriction ligation: The cloning method used in the cloning attempt 1 was now repeated with the difference that, instead of using the promoter, gene of interest and terminator as individual inserts, the fused DNA fragments constructed in the cloning attempt 4 were used as a single insert. However, a mistake in the new primers design was discovered. The new terminator reverse primer did not include the restriction site for the XhoI enzyme in its overhang. Therefore, the resulting terminator PCR product could not be cut by the restriction enzyme XhoI, which confers compatibility between the terminator and the digested pEC500E. To solve this problem, a new PCR was made using as DNA template the fused DNA fragments from the cloning attempt 4, as primers the old promoter forward and terminator reverse and the Q5 High-Fidelity DNA polymerase master mix. The idea consisted in obtaining the fused promoter-gene of interest-terminator fragments in which the 3' and 5'-ends contained the old promoter and terminator overhangs. These new fused fragments were shorter than before, since the old primers were designed to amplify a shorter promoter/terminator than the new ones. The PCR products were loaded in an electrophoresis gel (1% Agarose) and migrated at 80V for 40min. Their respective bands were cut out of the gel over an UV lamp and the DNA purified using the Gene JET Gel Extraction Kit. After the measurement of the DNA concentration in the purified solutions, using a NanoDrop Lite Spectrophotometer, the pEC500E and the new PCR products were digested by the restriction enzymes SphI HF and XhoI in a CutSmart buffer. The digestion was made at 37ºC for 60min. An enzymatic inactivation step followed the digestion, 20min at 65ºC. The pEC500E, as well as before, passed through the Gene JET Gel Extraction Kit to remove the 14bp fragments that were cut out from the pEC500E during the digestion. With all the DNA fragments properly digested with their ends compatible to each other, the ligation step took place. 100ng of the digested pEC500E and a ratio vector:insert of 1:3 was used. Two ligation solutions

were prepared, one with the DNA insert "old" promoter-CAETHG_3005-"old" terminator and the other with the "old" promoter-CAETHG_3899-"old" terminator. In addition to the DNA insert and the digested pEC500E, the solutions contained a T4 DNA ligase and T4 buffer. After 60min at room temperature the ligation products were ready to be transformed in *E. coli*.

Transformation in *E. coli*: The ligation products were transformed into Z-Competent *E.coli* XL-1 Blue, following the same procedure than before. In the next day, 7 colonies appeared in the selection plate containing the cells transformed with the ligation products where the gene CAETHG_3899 was used.

Colony PCR: All the 7 colonies were picked up and resuspended in 10µL of Mili-Q water. After the heating shock to lysate them, the same protocol used in the cloning attempt 5 colony PCR was followed. The expected bands were not revealed in the electrophoresis gel after the colony PCR.

Plasmids isolation: 1 of the 7 analysed colonies was chosen to reliable assurance its plasmids identity. The colony was inoculated in 10mL of a 25g/L LB liquid medium with 100µg/mL of ampicillin and left overnight at 37ºC and 250rpm. The same procedures than before were used to extract the plasmids from the liquid culture. The DNA concentration in the plasmids solution was measured using a NanoDrop Lite Spectrophotometer.

Restriction confirmation: The restriction digestion made in the cloning attempt 5 was equally performed for this plasmid identification. After the digestion products migrate for 40min at 80V onto an electrophoresis gel (1% Agarose).

Cloning attempt 7

Restriction ligation: The restriction ligation method was tried a last time. In this attempt, instead of the normal PCR made in the cloning attempt 6 to amplify the fused DNA fragments with the old primers, it was decided to do a touchdown PCR to obtain more precise PCR products and to avoid the amplification of undesired sequences. The same PCR solution was made, although, the PCR program was different. In this new PCR program, the annealing temperature decreased 1ºC/cycle in the first 14 cycles and remained at the minimum temperature during the next 11 cycles. The PCR products were equally analysed by electrophoresis, in the same conditions than in the cloning attempt 6, and gel purified using the Gene JET Gel Extraction Kit. The DNA concentration in the purified solutions was

measured using a NanoDrop Lite Spectrophotometer. The restriction digestion made in the cloning attempt 6 was repeated in this attempt, for both the insert and the pEC500E, with the digested pEC500E being posteriorly purified by the Gene JET Gel Extraction Kit. For the ligation step it was decided that a bigger amount of digested pEC500E and inserts could be a good solution to increase the probability of the desired plasmid be constructed. It was used 150ng of the digested pEC500E and a ratio vector:insert of 1:3. The same ligation solutions made in the cloning attempt 6 were prepared, with the difference that now the amount of vector and insert was higher than before. To be sure that the ligation reaction had enough time to be completed, the ligation solutions were left at room temperature for 90min.

Transformation in *E. coli*: Two selection plates were pre-heated at 37ºC for further inoculation. Aquacompetent *E. coli* Top 10, previously stored at -80ºC in a 15% glycerol solution, were used for transformation. Two tubes containing 50µL of these cells were thawed on ice and 5µL of the ligation products were added to each one of them (one ligation product per tube). After the solutions being gently mixed by pipetting, they were left on ice for 30min. At the end of the 30min, both solutions were subjected to a heating shock of 45 seconds at 42ºC, followed by 2 min on ice. Once finished the transformation step, 200µL of liquid LB medium were added to each solution and they were left for 60min at 37ºC and 250rpm, for regeneration of the cells. When the cells finished the regeneration step, each solution was spread into a pre-heated selection plate and left overnight at 37ºC.

Colony PCR: Both plates had around 20-30 colonies in the following day. 20 colonies from the plate that contained the cells transformed with the ligation products where the CAETHG_3005 gene was present were picked for colony PCR, as well as 15 colonies from the other plate. All the colonies were resuspended in 10µL of Mili-Q water and lysed by a short heat shock of 10min at 99ºC. The same pair of primers used in the cloning attempt 6 were also chose for this case. The PCR solutions were made by mixing the template DNA from the lysed cells with the primers and the DreamTaq Green PCR master mix. To enhance the PCR efficiency, it was done a touchdown colony PCR. For the first 12 cycles the annealing temperature decreased 1ºC/cycle and then it was maintained at the minimum temperature during the next 13 cycles. At the end, all the PCR products were loaded in an electrophoresis gel (1% Agarose) and migrated for 70min at 80V.

Cloning attempt 8

Gibson Assembly: For a new cloning attempt, it was decided that it could be a promising approach to repeat the Gibson assembly method using the fused DNA fragments constructed in the cloning attempt 4 as unique inserts. Therefore, instead of the Gibson assemblies made previously, in which four individual DNA fragments were used, in this attempt it was performed an assembly between just two DNA fragments, the vector and a single insert. According to the fusion PCR made in the cloning attempt 4, there were two fused DNA fragments that could be used for the Gibson assembly, the clusters promoter-CAETHG_3005-terminator and promoter-CAETHG_3899-terminator. The pEC500E was digested and gel purified following the same procedures made in the cloning attempt 6. Then, two assembly solutions were prepared, one for each fused DNA fragment. According to the NEBuilder® HiFi DNA Assembly kit, 50-100ng of the digested pEC500E were mixed with the inserts in a ratio 1:2 vector:insert, together with the HiFi assembly master mix and Mili-Q water. The assembly reaction occurred at 50ºC, in a block heater, for 30min.

Transformation in *E. coli*: Following the same protocol used in the cloning attempt 7, the assembly products were transformed into aquacompetent *E. coli* Top 10.

Cloning attempt 9

Silica Bead DNA Gel Extraction kit: After all the failed cloning attempts, it was necessary to carefully evaluate all the procedures made and try to find out what could be done differently to successfully construct the desired plasmid. It was concluded that, when the Gene JET Gel Extraction Kit was used, the concentration of DNA suffered a 5-fold drop comparing with the unpurified solutions. With such lower DNA concentrations in the purified solutions, it was necessary to use bigger volumes to reach the desired amount of DNA fragments for the assembly reaction. This was thought to be a source of error common to all the cloning attempts previously done. To minimize this problem, a new gel extraction kit was adopted for the further cloning attempts. The Silica Bead DNA Gel Extraction kit was known to allow bigger concentrations of DNA in gel purified solutions, when compared with the Gene JET Gel Extraction Kit. For this new attempt, the fusion PCR made in the cloning attempt 4 was repeated and the fused PCR products were purified from the electrophoresis gel using the Silica Bead DNA Gel Extraction kit. As well as the fused PCR products, the pEC500E was digested in the same way than in the cloning attempt 6 and gel

purified by the new gel extraction kit. After the measurement of the DNA concentration in the purified solutions, using a NanoDrop Lite Spectrophotometer, both solutions presented higher DNA concentrations when compared with the previous cloning attempts.

Gibson assembly: With the new concentrated DNA fragments, the NEBuilder® HiFi DNA Assembly kit was again used. The assembly protocol made in the cloning attempt 8 was repeated, but now strictly respecting the advisable volumes of purified DNA fragments.

Transformation in *E. coli*: The assembly products were transformed into aquacompetent *E. coli* Top 10 following the same protocol used in the cloning attempt 7.

Colony PCR: One day after the inoculation, 1 colony appeared in the selection plate inoculated with the cells transformed with the assembly products where the fused DNA fragment promoter-CAETHG_3899-terminator was used. The colony was picked and resuspended in 10µL of Mili-Q water in which it was lysed by exposure to 99ºC for 10min. The colony PCR was made by mixing the DNA from the lysed colony with a primer mix, composed by the promoter forward and terminator reverse primers, and the DreamTaq Green PCR master mix. The PCR products were directly loaded onto an electrophoresis gel (1% Agarose) and migrated for 40min at 85V.

Cloning attempt 10

Circular Polymerase Extension Cloning: Using the new purified DNA solutions, obtained in the cloning attempt 9, the CPEC was repeated. The same protocol used in the cloning attempt 5 was adopted, with the difference that now the PCR work volume was lower and the DNA fragments came from more concentrated solutions. The DNA concentration of the PCR product solutions was measured using a NanoDrop Lite Spectrophotometer and the solutions were loaded onto an electrophoresis gel (1% Agarose) and migrated for 50min at 85V.

Transformation in *E. coli*: The PCR products were transformed into aquacompetent *E. coli* Top 10 following the same protocol used in the cloning attempt 7.

Colony PCR: Two days after the selection plates inoculation with the transformed cells, both plates were full of colonies spread all over the agar medium. From the plate inoculated with the cells transformed with the PCR products that contained the CAETHG_3899 gene, 18 colonies were chosen for colony PCR. From the other plate, just 10 colonies were picked. The colony PCR made in the cloning attempt 9 was equally performed for these colonies.

Cloning attempt 11

Zero Blunt PCR Cloning Kit: After all the considered cloning methods had been repeatedly performed, it was decided that a new strategy should be adopted. The idea of using the Zero Blunt PCR Cloning Kit (Invitrogen) consists in inserting the fused DNA fragments into a pCR-Blunt plasmid that, after being amplified in *E.coli*, was going to be digested to release the insert with the assurance that it had compatible ends to ligate with a digested pEC500E. The advantage of this method relies on the fact that, after digestion of the pCR-Blunt, it is possible to see two bands in the electrophoresis gel, one correspondent to the pCR-Blunt linearized and the other to the insert already digested. Therefore, after gel extraction, it is possible to insert the digested fused DNA fragments into the pEC500E, eliminating any suspicion about the functionality of the restriction enzymes and compatibility between the inserts and the vector ends. First of all, the two fused DNA fragments, obtained in the cloning attempt 9, were inserted into two different pCR-Blunt plasmids. In order to do it, the needed amount of fused DNA fragments was estimated for a 10:1 molar ratio of insert to 25ng of pCR-Blunt vector. The pCR-Blunt came already as a linearized vector with blunt ends in the Zero Blunt PCR Cloning Kit, eliminating the need for a restriction digestion. The ligation reaction was made by mixing the fused DNA fragments with the linearized pCR-Blunt vector, together with a ExpressLink T4 DNA ligase, a 5X ExpressLink T4 DNA ligase Buffer and sterile water. The reaction occurred at room temperature for 30min.

Selection plates: Differently from the pEC500E, the pCR-Blunt has a gene that confers antibiotic resistance to kanamycin. Thus, the selection medium to inoculate cells transformed with this plasmid had to be supplemented with kanamycin. A stock solution of kanamycin (10mg/ml) was prepared and sterilized by filtration with a 0.2µm filter. The antibiotic stock solution was then diluted at 1:100 into a 35g/L LB Agar medium and petri dishes of 25mL of the resulting solution were prepared. The LB Agar medium was previously autoclaved and the antibiotic solution was just added when it cooled down to temperatures below 60ºC. All the steps were performed near the flame to avoid contaminations. The plates were left slightly open inside a foam hood, for 30 min, to let the agar solidified. Once the agar solidified the plates were stored at 4ºC.

Transformation in *E. coli*: The ligation products were transformed into aquacompetent *E. coli* Top 10, following the same protocol used in the cloning attempt 7, with the difference that it was used the kanamycin selection plates instead of the ampicillin ones.

Colony PCR: One day after the selection plates inoculation, both plates were full of colonies. 10 colonies from each plate were picked and resuspended in 10µL of Mili-Q water for further DNA extraction by lysis at 99ºC for 10min. Differently from the previous colony PCRs, it was used a mix of primers not designed in the present work. These primers, that came within the Zero Blunt PCR Cloning Kit, were made to amplify the pCR-Blunt region where the insert was supposed to be. If the insert was present, a band around the 2.8kb position should appear in the gel after electrophoresis. The primers were named as M13 forward and M13 reverse primers. To performed the colony PCR, the DNA extracted from the colonies was mixed with both the primers and the DreamTaq Green PCR master mix. The resulting PCR products were directly loaded onto an electrophoresis gel (1% Agarose) and migrated for 50min at 85V.

Cloning attempt 12

Zero Blunt PCR Cloning Kit: The cloning protocol, made in the cloning attempt 11, to insert the fused DNA fragments into the pCR-Blunt vector was repeated.

Transformation in *E. coli*: According to the transformation protocol followed in the cloning attempt 1, the ligation products were transformed into Z-Competent *E.coli* XL-1 Blue. However, due to the pCR-Blunt plasmids, it was used kanamycin selection plates instead of ampicillin.

Colony PCR: After the overnight incubation, around 20-30 colonies appeared in each selectin plate. 4 colonies from each plate were picked and lysed in 10µL of Mili-Q water by exposure to 99ºC for 10min. The colony PCR made in the cloning attempt 11 was repeated, using as template DNA the DNA derived from these lysed colonies. After an electrophoresis, using an 1% Agarose gel and the run made at 80V for 60min, unspecific bands appeared for all the colonies.

Plasmids isolation: To assertively confirm the plasmids identity, all the analysed colonies were inoculated in 10mL of a 25g/L LB liquid medium with 100µg/mL of kanamycin and left overnight at 37ºC and 250rpm. The same procedures used in the cloning attempt 1 were performed to isolate the plasmids from the colonies. At the end, the DNA concentration in the plasmid solutions was measured using a NanoDrop Lite Spectrophotometer.

Restriction confirmation: Once isolated, the plasmids were digested by the restriction enzyme EcoRI HF. The digestion was made in CutSmart buffer for 30min at 37ºC. An

enzymatic inactivation step was made, after the digestion, by exposure of the digestion solutions to 65ºC for 20min. The digested plasmids were then loaded onto an electrophoresis gel (1% Agarose) and migrated for 55min at 85V. The resulting gel did not contain the bands correspondent to the insert. Since it was just analysed 4 of the colonies presented in each selection plate, it was decided that 4 different colonies should be picked for their plasmids to be equally identified. Therefore, 4 new colonies from each plate were inoculated in 10mL of a 25g/L LB liquid medium with 100µg/mL of kanamycin and left overnight at 37ºC and 250rpm. The plasmids were isolated from the colonies using the same protocol mentioned above. When isolated, the plasmids were digested by the restriction enzymes SacI and XhoI in CutSmart buffer for 60min at 37ºC. The digested plasmids were directly loaded onto an electrophoresis gel (1% Agarose) and migrated for 60min at 85V.

Cloning attempt 13

TA cloning: For a last cloning attempt, a new approach was adopted. The TA cloning method takes advantages of basic cloning rules to ligate the inserts with a desired vector. This technique relies on the ability of an adenine and a thymine to complementary ligate to each other, even between different DNA fragments. When this ligation occurs between a vector and an insert, they hybridize and, in the presence of ligase, become ligated together. Firstly, it was necessary to convert the pEC500E into a T-vector (a linearized vector whose ends contain a free thymine). To do it, the pEC500E was digested with the restriction enzyme SmaI, in CutSmart buffer for 90min at 37ºC, with the purpose of linearize the plasmid and leave it with blunt ends. The enzymes were inactivated by exposure of the solutions to 65ºC for 20min. Then, 5µg of the digested pEC500E were mixed with 1 unit of Taq DNA polymerase, PCR buffer containing Mg^{+} and dTTPs. The solution was incubated at 70ºC for 120min. With the T-vector prepared, now it was necessary to add single 3'-adenine overhangs to the inserts ends. Once the fused DNA fragments were running out, the fusion PCRs made in the cloning attempt 4 were repeated, with the difference that a new fused DNA fragment was constructed with the cluster CAETHG_1608-1611 and the fused PCR products were purified from the electrophoresis gel using the Silica Bead DNA Gel Extraction Kit. Because the fused PCR products were amplified by a Q5 High-Fidelity DNA polymerase, an enzyme with proofreading activity, the resulting sequences did not include the 3' A-overhangs. Thus, these overhangs were added by mixing the fused DNA fragments with 1 unit of Taq DNA polymerase, dATPs and Taq DNA polymerase buffer. The solutions were

incubated for 20min at 72ºC. With the vector and the inserts ready for the TA-cloning, the TOPO TA-Cloning Kit (Invitrogen) protocol was followed. The T-vector was then mixed with the 3' A-overhangs inserts, one insert per reaction, together with a salt solution that came with the TOPO TA-Cloning Kit and Mili-Q water. The cloning reaction occurred at room temperature for 20min.

Transformation in *E. coli*: The ligation products were transformed into Z-Competent *E.coli* XL-1 Blue, following the same protocol used in the cloning attempt 1.

Plasmids isolation: In the day after the selection plates inoculation, both plates had several colonies. 5 colonies from each plate were picked and directly passed to 10mL of a 25g/L LB liquid medium solution, with 100µg/mL of ampicillin, and left overnight at 37ºC and 250rpm. Their plasmids were isolated, in the following day, by the same procedures adopted in the cloning attempt 1.

Restriction confirmation: The isolated plasmids supposed to have the gene CAETHG_3005 and the cluster CAETHG_1608-1611 inside, were digested with the restriction enzyme NcoI HF. Differently, the plasmids supposed to have the gene CAETHG_3899 inside, were digested with the enzyme PstI HF. Both digestions were made in CutSmart buffer and they took 30min at 37ºC.

Step 1 – Practical validation and control data;

Different fermentations were performed with *Clostridium beijerinckii* NCIMB 8052. All the fermentations occurred at 37ºC in sterilized and anaerobic conditions. A working volume of 50mL of CM2 medium was used and the fermentations were performed in pressure resistant Schott bottles of 250mL. The CM2 medium was always prepared with chemicals from the same recipients. Previously to the inoculation, all the cells were grown at the same conditions during the same period of time to guarantee that all the fermentations started with approximately the same concentration of cells. The results are presented in graphics and the considered values are the mean of two replicates (the data used to construct the graphics is organized in tables in the Appendices 8.2).

Heterotrophic fermentation, 60 g/L glucose (H1);

For all the heterotrophic fermentations, the overpressure created inside the bottles, along the fermentation, was constantly removed by a valve that was inserted with a needle in the rubber stopper. This valve allowed the release of the produced fermentation gases avoiding the entrance of air.

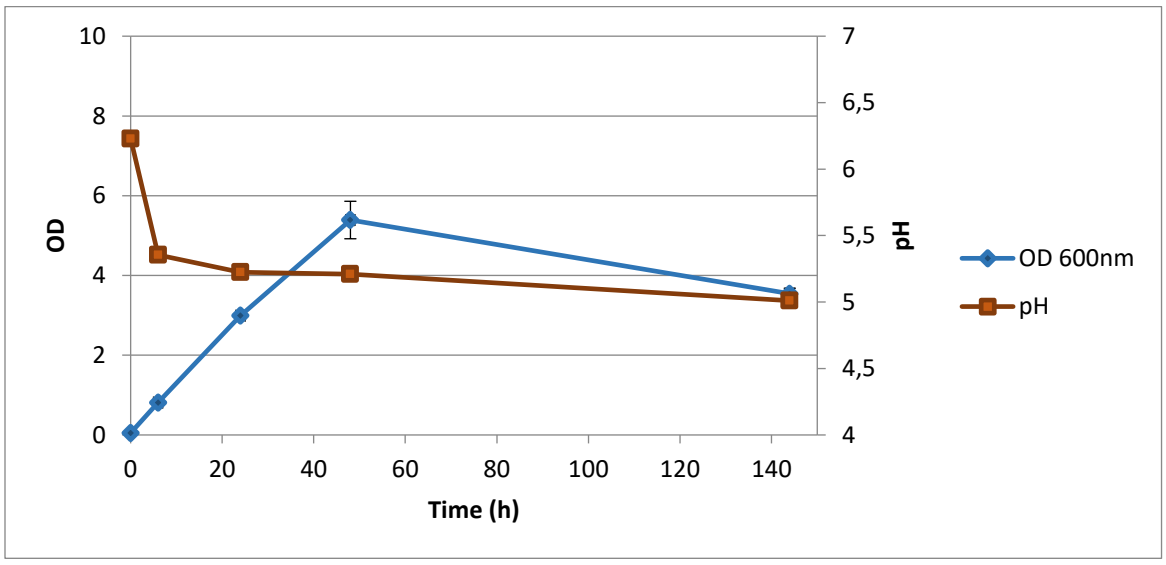


Figure 8 – The OD (600nm) and pH variation along the heterotrophic fermentation (H1);

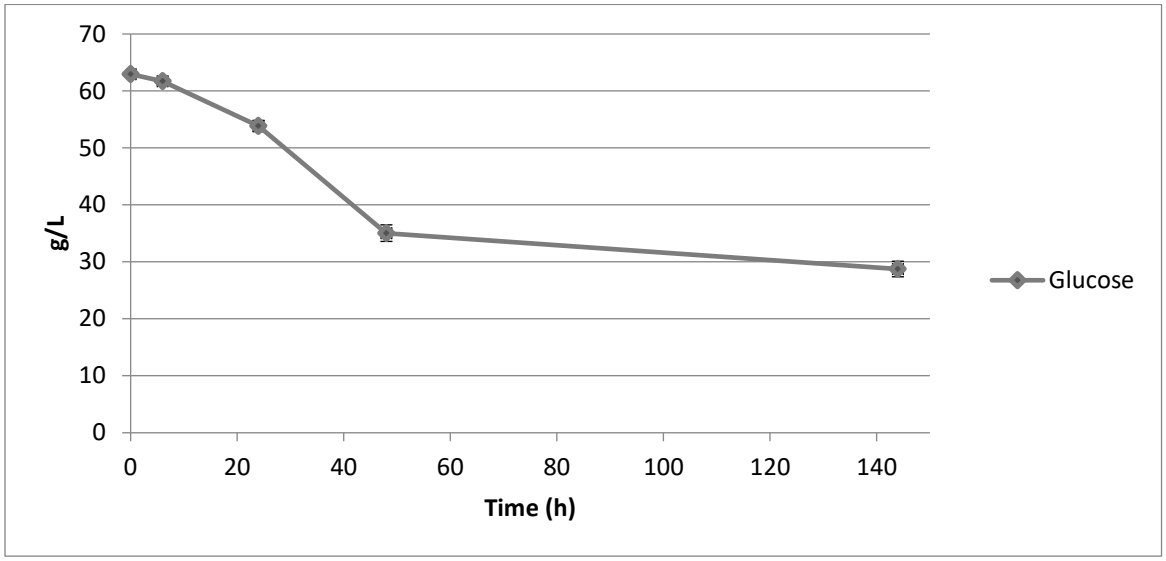


Figure 9 – The glucose content variation along the heterotrophic fermentation (H1);

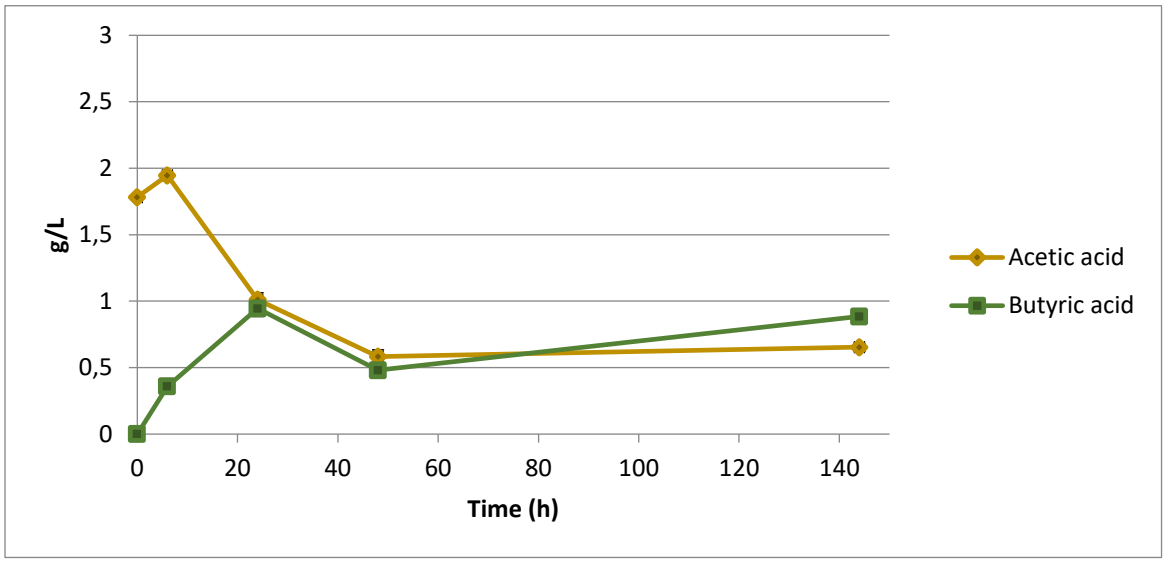


Figure 10 – The acetic and butyric acids content variation along the heterotrophic fermentation (H1);

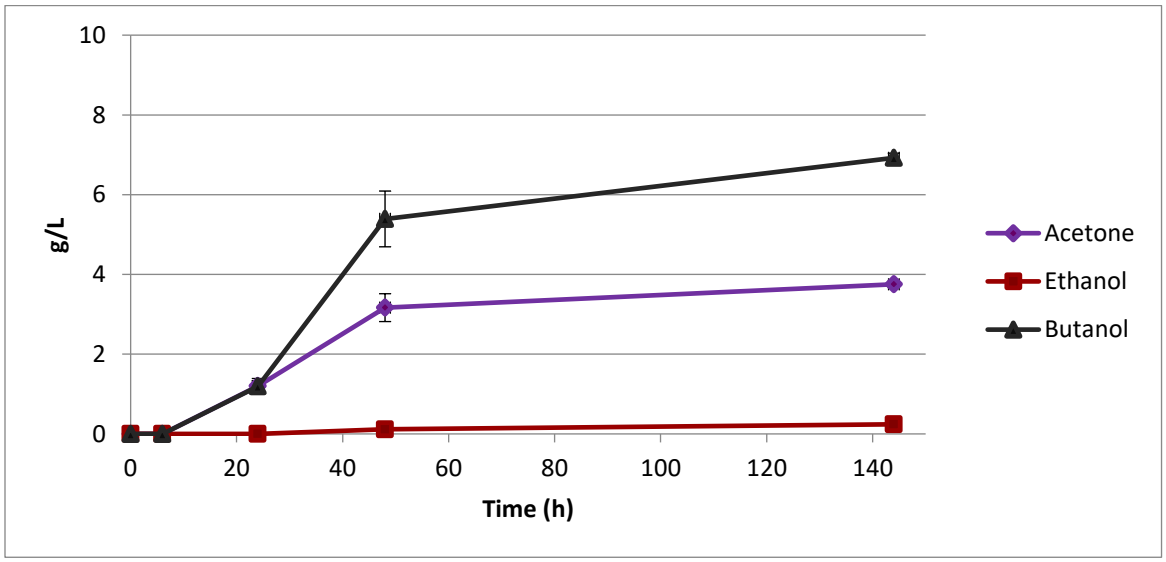


Figure 11 – The ABE content variation along the heterotrophic fermentation (H1);

Heterotrophic fermentation, 60 g/L glucose + 50rpm shaking (H2);

To implement agitation, it was used a shaking incubator at 37ºC. Every time that a fermentation condition included agitation it was performed in the shaking incubator.

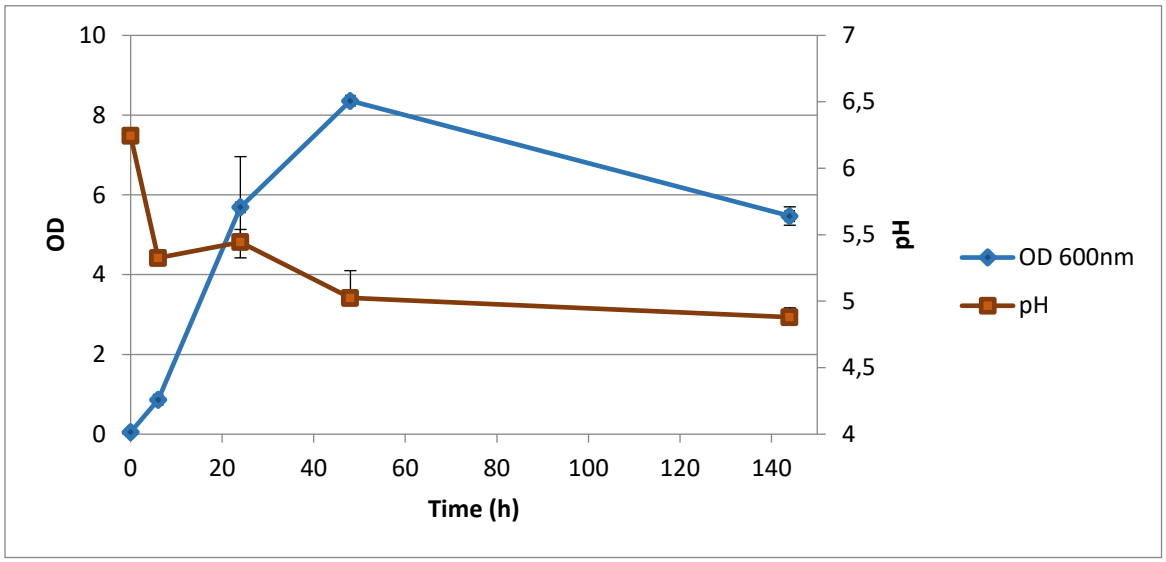


Figure 12 – The OD (600nm) and pH variation along the heterotrophic fermentation (H2);

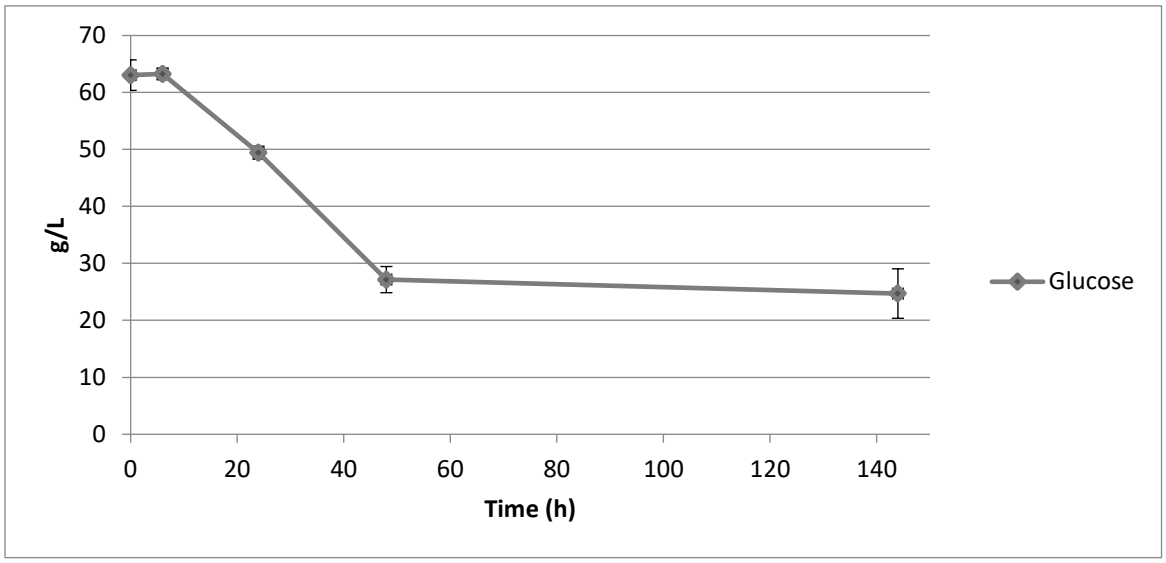


Figure 13 – The glucose content variation along the heterotrophic fermentation (H2);

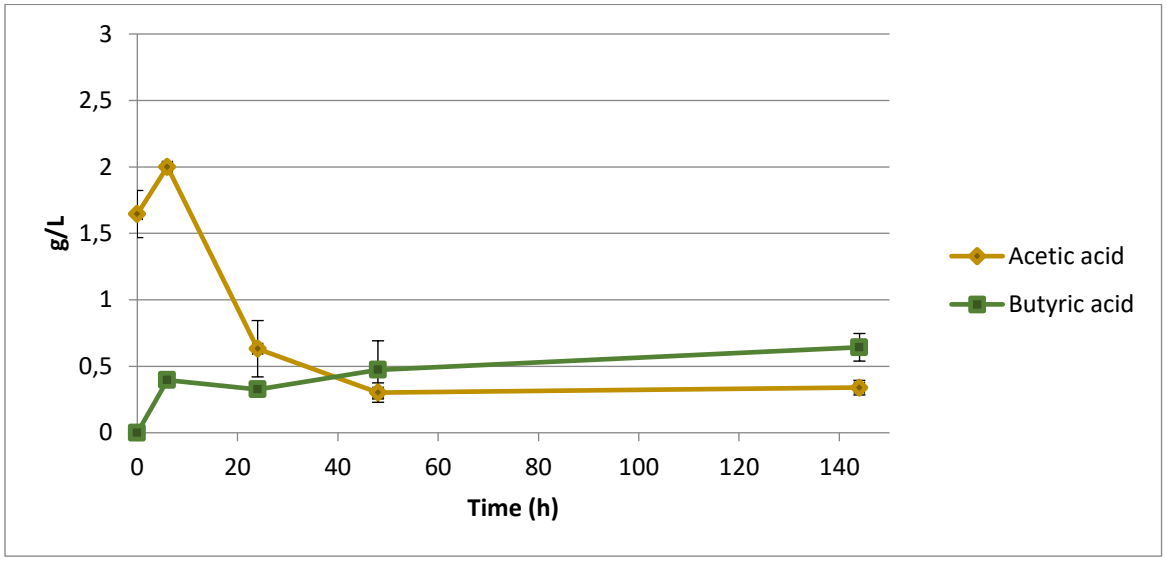


Figure 14 – The acetic and butyric acids content variation along the heterotrophic fermentation (H2);

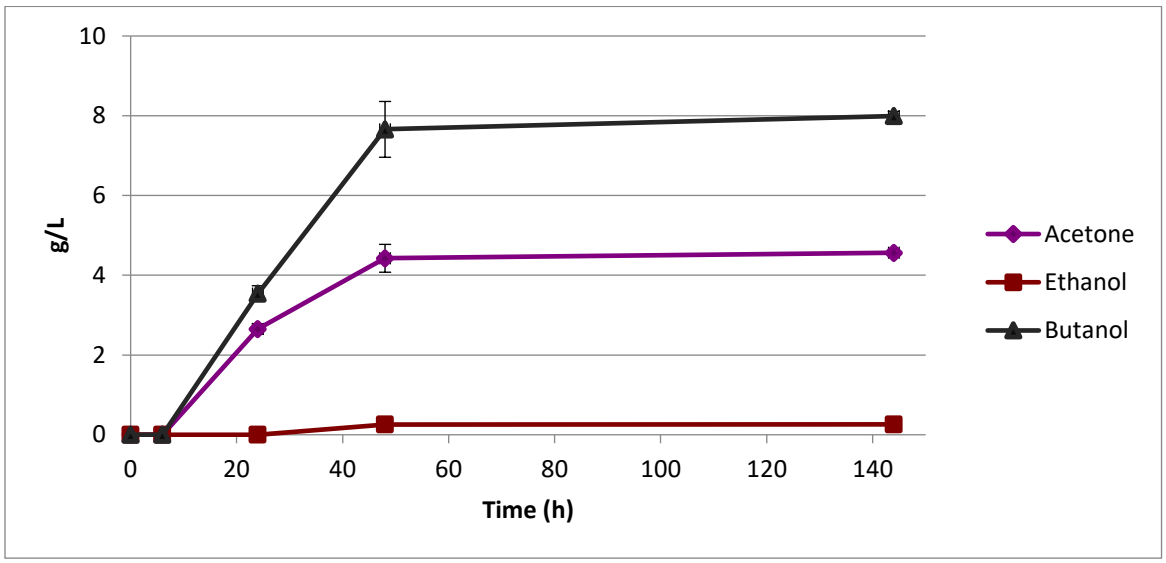


Figure 15 – The ABE content variation along the heterotrophic fermentation (H2);

Autotrophic fermentation, CO_2 (2bar) (A1);

For this case, no valve was used to constantly release the overpressure from the bottles. Differently from the previous fermentations, a barometer was used to precisely input 2bar of CO_2 in each bottle before the inoculation. The pressure inside the bottles was always measured prior to the samplings. After the samplings, the gas accumulated in the headspace of the bottles was discarded with a needle until the pressure inside the bottles reached 0,5bar, to avoid the entrance of external air. Then, the bottles were refilled again with 2bar of CO_2. This procedure was repeated three times to assure an efficient remove of all the fermentation gases produced and the existence of just CO_2 in the bottles headspace.

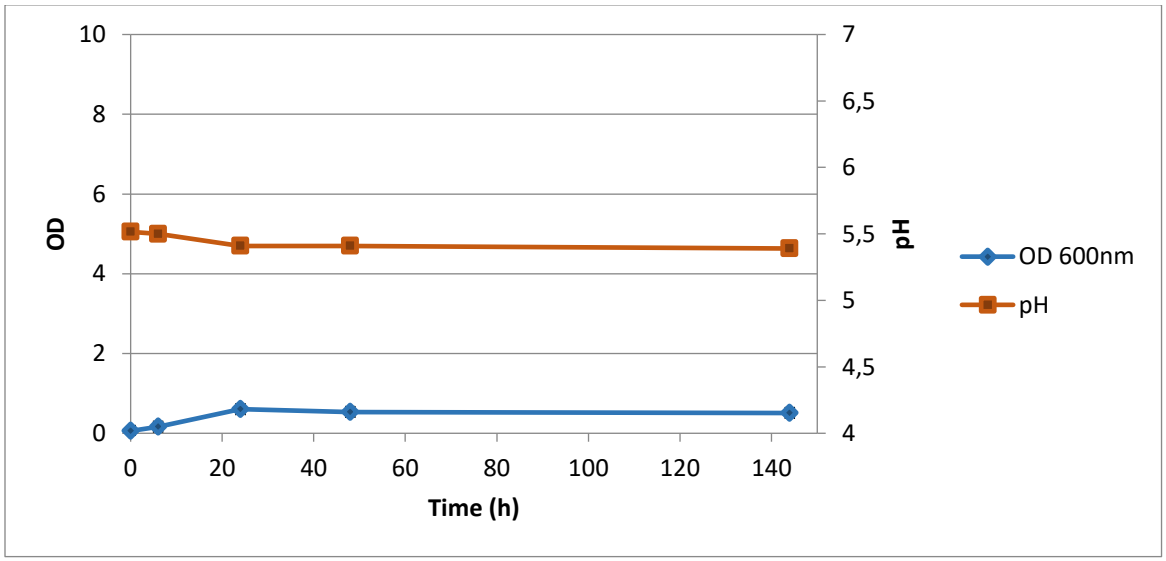


Figure 16 – The OD (600nm) and pH variation along the autotrophic fermentation (A1);

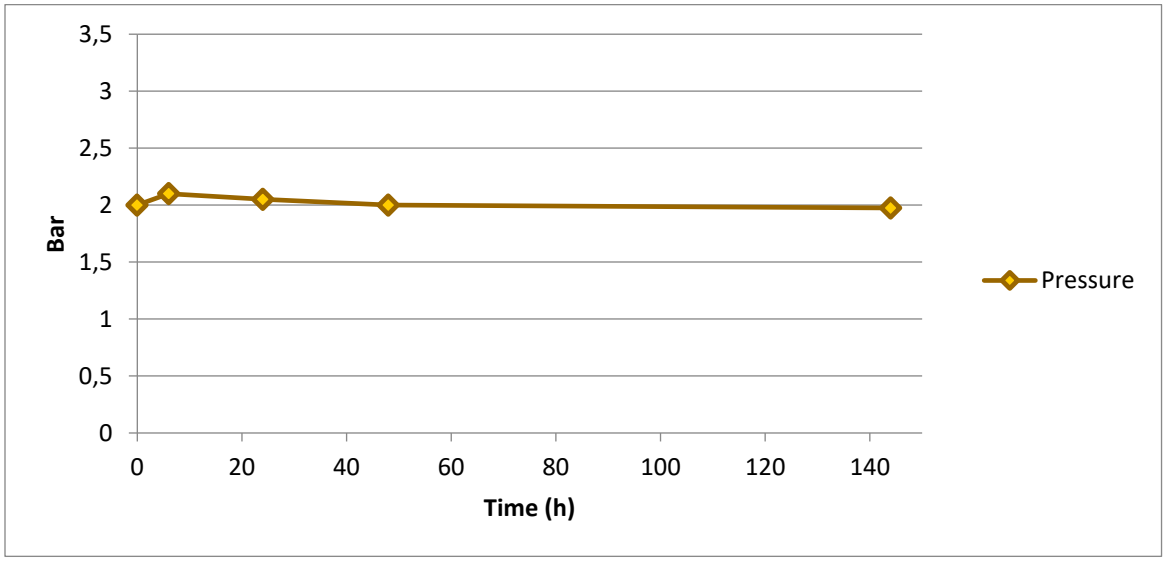


Figure 17 – The pressure variation inside the fermentation bottles along the autotrophic fermentation (A1);

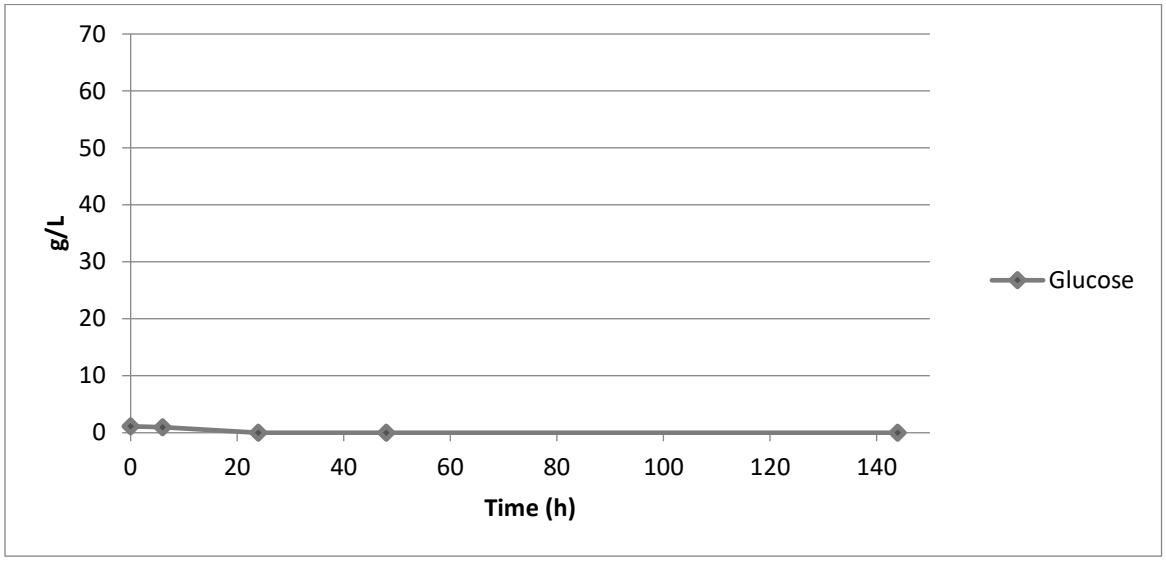


Figure 18 – The glucose content variation along the autotrophic fermentation (A1);

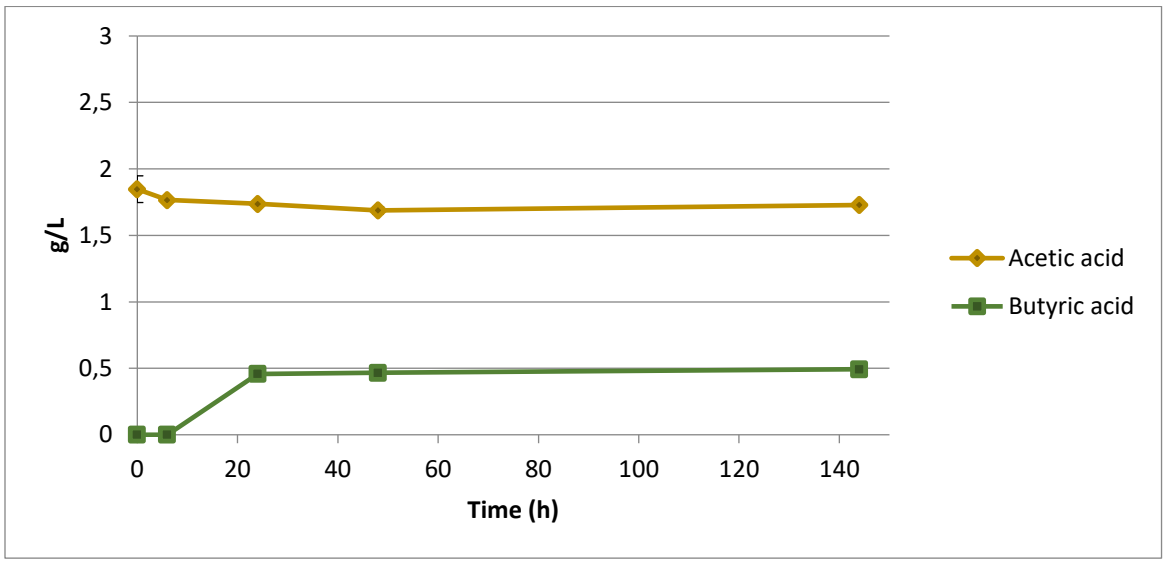


Figure 19 – The acetic and butyric acids content variation along the autotrophic fermentation (A1);

Autotrophic fermentation, CO_2 (15%) + H_2 (5%) (A2);

All the fermentations that were supplemented with H_2 were performed inside the anaerobic chamber (15% CO_2, 5% H_2 and 80% N_2). To allow the contact of the air inside the cabinet with the fermentation medium, the bottles were left with the lid unscrewed. Therefore, there was no need to release overpressures from inside the bottles. The samplings were always made inside the anaerobic chamber. Because there was not a shaking mechanism inside the chamber, the fermentations supplemented with H_2 were not tested with shaking.

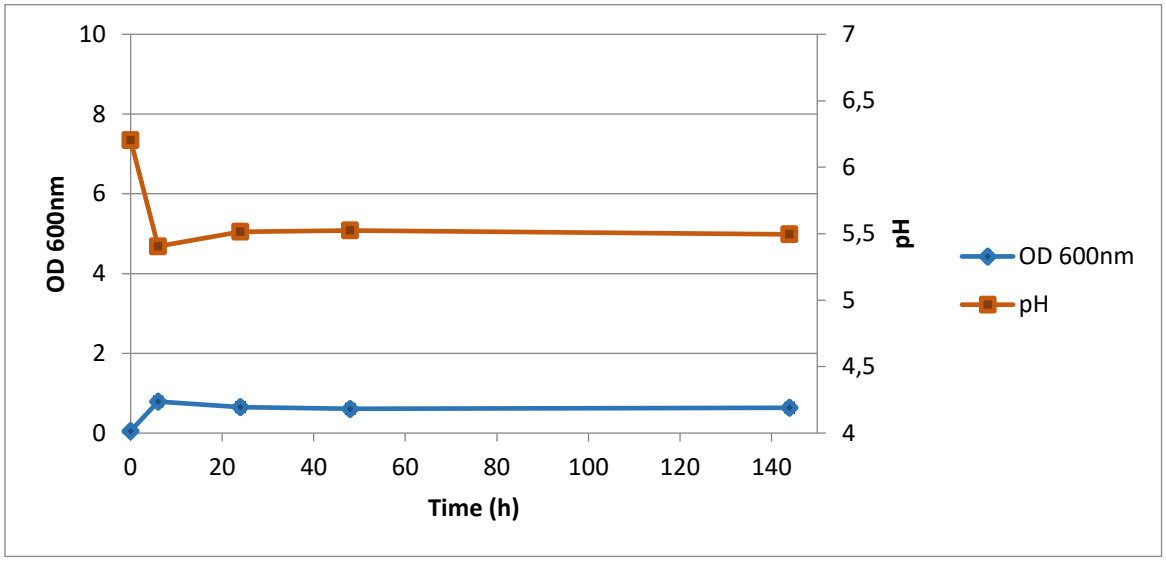


Figure 20 – The OD (600nm) and pH variation along the autotrophic fermentation (A2);

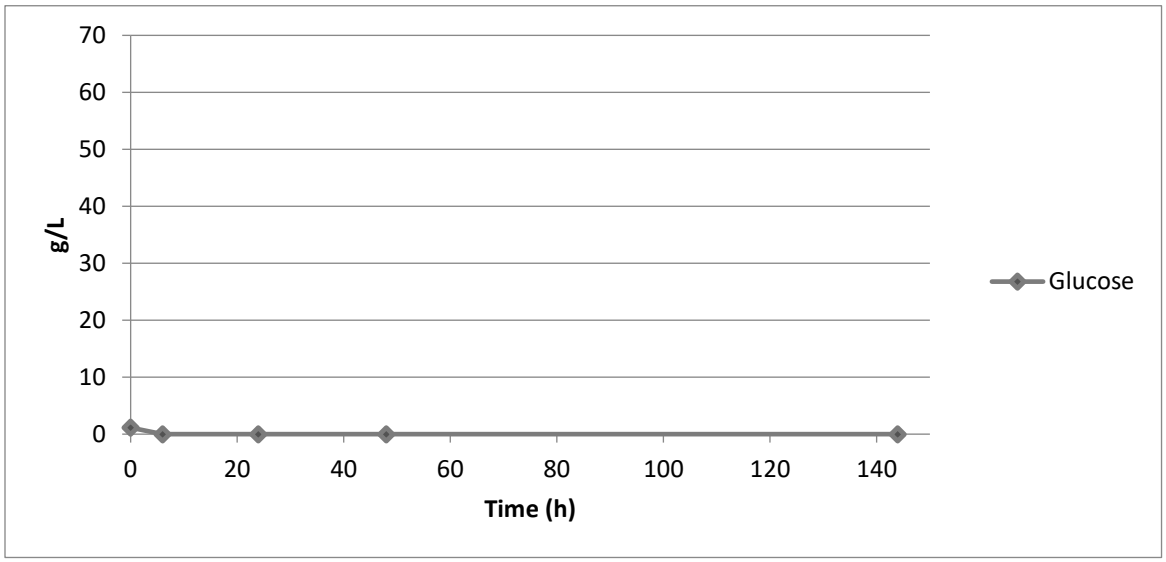


Figure 21 – The glucose content variation along the autotrophic fermentation (A2);

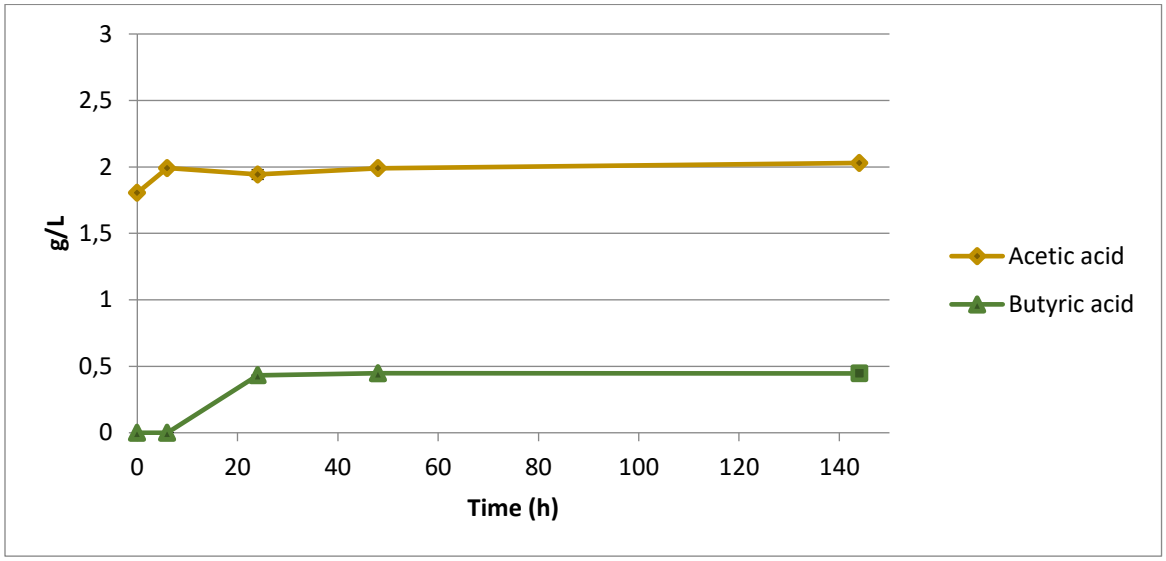


Figure 22 – The acetic and butyric acids content variation along the autotrophic fermentation (A2);

Mixotrophic fermentation, 60 g/L glucose + CO_2 (2bar) (M1);

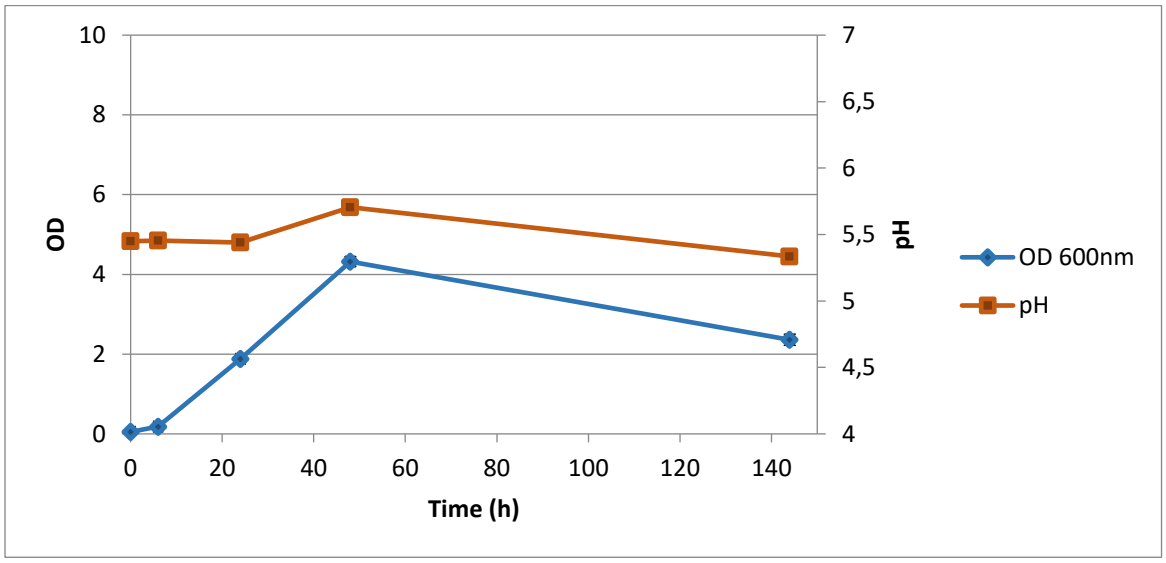


Figure 23 – The OD (600nm) and pH variation along the mixotrophic fermentation (M1);

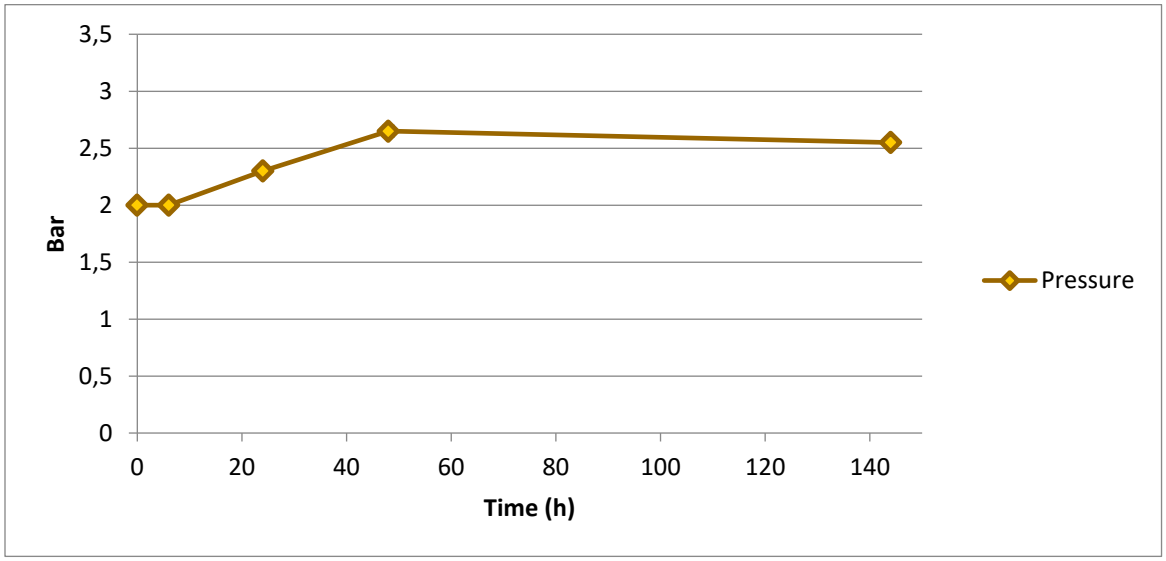


Figure 24 – The pressure variation inside the fermentation bottles along the mixotrophic fermentation (M1);

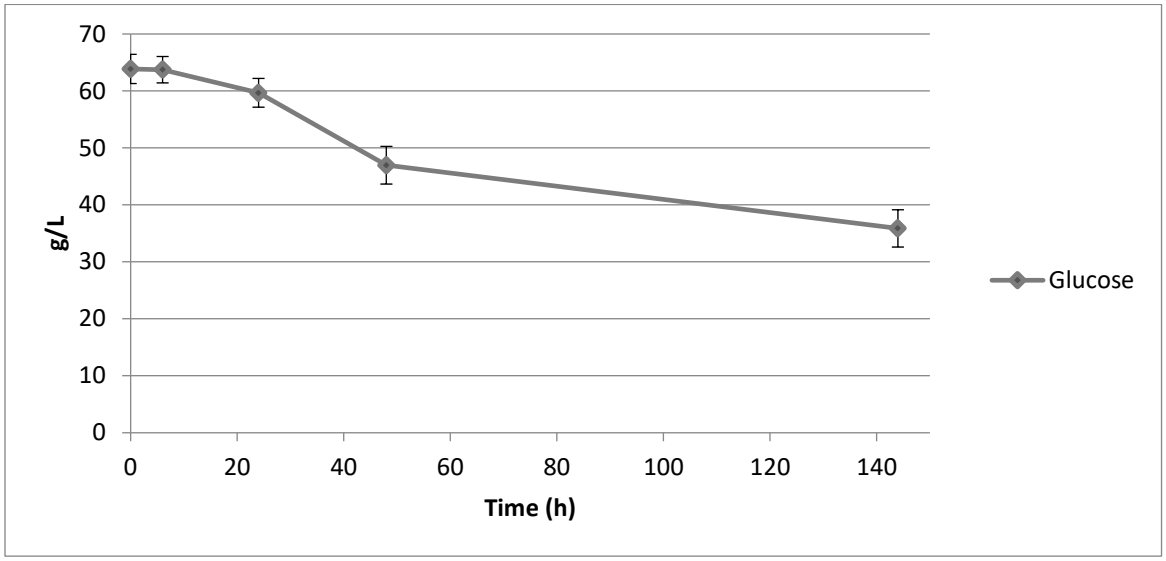


Figure 25 – The glucose content variation along the mixotrophic fermentation (M1);

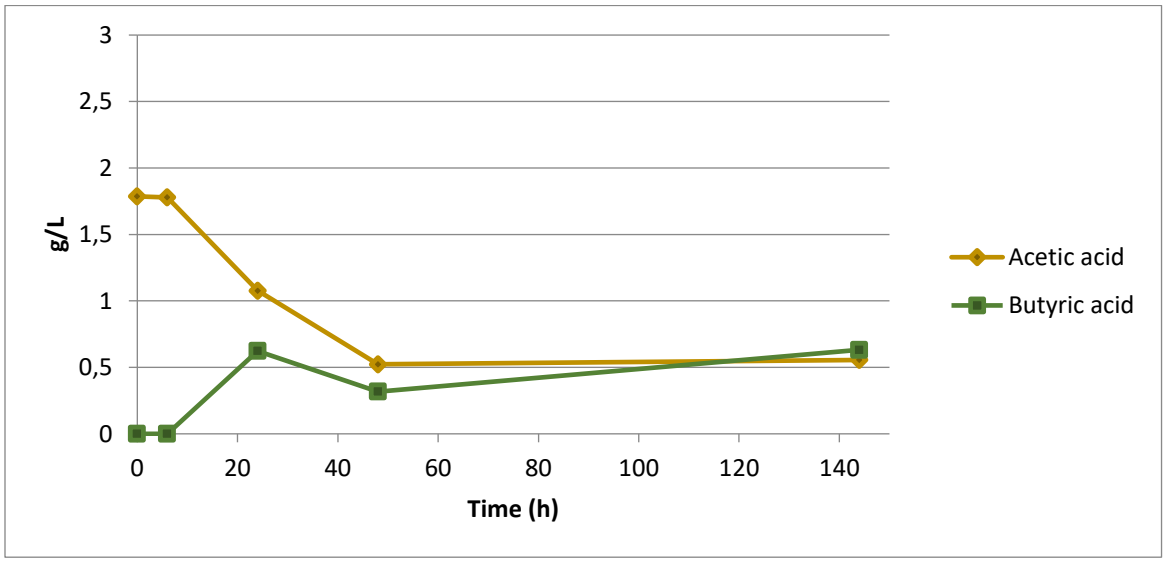


Figure 26 – The acetic and butyric acids content variation along the mixotrophic fermentation (M1);

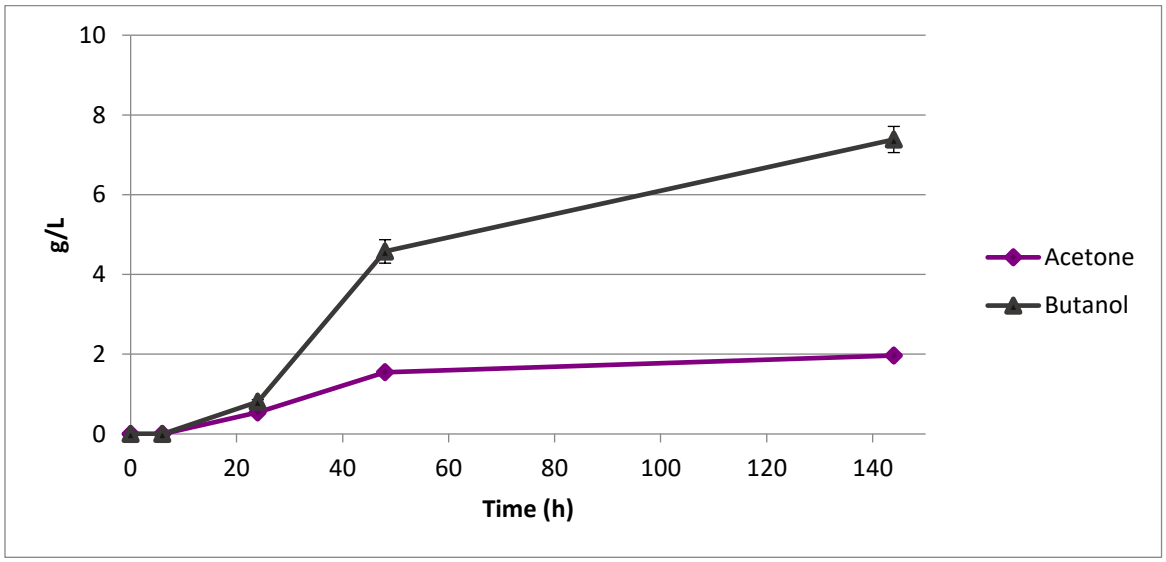


Figure 27 – The acetone and butanol content variation along the mixotrophic fermentation (M1);

Mixotrophic fermentation, 60 g/L glucose + CO_2 (2bar) + 50rpm shaking (M2);

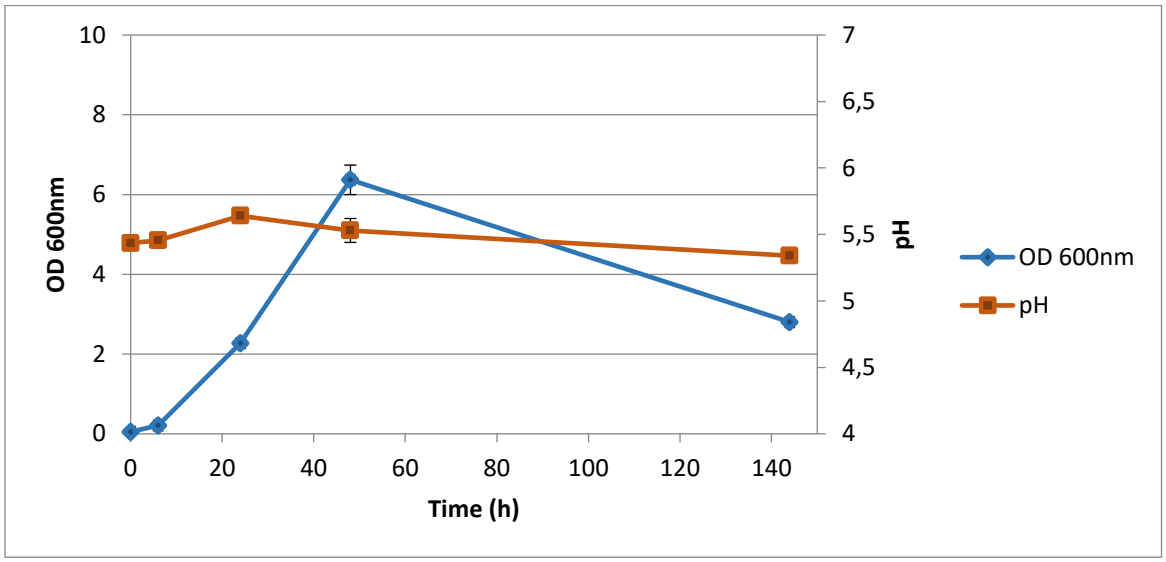


Figure 28 – The OD (600nm) and pH variation along the mixotrophic fermentation (M2);

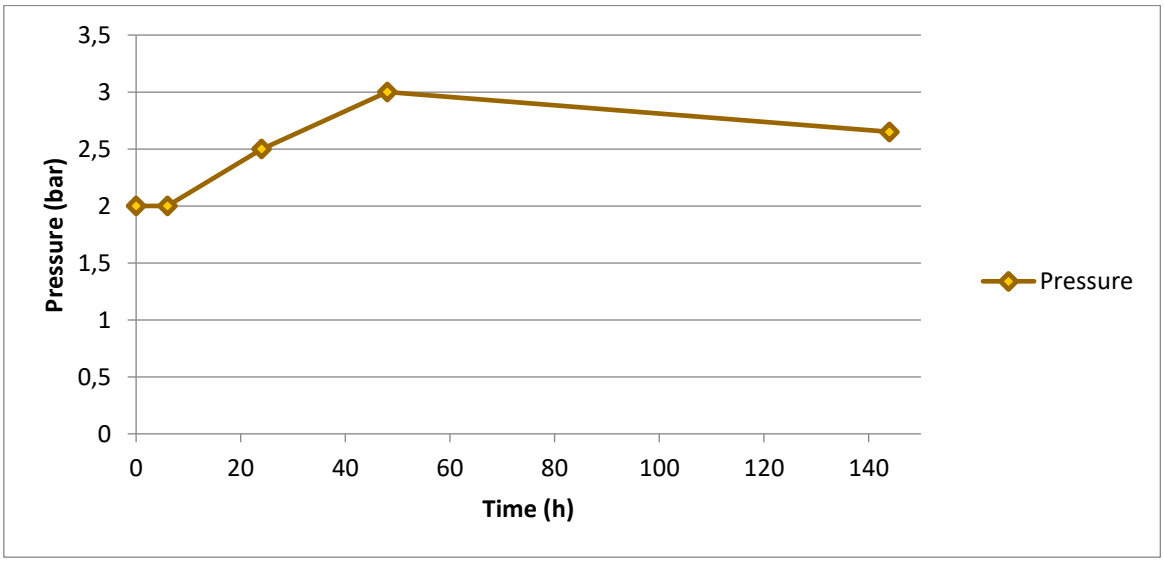


Figure 29 – The pressure variation inside the fermentation bottles along the mixotrophic fermentation (M2);

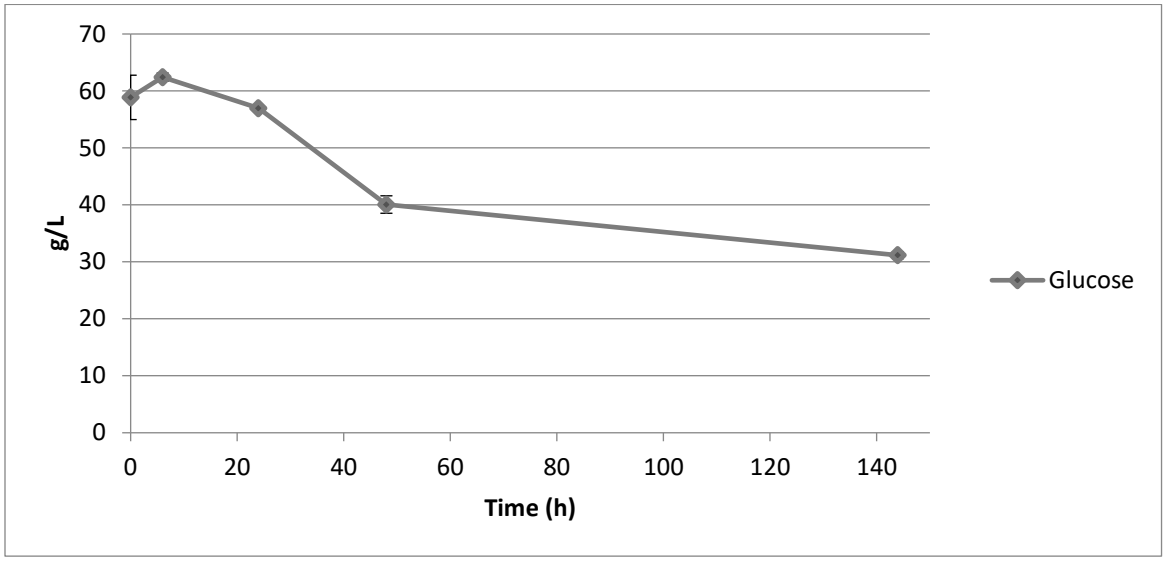


Figure 30 – The glucose content variation along the mixotrophic fermentation (M2);

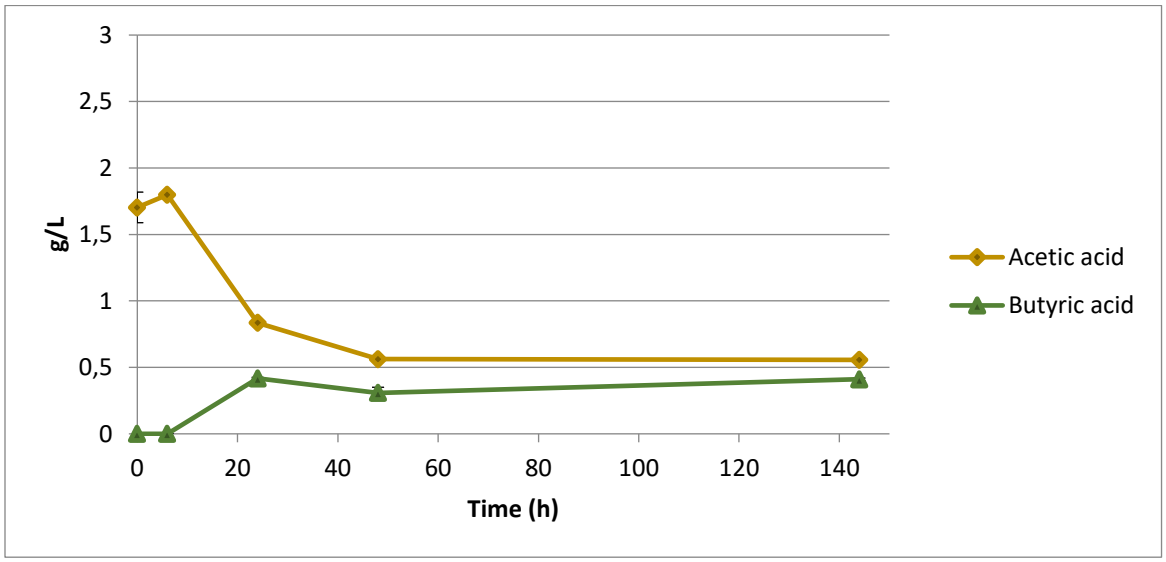


Figure 31 – The acetic and butyric acids content variation along the mixotrophic fermentation (M2);

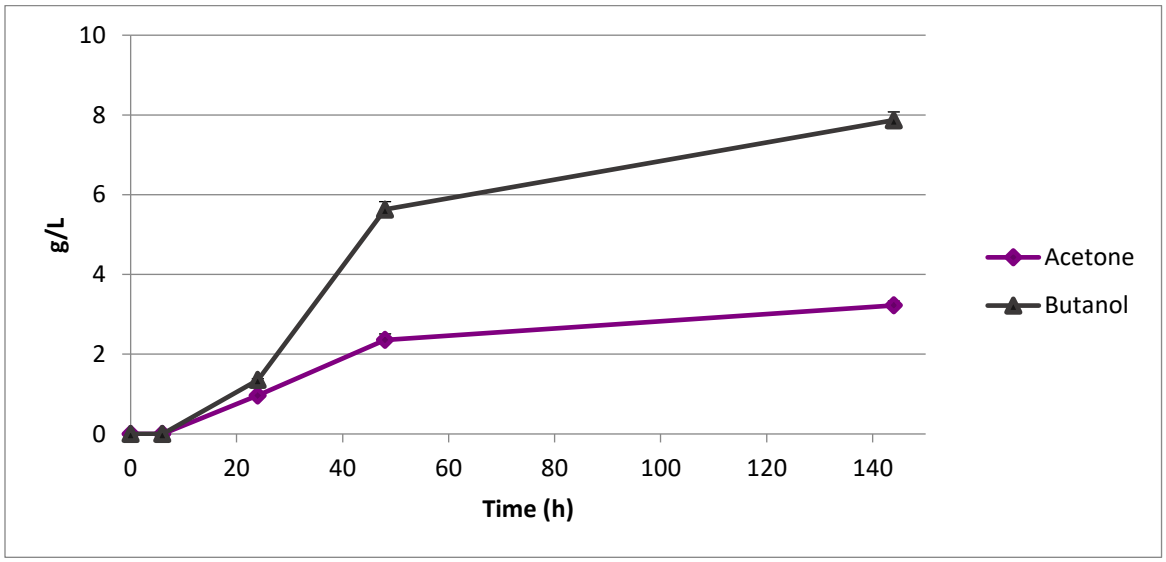


Figure 32 – The acetone and butanol content variation along the mixotrophic fermentation (M2);

Mixotrophic fermentation, 60 g/L glucose + CO_2 (15%) + H_2 (5%) (M3);

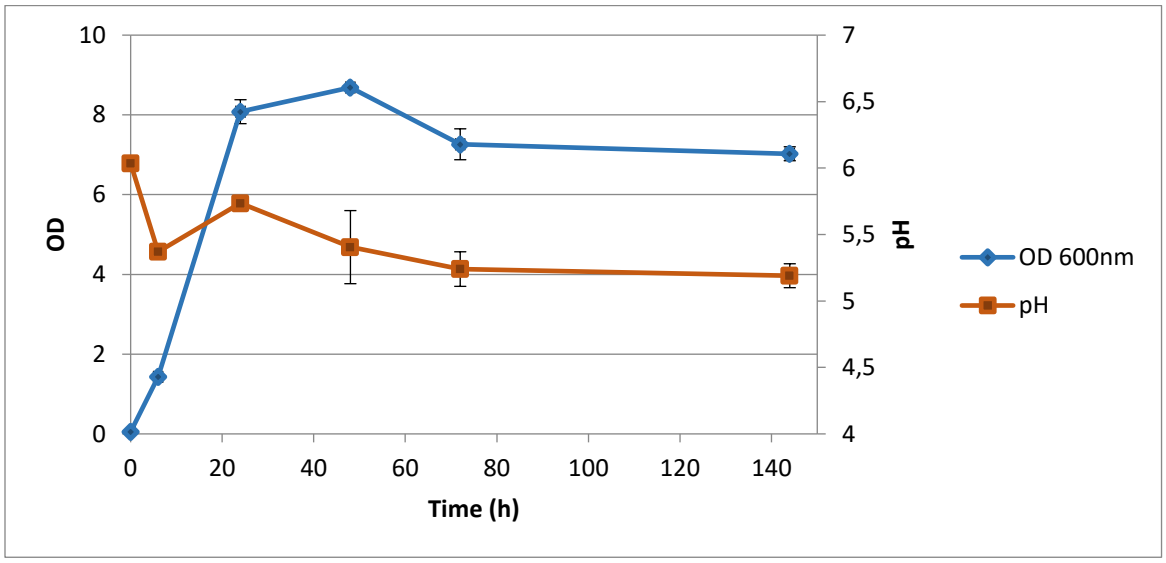


Figure 33 – The OD (600nm) and pH variation along the mixotrophic fermentation (M3);

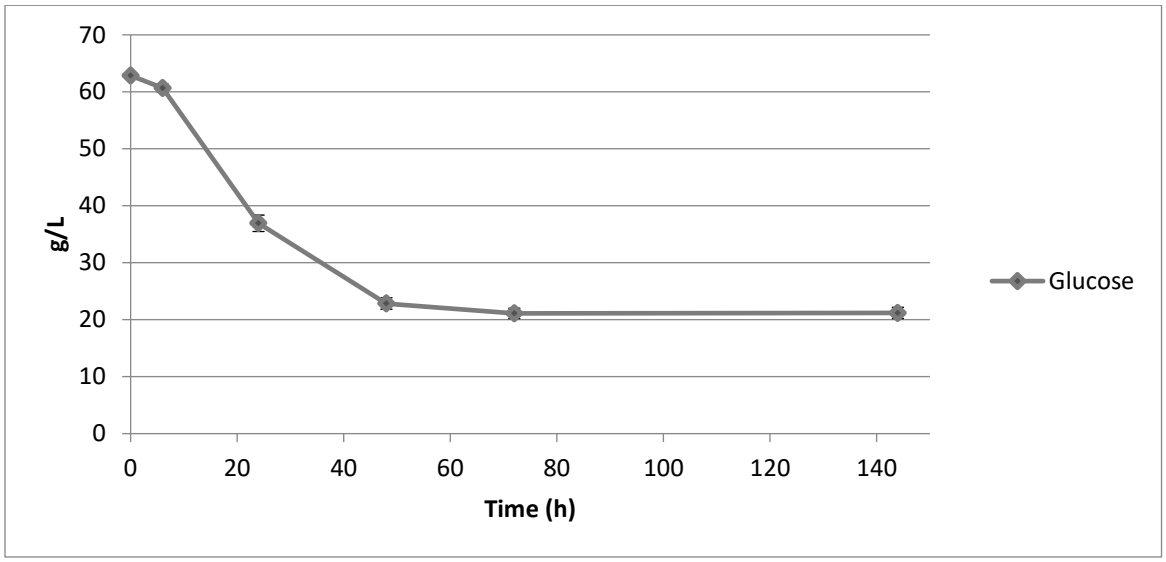


Figure 34 – The glucose content variation along the mixotrophic fermentation (M3);

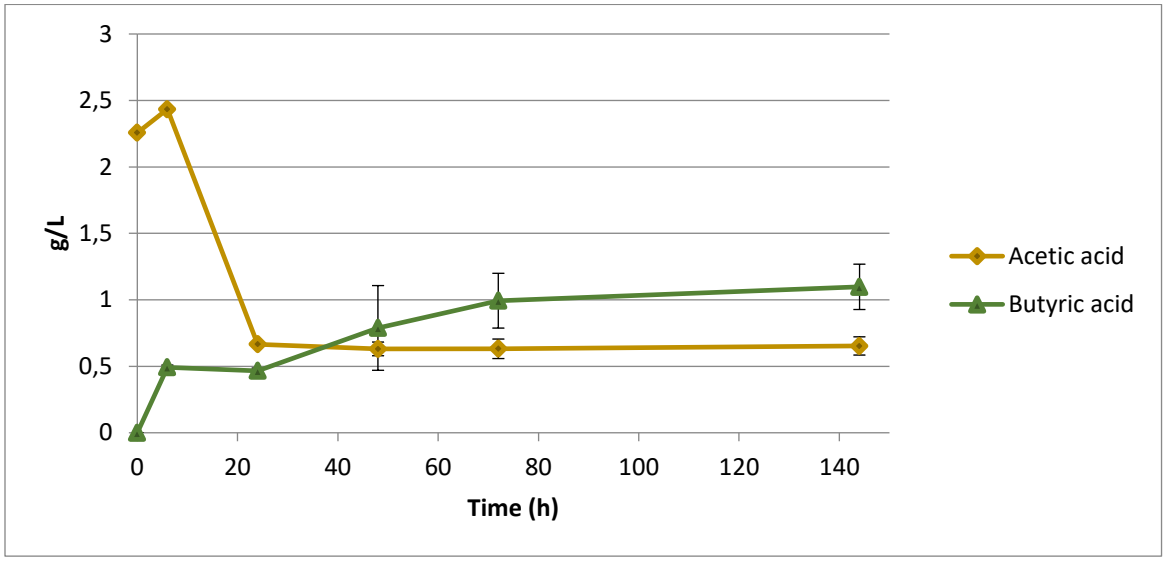


Figure 35 – The acetic and butyric acids content variation along the mixotrophic fermentation (M3);

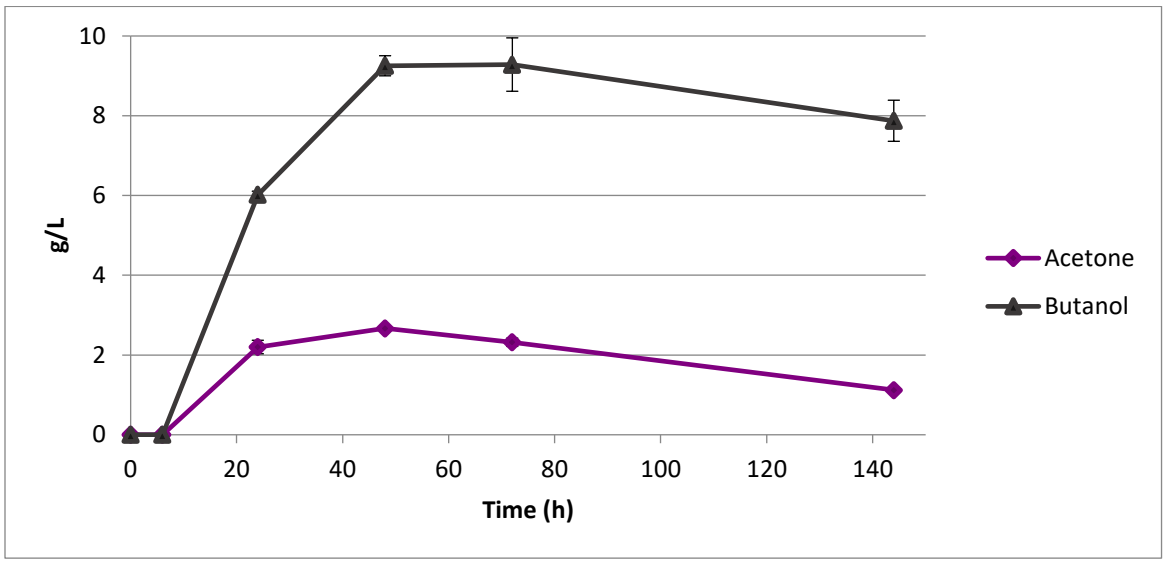


Figure 36 – The ABE content variation along the mixotrophic fermentation (M3);

Results overview;

To facilitate the results comparison between the different fermentation conditions, the results were compiled side by side in column charts. Data obtained from the different fermentation parameters analysed were submitted to hypothesis testing using Excel equations. The pseudo-F values in the Excel main tests were evaluated in terms of significance. When the main test revealed statistical significant differences ($p \leq 0.05$), pairwise comparisons were performed. The t-statistics in the pairwise comparisons were evaluated in terms of significance. Values lower than 0.05 were considered as significantly different.

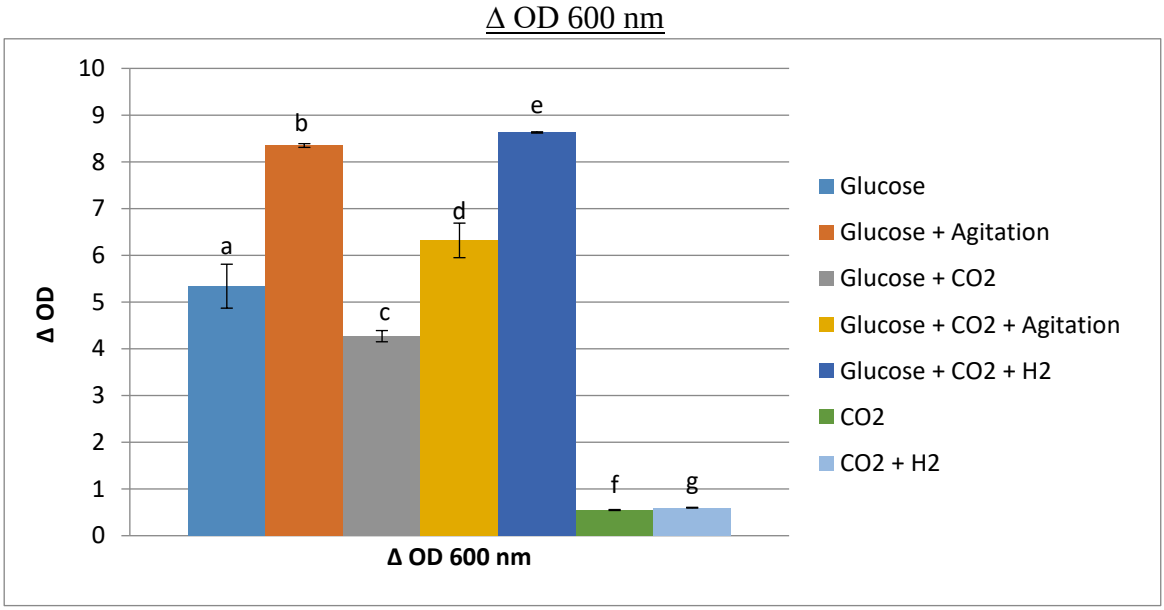


Figure 37 – The OD (600nm) variation for all the analysed fermentation conditions. Significant differences ($p \leq 0.05$) among values are presented with different letters;

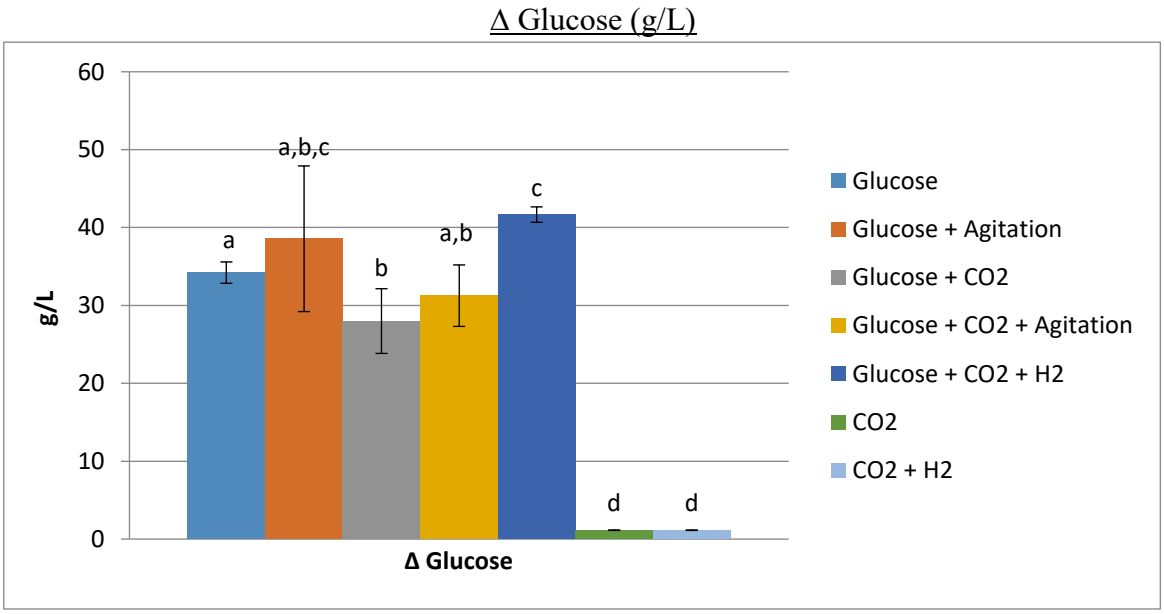


Figure 38 – The glucose content variation for all the analysed fermentation conditions. Significant differences ($p \leq 0.05$) among values are presented with different letters;

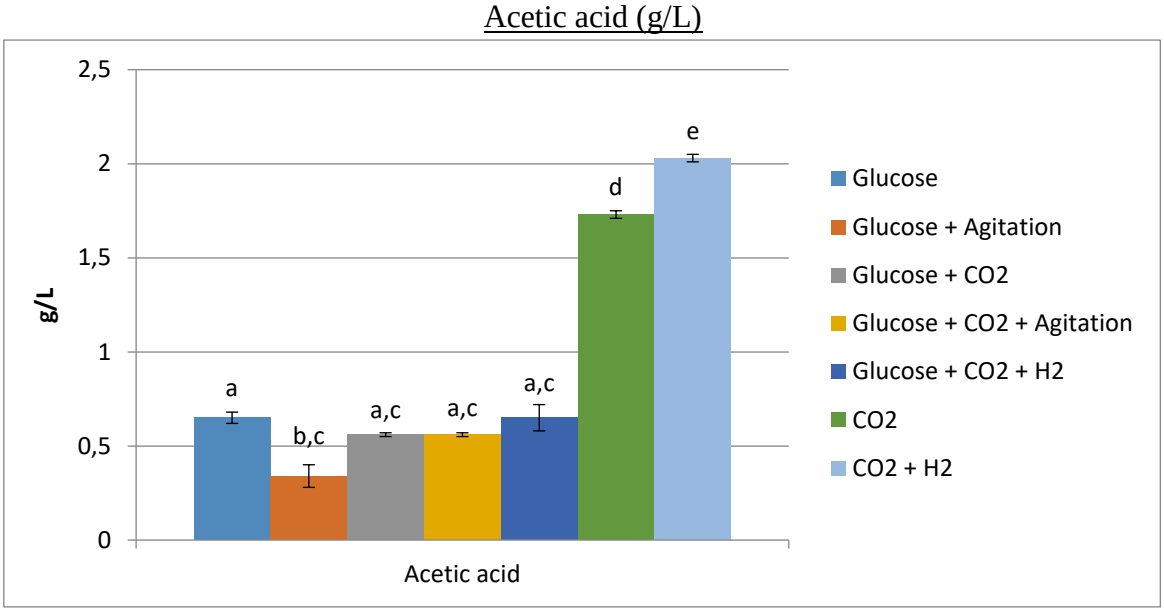


Figure 39 – The acetic acid content at the end of all the analysed fermentation conditions. Significant differences ($p \leq 0.05$) among values are presented with different letters;

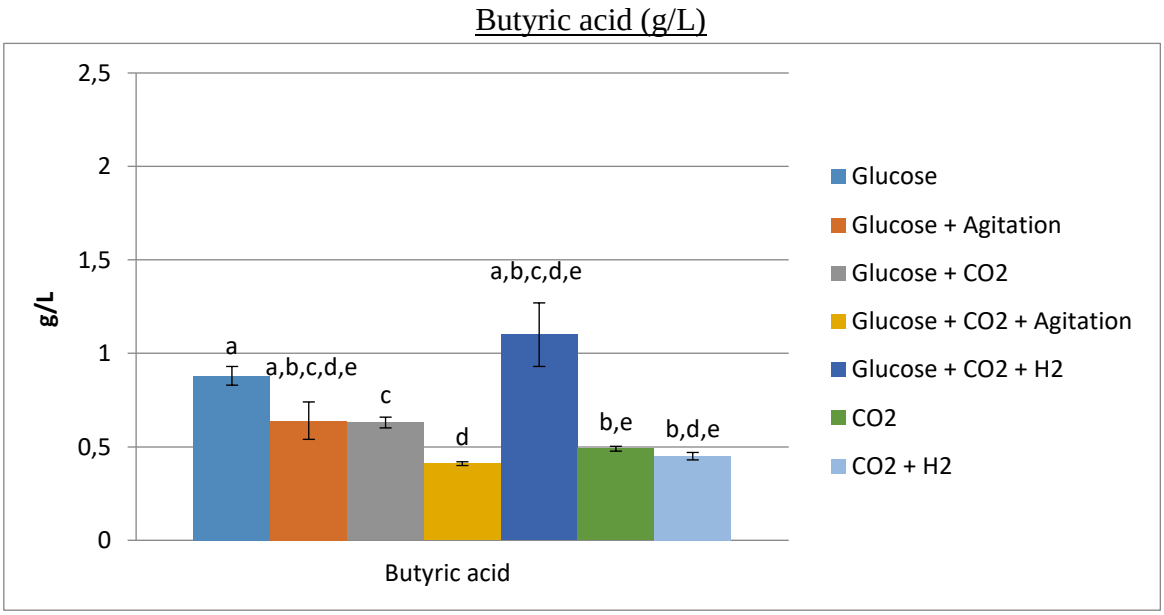


Figure 40 – The butyric acid content at the end of all the analysed fermentation conditions. Significant differences ($p \leq 0.05$) among values are presented with different letters;

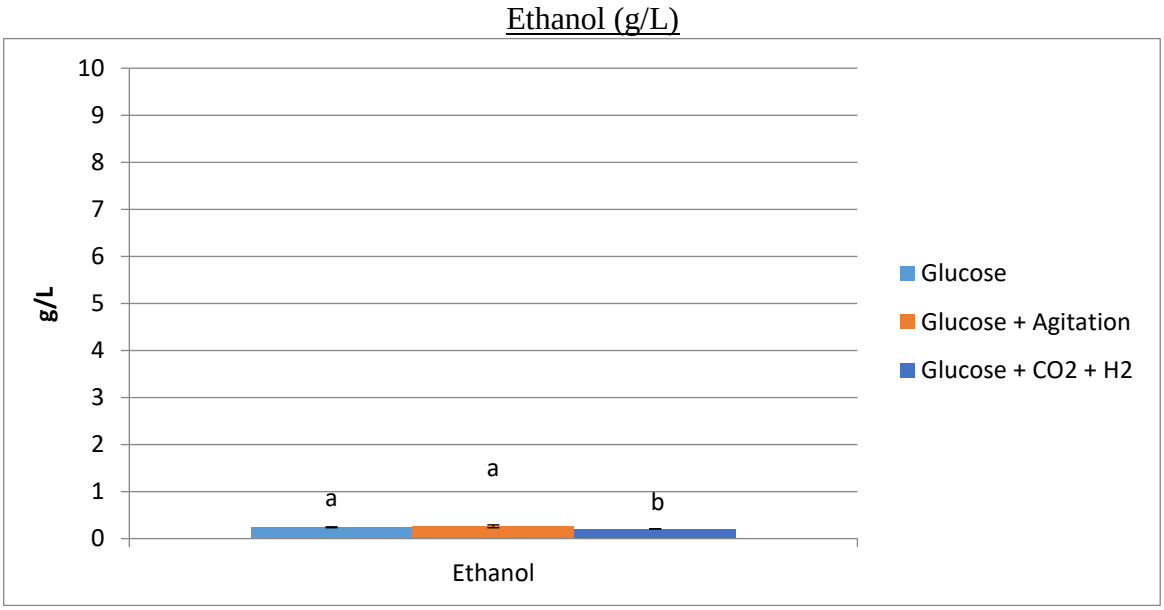


Figure 41 – The ethanol content at the end of all the analysed fermentation conditions. Significant differences ($p \leq 0.05$) among values are presented with different letters;

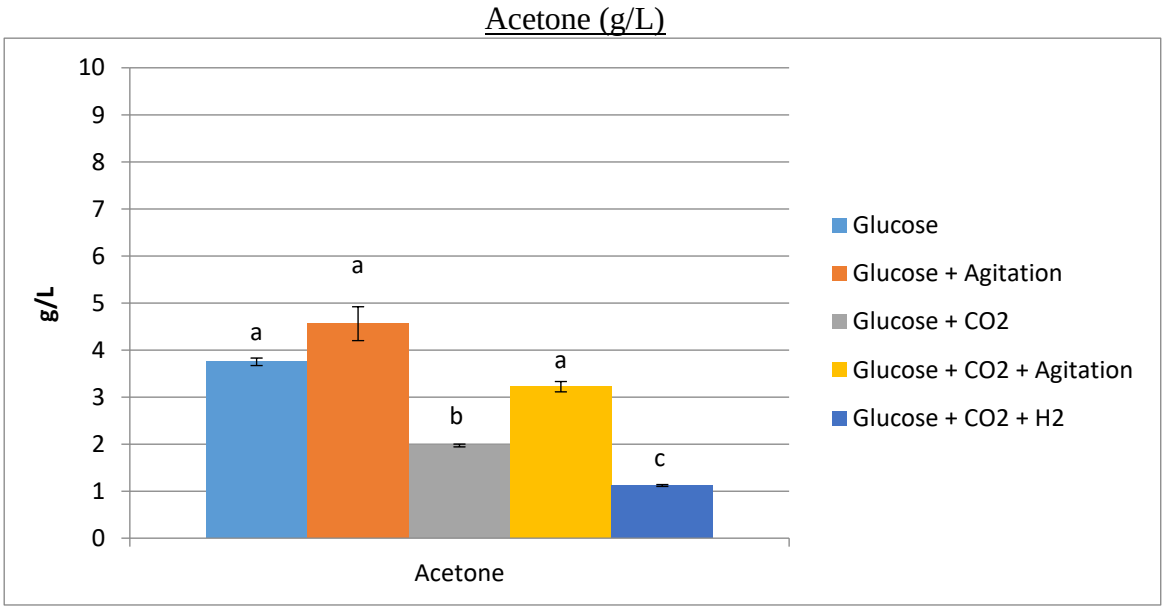


Figure 42 – The acetone content at the end of all the analysed fermentation conditions. Significant differences ($p \leq 0.05$) among values are presented with different letters;

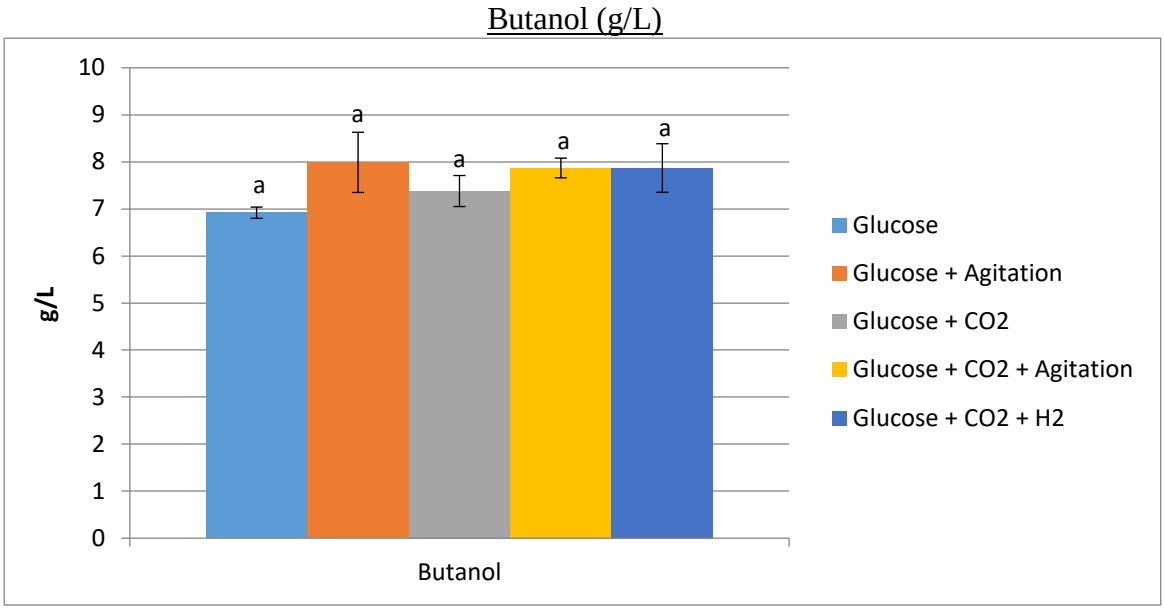


Figure 43 – The butanol content at the end of all the analysed fermentation conditions. Significant differences ($p \leq 0.05$) among values are presented with different letters;

	Mass concentration (g/L)						
	GLC	**Glc + Agitation**	**CO2**	**CO2 + H2**	**Glc + CO2**	**Glc + CO2 + Agitation**	**Glc + CO2 + H2**
Sugars at t = 0 h							
Glucose	62,95	63,26	1,13	1,13	63,87	62,41	62,85
Sugars at t = 144 h							
Glucose	28,74	24,7	0	0	35,88	31,16	21,19
Sugars consumed							
Glucose	34,21	38,56	1,13	1,13	27,99	31,25	41,66
Products at t = 144 h							
Acetate	0,65	0,34	1,73	2,03	0,56	0,56	0,65
Butyrate	0,88	0,64	0,49	0,45	0,63	0,41	1,1
Propionate	0	0	0	0	0	0	0
Acetone	3,75	4,56	0	0	1,97	3,22	1,12
Ethanol	0,24	0,26	0	0	0	0	0,21
Butanol	6,92	7,99	0	0	7,38	7,87	7,87
Total ABE	10,91	12,81	0	0	9,35	11,09	9,2
Yields							
g ABE / g substrate	0,319	0,332	0,000	0,000	0,334	0,355	0,221
g Butanol / g substrate	0,202	0,207	0,000	0,000	0,264	0,252	0,189

Table 7 - Representation, in g/L, of the glucose concentration at the beginning and at the end of the analysed fermentations and the respective glucose consumption. The fermentation products concentration, at the end of each fermentation, is also represented as well as the ABE and butanol yields, in g of product per g of glucose consumed;

	Molar concentration (mM)						
	GLC	**Glc + Agitation**	**CO2**	**CO2 + H2**	**Glc + CO2**	**Glc + CO2 + Agitation**	**Glc + CO2 + H2**
Sugars at t = 0 h							
Glucose	349	351	6	6	355	346	349
Sugars at t = 144 h							
Glucose	160	137	0	0	199	173	118
Sugars consumed							
Glucose	190	214	6	6	155	173	231
Products at t = 144 h							
Acetate	11	6	29	34	9	9	11
Butyrate	10	7	6	5	7	5	12
Propionate	0	0	0	0	0	0	0
Acetone	65	79	0	0	34	55	19
Ethanol	5	6	0	0	0	0	5
Butanol	93	108	0	0	100	106	106
Total ABE	163	192	0	0	133	162	130

Table 8 - Representation, in mM, of the glucose concentration at the beginning and at the end of the analysed fermentations and the respective glucose consumption. The fermentation products concentration, at the end of each fermentation, is also represented;

Carbon Balance

	Carbon Number	CO2 Released per mol of product	Total mmol Carbon						
			GLC	Glc + Agitation	CO2	CO2 + H2	Glc + CO2	Glc + CO2 + Agitation	Glc + CO2 + H2
Sugars at t = 0 h									
Glucose	6	0	2096	2107	38	38	2127	2078	2093
Sugars at t = 144 h									
Glucose	6	0	957	823	0	0	1195	1038	706
Products at t = 144 h									
Acetate	2	1	32	17	86	101	28	28	32
Butyrate	4	2	60	44	33	31	43	28	75
Propionate	3	0	0	0	0	0	0	0	0
Acetone	3	3	387	471	0	0	204	333	116
Ethanol	2	1	16	17	0	0	0	0	14
Butanol	4	2	560	647	0	0	597	637	637
mmol carbon in biomass			2,68	4,32	0,08	0,11	2,1	3,22	4
Carbon Recovery			93%	93%	318%	351%	94%	99%	63%

Table 9 - Representation of the carbon balance in all the analysed fermentations. These results allow the understanding of how much of the carbon consumed as glucose was effectively directed to the production of each fermentation product. The carbon balances were calculated according to the equation ((mM of the compound) x (carbon number of the compound)) + ((mM of the compound) x (CO_2 released per mol of the compound));

	OD t=0 h	OD t=48 h	ΔOD	Biomass (g/L)	Mass (g) /41 mL	C in biomass (mmol)
Glc	0,051	5,390	5,340	1,697	0,0696	2,68
Glc + Agitation	0,052	8,400	8,350	2,731	0,1120	4,32
CO2	0,058	0,605	0,550	0,051	0,0021	0,08
CO2 + H2	0,053	0,653	0,600	0,069	0,0028	0,11
Glc + CO2	0,050	4,320	4,270	1,330	0,0545	2,10
GLC + CO2 + Agitation	0,050	6,370	6,320	2,034	0,0834	3,22
GLC + CO2 + H2	0,071	7,510	7,439	2,419	0,0992	3,82

Assumptions:	1 g/L biomass = (OD-0.4)/2.91
	46.30% of biomass is carbon

Table 10 – Representation of the amount, in g/L, of biomass produced after the exponential phase in all the analysed fermentations, its respective mass and the amount of carbon directed to its production;

Step 2 - Bioinformatics;

Characterization and cataloguing of all the *Clostridium autoethanogenum* DSM 10061 genes involved in the WLP:

Table 11 – Characterization of all the genes involved in the *Clostridium autoethanogenum* DSM 10061 WLP;

***C. autoethanogenum* DSM-10061**			
Genes	**Protein**	**Predicted Function**	**Reference**
CAETHG_2790	U5RWP5	Formate dehydrogenase, alpha subunit	MaGe
CAETHG_2988	U5RWP7	Formate dehydrogenase, alpha subunit	MaGe/Liew et al., 2016
CAETHG_0084	U5RRY3	Nitrate reductase/ formate dehydrogenase major subunit	Kegg/Liew et al., 2016
CAETHG_2789	U5RW64	Molybdopterin oxidoreductase/ formate dehydrogenase major subunit	Kegg/Liew et al., 2016
CAETHG_1618	U5RWC6	Formate-tetrahydrofolate ligase	MaGe/Liew et al., 2016
CAETHG_1617	U5RWY6	Methenyltetrahydrofolate cyclohydrolase	MaGe/Liew et al., 2016
CAETHG_1616	U5RTD1	Bifunctional protein FolD	Kegg/Liew et al., 2016
CAETHG_1615	U5RSX6	Methylene-tetrahydrofolate reductase domain-containing protein	MaGe/Liew et al., 2016
CAETHG_1614	U5RT18	Methylenetetrahydrofolate reductase	MaGe/Liew et al., 2016
CAETHG_1608	U5RWA4	CO dehydrogenase/acetyl-CoA synthase complex, beta subunit	Kegg/MaGe/Liew et al., 2016
CAETHG_1609	U5RSZ7	5-methyltetrahydrofolate corrinoid/iron sulfur protein methyltransferase	MaGe/Liew et al., 2016
CAETHG_1610	U5RSW0	Acetyl-CoA decarbonylase/synthase complex subunit gamma	Kegg/MaGe/Liew et al., 2016
CAETHG_1611	U5RTB6	Acetyl-CoA decarbonylase/synthase complex subunit delta	Kegg/MaGe/Liew et al., 2016
CAETHG_1620	U5RSY1	Carbon-monoxide dehydrogenase (Acceptor)	Kegg/MaGe/Liew et al., 2016/Brown et al., 2014
CAETHG_1621	U5RTE2	Carbon-monoxide dehydrogenase (Acceptor)	Kegg/MaGe/Liew et al., 2016/Brown et al., 2014
CAETHG_3005	U5S0R8	Carbon-monoxide dehydrogenase, catalytic subunit	Kegg/MaGe/Liew et al., 2016/Brown et al., 2014
CAETHG_3899	U5RZD4	Carbon-monoxide dehydrogenase, catalytic subunit	Kegg/MaGe/Liew et al., 2016/Brown et al., 2014

Each colour in the previous table corresponds to a different metabolic step, within the WLP. In the top of the table, the first metabolic steps are represented with light colours that become darker along the table as it progresses to further metabolic steps. The next figure represents the WLP, in which each metabolic step is represented by the respective colour used in the table:

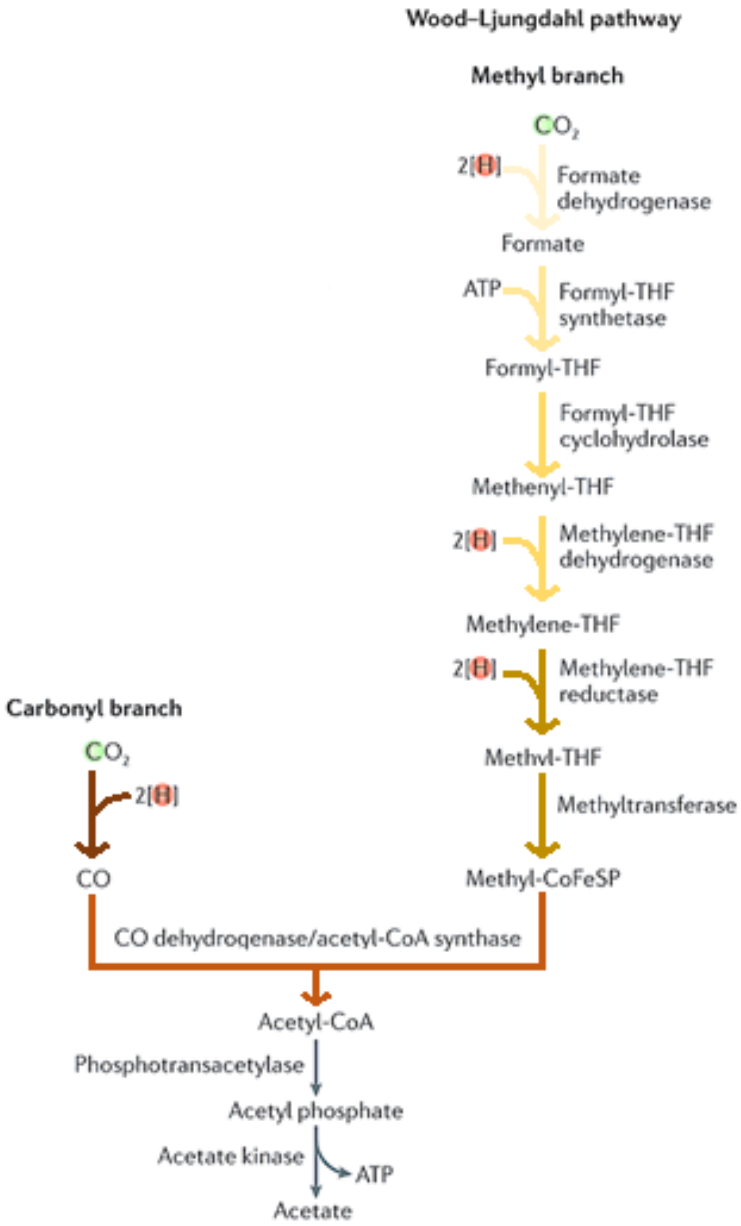


Figure 44 – Illustration of the WLP in which each metabolic step is represented with an arrow painted with the colour that represents the group of genes, in the previous table, responsible for performing the considered step. Figure adapted from (Fast et al., 2015);

Once identified all the *Clostridium autoethanogenum* DSM 10061 WLP genes, the GeneBank and MaGe platforms were used to obtain their FASTA sequences. BLASTn alignments were then made between the FASTA sequences and the *C. beijerinckii* NCIMB 8052 genome. The genes with statistical significant alignments ($E < e^{-10}$ and positive percentage above 50%) were selected and organized in a table (table 8).

Table 12 - *C. beijerinckii* NCIMB 8052 genes with statistical significant alignments ($E < e^{-10}$ and positive percentage above 50%) with the *Clostridium autoethanogenum* DSM 10061 WLP genes;

***C. Beijerinckii* NCIMB 8052**				
Homologous Protein	**Predicted Function**	**Identity**	**Positive**	**E-value**
Cbei_3801	Formate dehydrogenase subunit alpha	70.22	85.56	2.27007e-242
		72.47	85.81	0
		46.44	66.73	5.47466e-143
		81.88	90.58	5.62964e-67
Cbei_0101	Formate-tetrahydrofolate ligase	62.23	78.78	4.2217e-204
Cbei_1702	Bifunctional protein FolD [Includes: Methylenetetrahydrofolate dehydrogenase ; Methenyltetrahydrofolate cyclohydrolase]	42.61	60.56	1.79398e-58
Cbei_1828	Bifunctional homocysteine S-methyltransferase/5,10-methylenetetrahydrofolate reductase protein	29.29	54.29	5.68899e-31
Cbei_3020	Carbon-monoxide dehydrogenase, catalytic subunit	38.70	53.91	2.55565e-38
CAC2498	Carbon monoxide dehydrogenase, catalytic subunit (cooS)	38.70	51.30	9.71145e-38
Cbei_5054	Carbon-monoxide dehydrogenase, catalytic subunit	32.00	54.00	2.02966e-27
Cbei_5054	Carbon-monoxide dehydrogenase, catalytic subunit	28.68	50.79	2.99035e-44
Cbei_3020	Carbon-monoxide dehydrogenase, catalytic subunit	27.30	48.47	1.10063e-38
Cbei_5054	Carbon-monoxide dehydrogenase, catalytic subunit	71.75	86.52	9.50663e-269
Cbei_3020	Carbon-monoxide dehydrogenase, catalytic subunit	36.92	56.95	6.67944e-105
Cbei_3020	Carbon-monoxide dehydrogenase, catalytic subunit	78.16	89.08	1.45252e-296
Cbei_5054	Carbon-monoxide dehydrogenase, catalytic subunit	35.07	56.28	9.95388e-104

According to the BLASTn alignments, a group of genes, responsible for encoding the enzymes that catalyse one of the WLP steps, were completely missing from the *C. beijerinckii* NCIMB 8052 genome. The missing enzymes are responsible for catalysing a WLP key step, in which the convergence between the carbonyl branch and the methyl branch occurs to produce an acetyl-CoA (figure 45).

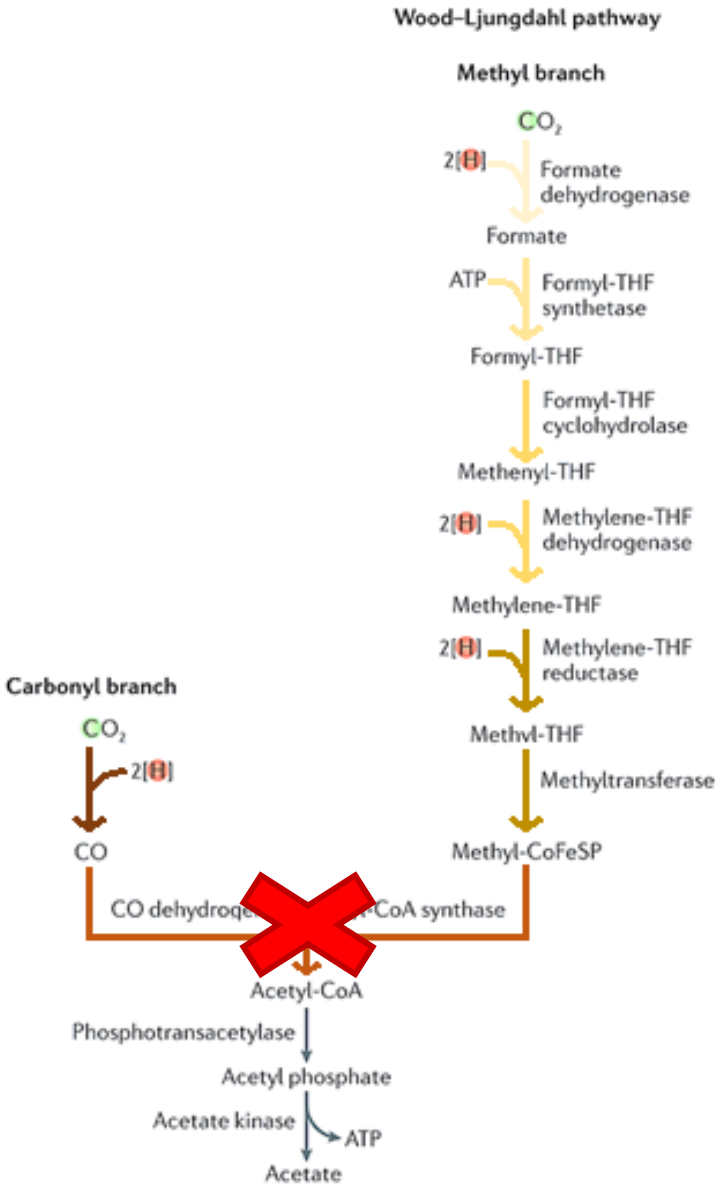


Figure 45 – Illustration of the WLP with a cross above the metabolic step considered as missing in *C. beijerinckii* NCIMB 8052. Figure adapted from (Fast et al., 2015);

Considering all the previous BLASTn alignments, some genes were chosen as the most promising to activate the WLP in *C. beijerinckii* NCIMB 8052. The selected genes are illustrated bellow in order of relevance:

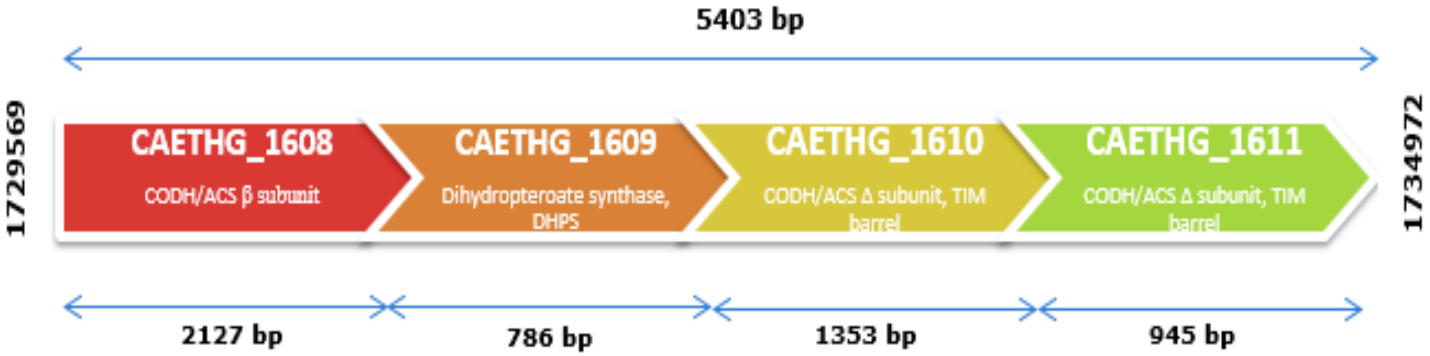


Figure 46 – Illustration of the cluster CAETHG_1608-1611, that includes all the genes responsible for encoding the bifunctional enzyme Carbon monoxide dehydrogenase/Acetyl-CoA synthase (CODH/ACS), that catalyses the metabolic step that is missing in *C. beijerinckii* NCIMB 8052;

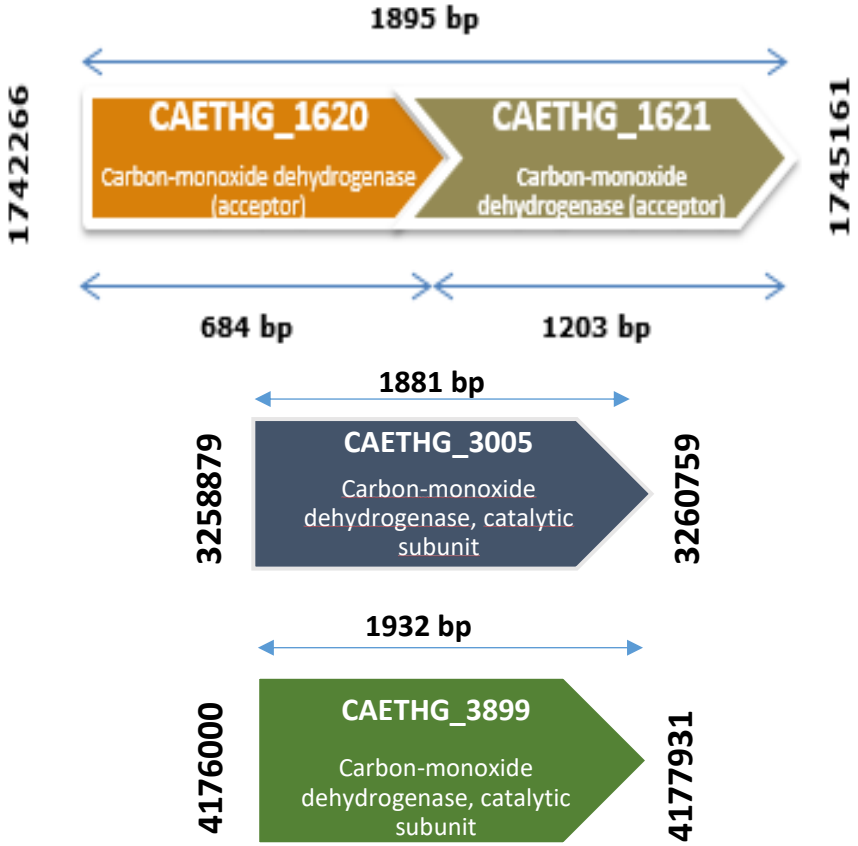


Figure 47 – Illustration of the cluster CAETHG_1620-1621 and the genes CAETHG_3005 and CAETHG_3899, that are responsible for encoding the enzymatic complex that catalyses the reduction of CO_2 to CO in the *Clostridium autoethanogenum* DSM 10061 WLP;

Step 3 – Genetic manipulation;

Once the genes of interest have been selected, the next step consisted in cloning and overexpressing the genes in *C. beijerinckii* NCIMB 8052. The results from this chapter are presented as pictures of the electrophoresis gels obtained in each cloning attempt. In all the electrophoresis, a GeneRuler DNA ladder mix (BIOGEN) was used to estimate the fragment's sizes. Prior to cloning, it was necessary to extract the C. *beijerinckii* NCIMB 8052 and *C. autoethanogenum* DSM 10061 genomes. The extracted genomes were analysed by electrophoresis (figure 48).

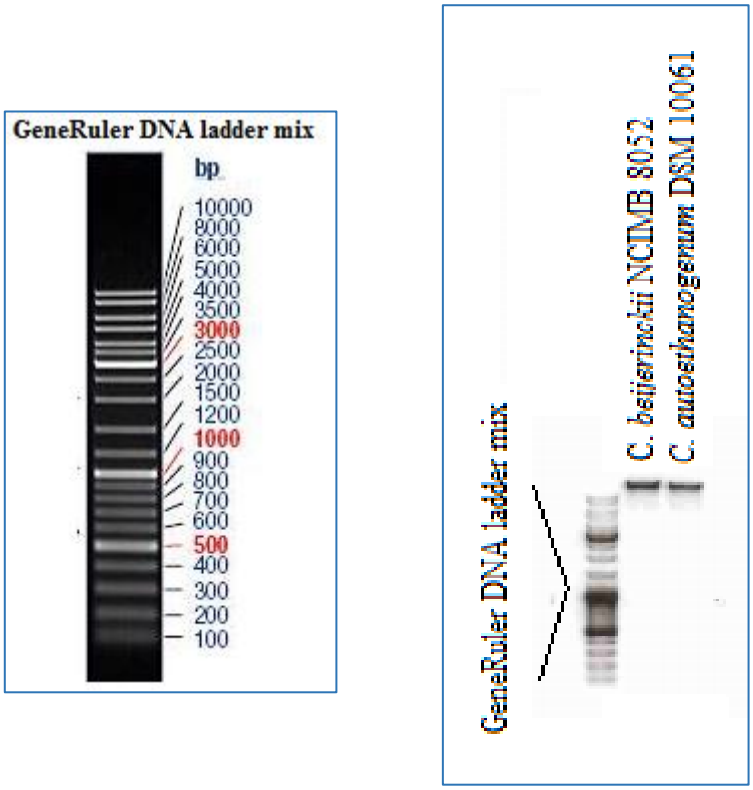


Figure 48 – Representation of the GeneRuler DNA ladder mix bands pattern and the respective bp number to which each band corresponds (in the left) and the electrophoresis gel with the extracted genomes from C. *beijerinckii* NCIMB 8052 and *C. autoethanogenum* DSM 10061 (in the right);

The DNA concentration in both PCR products solutions was measured with a NanoDrop Lite Spectrophotometer.

[C. *beijerinckii* NCIMB 8052 extracted genome] = 8,1 ng/µL
[*C. autoethanogenum* DSM 10061 extracted genome] = 6,5 ng/ µL

After 30min in the CentriVap Micro IR Vacuum Centrifugal Concentrator, at 60ºC with IR.

[C. *beijerinckii* NCIMB 8052 extracted genome] = 21 ng/µL
[*C. autoethanogenum* DSM 10061 extracted genome] = 19 ng/ µL

The *C. autoethanogenum* DSM 10061 genome contained the genes previously selected as essential to activate the WLP in C. *beijerinckii* NCIMB 8052. To isolate them, they were individually amplified by PCR. Specific primers were designed to precisely amplify the genes from *C. autoethanogenum* DSM 10061 genome. The PCR products were then loaded in different electrophoresis gels (figure 49).

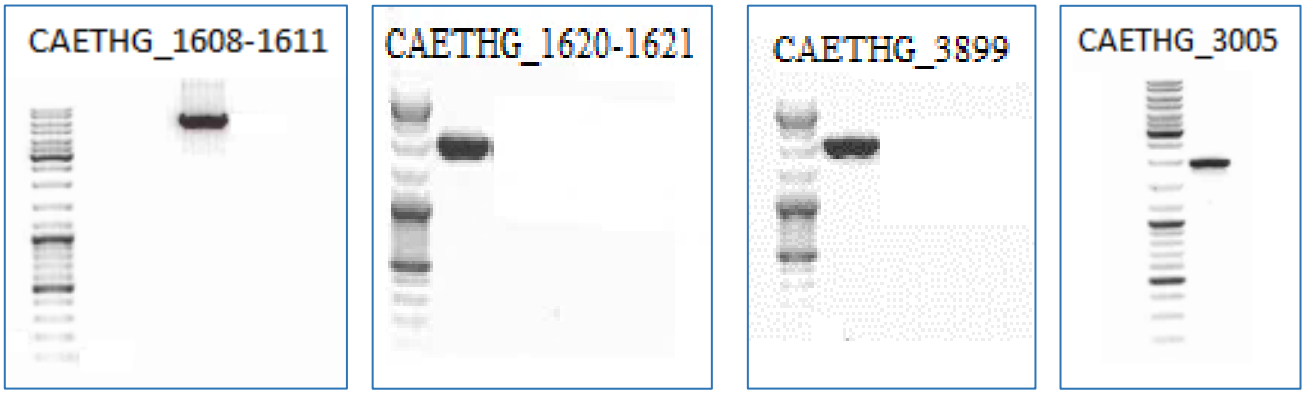


Figure 49 – Representation of the four electrophoresis gels in which the genes of interest, from *C. autoethanogenum* DSM 10061, were loaded and migrated, after being amplified;

Using the Gene JET Gel Extraction Kit, the genes of interest were purified from the gels. The DNA concentration in each purified solution was measured with a NanoDrop Lite Spectrophotometer. The respective DNA concentrations are presented below:

[CAETHG_1608-1611] = 19,8 ng/µL
[CAETHG_1620-1621] = 14,2 ng/µL
[CAETHG_3899] = 12 ng/µL
[CAETHG_3005] = 21,8 ng/µL

After 30min in the CentriVap Micro IR Vacuum Centrifugal Concentrator, at 60ºC with IR.

[CAETHG_1608-1611] = 28,8 ng/µL
[CAETHG_1620-1621] = 16,7 ng/µL
[CAETHG_3899] = 19,2 ng/µL
[CAETHG_3005] = 27,3 ng/µL

The C. *beijerinckii* NCIMB 8052 genome contained the thiolase promoter and terminator genes. Specific primers were designed and using the extracted genome as DNA template, a touchdown PCR was made to amplify them. The PCR products were loaded in an electrophoresis gel (figure 50).

Figure 50 – Representation of the electrophoresis gel where the promoter and terminator from the C. *beijerinckii* NCIMB 8052 thiolase gene were loaded and migrated after being amplified by touchdown PCR;

Equally to the genes of interest, the promoter and terminator PCR products were purified from the gel and their DNA concentration was measured.

[Promoter] = 7,4 ng/µL
[Terminator] = 6,8 ng/µL

After 30min in the CentriVap Micro IR Vacuum Centrifugal Concentrator, at 60ºC with IR.

[Promoter] = 9,7 ng/µL
[Terminator] = 11,3 ng/µL

Cloning attempt 1

For the first cloning attempt, the restriction ligation method was used. As explained in the materials and methods, the clusters CAETHG_1608-1611 and CAETHG_1620-1621 were used in this attempt, once they were the most relevant group of genes. After the restriction ligation, the products were transformed into Z-competent *E. coli* XL-1 Blue. In the next day, five colonies from each selection plate were selected for colony PCR and the resulting PCR products were analysed by electrophoresis (figure 51).

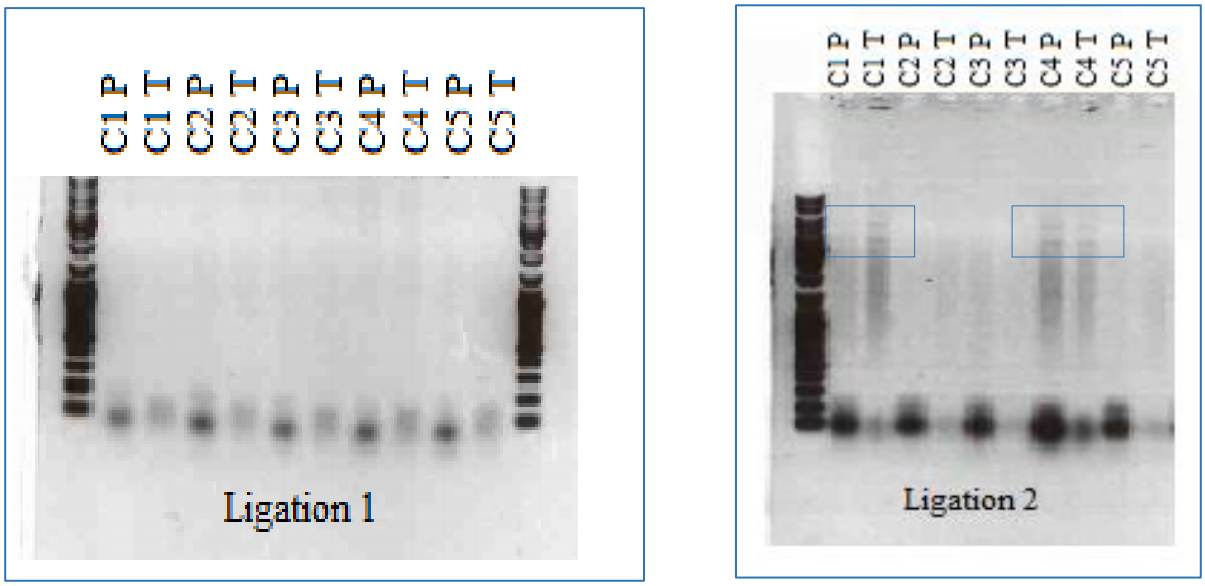


Figure 51 – Representation of the electrophoresis gels where the colony PCR products from the cloning attempt 1 (colonies 1 to 5) were loaded and migrated. The ligation 1 corresponds to the products from the restriction ligation where the cluster CAETHG_1608-1611 was used and the ligation 2 from the restriction ligation with the cluster CAETHG_1620-1621. The expected bands are underlined by two black boxes in the electrophoresis gel with the ligation 2 products;

The colonies 1 and 4, from the ligation 2, presented bands near to the expected positions. Therefore, it was decided that these two colonies, and in addition the colonies 2 and 3 from the same ligation, should be targets of restriction confirmation. After overnight cultures of these colonies, in liquid medium, their plasmids were isolated using the GeneJET Plasmid Miniprep Kit. The DNA concentration in the isolated plasmids solutions was measured by the NanoDrop Lite Spectrophotometer.

[Plasmids from C1] = 671,3 ng/µL
[Plasmids from C2] = 546 ng/µL
[Plasmids from C3] = 597 ng/µL
[Plasmids from C4] = 475,9 ng/µL

Restriction digests were performed to check for the plasmids identity. The restriction enzymes XcmI, HindIII and NdeI were used, individually and in pairs. The digested plasmids were then analysed by electrophoresis (figure 52).

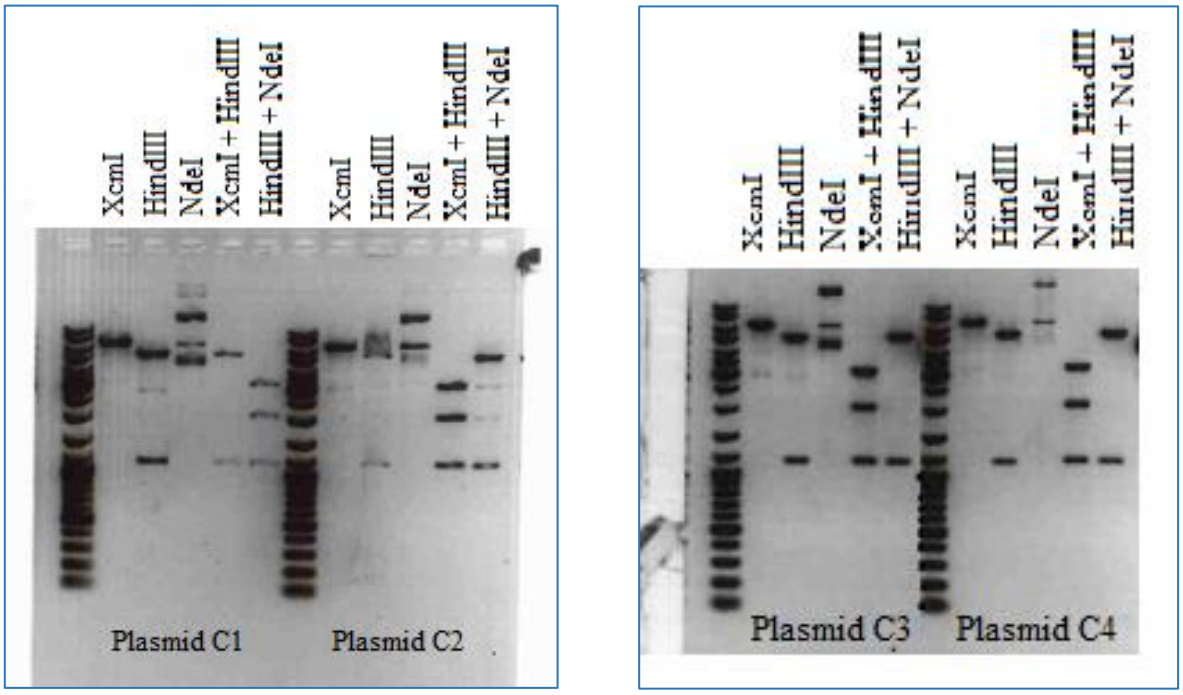


Figure 52 – Representation of the electrophoresis gels where the restriction digestion products were loaded and migrated. In the left gel are presented the digested plasmids from the colonies 1 and 2 and in the right gel from the colonies 3 and 4;

The observed bands pattern was the same regardless of the gel and colony analysed. However, this digestion pattern corresponded to the DNA fragments formed if the same digestion had been made with the pEC500E without the desired inserts. This means that all the analysed colonies possessed the same plasmid, which in turn did not correspond to the desired plasmid but to the original pEC500E.

Cloning attempt 2

The Gibson assembly method was adopted for the second cloning attempt. This time the cluster CAETHG_1608-1611 was used. A ratio 1:1:3:3 (vector : cluster : promoter : terminator) was chosen for the assembly. After the assembly reaction, the products were transformed into NEB 5-alpha Chemical Competent *E. coli* and 10 colonies were picked from the selection plate for a colony PCR. The colony PCR products were analysed by electrophoresis (figure 53).

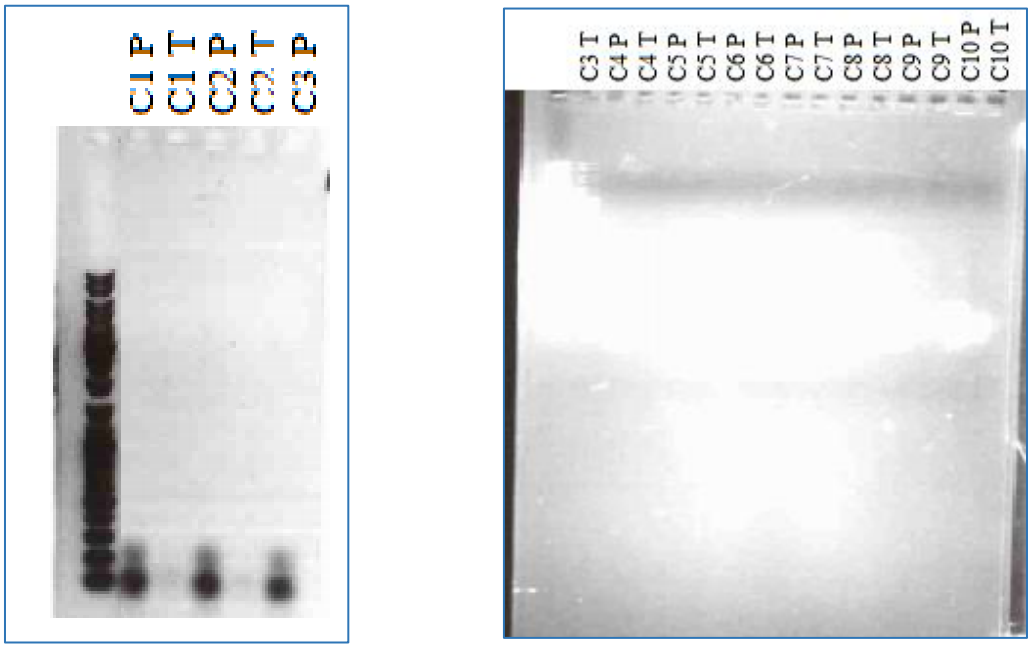


Figure 53 – Representation of the electrophoresis gels where the colony PCR products from the cloning attempt 2 were loaded and migrated. In the left gel are presented the colony PCR products from the colonies 1, 2 and 3 (for the colony 3 is just represented the colony PCR products where the primers for amplifying the promoter were used). In the right gel are presented all the other colony PCR products;

None colony PCR product revealed a band in the electrophoresis gel. Despite of the second gel picture does not be explicit about the existence of bands, the considered gel was observed under different intensities of UV radiation and no bands were visible.

Cloning attempt 3

After carefully looking into the promoter and terminator sequences, it was decided that they could be longer sequences so that cloning might be easier. New primers were designed and a touchdown PCR was made to precisely amplify the new promoter and terminator genes. They were both analysed by electrophoresis (figure 54).

Figure 54 – Representation of the electrophoresis gel where the new promoter and terminator PCR products were loaded and migrated, after being amplified by touchdown PCR;

The PCR products were gel purified with the Gene JET Gel Extraction Kit and the DNA concentration in the purified solutions was measured with the Nanodrop Lite Spectrophotometer.

[Promoter] = 14,6 ng/µL

[Terminator] = 10,2 ng/µL

The Gibson assembly method was repeated, with the difference that the new promoter and terminator genes were used. Additionally, it was decided that it would be better to work with the cluster CAETHG_1620-1621 and the gene CAETHG_3005, because the cluster CAETHG_1608-1611 is relatively big. The ratio vector : inserts was also different from the last cloning attempt. This time a ratio 1:1:2:2 (vector : gene of interest : promoter : terminator) was used. After the two assembly reactions, one with the cluster CAETHG_1620-1621 and other with the gene CAETHG_3005, the assembly products were transformed into Z-competent *E. coli* XL-1 Blue. After overnight incubation in selection plates, 8 colonies per plate were picked

for colony PCR. Two electrophoresis were made to analyse the colony PCR products (figure 55).

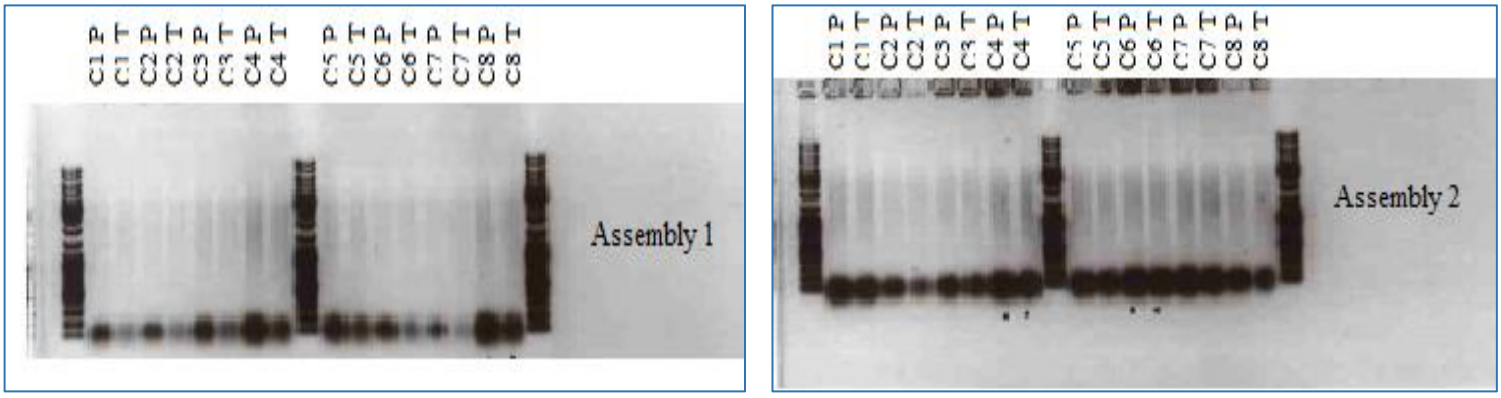


Figure 55 – Representation of the electrophoresis gels where the colony PCR products from the cloning attempt 3 (colonies 1 to 8) were loaded and migrated. The assembly 1 corresponds to the products from the assembly reaction where the cluster CAETHG_1608-1611 was used and the assembly 2 to the products from the assembly reaction made with the cluster CAETHG_1620-1621;

According to the gels, no bands appeared for none the colony PCRs. However, two colonies from each plate were chosen to further restriction confirmation, since there was quite some growth on the plates meaning that the cells contained a vector with the antibiotic resistance. These colonies were inoculated in LB liquid media, with ampicillin, and left overnight at 37ºC in an incubator. The next day, the plasmids were isolated from the cultures using the Gene JET Plasmid Miniprep Kit and the DNA concentration in the obtained solutions was measured with the NanoDrop Lite Spectrophotometer.

[C4 Assembly 1] = 327 ng/µL

[C8 Assembly 1] = 283 ng/µL

[C4 Assembly 2] = 742 ng/µL

[C8 Assembly 2] = 656 ng/µL

The plasmids were afterwards digested by the restriction enzymes SphI HF and NotI HF. The digested plasmids were then analysed by electrophoresis (figure 56).

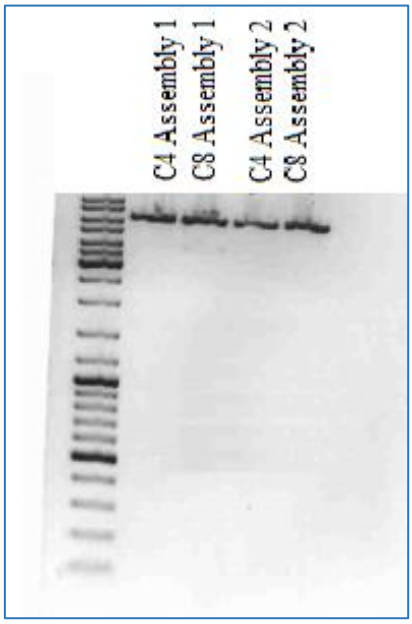


Figure 56 – Representation of the electrophoresis gel where the digested plasmids from the colonies 4 and 8, transformed either with the products from the assembly 1 either from the assembly 2, were loaded and migrated;

None of the bands corresponded to the digested pEC500E with the desired inserts, on the contrary, they belong to the digested pEC500E without the inserts.

Cloning attempt 4

To minimize the number of DNA fragments used as inserts in each assembly, it was decided to fuse them in order to have one sole insert instead of three. Firstly, a fusion PCR was made to ligate the promoter to the genes of interest. The cluster CAETHG_1620-1621 and the genes CAETHG_3005 and CAETHG_3899 were used. After the fusion PCR, the products were analysed by electrophoresis (figure 57), from whose gel they were afterwards extracted using the Gene JET Gel Extraction Kit.

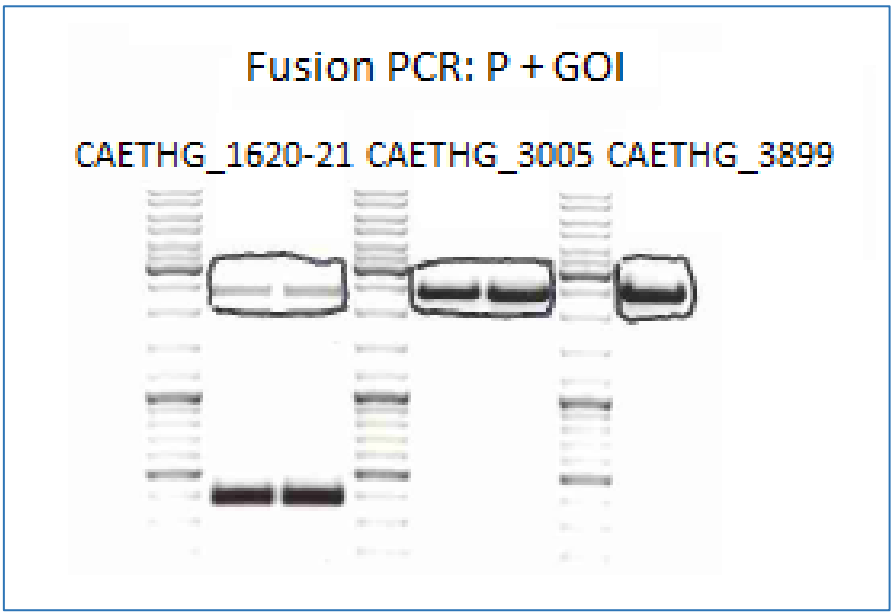


Figure 57 – Representation of the electrophoresis gel where the fusion PCR products were loaded and migrated. The black boxes underline the bands that correspond to the desired products;

The DNA concentration of the extracted DNA fragments was then measured with the NanoDrop Lite Spectrophotometer.

[Promoter - CAETHG_1620-1621] = 1,8 ng/µL

[Promoter - CAETHG_3005] = 6,8 ng/µL

[Promoter - CAETHG_3899] = 6,6 ng/µL

After 20min in the CentriVap Micro IR Vacuum Centrifugal Concentrator, at 60ºC with IR.

[Promoter - CAETHG_1620-1621] = 1,8 ng/µL

[Promoter - CAETHG_3005] = 6,8 ng/µL

[Promoter - CAETHG_3899] = 6,6 ng/µL

Once the fusion PCR products had been gel purified, the second fusion PCR to add the terminator was done. As well as previously, the PCR products were analysed by electrophoresis (figure 58) and the desired bands extracted with the Gene JET Gel Extraction Kit.

Figure 58 – Representation of the electrophoresis gel where the second fusion PCR products were loaded and migrated. The black boxes underline the bands that correspond to the desired products;

Despite of the first fusion PCR had been successful with all the genes of interest used, the second fusion PCR was not. Concerning the cluster CAETHG_1620-1621, it was possible to fuse it with the promoter in the first fusion PCR, however, it did not work so well in the second fusion PCR and the terminator was not ligated. For this reason, the fused DNA fragment promoter-CAETHG_1620-1621-terminator was excluded from the further cloning attempts and the successfully constructed DNA fragments promoter-CAETHG_3005-terminator and promoter-CAETHG_3899-terminator were used. As well as before, the DNA concentration in the fusion PCR products solutions, after the gel extraction, was quantified.

[Promoter - CAETHG_3005 - Terminator] = 4,3 ng/µL

[Promoter - CAETHG_3899 - Terminator] = 1,5 ng/µL

After 15min in the CentriVap Micro IR Vacuum Centrifugal Concentrator, at 65ºC with IR.

[Promoter - CAETHG_3005 - Terminator] = 15,5 ng/µL

[Promoter - CAETHG_3899 - Terminator] = 7,3 ng/µL

For the fourth cloning attempt a Circular Polymerase Extension Cloning (CPEC) method was performed. In order to do it, it was necessary to digest the pEC500E beforehand. The vector was digested by the restriction enzymes SphI HF and XhoI and to confirm that it was really digested, the digestion products were analysed by electrophoresis (figure 59).

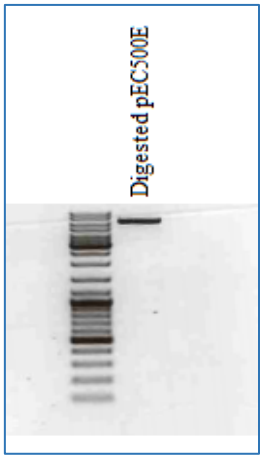


Figure 59 – Representation of the electrophoresis gel where the digested pEC500E was loaded and migrated;

Two CPECs were done, one with the fused DNA fragment promoter-CAETHG_3005-terminator and other with the promoter-CAETHG_3899-terminator. A ratio 1:1 (vector : insert) was used. After the CPECs, the products were directly analysed by electrophoresis (figure 60).

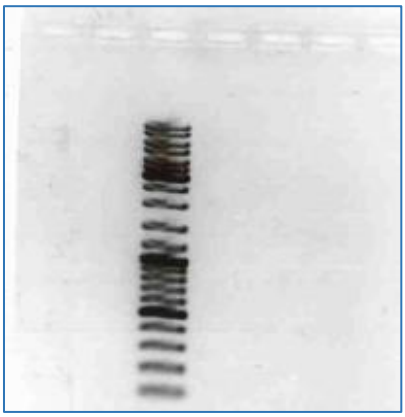


Figure 60 – Representation of the electrophoresis gel where the products from the two CPECs were loaded and migrated;

Strangely, no bands appeared in the gel. Despite it was certain that there was DNA inside the analysed samples, they were measured by the Nanodrop Lite Spectrophotometer to understand if the amount of DNA within the samples was enough to be noticed.

[CPEC with promoter-CAETHG_3005-terminator] = 414,6 ng/µL

[CPEC with promoter-CAETHG_3899-terminator] = 398 ng/µL

Since there was a considerable amount of DNA inside the samples, it was considered that the electrophoresis did not work properly and the CPEC products were transformed into Z-Competent *E.coli* XL-1 Blue. Even after two days, no colonies appeared in the selection plates.

Cloning attempt 5

The CPEC made previously was repeated in a new cloning attempt with the difference that this time, a bigger amount of vector and insert was used and the ratio vector : insert was changed to 1:2. The resulting products from the CPECs were directly analysed by electrophoresis (figure 61).

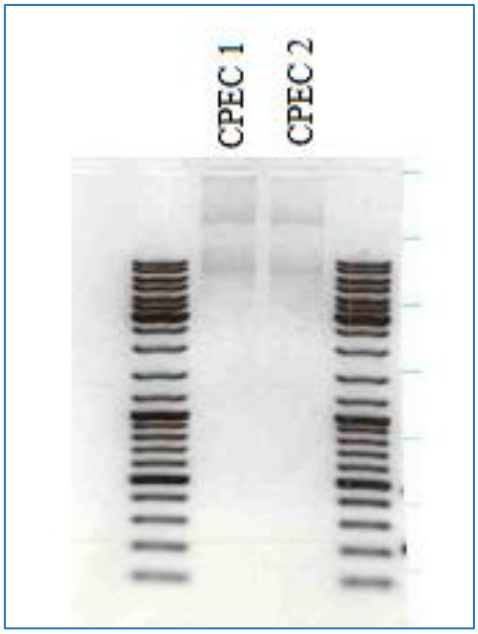


Figure 61 – Representation of the electrophoresis gel where the products from the two CPECs were loaded and migrated. The CPEC 1 corresponds to the CPEC made with the fused DNA fragment promoter-CAETHG_3005-terminator and the CPEC 2 to the CPEC made with the promoter-CAETHG_3899-terminator;

The DNA concentration in the product solutions was measured in a NanoDrop Lite Spectrophotometer.

[CPEC with promoter-CAETHG_3005-terminator] = 299 ng/µL
[CPEC with promoter-CAETHG_3899-terminator] = 367,2 ng/µL

Considering that the desired plasmids were apparently constructed, the CPEC products were transformed into Z-Competent *E.coli* XL-1 Blue. After two days both selection plates contained around 20 colonies. 16 colonies per plate were picked for colony PCR. The products were analysed by electrophoresis (figure 62).

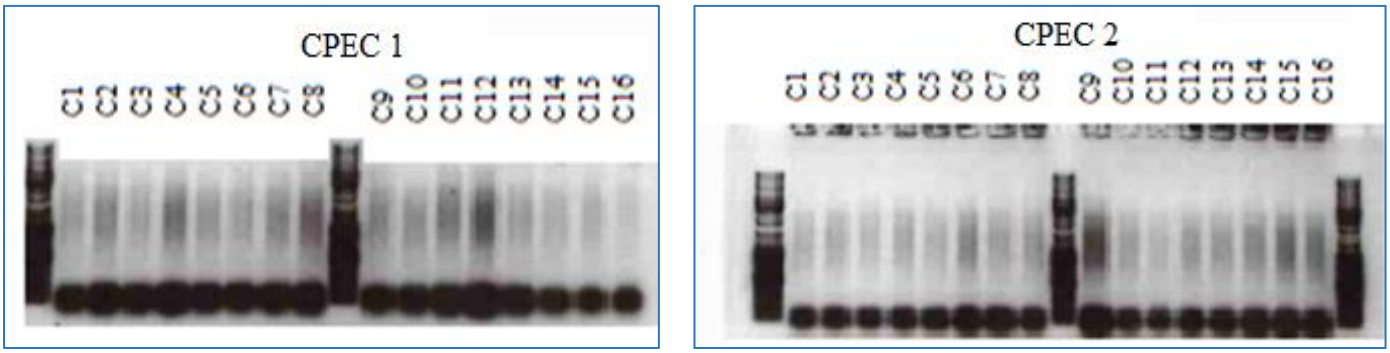


Figure 62 – Representation of the electrophoresis gels where the colony PCR products, made from the colonies transformed with the CPEC 1 and CPEC 2 products (from colonies 1 to 16), were loaded and migrated;

Despite of the lack of results from the colony PCR, three of the analysed colonies were chosen for a more detailed plasmid evaluation by restriction digest. Therefore, the colonies 8 and 12 from the CPEC 1 and the colony 1 from the CPEC 2, were inoculated in an overnight liquid culture and their plasmids were extracted with the Gene JET Plasmid Miniprep Kit. The DNA concentration in the after-extraction solutions was measured with the NanoDrop Lite Spectrophotometer.

[Colony 8, CPEC 1] = 245,4 ng/µL
[Colony 12, CPEC 1] = 337,9 ng/µL
[Colony 1, CPEC 2] = 302,3 ng/µL

The extracted plasmids were then digested by the restriction enzymes SphI HF and NotI HF. The resulting digestion products were loaded and analysed by electrophoresis (figure 63).

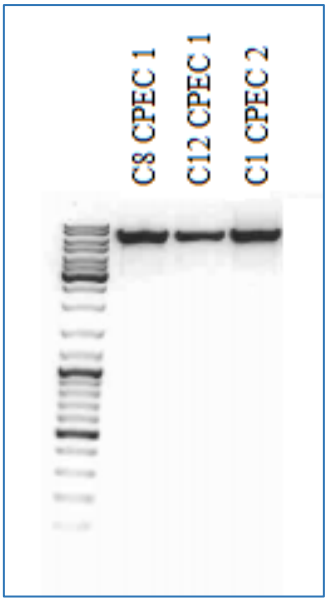


Figure 63 – Representation of the electrophoresis gel where the digested plasmids from the colonies 8 and 12, transformed with the CPEC1 products, and the colony 2, transformed with the CPEC 2 products, were loaded and migrated;

The observed bands did not correspond to the expected ones but to the original pEC500E, if it was digested by the same restriction enzymes used before.

Cloning attempt 6

The restriction ligation method was repeated with the difference that, instead of using the promoter, gene of interest and terminator as individual inserts, the fused DNA fragments were used. As explained in the materials and methods section, before the restriction ligation it was needed to perform a PCR. In this PCR, the fused fragments obtained in the cloning attempt 4 were used as DNA templates, together with the old promoter forward and terminator reverse primers and the Q5 High-Fidelity DNA polymerase master mix. At the end of the PCR, the products were analysed by electrophoresis (figure 64).

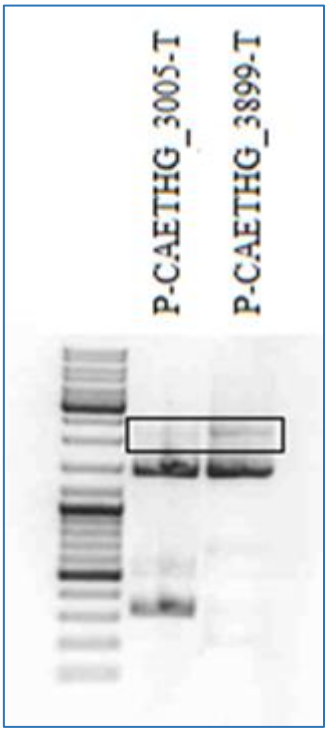


Figure 64 – Representation of the electrophoresis gel where the PCR products obtained from the fused DNA fragments P-CAETHG_3005-T and P-CAETHG_3899-T, using the old promoter forward and terminator reverse primers, were loaded and migrated;

With the Gene JET Gel Extraction Kit the considered bands were extracted from the electrophoresis gel. The DNA concentration of the resulting solutions was then measured with the NanoDrop Lite Spectrophotometer.

[P-CAETHG_3005-T] = 4,8 ng/µL
[P-CAETHG_3005-T] = 4,6 ng/µL

After 10min in the CentriVap Micro IR Vacuum Centrifugal Concentrator, at 60ºC with IR.

[P-CAETHG_3005-T] = 7,7 ng/µL
[P-CAETHG_3005-T] = 8,3 ng/µL

For the restriction ligation, the inserts, as well as the pEC500E, had to be digested before the ligation reaction. To create compatible ends between the inserts and the vector, both were digested by the restriction enzymes SphI HF and XhoI. After the digestion, two ligation reactions were performed, one per insert, using a ratio 1:3 (vector : insert). The ligation products were transformed into Z-Competent *E.coli* XL-1 Blue, that were afterwards inoculated in selection plates and incubated overnight at 37ºC. In the next day, 7 colonies appeared in the selection plate with the cells transformed with the ligation solution that contained the gene CAETHG_3899. All the colonies were picked for colony PCR. The resulting products were analysed by electrophoresis (figure 65).

Figure 65 – Representation of the electrophoresis gel where the colony PCR products, obtained from the colonies 1 to 7 transformed with the restriction ligation products where the gene CAETHG_3899 were used, were loaded and migrated;

There were no bands in the expected positions within the gel. The colony 1 was the only colony that presented an explicit band, although, it was a non-specific band. For that reason, this colony was picked and inoculated in liquid medium for further restriction confirmation of it plasmid identity. After an overnight incubation, the plasmids were isolated from the culture, using the Gene JET Plasmid Miniprep Kit, and the DNA concentration of the resulting solution was measured with the NanoDrop Lite Spectrophotometer.

[Colony 1] = 273,8 ng/µL

For the restriction confirmation, the plasmids were digested by the restriction enzymes SphI HF and NotI HF. The resulting DNA fragments were analysed by electrophoresis (figure 66).

Figure 66 – Representation of the electrophoresis gel where the colony 1 digested plasmid was loaded and migrated;

The observed band did not correspond to the expected one but to the original pEC500E, if it was digested by the same restriction enzymes used before.

Cloning attempt 7

The restriction ligation method was performed one last time. The same procedures than in the cloning attempt 6 were followed, with the difference that a touchdown PCR was performed to amplify the fused DNA fragments with the old promoter forward and terminator reverse primers. The resulting PCR products were analysed by electrophoresis (figure 67).

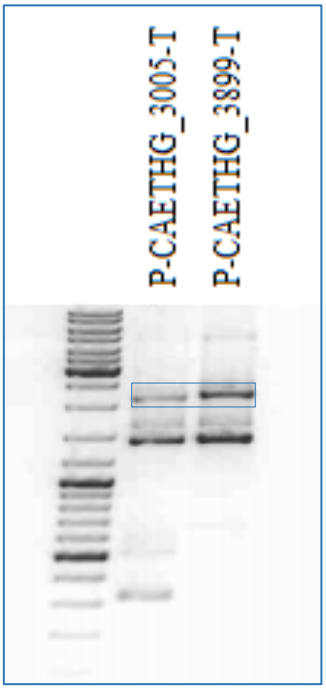


Figure 67 – Representation of the electrophoresis gel where the touchdown PCR products, obtained from the fused DNA fragments P-CAETHG_3005-T and P-CAETHG_3899-T, using the old promoter forward and terminator reverse primers, were loaded and migrated;

Equally to the last cloning attempt, the vector and the inserts were digested by the restriction enzymes SphI HF and XhoI. Two ligation solutions were made, maintaining the ratio 1:3 (vector : insert), but now using a bigger amount of both vector and inserts. The ligation products were then transformed in aquacompetent *E. coli* Top 10 and the cells were inoculated overnight at 37ºC on selection plates. In the following day, around 20-30 colonies appeared in each plate. To perform a touchdown colony PCR, 20 colonies from the plate containing the cells transformed with the ligation products that contained the P-CAETHG_3005-T were picked, together with 15 colonies from the other plate. After the colony PCR, the products were analysed by electrophoresis (figure 68).

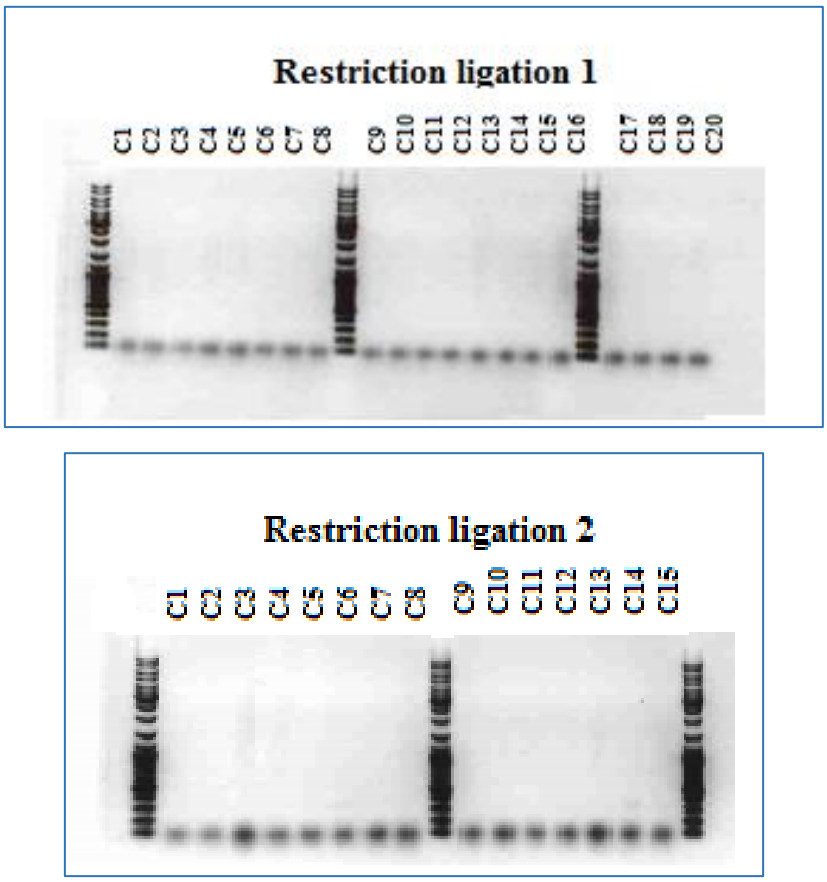


Figure 68 – Representation of the electrophoresis gels where the colony PCR products, obtained from the colonies where the ligation products from the restriction ligation 1 (colonies 1 to 20) and 2 (colonies 1 to 15) were transformed, were loaded and migrated. In the restriction ligation 1 it was used the fused DNA fragment P-CAETHG_3005-T and in the restriction ligation 2 the P-CAETHG_3899-T;

No band appeared for all the analysed colonies and the possibility of the desired plasmid be present was discarded.

Cloning attempt 8

The fused DNA fragments constructed in the cloning attempt 4, were used in this new cloning attempt to perform the Gibson assembly method. Firstly, the pEC500E was digested by the restriction enzymes SphI HF and XhoI and posteriorly purified using the Gene JET Gel Extraction Kit. Then, two assembly reactions were prepared, one for each fused DNA fragment, using a ratio 1:2 (vector : insert). Once finished the reaction, the assembly products were transformed into aquacompetent *E. coli* Top10, that were inoculated in selection plates. After two days, no colonies appeared.

Cloning attempt 9

Once more, the Gibson assembly method was performed with some modifications. There was a new kit, for the gel extraction, that ensured a bigger concentration of DNA in the resulting solutions. Therefore, both fusion PCRs made in the cloning attempt 4 were repeated and the PCR products analysed by electrophoresis (figure 69), from whose gel they were extracted using the Silica Bead DNA Gel Extraction Kit.

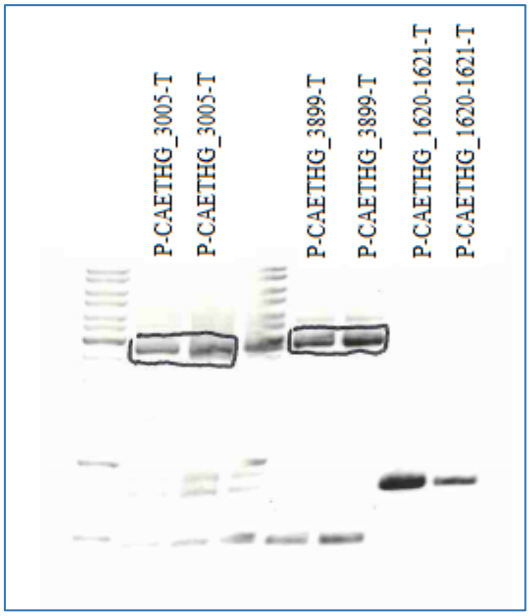


Figure 69 – Representation of the electrophoresis gel where the fusion PCR products, obtained from the fusion of the DNA fused fragments P-CAETHG_3005, P-CAETHG_3899 and P-CAETHG_1620-1621 with the terminator gene, were loaded and migrated;

As in the cloning attempt 4, the fusion PCR for the gene CAETHG_1620-1621 did not work. After gel extraction of the other fused DNA fragments, the DNA concentration in both solutions was measured with the NanoDrop Lite Spectrophotometer.

[Promoter-CAETHG_3005-Terminator] = 55,7 ng/µL

[Promoter-CAETHG_3899-Termnator] = 47,4 ng/µL

The pEC500E was digested by the same restriction enzymes used in the cloning attempt 8 but now it was purified with the Silica Bead DNA Gel Extraction Kit. With the vector and inserts properly prepared, the assembly reactions were made. When they finished, the assembly products were transformed into aquacompetent *E. coli* Top 10 and the cells were overnight

incubated in selection plates. In the next day, there was one colony in the selection plate that contained the cells transformed with the assembly products where the gene CAETHG_3899 was present. This colony was selected for colony PCR and the resulting products were analysed by electrophoresis (figure 70).

Figure 70 – Representation of the electrophoresis gel where the colony PCR products from the cloning attempt 9 were loaded and migrated. This colony PCR was made from the only colony that appeared in the selection plates and that was transformed with the assembly products where the gene CAETHG_3899 was used;

No band appeared in the gel.

Cloning attempt 10

With the new concentrated fused DNA fragments, obtained in the previous cloning attempt, the CPEC was repeated. It was followed the same protocol made in the cloning attempt 5. The CPEC products were analysed by electrophoresis (figure 71).

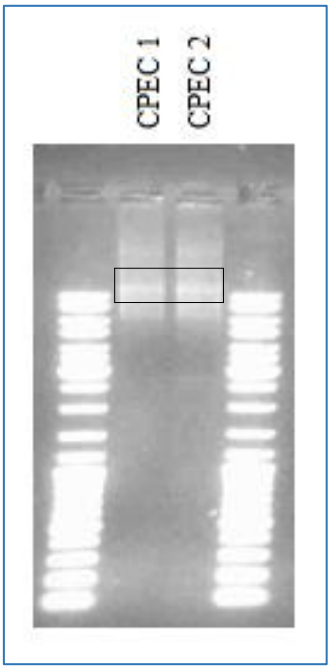


Figure 71 – Representation of the electrophoresis gel where the products from the CPEC 1 and 2 were loaded and migrated. The CPEC 1 corresponds to the CPEC made with the fused DNA fragment P-CAETHG_3005-T and the CPEC 2 to the CPEC made with the fused DNA fragment P-CAETHG_3899-T;

The observed bands seemed to be near the expected position. Then, the DNA concentration in the CPEC products solutions was quantified using the NanoDrop Lite Spectrophotometer.

[CPEC with promoter-CAETHG_3005-terminator] = 415 ng/µL
[CPEC with promoter-CAETHG_3899-terminator] = 327,9 ng/µL

Everything looked fine and the CPEC products were transformed into aquacompetent *E. coli* Top 10. The cells were inoculated on selection plates and two days later the plates were full of colonies. From the plate with the cells transformed with the CPEC products where the gene CAETHG_3899 was used, 18 colonies were selected for colony PCR. From the other plate 9 colonies. The colony PCR products were analysed by electrophoresis (figure 72).

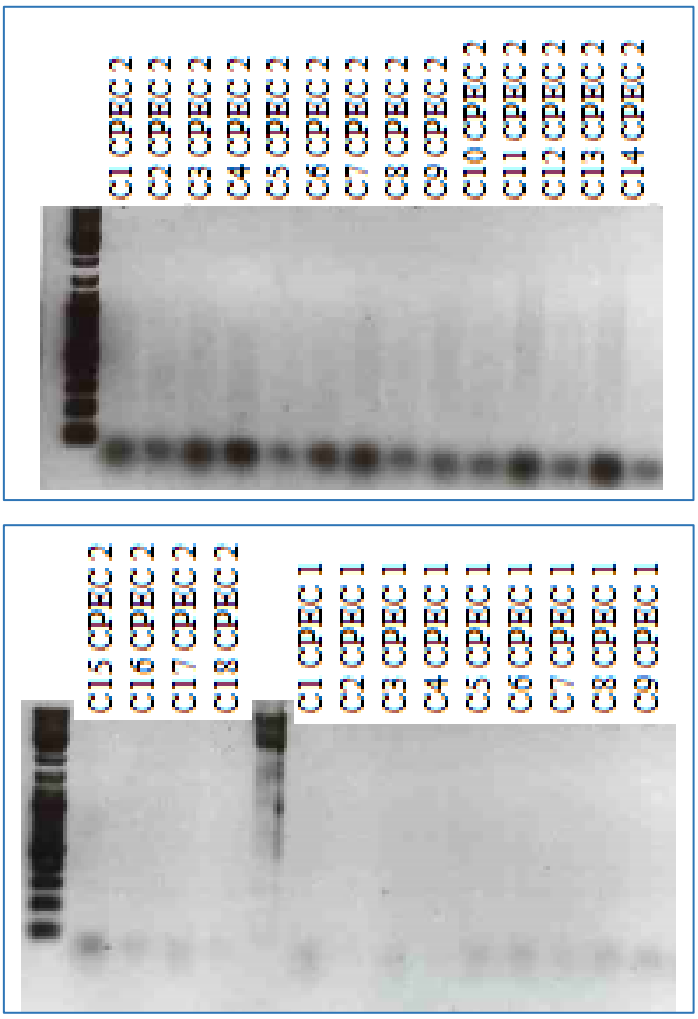


Figure 72 – Representation of the electrophoresis gels where the colony PCR products from the cloning attempt 10 were loaded and migrated. The first gel has the colony PCR products obtained from fourteen colonies transformed with the CPEC 2 products, as well as the second gel for the first four colony PCR products. In addition to these colonies, the second gel has nine more colony PCR products from the colonies transformed with the CPEC 1 products;

No bands appeared in any analysed colony.

Cloning attempt 11

A new method, called Zero Blunt PCR Cloning Kit, was now adopted. The fused DNA fragments obtained in the cloning attempt 9 were again used for this method. Firstly, the considered fused DNA fragments needed to be inserted into a pCR-Blunt plasmid. Therefore, it was prepared two ligation solution, one per insert, in which the fused DNA fragments were mixed together with linearized pCR-Blunt in a ratio 10:1 (insert : vector). Once the ligation reaction was finished the resulting products were transformed into aquacompetent *E. coli* Top 10 and, differently from all the previous attempts, the transformed cells were inoculated in selection plated containing the antibiotic Kanamycin. The plates were left overnight at 37ºC. In the following day, 10 colonies from each plate were picked for colony PCR. The PCR products were analysed by electrophoresis (figure73).

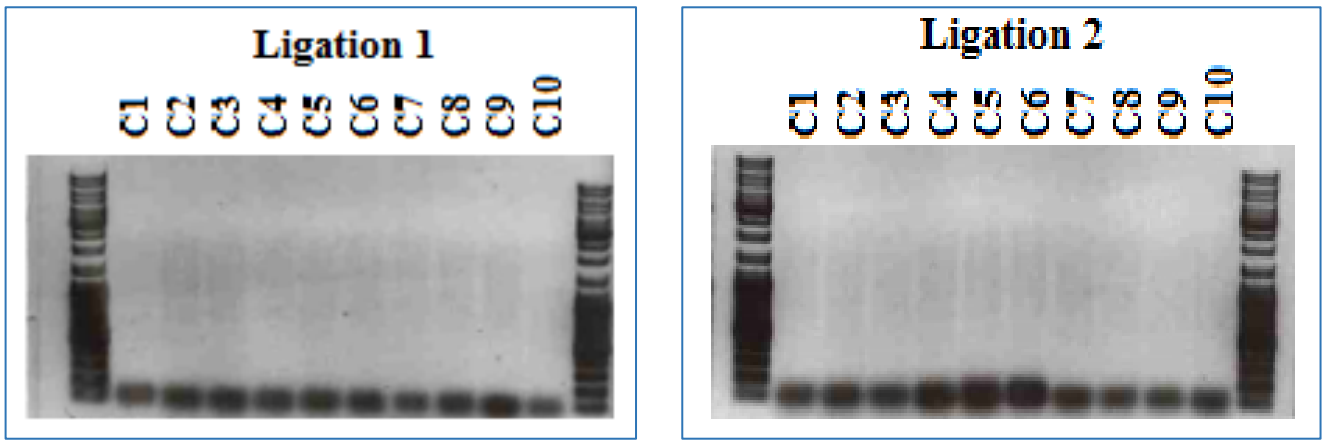


Figure 73 – Representation of the electrophoresis gels where the colony PCR products from the cloning attempt 11 were loaded and migrated. The ligation 1 (colonies 1 to 10) corresponds to the ligation reaction made with the fused DNA fragment P-CAETHG_3005-T and the ligation 2 (colonies 1 to 10) to the ligation reaction made with the P-CAETHG_3899-T;

No bands appeared in both electrophoresis gels.

Cloning attempt 12

The cloning protocol made in the cloning attempt 11, to insert the fused DNA fragments in linearized pCR-Blunt, was here repeated. The only difference, regarding the previous cloning attempt, was the fact that this time the ligation products were transformed into Z-Competent *E.coli* XL-1 Blue. The cells were equally inoculated in selection plates with Kanamycin and left overnight at 37ºC. In the next day, 4 colonies from each plate were picked for colony PCR. The same colony PCR was made and the products analysed by electrophoresis (figure 74).

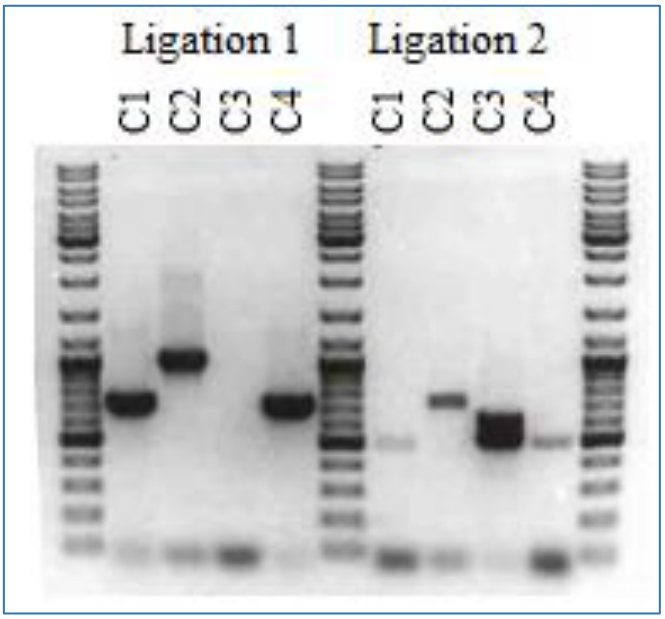


Figure 74 – Representation of the electrophoresis gel where the colony PCR products from the cloning attempt 12 were loaded and migrated. The ligation 1 (colonies 1 to 4) corresponds to the ligation reaction made with the fused DNA fragment P-CAETHG_3005-T and the ligation 2 (colonies 1 to 4) to the ligation reaction made with the P-CAETHG_3899-T;

Despite of the bands in the electrophoresis gel, they were all unspecific and did not correspond to the expected ones. Even so, all the analysed colonies were inoculated in liquid medium for further restriction confirmation. In contrast to the previous cloning attempt, the liquid medium contained the antibiotic kanamycin instead of ampicillin. After overnight incubation, plasmids were isolated from the cells using GeneJET Plasmid Miniprep Kit and the DNA concentration in the resulting solutions was measured with the NanoDrop Lite Spectrophotometer.

[C1 Ligation 1] = 728 ng/µL

[C2 Ligation 1] = 468 ng/µL

[C3 Ligation 1] = 571 ng/µL

[C4 Ligation 1] = 235,4 ng/µL

[C1 Ligation 2] = 396,7 ng/µL

[C2 Ligation 2] = 532 ng/µL

[C3 Ligation 2] = 639 ng/µL

[C4 Ligation 2] = 567 ng/µL

Once isolated, the plasmids were digested by the restriction enzyme EcoRI HF. The digestion products were analysed by electrophoresis (figure 75).

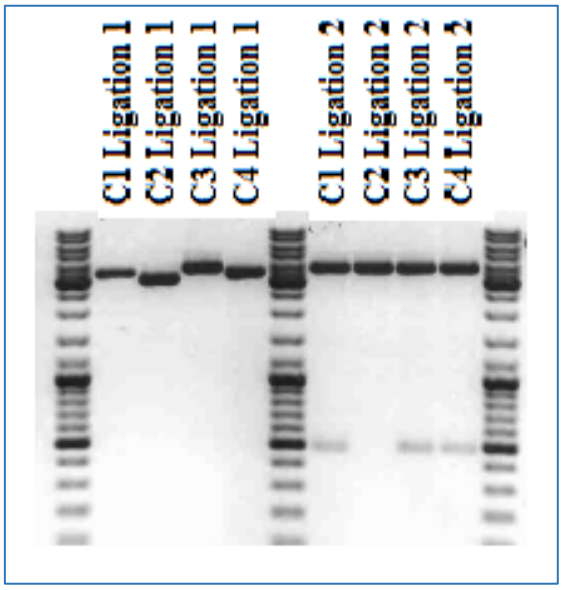


Figure 75 – Representation of the electrophoresis gel where the digested plasmids from the colonies 1 to 4, transformed either with the ligation 1 products, either with the ligation 2 products, were loaded and migrated;

Because just four of the colonies presented in each selection plate were analysed, it was decided that 4 different colonies should be picked for their plasmids to be equally identified. Therefore, 4 different colonies were picked from the selection plates and inoculated in liquid medium. In the next day, their plasmids were extracted with the GeneJET Plasmid Miniprep Kit and the

DNA concentration in the resulting solutions was measured with the NanoDrop Lite Spectrophotometer.

[C5 Ligation 1] = 687 ng/µL

[C6 Ligation 1] = 736 ng/µL

[C7 Ligation 1] = 523 ng/µL

[C8 Ligation 1] = 646,6 ng/µL

[C5 Ligation 2] = 493,1 ng/µL

[C6 Ligation 2] = 782 ng/µL

[C7 Ligation 2] = 756,4 ng/µL

[C8 Ligation 2] = 572,7 ng/µL

The plasmids were then digested by the restriction enzymes SacI and XhoI and the resulting DNA fragments were analysed by electrophoresis (figure 76).

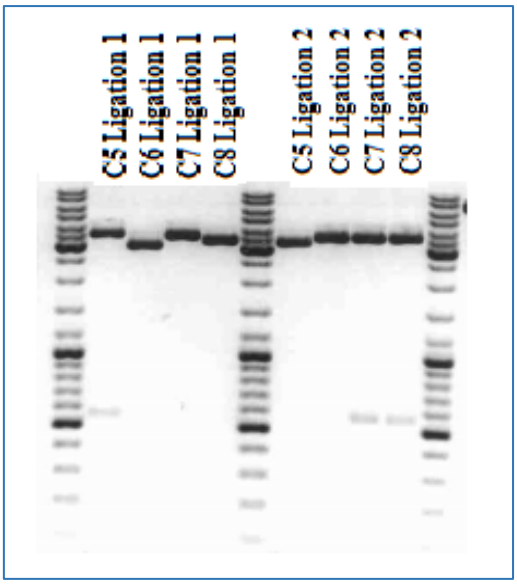


Figure 76 – Representation of the electrophoresis gel where the digested plasmids from the colonies 5 to 8, transformed either with the ligation 1 products, either with the ligation 2 products, were loaded and migrated;

A similar pattern to the last electrophoresis gel was noticed, although, it did not correspond to the expected bands.

Cloning attempt 13

After all the previous cloning attempts have failed, a new approach was adopted. This new cloning method is called TA cloning. To become feasible, there were some requirements that needed to be accomplish first. The pEC500E had to be a T-vector and the inserts to contain 3' A-overhangs. Therefore, to convert the pEC500E into a T-vector, the plasmid was firstly linearized by restriction ligation, in a way that allowed the formation of blunt-ends, and posteriorly, using a Taq polymerase, the dTTPs were added to the ends. Once the pEC500E was ready for cloning, it was just needed to take care of the inserts. Because the fused DNA fragments, obtained in the cloning attempt 4, were finishing, it was necessary to perform new fusion PCRs to construct more. Now, the fusion PCRs were done with all the genes of interest. The PCR products were analysed by electrophoresis (figure 77).

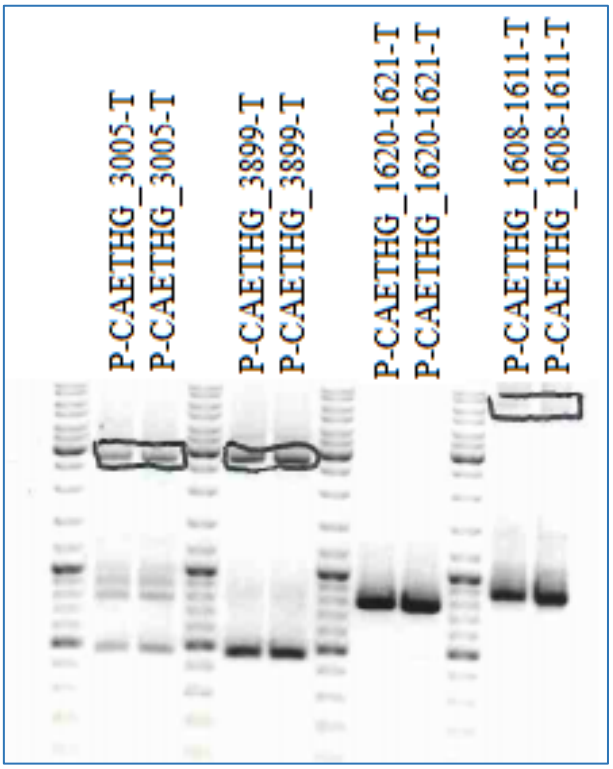


Figure 77 – Representation of the electrophoresis gel where the fusion PCR products, obtained from the fusion of the promoter gene with the genes of interest CAETHG_3005, CAETHG_3899, CAETHG_1620-1621 and CAETHG_1608-1611 and the terminator gene, were loaded and migrated. The black boxes underline the desired bands;

The fusion PCR worked out for all the genes of interest, except for the cluster CAETHG_1620-1621. Then, the underline fused DNA fragments were gel extracted using the Silica Bead DNA Gel Extraction Kit and the DNA concentration of the resulting solutions was quantified with the NanoDrop Lite Spectrophotometer.

[P-CAETHG_3005-T] = 154 ng/µL
[P-CAETHG_3899-T] = 115 ng/µL
[P-CAETHG_1608-1611-T] = 148,3 ng/µL

To add the 3' A-overhangs to the obtained fused DNA fragments, the Taq polymerase was used together with dATPs. When incubated at 72ºC, the Taq polymerase has the capacity to catalyse the addition of one dATP to each inserts ends. With the pEC500E converted into a T-vector and the inserts with the desired 3' A-overhangs, the TA cloning was performed. Three ligation solutions were made, one per fused DNA fragment, in which the inserts were mixed together with the T-vector and a salt solution that came with the TOPO TA-Cloning Kit. Because both ends, the T-vector and inserts ends, were compatible with each other, due to the complementarity between adenine and thymine, it was expected that the inserts, by collision with the T-vectors in the solution, automatically ligate with them. The ligation products were then transformed into Z-Competent *E.coli* XL-1 Blue. The transformed cells were overnight incubated at 37ºC. In the following day, the three plates were full of colonies. No colony PCR was made and 5 colonies per plate were directly inoculated in liquid medium with ampicillin for further restriction confirmation of the plasmids identity. After an overnight incubation in the liquid medium, the plasmids were isolated from the cultures using the GeneJET Plasmid Miniprep Kit and the DNA concentration of the resulting solutions measured with the NanoDrop Lite Spectrophotometer.

[C1 TA-cloning 1] = 601 ng/µL
[C2 TA-cloning 1] = 525 ng/µL
[C3 TA-cloning 1] = 414,8 ng/µL
[C4 TA-cloning 1] = 654 ng/µL
[C5 TA-cloning 1] =567 ng/µL

[C1 TA-cloning 2] = 397 ng/µL

[C2 TA-cloning 2] = 732 ng/µL

[C3 TA-cloning 2] =634 ng/µL

[C4 TA-cloning 2] =739 ng/µL

[C5 TA-cloning 2] =712 ng/µL

[C1 TA-cloning 3] = 47,9 ng/µL

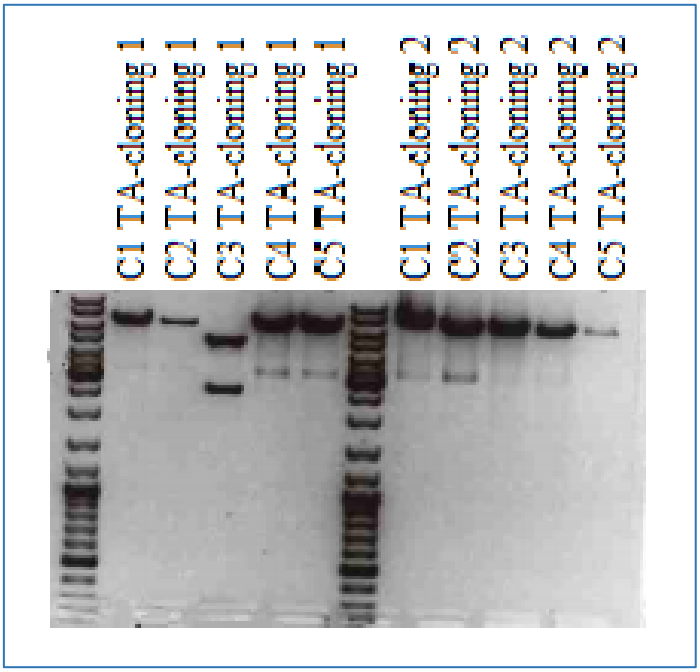
[C2 TA-cloning 3] = 89 ng/µL

[C3 TA-cloning 3] = 192 ng/µL

[C4 TA-cloning 3] = 143 ng/µL

[C5 TA-cloning 3] = 94 ng/µL

For restriction confirmation, the isolated plasmids, supposed to have the gene CAETHG_3005 and the cluster CAETHG_1608-1611 inside, were digested with the restriction enzyme NcoI HF. The plasmids supposed to have the gene CAETHG_3899 inside, were digested with the enzyme PstI HF. The digestion products were analysed by electrophoresis (figure 78).



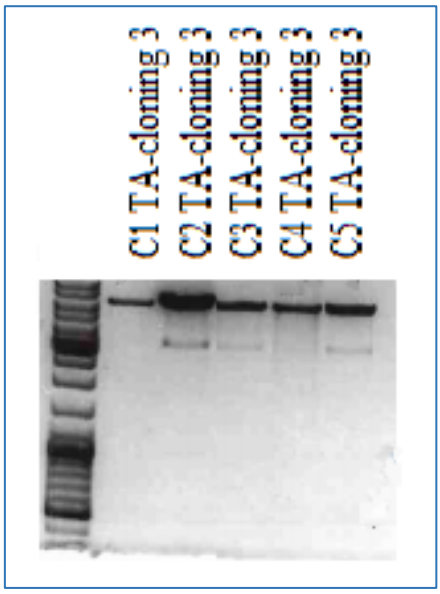


Figure 78 – Representation of the electrophoresis gels where the digested plasmids from the colonies 1 to 5, transformed either with the TA-cloning products 1, 2 and 3, were loaded and migrated. The TA-cloning products 1 were made using the fused DNA fragment P-CAETHG_3005-T, the TA-cloning products 2 using the P-CAETHG_3899-T and the TA-cloning products 3 using the P-CAETHG_1608-1611-T;

Some of the observed bands correspond to the original pEC500E, if it had been digested by the same restriction enzymes used in this procedure, while the others are considered unspecific bands. Concluding, none of the bands corresponded to the expected ones.

5.Discussion

Step 1 – Practical validation and control data;

The gathering of control data, from different fermentation conditions, for further comparison with results from the same fermentations with a genetic modified strain, was the driving force that motivated the work made in this chapter. An additional purpose was the demonstration that *C. beijerinckii* NCIMB 8052 was incapable of producing butanol when grown on CO_2.

To evaluate ABE fermentations with *C. beijerinckii* NCIMB 8052, under heterotrophic conditions, two conditions were tested: H1) CM2 medium supplemented with 60 g/L glucose; H2) CM2 medium supplemented with 60 g/L glucose + 50rpm shaking.

Previous works were already made with *C. beijerinckii* NCIMB 8052, in which the condition H1 was tested with the difference that, instead of CM2 medium, P2 medium was used (Liu et al., 2017; Zhang et al., 2012). In (Zhang et al., 2012), a control batch fermentation with *C. beijerinckii* NCIMB 8052 was performed using 60 g/L glucose as the sole carbon source. After 96 h fermentation, 15,8 g/L ABE were produced, of which butanol, acetone and ethanol were 9,4, 5,1 and 1,3 g/L, respectively, resulting in a productivity of 0,16 g/L.h and an ABE yield of 0,31 g/g. Acetic and butyric acids were mainly produced during the early exponential phase, reaching a maximum concentration of 2,6 and 1,3 g/L, respectively. In (Liu et al., 2017), after the same fermentation performed as in (Zhang et al., 2012), a glucose consumption of 230 ± 9,3 mM was observed with a production of 71,7 ± 1 mM and 140,7 ± 4,7 mM of acetone and butanol, respectively. When the same experiment was made in the present work, after 144h fermentation, 190mM of glucose were consumed and 10,91 g/L ABE were produced, from which, 6,92 g/L (93mM), 3,75 g/L (65mM) and 0,24 g/L (5mM) corresponded to butanol, acetone and ethanol, respectively. A productivity of 0,08 g/L.h and an ABE yield of 0,32 g/g was achieved. The acetic and butyric acids were mainly produced in the initial exponential phase and the highest concentrations were 1,95 g/L and 0,95 g/L, respectively. Despite of the slightly differences noticed between the experiment made in the present work and the other two mentioned, there was a substrate consumption and products formation pattern similar between all of them. The characteristic ABE fermentation acid crash, during the acidogenesis phase, and the following

pH stabilization, due to the solventogenesis phase, were clearly noticed in (Liu et al., 2017) as well as in this study. In both fermentations performed by Liu et al., 2017 and Zhang et al., 2012, there was a bigger amount of ABE produced in less than 48h of fermentation when compared with the experiment made in the present work. The fact that the fermentation here had been conducted for a longer period did not have a great influence in the fermentation parameters analysed, once, after 48h of fermentation, the cells entered in a stationary phase and did not grow anymore, as well as the ABE production that practically stabilized after the same time. Considering just the first 48h of fermentation, the ABE productivity increased to 0,18 g/L.h. Another reason to justify these differences can be related with the fermentation medium, that was different between the present work and the other two mentioned. It is known that a butanol concentration of 0,1-0,15 M causes a 50 % inhibition of both cell growth and sugar uptake (Moreira et al., 1981). This can be a reason for the cell growth stabilization and glucose consumption decreasing observed after 48h, when the butanol concentration in the fermentation broth was around 0,08 M. To confirm if a mixotrophic metabolism is being performed by *C. beijerinckii* NCIMB 8052, the cell growth and the ABE production must be bigger than the one described for this condition, when mixotrophic conditions are used.

In the condition H2, an agitation of 50rpm was added to the fermentation. Previous works (Company & Zeikus, 1988; County, 1985) were already made to determine the effects of agitation to fermentations where different strains of clostridium were used, namely *Clostridium thermocellum* and *Clostridium acetobutylicum*. (County, 1985) performed an experiment with *Clostridium acetobutylicum*. The fermentation medium was supplemented with 50 g/L glucose and four different stirring rates (25, 100, 200 and 300 rpm) were tested. The butanol productivity for each stirring rate was calculated. According to the results, the maximum butanol productivities were obtained with stirring rates of 25 and 100 rpm. When higher stirring rates were used, the butanol productivity significantly decreased. Based on these results, it was decided that a stirring rate of 50 rpm should be appropriated to notice an increasing in butanol productivity, comparing with the fermentation condition H1. As expected, a higher butanol productivity was achieved. After 144h of fermentation, 214 mM of glucose were consumed and 12,81 g/L ABE were produced. From the amount of ABE produced, 7,99 g/L (108mM) corresponded to butanol, 4,56 g/L (79mM) to acetone and 0,26 g/L (6mM) to ethanol. An ABE

productivity of 0,09 g/L.h and an ABE yield of 0,33 g/g were achieved. Considering just 48h of fermentation, the ABE productivity was as high as 0,26 g/L.h. The acetic and butyric acid were just produced in the exponential phase, as it was seen for condition H1. Despite of the cell growth had ceased after 48h, it was around 30% superior than in the condition H1, with twice the amount of consumed carbon being dedicated to biomass production. Summarizing, the agitation increased all the relevant ABE fermentation parameters. The reason for this phenomenon can be related with an increasing in the transfer of volatile fermentation products, like the solvents, from the fermentation broth to the headspace of the bottles. Thereby, an inhibition in growth is prevented and more sugar can be converted to products (Company & Zeikus, 1988; County, 1985).

To evaluate *C. beijerinckii* NCIMB 8052 capacity to fixate CO_2, two autotrophic conditions were tested: A1) CM2 medium supplemented with CO_2 (2bar); A2) CM2 medium supplemented with CO_2 (15%) + H_2 (5%).

There were no publications referring to autotrophic fermentations performed by *C. beijerinckii* NCIMB 8052. Therefore, bioinformatic searches in the KEGG platform were made to understand if there was a CO_2 fixation metabolic pathway described for this strain. According to the bioinformatic searches, no metabolic pathways, where inorganic compounds are used as initial substrates, were present in the *C. beijerinckii* NCIMB 8052 carbon metabolism. However, because it was mandatory to know if there was effectively a CO_2 fixation metabolism in *C. beijerinckii* NCIMB 8052, and because it was already described the existence of these pathways in other clostridium strains (Cotter et al., 2009; Henstra et al., 2007; Ragsdale & Pierce, 2008), for instance *C. ljungdahlii, C autoethanogenum C. carboxidevorans, C. thermoaceticum* (now named *Morella thermoacetica*) and *C. Formicoaceticum* , it was decided to perform a fermentation with the conditions described above.

For both conditions, no cell growth was noticed, except on the first hours of fermentation while some trace quantities of glucose were present in the medium. The heat shocked *C. beijerinckii* NCIMB 8052 spores were grown in liquid CM2 medium supplemented with 60 g/L glucose, before the inoculation. Despite of the glucose had been partially consumed during the cells growth, at the time of the inoculation there was some glucose that remained in the inoculum and

passed to the fermentation medium. In the first 24h, in which the fermentation medium still had some glucose, there was a slightly production of butyric acid but because the solventogenesis phase did not occurred, none of the acids were consumed and both acetic and butyric acids remained constant until the end of the fermentation. Since solventogenesis was not triggered, no ABE was produced. The major difference noticed between the fermentations performed with the conditions A1 and A2 was the pH during the first hours of fermentation. For the condition A1, it maintained practically unaltered since inoculation. For the condition A2, it showed a considerable decrease at the beginning and then stabilized at the same values than the condition A1. This difference can be attributed to the CO_2 acidification of the fermentation medium. Because the headspace of the bottles in condition A1 were pressurized with 2 bar of CO_2, the acidification occurred right at the beginning of the fermentation. For the condition A2, as it just contained 15% CO_2 and no pressure was applied, the accentuated acidification during the first hours of fermentation was attributed to the acetic and butyric acid production. In conclusion, there was no CO_2 fixation pathway being performed by *C. beijerinckii* NCIMB 8052, even when H_2 was added to the medium to be used as reducing agent, useful in other CO_2-fixing organisms (Jones et al., 2016).

At this point, it was already known that a fermentation under mixotrophic conditions, where CO_2 was going to be used as a co-substrate together with glucose, could not increase the butanol productivity when compared with the heterotrophic conditions. If the CO_2 was not going to be metabolized, the fermentation should run similarly to the fermentations made with the conditions H1 and H2. However, it was important to have a full control data background to better understand if a genetic modified strain would be able to improve the ABE production relative to these fermentations. Therefore, three mixotrophic conditions were tested: M1) CM2 medium supplemented with 60 g/L glucose + CO_2 (2bar); M2) CM2 medium supplemented with 60 g/L glucose + CO_2 (2bar) + 50rpm shaking; M3) CM2 medium supplemented with 60 g/L glucose + CO_2 (15%) + H_2 (5%).

Starting with the cell growth analysis, in the condition M1 the cells had the smallest growth of all the mixotrophic conditions, even smaller than the heterotrophic condition H1. The condition M2 allowed a cell growth significantly bigger than the condition M1, and in turn, in the

condition M3 the cell growth was even significantly bigger than the condition M2. For both conditions, the cells grew during the first 48h, entering in a stationary phase after that. Concordant with the cells growth, the glucose consumption was also the smallest one in the condition M1, 27,99 g/L (155mM), it increased in the condition M2, 31,25 g/L (173mM) and significantly increased in the condition M3, 41,66 g/L (231 mM). The pH variation along the fermentation was practically the same for the conditions M1 and M2. Both started with a low pH, due to the CO_2 acidification of the fermentation medium, that stabilized until the end of the fermentation, suggesting that the CO_2 has a strong buffer capacity that, despite of the acids production and consumption, maintained the pH almost constant. In condition M3, because no pressure was applied and the CO_2 was present in a smaller percentage, the pH at the beginning of the fermentation did not suffer the CO_2 acidification effect and started considerably higher than in the other two conditions, decreasing after 6h of fermentation and practically stabilizing until the end. Relative to the acids production and consumption, the condition M1 and M2 had a very similar pattern. The acetic acid started to be consumed right after 6h of fermentation until 48h, after which it stabilized. Differently, the butyric acid started to be produced after 6h until 24h of fermentation, after which it practically stabilized. For the condition M3 it was different, the acetic acid slightly increased in the first 6h of fermentation and decreased until 24h, after which it stabilized until the end. The butyric acid started to be produced directly until 6h of fermentation, after which it maintained stable during the following 20h, and then it was again produced for more 50h, from which it stabilized until the end. Curiously, the condition M3 was the one that achieved the lowest ABE productivity, 9,2 g/L (130 mM), from which 1,12 (19 mM), 0,21 (5 mM) and 7,87 g/L (106 mM) corresponded to acetone, ethanol and butanol, respectively. The ABE yield was also the lowest for this condition, namely 0,189 g/g. Similar to the heterotrophic conditions H1 and H2, the increment of agitation in the fermentation condition M2, led to an increase around 2 g/L (20 mM) of ABE, comparing with the condition M1). In the condition M1, an ABE productivity of 9,35 g/L (133 mM) was achieved, from which 1,97 g/L (34 mM) corresponded to acetone and 7,38 g/L (100 mM) to butanol, while, in the condition M2, an ABE productivity of 11,09 g/L (162 mM) was achieved, with 3,22 g/L (55 mM) corresponding to acetone and 7,87 g/L (106 mM) to butanol. No ethanol was produced in these two conditions. In (Fast et al., 2015), a theoretical approach based on stoichiometric

analysis of the ANP mixotrophic metabolism advantages for butanol production is described. It was predicted that under limited mixotrophic conditions (glucose + gas evolved (CO_2 released during the EMP glycolysis)), the butanol mass yield should increase 2%, comparing with the heterotrophic conditions. Under supplemented mixotrophic conditions (glucose + gas evolved + H_2) it should increase 20%. Considering the conditions M1 and M2 as equivalents to the limited mixotrophic condition (both glucose and CO_2 are used as co-substrates), it would be expected, if a mixotrophic metabolism was available, that the butanol yield increased 2% when compared with the conditions H1 and H2. An increase of around 5% was noticed, however, a 5% increase in the butanol yield is not such a big improvement that allows an assertive conclusion about this matter. Considering the condition M3 as an equivalent to the supplemented mixotrophic condition (glucose, CO_2 and H_2 are used as co-substrates), and comparing these results with the condition H1, it would be expected a butanol yield increase of 20%, which, once confirmed, would give a more consistent idea about the mixotrophic metabolism availability. However, the butanol yield decreased around 2% in the condition M3, comparing with the condition H1, reinforcing the idea that definitely the mixotrophic metabolism is not present in this strain.

Comparing the overall results, there are some conclusions that should be underlined. The autotrophic conditions were excluded from this analysis, since the cells were not able to grow when exposed to these conditions and consequently no ABE fermentation occurred. The conditions H2 and M3 were evidently the ones that allowed the biggest cell growth, being significantly higher than the other conditions. Concordantly, these conditions were also the ones where more glucose was consumed although it was just significant for the condition M3. Despite of the significant higher glucose consumption noticed in the condition M3, it was also the condition where more of the glucose carbon assimilated was directed to biomass production. Consequently, a lower amount of carbon was available for ABE production and the smallest ABE production was attributed to this condition. Because of that, the condition H2 was classified as the best condition to perform ABE fermentations with *C. beijerinckii* NCIMB 8052, because, despite of it had not been the condition that provided the most suitable environment for the cells to develop, the balance between the carbon dedicated to biomass and the one

dedicated to ABE production was the most efficient, allowing the biggest ABE production. No significant differences were noticed in the amount of butanol accumulated at the end of each fermentation, suggesting that the same metabolism was triggered regardless of the conditions to which the cells were exposed, excluding once more the possibility of the ANP mixotrophic metabolism. Either in the condition H2 or in the condition M2, the agitation apparently led to a bigger amount of ABE produced. However, as mentioned in (County, 1985), the agitation effect on the fermentation can be lost when pressure is added. Looking to the results obtained with the condition H1 and comparing it with the condition H2, where an increment of agitation was additionally added to the condition H1, a notorious increase in the ABE production and cell growth was noticed. Although, considering the condition M2, where more than the agitation a pressure of 2 bar was also applied to the condition H1, the results were similar to the condition H1, suggesting that the improvements noticed due to the agitation in the condition H2 were inhibit by the pressure.

Step 2 - Bioinformatics;

Based on the previous results, the absence of the ANP mixotrophic metabolism in *C. beijerinckii* NCIMB 8052 was attributed to the lack of the CO_2 fixation metabolism. It was important to find out if the dysfunctionality of the WLP was due to the absence of important genes in *C. beijerinckii* NCIMB 8052 genome or if all the necessary genes were present but for some reason they were not being expressed. Fortunately, the *C. beijerinckii* NCIMB 8052 genome was already fully sequenced, as well as genomes from other clostridial strains known to successfully perform the WLP. Thus, as starting point, the cataloguing of all the genes that can possibly be involved in the WLP was made. The *C. autoethanogenum* DSM 10061 genome was chosen to identify all the WLP genes, since this strain is used by other researchers in the institute as well. Because the WLP had already been described for *C. autoethanogenum* DSM 10061 as one of it carbon metabolisms, the KEGG platform created a schematic model of the WLP where the genes and enzymes responsible for each metabolic step were represented. The cataloguing of the genes of interest was then made according to the KEGG platform and additional publications to assure a complete description of all the genes. With the FASTA sequences of each gene, BLASTn analyses with the *C. beijerinckii* NCIMB 8052 genome were

conducted and it was revealed that some important WLP genes were missing. It was thought that if these genes were overexpressed in *C. beijerinckii* NCIMB 8052, all the WLP metabolic steps would be validated and this strain could start fixating CO_2.

Step 3 – Genetic manipulation;

After the genes of interest had been isolated, the promoter and terminator genes nominated and the vector selected, several cloning techniques were performed to transform the previous genes into *C. beijerinckii* NCIMB 8052. Most of the cloning techniques were repeated, according to some protocol changes that were thought to be relevant. However, after 13 cloning attempts, the construction of a pEC500E plasmid containing the genes of interest, together with the promoter and terminator, was not successful. The only technique that worked towards the progress to obtain the desired plasmid was the fusion PCR. It allowed the construction of fused DNA fragments, in the order Promoter – Gene of interest – Terminator, for all the genes of interest except the cluster CAETHG_1620-1621. Despite of all the cloning techniques protocols have been strictly followed, there were some mistakes and some procedures that could have been optimized. Starting with the primers design, there were some basic design rules (Dieffenbach et al., 1993) that were not strictly respected concerning some of the primers:

- The melting temperatures (Tm) between the forward and reverse primers should not exceed a difference of 5ºC.
- The annealing temperatures should be bigger than 50ºC.
- The annealing temperature should be 5-10ºC higher than the primers Tm.
- The primers GC content should be between 40-60%.

Primers should be designed with the goal of obtaining a balance between two characteristics, efficiency and specificity of amplification. When rules like the ones referenced above are not followed, the PCR products can contain unrelated and undesired amplicons that sometimes unable their capability of accomplish the objective for which they were designed.

Another important step for a successful cloning is a proper purification of the genetic elements from the electrophoresis gel. The gel extraction methods should guarantee that the DNA

sequences are not going to be damaged during the extraction as well as avoid the loss of DNA. The GeneJET Gel Extraction Kit, used to gel purify all the PCR products until the ninth cloning attempt, guaranteed recovery rates up to 95%. However, recovery rates of around 5% were obtained. These losses of DNA could have been one of the reasons that led to the unsuccessful plasmid construction attempts since too low amounts of DNA were used.

Other procedures could have been done differently regarding all the cloning attempts:

- In the restriction ligation method, as some enzymes can exhibit slower cleavage towards specific sites, sometimes the restriction digestion reaction should be conducted for a longer period (considering a maximum of 2h). Moreover, if the ligation reaction is not working at reduced molar ratios of vector to insert, like the ratios 1:1 to 1:3 used in the present experiment, bigger molar ratios should be used until a maximum of 1:20 (frequently recommended for short inserts). The time extension of the ligation reactions where T4 DNA ligases were used is also advisable (New England Biolabs, 2017b).

- Considering all the cloning attempts, the lack of results could also be attributed to a wrong choice of colonies, after they grow in the selection plates. Frequently, tiny colonies appeared surrounding bigger ones. They are called satellite colonies and they are formed by bacteria's that take benefits from the enzymes that are released by the antibiotic resistant colonies to stablish around them. These released enzymes have the purpose of degrade the antibiotics, allowing the appearance of cells that were not transformed with the pEC500E, and consequently, are not antibiotic resistant. Normally, when these colonies appeared in the selection plates, they were too close to each other and to the antibiotic resistant colonies, making it difficult to distinguish which colonies were the antibiotic resistant and which were the satellites (New England Biolabs, 2017b).

- When the cluster CAETHG_1608-1611 was used, because it was too long and formed large DNA fragments, it should have been selected competent cell strains that can be transformed efficiently with large DNA constructs (clusters) (New England Biolabs, 2017b).

- As advised for the restriction ligation method, for other cloning methods it should have been increased the molar ratio of insert to vector as well as the concentration of fragments and/or vector. The absence of enough inserts allows the vector to self-ligate (New England Biolabs, 2017a; invitrogen, 2013).

After a meticulous evaluation of which could be the cloning steps from where the errors could have arisen, it was decided that the protocol alterations mentioned above should be the main sources of error. If in the future someone decides to continue the present work it is advisable to follow the previous recommendation.

6.Conclusions

To limit greenhouse gas emissions and improving air quality, alternative energies, such as biofuels, have been target of an increasing demand from governments and several institutions all over the world. Butanol is a promising biofuel with superior properties when compared with other alcohols used as environmental friendly fuels alternative. However, butanol production from ABE fermentations, is not optimized in a way that allows cost-competitive prices between butanol and other fuels. In this work, the implementation of an ANP mixotrophic metabolism in *C. beijerinckii* NCIMB 8052 was suggested. Thereby, the carbon conversion of substrates into acetyl-CoA, the main building block for butanol should be increased. As a first approach, it was mandatory to realize how ABE fermentations are conducted in the laboratory conditions where this work was developed, in order to stablish a control fermentation database and to collect information about the natural capabilities of *C. beijerinckii* NCIMB 8052. After a set of fermentations, performed under different conditions, the following was concluded:

- ✓ C. *beijerinckii* NCIMB 8052 is not naturally capable of fixing CO_2, since it did not grow under autotrophic conditions, in the presence of CO_2 and absence of glucose;
- ✓ The lack of a CO_2 fixation pathway in C. *beijerinckii* NCIMB 8052 does not allow a natural ANP mixotrophic metabolism performance;
- ✓ The implementation of 50 rpm agitation to the fermentation conditions allows a significant cell growth increase, as well as glucose consumption, when compared with the same conditions without agitation. Consequently, although not significantly, a bigger production of ABE is also noticed;
- ✓ The input of 2 bar of pressure to the fermentation conditions, where agitation is also applied, inhibits the agitation positive effects;
- ✓ When the fermentation is supplemented with CO_2, there is not an accentuated acid crash during the acidogenesis phase, and the pH maintains stable along the fermentation avoiding cell growth inhibition by the medium pH;

The second step consisted in the identification and characterization of all the genes involved in the WLP, the metabolism that needed to be activated in *C. beijerinckii* NCIMB 8052 to enable the desired ANP mixotrophic metabolism. *C. autoethanogenum* DSM 10061, a clostridium strain known to perform the WLP, was selected as model organism to find out which genes are essential for the WLP. After the cataloguing of these genes and several BLASTn searches made between them and the *C. beijerinckii* NCIMB 8052 genome, it was concluded that a group of genes responsible for encoding a key enzyme of the WLP, the CODH/ACS, was missing in C. beijerinckii NCIMB 8052. These genes were successfully amplified and isolated. The promoter and terminator, from the thiolase gene of *C. beijerinckii* NCIMB 8052, were chosen as suitable to overexpress the genes of interest. It was successfully obtained after fusion PCRs, fused DNA fragments containing the previous genes, coupled with the promoter and terminator in the order Promoter – Gene of interest – Terminator. After several cloning attempts to insert the fused DNA fragments into a pEC500E vector, no results were obtained and the proposed genetic manipulation to enable the WLP remained unfinished.

Despite of the unsuccessful trials made in the present work, the adopted genetic engineering strategy remains feasible. Some alterations to the cloning attempts previously performed, suggested in the discussion, can be made if an interest of continuing this work arise.

7.Bibliography

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8. Appendices

8.1. Culture media

CM2 medium

Table 13 – Recipe to prepare CM2 culture media;

Component	Final concentration [mM]	Final concentration [g/L]	Mass [g]	
Yeast extract	-	2,50	2,50	**Basal medium (780 mL)**
KH2PO4	7,35	1,00	1,00	
K2HPO4	3,50	0,61	0,61	
NH4Ac	37,62	2,90	2,90	
Glucose **x H2O**	222,22	44,00	44,00	**5 x Glc (200 mL)**
MgSO4 x 7 H2O	4,06	1,00	5,00	**50 x trace elements (100 mL)**
FeSO4 x 7 H2O	0,00	0,0066	0,033	
pABA	0,73	0,10	0,50	

Prepare the three solutions separately. All solutions are de-oxygenated for approx. 20-30 min with N2 and autoclaved, expect the trace elements solution which is sterile-filtrated in an empty, de-oxygenated and sterilized flask.

Notes:
1. When using Glc instead of Glc x H2O, use 10 % less.
2. Dissolve Glc in approx. 100 mL heated water. Fill up to 200 mL as soon as the solution cooled down.
3. Add 20 µL antifoam to 1 L medium before de-oxygenating.
4. pABA is only soluble in an acidic milieu; therefore, acidify with H_2SO_4 to approximately pH 2.4 and let it stir for a few minutes.

DSM 10061 medium

Table 14 – Recipe to prepare DSM 10061 culture media;

Component	Final concentration (g/L)
NH_4Cl	0,90
NaCl	0,90
$MgCl_2$ x 6 H_2O	0,40
KH_2PO_4	0,75
K_2HPO_4	1,50
Trypticase peptone (BD BBL)	2,00
Yeast extract	1,00
Trace element solution SL-10	1,00
$FeCl_3$ x 6 H_2O solution (0,1% w/v in 0,2 N HCl)	2,50
L-Cysteine-HCl x H_2O	0,75
D-xylose	5,00

Dissolve ingredients except cysteine and xylose. Sparge medium with 100% N_2 gas for 30 – 45 min to make it anoxic, then add cysteine and adjust pH to 6. Distribute medium under same gas atmosphere into anoxic Hungate-type tubes or serum vials and autoclave. Add xylose after autoclaving from an anoxic stock solution prepared under 100% N_2 gas atmosphere and sterilized by filtration. Adjust pH of the complete medium to 6, if necessary.

Trace element solution SL-10	
HCl (25%; 7,7 M)	10.00 mL
$Fecl_2$ x 4 H_2O	1,50 g
$ZnCl_2$	70.00 mg
$MnCl_2$ x 4 H_2O	100,00 mg
H_3BO_3	6,00 mg
$CoCl_2$ x 6 H_2O	190,00 mg
$CuCl_2$ x 2 H_2O	2.00 mg
$NiCl_2$ x 6 H_2O	24.00 mg
Na_2MoO_4 x 2 H_2O	36.00 mg
Mili-Q H_2O	990,00 mL

First dissolve $FeCl_2$ in the HCl, then dilute in Mili-Q H_2O and add the other salts. Finally make up to 1000,0 mL and filter-sterilize the solution.

8.2. Fermentation data

Heterotrophic fermentation, 60 g/L glucose (H1);

Table 15 – Results from the OD (600nm) and pH measurements made along the heterotrophic fermentation (H1);

			Replicate				***Replicate***			
			1	**2**			**1**	**2**		
Day	**Hour**	**Time (h)**	**OD**		**Average**	**SD**	**pH**		**Average**	**SD**
07/12/2016	11:00	0	0,051	0,050	0,0505	0,0005	6,23	6,23	6,230	0
07/12/2016	17:00	6	0,845	0,780	0,8125	0,0325	5,34	5,37	5,355	0,015
08/12/2016	11:00	24	3,060	2,920	2,9900	0,0700	5,25	5,20	5,225	0,025
09/12/2016	11:00	48	5,860	4,920	5,3900	0,4700	5,20	5,22	5,210	0,010
12/12/2016	11:00	144	3,400	3,680	3,5400	0,1400	5,03	4,99	5,010	0,020

Table 16 - Results from the fermentation products quantification made along the heterotrophic fermentation (H1). The considered values are the mean of two replicates;

	Glucose		***Acetic acid***		***Butyric acid***		***Propionic acid***		***Acetone***		***Ethanol***		***Butanol***	
Time (h)	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
0	62,95	0,15	1,78	0,00	0,00	0,00	-	-	0,00	0,00	0,00	0,00	0,00	0,00
6	61,71	0,02	1,95	0,02	0,36	0,01	-	-	0,00	0,00	0,00	0,00	0,00	0,00
24	53,85	0,99	1,01	0,05	0,95	0,06	-	-	1,20	0,13	0,00	0,00	1,19	0,20
48	35,04	1,45	0,58	0,05	0,48	0,01	-	-	3,17	0,35	0,11	0,11	5,39	0,70
144	28,74	1,36	0,65	0,03	0,88	0,05	-	-	3,75	0,08	0,24	0,01	6,92	0,12

Heterotrophic fermentation, 60 g/L glucose + 50rpm shaking (H2);

Table 17 – Results from the OD (600nm) and pH measurements made along the heterotrophic fermentation (H2);

			Replicate				***Replicate***			
			1	**2**			**1**	**2**		
Day	**Hour**	**Time (h)**	**OD**		**Average**	**SD**	**pH**		**Average**	**SD**
07/12/2016	11:00	0	0,052	0,050	0,051	0,001	6,25	6,24	6,245	0,005
07/12/2016	17:00	6	0,830	0,890	0,860	0,030	5,35	5,30	5,325	0,025
08/12/2016	11:00	24	4,420	6,960	5,690	1,270	5,54	5,35	5,445	0,095
09/12/2016	11:00	48	8,400	8,320	8,360	0,040	5,23	4,82	5,025	0,205
12/12/2016	11:00	144	5,700	5,240	5,470	0,230	4,95	4,81	4,880	0,070

Table 18 - Results from the fermentation products quantification made along the heterotrophic fermentation (H2). The considered values are the mean of two replicates;

	Glucose		*Acetic acid*		*Butyric acid*		*Propionic acid*		*Acetone*		*Ethanol*		*Butanol*	
Time (h)	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
0	57,45	2.68	1,65	0,18	0,00	0,00	-	-	0,00	0,00	0,00	0,00	0,00	0,00
6	63,26	1,03	2,00	0,01	0,40	0,02	-	-	0,00	0,00	0,00	0,00	0,00	0,00
24	49,43	1,14	0,63	0,21	0,33	0,01	-	-	2,65	0,62	0,00	0,00	3,54	1,26
48	27,15	2,30	0,30	0,07	0,47	0,22	-	-	4,43	0,25	0,26	0,03	7,66	0,30
144	24,70	4,34	0,34	0,06	0,64	0,10	-	-	4,56	0,36	0,26	0,03	7,99	0,64

Autotrophic fermentation, CO_2 (2bar) (A1);

Table 19 – Results from the OD (600nm), pH and pressure measurements made along the autotrophic fermentation (A1);

			Replicate				***Replicate***				***Replicate***			
			1	**2**			**1**	**2**			**1**	**2**		
Day	**Hour**	**Time (h)**	**OD**		**Average**	**SD**	**pH**		**Average**	**SD**	**Presure (bar)**		**Average**	**SD**
18/01/2017	11:00	0	0,056	0,060	0,058	0,002	5,53	5,50	5,515	0,015	2,00	2,00	2,000	0
18/01/2017	17:00	6	0,175	0,155	0,165	0,010	5,50	5,50	5,500	0	2,10	2,10	2,100	0
19/01/2017	11:00	24	0,595	0,615	0,605	0,010	5,42	5,40	5,410	0,010	2,05	2,05	2,050	0
20/01/2017	11:00	48	0,535	0,535	0,535	0	5,40	5,42	5,410	0,010	2,00	2,00	2,000	0
23/01/2017	11:00	144	0,515	0,505	0,510	0,005	5,39	5,39	5,390	0	2,00	1,95	1,975	0,025

Table 20 - Results from the fermentation products quantification made along the autotrophic fermentation (A1). The considered values are the mean of two replicates;

	Glucose		*Acetic acid*		*Butyric acid*		*Propionic acid*		*Acetone*		*Ethanol*		*Butanol*	
Time (h)	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
0	1,13	0,02	1,85	0,10	0	0	-	-	-	-	-	-	-	-
6	0,97	0,02	1,77	0,01	0	0	-	-	-	-	-	-	-	-
24	0,00	0,00	1,74	0,01	0,46	0,02	-	-	-	-	-	-	-	-
48	0,00	0,00	1,69	0,03	0,47	4,95E-06	-	-	-	-	-	-	-	-
144	0,00	0,00	1,73	0,02	0,49	0,01	-	-	-	-	-	-	-	-

Autotrophic fermentation, CO_2 (15%) + H_2 (5%) (A2);

Table 21 – Results from the OD (600nm) and pH measurements made along the autotrophic fermentation (A2);

			Replicate				*Replicate*			
			1	2			1	2		
Day	Hour	Time (h)	OD		Average	SD	pH		Average	SD
07/12/2016	11:00	0	0,051	0,054	0,0525	0,0015	6,20	6,21	6,205	0,005
07/12/2016	17:00	6	0,820	0,760	0,7900	0,0300	5,40	5,41	5,405	0,005
08/12/2016	11:00	24	0,660	0,645	0,6525	0,0075	5,51	5,52	5,515	0,005
09/12/2016	11:00	48	0,590	0,625	0,6075	0,0175	5,56	5,49	5,525	0,035
12/12/2016	11:00	144	0,620	0,650	0,6350	0,0150	5,50	5,49	5,495	0,005

Table 22 - Results from the fermentation products quantification made along the autotrophic fermentation (A2). The considered values are the mean of two replicates;

	Glucose		*Acetic acid*		*Butyric acid*		*Propionic acid*		*Acetone*		*Ethanol*		*Butanol*	
Time (h)	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
0	1,13	0,02	1,81	0,00	0,00	0,00	-	-	-	-	-	-	-	-
6	0,00	0,00	1,99	0,01	0,00	0,00	-	-	-	-	-	-	-	-
24	0,00	0,00	1,94	0,04	0,43	0,00	-	-	-	-	-	-	-	-
48	0,00	0,00	1,99	0,01	0,45	0,01	-	-	-	-	-	-	-	-
144	0,00	0,00	2,03	0,02	0,45	0,00	-	-	-	-	-	-	-	-

Mixotrophic fermentation, 60 g/L glucose + CO_2 (2bar) (M1);

Table 23 – Results from the OD (600nm), pH and pressure measurements made along the mixotrophic fermentation (M1);

			Replicate				*Replicate*				*Replicate*			
			1	2			1	2			1	2		
Day	Hour	Time (h)	OD		Average	SD	pH		Average	SD	Presure (bar)		Average	SD
07/12/2016	11:00	0	0,049	0,050	0,0495	0,0005	5,45	5,45	5,450	0	2,0	2,0	2,00	0
07/12/2016	17:00	6	0,185	0,180	0,1825	0,0025	5,46	5,45	5,455	0,005	2,0	2,0	2,00	0
08/12/2016	11:00	24	1,800	1,960	1,8800	0,0800	5,45	5,43	5,440	0,010	2,3	2,3	2,3	0
09/12/2016	11:00	48	4,200	4,440	4,3200	0,1200	5,69	5,72	5,705	0,015	2,6	2,7	2,65	0,05
12/12/2016	11:00	144	2,500	2,220	2,3600	0,1400	5,39	5,28	5,335	0,055	2,6	2,5	2,55	0,05

Table 24 - Results from the fermentation products quantification made along the mixotrophic fermentation (M1). The considered values are the mean of two replicates;

	Glucose		*Acetic acid*		*Butyric acid*		*Propionic acid*		*Acetone*		*Ethanol*		*Butanol*	
Time (h)	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
0	63,87	2,56	1,79	0,01	0	0	-	-	0,00	0,00	-	-	0,00	0,00
6	63,73	2,32	1,78	0,01	0	0	-	-	0,00	0,00	-	-	0,00	0,00
24	59,68	2,52	1,08	0,02	0,620	0,030	-	-	0,54	0,03	-	-	0,81	0,05
48	46,97	3,30	0,52	0,02	0,320	0,023	-	-	1,55	0,05	-	-	4,58	0,29
144	35,88	3,27	0,56	0,01	0,630	0,029	-	-	1,97	0,03	-	-	7,38	0,33

Mixotrophic fermentation, 60 g/L glucose + CO_2 (2bar) + 50rpm shaking (M2);

Table 25 – Results from the OD (600nm), pH and pressure measurements made along the mixotrophic fermentation (M2);

			Replicate				*Replicate*				*Replicate*			
			1	**2**			**1**	**2**			**1**	**2**		
Day	**Hour**	**Time (h)**	**OD**		**Average**	**SD**	**pH**		**Average**	**SD**	**Presure (bar)**		**Average**	**SD**
07/12/2016	11:00	0	0,050	0,049	0,0495	0,0005	5,44	5,43	5,435	0,005	2,0	2,0	2,0	0
07/12/2016	17:00	6	0,200	0,220	0,2100	0,0100	5,47	5,44	5,455	0,015	2,0	2,0	2,0	0
08/12/2016	11:00	24	2,340	2,200	2,2700	0,0700	5,64	5,64	5,640	0	2,5	2,5	2,5	0
09/12/2016	11:00	48	6,000	6,740	6,3700	0,3700	5,62	5,44	5,530	0,090	3,0	3,0	3,0	0
12/12/2016	11:00	144	2,800	2,800	2,8000	0	5,35	5,33	5,340	0,010	2,6	2,7	2,65	0,05

Table 26 - Results from the fermentation products quantification made along the mixotrophic fermentation (M2). The considered values are the mean of two replicates;

	Glucose		*Acetic acid*		*Butyric acid*		*Propionic acid*		*Acetone*		*Ethanol*		*Butanol*	
Time (h)	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
0	58,87	3,90	1,70	0,12	0,00	0,00	-	-	0,00	0,00	-	-	0,00	0,00
6	62,42	0,72	1,80	0,01	0,00	0,00	-	-	0,00	0,00	-	-	0,00	0,00
24	56,96	0,43	0,84	0,01	0,42	0,01	-	-	0,97	0,02	-	-	1,36	0,03
48	40,06	1,53	0,56	0,01	0,31	0,04	-	-	2,36	0,15	-	-	5,63	0,20
144	31,16	0,59	0,56	0,01	0,41	0,01	-	-	3,22	0,11	-	-	7,87	0,21

Mixotrophic fermentation, 60 g/L glucose + CO_2 (15%) + H_2 (5%) (M3);

Table 27 – Results from the OD (600nm) and pH measurements made along the mixotrophic fermentation (M3);

			Replicate				*Replicate*			
			1	**2**			**1**	**2**		
Day	**Hour**	**Time (h)**	**OD**		**Average**	**SD**	**pH**		**Average**	**SD**
22/11/2016	12:00	0	0,053	0,052	0,0525	0,0005	6,04	6,03	6,035	0,005
22/11/2016	18:00	6	1,410	1,450	1,4300	0,0200	5,36	5,38	5,370	0,010
23/11/2016	12:00	24	7,780	8,380	8,0800	0,3000	5,74	5,73	5,735	0,005
24/11/2016	12:00	48	8,700	8,675	8,6875	0,0125	5,13	5,68	5,405	0,275
25/11/2016	12:00	72	7,650	6,875	7,2625	0,3875	5,11	5,37	5,240	0,130
28/11/2016	12:00	144	7,200	6,850	7,0250	0,1750	5,10	5,28	5,190	0,090

Table 28 - Results from the fermentation products quantification made along the mixotrophic fermentation (M3). The considered values are the mean of two replicates;

	Glucose		***Acetic acid***		***Butyric acid***		***Propionic acid***		***Acetone***		***Ethanol***		***Butanol***	
Time (h)	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
0	62,85	0,14	2,26	0,02	0,00	0,00	-	-	0,00	0,00	0,00	0,00	0,00	0,00
6	60,67	0,68	2,43	0,00	0,49	0,02	-	-	0,00	0,00	0,00	0,00	0,00	0,00
24	36,93	1,45	0,67	0,01	0,47	0,02	-	-	2,20	0,17	0,00	0,00	6,02	0,09
48	22,84	1,00	0,63	0,05	0,79	0,32	-	-	2,67	0,03	0,23	0,01	9,25	0,25
72	21,11	0,91	0,63	0,07	0,99	0,21	-	-	2,32	0,10	0,22	0,01	9,35	0,67
144	21,19	0,98	0,65	0,07	1,10	0,17	-	-	1,12	0,02	0,21	0,00	7,87	0,52

Results overview;

Table 29 - Representation of the OD (600nm) and glucose content variation for all the analysed fermentation conditions, together with the fermentation products content at the end of them;

			[g/L] at the end of fermentation					
	Δ OD 600 nm	**Δ Glucose (g/L)**	**Acetic acid**	**Butyric acid**	**Ethanol**	**Acetone**	**Butanol**	**Total ABE**
Glucose (H1)	5,34	34,21	0,65	0,88	0,24	3,75	6,92	10,91
Glucose + Agitation (H2)	8,35	38,56	0,34	0,64	0,26	4,56	7,99	12,81
CO2 (A1)	0,55	1,13	1,73	0,49	0	0	0	0
CO2 + H2 (A2)	0,60	1,13	2,03	0,45	0	0	0	0
Glucose + CO2 (M1)	4,27	27,99	0,56	0,63	0	1,97	7,38	9,35
Glucose + CO2 + Agitation (M2)	6,32	31,25	0,56	0,41	0	3,22	7,87	11,09
Glucose + CO2 + H2 (M3)	8,63	41,66	0,65	1,10	0,21	1,12	7,87	9,20