Universidade de Aveiro Departamento de Química

Ano 2019

Alfredo Pina de Macedo

Produção de ácido succínico a partir de hidrolisados de pasta kraft do eucalipto

Production of succinic acid from eucalyptus kraft pulp hydrolysates

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Industrial e Ambiental, realizada sob a orientação científica da Doutora Ana Maria Rebelo Barreto Xavier, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e do Doutor Dmitry Evtyugin, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro.

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palavras-chave

Ácido succinico, Actinobacillus succinogenes, Batch, Fed-batch, Kraft, Pasta de papel, Hidrolisados, Resíduos lenhocelulósicos, Anaerobiose

resumo

O ácido succínico é um importante "*building block*" interveniente na indústria química, sendo uma molécula base na síntese de uma vasta gama de produtos. Apesar das suas origens biológicas, o ácido succínico era produzido exclusivamente pela indústria petroquímica, acarretando grandes desvantagens ambientais tais como a libertação de CO_2. Presentemente, com o desenvolvimento da biotecnologia e devido a um aumento de pressões ambientais e económicas, uma parte consideravel da produção de ácido succínico passou a ser feita através de bioprocessos.

A produção biológica de ácido succínico contrasta com a petroquímica ao consumir CO_2, permitindo assim contribuir para uma redução das emissões. Adicionalmente, já se encontra documentada a possibilidade de utilizar resíduos agroindustriais, nomeadamente lenhocelulósicos, como fonte de substrato para a produção de ácido succínico. Destes, é possível destacar os resíduos produzidos pelas indústrias papeleiras, nomeadamente resíduos da madeira onde o processo kraft se poderá revelar um forte candidato como pré-tratamento para bioprocessos.

O objetivo deste trabalho centrou-se na produção de ácido succínico a partir de pasta celulósica obtida pela deslenhificação de madeira de *Eucalyptus globulus* através do cozimento kraft. Para isso, fez-se a hidrólise enzimática das celulose e hemiceluloses presentes nas pastas kraft, para produção de um hidrolisado formado pelos respetivos monossacáridos. Este foi fermentado pela bactéria *Actinobacillus succinogenes,* biocatalisador deste processo, em operação batch e fed-batch.

Através da fermentação do hidrolisado de pasta de madeira obteve-se uma concentração de ácido succínico de 19.93 g/L, com produtividade e rendimento de 0.399 g/L.h e 0.331 $g_{acido\ succínico}/g_{açucares}$ respetivamente. Estes valores foram melhorados após a adição de carbonato de magnésio, apesar de se ter verificado um aumento na produção de subprodutos. No caso do *fed-batch,* verificou-se uma elevada produção de ácido láctico que a certa altura, superou a produção de ácido succínico. Não obstante, estes resultados podem ser utilizados a nível industrial, com o objetivo de produzir ambos os ácidos orgânicos, uma vez que estes poderão ser separados.

Este trabalho serviu de referência para estudos futuros utilizando resíduos de madeira como substrato, de forma a proceder à sua integração numa biorrefinaria de pasta de papel que se enquadre num modelo de Economia Circular.

keywords

Succinic acid, Actinobacillus succinogenes, Batch, Fed-batch, Kraft, Paper Pulp, Hydrolysate, Lignocellulosic wastes, Anaerobiosis

abstract

Succinic acid is an important “building block” to the chemical and polymer industries, being the base molecule for the synthesis of a vast array of products. Despite its biological origin, the succinic acid was initially produced by the petrochemical industry, having high environmental impacts like the release of CO_2. Nowadays, with the increasing development of biotechnology and due to environmental and economic concerns, there is a significant production of succinic acid by bioprocesses, within the biobased industry.

The biological production of succinic acid includes the consumption of CO_2 and contributes for reducing the emissions. Moreover, it is well documented the possibility of using agro-industrial wastes such as lignocellulosic, as feedstocks to produce succinic acid. From those it is possible to evidence the residues originated by the pulp and paper industry, namely wood residues, for which the kraft process could be used as a pre-treatment candidate for bioprocesses.

The aim of this work was to produce succinic acid from cellulose pulp obtained by delignification of Eucalyptus globulus wood through kraft cooking. The first step was an enzymatic hydrolysis of the celluloses and hemicelluloses to produce a hydrolysate composed by the respective monosaccharides. This hydrolysate was fermented by the bacterium *Actinobacillus succinogenes,* the process biocatalyst operated under batch and fed-batch conditions.

Using the kraft wood pulp hydrolysate, it was possible to attain a succinic acid concentration of 19.93 g/L, with a yield and productivity of 0.399 g/L.h and 0.331 $g_{succinic\ acid}/g_{sugars}$, respectively. These values were improved with the addition of magnesium carbonate, however, this salt also led to the production of a higher concentration of byproducts. In the fed-batch fermentation, it was verified that the lactic acid production surpassed the succinic acid production. Nonetheless, those results further evidenced the possibility of exploiting this fed-batch operation to produce both succinic and lactic acid.

This study served as a reference for future studies fed with wood residues, in order to their integration into a pulp and paper biorefinery working according to the Circular Economy model.

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List of Abbreviations

μmax – specific growth rate

acK – acetate kinase

ACoA – acetyl coenzyme A

ATP – adenosine triphosphate

CA – carbonic anhydrase

FR – fumarate redutase

Fse – fumarase

Hace – acetic acid

HC – hydrocarbons

Hfor – formic acid

Hlac – lactic acid

HPLC – high-performance liquid chromatography

Hsuc – succinic acid

LAB – lactic acid bacteria

LDH – lactate dehydrogenase

LEX/SEX – Ligand exchange/Size exclusion

MDH – malate dehydrogenase

MO – microorganism

MRS - Man, Rogosa and Sharpe medium

NAD – nicotinamide adenine dinucleotide

OD – optical density

P – product

PDH – pyruvate dehydrogenase

PEP – phosphoenol pyruvate

PEPCK – phosphoenol pyruvate carboxykinase

PFL – pyruvate-formate lyase

PK – pyruvate kinase

RI – refractive index

S – substrate

t – time

TSB – tryptic soy broth

WPH – wood pulp hydrolysate

X – biomass

Y – yield

1 Introduction

The oil is one of the main resources used by the humanity. It is utilized for both energy related applications and for the synthesis and extraction of important base molecules, commonly referred as building blocks[1]. These molecules are used in a wide variety of industries to produce more complex molecules and polymers. Building blocks can be obtained directly from the fractionated distillation of oil (Figure 1), or by processing each fraction.

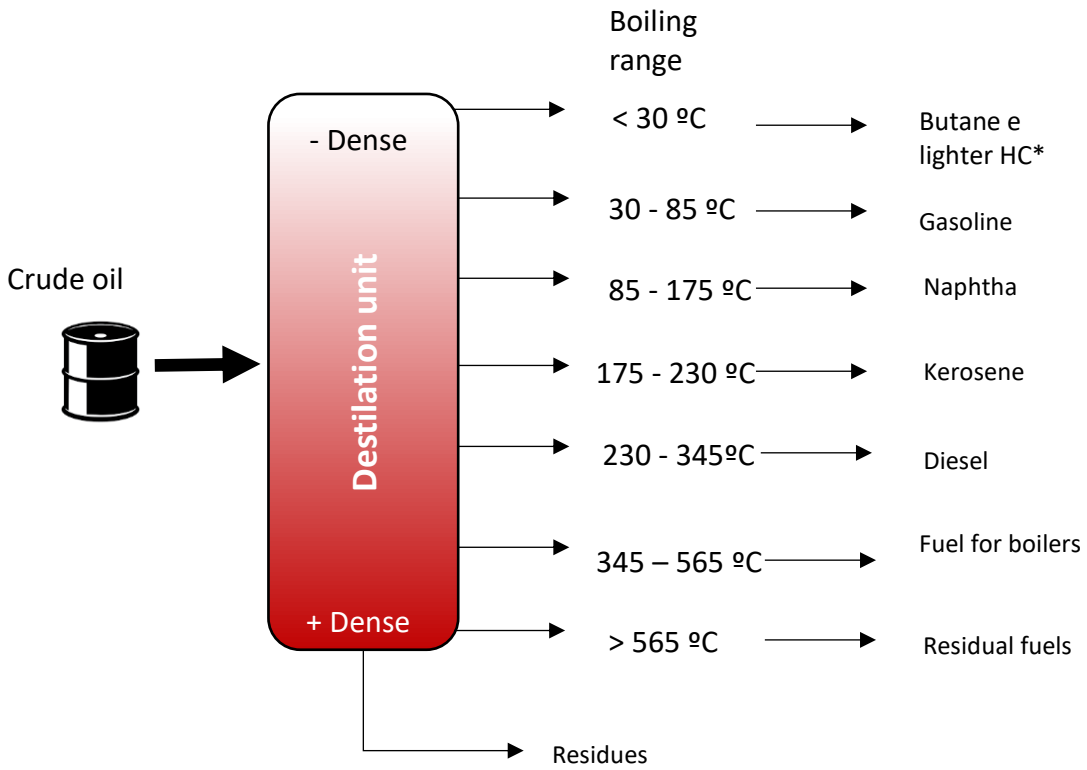


Figure 1 - Destilation process of crude oil (HC - hidrocarbonets). (Adapted from U.S. Energy Information Administration (2008))

The advantage associated with the molecules produced from crude oil relies on their low cost and high efficiency of the associated processes[2]. Nevertheless, the high carbon footprint is a major disadvantage associated with that type of industry. This can happen directly due to the consumption of fuels to generate the heat needed for the distillation process used in the oil fractioning. It can also happen indirectly, namely when using the fuels produced from the oil for transport aplication[3]. Another disadvantage, even though not directly linked to the building blocks, is the long cycle needed for regeneration of the oil. The crude oil consumption is already higher than the rate at which new reserves are discovered, as well as its regeneration rate[3] and its demand has been increasing each year (Figure 2)[4]. Such can lead to the economical depletion of oil since deeper reserves can be unviable to be explored[5].

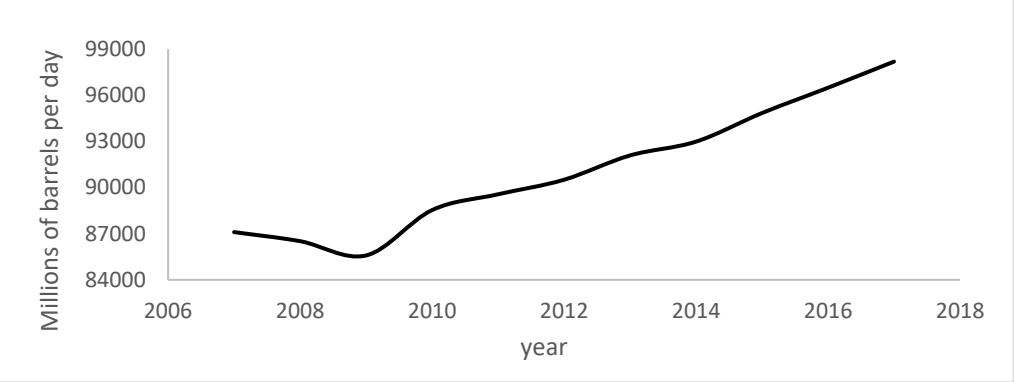


Figure 2 -Worldwide oil consumption evolution. (Data from BP (2018))

A suitable alternative to face the oil dependence can be found in vegetal biomass. Besides many added-value molecules, there are also a large number of sugars, mainly polysaccharides, present in it. Those can be suitable to feed industrial bioprocesses, using bacteria, yeasts or fungi as biocatalysts to produce metabolites, either to be used as biofuels or building blocks[6–8]. Additionally, all these processes can be associated, which result in the creation of different fraction that can be further processed, like what happen with the sugars that feed the fermentations. This led to the creation of the term "biorefinery", due to the similarities that the biomass fractioning shows, when comparing with the oil distillation that occur in the refineries.

Biorefineries which operates with food crops as feedstock, are categorized as the first generation. The most common crops used are cereals, mainly corn, due to their low price and high productivity. They also produce seeds with a high content on starch, a reserve polysaccharide[9]. This sugar, contrarily from what happens with most of the mono and disaccharides, is not directly fermented by many microorganisms (MOs), the reason why it is necessary a previous hydrolysis process[9,10]. In addition to cereals, the use of sugar cane and sugar beet has also been explored by some countries. They possess the advantage of containing sucrose, which is readily fermented by the majority of MOs[9]. However, due to their lower price, the use of cereals is more cost-effective, even with the hydrolysis process being considered[10].

The main disadvantage related to the first generation of biorefineries is their competition with the food industry. This can lead to socioeconomic impacts, such as rioting or starvation related to food price inflation, as illustrated in the Figure 3[11].

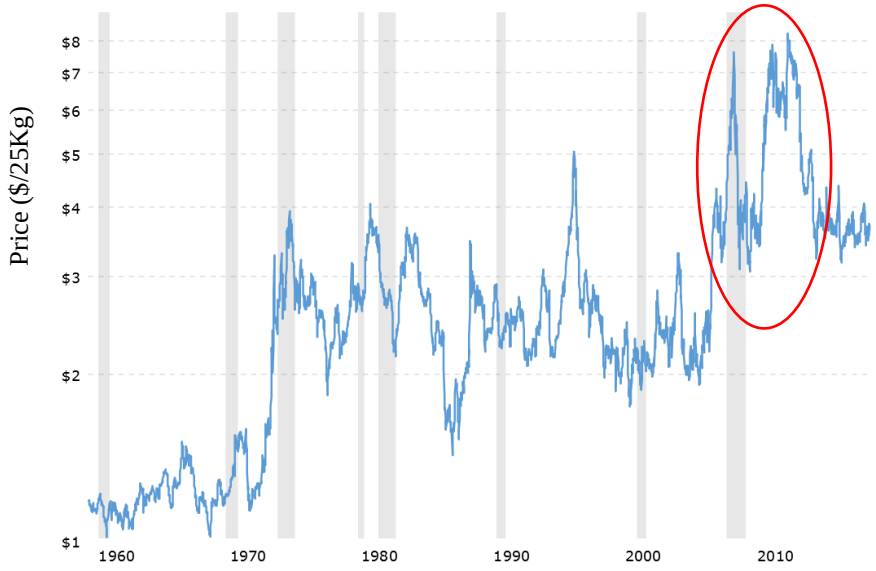


Figure 3 - Corn price evolution. The red circle represents a high price inflation, despite the higher abundance of corn (adapted from Macrotrends (2018))

It is visible that close to the year of 2010, there was a substantial inflation in the cereal prices and, as expected, starvation cases were verified in some producing countries. Paradoxically, this was also the year when there was the most considerable abundance of cereals worldwide, and those cases of starvation were being caused by the channeling the food resources into industrial procsses[11,12]. To avoid such problems, the uses of cereals and other food crops were put under restrictions by some countries.

The second generation of biorefineries operates with lignocellulosic residues. Therefore, the competition between food and industry is avoided. This type of biorefinery is also more sustainable, since the use of residues promote a circular economy model[13]. However, the lignocellulosic residues are complex, which lead to the necessity of additional processes to allow its use. Analyzing the chemical composition of lignocellulosic material, it can be highlighted three major components, cellulose, hemicelluloses and lignin[14].

Cellulose is a macromolecule composed by subunits of β-D-glucopyranose, linked by β(1→4) bonds (Figure 4)[9,14]. This polysaccharide only forms a linear rigid chain due to the strong intramolecular hydrogen bonds. These are bind by hydrogen bonds or by Van der Waals forces (intermolecular bonds), forming supramolecular structures, such as hydrogen bonds or by Van der Waals forces (intermolecular bonds), forming supramolecular structures, the fibrils and the microfibrils (Figure 5)[14]. Both of these interactions (intermolecular and intramolecular) are the reason why the cellulose has high tensile strength and low solubility [9,14]. The molecular weight of plant cellulose can reach values of 106 kDa or higher[15].

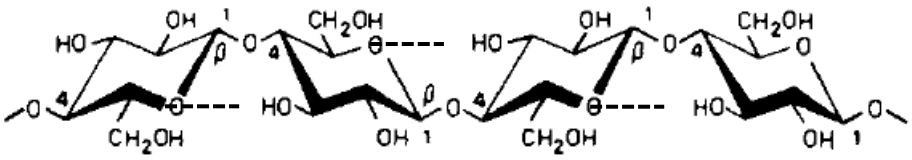


Figure 4 - Cellulose structure. The dotted lines represent the intramolecular hydrogen bonds (adapted from Sjöström (1992))

Along the microfibrils, two domains of cellulose can coexist in variable proportions, the amorphous domain, and the crystalline domain. The main difference between those domains relies to intensity and regularity of interaction by hydrogen bonds [14]. Therefore, the crystalline domain where the level of organization is higher, correspond to more hydrogen bonds interaction, being the opposite of the amorphous domain, where the level of organization is less regular[14]. Consequently, the last domain referred to represent the weak point in the hydrolysis, both chemical and enzymatic[14,16,17]. Microfibrils can ultimately associate themselves to form the cellulose fibers, the superstructure that contributes the most for the plant physical support[14,17].

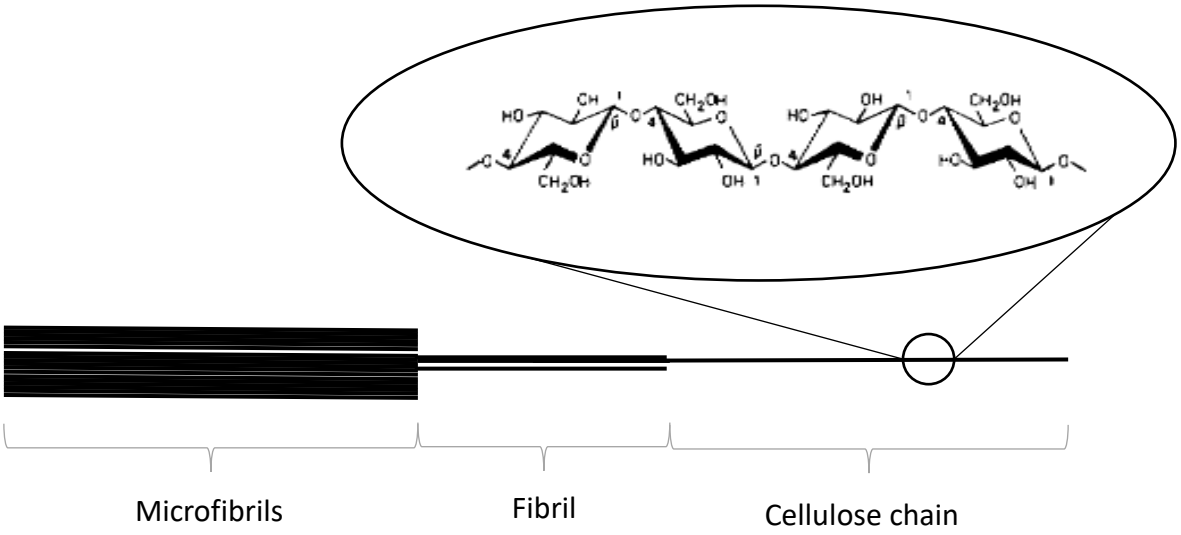


Figure 5 - Supramolecular hierarchical structure of a cellulose fiber

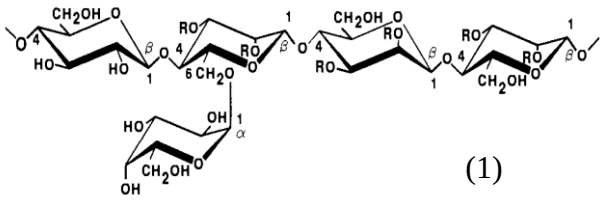
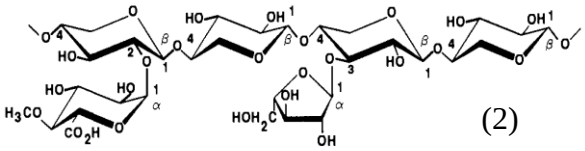
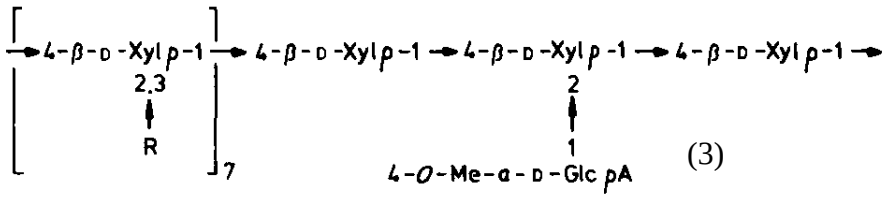
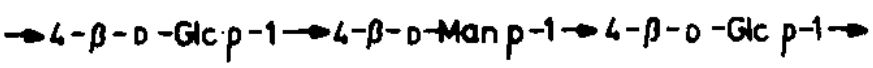
Hemicelluloses are structural polysaccharides of non-cellulosic origin composed by both pentoses or hexoses, linked essentially by (1→4) or (1→3) bonds[14,18]. However, the main backbones in hemicelluloses, unlike to cellulose, may possess ramifications such as α(1→2), α(1→3) and α(1→6)[9,14,19]. This group of polymers always has an amorphous structure, thus contrasting with the alternating structure of the cellulose. Moreover, the

morphologic and molecular characteristics of the hemicelluloses can give them some solubility in in water[14]. The average molecular weight of this kind of polymers is usually lower than 105 kDa[15].

Hemicelluloses are responsible for the structural association between the cellulose chains, allowing a better cohesion between them[14,17]. Also, they can interact with the lignin, further increasing its bonding strength to the cellulose microfibrils[9,14].

Another basic characteristic of the hemicelluloses is the difference between them from plant to plant. It can be emphasized two groups of plants based on the similarities on their constituent hemicelluloses, the softwoods and the hardwoods (Table 1)[14].

Table 1 - Most abundant polysaccharides among hemicelluloses. (1) Galactoglucomanan. (2) arabinoglucuronoxylan. (3) Glucuronoxylan. (4) Glucomanan. (Adapted from Sjöström (1992))

Softwoods	 (1)  (2)
Hardwoods	 (3)  (4)

The lignin is a rigid three dimensional aromatic polymer, composed by molecules derived from p-coumaryl, coniferyl and sinapyl alcohols (Figure 6)[14]. The lignin establishes covalent bonds with the hemicelluloses and non-covalent bonds with cellulose chains, which contributes to higher mechanical strength of the lignocellulosic material. Moreover, it can difficult the action of enzymes that would eventually degrade this material, such as cellulases, hence protecting the plants against the action of MOs[14,17,19]. Also, the phenolic nature of this polymer has an inherent antimicrobial activity, giving the plants an additional layer of protection against MO[20,21].

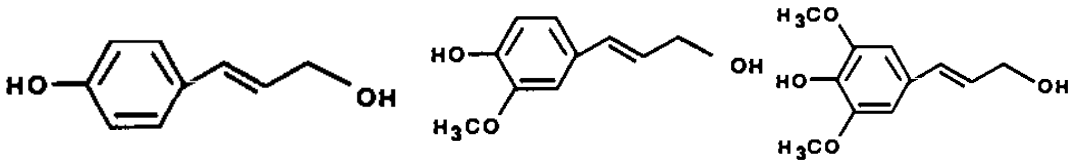


Figure 6 - Lignin precursors. Left: p-coumaryl alcohol; Center: coniferyl alcohol; Right: sinapyl alcohol (Sjöström (1992))

The presence of the lignin can have negative impacts on the use of lignocellulosic materials for the bioprocessing purposes. This polymer can thus led to reduction of both the hydrolysis efficiency and the fermentations yields[13,21]. Therefore, the integration of pre-treatment steps to remove the lignin can be useful in a biorefinery concept, improving the hydrolysis and fermentation of the lignocellulosic material. Additionally, the removed lignin can be recovered, hence valorizing the feedstock used.

The pulp and paper industry is an example of an activity which produces large quantities of residues[22]. In Portugal for example, The Navigator Company alone produces more than 80 kg of residues per ton of product[23]. However, many of these residues are simply discarded or burned for the production of electricity, instead to be involved in more cost

attractive processing. Consequently, the pulp and paper industry can be a suitable candidate to be integrated in a biorefinery concept[24].

A key step in wood pulp production is the dispersion of the cellulose fibers in water, which can be done mechanical or chemically. The principle beyond the chemical processes is the dissolution of lignin, causing the release of cellulosic fibers into the aqueous medium. Among these chemical processes it is possible to highlight the kraft cooking process, one of the most used worldwide for pulp making[25]. This process uses sodium hydroxide and sodium sulfide, which turns the medium alkaline ,usually with pH higher than 12[25,26]. These conditions, alongside the high pressures and temperatures, dissolve most of the lignin, forming a cellulosic pulp[25–27].

Although the kraft process is mainly intended to produce pulp for paper, its ability to delignifying the lignocellulosic material makes it a suitable candidate for the pretreatment needs. This can be an advantage for a pulping industry since they do not need to invest in additional operation units. Additionally, the kraft process can deal better with higher quantities of lignin than other chemical processes, making it more suitable to be integrated into a biorefinery concept[25]. Ultimately, the kraft process should be suitable to delignify lignocellulosic wastes, with higher lignin content to be used in papermaking. Those treated wastes are the suitable to be hydrolyzed and used as substrate for bioprocesses.

To produce the fermentable sugars from the kraft pulp, a previous hydrolysis step is needed. This step can be performed by acidic or enzymatic hydrolysis. The acidic hydrolysis is the quickest method to produce fermentable sugars. Nevertheless, it has the disadvantage to degrade the resulting monosaccharides into furfural and hydroxymethilfurfural, both strong inhibitors of MO[28,29]. Moreover, in the end of the hydrolysis the pH needs to be heavily corrected to make the substrate suitable for fermentation. On the other hand, the enzymatic hydrolysis uses mild conditions at physiologic values of pH. This avoids the production of inhibitors and lead to less corrections of the pH[13,30]. Therefore, this was the chosen hydrolysis process used in this thesis.

Various metabolites can be produced with monosaccharides originated by lignocellulosic residues, in several types of bioprocesses. In this case, the studied process was the production of succinic acid (Hsuc). This molecule has significant importance in the chemical industry as a precursor for other molecules and easily degradable polymers[31,32]. It

is classified as one of the most essential base molecules in the chemical industry, being additionally used in the formulation of pharmaceutical and food products[33,34]. The Hsuc was a building block which was produced exclusively by the petrochemical industry. However, do to environmental and economic pressures a considerable part of its production shifted to a biobased industry, using renewable resources as feedstock[35,36]. The Hsuc originated by renewable sources, like the lignocellulosic biomass, is also linked with the consumption of CO_2, both in the plants' growth and in the Hsuc metabolic production itself. Such makes this bioprocess for Hsuc production highly sustainable[37].

There are many organisms, mostly prokaryotic capable to naturally produce and accumulate Hsuc. An example of that is the bacterium *Actinobacillus succinogenes.* This was first described in 1999 by Geutler et al. and it is still a reference MO for study the production of Hsuc in different substrates, due to its accessibility and easiness to operate.

Overall the aim of this thesis was to produce Hsuc in a bioprocess fed by delignified *Eucalyptus globulus* pulp, generated by the kraft pulping process and posteriorly hydrolyzed. Such can allow to understand the possibility to integrate the kraft process into a biorefinery concept. The MO used was the *A. succinogenes*, which was operated under anaerobic conditions.

2 Overview

2.1 Pulp and paper industry biorefinery potential

Due to the growth of pulp and paper industry, a crescent residue production is being verified. To attenuate this problem, new sustainable strategies are being developed. One strategy is to integrate the pulp and paper process into a biorefinery concept.

The chemical processes are the main pulping methods used in this type of industry. Their objective is to chemically remove the lignin from the lignocellulosic feedstock used for the papermaking[25]. Due to their capacity to fractionate the raw material used, the pulping processes are a suitable candidate to be used as a biorefinery unit. Furthermore, the carbohydrate fraction obtained can be used to feed bioprocesses, such as the Hsuc production. Just an example, kraft delignification can be used for the production of fibrous material from bark that nowadays is underutilized waster being burned for the energy production.

2.1.1 Kraft process

The pulping process is an essential step in papermaking. It has encouraged the development of various methods, being the chemical ones used more often in the industry due to their higher efficiency. From those, it is possible to evidence the kraft process. This method was developed in 1879 and nowadays is one of the most used chemical pulping process[25]. The main advantage of it is the higher tolerance to wood where the lignin content when compared to other chemical pulping process, which makes it more efficient in terms of feedstock usage. The kraft process uses sodium hydroxide and sodium sulfide, which determines its characteristic high pH, usually higher than 12[25,26]. These alkaline conditions,

in conjunction with high pressures and temperatures, dissolve most of the lignin, leaving a wood pulp composed almost exclusively by polymeric sugars.

In the papermaking industry contest, the pulping step is what allows the creation of raw material to produce the paper[25–27]. However, in a biorefinery point of view, this can constitute a suitable pretreatment for the lignocellulosic material present in this work. As such, the polysaccharides can be easily hydrolyzed without having the lignin, which increases the process efficiency[13]. Additionally, without lignin it is easier to use the hydrolyzed polysaccharides present in the lignocellulosic material as substrate for biomass, since the possibilities of inhibition are reduced[20,38].

It is possible to conclude that a pulping facility that uses the kraft process, can be easily converted into a biorefinery. Furthermore, the ability of this process to tolerate larger quantities of lignin allows to process the wastes generated from the respective pulping industry, thus valorizing them.

2.1.2 Wood pulp hydrolysis

The delignified wood pulps can be a suitable substrate for microbial bioprocesses due to their sugar content. However, these sugars, are present in the polymeric form, and thus cannot be directly metabolized in biorefinery processes[13,14,17,39]. Nonetheless, a previous hydrolysis step can turn this polysaccharides into their monomeric forms, making them suitable for biorefinery processes, such as the Hsuc bioproduction[14,40].

There are two main hydrolysis technics used nowadays, the acid hydrolysis and the enzymatic hydrolysis[14]. The first one uses strong mineral acids, such as H_2SO_4 and HCl, at high temperatures (higher than 150 ºC)[14]. This method, however, can lead to the degradation of the resulting monosaccharides to furfural and hydroxymethyl-furfural[14], both strong MOs inhibitors. Even with the low concentration of acid, the high temperatures presents in the process can lead to the formation of those inhibitory molecules[14,41]. Consequently, these factors are the main disadvantage of acid hydrolysis and the reason why this process is being replaced.

The enzymatic hydrolysis is, therefore, a suitable alternative for the acidic processes. Contrarily to these, the pH and temperature conditions are relatively moderate, since many

enzymes operate around 50 ºC and the pH values commonly never drop below 4 [30,42]. This way, the enzymatic hydrolysis presents a higher efficiency than the acid hydrolysis, due to less energy consumption and lower need of pH correction on the final hydrolysate. The majority of enzymes used in these processes are expressed by genes found in white rot fungi, especially *Thrichoderma* species[14,28]. Those enzymes can be divided into two groups: a) The cellulases, which are responsible for the degradation of the cellulose; b) The enzymes that degrade hemicelluloses (also referred as hemicellulases for some authors)[14,28,30]. In the cellulases group is possible to highlight three important types of enzymes, the endo-glucanase, responsible for hydrolyzing lower crystallinity spots on the cellulose, the exo-glucanase, responsible for the degradation of the cellulose chains' ends releasing cellobiose units, and the β-glucosidase, which specifically degrades the β links in the cellobiose units, hence releasing its glucose (Glu) subunits[14,30]. As for the enzymes responsible for the degradation of hemicelluloses, their diversity is higher, being possible to highlight the xylanases, which can hydrolyze the xylans, present in high quantities in the hemicelluloses[30].

2.2 Succinic acid

Hsuc (IUPAC name: butanodioic acid) (Figure 7) is an organic dicarboxylic acid, naturally produced by many organisms[43]. It is a colorless and water-soluble molecule, with the capability to buffer a solution at pH values of 4.21 and 5.64 [43]. The most referred metabolic pathway in the formation of Hsuc is the respiration (both aerobic and anaerobic), beginning with the degradation of its carbon source to form acetyl coenzyme A (ACoA) to initiate the citric acid cycle[8]. Besides, it is an important electron donor of electrons, hence playing an important role in cellular respiration and other metabolic pathways of anaerobic organisms[44,45].

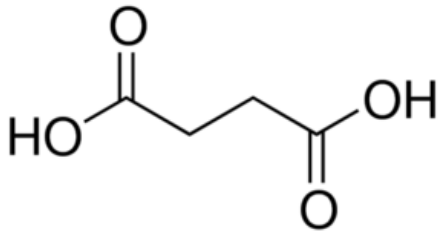


Figure 7 - Hsuc structure. (NCBI)

The importance that Hsuc acquired in the XX century was due to its first uses as chemical building block[40,46,47], base molecules in the chemical industry. These are known for their ability to produce more complex molecules, including polymers, hence being able to originate a vast array of products[46,48].

Hsuc was one of the first molecules to be used as a building block. It can originate both commodity chemicals[47], used to satisfy worldwide demands[47,48], and specialty chemicals[34,47], capable of attributing a differentiating property to a certain product. The main examples of commodity chemicals produced by Hsuc are, a) the 1,4-butanediol, an important solvent and precursor of polymers such as polyester and polybutylene[8], b) the adipic acid, used as lubricant, as food additive and also a precursor to nylon, c) the γ-butyrolactone [6,8], used in the textile industry and as paint remover, d) the tetrahydrofuran, main component of adhesives and inkjet for printers and the e) linear aliphatic esters[8,34], which are the base molecules in the production of polymers degraded by industrial composting[31,32], such as the polybutylene succinate[33,35,49–51].

Regarding the specialty chemicals, there are predominantly formulations which integrate the Hsuc itself. As main examples are a) the animal rations, in which the Hsuc can promote the production of propionate, hence increasing the protein synthesis of the animals[52], b) the sodium succinate, as flavor enhancer, c) the diethyl succinate, used as solvent in cleaning products especially for metallic surfaces, the d) ethylene diaminodisuccinate, an ion chelator which potentially can substitute the EDTA, and e) the Hsuc itself as supplement for plant growth[34]. Due to all these applications of this C4 organic acid, it is comprehensible the broad market created around it.

Even though it was previously mentioned that the Hsuc is present in many organism's metabolisms, its commercial production started in the petrochemical industry (Figure 8)[6,36].

Some processes were a) the oxidation of 1,4-butanediol, b) catalytic hydrogenation of C4 dicarboxylic anhydrides, or unsaturated acids and c) carbonylation processes using ethylene or acetylene. From these, the more direct approach and still present nowadays, is the oxidation of maleic anhydride (based on b)), extracted from naphtha to succinic anhydride, which is further hydrated to Hsuc [6,36]. In this process, the first step requires temperatures ranging from 120 ºC to 180 ºC, with pressures reaching the 4 MPa in the presence of a metal catalyst such as nickel or palladium[6]. In the second part of the process, where the succinic anhydride is hydrated, methods can be implemented to increase sustainability, such as water recirculation[6]. This two-step process has the advantage of being a highly efficient one, with conversion rates close to the theoretical values, allowing for production costs around 6-9 $/Kg Hsuc. Nonetheless, it has some disadvantages such as the carbon footprint related to the petrochemical industry and the energetic demand of the process itself[36].

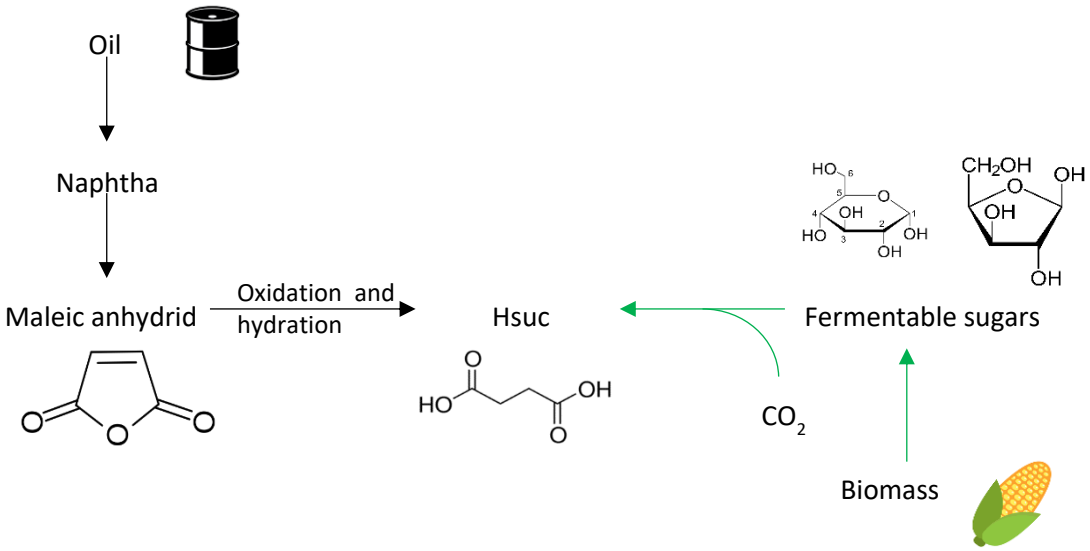


Figure 8 - Hsuc petrochemical production vs biotechnologic production. (Adapted from Bechthold et al. (2008))

Nowadays the with the development of biotechnology the petrochemical rout to produce is close with the biobased production. Therefore, even though the biological approach is less efficient, leading to the formation of unwanted products, it allows for production costs which in time might turn out to be competitive[36]. Additionally, the spike of the oil price in 2007, led to an increasing investment on biological production of Hsuc[4,31,36].

Nowadays, recent decreases in oil prices, caused by the large quantities of shale oil entering the market, led to pressure on the biotechnological Hsuc industry[53]. Therefore, novel approaches shall be studied, thus improving the competitiveness of the biotechnological produced Hsuc.

2.2.1 Producers of Hsuc

There is a large number of MO capable of metabolically producing Hsuc. Normally, the exclusive presence of the citric acid cycle's reductive branch is what cause these MO to produce large quantities of Hsuc (Figure 9)[37,54]. This pathway allows the oxaloacetate (OAA) to originate Hsuc. Since this organic acid cannot be further metabolized, it accumulates and is excreted to the fermentation medium[54]. This type of metabolic pathway is more common in prokaryotic organisms, which are anaerobic. However, it is possible to highlight few eukaryotic organisms as potential Hsuc producers, like fungi from the genus *Aspergillus* or *Penincillium*[51], which are less reported in the literature.

Naturally, the Hsuc producers also present alternative pathways to produce other metabolites. However, those can be undesirable from a bioprocess point of view[37,55,56]. Some common examples of Hsuc alternative pathways can be the ones responsible for the formation of acetic acid (Hace), formic acid (Hfor), lactic acid (Hlac) and even ethanol (EtOH). Therefore, to counteract these disadvantages of performing undesirable metabolic pathways, genetically modified organism were developed[49], as further explored.

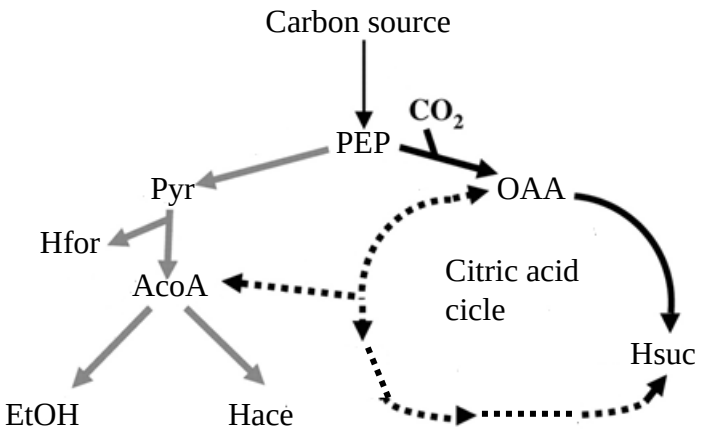


Figure 9 - General representation of the metabolism associated with Hsuc producers. The dotted lines represent the oxidative branch of the citric acid cycle, absent in these organisms; the black continuous line represents the reductive branch of the citric acid cycle; the gray lines represent some competitive pathways.

In the following table, it will be presented organisms already investigated with the ability to produce Hsuc naturally:

Table 2 - Hsuc natural producers, substrates, types of operation and kinetic and operational parameters

Specie	Strain	Substrate ([g/L])	Operation type	Hsuc			Ref.
				Concentration (g/L)	Productivity (g/L.h)	Yield (g/g)	
Actinobacillus succinogenes	130Z	Glu (9)	Batch	4.2	0.28	0.46	49, 59
		(nm)	Batch	67.2	0.8	0.7	58
		(60)	Continuous	28.9	nm	nm	59
		Spent sulfite liquor (SSL) (5-20)	Fed-batch	27.4	0.39	0.52	62, 63
		(5-20)	Fed-batch	25.2	0.47	0.57	62, 63
		Whey (50)	Batch	21.5	0.44	0.57	62
		Residual Biomass (65.8)	Batch	48	0.94	0.75	63
	CGMCC 1593	Glu (25)	Batch	60.2	1.3	0.75	64
		Melaço da cana (65)	Batch	50.6	0.84	0.8	65
Basfia succiniciproducens	DD1	Glu (50)	Batch	20.00	nm	0.49	66
	JF 4016	SSL (5-20)	Fed-batch	33.8	0.48	0.58	62, 63
		(5-20)	Fed-batch	39	0.31	0.54	62, 69
	BPP7	*A. donax* Hydrolizate (59)	Batch	9	nm	0.84	68

n.m - not mentioned

Table 2 cont.

Specie	Strain	Substrate ([g/L])	Operation type	Hsuc			Ref.
				Concentration (g/L)	Productivity (g/L.h)	Yield (g/g)	
Anaerobiospirillum succiniciproducens	ATCC 53488	Glu (20)	Batch	34.4	1.8	0.86	69
		(50)	Batch	32.2	1.2	0.99	70
		(19)	Continuous	3.91	2.03	0.85	71
	ATCC 29305	Galactose (27)	Batch	15.3	1.46	0.87	72
		Whey (48.7)	Fed-batch	34.7	1.02	0.91	73
		(48.7)	Continuous	19.8	3	0.64	73
		Wood hydrilysate (27)	Batch	24	0.74	0.88	72
Mannheimia succiniciproducens	MBEL55E	Glu (20)	Batch	14	1.87	0.70	74
		Whey (28)	Batch	13.4	1.18	0.71	75
		(22)	Continuous	6.4	3.9	0.69	75
Corynebacterium glutamicum	R	Glu (18)	Batch	5	2.7	0.288	76
Enterobacter sp.	LU1	Glycerol (50) and lactose (25)	Batch	35	0.51	0.11	50

From Table 2, it is possible to observe the *A. succinogenes* as the most prominent natural producers of Hsuc, being also one of the first to be isolated[77]. With this species, there was already achieved the production of considerable Hsuc concentrations as well as high productivities and yields in its bioprocesses. Moreover, works such as the one done by Alqvist et al. (2016) have shown its ability to ferment lignocellulosic wastes[78,79]. In Table 2 the highest concentration of Hsuc (67.2 g/L) was achieved by *A. succinogenes*, being this strain one of the most readily available. The *M succiniciproducens* is also a good producer due to its high Hsuc productivity. Such factor makes this bacterium a suitable candidate to be operated in continuous fermentation. However, laboratory studies with the *M succiniciproducens* can be difficult, since its use is currently limited by a patent[80].

The bacterium *B. succiniciproducens* that was more recently isolated has received interest due to its genetic similarity with the M. succiniciproducens[80]. Nonetheless, its use to produce Hsuc is still relatively recent, being necessary further investigation, to ensure the optimization of the productivity and yield parameters. The *A. succiniciproducens* is another natural Hsuc producer with strong perspectives to be operated in continuous, presenting one of the highest yields achieved. However, this species presents the operational disadvantage of being a restricted anaerobe[31,69], which implies additional care in its handling as well as additional costs in case of industrial use. Furthermore, there is not a total understanding of the *A. succiniciproducens* and therefore there are no optimized processes reported [31].

C. glutamicum, a bacterium already explored by the industry to produce amino acids, acetone, butanol, and ethanol (the last three are produced in what is known the ABE process), was already studied as a possible biocatalyst for Hsuc production. However, this bacterium also generates large quantities of byproducts, which are the reason for its low Hsuc yields[76]. Nevertheless, genetic editing of the *C. glutamicum* can make it suitable to be use as a Hsuc producer at industrial level[81].

The last bacterium of Table 2 is the *Enterobacter sp.* and was only recently described as Hsuc producer, using glycerol. This substrate is an abundant byproduct from biodiesel production and hard to be further process[50]. Despite this bacterium showed the capacity to ferment glycerol, respective yields were low and lactose was assayed as co-substrate[50]. However, to get an economically viable production further research is need.

Besides these natural producers, genetic engineering allows for the creation of fine-tuned MOs to produce Hsuc, as presented in the next subtopic.

2.2.2 Biotechnological production of Hsuc by *A. succinogenes*

The bacterium *A. succinogenes* was isolated from the bovine rumen, and firstly described in 1999[77]. Its name reflects into the bacterium elongated cylindrical shape and its ability to produce Hsuc[77]. It is a Gram-negative bacterium, belonging to the *Pasteurellaceae* family, which is believed to create innumerous symbiotic relations with their host organism[82]. Also, the species of this family are known to be responsible for the degradation of cellulosic material present in their host's foods, with the benefit of producing important metabolites for them [77,82]. Consequently, that characteristic can indicate the possibility to use this family's species in lignocellulosic biomass based bioprocesses[77].

Looking to its physiology, the *A. succinogenes* has an optimal temperature of 37 ºC where both of its growth and Hsuc production are maximum. This temperature is also close to that registered in the cow's rumen (38-38.5 ºC)[83]. It is a capnophilic facultative anaerobic bacterium, preferring environments of microaerobiosis, especially the ones that contain around 40% of CO_2[77,84]. This characteristic also relate this bacterium with its host organism, since the cow's rumen present low concentrations of O_2 and relatively high concentrations of CO_2[85,86].

A. succinogenes can have a stable production of Hsuc at pH values ranging from 6.0 to 7.2, being the optimal values often described close to 6.7 [59,85]. Moreover, below the pH value of 5.8 an inhibitory effect starts to be noticed, either in the *A. succinogenes* growth rate or in the production of Hsuc. This metabolite, along with other organic acids, namely the Hace, contributes to a decrease in the fermentation medium pH, being necessary a constant addition of base to ensure the best condition for the Hsuc production[64,85,87]. To maintain the optimal pH values, it is common to use NaOH or, as some studies suggest, carbonated salts, being ensured that they could not cause an inhibitory effect, which could interfere with the process[64,69]. Previous studies had already appointed the ion Ca^{2+} present in the calcium carbonate as a strong inhibitor of the Hsuc production. Moreover, there was also verified that Mg^{2+} present in $MgCO_3$, is described to have a positive effect in the *A.*

succinogenes metabolism, thus being commonly used to adjust the pH[88]. Other carbonated salts that are regularly used are the sodium carbonate and sodium hydrogenocarbonate, however they are known to promote the *A. succinogenes* to aggregation, hence adding difficulties in mass transfer and the overall process efficiency[64].

The *A. succinogenes* was previously identified as capable of consuming the majority of monosaccharides present in the lignocellulosic biomass[13,21,67,89]. However, in contrast to other organisms from the *Pasteurellaceae* family, the *A. succinogenes* does not have the enzymes needed to hydrolyze polysaccharides. Inside the bovine rumen, those polysaccharides are normally degraded by other bacteria. From the resulting monosaccharides, the *A. succinogenes* can metabolize both pentoses and hexoses[31,79,82]. Nonetheless, when operating in pure culture, this biological relation cannot be maintained and a previous hydrolysis step when using lignocellulosic biomass[7,13]. The concentration of monosaccharides in the fermentation must then be controlled to prevent the described substrate inhibition of the A. succinogenes. In the literature, the often-described optimal concentration of monosaccharides for the Hsuc production is normally between 50-60 g/L[87].

A key step in the *A. succinogenes* Hsuc production metabolism is the formation of Oxaloacetate directly from phosphoenolpyruvate (PEP), alternative to its formation from ACoA[31,37,57]. This molecule then follows the reductive branch of the citric acid cycle, which is interrupted after the formation of Hsuc, blocking its consumption and thus leading to its accumulation[35,37]. It is essential to notice that this metabolic pathway will eventually compete with others, such as the acetogenesis , also the ethanol and lactic acid fermentation, which can consequentially lower its yield[37,90].

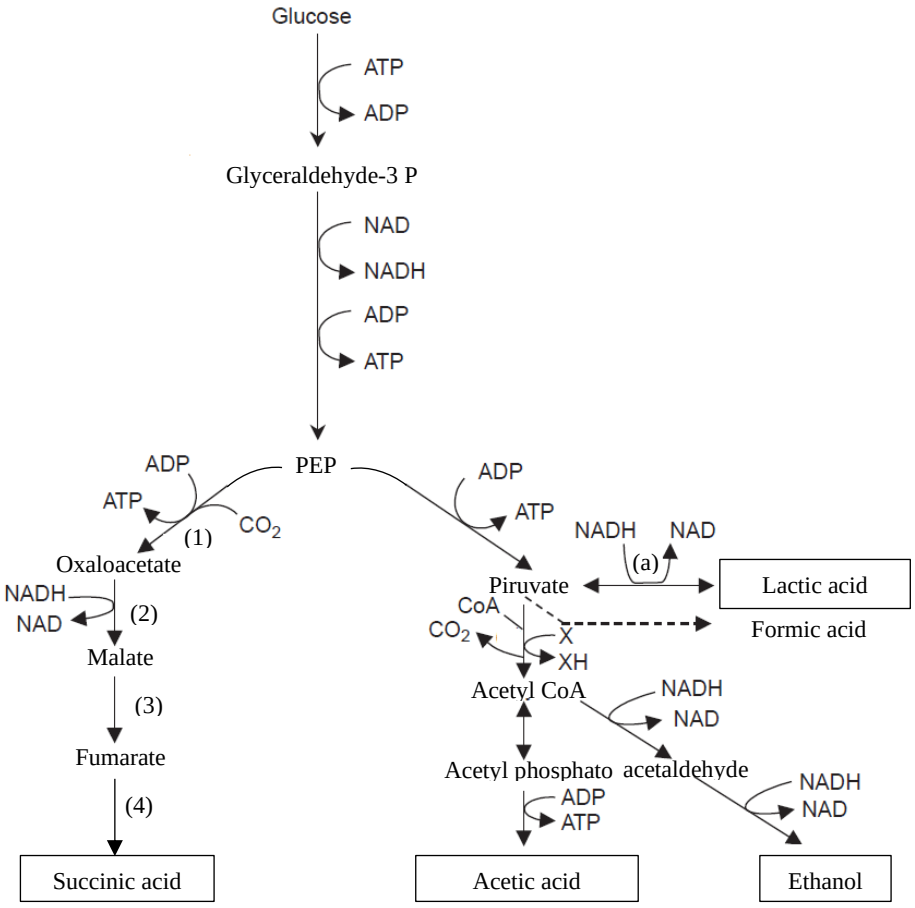


Figure 10 - Simplified metabolism of the *A. succinogenes*. (1) PEPCK, (2) MDH, (3) Fse, (4) FR, (a) LDH. The numbers represent the metabolic pathways of Hsuc production, and the letter represent the Hlac formation that can supplant it. (adapted from Samuelov (1991))

There are four vital enzymes in the *A. succinogenes* worth mentioning in the production of Hsuc: 1) the phosphoenolpyruvate carboxykinase (PEPCK); 2) the L-malate dehydrogenase (MDH); 3) the fumarase (Fse) and 4) the fumarate reductase (FR) (Figure 10). The first one (1) is known mainly for catalyzing the transformation of oxaloacetate into PEP with the release CO_2[91–94]. However, it can also promote the inverse reaction, which is the base of Hsuc natural production, by consuming CO_2. Additionally, the activity of the PEPCK increases with the concentration of this molecule, since it will be the source of inorganic carbon to transform the PEP (three carbons) into the oxaloacetate (four carbons)[37,57]. The concentration of CO_2 is therefore a factor that can be exploited to favor

the *A. succinogenes* metabolism in producing Hsuc[95]. It is even possible to find studies, which suggest the use of waste CO_2 in Hsuc production bioprocesses, ultimately increasing its overall sustainability[85,86,96]. The other three enzymes, the MDH (2), Fse (3), and FR (4), are responsible for catalyzing the conversion of oxaloacetate to malate, malate to fumarate and, finally, the fumarate to succinate, respectively. It is also worth mention that all these key enzymes are more active at slightly acidic values of pH[57,85].

2.2.3 Acetogenesis

In the *A. succinogenes* metabolism, the Hsuc production main competitor pathway generally is the acetogenesis, which makes the Hace its major fermentation byproduct This acid, besides its difficulty to be separated from the Hsuc, also contributes to the inhibition of the bacterium metabolism[37,88]. Some reports suggest that the production of Hace is increased when the CO_2 concentration in the medium is low[57,97]. Nonetheless, the formation of Hace can still be considerable at high CO_2 concentrations.

The acetogenesis is, therefore, one of the biggest challenges related with the use of *A. succinogenes* to produce Hsuc. Moreover, this pathway cannot be suppressed from the bacterium metabolism, since it constitutes a major source of ATP for the *A. succinogenes* and has a crucial role on the formation of pyruvate and ACoA[35,37], essential for the bacterium. Therefore, it is only possible to mitigate the production of Hace, thus a process to separate the Hsuc from the Hace will always be needed[37].

Despite the acetogenesis being the metabolic pathway which is the most competitive with the production of Hsuc, it is important to mention the existence of other byproducts of the *A. succinogenesis* metabolism, such as the Hfor, which can be more unwanted than the Hace, due to its inhibitory effect on the cellular growth, and the Hlac, which the metabolic pathway can, in specific conditions, supplant the production of Hsuc[88,90].

2.2.4 The effect of oxygen in the *A. succinogenes* metabolism

The lactic fermentation is the metabolic pathway that can supplant the formation of Hsuc[17,90]. Similarly, from what happens with acetogenesis, this pathway expression is increased when the CO_2 concentration is low. However, another factor is believed to alter the *A. succinogenes* metabolism almost exclusively towards the production of Hlac: The presence of oxygen[90,97].

The metabolism of the *A. succinogenes* is still not fully understood, and so the reason why the oxygen can trigger the production of Hlac is mostly unknown. However, studies have already proven the ability of the *A. succinogenes* to generate large quantities of Hlac[17,90]. The best results were achieved by implementing a two-phase fermentation, where the first phase was carried out under aerobiosis and a second, where the anaerobic condition were maintained by CO_2 sparging[90,98]. The first phase is used to promote a metabolic shifting of the *A. succinogenes* to produce Hlac, while the second phase potentiate the overall metabolism of the bacterium, thus increasing the Hlac production[90]. Additionally, the last phase do not make the *A. succinogenes* restart the production of Hsuc, despite that pathway being favored by the CO_2 concentration[35,37].

The study made by Li et al. has suggested that some enzymes are the root cause for the metabolic shift in the *A. succinogenes* that cannot be reversed by the posterior adding of CO_2. Two of the identified enzymes in which the activity is affected by the presence of O_2 are the carbonic anhydrase (CA) and the LDH[90]. The first one is responsible for the passage of CO_2 to the bacterium interior. However, it was reported a diminish in the activity of the CA, when in contact with O_2, which consequently led to less CO_2 available for the bacteria[90,97]. Ultimately, this contributes to a repression on the metabolic pathway responsible for the formation of Hsuc, since the PEPCK has less co-substrate to produce oxaloacetate[85,97].

The second referred enzyme, the LDH, is the responsible for catalyzing the transformation of pyruvate into Hlac. Contrarily from what happens with the CA, its activity increases in aerobic environments. Moreover, this increment do not change in the *A. succinogenes* metabolism when the conditions become anaerobic[90].

From this information it can be concluded that there are variations in some enzymes of the *A. succinogenes*, when the bacterium is exposed to O_2, being the CA and the LDH the most described. Therefore, the O_2 is possibly responsible by altering the metabolism of the *A. succinogenes*, shifting the production of Hsuc to Hlac. Also, further adding CO_2 seems to not be enough to reset the bacterium metabolism to produce Hsuc[90]. Thus, it can be concluded that maintaining anaerobiosis conditions when operating with *A. succinogenes*, can be advantageous for producing Hsuc.

2.2.5 Bioreactor operation modes

Some important parameters to define for a bioprocess are the operation modes. In the Hsuc production by *A. succinogenes*, it was already studied the feasibility to operate in batch, fed-batch and continuous, with the last two being the least studied[59,60,88,95]. Nevertheless, it is vital to notice that in types of operation enunciated, the addition of CO_2 is a common factor due to this molecule importance in the production of Hsuc[35,96].

The batch fermentations are the simplest and easiest way to produce Hsuc[99,100]. Innumerous studies have been carried out under this type of operation strategy, namely for the determination of *A. succinogenes* kinetic parameters[87,96]. However, this type of operation has inherent disadvantages, the principal reason for the batch's low productivity and yield when compared with the continuous and fed-batch operations[7]. The accumulation of *A. succinogenes* fermentation products is the most notorious, and is due to bacterial organic acids production and growth inhibition[88]. Also, the initial substrate concentration can have an inhibitory role on the *A. succinogenes*[87]. Lin et al. (2008), have shown that this effect is evident for Glu concentrations above 70 g/L[87]. When this is applied at an industrial level, can be disadvantageous since the maximum mass of Hsuc will be highly dependent on the reactor volume.

Working on fed-batch allows to avoid substrate inhibition while using a higher quantity of total sugar. This is made by feeding more substrate throughout the process, keeping the overall concentration bellow values that can be inhibitory[87,100,101]. This way, it is possible to achieve a higher overall consumption of sugars per fermentation, consequently producing a higher concentration of Hsuc[99,100]. Despite those pointed advantages, this

process can be hard to operate, typically requiring a previous comprehension of the fermentation profile of the *A. succinogenes*.

The operations in continuous are currently being perceived as the best way to conduct fermentations using *A. succinogenes*. The main advantage for that is the maintenance of optimum substrate concentration, while simultaneously eliminating the fermentation products, thus avoiding product inhibition[66,100–102]. The main difficulty this mode of operation, besides the more expensive equipment, is the difficulty to accumulate enough biomass and prevent washout in the bioreactor. This can ultimately, compromise the yield and productivity of process[40,59,103,104]. Therefore, complementary strategies like immobilization and biomass recirculation were already studied to address this problem[40,44,60,101,102].

The immobilization explores the capacity of the *A. succinogenes* to form biofilms, which not only enhance the growth and Hsuc production, but also avoid the washout of the biomass from the bioreactor[40,78,95,102]. To explore that capacity, addition of physical supports with high surface areas to the process is made to promote the *A. succinogenes* biofilm formation[60,102,104,105]. These supports are usually polymers and biopolymers, such as lignocellulosic material, that can sit freely on the reactor or being packed into it[60,103,104]. Overall, this strategy allows a higher cell growth, due to the biofilm properties of the *A. succinogenes*. It can also avoid the biomass washout, leading to high biomass accumulation in the reactor when compared with cell free processes[40,95,102]. Bioreactors with biomass membrane recirculation retain the *A. succinogenes* inside the reactor since fermentation medium is filtered[51].

Those there were if two strategies to enhance the production of Hsuc, are hard to compare due to different variables between fermentations. However, they were already achieved productivity values higher than 1 g/L.h when applied immobilization technics. Finally, it is essential to mention that also batch and fed-batch fermentations can be optimized with biomass immobilization to improve the production of Hsuc[60].

In the Table 3 it is possible to compare those different modes of operations applied to the Hsuc production using *A.succinogenes*:

Table 3 - Different operation modes with *A. succinogenes*, substrates, kinetic and operational parameters.

Operation type	Substrate	Imobilization type	Recirculation	Hsuc			ref
				Titer (g/L)	Productivity (g/L.h)	Yield ($g_{Hsuc}/g_{substrate}$)	
Batch	Glu	n.a.	no	33.8	1.35	0.62	84
	SSL	Delignified wood sawdust	no	13.4	0.39	0.57	60
		Alginate beads	no	12.3	0.51	0.71	60
Fed-batch	Glu	n.a.	no	52.1	1.1	0.909	64
	SSL	Delignified wood sawdust	no	27.3	0.32	0.65	60
		Alginate beads	no	36.8	0.39	0.81	60
	Cane molasses	n.a.	no	55.2	1.15	nm	65
continuous	Glu	Groperl particles	yes	5-10	6.35	0.71	102
	Glu	Steel wool	yes	48.5	n.m	0.91	95
	SSL	n.a.	no	19	0.5	0.48	79
	Xylose (Xyl)-enriched hydrolysate	Porous polypropylene	no	26.4	2.64	0.77	40

n.a. – Not availables

Observing the data of the Table 3, it is clear that the fed-batch fermentations are capable to achieve a higher Hsuc concentration than the batch, related to its higher total sugar that is consumption. However, since the fed-batch fermentations can maintain the sugars concentration bellow inhibitory levels, they allow the production of higher concentrations of Hsuc[64,88,100]. With this type of fermentation it is possible to attain higher Hsuc yields than in batch by keeping the sugar concentration at values, which do not promote the production of byproducts[84,88]. Finally, the productivity appears to be slightly lower in the fed-batch than in batch. However, its larger fermentation time can lead to the inclusion of points with low variation of Hsuc concentration, thus making the fed-batch fermentation look less productive than their batch counterparts.

Continuous operations have the ability to maintain optimal conditions as illustrated in the Table 3. Therefore, it can be seen this fermentation mode allows higher achievement on productivity and yield[87,100]. The Hsuc concentration is normally lower when compared with fed-batch or even batch fermentations, due to the constant dilution caused by the continuous substrate feeding. However the overall Hsuc production is higher in continuous[100].

With this information, it is possible to admit that all the previous types of fermentations are suitable for producing Hsuc. It is necessary to understand that each one has its own advantages, such as the simple design and low cost of the batch fermentation, the high Hsuc concentrations achieved with fed-batch and the high productivity attained with the continuous operations. Therefore, choosing the adequate configuration of fermentation can be as important as choosing the right Hsuc producer.

2.3 Genetically modified producers

Sometimes the natural organisms do not have the desirable characteristics needed to be viable in an industrial Hsuc fermentation process. Therefore, genetic engineering can be used in those organisms to enhance their overall productivity, thus making them suitable for industrial use. Moreover, there are well-understand organisms, in which the Hsuc production is scarce or cannot accumulate at all, like what happen with *Escherichia coli* and *S. cerevisiae* respectively. In this case genetical engineering is vital in turning MOs like those into efficient Hsuc producers[106,107].

In the following table, there will be presented genetically altered MO already tested for Hsuc production:

Table 4 - Genetically modified organisms operated in batch, their modifications, substrates, and kinetic and operational parameters.

Specie and strain	Overexpressed/added pathways	Underexpressed genes/knocked out pathways	Substrate	Hsuc			Ref
				Titer (g/L)	Productivity (g/L.h)	Yield ($g_{Hsuc}/g_{substrate}$)	
A. succinogenes FZ53	none	Production of Hfor and Hace	Glu	105.8	1.36	0.83	108
C. glutamicum CRZ1	Pyruvate carboxylase reaction	none	Glu	n.m.	3.7	0.71	81
B. succiniciproducens LU15224	none	Production of Hlac and Hfor	Glycerol	36.20	1.51	1.26	109
M. succiniciproducens LPK7	None	Production of Hlac and Hfor	Glu	52.43	1.80	0.76	110
E. coli NZN111	Malate dehydrogenase reaction	Production of Hlac and Hfor	Glu	15.2	1.01	0.72	111
Yarrowia lipolytica	none	Succinate dehydrogenase reaction	Glu	10.7	0.22	0.23	112
Saccharomyces cerevisiae AH22ura3	None	Succinate dehydrogenase and isocitrate dehydrogenase reactions	Glu	3.62	n.m.	0.072	106

In the first example (Table 4 - Genetically modified organims operated), the *A. succinogenes* FZ53, a patented strain, was genetically modified in order to produce less Hace and Hform. It is possible that the modified genes were the ones responsible for expressing the acetate kinase (AcK) and the pyruvate formate-lyase (PFL)[37,57], despite this information not being described in the patent. Having less byproducts led to productivities higher than any other wild type strain presented in the table 2. Other possible editions that can be done in this bacterium genome, could be the deletion of the gene responsible for the lactate dehydrogenase (LDH) expression[49,90]. Some studies describe the gene responsible for the LDH expression present in the *A. succinogenes* metabolism, though it is normally unexpressed. However, when expressed it can compromise the Hsuc production as shown in the topic 2.2.4. Therefore, suppressing the metabolic pathway linked to the LDH could be a crucial step to ensure the Hsuc production. The reported results are the best presenting the highest concentration and yield for Hsuc respectively of 105.8 g/L and 0.83 $g_{Hsuc}/g_{substrat}$[112].

C. glutamicum can also be enhanced to produce Hsuc. In the example represented in the Table 4, the gene which expresses the Pyruvate Carboxylase was overexpressed. This shifted the *C. glutamicum* CRZ1 metabolism towards the production of Hsuc[81], thus leading to an increase in both the productivity and yield when compared to the wild type. In addition, there are additional gene editing that can be used to improve the Hsuc production, such as knocking out the gene responsible to express the LDH, disabling the Hlac production[49].

The bacteria *B. succiniciproducens* and *M. succiniciproducens* have a similar metabolism, characterized by commonly producing Hlac. It is, therefore, advantageous to knock out the genes responsible for expressing the LDH, allowing to shift most of the metabolism towards the Hsuc production[49,110,113,114]. This is verified in the *M. succiniciproducens* LPK7 when compared with respective wild type strain. For the example of *B. succiniciproducens*, it was possible to achieve a higher yield, however there was the use of a different substrate for this fermentation, whichmust be also highlighted. The *B. succiniciproducens*, also achieved a productivity much higher when compared to the wild type. Finally, it is important to mention that in both *M. succiniciproducens* LPK7 and *B. succiniciproducens* LU15224, the gene responsible for the expression of PFL was slightly repressed. This eventually led to decreased production of Hfor, thus enhancing the production of Hsuc.

In an example present on the Table 4, a *E. coli* was submitted to a less common genetic alteration. Normally in that bacterium, only the genes responsible for expressing the LDH and PFL are knocked out (characteristic of the NZN111 strain), hence stopping the production of Hlac and Hfor[49,88]. However, this study overexpressed the gene responsible for expression of malate dehydrogenase (MDH), which was expected to increase the metabolic flux towards the production of Hsuc. Nevertheless, that led to a higher $NADH/NAD^+$ ratio, which impaired the recombinant strain growth[111]. It was, however, possible to attain higher yields with this *E. coli* NZN111 than with other recombinant strains without the MDH gene editing. Still, those values are bellow other experiments where the PyC was overexpressed[49,111,115].

In the eukaryotic specter there were works already made for the production of Hsuc namely in *S. cerevisiae* and *Y. lipolytica*. In both examples the gene responsible for expressing the succinate dehydrogenase was knouckout, otherwise the Hsuc would be normally consumed in the citric acid cycle[49,106,112]. Even with the aid of genetic engineering, the yield and productivity in both MOs was low compared to natural producers. In the case of the *S. cerevisiae* the gene responsible for expressing the isocitrate dehydrogenase, was also knocked out, allowing to further shift the metabolic flux to a more direct pathway for Hsuc production[106].

In conclusion, it is possible to assert that genetically modified Hsuc producers have usually better performance than their parental wild types. The cost and know-how associated with their creation can, however, constitutes a difficult challenge.

2.4 Biotechnological Hsuc downstream processes

The extraction and purification processes constitute the biggest challenges that the Hsuc biotechnological production has to face[31,116]. They can make up 50% of the total Hsuc production cost, due the expensive and sometimes complex processes. Moreover, in bioprocesses that use complex substrates such as the *E. globulus* kraft pulp hydrolysate, some molecules can eventually interfere with the downstream processes[19,117,118]. The pH values in which the bioprocess is operated can also influence the Hsuc separation and purification processes, since it can alter the Hsuc protonation[119,120]. All of this can ultimately lead to the addition of more separation and purification steps, increasing the overall Hsuc production cost [118].

In other perspective, the byproducts of the *A. succinogenes* increase the difficulty of downstream processes. This can be associated with the somehow similar characteristics between Hsuc and the other organic acids, which can cause interference in the separation process. Originally the separation was achieved through precipitation and liquid-liquid extraction. However, they presented serious disadvantages like sludge formation and low partition coefficients, respectively[116,121]. The use of reactive liquid-liquid extraction (including supercritical CO_2 and the usage of liquid emulsion membranes) is presently described as a useful alternative[122,123]. The Hsuc combination with other molecules allows for higher partition coefficients without sludge formation[116,121,124–129]. However, in order to achieve higher purities it is necessary the use of additional purification processes such as chromatography, which inevitably increases the production costs [116,121,128,130,131]. Despite this it is important to mention that some Hsuc applications require its high purity, thus justifying the higher cost of additional purification processes.

Concluding, the downsteam processes can be the limiting issue in the Hsuc production, making up around half of its production cost. To change this, new technics have been developed to allow better separation of Hsuc from undesirable molecules. Finally, it is important to take in mind that the choice of the substrate can influence the efficiency and cost of the whole downstream process.

2.5 Biotecnological Hsuc perspectives

Nowadays the biotechnological Hsuc production is facing a major challenge, the decreasing of the oil prices[53]. The main factor that promoted a substantial shift from the petrochemical industry to the biotechnology was the high oil prices verified in 2007, which suggest the inverse can happen[36]. Presently the main companies that produce bio Hsuc are Succinicity, Roquette and Myriant, after the largest producer, Bioamber opened bankruptcy in 2018 [53,132]. Additionally, the joint venture where the company Roquette participate was dissolved leaving that company with the production rights of bio Hsuc.

Despite the dreaded forecast for the biotechnological produced Hsuc, it is important to remember its potential as alternative to the petrochemical industry[36]. The main justification is the advantage CO_2 consumption during the fermentation, hence being an industrial process that can balance CO_2 emissions[36,46,51]. Also, as new downstream technologies and specialized producing strains are being developed[133], Hsuc production cost will probably decrease and eventually compete again with the price of petrochemical produced Hsuc.

All in all, bio Hsuc production concept was designed to face the increasing prices of oil verified in 2007[53], without having in mind the recent petrochemical market evolution. Nonetheless, environment concerns can be the leading reason to develop more efficient bio Hsuc production plants, more sustainable than the petrochemical ones.

2.6 Objectives

This present work aimed to evaluating the production of Hsuc, using a lignocellulosic material as feedstock. The lignocellulosic biomass used was obtained from *E. globulus*, pretreated with the kraft process. This step was a fundamental pretreatment for delignify the lignocellulosic biomass. The pretreated and hydrolyzed pulp was used to produce Hsuc using the bacterium *A. succinogenes* as biocatalyst, due to its high Hsuc productivity. Preliminary fermentations to acquire reference points were done in anaerobic shake flasks. The batch fermentations were carried out in bioreactor with pH control and CO_2 sparging. In order to optimize the Hsuc production different concentrations were evaluated. It was also conducted a fed-batch fermentation to achieve higher Hsuc concentrations without having inhibition by substrate.

3 Materials and Methods

3.1 Enzymatic Hydrolysis

The enzymatic hydrolysis of kraft pulp was performed using250 g (DW) of unbleached *E. globulus* kraft pulp provided by RAIZ (Forest and Paper Research Institute) mixed with citrate buffer 0.05 M (sodium citrate) to obtain a total volume of 3 L. The enzyme consortium was added aiming to obtain a total enzymatic load and activity of 20 FPU/g and 242 FPU/mL, respectively.

The reaction was performed in a vessel inside a 50 ºC heated bath, submitted to a constant mechanical stirring of 100 rpm and pH was kept between 5.5 and 4.5. When the temperature inside the vessel reached 50 ºC, the right amount of the enzyme consortium was added. To monitor the reaction, samples were taken and both the pH and the concentration of reducing sugars were analyzed. To adjust the pH to the pre-established values, concentrated NaOH or H_2SO_4 were added manually, until the pH was within the intended range. The hydrolysis was finished after 24 h, and the hydrolysate was centrifuged (Herceus, Megafuge 16 R Centrifugue) at 4 ºC and 3000 rpm for 30 min, to remove the suspended solids. The hydrolysate was sterilized afterwards at 121 ºC for 20 min and centrifuged again at the same conditions to remove any formed precipitate. Elemental analysis to the magnesium was carried by the Central Analysis Laboratory of the University of Aveiro, to quantify the Mg^{2+} present in the WPH.

The hydrolysis reaction was repeated 4 times in order to obtain nearly 10 L of hydrolysate. The final hydrolysate was then frozen in 500 mL bottles at -18 ºC.

3.2 Fermentations

All the fermentations were done in asepsis conditions provided by a laminar flow hood (B. Braun Horizontal 4). All the materials and media were sterilized at 121 ºC for 20 min (Uniclav 88), always keeping the sugars separated from the other medium components to avoid Maillard reactions. The separated components were then mixed inside the laminar flow hood to avoid contaminations. For flask anaerobic fermentations the flasks were encapsulated with chlorobuthyl stoppers allowing for purging and the maintenance of anaerobiosis.

3.2.1 Media preparation

In this work, three different media for growth and fermentation were used. The Tryptic Soy Broth (TSB) medium (Fluka), the synthetic medium and the wood pulp hydrolysate (WPH) medium.

The TSB medium (Table 5) was used for growing pre-inocula and inocula. It was prepared by diluting a commercial TSB mixture with water to achieve the concentrations presented in Table 5, and the pH was adjusted to 7.3 with H_2SO_4 or NaOH.

Table 5 - TSB medium composition

Components	**Concentration (g/L)**
Casein peptone	17.00
Dipotassium hydrogen phosphate	2.50
Glu	2.50
Sodium chloride	5.00
Soya peptone	3.00

The synthetic medium was produced to mimic the Glu/Xyl ratio present in the wood pulp hydrolysate medium. This medium was prepared similarly to Xi et al. (2011), using both Glu and Xyl (Table 6). The total concentration of both monosaccharides was 50 g/L, with Glu making up 80% (w/w) of the total sugars and Xyl the remaining 20% (w/w).

Table 6 - Composition of the fermentation media

Components	Media	
	Synthetic	WPH
Substrate		
Glu (g/L)	40.0	-
Xyl (g/L)	10.0	-
Hydrolysate (% v/v)	-	74
Supplement		
KH_2PO_4 (g/L)	3.0	3.0
$MgCl.6H_2O$ (g/L)	0.2	0.2
$CaCl_2.2H_2O$ (g/L)	0.2	0.2
NaCl (g/L)	0.1	0.1
Yeast extract (g/L)	10.0	10.0

The medium with WPH was prepared to match the concentration of each component according to Xi et al. (2011), as shown in Table 6. Both the hydrolysate and the supplement solution were mixed in the laminar flow hood into a sterile bottle, and the pH was corrected with concentrated H_2SO_4 or NaOH.

3.2.2 Microbial culture

The MO used in this work was the bacterium *A. succinogenes* 130Z provided by DSMZ in the freeze-dried state.

The bacterium was reactivated with 0.5 ml of TSB medium previously purged with N_2 to ensure anaerobic conditions. The 0.5 ml containing the bacterium were then, equally divided by two 10 ml flasks, encapsulated with clorobuthyl stoppers, containing 5 m/L of TSB medium and purged with N_2 (filtered through a membrane with 0.22 µm porous

diameter). All this procedure was done in an anaerobic chamber (in Biology Department), to avoid any contact with oxygen.

Finally, the flasks were introduced in an orbital incubator (Stuart SI500) at 37 ºC and 140 rpm for 24h, being then transferred for a fresh TSB medium.

3.2.3 Preparation of the pre-inoculum and inoculum

The pre-inoculum was prepared in sterilized 100 mL anaerobic encapsulated flasks, containing 50 mL of TSB medium, purged with N_2 (filtered through a membrane with 0.22 µm porous diameter) for 5 min at 1 vvm. 1 mL of a stock culture of *A. succinogenes* was introduced in it and incubated for 24 h. 0.5 mL of this culture was then used in the inoculum, also done in 100 mL anaerobic flask with 50 mL of TSB medium, already purged. The inoculum was incubated for 14 h before being transferred for each respective fermentation. For each experiment it was inoculated a culture percentage of 5.8% ($v_{inoculum}/v_{fermentation\ medium}$).

3.2.4 Fermentations in shake flasks

The inoculum was prepared as described in 3.4 and used to inoculate the shake flasks after the 14 h of incubation

Anaerobic fermentations were conducted in 100 ml anaerobic shake flasks with 85 ml of the respective media (synthetic or WPH), containing an additional 4.25 g of $NaHCO_3$. The flasks with the media were encapsulated with chlorobutyl stoppers and purged with 1 vvm of N_2 filtered through a membrane, with a porous diameter of 0.22 µm for 5 minutes. After inoculation all the flasks were incubated at 37 ºC and 140 rpm.

In the case of the aerobic fermentations the conditions besides the purge with N_2 were similar, using 100 mL Erlenmeyer flasks instead of anaerobic shake flasks.

3.2.5 Fermentations in Bioreactor

All bioreactor fermentations were carried out in a B Braun bioreactor using a custom built 1260 mL vessel, working with constant agitation of 200 rpm. The pH was automatically controlled by an analog controller (Hannah Instruments 8711) linked to a pump (B. Braun). The solution used for correcting the pH values was NaOH 10 M. Temperature was maintained at 37 ºC by a circulating thermostatic bath and CO_2 was continuously sparged, at a constant flowrate of 0.1 vvm.

One Batch fermentation was promoted using synthetic media and three using WPH with addition of 0 g/L, 10 g/L or 20 g/L of magnesium carbonate (light, Panreac). The working volume was 700 mL.

One Fed-batch fermentation was conducted with WPH (0 g/L $MgCO_3$) starting with a volume of 600 mL. The feeding pulses were composed by 200 mL WPH (with no added salts), concentrated to a factor of three by rotary evaporation (60 ºC and 200 rpm), with latter addition of 20 g/L of yeast extract (40 mL). These feeding solutions were introduced by a peristaltic pump (Watson-Marlow) at defined times, according to the previous batch results.

3.3 Analytical Methods

Samples were taken periodically, to monitor the optical density (OD) at 660 nm and after 5 min of centrifugation at 10000 rpm, each sample supernatant was collected and frozen at -18 ºC to further analysis.

3.3.1 Cell concentration

Cell concentration was estimated by OD (UV Mini-1240, Shimadzu) at 660 nm. Appropriate dilutions were made with the medium itself for the TSB fermentations, and a 0.9% solution of NaCl was used for the other media fermentations. A calibration curve for each media was made to relate OD values with biomass concentration: the OD of the appropriate dilutions of standard culture (in early stationary phase) were used in each

calibration curve and related with cell dry weight. 9.00 mL of each culture were filtered through a 0.22 µm membrane and then dried at 105 ºC, until constant weight, duplicates were evaluated. The dry weight was used, for calculating cell concentration and determination of respective calibration curves.

3.3.2 Chromatography

High-performance liquid chromatography (HPLC):

Before being loaded in the HPLC equipment, the samples from all fermentations were diluted by a factor of 20 and filtered through a 0.22 µm membrane (Eppendorf minispin). The samples constituents were separated with an ion-exchange Rezex™ ROA-Organic Acid column (internal diameter of 7.8 mm and length of 300 mm), able to separate organic acids, monosacharides and alcohols analyzed by a refractive index (RI) detector (Hitachi L-2490) module. The eluent used was a 0.005 N solution of H_2SO_4, pumped through the system at 500 µL/min (40 ºC), and 10 µL of each sample and standards were automatically injected by the auto-sampler (Hitachi L- 2200) and ran through the system for 45 min.

The peaks in the chromatogram were converted into concentration values by the calibration curves of the respective compounds. These curves were determined using standards of Hace, Hace, Hfor, Hsuc, Glu, Xyl and EtOH, prepared from a stock solution of 5 g/L of each component. Obtained data was then processed in Microsoft Excel. The linear regression for the calibration curves were made using the function “LINEST”, assuming the X values as the known concentration and the Y values as the determined area. The obtained equations allowed conversion of the peak area, calculated by the equipment software, into concentration values.

Ligand exchange/Size exclusion (LEX/SEC) chromatography:

A different chromatography was used to evaluate the oligomers present in the WPH. The column was a semi-preparative LEX-SEC Shodex sugar KS 2002 column (internal diameter of 20 mm and length of 300 mm), coupled to a RI and UV detector (Knauer K-2401). The eluent used was water, with a pH value of 6.5 and it was pumped through the

system with a 2.80 mL/min flowrate. In each analysis 0.5 mL of a previously filtered and diluted sample, was used.

3.7.3 WPH reducing sugars determination by dinitrosalicylic (DNS) acid

100 mL of the reagent were prepared by dissolving 1.0 g of DNS acid (Acros Organic) in 20 mL of NaOH 2 M solution at 70 ºC. This step was done slowly to avoid the DNS precipitation. After the dilution had been completed, 30 g of potassium and sodium tartarate was added to the solution. The final volume was adjusted with water to achieve 100 mL. DNS reagent was then filtered to remove any suspended particles and it was stored in a dark bottle to preserve it from photodegradation.

The determination of reducing sugars was done by adding 1 mL of the DNS reagent to 1 mL of a properly diluted WPH sample, in a test tube. This mixture was then allowed to react for 5 min in a boiling water bath, being then stopped by chilling the test tubes in ice. Lastly, 8 mL of water were added and the absorbance at 540 nm was analyzed. The results were converted into reducing sugars concentration using a calibration curve, where the standards were solutions of Glu.

3.3.4 Initial WPH sugars determination by anthrone method

Anthrone method was used to determine the total concentration of sugars in the beginning of the fermentations in WPH. The 0,2% (w/v) reagent was prepared by dissolving the anthrone in 98% (v/v) H_2SO_4. This step was done in an iced bath to assure the stability of the anthrone reagent. To evaluate the total sugar concentration, 1.0 mL of a properly diluted samples were cooled in test tubes, to which were added 4.0 mL of cold anthrone reagent. The samples were allowed to react for 10 min in a boiling water bath, being cooled in an ice bath, to stop the reaction.

The absorbance at 620 nm was then measured and compared with the respective calibration curve, for determining the concentration of total sugars. The standards used in the calibration curve were prepared using 80 % (w/w) Glu and 20 % (w/w) Xyl.

3.3.5 Calculation methods of fermentation parameters

- Volumetric productivity rate (r_p), was calculated according to Equation 1, where P_0 and P are respectively the initial and highest concentrations of Hsuc, Hlac, Hfor or Hace and t is the time until P is attained:

$$r_p(g/L \cdot h) = \frac{P(g/L) - P_0(g/L)}{t(h)} \quad \text{Equation 1}$$

- Product or biomass yields ($Y_{P/S}$ or $Y_{X/S}$) were calculated according to Equation 2, where P_0 and P are respectively the initial and final concentrations of Hsuc, Hlac, Hfor, Hace or biomass, and the S_0 and S_f are respectively the initial and final substrate concentrations. It is also important to mention that, for the biomass yield calculation, the initial and final points selected were within the exponential phase:

$$Y_{(g_{P\ or\ X}/g_S)} = \frac{P(g/L) - P_0(g/L)}{S_0(g/L) - S_f(g/L)} \quad \text{Equation 2}$$

- Maximum specific growth rate (μ_{max}) was calculated using the linearization of Equation 3. The t_0 and t_x represent the initial and final of the exponential phase. The X_0 and X_{tx} represent the respective initial biomass concentration and the corresponding concentration on the final time point, of the exponential phase:

$$X_t = X_0 \cdot e^{\mu_{max}(t_x - t_0)} \quad \text{Equation 3}$$

- Succinic acid to total organic acids ratio (Hsuc/organic acids ratio) was calculated using the Equation 4. Each organic acid abbreviation represents each product

concentration. It should be mentioned that the Hsuc was considered for the total organic acid concentration:

$$\text{Hsuc/organic acids ratio} = \frac{Hsuc}{Hsuc + Hlac + Hace + Hfor} \quad \text{Equation 4}$$

4 Succinic acid production using lignocellulosic wastes: Feasibility to integrate kraft pulp hydrolysates as feedstock

Pulps hydrolysis was promoted to produce the hydrolysate used in this work and the study started with a synthetic medium assay for evaluating *A. succinogenes* kinetic behavior. The results were used as reference for the fermentations with WPH. This assay allowed to analyze possible inhibitory effect caused by the hydrolysate, or the lack of micronutrients, which could influence the bacterial growth and productivity.

4.1 Enzymatic hydrolysis

Prior to the hydrolysis the pulps were characterized in RAIZ (Forest and Paper Research Institute) being the respective composition shown in the Appendix V.1

In the end of the hydrolysis 10.725 L of WPH were obtained, with a reducing sugar concentration of 75.0 g/L (Figure 11), corresponding to a final mass of 803.0 g and an enzymatic yield of 73.8 %. Also, the HPLC results shown that the final hydrolysate is composed by 82.3% of Glu and 17.7 % of Xyl.

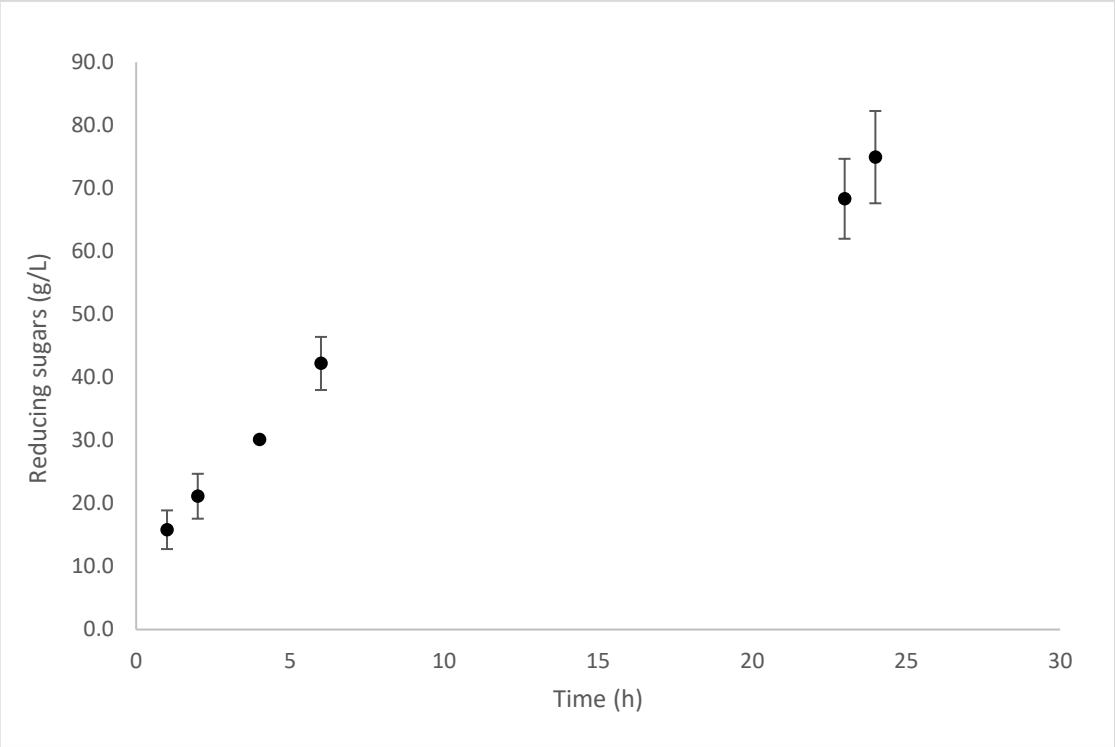


Figure 11 - Evolution of the reducing sugars concentration of the kraft pulp hydrolysis

Documented studies by De Bari et al. (2013) of enzymatic hydrolysis in *A. donax* (with a similar enzymatic load) revealed similar yields. However, these results also revealed that the hydrolysis yield obtained in the present study could be improved, if the reaction time would be increased[134]. Despite this, it is important to take into consideration the industrial context explored in this study. In that the productivity is one of the most key factors in the industry and it must be taken into consideration. Therefore, the enzymatic reaction was deliberately stopped after 24 h to avoid unnecessary energy losses and to ensure the highest productivities.

A LEX/SEC chromatography was used to identify the level of polymerization of the sugars present in the WHP. The results presented in Figure 12 revealed that some oligomers, mainly dimmers, could certainly remain in the WPH after the hydrolyses step. These are not detected by the HPLC, thus showing an apparent lower sugars concentration. Since the *A. succinogenes* can degrade small oligomers, such as cellobiose[77,82], this factor can lead to apparent increases in the sugar concentration, which are later evident in the WPH time

courses. This variation can induce default calculation errors on consumed sugars, thus resulting in apparent higher yield. Additionally, with the LEX/SEC chromatography it was possible to confirm by comparison with previous studies in the same LEX/SEC column, the presence of phenolic compounds[135].

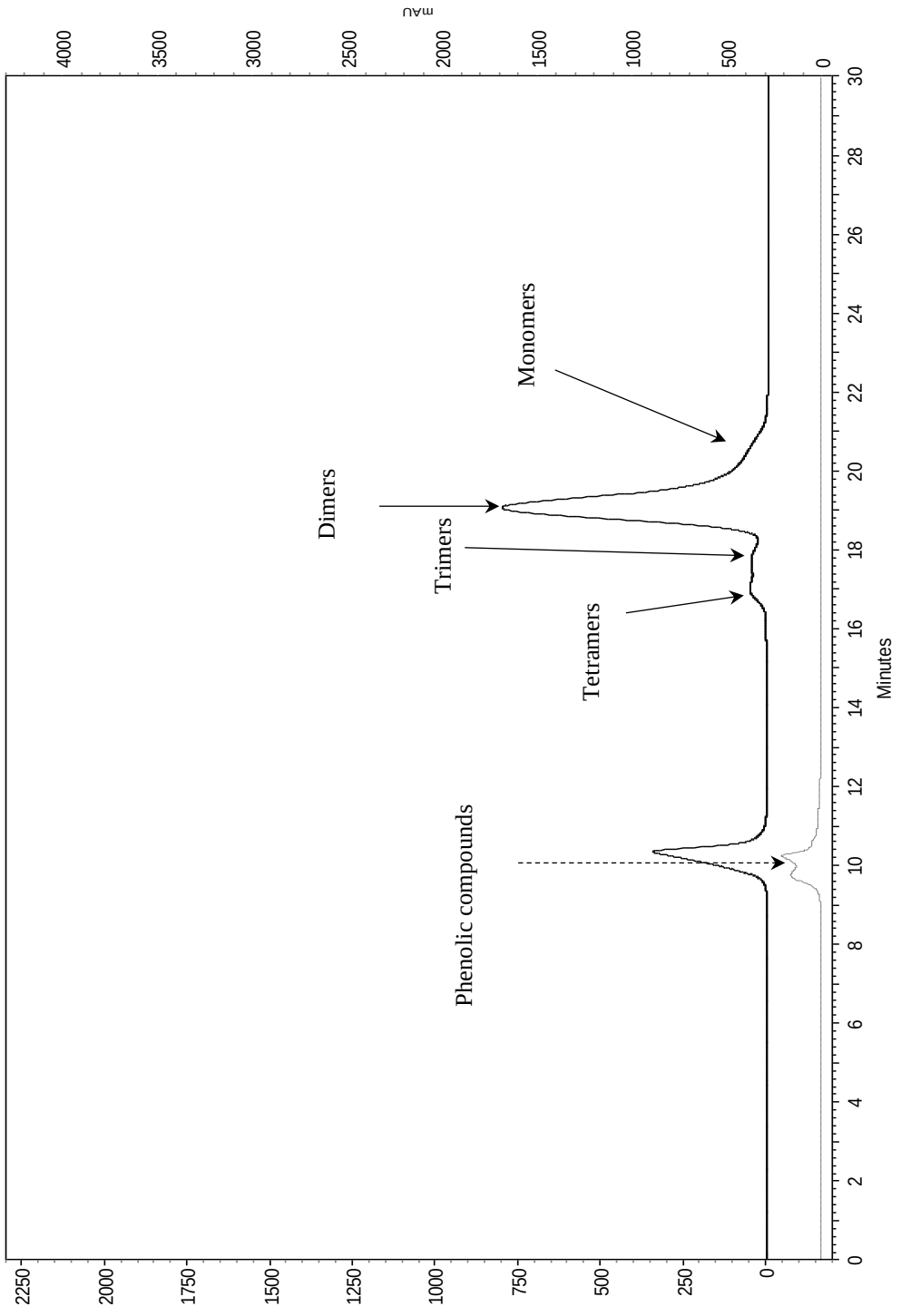


Figure 12 - Result of the LEX-SEC chromatography. The continuous line represents the chromatogram obtained by the RI detector in the Knauer K-2401 module and the dotted line represents the chromatogram obtained by the UV detector also present in the Knauer K-2401 module.

4.2 Fermentation in anaerobic shake flasks

The fermentations in anaerobic flasks were performed to test the feasibility of producing Hsuc in both the synthetic and WPH medium. As inorganic CO_2 source it was used $NaHCO_3$.

Figure 13 shows the time course of the synthetic medium fermentation. There was a lag phase of 5 h and the exponential phase until 10 h of fermentation. After that, the *A. succinogenes* faced a pH inhibition[64] since biomass production was impaired, in contrast to the organic acid production. The maximum Hsuc concentration obtained was 8.0 g/L and there was still more than 20 g/L of Glu remaining in the medium. Certainly, no more Glu was converted in Hsuc because pH has decreased below 5.5, (5.2 in the end of the fermentation) inhibiting *A. succinogenes* fermentation. As for the Hfor and the Hace, their respective concentrations were 3.0 g/L and 3.5 g/L.

This result can be compared with the studies made by Liu et al. (2008)[64] carried out with an initial pH value of 7.2, achieving a maximum concentration of 8.0 g/L of succinic acid similar to the value attained by the present study[64].

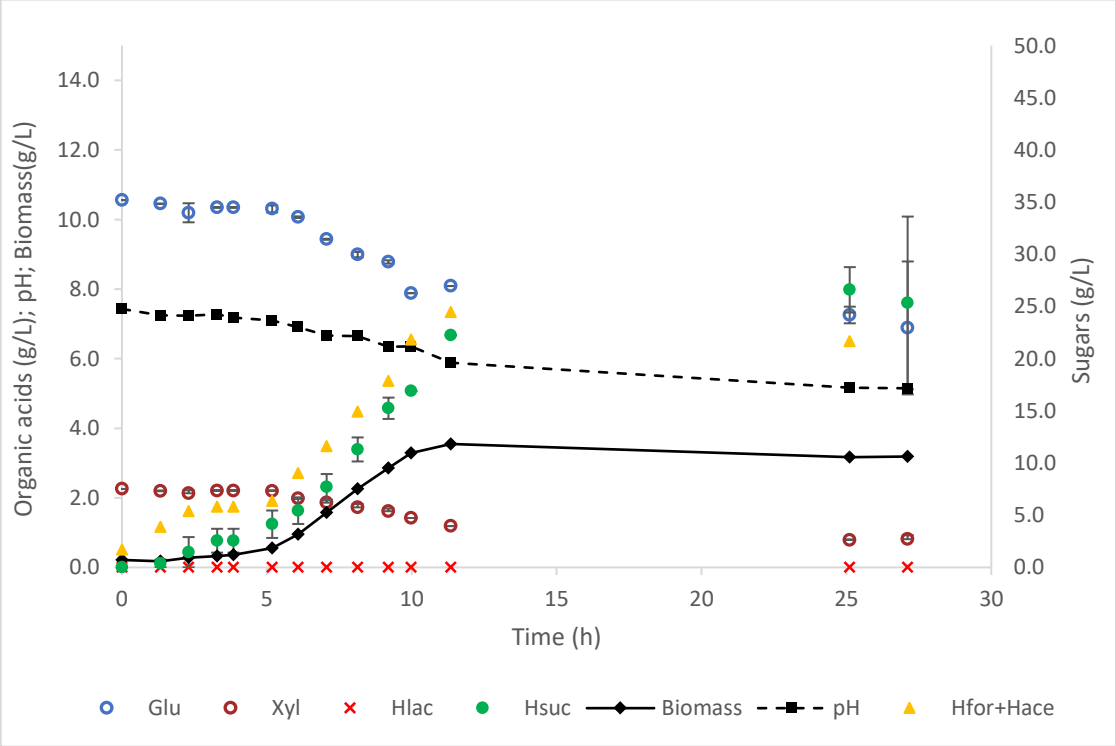


Figure 13 - Evolution of the sugars, organic acids, pH and biomass in the shake flask fermentation in synthetic medium in anaerobic flask

The results obtained with the WPH (Figure 14), demonstrated a final Hsuc concentration of 7.5 g/L at a final pH of 5.4, with a biomass concentration of 1.9 g/L. However, these results show a significant difference compared to the synthetic medium, due to Hlac production (7.1 g/L). Such fact was firstly attributed to the presence of a contaminant bacterium. Previous reported studies shown the vulnerability of *A. succinogenes* cultures towards contaminations, specially by lactic acid bacteria (LAB), such as *Lactobacillus* and *Streptococcus*[136]. To evaluate this issue, a final sample was taken from the fermentation and used to inoculate two distinct media: The TSB agar and the De Man, Rogosa and Sharpe (MRS) agar, a selective medium where the contaminant bacteria are able to grow, while the *A. succinogenes* would be inhibited[137]. This experiment however, revealed a total absence of colonies in the MRS medium, in contrast to what happened with the TSB medium, where a large number of colonies were observed in pure culture. This led to conclude that the Hlac production was in fact being caused by the *A. succinogenes* metabolism. It has been already shown that this bacterium contains the genes necessary to express the enzyme LDH, ultimately responsible for the Hlac production[138]. Although these genes are normally inactive, the presence of oxygen can induce their expression. During the inoculation and culture transference it can be possible that a small quantity of oxygen entered in contact with the bacterium, thus changing part of its metabolism[90,139]. The whole fermentation was repeated with another cryotube of this bacterium, in order to avoid the Hlac production. Although, this assay was unsuccessful in avoiding its production.

The lower biomass concentration observed in the WPH fermentation was predictable since this substrate demonstrated residual amounts of phenolic compounds. These are known to inhibit the *A. succinogenes* growth[20,140]. The higher concentration of byproducts, including the Hace (5.3 g/L) can be attributed to the possible Mg^{2+} ions present in the WPH. These ions occur naturally in the *E. globulus* and can potentiate metabolic pathways which can compete with the Hsuc production. Further WPH ion-mobility spectrometry analysis carried out by the University's Central Analysis Laboratory revealed that the Mg^{2+} concentration was unintentionally twice as much, compared with the synthetic medium.

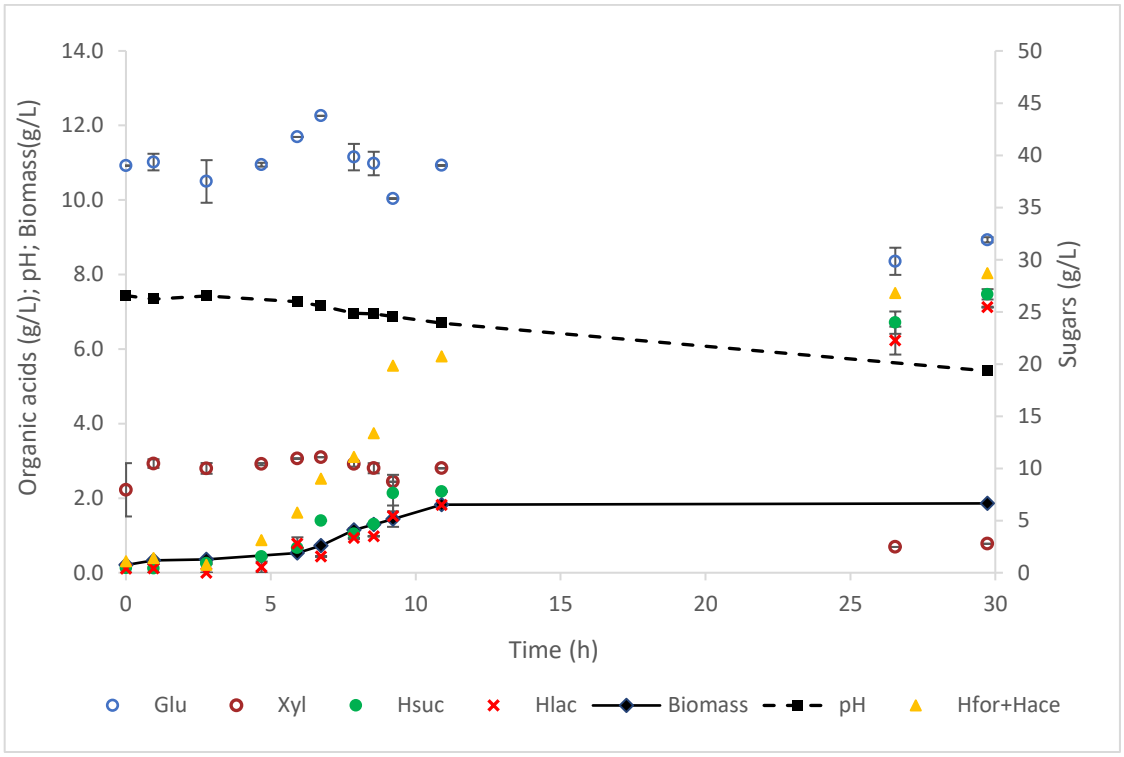


Figure 14 - Evolution of the sugars, organic acids, pH and biomass in the shake flask fermentation in WPH medium in anaerobic flask

In Table 7 the results regarding both fermentation are compared and the obtained μ_{max} of 0.558 h^{-1} in synthetic medium was higher than the value obtained for the WPH (0.431 h^{-1}). This agrees with the lower concentration of biomass attained and can be attributed to the inhibitory effect caused by residual phenolic compounds certainly present in the WPH[20,140]. Additionally, both fermentation results were according to the review made by Brink & Nicol (2014), in which the normal μ_{max} values for *A. succinogenes* were described between 0.400 h^{-1} and 0.800 h^{-1} [105].

Table 7 - Results of the fermentations in anaerobic flask comparing all the kinetic parameters, related to the Hsuc, and the sugar consumption

Fermentation	μ_{max} (h^{-1})	Hsuc			Sugars consumed (g/L)	Hsuc/organic acids ratio
		Concentration (g/L)	Productivity (g/L.h)	Yield (g_{Hsuc}/g_{sugars})		
Synthetic medium	0.558	8.0	0.318	0.502	15.9	0.551
WPH medium	0.431	7.5	0.251	0.608*	12.3	0.330

* Determined with errors induced by the oligomers present in the WPH

The obtained productivity and yield in the synthetic medium were 0.318 g/L.h and 0.502 g_{Hsuc}/g_{sugars}, respectively. These results confirm that *A. succinogenes* culture was similar to those presented in the literature. Regarding the WPH medium, Hsuc productivity was negatively affected however, the obtained yield, 0.608 g_{Hsuc}/g_{sugars}, was unexpectedly higher than that obtained with the synthetic medium (0.502g_{Hsuc}/g_{sugars}), due to the presence of oligomers.

In both fermentations it was observed more than 70% of unconsumed sugars, which happened due to an early ending of the fermentation. The reason of this is the acidogenic fermentation responsible for the dropping of the pH for values bellow 5.5, which is considered the lowest value for *A. succinogenes* metabolic activity[85]. This led to the inhibition of the cell growth and organic acids production, reason why no further sugar consumption was verified. These results suggest that an operation conducted in bioreactor, with pH control would increase the overall production of Hsuc.

4.3 Fermentation in aerobic shake flask

To evaluate the effect of oxygen in the metabolism of the *A. succinogenes* in WPH, a fermentation was conducted in an Erlenmeyer flask with a cotton stopper. As expected when subjected to aerobiosis it was verified that *A. succinogenes* could not produce any Hsuc, and besides Hace and Hform it produced Hlac (Figure 15). Furthermore, this result obtained in this experiment is according to that described by Li et al. (2010), after exposing the *A. succinogenes* to different aeration conditions Regarding the biomass growth, the results were lower than those obtained in the anaerobic fermentation since a maximum value of 1.3 g/L was attained instead of 1.8 g/L. This maximum here obtained is not possible to compare with the literature, due to the lack of studies in aerobiosis. However, it is possible to associate the low biomass production with the absence of production of Hsuc, which is a major contributor for the *A. succinogenes* metabolism of biosynthesis[37].

The concentration of Hlac was similar in both anaerobic and aerobic fermentations (7.1 g/L). The yield of Hlac in the aerobic fermentation (0.907 g_{Hlac}/g_{sugars}) was higher when compared with the anaerobic fermentation (0.578 g_{Hlac}/g_{sugars}). This result may lead to the assumption that the Hlac concentration should be higher in the aerobic fermentation. Such did not happen, because $NaHCO_3$ was not present in this fermentation. Without the buffer effect of that salt, the pH values rapidly dropped to critical levels, thus repressing the bacterium metabolism. Without the buffer effect of that salt, the pH values rapidly dropped to critical levels, thus repressing the bacterium metabolism. With both the aerobic and anaerobic experiments it is possible to observe the result of the gene expression described by Zang et al. (2019)[138]. In their study it was evidenced that under aerobiosis, the genes responsible for the Hsuc production were repressed, whilst the genes responsible for the Hlac production were activated. However, when subjected to an aerobic phase followed by an anerobic phase, the *A. succinogenes* expressed both Hsuc and Hlac production pathways. This reported experiment led to the conclusion, that by exposing the *A. succinogenes* to oxygen, can induce production of Hlac from the sugars, in the WPH. Additionally, it is suggested that a small amount of O_2 can be enough to induce a metabolic shift throughout the successive bacterium generations, leading to the increase in Hlac production.

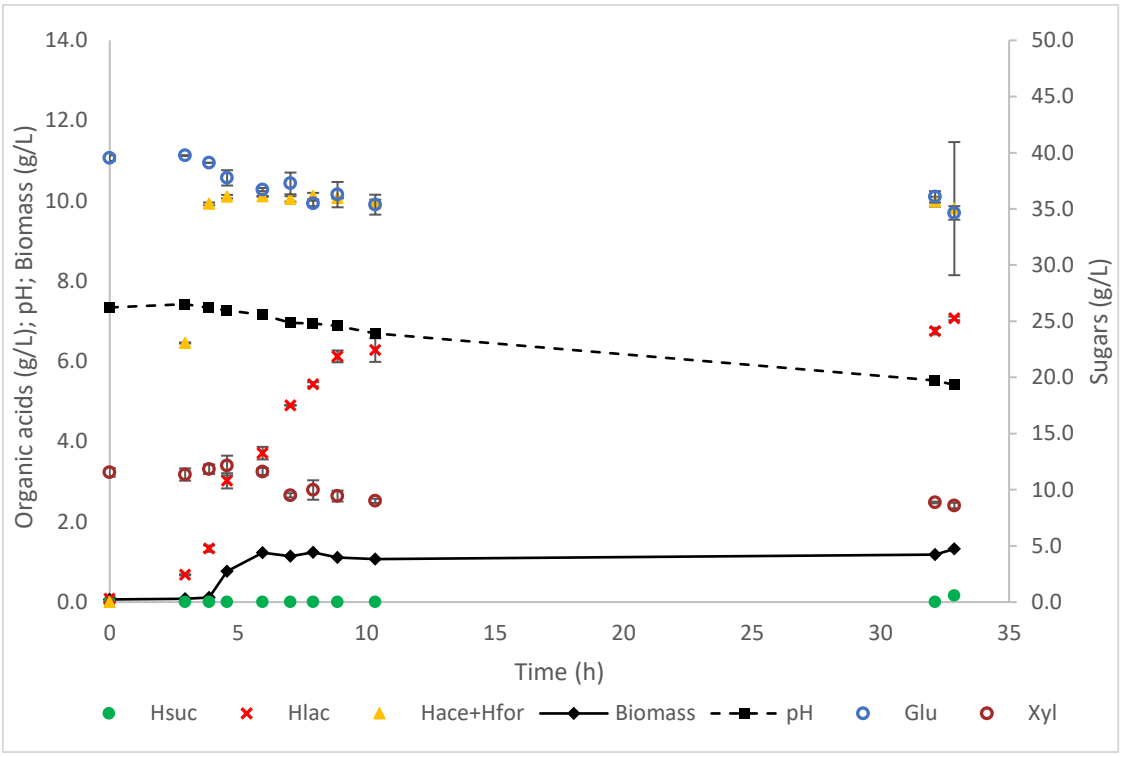


Figure 15 - Evolution of the sugars, organic acids, pH and biomass in the shake flask fermentation in WPH medium under aerobic conditions

4.4 Fermentation in bioreactor

Experiments with pH control were conducted in a bioreactor also with CO_2 sparging. The main objective was to extend fermentation since the pH was controlled promoting efficiency and to increase the fermentation scale. In the other hand CO_2 was used as one of the reactants for metabolic production of Hsuc and to input a positive pressure inside the bioreactor preventing O_2 entrance.

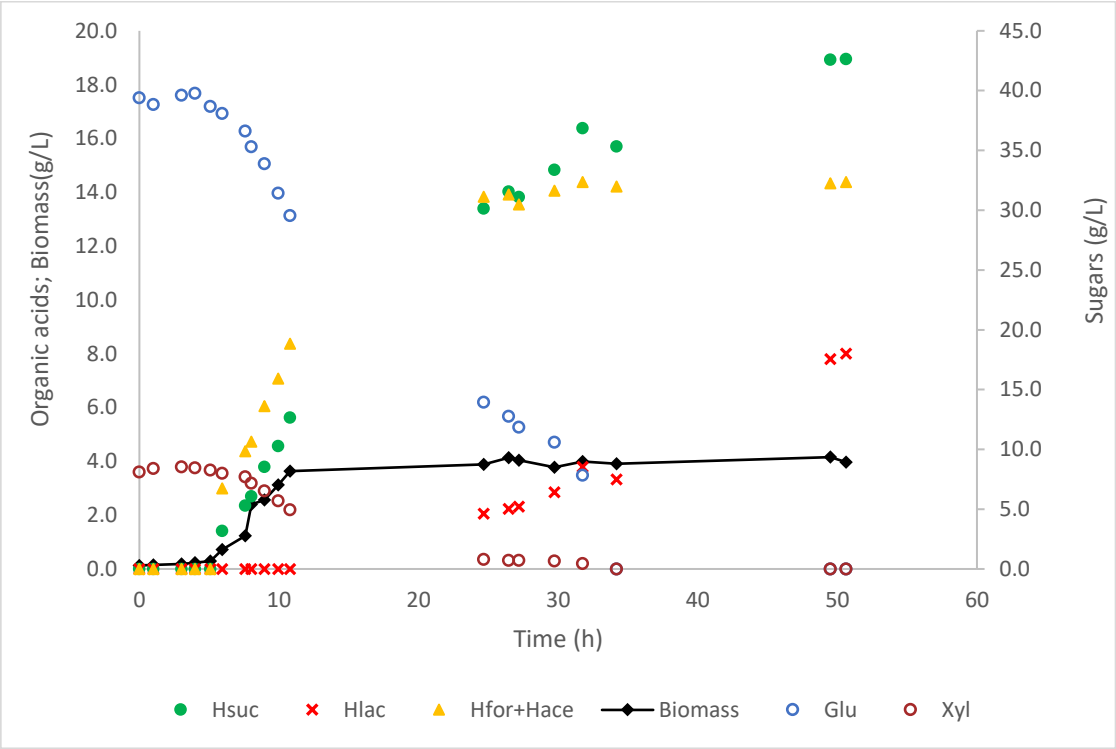


Figure 16 - Evolution of the sugars, organic acids and biomass in the bioreactor fermentation in synthetic medium with pH control at 6.7 and CO_2 sparging

In Figure 16 it is represented the time course relative to the bioreactor experiment using synthetic medium. 19.0 g/L of Hsuc and 4.0 g/L of biomass were, however it was also observed the production of Hlac (8.0 g/L) with a negative impact in the final Hsuc concentration, as can be noticed when compared to the literature data. Regarding the concentrations of the Hace and Hfor, they were 7.8 g/L and 6.5 g/L, respectively. As expected, the pH control extended the fermentation resulting in complete sugars consumption by the *A. succinogenes* and such led to the higher Hsuc and biomass relative to

the experiments in the anaerobic flasks. This experiment was also easier to compare with the literature since there is a considerable number of studies involving similar conditions and with pH control. It is common to find biomass concentration values between 3.7 g/L and 5.5 g/L, corresponding respectively to the lowest value obtained with the high content of sodium utilized, by Corona-Gonzalez et al. (2008) and the highest value, associated with the use of a higher concentration of yeast extract obtained by Kim et al. (2009)[84,141]. The biomass concentration in the present study (4.0 g/L) fits inside such range assuring again that this *A. succinogenes* culture was according to what was expected. However, the same was not verified for the Hsuc concentration since regarding this product it is common to find attained concentrations above 30 g/L[56,142], higher than the obtained in this experiment (19.0 g/L). Nonetheless, these differences can be justified by the unexpected production of Hlac, which consequently was a product from sugars utilization metabolic carbon shift from the Hsuc production pathway to the Hlac fermentation pathway.

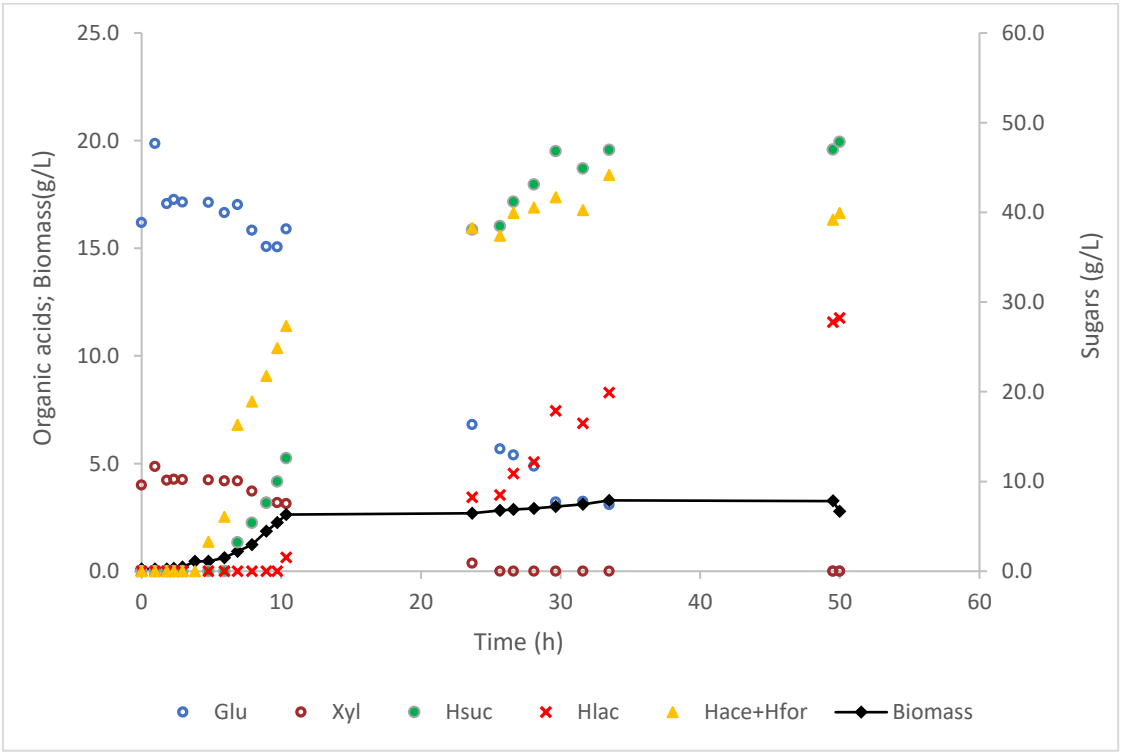


Figure 17 - Evolution of the sugars, organic acids, and biomass in the bioreactor fermentation in WPH medium with pH control at 6.7 and CO_2 sparging.

The second phase of the bioreactor study was to evaluate the fermentation in the WPH medium (Figure 17). As previously mentioned, this substrate naturally can have an inhibitory effect due to residual phenolic compounds left over by the kraft process. Consequently, this caused a lower biomass concentration compared to the bioreactor fermentation in synthetic medium, thus obtaining a value close to 3.0 g/L. Nonetheless, the attained Hsuc concentration of 19.9 g/L was higher than the value obtained by the synthetic medium fermentation in the same conditions. Certainly, the reason for that was caused by the unintentionally higher concentration of sugar, an error produced by the presence of small oligomers, not quantified by the HPLC. That reason is further evidenced by the apparent increment in the sugar concentration illustrated in the graphic, which was linked to the degradation of those oligomers by the *A. succinogenes*. To solve the issue related to monosaccharides concentration, the anthrone total sugar quantification method was explored. Resorting to that method, the initial concentration determined was 60.2 g/L, instead of the 48 g/L observed in the Figure 17. This higher amount of substrate is the only reason why the *A. succinogenes* produced a larger concentration of Hsuc in the WPH medium, despite the apparently similar conditions relative to the synthetic medium fermentation. Regarding the Hlac (11.6 g/L), Hfor (6.8 g/L) and Hace (9.8 g/L), their values were higher than the obtained in the fermentation with the synthetic medium. Howvere this occurrence can be again explained by the presence of higher Mg^{2+} concentrations in the WPH.

Table 8 – Results of the fermentations in bioreactor comparing all the kinetic parameters, related to the Hsuc, and the sugar consumption

Fermentation	μ_{max} (h^{-1})	Hsuc			Sugars consumed (g/L)	Hsuc/organic acids ratio
		Concentration (g/L)	Productivity (g/L.h)	Yield (g_{Hsuc}/g_{sugars})		
Synthetic medium	0.561	19.0	0.459	0.399	47.5	0.458
WPH medium	0.459	19.9	0.399	0.331	60.2	0.413

In the Table 8 the results relative to both fermentations are compared. Regarding the yield values, the competition for sugar between the Hlac and Hsuc pathways becomes evident. Compared to the literature, where in the worst cases the yield is normally above 0.500 g_{Hsuc}/g_{sugars}[35], the attained yield in both experiments was only 0.399 g_{Hsuc}/g_{sugars} and 0.331 g_{Hsuc}/g_{sugars}, for the synthetic and WPH media respectively. As for the Hsuc productivity, the value obtained in bioreactor was higher than the obtained in the fermentation flasks, due to the higher sugar consumption, but still lower than that described in the literature. Such could be attributed to the production of Hlac or due to the point selected to calculate this parameter. It is important to refer that the way this kinetic parameter is calculated can heavily influence its result. In this case, the final point considered for the Hsuc productivity determination was the first where the sugar concentration was 0 g/L. That point represents the instance where the Hsuc production stops, due to the lack of carbon source, thus being inefficient to prolong the process after that instant. The result regarding the WPH medium (0.399 g/L.h) was lower than the obtained in the synthetic medium, again due to the inhibition caused by the residual phenolic compounds.

Analyzing the μ_{max} in both fermentations the results were 0.561 h^{-1} and 0.459 h^{-1}, for the synthetic and WPH media respectively. Both results were similar to the ones obtained in the anaerobic shake flasks. That happened because all the conditions besides the pH control were equal in both scales of the fermentation. Therefore, the only difference between them was the duration of the lag phase, fact that cannot influence the result of the μ_{max}. Additionally, the μ_{max} obtained in both media was within the range of those described by Brink and Nicol (2014). Ultimately, it was possible to assert that the WPH medium has an inhibitory effect in the *A. succinogenes* growth and consequently in how quickly the bacterium can produce Hsuc.

Comparing both fermentations, the points where the Hlac production started, they were coincident with the points where the maximum biomass concentrations were achieved. Such can lead to the hypothesis that certainly at those corresponding times the organic nitrogen was depleted, with still significant sugars concentrations. This fact may have promoted the cease of cell growth with associated Hsuc production and instead the beginning of Hlac production, besides the oxygen contact. However, it is necessary to make more experiments, where different concentrations of organic nitrogen are compared, to confirm this hypothesis, since there is no data reported in the literature.

In the studied fermentations there was no visible difference between the supplementation of inorganic carbon through CO_2 sparging and through the use of $NaHCO_3$, in the anaerobic shake flask fermentations.

5 Evaluation of succinic acid production in kraft wood pulp hydrolysate using different strategies

In order to optimize the conditions used to produce Hsuc by *A. succinogenes*, two different strategies were employed: the first was to add different concentrations of $MgCO_3$, in order to improve the Hsuc production pathway of the *A. succinogenes* and the second was a fed batch operation, to test if a higher overall sugar quantity and the longer fermentation time could potentiate the Hsuc production.

5.1 Effect of magnesium carbonate in the WPH medium

According to the literature carbonated salts, such as $MgCO_3$, can have a major impact in the Hsuc production[35,78]. This variable was assessed by conducting fermentations in WPH with different concentrations of $MgCO_3$. It was initially expected that the $MgCO_3$ contributed as an additional inorganic carbon source, thus promoting the Hsuc production.

In the fermentation with 10 g/L of $MgCO_3$ (Figure 18), it was verified that the production of organic acids started earlier than in the previous experiments. Additionally, the Hsuc concentration was higher compared to the experiment without $MgCO_3$ achieving a final value of 23.2 g/L. In contrast the biomass decreased when compared to the fermentation without $MgCO_3$. However, the biomass could not be accurately estimated due to turbidity caused by the $MgCO_3$, which increased the OD measurements[84]. Moreover, the organic acids released by the *A. succinogenes* throughout the fermentation, reacted with the $MgCO_3$, thus decreasing the OD during the fermentation time. These fluctuations in the measured OD led estimation errors in the biomass. Therefore, it was considered the 2.6 g/L as the value for the final biomass concentration, due to its proximity to the value estimated through the cell dry weight. The concentrations of Hlac (15.9 g/L), Hfor (9.0 g/L) and Hace (12.4 g/L) showed an increase relative to the experiment without $MgCO_3$.

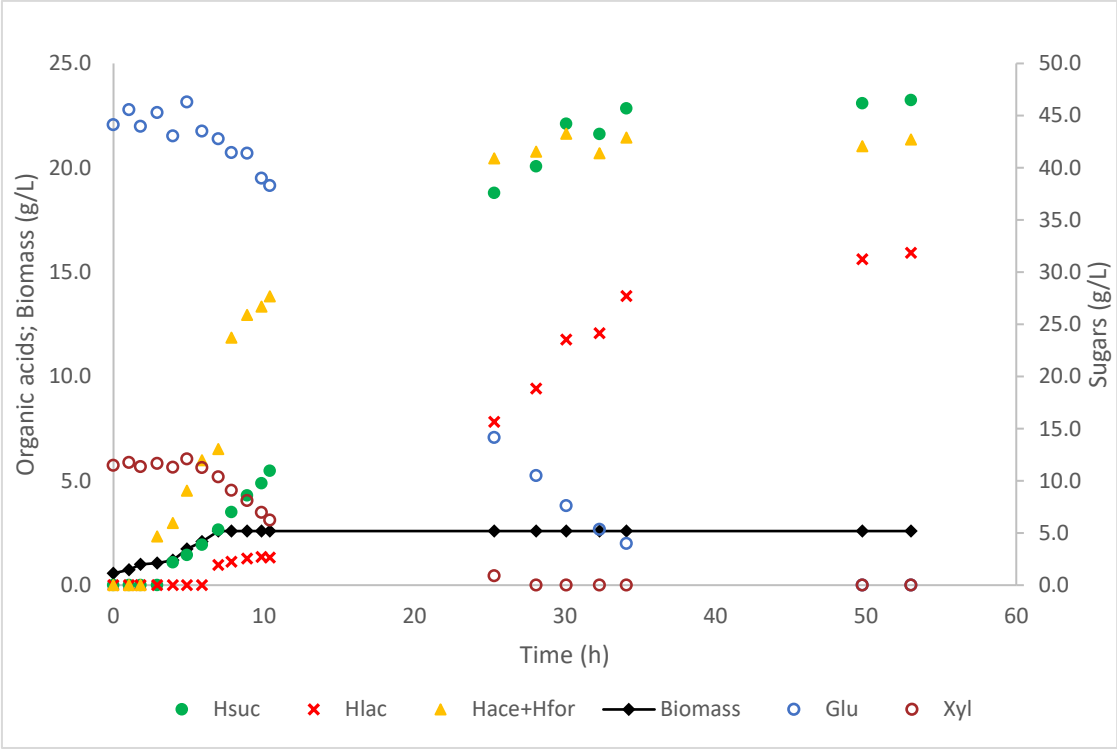


Figure 18 - Evolution of the sugars, organic acids and biomass in the bioreactor fermentation with the addition of 10 g/L of $MgCO_3$, pH 6.7 and CO_2 sparging.

In the experiment with 20 g/L of $MgCO_3$ (Figure 19) samples were diluted with HCl, to mitigate the OD errors caused by the carbonated salt. With this strategy it was possible to estimate more accurately the biomass concentration. The results obtained were similar to the fermentation without any addition of $MgCO_3$, attaining a maximum biomass concentration of around 3g/L. However, this value decayed over time, as commonly shown in the literature after adding carbonated salts. Although the reason of why such happened is not evidenced, it is possible to assume, based on results of the fermentation with 10 g/L of $MgCO_3$, that the salt contributed more for the production of organic acids than for the production and maintenance of biomass. Accordingly, the biomass yield was lower when compared to the fermentation in WPH, without $MgCO_3$. Hsuc concentration obtained was 23.2 g/L, similar to the result achieved by the fermentation carried out with 10 g/L of $MgCO_3$. Nonetheless, it was again verified an increase of other organic acids production. This fact indicates that increasing the concentration of $MgCO_3$ may not lead to an increase on the Hsuc production.

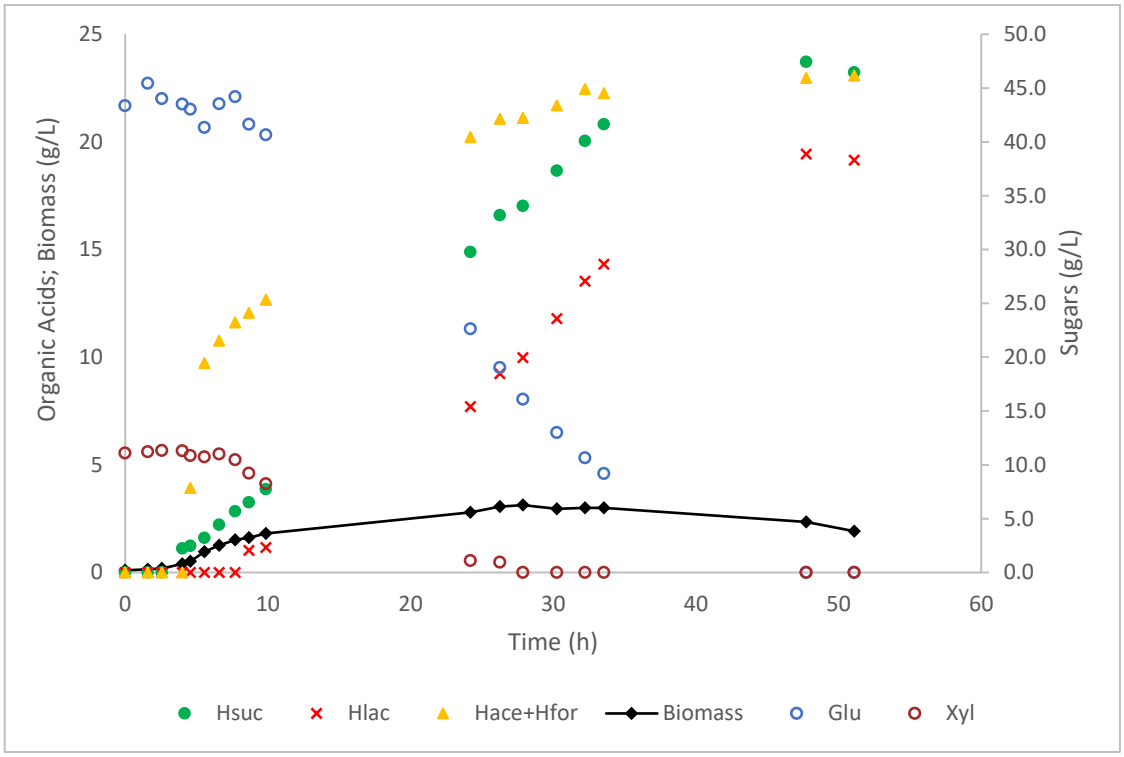


Figure 19 - Evolution of the sugars, organic acids and biomass in the bioreactor fermentation with the addition of 20 g/L of $MgCO_3$, pH 6.7 and CO_2 sparging.

In Table 9 a comparison is made between different concentrations of $MgCO_3$ used in the WPH medium. Regarding the μ_{max} the results obtained were 0.307 h^{-1} or 0.455 g/L.h, with 10 g/L or 20 g/L of $MgCO_3$ respectively. The lower value obtained with 10 g/L of $MgCO_3$ was associated with the turbidity caused by the salt, which led to errors in the μ_{max} determination. Nonetheless the value of μ_{max} obtained with 20 g/L $MgCO_3$ was almost identical to the obtained in the fermentation without $MgCO_3$. Such was not expected, due to some studies reporting a decrease in the bacterium growth, in contrast to the increased production of organic acids, when carbonated salts are added[143]. In all of these fermentations the Hlac production was again consistent with the cease of biomass production, thus suggesting that a depletion on organic nitrogen can favor the formation of this byproduct.

Table 9 – Results of the fermentations with different concentrations of $MgCO_3$, comparing all the kinetic parameters, related to the Hsuc, biomass yield and the sugar consumption

$MgCO_3$ (g/L)	μ_{max} (h^{-1})	Biomass yield ($g_{biomass}/g_{sugars}$)	Hsuc			Sugars consumed (g/L)	Hsuc/organic acids ratio
			concentration (g/L)	Productivity (g/L.h)	Yield (g_{Hsuc}/g_{sugars})		
0.0	0.459	0.180	19.9	0.399	0.331	0.413	0.413
10.0	0.307	0.126	23.2	0.459	0.377	61.6	0.384
20.0	0.455	0.123	23.2	0.497	0.370	62.7	0.354

Regarding the productivity, it was observed an improvement associated with increase of the $MgCO_3$ concentration. These improvements were expected since comparable results were described for other substrates after the addition of $MgCO_3$. Moreover, both the Mg^{2+} and the CO_3^{2-} can positively influence the *A. succinogenes* metabolism. The yield was also higher when comparing 10 g/L and 20 g/L of $MgCO_3$ to the absence of this salt. Despite this result obtained in both fermentations with $MgCO_3$ addition it was verified a higher concentration of byproducts associated with a decrease in the biomass, as it was suggested by the literature[139].

Concerning these results with different concentrations of $MgCO_3$, it is important to understand how the carbonated salt interfere with the *A. succinogenes* metabolism, thus producing higher concentrations of Hsuc as well as the other byproducts. According to the literature, it is possible to see that the use of a carbonated salt is normally associated with the release of CO_2 which will be used as co-substrate by the PEPCK. However, there are few studies where the influence of the metal ion associated with the carbonated salt is considered. This factor, however, can have a significant impact in the *A. succinogenes* enzymes since some metal ions can enhance or reduce their specific activities. Moreover, there are studies

which show the Mg^{2+} is a cofactor for the pyruvate kinase (PK) and the pyruvate dehydrogenase (PDH)[144]. These enzymes are responsible for catalyzing the transformation of PEP into pyruvate and catalyzing the transformation of pyruvate into ACoA, respectively. This implies that Mg^{2+} can promote the metabolic pathways, which will compete with the production of Hsuc. Consequently, it can be the key factor causing the decrease of Hsuc/organic acids ratio when higher amounts of $MgCO_3$ were added into the fermentations. Regardless this information, the effect of the CO_2 released from the $MgCO_3$ or any other carbonated salts cannot be completely neglected, since it was demonstrated that a higher concentration of CO_2 can indeed enhance the Hsuc production[139].

Further comparison of the $MgCO_3$ to other carbonated salts described in the literature, show that the metal ion plays a significant role in enzyme regulation. Therefore, an experiment using different concentrations of $MgCl_2$, could show how this specific metal ion would influence the fermentation, without having the interference of the CO_2 associated with the carbonate. It could also be useful to promote a similar test using a salt containing Mn^{2+}, since it is a known cofactor for the enzyme PEPCK[145]. This could allow activating the metabolic pathway responsible for the production of Hsuc.

5.2 Fed-batch fermentation in WPH medium

A fed-batch strategy was employed in order to achieve a higher concentration of Hsuc. Based on results obtained with different concentrations of $MgCO_3$, it was chosen to avoid its usage in this experiment. All the kinetic parameters were calculated for each feeding pulse and for the whole process (Table 10)

Table 10 – Fed-batch results in each pulse of operation with in WPH medium, presenting all the kinetic parameters, related to the Hsuc and the Hlac, and the sugar consumption

Pulses	Hsuc			Hlac			Consumed sugars (g/L)
	Concentration (g/L)	Productivity (g/L.h)	Yield (g_{Hsuc}/g_{sugars})	Concentration (g/L)	Productivity (g/L.h)	Yield (g_{Hlac}/g_{sugars})	
0	22.7	0.652	0.392	20.3	0.583	0.352	57.7
1	4.4	0.180	0.170	35.7	1.471	1.388	27.0
2	2.2	0.070	0.108	32.6	1.032	1.589	20.5
Whole process	29.3	0.317	0.276	88.6	0.968	0.84	105.2

The fed-batch successfully increased the overall organic acid production. Additionally, the final biomass concentration past beyond the 5 g/L (Figure 20), due to the higher overall quantity of substrate used in this assay. In the first stages of the fermentation, prior to any feeding pulse, the biomass evolution was similar to the observed in WPH batch made in a previous fermentation, achieving a concentration close to 4 g/L. After the first feeding pulse it was observed an increase in the production of Hlac contrasting to the production of Hsuc, Hace and Hfor being stalled and, consequently, their concentrations remaining relatively constant throughout the fermentation. Because this event could be interpreted as a contamination, a sample from the end of the fermentation was inoculated in two distinct petri dishes with solid medium namely TSB, suitable for the growth of *A. succinogenes*, and MRS, for its inhibition, while promoting the growth of common

contaminant bacteria (Lactobacillus) described in the literature. The presence of bacterial colonies in TSB and absence on MRS, respectively, showed that the production of Hlac was not caused by any contamination.

A study conducted aiming to evaluate *A. succinogenes* metabolic fluxes and enzymatic activities under different aeration strategies, has shown a relative decrease in the expression of the main genes contributing to the Hsuc production pathway. Such happens when the *A. succinogenes* is exposed to O_2, which repress the expression of the gene responsible to produce the PEPCK[138]. Moreover, this exposure induces the expression of other genes capable of promoting the production of Hlac. This led to the common described conclusion which indicates that enzymes, such as the LDH and the PK, suffer an increase on their activity, due to their higher concentration. In the case of this fed-batch fermentation, it is possible that the Hlac production was induced by O_2 contact in the stock culture. This metabolic shift was eventually improved throughout the fermentation. Additionally, a northern blot technic could be carried out to determine the expression of the key genes of the *A. succinogenes,* to further understanding this Hlac production.

It should be also mentioned that in this experiment, the addition of organic nitrogen together with the feeding solution was unable to change the metabolic pathways to the Hsuc production.

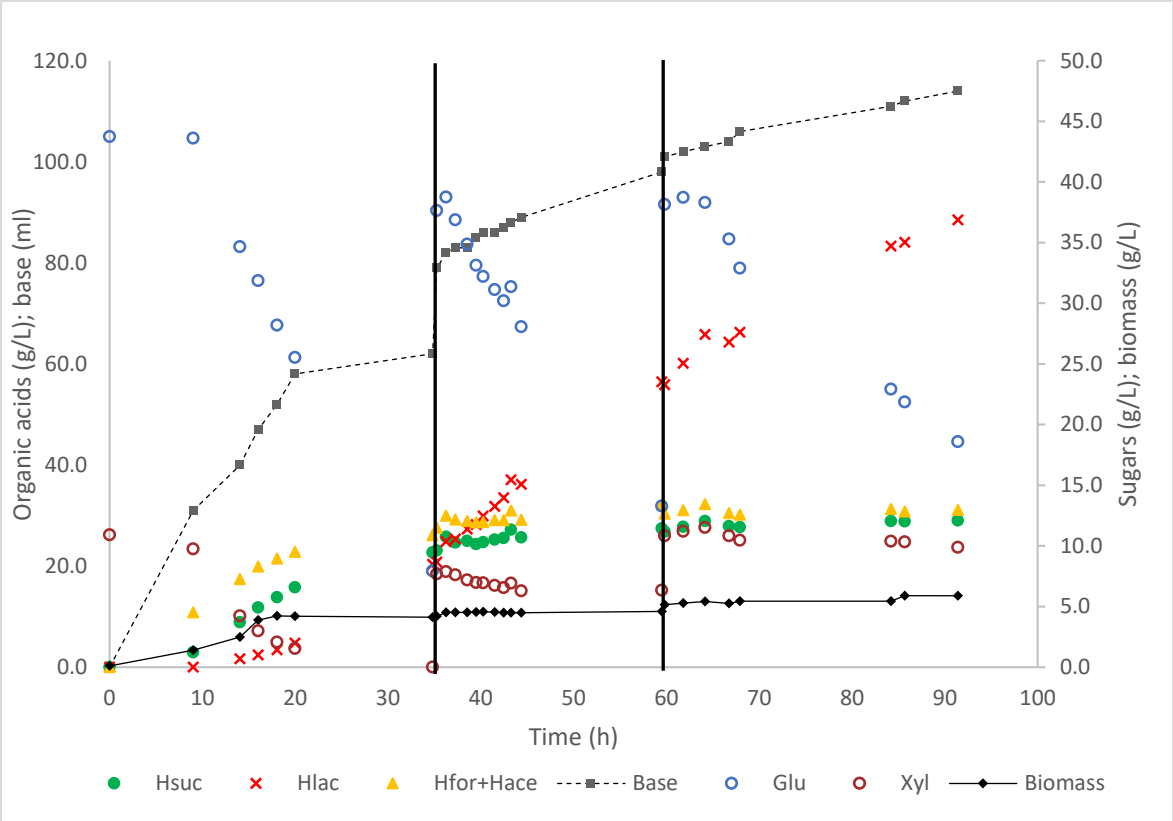


Figure 20 - Time course of the Fed-batch fermentation in WPH medium. The vertical bars represent the feeding pulses. The concentrations of sugars and organic acids were calculated considering their respective masses and assuming a constant initial volume.

In this fermentation it was not possible to accurately determine the maximum specific growth rate due to insufficient points in the exponential phase. The Hsuc yield obtained was calculated for each feeding pulse and for the whole process (Table 10). Prior to the feeding pulses the results were consistent with the batch fermentation without $MgCO_3$. It was also obtained a Hsuc yield of 0.392 g_{Hsuc}/g_{sugars} with a productivity of 0.652 g/L.h. After the first pulse those values decreased to 0.123 g_{Hsuc}/g_{sugars} and 0.180 g/L.h, dropping further on the subsequent pulse. Certainly, this drop is attributed to the increasing expression of the genes responsible for the metabolic pathway, opposed to the Hsuc production namely the one producing Hlac. In the final pulse the Hsuc production was almost nonexistent, achieving a final total Hsuc concentration of 29.3 g/L. As consequence of the Hlac production, the total yield and productivity of Hsuc were 0.276 g_{Hsuc}/g_{sugars} and 0.317 g/L.h, respectively. These were lower than the values obtained in all batch fermentations and those reported in the literature. Thus, showing the inefficiency of producing Hsuc in fed-batch when the

production of Hlac is verified. It can be possible that a small activation of the gene responsible for the expression of LDH could have changed the *A. succinogenes* metabolism, leading to the increasing Hlac production.

Since the Hlac can also be a valuable bioproduct, it was important to evaluate the kinetic parameters associated with its production. Prior to any pulse, both the Hlac productivity and yield were below those already presented in the literature. However, after the first pulse it was verified an increase in such parameters, obtaining a yield of 1.388 g_{Hlac}/g_{sugars} and a productivity of 1.008 g/L.h. After the last pulse, the yield was increased to 1.589 g_{Hlac}/g_{sugars}, due to the drastic reduction on the production of other organic acids. Attending to the overall results, it is possible to see that the Hlac yield was lower than the obtained by Li et al. (2010). However, both productivities were similar. The proximity between the yields in both studies, might suggest that the WPH could be a suitable substrate to promote the Hlac production, using the same strategy as Li et al. (2010). In addition, the presence of $MgCO_3$ in the WPH can be benefic for Hlac production.

Despite the higher production of Hlac compared to Hsuc, the results obtained with the *A. succinogenes* suggest that this MO can compete with Hlac producing bacteria. However, it should be considered that some of these bacteria are homolactic, and consequently produce exclusively Hlac, thus requiring less downstream processing. Nonetheless, since the concentration of Hsuc was close to the produced in the batch processes, it is possible to explore the simultaneous production of both the Hlac and Hsuc using a fed-batch strategy.

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6 Conclusions

In this work, it was established the ability of the bacterium *A. succinogenes* to produce succinic acid (Hsuc), from *Eucalyptus globulus* wood pulp originated from the kraft pulping process and hydrolyzed afterwards. Small scale experiments in anaerobic shake flaks showed the consumption of pentoses besides hexoses for the production of high concentrations of organic acids, being the Hsuc the predominant one. These concentrations consequently decreased the pH values, which led to an early fermentation stop, thus leaving unconsumed sugars in the fermentation medium. In terms of biomass, the synthetic medium results were higher than the obtained with WPH, both in succinic acid concentration and productivity as well as in specific growth rate. This was expected due to the presence of any inhibitors coming from lignin, such as residual phenolic compounds, which were evidenced by the LEX-SEC chromatography. The yield however was unexpectedly higher in the WPH. The explanation for that was obtained again through the LEX-SEC chromatography where the results showed the presence of dimers which were not detected by HPLC. However, they could be used as carbon source by *A. succinoges* for producing organic acids. It must be also accounted the significant production of Hlac verified in the WPH fermentation, which certainly was due to changes in the *A. succinogenes* metabolism linked to an oxygen exposure.

In the fermentations made in bioreactor, the effects made by the WPH in the *A. succinogenes* metabolism became evident. The pH control allowed the complete sugar consumption by the *A. succinogenes* and due to the presence of phenolic compounds all kinetic parameters decreased, while byproducts formation occurred due to the Mg^{2+} abundance in the WPH. Certainly, there was interaction of Mg^{2+} with the enzymes that favor other organic acids biosynthesis, which compete with the succinic acid production pathway. The production of high Hlac was verified, contrary to what is commonly reported in the

literature. Such happen due to a possible interaction of the culture with the oxygen, being possibly enhanced afterwards by the organic nitrogen depletion.

To evaluate if the kinetic parameters could be somehow improved, by different concentrations of $MgCO_3$ were evaluated. It was confirmed that this salt enhanced all kinetic parameters except the specific growth rate. In the literature, the carbonate present on the $MgCO_3$ is pointed as the principal factor, due to its interaction with the PEPCK. However, the $MgCO_3$ also led to the production of more byproducts. Such result can also support the previous theory, about the magnesium ion present in the wood, having a major influence in the byproducts production. It is already known that this metal ion is an important cofactor for the pyruvate kinase, thus explaining the higher byproducts concentration obtained. Furthermore, the use of cofactors of the Hsuc production pathway, could improve the overall production of this organic acid, hence manganese should be studied.

Finally, due to the shift in the *A. succinogenes* metabolism that led to the production of Hlac, the fed-batch fermentation was not suitable for producing Hsuc exclusively. Nonetheless, before the first feeding pulse, the concentration of Hsuc was similar to the attained in batch. However, after the feeding pulses it was only observed the production of Hlac. This can lead to the conclusion that this fed-batch could be suitable for producing both organic acids.

Despite the production of Hlac, the objective of this work was successfully accomplished since WPH is a suitable feedstock to produce Hsuc using *A. succinogenes.* This study served as a reference for future bioprocess studies fed with wood residues, in order to their integration into a pulp and paper biorefinery working according to the Circular Economy model. Moreover, this process has the particularity of consuming CO_2, allowing to simultaneously decrease the emissions. It could be also possible to exploit the use of $MgCO_3$ and fed-batch operations to improve the production of both Hsuc and Hlac.

7 Future work

Due to the production of Hlac, the main focus of future work should be to find specific process conditions of *A. succinogenes* to direct the carbon flux to Hsuc pathway in order to guarantee the production of Hsuc, without needing genetic manipulation. This objective was never attempted, but some strategies may allow this performance. Different studies could be carried out as the use of LDH inhibitors, such as copper ions, the use of manganese ions to promote de activity of the PEPCK and the use of small concentrations of benzoic acid, which some authors described as an inhibitor for the expression of the gene responsible for the LDH production. Also, the importance of the concentration of nitrogen and a reductive substrate should be tested in order to enhance the Hsuc production.

With *A. succinegenes* producing high concentrations of Hsuc, the next step should be the use of WPH originated from bark pulps of the *E. globulus*. The process conditions shall be optimized in a bioreactor with pH control and constant CO_2 sparging. Fed-batch and continuous operations could also be assessed to ensure the highest productivities and concentrations of Hsuc. If possible, technics such as genetic engineering and *in silico* simulations could be useful to improve the Hsuc bioprocess.

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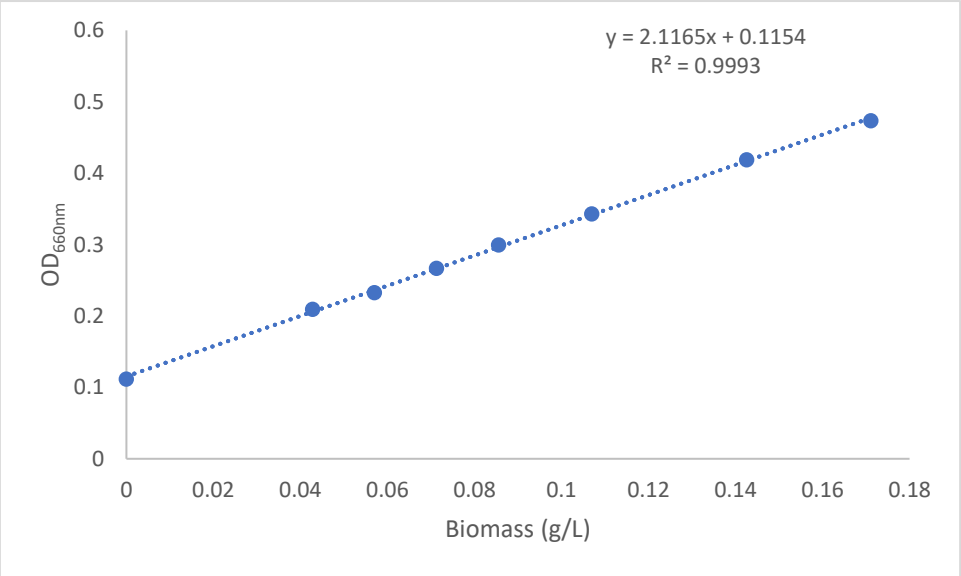
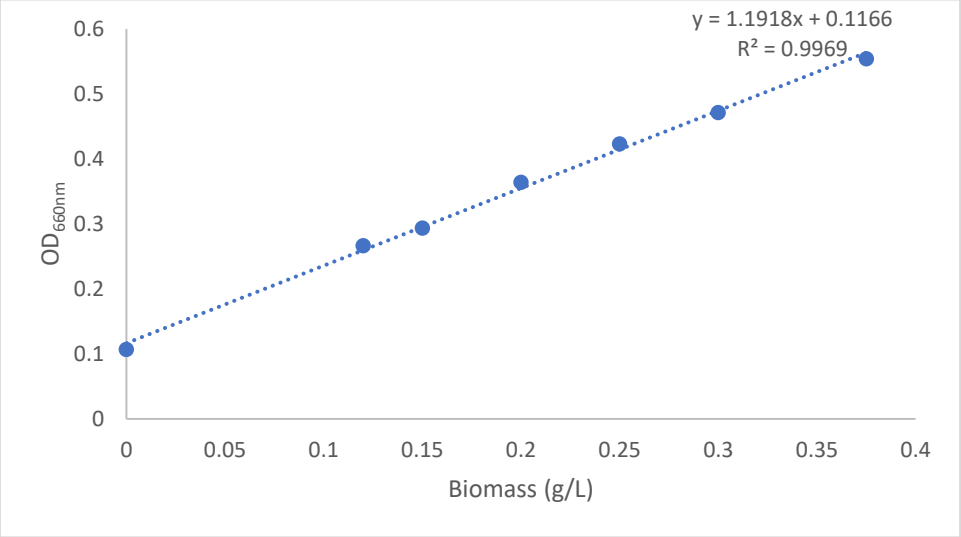
8 Appendix

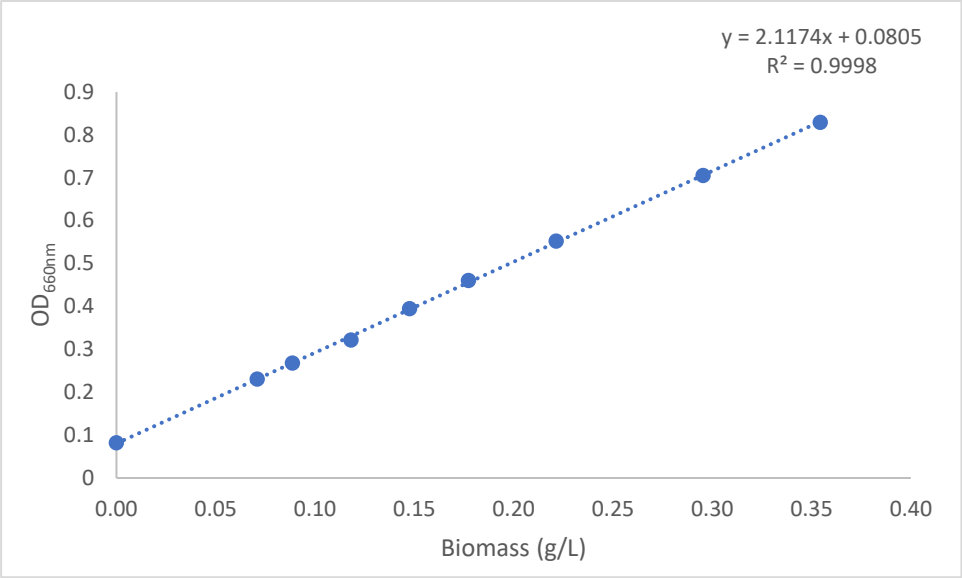
APPENDIX I – Calibration Curves of the Biomass



I.1 – Calibration curve of the biomass concentration in TSB medium

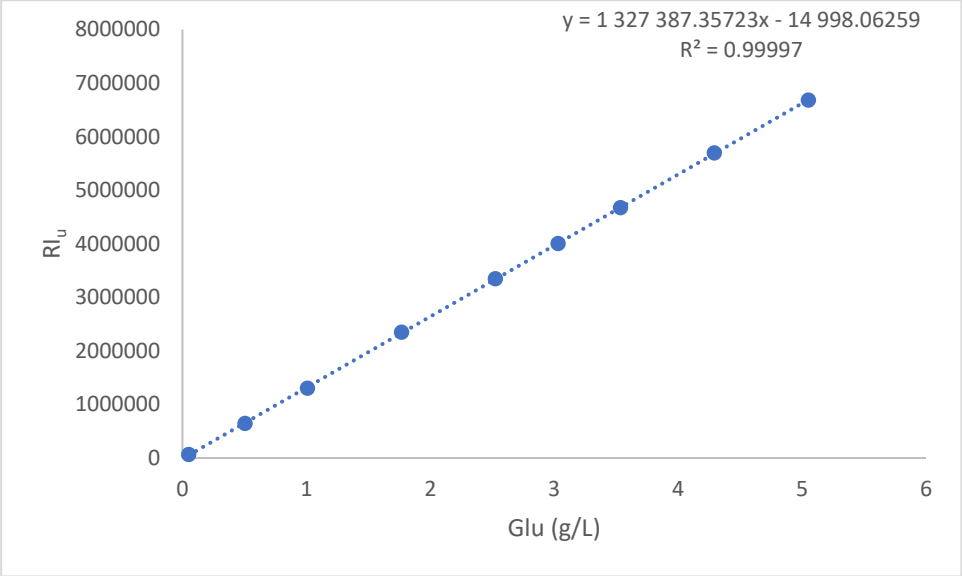


I.2 – Calibration curve of the biomass concentration in synthetic medium

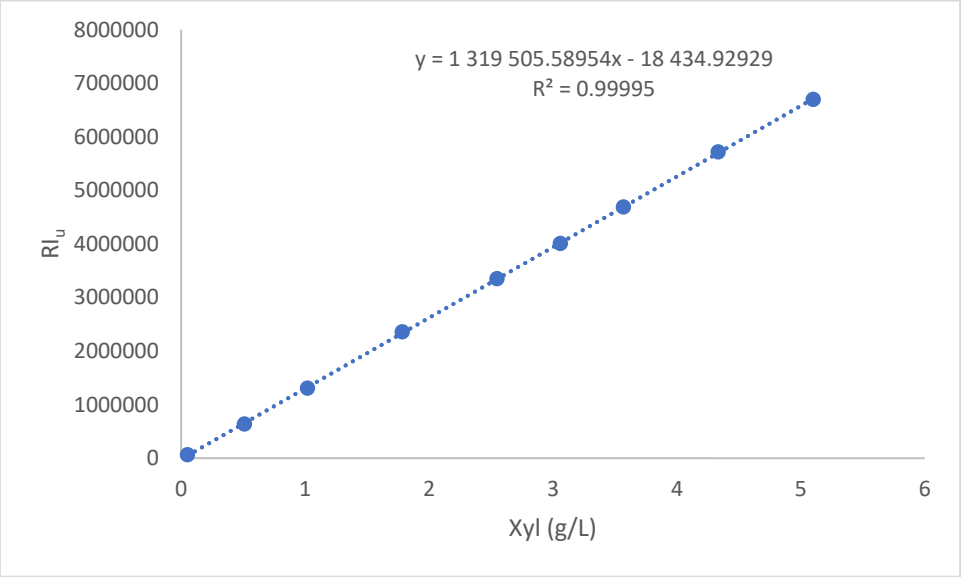


I.3 – Calibration curve of the biomass concentration in WPH medium

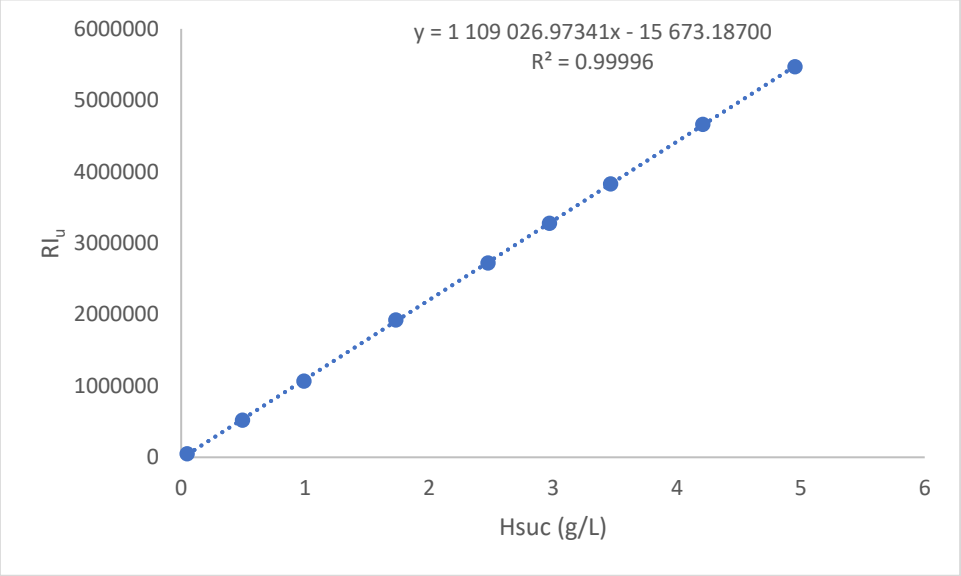
APPENDIX II - Calibration Curves of the HPLC



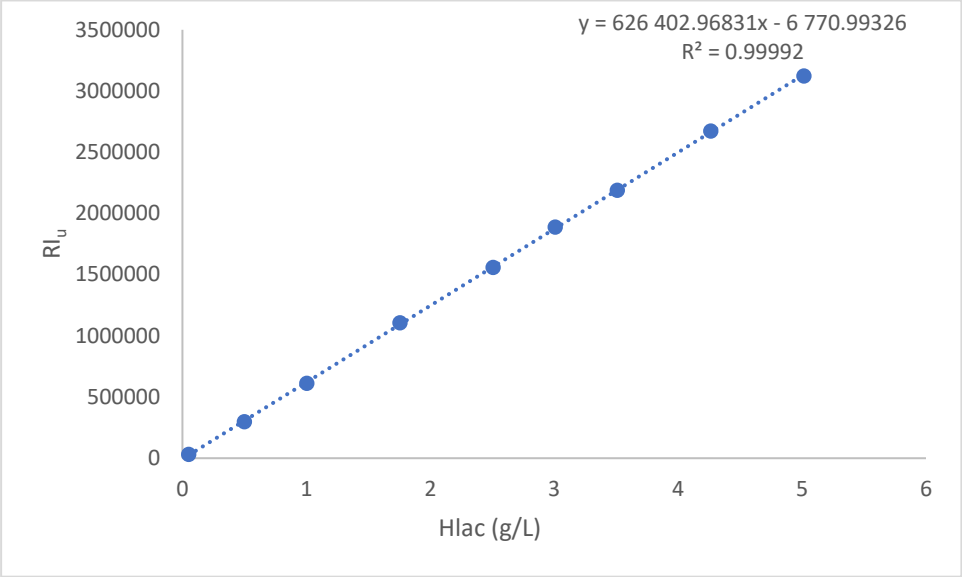
II.1 – Calibration curve of the Glu



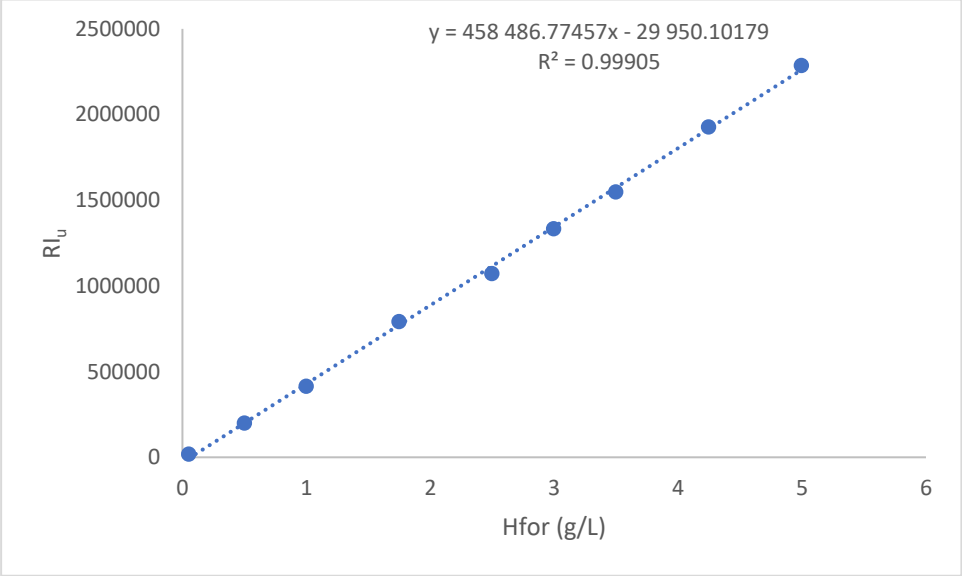
II.2 – Calibration curve of the Xyl



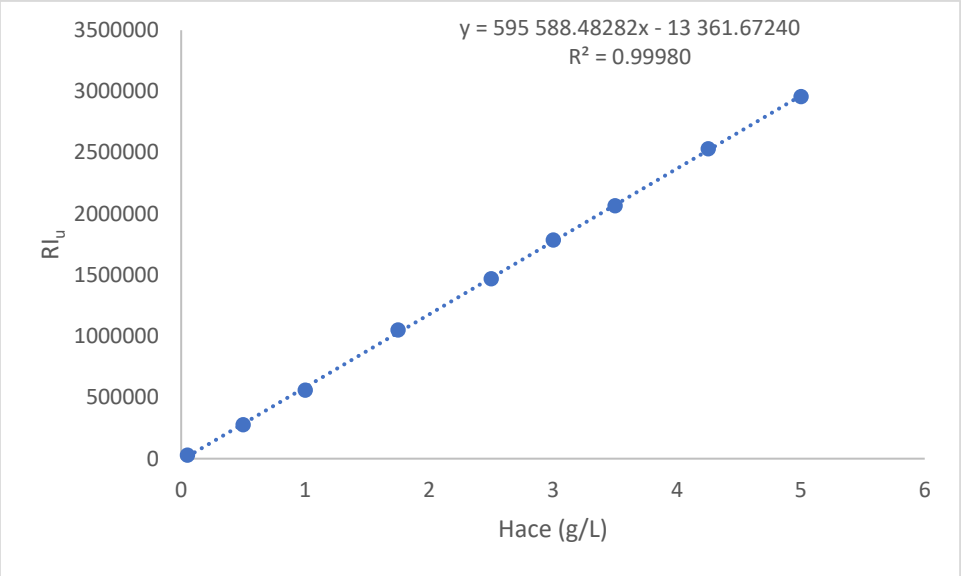
II.3 – Calibration curve of the Hsuc



II.4 – Calibration curve of the Hlac

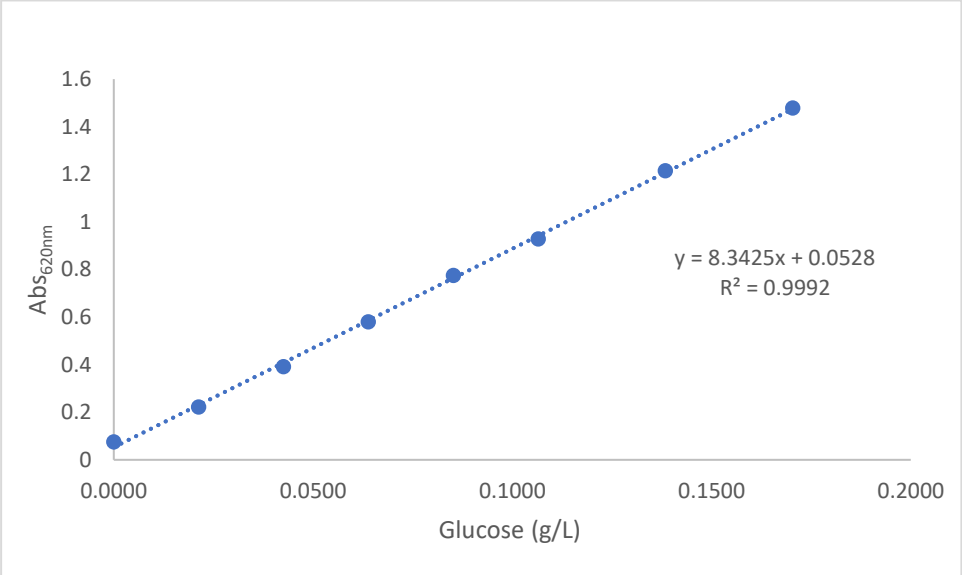


II.5 – Calibration curve of the Hfor



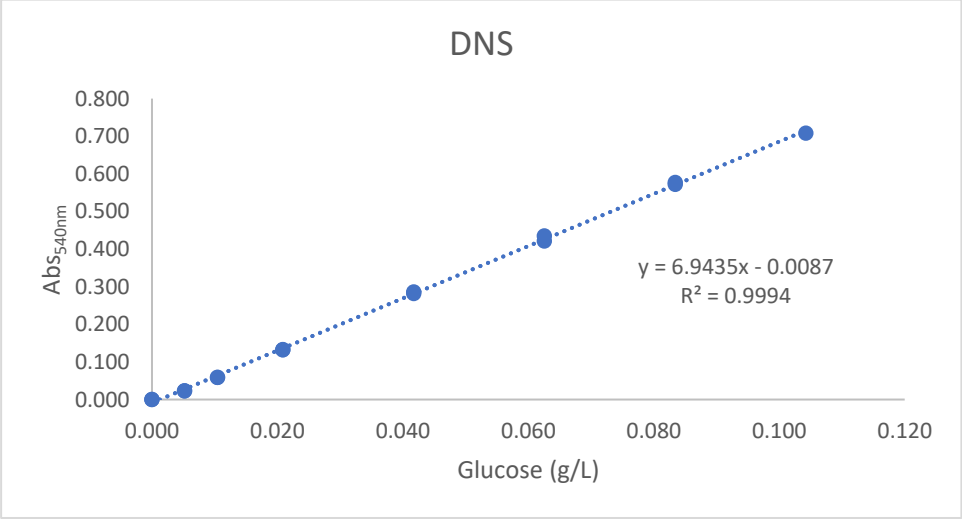
II.6 – Calibration curve of the Hace

APPENDIX III - Calibration curve of the anthrone method



III.1 – Calibration curve of the anthrone

APPENDIX IV - Calibration curve of the /DNS method



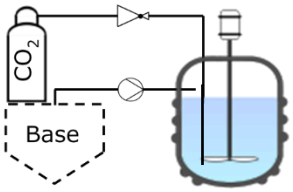
IV.1 – Calibration curve of the DNS

APPENDIX V – Wood Pulp composition

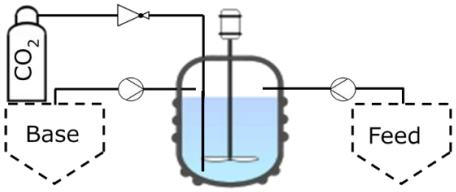
V.1 – Wood Pulp composition

Sugars (%)	Lignin (%)	Humidity (%)	Cellulose (%)	Hemicelluloses (%)
97.9%	2.1%	70.66%	83	15

APPENDIX VI – Bioreactor setup scheme



VI.1 – batch setup



VI.2– fedbatch setup