Tatiana Soares Costa

Degree in Biochemistry

Biovalorization of lignocellulosic wastes for sugar alcohols production by the yeast *Komagataella pastoris*

Dissertation for the degree of Master in Biotechnology

Supervisor: Professor Maria da Ascensão Carvalho Fernandes Miranda Reis, Full professor, FCT NOVA

Jury
President: Professor Carlos Alberto Gomes Salgueiro
Examiner: Doctor Maria Teresa Ferreira Cesário Smolders
Vogal: Professor Maria da Ascensão Carvalho Fernandes Miranda Reis

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

September, 2019

Tatiana Soares Costa

Degree in Biochemistry

Biovalorization of lignocellulosic wastes for sugar alcohols production by the yeast *Komagataella pastoris*

Dissertation for the degree of Master in Biotechnology

Supervisor: Professor Maria da Ascensão Carvalho Fernandes Miranda Reis, Full professor, FCT NOVA

Jury
President: Professor Carlos Alberto Gomes Salgueiro
Examiner: Doctor Maria Teresa Ferreira Cesário Smolders
Vogal: Professor Maria da Ascensão Carvalho Fernandes Miranda Reis

September, 2019

Biovalorization of lignocellulosic wastes for sugar alcohols production by the yeast *Komagataella pastoris*

Agradecimentos

Em primeiro lugar um agradecimento especial à professora Ascensão Reis, que com as suas aulas de Engenharia Bioquímica, me fascinou com os bioprocessos sustentáveis que são desenvolvidos no seu grupo de investigação e me deu a oportunidade de fazer parte desse universo durante este último ano. Agradeço também à professora Filomena Freitas por me ter recebido tão bem no seu laboratório, pela disponibilidade e preocupação constantes, por todos os ensinamentos, conselhos e motivação que foram realmente importantes ao logo deste trabalho.

A todo o grupo BioEng, em especial, aos colegas do laboratório 407, por estarem sempre prontos a ajudar e a ensinar, foram uma peça essencial neste processo.

À Diana Araújo pela força e energia contagiantes, pelas longas conversas e por todo apoio a nível laboratorial e também pessoal, muito obrigada!

À Fundação Lapa do Lobo, pelo apoio financeiro ao longo de todo o meu percurso académico, que me permitiu focar apenas nos estudos.

Aos meus amigos, que apesar das ausências prolongadas continuam sempre lá para tudo. Obrigada por todos os momentos e histórias que nunca esquecerei!

Ao Nuno por tudo e por nada! Principalmente por ainda continuar a aturar o meu mau feitio (e não é nada fácil!).

E por fim, o mais importante! À minha mãe, ao meu pai, à avó Aurora e ao avô Quim, que por mais vontade que tenha é impossível agradecer tudo o que fazem por mim. Sou realmente uma sortuda e tenho muito orgulho em vocês!

Um grande bem-haja a todos!

Resumo

Xilitol e arabitol são álcoois de açúcar naturais usados como alternativas à sucrose e adoçantes artificiais, que pertencem à lista de compostos de valor acrescentado a serem produzidos a partir de biomassa de baixo custo. As suas ótimas propriedades e benefícios para a saúde têm atraído a atenção das indústrias alimentares e farmacêuticas, mas as suas aplicações continuam limitadas pelo preço e baixa disponibilidade.

O objetivo principal desta tese foi avaliar, pela primeira vez, a capacidade de levedura *Komagataella pastoris* DSM 70877 para produzir xilitol e arabitol utilizando resíduos lignocelulósicos como substrato, particularmente, cascas de banana, dreche, carolos de milho, bagaço e engaço de uva e serradura. Uma via biotecnológica promissora e alternativa à produção química em larga escala.

A primeira parte do trabalho focou-se na obtenção de licores ricos em açúcares e fermentáveis, a partir dos diferentes resíduos lignocelulósicos, submetendo-os a uma hidrólise ácida diluída. Na dreche, engaço de uva e serradura revelou-se uma maior recuperação de açúcares, originando hidrolisados com um teor total de monossacarídeos entre 12.9 e 21.5 g/L e diferentes rácios de glucose, xilose e arabinose. Os inevitáveis subprodutos tóxicos do processo foram também detetados nos hidrolisados, nomeadamente, furfural, 5-HMF e ácido acético, e, portanto, na tentativa de os reduzir/eliminar recorreu-se a tratamentos com carvão ativado.

Na segunda parte deste trabalho, os hidrolisados foram testados como substratos para o cultivo de *K. pastoris* e produção de álcoois de açúcar. Entre os ensaios em Erlenmeyer, o hidrolisado da dreche levou à maior produção de xilitol, 3.97 g/L, com um rendimento de xilitol/xilose de 0.47 g/g. Ensaios em bioreator também foram realizados, em batch e fed-batch, testando diferentes substratos e condições de arejamento. Os substratos selecionados foram dreche, cascas de banana, mistura de dreche (ou engaço de uva) com alimentação de serradura e ainda, uma mistura de três substratos: dreche (ou engaço de uva) e cascas de banana com alimentação de serradura. A levedura alcançou uma produção máxima de xilitol de 1.33 g/L (rendimento 0.18 $g_{xilitol}/g_{xilose}$) num ensaio em batch, com uma taxa de fluxo de ar de 0.5 L/min e usando como única fonte de carbono o hidrolisado de dreche concentrado 2 vezes e destoxificado.

Apesar das baixas produções alcançadas, neste trabalho provou-se o potencial da levedura para produzir xilitol a partir de hidrolisados hemicelulósicos. Assim, este estudo terá de ser validado e aprofundado para desenvolver e otimizar o processo.

Curiosamente, percebeu-se que a cultura estava a canalizar parte do carbono fornecido pelos hidrolisados hemicelulósicos para outras vias metabólicas e/ou produção de outros compostos, como detetado pela análise de HPLC. Abre-se assim a possibilidade da levedura *K. pastoris* ser capaz de produzir compostos de grande interesse e valor comercial.

Palavras-chave: Resíduos lignocelulósicos, hidrolisados hemicelulósicos, álcoois de açúcar, xilitol, *K. pastoris*

Abstract

Xylitol and arabitol are natural sugar alcohols used as alternatives to sucrose and artificial sweeteners, that belong to the list of value-added compounds to be produced from low-cost biomass. Their great properties and health benefits have attracted the attention of food and pharmaceutical industries, but their applications are still limited by cost and lack of availability.

The main goal of this thesis was to evaluate, for the first time, the ability of the yeast *Komagataella pastoris* DSM 70877 to produce xylitol and arabitol by using lignocellulosic waste materials as feedstocks, namely, banana peels, brewers' spent grains (BSG), corncobs, grape pomace, grape stalks and sawdust, a promising biotechnological route as an alternative to the chemical large-scale production.

The first part of this work was focused on obtaining sugar-rich and fermentable liquors from different lignocellulosic waste materials, by subjecting them to dilute acid hydrolysis. The higher sugar recoveries were achieved for BSG, grape stalks and sawdust, leading to hydrolysates with total monosaccharides' contents between 12.9 and 21.5g/L, with different ratios of glucose, xylose and arabinose. The inevitable toxic by-products generated were also detected in the hydrolysates, furfural, 5-HMF and acetic acid, and attempts were done to reduce/eliminate them by treatment with activated charcoal.

In the second part of this work, the hydrolysates were tested as substrate for cultivation of *K. pastoris* and sugar alcohols production. Within shake flask assays, BSG hydrolysate attained the highest xylitol production of 3.97 g/L, with a xylitol/xylose yield of 0.47 g/g. Bioreactor cultivations were also performed in batch and fed-batch modes, testing different feedstocks and aeration conditions. The selected feedstocks were BSG, banana peels, a mixture of BSG (or grape stalks) with feeding of sawdust and triple mixture of BSG (or grape stalks) and banana peels with feeding of sawdust. The yeast achieved a maximum xylitol production of 1.33 g/L (yield of 0.18 gxylitol/gxylose) in a batch cultivation with an airflow rate of 0.5 L/min, using twofold concentrated and detoxified BSG hydrolysate as the sole carbon source.

Despite the low productions achieved, this work proved the yeast potential to produce xylitol from hemicellulosic hydrolysates. Thus, further research is required in order to develop and optimize the process.

Interestingly, it was noticed that the culture was channeling part of the carbon provided by the hemicellulosic hydrolysates into other metabolic pathways and/or different products, as detected by HPLC analysis. This opens the possibility of *K. pastoris* to be able to produce other compounds of great interest and commercial value.

Keywords: Lignocellulosic wastes, hemicellulosic hydrolysates, sugar alcohols, xylitol, *K. pastoris*

List of contents

List of Figures

List of Tables

List of Abbreviations

5-HMF	5-Hydroxymethylfurfural
AIL	Acid Insoluble Lignin
AR	Arabinose Reductase
ASL	Acid Soluble Lignin
BSG	Brewers' Spent Grain
CDW	Cell Dry Weight
CGC	Chitin–Glucan Complex
DOC	Dissolved Oxygen Concentration
DW	Dry Weigh
FDA	Food and Drug Administration
GI	Glycemic Index
GMO	Genetically Modified Organism
GRAS	Generally Recognized As Safe
HPLC	High Performance Liquid Chromatography
LAD	Arabitol Dehydrogenase
LXR	Xylulose Reductase
n.a.	data not available
n.d.	not detected
NREL	National Renewable Energy Laboratory
OD	Optical Density
ODW	Oven Dry Weight
QAS	Quality Assurance Standards
rpm	rotation per minute
TP	Total Phenol Concentration
XDH	Xylose Reductase
XR	Xylitol Dehydrogenase

Chapter 1

Introduction and Motivation

1. Introduction

1.1 Sugar alcohols

Nowadays, population lifestyle is changing dramatically. An increasingly health conscious consumer, along with the increased rate of diabetes and obesity, led to a strong demand for energy-reduced products, containing low caloric sweeteners. Sweeteners are now present as additives in a wide range of food products [1].

Sweeteners can be divided into nutritive sweeteners, including sugar alcohols, and non-nutritive (artificial) sweeteners [1]. The main difference between artificial sweeteners and sugar alcohols is that the first ones contain zero calories, being 30 to 13 000 times sweeter than sucrose and may present an unpleasant aftertaste. On the other hand, sugar alcohols supply 0.2 to 2.7 kilocalories per gram (kcal/g) and have lower or similar sweetness than sucrose [2]. Although some sweeteners from both classes are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA), the artificial ones have maximum amounts allowed for consumption, while for sugar alcohols, no maximum values are established. This is supported by the fact that overconsumption of sugar alcohols can only cause transient laxation and gastrointestinal discomfort as side effects [1].

Sugar alcohols, which are also known as polyhydric alcohols, polyols or alditols, derive from carbohydrates. They are produced by reduction of the carbonyl groups of the corresponding carbohydrates to primary or secondary hydroxyl groups [3]. Most of them are naturally present, in small amounts, in fruits, some vegetables and other food products [1].

Polyols can be divided in tree main groups: hydrogenated monosaccharides (including xylitol, arabitol, sorbitol, mannitol and erythritol), hydrogenated disaccharides (namely maltitol, lactitol and isomalt) and hydrogenated oligosaccharides (hydrogenated starch hydrolysates) [1]. Besides being mainly used as natural sweeteners, polyols can also be used to improve product texture (as thickener and emulsifier agent), preservation and holding moisture [1]. Among other properties, polyols have high thermal, chemical and microbial stability and do not undergo Maillard-type reactions when heated [3]. Given their properties, they have attracted the attention of several industries being widely used in food, cosmetic and pharmaceuticals areas.

1.1.1. Xylitol and arabitol: characteristics, properties and applications

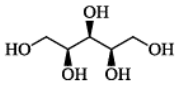
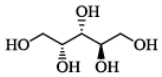
Xylitol ($C_5H_{12}O_5$) is a naturally occurring pentahydroxy sugar alcohol, obtained from D-xylose conversion [4]. It can be found in nature, in amounts of less than 1%, in a variety of fruits, vegetables, berries, oats and mushrooms and it is produced, in small quantities, in the human organism during glucose metabolism [5]. It was first obtained in the form of syrup from beech

wood by Emil Fischer, in 1891, and since 1984 it has been recognized as safe in the European Economic Community, known as E967 food additive [1, 4]. Xylitol is perhaps the most popular sugar alcohol. It is the sweetest sugar alcohol, with a visual appearance really similar to sucrose and the same sweetening power (Table X).

Arabitol ($C_5H_{12}O_5$), also named arabinitol or lyxitol, belongs to the pentitol family and it is obtained from arabinose conversion. It exhibits optical isomerism, L-arabitol or D-arabitol and it is found naturally in many lichens and mushrooms [6].

According to the US department of energy, xylitol and arabitol belong to the top 12 value-added compounds to be obtained from biomass [7]. Xylitol is a well-known substitute for sucrose, while arabitol has not been so investigated so far. One gram of arabitol contains 0.2 kcal, a lower caloric content when compared to 2.4 kcal/g of xylitol and 4.0 kcal/g given by sucrose [5]. Chemically, arabitol and xylitol are stereoisomers which give them similar properties that are presented in Table 1.

Table 1 – Physical and chemical properties of xylitol and arabitol. (Adapted from [5, 8]**)**

	Xylitol	**Arabitol**
Chemical structure		
Molecular weight	152.15 g/mol	152.15 g/mol
Appearance	White crystalline powder	Fine white crystalline powder
Specific rotation	Optically inactive	+10.5 °C to +13.5 °C (2%, Borax soln.2 %)
Melting range	92 - 96 °C	98 – 104 °C
Solubility at 25 °C	63 g/100g H_2O; sparingly soluble in ethanol and methanol	50 g/100g H_2O
Heat stability	>160 °C	>160 °C
Relative sweetness	Equal to sucrose	Not reported
Calorie content	2.4 kcal/g	0.2 kcal/g

Xylitol is extremely well tolerated by the human organism, but it can cause laxative effects after intakes higher than 60 g per day [4]. Moreover, it has an extremely low glycemic index (GI) of 7 (low GI $\leq$ 55) and two main absorption pathways (liver and intestinal flora) which are independent of insulin, representing a suitable alternative for diabetics [9]. It also possesses a high cooling power (the dissolution of xylitol is an endothermic reaction), causing a cooling sensation in mouth without interfering with food nutritional value [1]. The impact of xylitol on teeth is well studied, demonstrating anticariogenic properties that inhibits bacterial growth, due to its non-fermentable origin, preventing the deposition of oral plaque [4]. Thus, sugar-free chewing gum is the main application of xylitol. Additionally, it is used in other types of food such

as candies, chocolates, caramels and beverages, in pharmaceutical preparations and in odontological products as toothpaste and mouthwash [5, 9]**.**

Xylitol and arabitol have identical physiological effects, so probably arabitol has high potential to be used for similar applications. Studies have shown that arabitol is able to prevent the accumulation of fat and reduce the adipose tissue in the body [10]. It was also demonstrated that rumen bacteria of sheep are only able to metabolize 0.24 g/L arabitol from 1 g/L of feed [10]. Some of these bacteria cause dental caries, so the results also hypothesized the potential application of arabitol as anticariogenic agent.

Owing the rising popularity of sugar substitutes, in 2018, their global market reached a value of US $ 15 billion and it is further expected to reach US $ 19 billion by 2024 (www.prnewswire.com/news-releases/global-sugar-substitutes-d-tagatose-sorbitol-xylitol-stevia-maltitol-acesulfame-and-neotame-markets-2011-2018--2019-2024-300851649.html). In 2015, the global xylitol market size was valued at US $ 737 million, of which 66% belongs to chewing gum. According to the Research and Market Report, the global xylitol market has generated more than US $ 823.6 million in 2017 [11]. In 2021, it is expected an annual demand of 250 million metric tons, with an overall market value of US $ 1 billion. Following the growing trend, it is estimated that the value reaches US $ 1.37 billion by 2025 [12].

1.1.2. Commercial production

In 1957, it was reported for the first time the chemical production of xylitol, which was originally created to be used as a sweetener for diabetics [13]. Later, back in the 70s, a company known as Finnish Sugar Co. Ltd, in Finland, started xylitol industrial-scale production.

Globally, the leading xylitol supplier is the company DuPont Danisco, located in Finland, China and USA (www.dupont.com), followed by Sweet Natural Trading Co. Limited, Cargill, CSPC Shengxue Glucose, Ingredion and Mitsubishi Shoji Food tech [8]. Asia is responsible for half of the world's xylitol production [11].

Currently, xylitol demand in the market is fulfilled by chemical synthesis. The chemical process is characterized by hydrogenation of pure xylose, obtained from birch trees, other hardwood and corncobs, or any source rich in xylose [8]. The currently large-scale production can be mainly divided into four steps. First, the xylan-rich raw materials are subjected to an acid hydrolysis to obtain a mixed of pentose sugars. Then, in step 2, a combination of ion exchange chromatography and activated charcoal is used to hydrolysate purification and color removal, in order to obtain a solution of pure xylose, which undergoes a catalytic hydrogenation process in the presence of a metal catalyst (Raney Nickel) under severe temperatures (140 – 200 ºC) and high-pressure (50 – 60 bar) conditions until xylitol production is achieved (3-5 h). Finally,

chromatography and crystallization processes are required for xylitol purification [4]. In this chemical process, a 98% yield of xylitol recovery from substrate can be reached from pure xylose. From the biomass, it is possible to convert 50-60% of the xylans into xylitol or 8-15% of the initial raw material [8].

Similar to the industrial production of most sugar alcohols, arabitol can be produced by the catalytic reduction of sugars such as arabinose and lyxose, or araninonic and lyxonic acids [6]. These reactions require expensive ruthenium catalysts and high temperatures [10]. Besides that, in order to remove the by-products of the reaction, extensive purification steps must be performed.

Despite the high yields and high conversion efficiency achieved through these chemical processes, they suffer from major drawbacks and the overall process remains very costly, energy intensive and non-environmentally friendly. The high cost of chemical production process boosts the high price of xylitol, reaching values of $ 4.50-5.50 per kg for bulk purchase by companies and $ 20.00 per kg in supermarkets [4].

Over the last decade, as a potential alternative to overcome the drawbacks of the traditional processes, intensive research has been focused on the biotechnological production of xylitol and arabitol.

1.1.3. Biotechnological production

Biotechnological processes, i.e. xylose fermentation to xylitol by microorganisms has attracted a lot of attention owing to the increased awareness about ecological practices. In comparison to the chemical process, it involves much milder conditions, less discharges of chemicals into the environment and extensive xylose purification is not required [7]. Figure 1 shows the main differences between chemical and biotechnological routes for xylitol production.

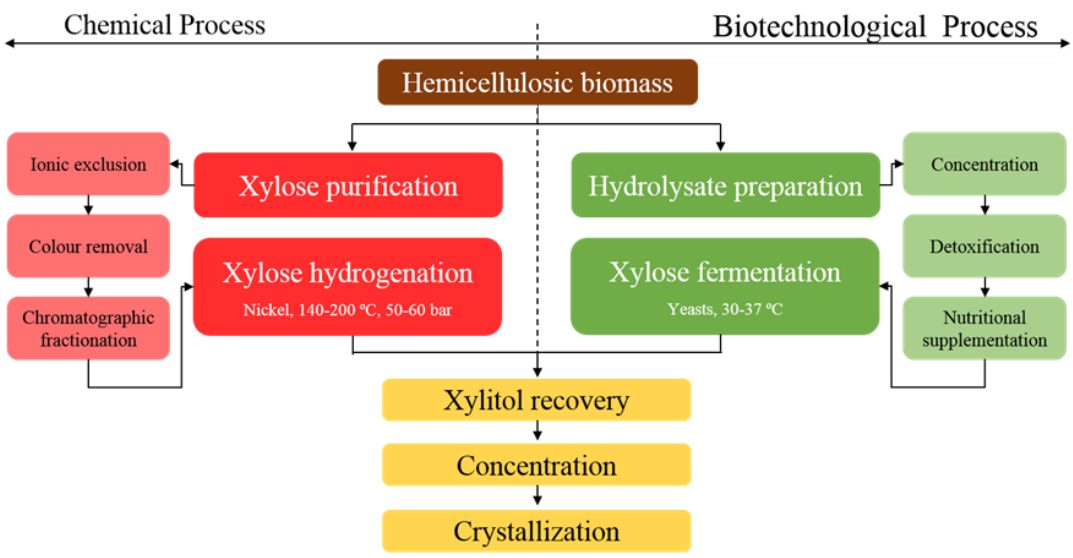


Figure 1 – Comparison of the main steps involved in chemical and biotechnological production of xylitol. (Adapted from [7])

Generally, in biotechnological production, the microorganisms, including bacteria, fungi, yeast and/or recombinant strains work as biocatalysts. Besides that, enzymatic production of xylitol is also a biotechnological alternative, mainly, due to the high yield (around 100 %) achieved in laboratory. However, for large scale production, the expensive cofactor requirement for enzymatic activity is a big drawback and it might not be economically feasible [7, 9]. On the other hand, in microbial production, the microorganisms are able to generate the cofactors required by the enzymes involved in the bioconversion of xylose into xylitol, which is a great advantage.

The first microorganism known as able to reduce xylose to xylitol was the fungus *Penicillium chrysogenum*, reported by Chiang et al. (1958) [15]. Later, in 1966, Onishi et al. [16] described the assimilation of xylose and arabinose by the yeast *Candida polymorpha*, and their conversion into xylitol and arabitol, respectively. The same authors, in 1969, proved that it was also possible to produce xylitol from glucose by certain yeasts, such as, *Candida guilliennondii*, *C. guilliermondii var. soya and D. hanseni* [17]. Some studies based on glucose conversion into xylitol through the pathway glucose-arabitol-xylulose-xylitol have been reported in literature, but the focus is still the microbial production of xylitol through xylose [7].

Few bacteria have been reported to produce xylitol, *Enterobacter liquefaciens* and *Corynebacterium sp*. are two examples. Studies regarding xylitol production with filamentous fungi are also difficult to find [9]. Some examples of microbial production among the different genera are presented in Table 2.

Table 2 – Microbial production of xylitol among the different genera and using commercial xylose as carbon source. (Adapted from [9])

Microorganism	Xylose initial (g/L)	Xylitol (g/L)
Bacteria		
Enterobacter liquefaciens 553	100.00	33.3
Corynebacterium sp. B-4247	100.00	40.00
Fungus		
Penicillium crustosom CCT 4034	11.50	0.52
Aspergillus niger PY11	30.00	1.39
Yeast		
Candida tropicalis IF0 0618	172.00	-
Candida tropicalis KCTC 7221	150.00	110.00
Candida parapsilosis NCIM-3323	60.00	28.14
Pichia sp.	40.00	25.00
Debaromyces hansenii UFV-170	10.00	5.84
Hansunela anomala NCAIM Y.01499	50.00	21.70
Kluyveromyces marxianus IMB2	50.00	7.78

Among microorganisms, yeasts are the best xylitol and arabitol producers due to their higher pentose assimilation rates [18]. The mechanism for converting xylose into xylitol by yeasts was first reported in 1960, as an alternative path for pentose metabolism [4]. The conversion occurs in two steps and involves two enzymes: xylose reductase (XR) and xylitol dehydrogenase (XDH). First, xylose is reduced to xylitol by a NADPH-depending XR, then xylitol is either secreted from the cell or oxidized to xylulose by XDH, requiring NAD+ [4]. Similarly, arabinose is transported across the cell membrane. The metabolism of arabinose in yeasts starts with its conversion into arabitol by arabinose reductase (AR), followed by arabitol oxidation to xylulose by arabitol dehydrogenase (LAD). The xylulose formed is converted into xylitol by xylulose reductase (LXR) [7]. Thus, good yields of polyols production depend on controlled high intracellular NADPH/NAD^+ ratios. The metabolism of both sugars assimilation and polyols production are schematically represented in Figure 2.

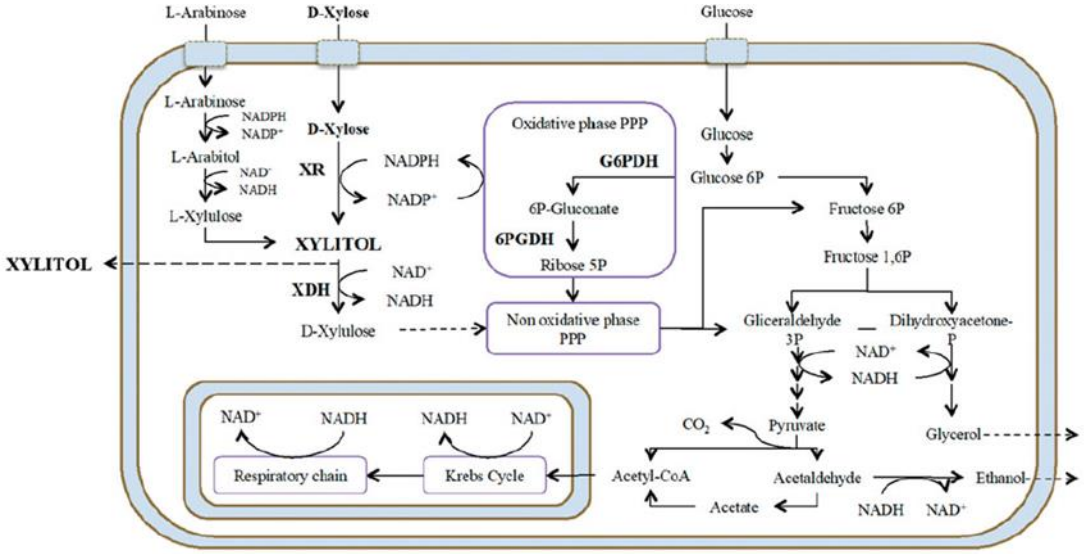


Figure 2 – Schematic representation of the metabolism involved on the assimilation of xylose and arabinose and the respective polyols production (xylitol and arabitol). (Adapted from [7])

Yeasts belonging to Genus *Candida* are reported as the best xylitol producers, since they can reach conversion efficiencies of 65–85% [9].

Regarding arabitol production, there are different carbon sources that some yeasts can convert into arabitol, namely, arabinose, glucose and glycerol [10]. Besides that, arabitol accumulation is typically observed in osmophilic yeast as a protection mechanism in response to their exposure to several environmental stresses, such as osmotic stress [6]. Arabitol production from glucose was first patented by Kiyomoto et al. (1971), using the yeast *Pichia ohmeri* (US Patent US3607652). Other yeasts such as *Zygosaccharomyces rouxii, Debaryomyces hansenii, Candida albicans, Candida pelliculosa, Candida famata* and *Pichia miso* can also convert glucose into arabitol. However, the yields are usually small, and the processes are time-consuming [6].

The best arabitol producers are *Candida tropicalis* strains, such as, *C. shehatae, C. tropicalis, P. stipitis, P. tannophilus and Torulopsis sonorensis* [10].

1.2. Lignocellulosic waste

The cost of synthetic sugars for the biotechnological production of polyols is one of the major drawbacks for an economically sustainable process. To overcome this disadvantage, lignocellulosic wastes stand out as promising low-cost feedstocks.

Lignocellulosic biomass is unarguably the most abundant and renewable organic carbon source on earth [19]. Less than 5% of the total lignocellulose produced annually (170 billion metric tons), are utilized, mainly for food and energy generation applications [20]. Moreover, millions of tons of lignocellulosic waste are generated due to the activity of food, agricultural and forestry industries. Significant efforts have been made to convert this waste into valuable chemicals (e.g. succinic, acetic, lactic, levulinic and butyric acids, propanol, furfural, xylitol), biofuels and polysaccharides (e.g. chitosan, xanthan, bacterial cellulose and microbial polyhydroxyalkanoates) [20].

1.2.1. Composition and structure

Lignocellulose comprises cellulose, hemicellulose, and lignin components (Figure 3) in its supramolecular structure, representing about 80 to 95 % of dry lignocellulose [19]. Depending on the biomass type, cultivation and climate conditions, there are differences in their composition. Usually, the cellulose fraction varies between 20 and 55%, hemicellulose from 15 to 40 % and lignin 10 to 35 %, proteins and extractives represent a minor fraction (Table 3).

Table 3 – Chemical composition of several representative lignocellulosic biomass. (Adapted from [19, 21, 22])

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood	40-55	20-40	18-25
Softwood	35-50	25-35	25-35
Sugarcane bagasse	32-45	27-35	19-24
Sweet sorghum bagasse	34-45	18-27	14-21
Wheat straw	33-40	20-25	15-20
Rice straw	36-36	23-28	12-19
Corn stover	38-40	24-26	7-19
Grasses	25-40	25-40	10-30
Nut shells	25-30	25-30	20-35

Cellulose is a linear homopolymer of glucose units linked by β-1,4-glycosidic bonds, exhibiting crystalline and amorphous regions. Microfibrils of cellulose, arranged parallel to each other, confers strength and resistance to the lignocellulose structure. Hemicellulose is the second most abundant renewable polymer in nature. It is an heteropolymer which may contain pentoses (xylose and arabinose), hexoses (mannose, glucose, and galactose) and/or uronic acids monomers. Based on the main sugar in the backbone, it is roughly classified as xylan, mannan or glucan. Xylans, including arabinoxylans, are the most relevant and large amounts are generated as by-products of agroindustries. Lignocellulose consistency and flexibility are given by hemicellulose that binds the cellulose microfibrils units together, through hydrogen linkages and interacts covalently with lignin. Lastly, lignin is a complex, dense and amorphous polyphenolic biopolymer composed by aromatic units [19, 23].

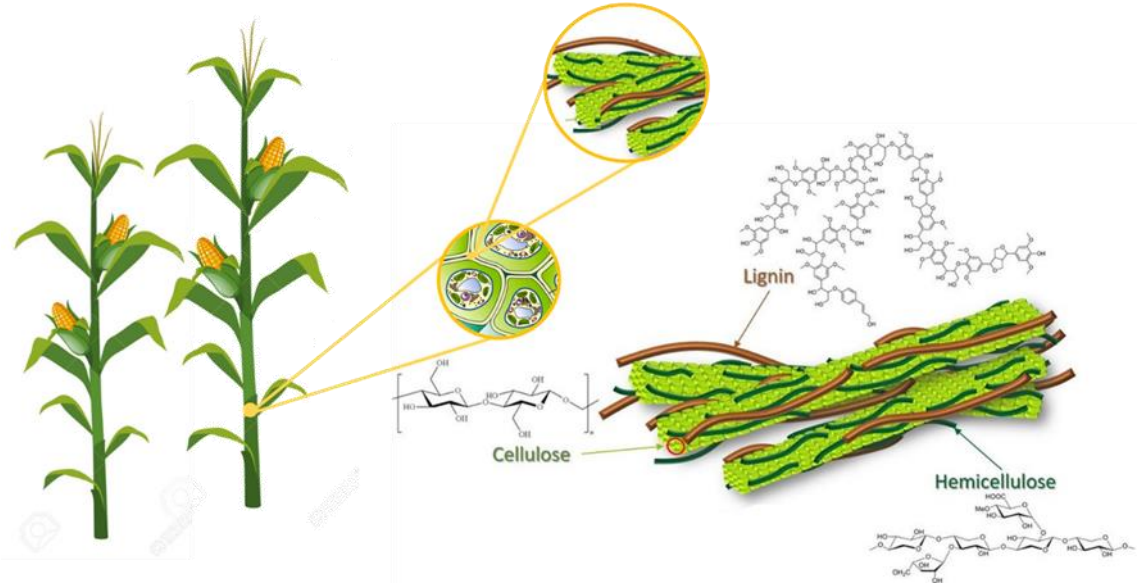


Figure 3 – Structure of lignocellulosic biomass.

1.2.2. Lignocellulose fractionation and saccharification

The strong physical and chemical structure of lignocellulose is a major barrier for microbial digestibility. Consequently, pretreatment of raw materials is an inevitable step to modify their properties and allow the release of fermentable sugars**.** These sugars can then be used to produce value-added compounds by the appropriate microorganisms. Several pretreatment technologies have been developed and can be divided into four categories: physical, chemical, physicochemical and biological [21].

Techniques including milling, extrusion, microwave and ultrasonication belong to physical pretreatment. Physical methods involve size reduction by breaking down the structural integrity of lignocellulose, and, consequently, increase the surface area and reduce the cellulose crystallinity. In these processes, the major disadvantage is related with the high energy

consumption associated. On the other hand, chemical techniques exploit the use of chemicals such as acids, alkali, salts, oxidants, organic solvents and deep eutectic solvents. These chemicals can affect the degree of hemicellulose polymerization, the cellulose crystallinity, the covalent linkages and the lignin breakage. Physicochemical techniques, which include steam explosion, ammonia fiber explosion, CO_2 explosion and liquid hot water, are typically a combination of both mechanical forces and chemical effects. In the biological methods, specific bacteria and predominantly fungi, are used to treat lignocellulose biomass, due to their capacity to produce enzymes able to break the lignocellulose structure [19, 21].

An effective pretreatment technology, suitable for different types of lignocellulose, energy-effectivity, cost-effectivity and sustainable is still a challenge.

Considering sugar alcohols production, hemicellulose is the most important fraction since after undergoing a pretreatment it can release xylose and arabinose which are the main carbon sources for this production. To achieve high xylose and arabinose recovery from the biomass, acid hydrolysis, autohydrolysis and steam explosion stand out as suitable pretreatments since lignin and cellulose remained mostly in the solid fraction while hemicellulose is solubilized in the hydrolysate liquor (Figure 4) [24].

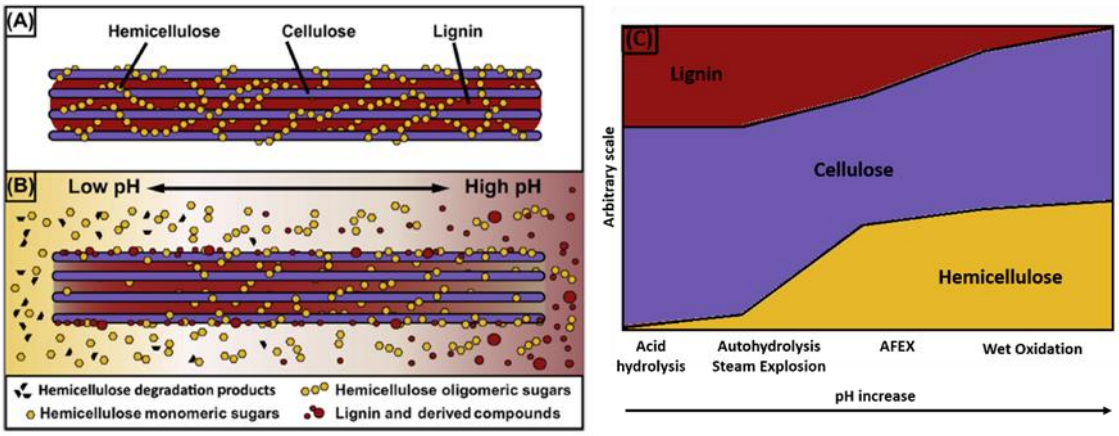


Figure 4 – (A and B) Effect of pH on hemicellulose and lignin solubilization. **(A)** Untreated cell wall and **(B)** Cell wall during pretreatment. (Adapted from [23]). **(C)** Typical solid fraction composition that remains after different biomass pretreatments. (Adapted from [24])

Acid hydrolysis is the most studied pretreatment for xylitol production allowing to achieve reasonably high sugar yields from hemicellulose. [7]. Several acids such as H_2SO_4, HCl, HNO_3, and H_3PO_4 have been employed in this technique. In addition to the high hydrolysis rate, several other advantages are associated with this method, such as simple, low-cost, easy-operation mode and it is not time-consuming [19]. However, the toxicity and corrosive nature of the catalysts is a drawback for a sustainable technology.

Autohydrolysis has a mechanism similar to dilute acid hydrolysis, however no catalyst is required and water is the only compound added. In this case, hemicellulose is mainly converted, with high yield, into soluble oligosaccharides. Due to the absence of acid, the disadvantages mentioned above are overcome and the cost and the environmental impact of the process decrease [24].

In steam explosion process, lignocellulose is heated under high-pressure steam (20-50 bar, 210-290 °C) for a short period of time followed by sudden decompression. Thus, desegregation of lignocellulosic matrix occurs and hemicellulose is partially hydrolyzed into water-soluble oligomers and simple sugars (yields around 45-65%). Additionally, acid catalyst is required to increase the process efficiency [7, 24].

Depending on the method and conditions performed, the formation of undesirable inhibitory by-products is inevitable. These compounds can be directly released from the raw material or in consequence of the sugar degradation. The main inhibitors possible to be generated in lignocellulosic hydrolysate are: furan derivatives (furfural and 5-hydroxymethylfurfural (5-HMF)), phenolic compounds (p-hydroxybenzoic, vanillic, syringic, p-coumaric and ferulic acids), weak acids (acetic, levulinic and formic acid) and traces of heavy metals (chromium, copper, iron and nickel) [4]. Furfural and 5-HMF are released from pentose and hexose degradation, respectively. These compounds can act as potential microorganism inhibitors, disturbing enzymatic activity and inhibiting protein synthesis [25]. On the other hand, phenolic compounds can damage the integrity of biological membranes and acid acetic, released from the acetyl-hemicellulose may affect the gradient of protons in nutrient transport and energy production [25].

It is important to highlight that the effect of these toxic compounds depends on their concentration, the synergy between them or not, and the capacity of the microorganism to adapt and transform the compounds into less toxic substances [26] .

Inhibitors, even present in small amounts, can be a limitation for carbon uptake and microbial growth in the fermentation process. Therefore, removal of these compounds may be crucial. Detoxification methods can be divided into four types: physical (mediated by evaporation), chemical (addition of alkaline agents), adsorption (involving ion-exchange resins and activated charcoal) and biological (microbial and enzymatic) [26]. Among others, activated charcoal and ion-exchange resins have been reported as the most successful strategies to eliminate inhibitors [27].

1.2.3. Microbial production using waste as feedstock

Several hemicellulosic hydrolysates are the major raw materials that have been studied for the biotechnological production of xylitol and arabitol. Besides being efficient carbon sources,

these hydrolysates can also be used as source of nitrogen, vitamins and minerals, conferring it other advantage [7].

Several lignocellulosic waste including corncobs, sugarcane bagasse, hardwoods, sawdust, brewers' spent grain (BSG) and rice straw have been widely studied for microbial production of xylitol [7]. In contrast, the use of waste for arabitol production by yeasts is still poorly investigated.

Different approaches, including free cells or immobilized cells in batch, fed-batch or continuous processes, have been studied for xylitol production [29]. Most of the studies were carried out with *Candida* sp.

In addition to the several studies about xylitol-producing native yeasts, metabolic engineering strategies have also been studied to enhance the production of polyols. Some strategies already reported include increased expression of the XR enzyme, reduction of XDH activity, increased availability of cofactor NADPH and heterologous expression of xylose transporters [7, 28]. One practical example is the possibility to use *Saccharomyces cerevisiae*, that is not able to use xylose as a carbon source (low-level expression of xylose assimilation genes), but is more tolerant to toxic compounds than *Candida sp*. Implementation of an efficient enzyme system, developing a recombinant strain of *S. cerevisiae* with cloned XYL1 genes from *Candida* sp., led to bioconversion efficiency [28]. However, although the ability of genetically modified organism (GMO) to achieve higher polyols productivities, these processes have a major drawback that prevents the commercialization of GMO-related xylitol, due to legislation concerning safety issues implemented in several countries.

Several variables have influence on microbial growth and polyols production. To optimize the processes and achieve high quality polyols with high efficiency, the control of variables is fundamental and must be designed specifically for each microorganism. Optimization of cultivation conditions, such as temperature, pH, aeration rate, inoculum concentration and nutritional composition (carbon, nitrogen, and vitamin sources) have been vastly investigated and discussed below in Chapter 3 [18].

The final step of polyols production is their recovery from the cultivation broth and purification. Depending on the type of lignocellulosic waste used and processing conditions, the final fermentation broth is a mixture of sugars, polyols and other compounds, which justifies the difficulty of the recovery and purification steps. Scientific papers exploring this topic are scarce. However, Wei et al. (2010) developed an economical method, reaching xylitol with 95% purity, produced from corncobs. It involved colour removal with activated charcoal, salt removal with two ion exchange resins, sugar separation with other resin, vacuum-concentration to super saturate xylitol and, finally, cooling (-20 °C, 48 h) to obtain crystalline xylitol [29].

Consolidation and scale-up of biotechnological routes for polyols production are still required. Biotechnological disadvantages need to be overcome towards to the develop of a pretreatment with high sugar recovery yields, a cost-efficient detoxification procedure to improve microbial bioconversion and an efficient downstream processing.

1.3. Komagataella pastoris

The yeast *Komagataella pastoris*, formerly named *Pichia pastoris*, is a single-celled and monoploid microorganism. *K. pastoris* is a methylotrophic yeast, able to obtain all the carbon and energy sources needed for growth from one-carbon compounds (methanol) [30].

It has become a preferred workhouse for biotechnology and pharmaceutical industry, especially for production of recombinant heterologous proteins [31]. The success is explained by its capacity to achieve fast growth rates (high cell density in shorter cultivation times) and high yields of protein secretion, which is a benefit for downstream protein recovery [31]. Additionally, it was recognized as safe, given by the GRAS status for a protein used in animal feed and the FDA approval of Kalbitor®, a recombinant biopharmaceutical product [32]. Moreover, a huge advantage of *K. pastoris* is its ability to use efficiently a wide range of substrates such as glycerol, methanol and glucose, including inexpensive raw materials, as waste glycerol from biodiesel process. Using waste as carbon source, the overall production becomes economically viable and sustainable [31]. *K. pastoris*, as any other yeast and fungi, has the chitin–glucan complex (CGC) as the main component of cell wall, which gives stability and rigidity to the cells [33]. CGC is as copolymer composed of chitin (N-acetyl-d-glucosamine polymer) and β-glucan (glucose homopolymer), with water-insoluble characteristics [31].

CGC is a valuable cell-wall polysaccharide for pharmaceutical, biomedical, cosmetic and food applications, since it combines the properties of its two bioactive polymers. Therefore, its antioxidant, antimicrobial and anti-inflammatory properties as well as heavy metal removal ability have been demonstrated. In addition, yeast/fungi CGC is biocompatible and biodegradable, representing an alternative chitin source to the one from crustaceans, algae and marine invertebrates, avoiding risk of allergy [31, 34].

Production of CGC using glycerol as carbon source is well-stablished. For example, relatively high amounts of CGC were extracted from the biomass (11-21 wt. % of biomass), in a patented bioprocess using *K. pastoris* and biodiesel waste glycerol as the sole carbon source (International Patent WO2010013174) [33].

In addition, production of CGC using alternative carbon sources are scarce in literature. Araújo et al. (2017) tested, for the first time, CGC production using different ratios of glucose

and xylose, which had influence on CGC composition, since increased xylose content led to a chitin-enriched CGC [33].

1.3.1. Value-added compounds: Xylitol and arabitol

Potential production of primary and secondary metabolites using *K. pastoris* as a cell factory has aroused a lot of interest. It has been increasingly employed as a platform to produce some fine chemicals and active pharmaceutical compounds [32]. Metabolic engineering strategies have been described for production of different compounds, such as S-adenosyl-L-methionine (with potential for treatment of Alzheimer's, osteoarthritis and liver disease) by *K. pastoris* GS115 (his4), isobutanol and isobutyl acetate by *K. pastoris* KM71, dammarenediol-II (pharmaceutically relevant triterpenoide) and 6-methylsalicylic acid (bioactive compound) by *K. pastoris* GS115, and lactic acid by the strain X-33 [32].

Additionally, natural arabitol accumulation of *K. pastoris* X-33 was described by Baumann et al. (2010) as protective response to osmotic stress and redox imbalance [35]. Then, Cheng et al. (2014) exploited the same strain of *K. pastoris* for xylitol production, co-expressing cloned arabitol and xylitol dehydrogenases. The recombinant strain was able to produce xylitol from glucose, resulting in the direct synthesis of 0.29 g/(L.h) and a yield of 0.078 $g_{xylitol}/g_{glucose}$ [36].

However, xylitol production by *K. pastoris* using a wild type strain DSM 70877 was only recently reported by Araújo et al. (2017) [33]. The cultivation of the yeast in bioreactor experiments, using glucose/xylose mixtures reached a production of 7.64 g/L, with a yield of 0.52 $g_{xylitol}/g_{xylose}$ [33]. Cultivation conditions were posteriorly optimized in shake flask assays and the highest production achieved was 12.00 g/L of xylitol, with a yield of 0.35 $g_{xylitol}/g_{xylose}$ [37]. In that study, *K. pastoris* DSM 70877 proved also to be able to produce 3.15 g/L of arabitol using arabinose as substrate, a feature not reported before [37].

In fact, it becomes evident that the capacity of *K. pastoris* DSM 70877 to use glucose/xylose/arabinose mixtures to produce xylitol and arabitol, opens the possibility to use lignocellulosic wastes as feedstocks.

2. Motivation

The negative health effects of sugar, alongside with the increased prevalence of some diseases (obesity and diabetes) are increasing consumer awareness regarding calorie intake. The food industry is following this tendency, and sugars added to food and beverages are being constantly replaced by low-caloric sugar substitutes. Consequently, the sweetener market is growing and the increased demand for xylitol stands out due to its important applications in food, odontological and pharmaceutical industries. However, the high commercial value of xylitol is a limitation for its expansion in terms of applications [8].

Around the world, significant steps are being taken towards a more sustainable economy. In addition, consumers habits are also changing and becoming more sensible to the environmental issues, preferring the consumption of foods produced with less impact on the environment. Reduction of pollution generated from industrial activities has been under a huge social and political pressure. Microbial production of polyols from lignocellulosic waste is definitely a solution for sustainability, because it involves clean technologies, recycling and reuse the huge amounts of biomass generated. It is noteworthy that the biggest commercial xylitol producers are becoming aware of the benefits given by the biotechnological processes and they are focused on its implementation. DuPont Danisco, the leading xylitol supplier, is now reducing its environmental footprint by using xylose from the waste side stream of paper industries [8]. Another example is the company Thomson Biotech, which is at the vanguard of searching for biotechnological alternatives. This company has patented a process using the yeast *C. tropicalis* for bioconversion of xylose to xylitol (US patent 20120021467) [38].

Different microorganisms and several raw materials have been evaluated for biotechnological production of xylitol and arabitol. However, the pathogenic nature (*Candida* sp.) or genetic modifications of the best producer yeasts are a barrier for its implementation at an industrial scale, due to safety issues and legislation that prohibits sales of GMO-related products in several countries.

Therefore, the use of a wild-type yeast strain, namely, *K. pastoris* DSM 70877 for the bioconversion of hemicellulosic hydrolysates into xylitol and arabitol becomes a promising alternative. It was previously proven that *K. pastoris* can use pure glucose/xylose/arabinose mixtures to produce polyols, xylitol and arabitol. Besides that, the GRAS status was granted to *K. pastoris*, a fact that favors the use of xylitol and arabitol produced by this yeast in edible.

The main objective of this thesis was to study the production of xylitol and/or arabitol by the wild-type *K. pastoris* DSM 70877 using six selected lignocellulosic waste (banana peels, BSG, corncobs, grape pomace, grape stalks and sawdust). The work is divided in two parts: (1) characterization of lignocellulosic waste and their conversion into fermentable sugars and (2)

utilization of the lignocellulosic hydrolysates previously obtained as culture medium for xylitol and arabitol production by the yeast *K. pastoris*. Different strategies were evaluated in order to define the best feedstocks for cell growth and sugar alcohols production. The concentration of the raw hydrolysates, detoxification treatments (neutralization with $Ca(OH)_2$ and activated charcoal), the mixture of different lignocellulosic hydrolysates and aeration conditions were studied as well. CGC production in bioreactor cultivations was also studied to understand if *K. pastoris* cultivation in lignocellulosic hydrolysates impacted CGC content in the cell-wall and its composition.

Chapter 2

Characterization and treatment of lignocellulosic wastes

2.1. Introduction

As mentioned in Chapter 1, lignocellulosic wastes have been reported as the most promising alternative sources to produce xylitol and arabitol, through either chemical or biotechnological routes.

In this work, six lignocellulosic waste materials, including banana peels, BSG, corncobs, grape pomace, grape stalks and sawdust, were studied as potentially valuable resources for xylitol and arabitol production.

These wastes are regenerated in large amounts by sectors of activity with great impact on the Portuguese economy. Portugal has a strong wine tradition, with several wine regions and great wine varieties spread all over the country. Woodworking industries (paper industry, engineered wood industry, furniture industry and cork industry) play also an important economic role, as well as, the large banana production in Madeira island.

Banana is a highly nutritional fruit and it is considered the fourth most important food in the world nowadays [39]. It is produced in many countries, but the largest crops are in tropical and sub-tropical regions (Asia, Latin America and Africa) [40]. Banana reached a record production of 114 million tons in 2017, in a total of 5.6 million hectares of land (www.fao.org/economic/est/est-commodities/bananas/bananafacts/en/). As 40% of fresh bananas' total weight is peel, several tons of banana peel waste are produced every day, as a result of its consumption as a fruit, supermarkets and restaurants waste, and fruit processing industries waste (production of banana chips and other food products) [41]. Typically, banana peels are disposed in landfills. However, they contain carbon-rich organic compounds, such as cellulose, hemicellulose, pectin and proteins, representing a promising raw material [41]. It has been reported their potential application as food thickeners and gelling agent (using the pectin), high nutritional quality medium for microbial growth (especially for fungi), substrate for ethanol production, low-cost banana briquettes (as a substitute for solid fossil fuels) and heavy metals sorption [39, 40].

Corn is one of the largest cereal crops worldwide. In 2017, there were 192 million hectares of corn plantations, generating around 1 billion tons of corn. The leading producers are United States and China [42]. Corncobs are the by-products that remain after removing the grains, representing 30% of biomass waste [42]. Corncobs are destroyed by fire or used as an energy source, but air pollution is a major consequence. Currently, due to their high hemicellulosic content (around 35%), researchers have been exploring their potential as a sustainable alternative to commercial production of several valued-added compounds, such as lactic acid, succinic acid, ethanol and industrial enzymes [43].

Around the world, in several countries, winemaking represents another agricultural activity with a significant impact on their economies. Italy, France and Spain produce 51% of the total world wine. Wine consumption has increased over the years. In 2018, 292 million hectoliters of wine were produced worldwide, and the world vineyards filled an area of 7.4 million hectares. Portugal is on the top 12 of the biggest wine producers, with 6.1 million hectoliters produced in 2018 (www.forbes.com/sites/karlsson/2019/04/14/record-global-wine-harvest-in-2018-stable-consumption/). Wine industry wastes are mainly grape pomace and stalks, which consist in 25-35 kg per 100 L of produced wine [44]. Grape stalks are the skeleton of the grape bunch. It is a lignocellulosic by-product with an important concentration of cellulose (30-35%), hemicellulose (10-15% of xylans), lignin, tannins and several polyphenolic compounds [44]. Normally, during the red winemaking process, the grape stalks are first removed by means of a mechanical destemmer, with an average production of 5 tons per hectare (12% of the total mass of waste) [45]. On the other hand, red grape pomace is generated after fermentation and pressing. Thus, grape pomace is typically composed of seeds and skin, representing the main solid waste (up to 45%) [46]. Grape pomace is made of lignocellulosic fiber (approximately 40 %), reduced sugars, pigments, polyphenols, tartrate acid and some fatty acids [47]. For a long time, wine industry wastes have been used in an inefficiently and uneconomically way. Grape stalks are currently employed as fertilizers, incinerated or discarded in landfills [46]. Only a small fraction of grape pomace is reused to produce distilled spirits and liquors, fertilizers or animal feed [47]. Due to the environmental concerns and the composition of these wastes, several studies have been focused on their valorization. Grape stalks have been studied for their potential as biosorbents, dietary fibers, antioxidant supplements, activated charcoal and cellulosic pulp. Besides that, several studies have investigated the extraction of phenolics, pigments and antioxidants from grape pomace and, the conversion of grape pomace into high-value products such as lactic acid, bioethanol, biogas, charcoal, biosurfactants, among others [47].

BSG is a hemicellulosic rich waste, representing the most abundant by-product generated from the beer-brewing process, accounting approximately 85% of the total by-products generated [48]. Briefly, during the process of beer manufacturing, malted barley grains are dried, crushed and treated in hot water (70-74 °C) to obtain fermentable sugars. Then, the mixture is filtered and the sugar-rich liquor (wart) is fermented to produce beer and the remaining retentate (BSG) is rejected [49]. Beer production is increasing. In 2017, 191 billion liters were produced globally, for which, Portugal contributed with 7.5 billion liters. China, the United States and Brazil are the biggest world beer producers [50]. Considering that the manufacture of 100 L of beer leads to 20 Kg of wet BSG, it becomes evident that massive amounts of BSG are produced throughout the world [48]. BSG composition can vary depending on the type of grain and processing conditions employed. The main BSG constituents include fiber (30–70% w/w) and protein (19–30% w/w),

but it also contains several vitamins and minerals [49]. The main practices for BSG elimination have been animal feed and its disposal in landfills, but they are not economical neither ecological. Therefore, upgrading solutions have been investigated, such as, energy, bioethanol and activated carbon production, and BSG potential as a brick component, culture media for mushroom growth and fertilizer for agriculture [49].

Finally, sawdust is a waste composed of fine particles of wood, available in sawmills, pulp plants, paper industries and all woodworking industries. For example, sawmills generate enormously quantities of sawdust throughout the year, since only about a half of all wood consumed is converted into products [51]. Composition of sawdust is similar to that of dry wood, mostly cellulose, lignin and hemicellulose (85-90%). The disposal of sawdust represents a serious economic problem for industries and a threat to the environment. Sawdust is commonly used in thermal processes as fuel (high content of organic matter), in energy industries. Little developments have been done to reuse sawdust, but it has been suggested as a valuable raw material in manufacturing industries for wood boards, light weight construction materials, insulator materials (very low thermal conductivity), briquettes and pellets [52].

Due to the intrinsic recalcitrant properties of lignocellulose, a pretreatment step is mandatory to break down the structure and release monomers, becoming accessible to the microorganisms [53].

All pretreatment methods for lignocellulosic materials have their pros and cons. Diluted acid hydrolysis is a well-established and low-cost process that achieves reasonably high sugar yields from hemicellulose depolymerization (20-90 %), depending on the process conditions [54]. The other pretreatments require an additional enzymatic hydrolysis, because they release non fermentable sugars. As dilute acid hydrolyzes biomass into fermentable sugars, the enzymatic step is not necessary, being a key advantage [24].

Dilute acid hydrolysis, using sulfuric acid as catalyst, has been most favored for industrial pretreatment of a variety of biomass types [23]. H_2SO_4 is inexpensive, less volatile and does not corrode equipment at low concentrations [7]. The efficacy is influenced by sulfuric acid concentration, pressure, temperature and reaction time [27]. A problem of this pretreatment is the formation of toxic compounds, which can be a major factor limiting further bioconversion processes. Slow kinetics, with limited yield and productivity are usually associated to non-detoxified hemicellulosic hydrolysates.

Adsorption on activated charcoal and on ion-exchange resins are successful techniques for detoxification, but resins are expensive whereas activated charcoal is a low-cost method [27]. In addition, a positive impact on xylitol production using activated charcoal detoxification has been reported in different studies. For example, Dominguez et al. (1996) subjected sugarcane

bagasse to dilute sulphuric acid (3%, w/v) hydrolysis and after tested activated charcoal and ion-exchange resins as detoxification methods. Xylitol production was higher using activated charcoal (10.54 g/L) in comparison with use of resins (8.32 g/L) [55].

In this chapter, conversion of the selected lignocellulosic wastes (banana peels, BSG, corncobs, grape pomace, grape stalks and sawdust) into fermentable sugars by dilute sulphuric acid hydrolyses was evaluated. Besides that, in order to investigate the impact of the detoxification methods in the removal of toxic compounds, some of the hemicellulosic hydrolysates obtained were treated with activated charcoal.

2.2. Materials and methods

2.2.1. Raw materials

Corncobs and sawdust used in this study were collected from a local farm and a local sawmill, respectively, in Carregal do Sal (Portugal) and were kept at room temperature in a plastic bag. Grape pomace and grape stalks were kindly provided by a wine-making factory, Quinta do Ribeiro Santo, in Carregal do Sal (Portugal) during the red wine vintage 2018. Samples of both materials were milled using a domestic coffee grinder (Selecline, France) to achieve a particle size suitable for posterior hydrolysis treatment, and stored in sealed bags at -20°C, until further use. Brewers' spent grain (BSG) was supplied by a brewery factory named Sociedade Central de Cervejas, in Vialonga (Portugal). Contrary to the others raw materials, BSG was subjected to a heat treatment (70 °C for one week), to avoid fungi contamination, milled and stored in sealed bags at room temperature. Banana peels were provided by Sonae Mc, a market leader of food retail in Portugal, and preserved at -20°C until further use.

2.2.2. Characterization of raw materials

2.2.2.1. Moisture and inorganic content

To determine the raw materials moisture content, 1.0 g of each raw material was placed in a convection drying oven at 105 °C (Memmert), until constant weight. The dry weights were obtained gravimetrically, and the moisture percentages were calculated by equation 1:

$$\%\ Moisture = \left(\frac{weight_{sample\ as\ received} - weight_{dry\ sample}}{weight_{sample\ as\ received}}\right) \times 100 \qquad (1)$$

The inorganic content on a dry basis was evaluated from the dried samples, obtained from moisture analysis, by subjecting them to pyrolysis at 575 °C (Nabertherm, Germany), until constant weight (approximately for 24 h. The values were determined by equation 2:

$$\%\ Inorganic\ content\ on\ dry\ basis = \left(\frac{weight_{ash}}{weight_{dry\ sample}}\right) \times 100 \qquad (2)$$

The determinations were performed in duplicate.

2.2.2.2. Protein quantification

The protein content of the raw materials was determined by elemental analysis, through the quantification of the total nitrogen, using an CHNS analyzer Thermo Finnigan-CE Instruments Flash EA 1112 CHNS series. The method is based on a flash dynamic combustion of

samples (2.0 - 3.0 mg), within a very high temperature oxidation reactor (about 1800 °C) that allows the complete conversion of samples to elemental gases. This process is followed by a precise determination of these elemental gases through a gas chromatography column, connected to a highly sensitive thermal conductivity detector. The protein content was estimated by the following equation (3):

$$Protein = N \times 6.25 \quad (3)$$

where N represents the total nitrogen and 6.25 is a nitrogen-to-protein conversion factor [61].

All the samples were analyzed in duplicated.

2.2.2.3. Hemicellulose, cellulose and lignin quantification

The hemicellulose, cellulose and lignin contents were determined based on the well-established laboratory analytical procedures developed by the National Renewable Energy Laboratory (NREL) [62]. All the raw materials were used as stored, except grape pomace and banana peels that were oven dried at 40 °C prior to this procedure. As quality assurance standards (QAS), cellulose microgranular (Sigma-Aldrich) and xylan from oat spelts (Fluka) were also subjected to this method. The procedure consisted in a two-step acid hydrolysis. First, 300.0 mg of the raw materials and QAS, in duplicate, were hydrolyzed using 3 mL of 72 wt% sulfuric acid (H_2SO_4, Honeywell Fluka, 97.0%), in a water bath at 30 °C for 1 h. During this time, the samples were stirred every 5 minutes to ensure a uniform contact between particles and the acid. The second step consisted in samples dilution to 4 wt% acid by adding 84 mL of deionized water, for additional hydrolysis by autoclaving at 121 °C and 1 bar for 1 h. To quantify the sugars losses due to possible degradation during the second acid hydrolysis, a set of sugar recovery standards (SRS), including D-(+)-glucose (Merck KGaA), D-(+)-xylose (Sigma-Aldrich, 99%), D-(+)-galactose (Fluka), L-(+)-arabinose (AppliChem *Panreac)* and D-(+)-mannose (Sigma-Aldrich, 99%) were also included in this procedure. The SRS were prepared using 104.0 mg and 204.0 mg of each sugar plus 10 mL of deionized water, adding 348 µL of 72 wt% sulfuric acid.

After hydrolysis, the solutions were vacuum filtered: the hydrolysis liquors obtained were used to quantify sugars, as well as acid soluble lignin (ASL), and the remaining solid residues were analyzed for acid insoluble lignin (AIL), being exposed to a convection drying oven, with temperature control of 105 °C for 4 h and then, to pyrolysis at 575 °C for 24 h. The AIL (%) content was determined by equation 4:

$$\%\, AIL = \frac{(weight_{sample\ after\ 105\,^{\circ}C} - weight_{sample\ after\ 575\,^{\circ}C})}{ODW_{sample}} \times 100 \quad (4)$$

where ODW_{sample} represents oven dry weight (g) of each sample calculated by using the moisture content (%) described above.

For determination of acid soluble lignin (% ASL), samples were diluted in deionized water and the absorbance was measured on a UV-Visible spectrophotometer (Evolution 201, Thermo Scientific) at 240 nm, using a high precision quartz cuvette (Hellma Analytics), into the range of 0.7-1.0. Each sample was analyzed in duplicate and the amount of ASL (%) was given by the following equation 5:

$$\% \, ASL = \frac{Abs_{UV} \times V_{filtrate} \times Dilution \; factor}{\varepsilon \times ODW_{sample} \times Pathlengh} \times 100 \qquad (5)$$

where Abs_{UV} is the average of UV-Vis absorbance for each sample (nm), $V_{filtrate}$ corresponds to the volume of hydrolysis liquor (L), ε is the absorptivity of biomass at 240 nm (L/g cm) and pathlength is the pathlengh of UV-Vis cell (cm). The ε were selected based on the biomass types, considering the values 12 for sawdust and grape stalks, 25 for grape pomace, banana peels and BSG and 30 for corncobs [62].

Thereby, the total amount of lignin (%) is given by the sum of AIL and ASL, represented by equation 6:

$$\% \, Lignin \; = \% \, AIL + \% \, ASL \qquad (6)$$

For the analysis of carbohydrates, the pH of the hydrolysis liquors obtained were set to values between 5 and 6 (pH1100L, VWR pHenomeral™) with calcium hydroxide ($Ca(OH)_2$). The precipitate that was formed in each sample was discarded and the supernatants were used for quantification of monosaccharides by High Performance Liquid Chromatography (HPLC), as described below (see section 2.2.4.2).

Using the SRS, the percentage of recovery sugars (% R_{sugar}) was calculated considering the concentration of each sugar after dilute acid hydrolysis (mg/mL) and the known concentration of sugar before hydrolysis (mg/mL), as expressed by equation 7:

$$\% \, R_{sugar} = \frac{conc.detected \; by \; HPLC}{known \; conc.of \; sugar \; before \; hydrolysis} \times 100 \qquad (7)$$

Then, % R_{sugar} was useful to correct the corresponding sugar concentration values, obtained by HPLC for each hydrolyzed sample, C_{HPLC}. The concentration of a sugar in the hydrolyzed sample after correction (mg/L), C_x, was calculated as indicated by equation 8:

$$C_x = \frac{C_{HPLC} \times dilution \; factor}{\% \, R_{sugar}/100} \qquad (8)$$

Finally, the glucan, xylan and arabinan contents (%) were calculated by the following equations 9, 10, 11 and 12:

$$Glucan\ content\ (\%) = 100 \times \frac{GH\ /\ 1.111}{sample\ weight/\ V_{filtrate}} \quad (9)$$

$$Xylan\ content\ (\%) = 100 \times \frac{XH\ /\ 1.136}{sample\ weight/\ V_{filtrate}} \quad (10)$$

$$Arabinan\ content\ (\%) = 100 \times \frac{AH\ /\ 1.136}{sample\ weight/\ V_{filtrate}} \quad (11)$$

$$Galactan\ content\ (\%) = 100 \times \frac{GaH\ /\ 1.111}{sample\ weight/\ V_{filtrate}} \quad (12)$$

in which GH, XH, AH and GaH a represent glucose, xylose, arabinose and galactose concentrations (g/L), respectively, and sample weight (g) is the amount of raw materials used. The values 1.111 and 1.136 are the mass conversion factors for glucose (galactose) to cellulose (galactan), and xylose (arabinose) to xylan (arabinan), respectively [62]. The content of hemicellulose was estimated by the sum of xylan, galactan and arabinan.

2.2.2.4. Particle size analysis

Determination of particle size distribution was only performed for four of the raw materials, namely, grape pomace, grape stalks, sawdust and BSG. Corncobs and banana peels were not subjected to this analysis because corncobs were too hard to ground, so, in this work, they were used in their natural form, while banana peels were smashed by a domestic blender becoming a puree. Thus, 100.0 g of each selected raw material, except corncobs and banana peels, were sieving through a mechanical sieve shaker (Tecnilab), using sieves (Laboratory Test Sieve, Endecotts LTD., England) with pores sizes between 2360 μm and 106 μm, for 15 min. The fraction collected by each sieve was weighed separately.

2.2.3. Preparation of the hemicellulosic hydrolysates

2.2.3.1. Dilute acid hydrolysis treatment

The selected raw materials were subjected to a dilute acid hydrolysis using H_2SO_4 in order to obtain liquors rich in simple sugars, mainly glucose, xylose and arabinose, which were subsequently used as culture medium. As described in section 2.2.1, prior to this treatment, grape pomace, grape stalks and BSG were milled to increase the efficiency of the process. Likewise, banana peels were mashed using a domestic blender (Selecline, France). The hydrolysis was carried out in an autoclave at 121°C and 1 bar. The concentration of H_2SO_4 and the hydrolysis time of each residue was selected based on different works reported in literature that best fitted

the purpose of this work. Table 4 summarizes the conditions applied to each raw material. After hydrolysis, the solid fractions were separated by centrifugation (9000 rpm, 4 °C, for 30 min) and the formed pellets were discarded. The pH of the supernatants were raised to 5 by the addition of $Ca(OH)_2$ powder for BSG, sawdust, corncobs and banana peels hydrolysates and by the addition of calcium carbonate ($CaCO_3$) powder for grape pomace and grape stalks. This pH adjustment was made since the destination of hydrolysates was their use as culture media for *K. pastoris* to produce polyols (Chapter 3). The pH of hydrolysates after hydrolysis was extremely low (bellow pH 1.0), that would not allow the yeast growth, since it is reported that *K. pastoris* is able to grow in pH range from 3.0 to 7.0 [63]. In this way, pH 5 was selected since it is a well establish parameter for cultivations with *K. pastoris* [31, 33, 63].The formed precipitates were removed by centrifugation (9000 rpm, 4 °C, for 20 min) and the supernatants were collected, representing the raw hydrolysates.

Table 4 – Hydrolysis conditions selected for the different lignocellulosic materials.

Raw material	Concentration of H_2SO_4 (% w/w)	Liquid to solid ratio (% w/w)	Time of hydrolysis (min)	Reference
Grape pomace	3.3	8	30	[64, 65]
Grape stalks	3	8	20	[66]
BSG	3	8	20	[67]
Sawdust	6	8	20	[68]
Corncobs	6	10	40	[69]
Banana peels	1	10	40	[70]

2.2.3.2. Concentration of the hydrolysates

In order to increase the concentration of sugars in the raw hydrolysates, the solutions were placed in a convection drying oven (Venticell, Eco line), with temperature control of 70 °C for a period of time necessary to remove the amount of water required (24 h - 48 h). It was not possible to use a rotatory evaporator, due to the high amount of foam formed as a result of the increased pressure.

2.2.3.3. Detoxification treatment

The detoxification of the concentrated hydrolysates was based on a simple and cheap procedure, adapted from Kamal et al. (2011) [71]. Briefly, powdered activated charcoal (Sigma-Aldrich) was mixed with the selected hydrolysates at a concentration of 2.5 % (w/v) and stirred at 800 rpm, for 1 h at room temperature. To remove the activated charcoal, the solutions were centrifugated at 9000 rpm, for 30 min at 4°C.

2.2.4. Characterization of the hemicellulosic hydrolysates

2.2.4.1. Identification and quantification of sugars

Both hydrolysates, raw and treated, were analyzed for determination and quantification of soluble sugars. The samples diluted with deionized water were filtered through a 0.2 µm nylon syringe filter into a sampler vial before injection. The samples were run on a HPLC (Dionex) with a CarboPac PA10 (250 mm x 4 mm) column and AminoTrap, equipped with a Pulsed Amperometric *Detector* (PAD). The column was eluted with gradient eluent of NaOH and CH_3COONa, at a flow rate of at 1 mL/min and it was operated at 25 ºC. D-(+)-glucose (Merck KGaA), D-(+)-xylose (Sigma-Aldrich, 99%), D-(+)-galactose (Fluka), L-(+)-arabinose (AppliChem *Panreac*), D-(+)-mannose (Sigma-Aldrich, 99%), D-(-)-Fructose (Scharlau) , D-(+)-Sucrose (Fluka) and D-(+)-Maltose (VWR, Prolabo) were used as standards in a concentration range from 5 ppm to 100 ppm.

2.2.4.2. Quantification of 5-HMF, furfural and carboxylic acids

Identification and quantification of degradation products (furfural, HMF and acetic acid) were performed by HPLC, through a Biorad Aminex (Knauer) HPX-87H (300 x 7.8 mm) ion exclusion column at 30 ºC, equipped with a UV/Vis detector (210 nm / 280 nm), using a 10 mM sulfuric acid solution as the eluent, a flow rate of 0.6 mL/min and a sample volume of 20 µL. All runs were performed at 210 nm for the first 25 min, for the detection of acetic acid, and changed for 280 nm during 50 min in order to detect furfural and 5-HMF.

2.2.4.3. Quantification of total phenols

For the total phenols determination, the method described by Mussatto et al. (2011) was used [72]. For that, the pH of the raw hydrolysates was raised to 12.0 with a solution of NaOH 6.0M and the obtained solutions were diluted with deionized water in order to have an absorbance value below 0.5. The total phenol concentration (g/L), TP, was given by equations 13 and 14:

$$TP = 4.187 \times 10^{-2}(Abs_{LIG280} - Abs_{PD280}) - 3.279 \times 10^{-4} \quad (13)$$

$$Abs_{PD280} = [(C_F \varepsilon_F) + (C_{HMF} \varepsilon_{HMF})] \quad (14)$$

where A_{LIG280} is the absorbance at 280 nm, C_F and C_{HMF} are the concentrations (g/L) of furfural and HMF, respectively determined by HPLC. ε_F and ε_{HMF} are the extinction coefficients (L/g cm) of furfural (146.85) and HMF (114.00) previously determined by ultraviolet spectroscopy at 280 nm. The measurements were performed in duplicate.

2.2.5. Recovered sugar yield and hydrolysis efficiency

The amount of sugars, namely, glucose, xylose or arabinose (g) that can be achieved from 100.0 g of dry lignocellulosic matter were represented by recovered sugar yields ($Y_{G,}$ Y_X, Y_A) and calculated using the following equation 15:

$$Y_{sugar} = \left(C_{sugar} \times \frac{V}{M}\right) \times 100 \qquad (15)$$

in which C_{sugar} is the concentration of the sugar in the liquid phase (g/L), M represents the amount of dry matter used (g) and V is the volume of acid solution (L) employed in each dilute acid hydrolysis.

The hydrolysis efficiency, i.e., the percentage of glucan, xylan and arabinan converted into glucose, xylose and arabinose, represented by $Glu_{R,}$ Xyl_R and $Ara_{R,}$ respectively, were obtained by using equations 16 and 17, adapted from Carvalheiro et al. (2004b) [73].

$$Glu_R = \frac{135}{150} \times \frac{C_{sugar} \times V}{\%\ glucan\ content \times M} \times 100 \qquad (16)$$

$$Xyl_R\ or\ Ara_R = \frac{132}{150} \times \frac{C_{sugar} \times V}{\%\ Xylan\ or\ arabinan\ content \times M} \times 100 \qquad (17)$$

where xylan and arabinan contents (%) vary depending on the type of lignocellulosic biomass used, as previous calculated.

2.3. Results and discussion

2.3.1. Characterization of the raw materials

The chemical composition, in terms of moisture, ash, hemicellulose (xylan, arabinan, galactan), cellulose, lignin (ASL and AIL) and protein of the six selected lignocellulosic wastes (Figure 5) used in this work is presented in Table 5, as well as the comparison with data found in literature. In an overview, the chemical composition determined for all raw materials, in this work, is in accordance with the range of values reported in literature for the same biomass type.

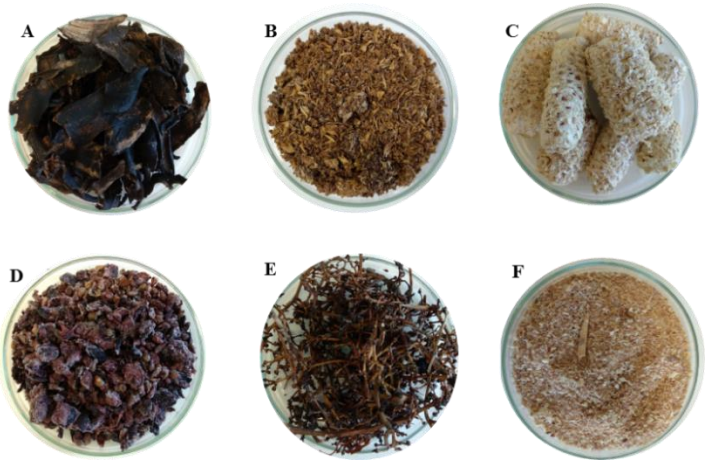


Figure 5 – Lignocellulosic waste materials used in this work. A – Banana peels; B – BSG; C – Corncobs; D – Grape pomace; E – Grape stalks; F – Sawdust.

Literature data clearly reveal some differences in the composition within each type of biomass. The chemical composition of the biomass depends on several factors, such as climate and cultivation conditions, harvesting and industrial processes to which they are subjected. Moreover, accurate determinations of cellulose, hemicellulose and lignin have some difficulties, because they are based on quantitative analysis of the corresponding monomers and lignocellulose has recalcitrant structural characteristics and wide structural variations [74].

As expected, in line with literature data, lignocellulosic polymers (cellulose, hemicellulose and lignin) represent a large percentage of the total dry weigh (DW) of the materials used in this work (Table 5) [21].

Corncobs have the highest average content of lignocellulosic polymers that account for 85.1% of the material's DW, followed by sawdust which sums 84.1%. The remaining raw materials exhibit lower percentages, around half of the former, namely 47.7, 57.4, 40.9 and 46.5%, corresponding to banana peels, BSG, grape pomace and grape stalks, respectively. Hemicellulose, the most relevant component for polyols production due to its xylan-rich backbone, is present in great quantity in BSG (22.1%), corncobs (39.6%) and sawdust (23.4%). Banana peels, grape pomace and stalks hold between 10 and 12.5% of hemicellulose.

Table 5 – Chemical composition of the lignocellulosic waste materials used in this work.

	Banana peels		**BSG**		**Corncobs**		**Grape pomace**		**Grape stalks**		**Sawdust**	
	This work	Literature values	**This work**	Literature values	**This work**	Literature values	**This work**	Literature values	**This work**	Literature values	**This work**	Literature values
Moisture (%)	**89.7**±0.4	62.3 - 89.1	**72.1**±0.8	72.6 - 81	**8.9**±0.9	5.1 - 15	**58.9**±0.6	64.4 - 73.6	**9.7**±0.4	7.09 - 20	**9.1**±0.5	10.0 - 10.8
Inorganic content (% [a])	**13.4**±1.7	3.5 - 13.6	**1.3**±0.5	1.1 - 4.6	**4.1**±0.5	1.3 - 8.5	**8.7**±0.8	4.6 - 7.8	**5.4**±0.7	5.1 - 7.7	**0.5**±1.1	0.43 - 12.3
Hemicellulose (% [a])	**12.5**±1.0	1.8 - 7.94	**22.1**±2.1	19.2 - 41.9	**39.6**±1.1	34.3 - 44.4	**10.6**±0.8	11.3 - 12.5	**10.3**±1.2	13.9 -25.7	**23.4**±0.8	20.1 - 30.6
Xylans (% [a])	**4.8**±0.4	n.a.	**10.7**±1.0	9.4	**30.2**±0.9	30.2	**5.9**±0.4	8.3	**3.9**±0.5	9.9	**17.5**±0.6	29.22
Arabinans (% [a])	**2.5**±0.3	n.a.	**6.9**±0.7	3.1	**3.9**±0.8	3.8	**1.4**±0.2	1.5	**3.9**±0.9	3.9	**0.5**±0.1	n.a.
Galactans(% [a])	**5.2**±0.3	n.a.	**4.5**±0.3	n.a.	**5.5**±0.4	n.a.	**3.3**±0.6	n.a.	**2.5**±0.9	n.a.	**5.4**±0.1	n.a.
Cellulose (% [a])	**21.1**±1.3	7.0 - 24.8	**19.0**±1.7	12 - 26	**28.4**±0.5	28.9 - 45.1	**12.2**±0.4	13.7 - 20.8	**17.6**±2.2	12.2 - 37.8	**42.8**±1.1	41.1 - 44.7
Total lignin (% [a])	**14.1**±0.5	9.7 - 12.1	**16.3**±0.6	5.4 - 27.8	**17.1**±2.4	11.9 - 18.4	**18.1**±0.7	20.5	**18.6**±0.9	15.5 - 32.9	**17.9**±0.5	25.2 - 29.2
ASL (% [a])	**6.8**±0.5	n.a.	**11.3**±0.2	n.a.	**6.3**±2.3	0.9	**3.9**±0.7	n.a.	**7.1**±1.3	n.a.	**9.6**±0.6	n.a.
AIL (% [a])	**7.3**±0.1	n.a.	**5.0**±0.2	n.a.	**10.8**±2.4	14.3 - 17.5	**14.2**±0.2	n.a.	**11.5**±0.8	17.4	**8.3**±0.7	n.a.
Protein (% [a])	**6.6**±0.3	1.2 - 6.0	**27.5**±0.5	10 - 31	**3.4**±0.4	3 - 5	**9.8**±0.6	8 - 18.8	**9.2**±0.2	6.1	**0.5**±0.1	1.8 - 3.5
Reference		[75, 76]		[77, 78, 79]		[42, 56, 57, 58, 59, 60]		[47, 65,80]		[81, 82, 83]		[68, 84]

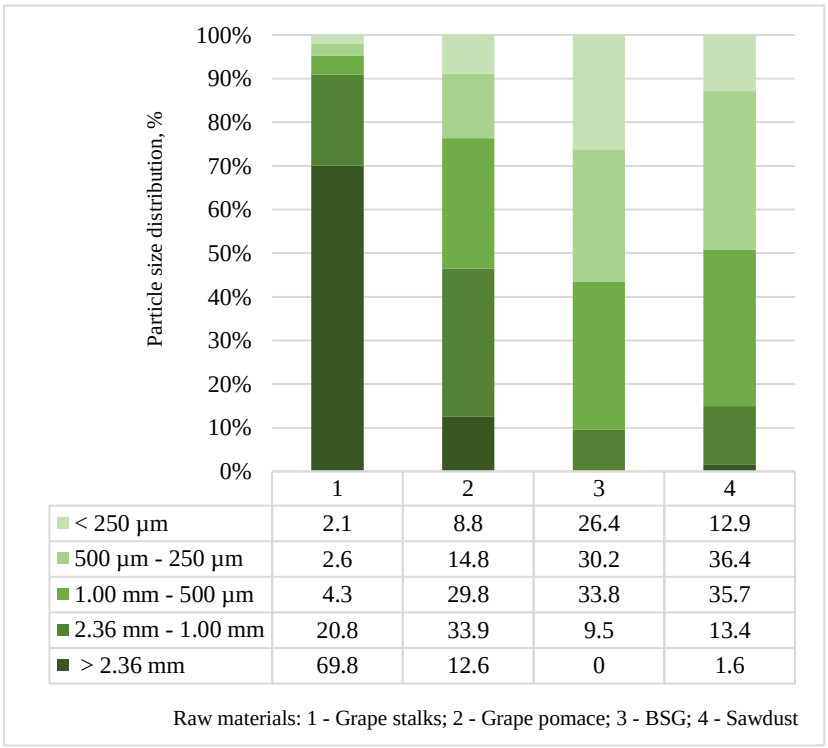
[a] - on a dry weight basis; n.a. – data not available

Other components are present in small amounts in lignocellulose, namely pectin, fats, oils, proteins, extractives and ash [21]. As expected, BSG revealed to be a protein-rich waste, reaching 27.5 %, the highest amount of all wastes, whereas, sawdust and corncobs presented the lower protein content of 0.5 and 3.4 %, respectively (Table 5). This is in accordance with literature data and, curiously, BSG has been incorporated in the human diet, in protein rich foodstuff [85].

The moisture level of lignocellulosic wastes is an important factor for the success of the implementation of biotechnological technologies in large-scale. High moisture contents may involve high costs of transportation and increase the susceptibility to microbial contamination and spoilage. Moisture below 10% has been suggested to avoid these problems, extending the storage time [85]. Table 5 shows that for corncobs, grape stalks and sawdust, moisture content is not problematic since the values are below 10%, whereas, banana peels, BSG and grape pomace have large amounts of water in their composition (89.7, 72.1 and 58.9 % respectively). In order to preserve the lignocellulosic waste with high moisture content, additional methods must be studied for an economical preservation.

In addition, another important factor is the particle sizes of the raw materials, which have impact on the efficiency of the subsequent pretreatments applied to lignocellulosic biomass. In the present work, BSG, grape stalks, grape pomace and sawdust were analysed about their particle size distribution and the results are expressed in Figure 6. Corncobs and banana peels were excluded from this analysis, due to their physical characteristics, since corncobs were too hard to grind and banana peels to soft being reduced to a puree. Grape stalks have the highest percentage of particles larger than 2.0 mm (about 70%). BSG and sawdust are mainly composed of particles smaller than 1.0 mm (about 85-90 %). In grape pomace, approximately 79% of the particles' size varies between 0.5 and 2.3 mm. It is reported that smaller particle sizes improve hydrolysis

efficacy, because the surface/volume ratio increases, decreasing mass transfer limitation [86]. For example, Barakat et al. (2013), reviewed dry fractionation of lignocellulose as an important step and they concluded that lignocellulosic materials must have particle sizes between 0.5 and 2 mm to reach a well-accepted level of digestibility [86]. Taking this into account, BSG, grape pomace and sawdust used in this work fulfill the prerequisites in terms of particle size to enhance the effectiveness of dilute acid hydrolysis performed posteriorly. However, alternative technologies are necessary to replace mechanical size reduction processes. Currently, milling operations require high energy consumption, which is not cost-effective. A suitable alternative would be steam explosion pretreatment, which has been considered one of the most energy efficient ways to treat lignocellulose [19]. In steam explosion, biomass undergoes to hot steam (180 to 240 °C) and high pressures (1 to 3.5 MPa), followed by a sudden pressure reduction. This process induces an explosive decompression of lignocellulose, breaking down inter- and intra-molecular linkages, i.e., hemicellulose is hydrolyzed and extracted in the water-soluble fraction [19, 24].



	1	2	3	4
< 250 µm	2.1	8.8	26.4	12.9
500 µm - 250 µm	2.6	14.8	30.2	36.4
1.00 mm - 500 µm	4.3	29.8	33.8	35.7
2.36 mm - 1.00 mm	20.8	33.9	9.5	13.4
> 2.36 mm	69.8	12.6	0	1.6

Figure 6 – Particle size distribution of BSG, grape stalks, grape pomace and sawdust used in this work.

2.3.2. Raw material hydrolysates

To establish the potential to recover hemicellulosic sugars, specially xylose and arabinose, from the selected lignocellulosic wastes, they were subjected to a dilute sulphuric acid hydrolysis treatment. The hydrolyses were carried out in an autoclave at 121 °C and 1 bar, using specific H_2SO_4 concentrations and reaction times, as mentioned in Section 2.2.

The sugar composition of the raw hydrolysates obtained are shown in Table 6, along with the recovery sugar yields (Y_G, Y_X, Y_A), which express the amount of glucose, xylose and arabinose (g) that can be obtained from 100 g of dry matter of each lignocellulose waste and, Glu_R, Xyl_R and Ara_R that represent the percentage of glucan, xylan and arabinan converted into glucose, xylose and arabinose, respectively.

Table 6 – Sugar composition, sugar recovery yields (Y_G, Y_X, Y_A) and hydrolysis efficiencies (Glu_R, Xyl_R and Ara_R) for glucose, xylose and arabinose, respectively, after lignocellulosic waste pretreatment with H_2SO_4.

	Glucose (g/L)	**Xylose (g/L)**	**Arabinose (g/L)**	**Y_G ($g_{glucose}$/ 100g $_{dry\ matter}$)**	**Y_X (g_{xylose}/ 100g $_{dry\ matter}$)**	**Y_A ($g_{arabinose}$/ 100g $_{dry\ matter}$)**	**Glu_R (%)**	**Xyl_R (%)**	**Ara_R (%)**
BSG raw hydrolysate	6.44 ± 2.3	8.85 ± 2.6	6.19 ± 2.1	6.92 ± 1.2	12.65 ± 0.6	8.84 ± 0.7	22.8 ± 0.8	91.0 ± 1.8	98.7 ± 2.5
Grape stalks raw hydrolysate	8.03 ± 0.7	5.53 ± 1.1	1.90 ± 0.7	9.03 ± 0.9	7.89 ± 0.8	2.71 ± 1.1	28.8 ± 1.3	99.6 ± 0.8	53.6 ± 1.4
Grape pomace raw hydrolysate	1.71 ± 0.5	3.10 ± 1.3	0.85 ± 0.3	2.14 ± 0.5	4.43 ± 1.1	1.21 ± 0.9	10.5 ± 0.9	57.8 ± 0.4	66.8 ± 0.8
Corncobs raw hydrolysate	2.27 ± 0.4	8.24 ± 1.5	0.94 ± 0.2	2.27 ± 0.9	8.24 ± 0.3	0.94 ± 0.2	4.8 ± 0.3	24.0 ± 0.7	21.2 ± 0.9
Sawdust raw hydrolysate	1.72 ± 0.2	10.78 ± 1.9	0.43 ± 0.1	1.73 ± 1.1	15.40 ± 1.5	0.62 ± 0.4	3.7 ± 0.4	67.8 ± 0.3	95.1 ± 2.3
Banana peels raw hydrolysate	8.20 ± 0.6	1.15 ± 1.1	1.41 ± 0.3	10.25 ± 1.6	1.15 ± 1.4	1.41 ± 0.6	23.3 ± 0.8	21.1 ± 0.9	49.6 ± 0.6

BSG raw hydrolysate contained about 21.5 g/L of glucose, xylose and arabinose together (at a ratio of 30:41:29, on a weight basis), being the hydrolysate with the highest concentration of total fermentable monosaccharides. From 100.0 g of dry BSG, it is possible to obtain 6.9 g of glucose, 12.7 g of xylose and 8.8 g of arabinose (Table 6). In addition, more than 90% of BSG's xylan and arabinan were converted into xylose and arabinose, respectively, which is in agreement with literature data. For example, Carvalheiro et al. (2004) applied similar operational conditions and, reported the recovery of 95% of the BSG's xylan as xylose and 96% of the arabinan as arabinose [87]. Dilute sulphuric acid hydrolysis of BSG was also evaluated by Mussato et al. (2004), under different acid concentrations, liquid/solid ratios and reaction times [48]. In that work, all tested conditions got high hydrolysis efficiencies for xylan and arabinan, varying from 88.7 to 96.5%. These results seem to be more favourable in comparison with the results obtained by exposing BSG to autohydrolysis combined with enzymatic pretreatments. Duarte et al. (2004) revealed an incomplete degradation of the arabinoxylan backbone (only 63% for xylan and 70%

for arabinan) of BSG hemicellulose, applying enzyme treatments, possibly due to inhibitory effects on enzymatic activities [89]. Raw hydrolysates from grape stalks, corncobs and sawdust revealed a total concentration of sugars around 10 and 15 g/L (Table 6), with a very high xylose to arabinose ratio of 74:26, 90:10 and 96:4 %, respectively, due to the low amount of arabinose present in each hydrolysate.

Dilute acid hydrolysis of grape stalks was very efficient in the conversion of xylan into xylose (99.6%) (Table 6). However, the xylose concentration in the hydrolysate (5.5 g/L) was lower than the values (9.8 and 11.9 g/L) reported by Egüés et al. (2013) and Moldes et al. (2007), for similar hydrolysis conditions [90].

Overall, dilute acid hydrolysis was not effective in the removal of glucose from cellulose, since the hydrolysis efficiency did not exceed the 30% (Table 6). This finding may be explained due to cellulose structure, namely, its crystallinity and degree of polymerization, that require severe conditions to be hydrolyzed, whereas, the amorphous hemicellulose structure is more favorable to degradation [18].

The pretreatment of corncobs was not effective. The hydrolysates obtained had 8.3 g/L of xylose (Table 6), whereas literature data present values up to four times higher, such as 31.3 g/L, 28.7 g/L and 21.7 g/L in similar works done by Li et al. (2011), Cheng et al. (2009) and Ping et al. (2013), respectively [59, 69, 91]. In the present work, corncobs were used at their normal size during hydrolysis process, because it was not possible to mill them due to their rigid structure. Thus, the large size of corncobs is a probable explanation for the low conversion percentage of xylan into xylose (24%), as it may increase mass transfer limitations, interfering with digestibility.

Hydrolysis of banana peels was not very successful either, xylans were not properly digested because it was a challenge to mix the blended fresh banana peels and the acid solution. Consequently, only a small portion of banana peels were in direct contact with the acid solution and, only 1.2 g/L of xylose were released to the hydrolysate (Table 6). However, in this hydrolysate 10.3 g of glucose were obtain from 100.0 g of dry matter, presenting the highest glucose recovery, which may be explained by the fact that banana peels have high free sugars contents. For example, Emaga et al. (2011) found out values from 23.5 to 38.3 % (dry matter) of free sugars depending on the variety and maturation stage of the banana peels [92].

The highest xylose concentration (10.8 g/L) was obtained by using sawdust as raw material (Table 6). Nevertheless, Xyl_R only achieved 67.8%, suggesting that the operational conditions used in the dilute acid hydrolysis of sawdust are not fully optimized. The operational conditions used were the optimal ones proposed by Rafiqul et al. (2012) in a kinetic study for xylose production from sawdust by acid hydrolysis. However, Rafiqul et al. (2012) obtained a maximum recovery of xylose of 18.65 g/L, corresponding to an efficiency of 89.9% [68].

Since the main goal of this work was to use the hydrolysates for *K. pastoris* growth and polyols production, special attention was paid to acid hydrolysis by-products, which may have a negative impact on fermentation, depending on the overall composition of the hydrolysates and the capacity of the chosen microorganism to adapt to the toxic compounds.

In this work, the obtained hydrolysates contained not only a mixture of sugars, as shown in Table 6, but also some compounds that would potentially interfere with microbial growth, such as furfural and 5-HMF (sugar-decomposition products), acetic acid and phenolic compounds, and some amino acids, as indicated in Table 7.

Furan derivates were found in residual concentrations in all hydrolysates, with values lower than 0.5 g/L, which are in accordance with literature data (0.03-0.55 g/L) [65, 69, 89, 90]. An important step that may lead to such low results was the prior pH adjustment of raw hydrolysates with $Ca(OH)_2$, which induced the precipitation and instability of some toxic compounds. Furfural is the main toxic compound formed from pentoses degradation, whereas 5-HMF is the degradation product of hexoses. This is particularly noticed in the raw hydrolysates of sawdust and banana peels, because they have the highest amounts of xylose and glucose, respectively, and consequently sawdust hydrolysate had the highest concentration of furfural and banana peels hydrolysate the highest concentration of 5-HMF (Tables 6 and 7).

Acetic acid generation occurs due to the release of acetyl groups from hemicellulose [68]. As indicated in Table 7, corncob and sawdust were the richest acetic acid hydrolysates, probably because they have the highest contents of hemicellulose (Table 5) and it is described that hemicellulose of hardwoods is highly acetylated [91, 93].

The release of phenolic compounds has also been reported during hydrolysis processes, as well as, their negative influence on fermentation [25]. In this work, grape pomace and grape stalks hydrolysates showed the most abundant contents of total phenol, namely, 1.85 and 1.62 g/L, respectively. It is well known that phenols are the bioactive compounds present in largest quantities in grape composition [94]. For example, grape pomace is especially rich in anthocyanins, flavonols, hydroxibenzoic acids, hidroxycinnamic acids and stilbenes [94]. Additionally, banana peels hydrolysate also revealed a significant amount of total phenols (1.23 g/L), which is accordance with literature data (1.35 g/L) [70]. Surprisingly, forty different phenols have been identified from banana peels [95].

It is widely reported that high sugar concentrations favoured polyols production. In this way, a concentration step of some raw hydrolysates was performed to evaluate the effect of substrate concentration on fermentation. This concentration step also leads to an increased level of potential toxic/interfering compounds. Therefore, to reduce the concentration of inhibitors, a detoxification treatment using activated charcoal, was also evaluated in order to improve the

fermentability of the media. Activated charcoal has been reported as the most effective method for removal of furan derivates and low molecular weight phenols [87].

BSG, grape stalks and sawdust were the selected raw hydrolysates subjected to these treatment steps, since it was possible to extract the highest amounts of total sugars (glucose+xylose+arabinose) from 100.0 g of the original dry matter, namely, 28.41, 19.63 and 17.75 g, respectively (Table 6). By opposition, from 100.0 g of grape pomace, corncobs and banana peels only 7.78, 11.45 and 12.81 g of total sugars were respectively obtained (Table 6).

Table 7 – Toxic compounds present in raw hydrolysates.

	5-HMF (g/L)	**Furfural (g/L)**	**Acetic acid (g/L)**	**Total phenols (g/L)**
BSG raw hydrolysate	0.12	0.18	0.78	0.78
Grape stalks raw hydrolysate	0.08	0.01	1.15	1.62
Grape pomace raw hydrolysate	n.d.	n.d.	1.42	1.85
Corncobs raw hydrolysate	0.03	0.11	2.90	0.19
Sawdust raw hydrolysate	0.03	0.32	2.75	0.58
Banana peels raw hydrolysate	0.51	0.01	0.47	1.23

n.d., not detected

During activated charcoal treatment, it was observed a loss of colour in sawdust and grape stalks hydrolysates, but not in BSG hydrolysate (Figure 7). This colour reduction may be related with the removal of phenolic compounds present in the hydrolysates. It is described that hydrolysate color is directly related to the presence of phenolic compounds. Activated charcoal promotes the packing of phenolic molecules in the its pores, removing the phenolics from the hydrolysates and, consequently losing color [83].

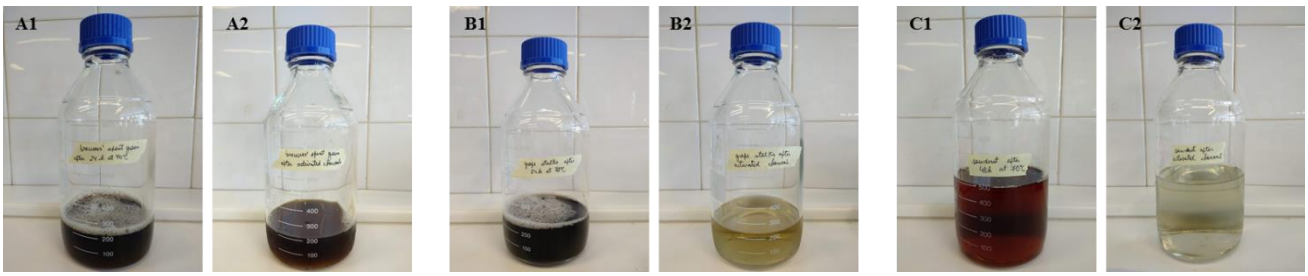


Figure 7 – Effect of activated charcoal on hydrolysates color. A1 – BSG before charcoal treatment; A2 – BSG after charcoal treatment; B1 – Grape stalks before charcoal treatment; B2 – Grape stalks after charcoal treatment; C1 – Sawdust before charcoal treatment; C2 – Sawdust after charcoal treatment.

Table 8 shows the variation of concentration of toxic compounds of BSG, grape stalks and sawdust hydrolysates along the steps considered (concentration and detoxification).

For the 2-fold concentrated BSG and grape stalks hydrolysates and the 5-fold concentrated sawdust hydrolysate, the detoxification step with activated charcoal allowed to completely remove the furan derivates (Table 8). Nevertheless, the amounts of acetic acid have undergone only minor changes (removal below 36.8%). It is possible to establish a relation between the amount of acid acetic present in the concentrated hydrolysates and the percentage of removal after detoxification step (Table 8). The hydrolysate with the highest amount of acetic acid (12.0 g/L) had the lowest removal efficiency (20.1%), whereas the hydrolysate with the lowest concentration of acid acetic (1.14 g/L) had the highest removal efficiency (37%).

Table 8 – Composition in sugars and toxic compounds of lignocellulosic hydrolysates along concentration and detoxification steps.

	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	5-HMF (g/L)	Furfural (g/L)	Acetic acid (g/L)
BSG						
After pH adjustment with $Ca(OH)_2$	6.44	8.13	5.72	0.15	0.15	0.76
After twofold concentration	11.71	15.22	10.30	0.23	0.30	1.14
After activated charcoal treatment	9.57	13.00	8.85	0.01	0.00	0.72
Sugars loss/toxic compounds removal (%)	**18.3**	**14.6**	**14.1**	**95.7**	**100.0**	**36.8**
Grape stalks						
After pH adjustment with $Ca(OH)_2$	8.03	5.83	1.90	0.08	0.01	1.15
After twofold concentration	15.85	11.12	3.92	0.18	0.02	2.73
After activated charcoal treatment	11.00	7.96	2.77	0.00	0.00	2.04
Sugars loss/toxic compounds removal (%)	**30.6**	**28.4**	**29.3**	**100.0**	**100.0**	**25.3**
Sawdust						
After pH adjustment with $Ca(OH)_2$	1.72	10.72	0.57	0.05	0.32	2.99
After fivefold concentration	8.71	42.09	3.635	0.26	0.56	12.00
After activated charcoal treatment	6.54	33.83	2.85	0.00	0.00	9.59
Sugars loss/toxic compounds removal (%)	**24.9**	**19.6**	**21.6**	**100.0**	**100.0**	**20.1**

In addition, there was also a loss of the fermentable sugars content, which is an inevitable side effect of the detoxification procedure. Reduction of each sugar was similar within each hydrolysate. There was a reduction of around 15, 20 and 30% for sugars present in BSG, sawdust and grape stalks hydrolysates, respectively (Table 8). Similar detoxification treatment and results were reported by Salgado et al. (2012) for BSG hydrolysates, in which there was a 99% removal of phenolic compounds, along with a small percentage (around 16%) of sugars, whereas acetic acid remained constant before and after detoxification. [65]. A possible explanation for the low activated charcoal efficiency in the removal of acetic acid may be attributed to the charcoal preference for adsorbing other substances from the hydrolysate. This theory is supported by Berson el al. (2005), that studied the adsorption of acetic acid by activated charcoal from corn stover hydrolysates and from acetic acid synthetic solutions. They observed an effective removal of acetic acid from the synthetic hydrolysate and a less effective result from the corn stover hydrolysate [96]. In a different study with hardwood wastes, Ko et al. (2008) suggested that the bond strength between acetic acid and activated charcoal is weak [84]. Although they observed

the reduction of furfural, 5-HMF and total phenols by activated charcoal, the concentration of acetic acid remained also nearly constant. Thus, they tested the removal of acetic acid by sequential adsorption in an ion exchange column (DIAION PA408 strong anionic exchange resin) and found out that it is possible to obtain an effective separation [84].

2.4. Conclusion

Dilute acid hydrolysis enabled a high solubilization and hydrolysis of hemicellulose with high pentose recovery for BSG and grape stalks, 91.0 and 99.6% of xylose from xylan and 98.7 and 53.6% of arabinose from arabinan, respectively. The recovery of glucose from cellulose was not effective, as expected under the hydrolysis conditions applied. However, BSG, grape stalks and banana peels revealed reasonable amounts of glucose (6.4, 8.0 and 8.2 g/L, respectively). Probably, part of glucose came from free sugars present in the wastes. Optimization of the hydrolysis conditions for corncobs and sawdust would be interesting, because although xylose recovery yields were around 60%, the hydrolysates are in the top 3 of the highest xylose concentrations obtained. The pentose-rich hydrolysates were found to exhibit a low level of furan derivates, however acetic acid was present in significative amounts (especially in sawdust, 2.75 g/L, and corncob, 2.90 g/L, hydrolysates). Through a concentration step, aiming the increase of sugars in the hydrolysates, acetic acid concentrations also increased and, activated charcoal detoxification procedure was not efficient in its removal.

Considering the main purpose for which these hydrolysates were obtained, their use as substrate for the cultivation of *K. pastoris* and polyols production, BSG and grape stalks demonstrate, in advance, a promising potential due to their composition. They present the highest total sugars content of all hydrolysates (21.5 and 15.5 g/L, respectively), with glucose to xylose/arabinose ratios of $6.5g_{glucose}$:$8.9g_{xylose}$:$6.2g_{arabinose}$ for BSG and $8.0g_{glucose}$:$7.4g_{pentoses}$ for grape stalks. These balanced ratios are important because glucose is needed for growth and xylose/arabinose for sugar alcohols production. In addition, these hydrolysates did not reveal considerable high amounts of toxic compounds, of which acetic acid and phenolics were the majority.

The activated charcoal treatment tested to reduce/eliminate the toxic compounds was not effective in the removal of acetic acid, but the colour loss observed in some hydrolysates was an indicator of phenolic removal. Cultivation studies will be needed to conclude if this detoxification treatment is beneficial for yeast growth and polyols production, because the tolerance for these compounds depends on yeasts ability to adapt.

Chapter 3

Bioproduction of sugar alcohols from lignocellulosic wastes

3.1. Introduction

Sugars hydrolyzed from hemicellulose of lignocellulosic waste materials have been reported as suitable substrates for cultivation of different microorganisms that transform them into value-added products [24]. In Chapter 2, dilute acid hydrolysis of the selected lignocellulosic waste materials led to pentose-rich hydrolysates, revealing their great potential to be converted into polyols by yeasts.

The genetic nature of the microorganism is, undoubtedly, very relevant for its potential to convert xylose/arabinose into xylitol/arabitol [38]. However, there are also several fermentation parameters playing an important role in the bioconversion of sugars into polyols by yeasts. These parameters include process and culture conditions, such as temperature, pH, aeration, inoculum concentration, mode of reactor operation and possibility of cell recycling and, also, nutritional conditions, namely, carbon source, substrate concentration, nitrogen source and media supplementation [18, 97]. Several researches are focused on their optimization because a proper synergy of these factors is of great importance for the viability of the bioprocess, attaining high-quality polyols with high efficiency [4, 98].

The initial step for xylitol/arabitol production is xylose/arabinose uptake by the yeast cell. The initial sugars concentration may affect polyols production. Generally, increased xylitol production rates are obtained through higher sugar concentrations, but the sugar concentration and the osmotic pressure cannot exceed the microorganism tolerance [99]. On the other hand, arabitol production by osmophilic yeasts, such as *Kodamaea ohmeri*, has been reported and, thus, high osmolarities are advantageous to achieve higher arabitol productions [6].

Most studies employing xylitol producers have been performed in a wide pH range [9]. It is suggested that pH plays an important role in sugars transportation, influencing, for example, the activity of the permeases present in the cytoplasmic membrane and, it is also significant for the catalytic activity of XR [100].

Aeration is seen has a major factor in the pentose-polyol bioconversion by pentose-fermenting yeasts. Oxygen availability is determinant to choose the pathway in which xylose/arabinose will be take part in, fermentation or respiration, with the two pathways also able to occur simultaneously [4]. The mechanism of xylose conversion into xylitol, by means of reduction by XR, and its oxidation into xylulose, catalyzed by XDH, as illustrated in Chapter 1 (Figure 2), is limited by a redox imbalance of NADPH/NAD^+. Theoretically, the redox imbalance is promoted by restricting the oxygen availability, leading to the reduction of XDH, thus preventing the conversion of xylitol into xylulose, representing a key factor for xylitol accumulation [7]. Arabinose conversion into arabitol is followed by a subsequently oxidation into xylulose by a NAD dependent enzyme. Since there is a limited regeneration of NAD under low

oxygen conditions, arabitol accumulation is also favoured by restricting the oxygen availability [10]. However, yeast strain and culture medium conditions, especially lignocellulosic hydrolysates, which may contain glucose and toxic compounds, are fundamental to determine the amount of oxygen necessary for bioconversion [99]. Due to the importance of aeration on pentoseassimilation and polyols production, there are numerous studies focused on finding suitable aeration rates, especially under oxygen-limited conditions, to increase polyols productivity for each microorganism and carbon source chosen [97].

Production of polyols have been performed using free cells or immobilized cells in batch, fed-batch and continuous configurations [4]. Batch mode (flasks or bioreactor) is the most common configuration implemented for polyols production in literature [4]. The major advantage is the reduced risk of contamination. However, inhibition of cell growth and polyols production, in consequence of high concentrations of the carbon source or the product formed, is a problem usually linked with this type of fermentations [101]. Alternatively, fed-batch fermentations mitigate the toxic effects of high concentrations and, generally, present better yields and productivities compared to batch fermentations [102]. Continuous fermentations are characterized by maintaining high productivities, stability in product synthesis, with a substantial reduction of processing time by eliminating the time for charge, discharge, cleaning and sterilization [101]. Batch and fed-batch processes are more advantageous at bench-scale, because of the flexibility with varying biological systems, whereas, continuous mode may be more profitable at large-scale production.

This chapter presents different shake flask assays and bioreactor cultivations using the lignocellulosic hydrolysates, obtained as described in chapter 2, as feedstock for the production of xylitol and/or arabitol by the yeast *K. pastoris DSM 70877*. Raw hydrolysates, concentrated hydrolysates, hydrolysates subjected to detoxification treatments, mixture of different hydrolysates and aeration conditions were the approaches used aiming to achieve higher polyols productions.

3.2. Materials and methods

3.2.1. Yeast strain and media

In this work, all assays were performed with *Komagataella pastoris* strain DSM 70877. The microorganism was cryopreserved in 20% (v/v) glycerol and stored at -80 °C.

Medium K was used for inocula preparation. Medium K had the following composition (per litter of deionized water): KH_2PO_4, 37.33 g; $CaSO_4.2H_2O$, 0.1667 g; $MgSO_4 \cdot 7H_2O$, 2.67 g; $(NH_4)_2SO_4$, 18.07 g and 2 mL of a trace mineral solution comprising the following composition (per litter of deionized water): $CuSO_4 \cdot 5H_2O$, 0.20 g; $MnSO_4 \cdot H_2O$, 3.00 g; $ZnCl_2$, 7.00 g; $FeSO_4 \cdot 7H_2O$, 22.00 g; biotin, 0.20 g; H_2SO_4, 5 mL. The pH of the medium was adjusted to 5.0 with a 5 M NaOH solution and it was sterilized by autoclaving at 121 °C and 1 bar for 20 min. The trace mineral solution was filter-sterilized (0.2 μm, Sartorius Stedim Minisart) and stored at 4 °C.

The shake flasks and bioreactors experiments were performed with the hydrolysates obtained after dilute acid hydrolysis of all raw materials as cultivation media. For these experiments, the hydrolysates were filter-sterilized (0.2 μm, Sartorius Stedim Minisart) and supplemented with $(NH_4)_2SO_4$ to reach a concentration of 13.55 g/L.

3.2.1.1. Inocula preparation

For all the experiments, inocula were prepared in a 500 mL baffled shake flask by inoculating 1 mL of the cryopreserved culture, in 150 mL of medium K enriched with a glucose solution (4.00 g in 15 mL of deionized water) and 1 mL of the trace mineral solution. The pH was set to 5.0 by addition of a 25% (v/v) ammonium hydroxide solution (Scharlau). The inocula were incubated in an orbital shaker at 30 °C and 200 rpm for 40 h, to ensure that the culture is at exponential growth phase before transferring to the shake flasks and bioreactors containing the selected culture medium.

3.2.1.2. Shake flask assays

In order to select the best carbon sources for the purpose of this work, the hydrolysates obtained after dilute acid hydrolysis of all raw materials, prepared as described in section 2.2.3.1., were used as culture medium in shake flask assays. For these experiments, 80 mL of each hydrolysate were filter-sterilized (0.2 μm, Sartorius Stedim Minisart) to baffled shake flasks of 250 mL and supplemented with 10 mL of a solution of $(NH_4)_2SO_4$ (13.55 g/L). The media were inoculated with 10% (v/v) of inocula, prepared as described above. All cultures were incubated in an orbital shaker at 30 °C and 200 rpm for 120 h. Samples of 5 mL were daily collected for

measurement of the optical density at 600 nm (OD_{600nm}) and pH, determination of biomass (CDW) and for the quantification of sugars, alcohols and organic acids. All the experiments were performed in duplicate in order to improve cellular growth and sugar alcohols production, different conditions were tested in shake flask assays. The effect of concentration, detoxification and mixture of the raw hydrolysates were studied, as well as the effect of aeration during the production phase. The aeration effect was tested by stopping the oxygen supply after 72 h of cultivation, by replacing the filter cap by a closed cap.

For these experiments, all the raw hydrolysates used as culture media were filter-sterilized, while the hydrolysates subjected to concentration and/or activated charcoal treatment were sterilized by autoclaving at 121 °C and 1 bar, for 20 min. The medium supplementation and the inoculations were performed as described above in the screening assay, along with the sample collection and processing. The shake flasks were kept in the orbital shaker at 30 °C and 200 rpm, for 168 h. All assays were performed in duplicate.

3.2.1.3. Bioreactor assays

Due to their promising results, some of the conditions tested in the shake flask assays were selected and the processes were scaled-up to bioreactor cultivation. The differences in the tested conditions used in the bioreactor fermentations were based on culture medium, operation mode, pH control and airflow rate.

The experiments were carried out in a BioStat® B-plus bioreactor (Sartorius) with working volume of 2 L and in a Jupiter 4.0 bioreactor (Solaris) with working volume of 3 L.

The selected lignocellulosic media (1.6 L) were supplemented with 10 % (v/v) of a solution of $(NH_4)_2SO_4$ to give a final concentration of 13.55 g/L, and were inoculated with 10% (v/v) of the inocula, prepared as mentioned before. In all the experiments, the temperature was maintained at 30 °C and the initial pH was adjusted to 5.0. The pH and the dissolved oxygen concentration (DOC) in the medium were continuously monitored using a pH probe (Mettler Toledo) and a DO probe (Mettler Toledo), respectively. Control of the DOC at 15% of the air saturation was achieved by the automatic variation of the stirring rate, from 300 to 1000 rpm. Foaming was automatically controlled by addition of Antifoam Y-30 (Sigma-Aldrich), diluted by a factor of 2. Similarly to shake flask assays, samples of 15 mL were periodically taken for measurement of OD_{600nm} and pH, determination of CDW and for the quantification of sugars, sugar alcohols and organic acids.

3.2.2. Analytical techniques

3.2.2.1. Cell growth

Cell growth was monitored during the experiments by determining the optical density of the cultivation broth at 600 nm (OD_{600nm}), in a UV-Vis spectrophotometer (VWR, V-1200 Spectrophotometer). The samples were diluted with deionized water in order to obtain an absorbance value below 0.3, using deionised water as zero reference. All the measurements were performed in duplicate.

3.2.2.2. Biomass quantification

The dry weight of cells per litre of fermentation broth, cell dry weight (CDW), was determined gravimetrically. Broth samples were centrifuged at 10 000 rpm, for 20 min at 4 °C. The cell-free supernatant obtained was stored at -20 °C for further analysis and the cell pellet was washed twice by resuspension in deionized water and centrifugated at 10 000 rpm, for 20 min at 4 °C. After washing, the pellets were lyophilized (ScanVac CoolSafeTM, LaboGene) at -110 ˚C for 48 h. The CDW was determined by the weight of the lyophilized cell pellets. All the determinations were performed in duplicate.

3.2.2.3. Quantification of the composition of media and formed products

The quantification of sugars, furfural, 5-HMF, acetic acid and some organic acids during the different assays were performed by HPLC. The cell-free supernatants of all collected samples were diluted to fit the calibration curves and prepared and analyzed as described in sections 2.2.4.2. and 2.2.4.3., respectively. Alcohols, mostly the sugar alcohols xylitol and arabitol, were also identified and quantified by HPLC, in a Dionex ICS 3000 system with a CarboPac MA1 column (4 mm x 25 mm) and a pre-column (4 mm x 25 mm), equipped with an electrochemical detector (ED). The columns were operated at 25 °C using a 612 mM NaOH solution as the eluent, a flow rate of 0.4 mL/min and a sample injection volume of 10 µL. Standard solutions including xylitol (Sigma-Aldrich, 99%), L-(-)-arabitol (Tokio Chemical Industry), D-(+)-sorbitol (Sigma-Aldric*)*, D-(+)-manitol (Alfa Aesar, 99%) were prepared in deionized water in a concentration range from 5 ppm to 100 ppm.

3.2.3. Kinetic parameters

The biomass yield ($Y_{X/S}$, g_{CDW} / $g_{substrate}$) was calculated using equation 18:

$$Y_{X/S} = \frac{dX}{dS} \qquad (18)$$

where dX (g/L) is the CDW at time t (h) and dS (g/L) is the substrate consumed at time *t* (h).

The product yield ($Y_{P/S}$, $g_{sugar\ alcohol}$ / $g_{substrate}$) was obtained using the equation 19:

$$Y_{P/S} = \frac{dP}{dS} \qquad (19)$$

where dP (g/L) is the product formed (xylitol or arabitol) at the time t (h) and dS (g/L) is the substrate consumed (xylose or arabinose, respectively) at time *t* (h).

The volumetric productivity (r_P) of sugar alcohols productions was obtained using equation 20:

$$r_P = \frac{dP}{dt} \qquad (20)$$

where dP represents the variation of the concentration of products (g/L) and dt corresponds to the time interval necessary for the production (days).

3.2.4. CGC extraction

Prior to the extraction, 200 mL of each bioreactor's fermentation broth were centrifuged (9000 rpm and 4 °C, for 30 min) and the supernatant was discarded. The cell pellets were washed twice by resuspension in deionized water and centrifugated at 9000 rpm at 4 °C, for 20 min. For extraction of CGC from *K. pastoris* biomass, 1.20 g of each cell pellet was treated with 30 mL of NaOH 5 M, in a water bath at 65 °C for 2 h, under constant stirring. After cooling, the suspension was centrifuged (10 000 rpm for 15 min) and the pellet containing the alkaline insoluble material, CGC included, was resuspended in deionized water and neutralized with HCl 5 M. The obtained suspension was washed twice by resuspension in deionized water and centrifugated in the conditions described before. The polymer, free of alkali soluble components, was freeze dried for the gravimetric quantification of the polymer content in the biomass.

3.2.4.1. CGC composition

CGC sugar composition was determined following a procedure reported by Barrera et al. (2016) (Patent US 2016/0122444 A1). The method requires the determination of total nitrogen of CGC samples, using an elemental analyzer Thermo Finnigan-CE Instruments Flash EA 1112 CHNS series. The chitin content (Q, %) of each polymer was determine following equation 21 which is function of the amount of nitrogen (N, %) quantified by elemental analysis.

$$Q = 14.199\ N \qquad (21)$$

It is important to highlight that the nitrogen quantified comes only from chitin and that all proteins were removed during centrifugation and washing steps.

3.3. Results and discussion

3.3.1. Screening assays

In order to determine the most suitable hemicellulosic hydrolysates for polyols production, using the selected *K. Pastoris* strain, a set of screening assays were performed in shake flasks, under aerobic conditions and incubated in an orbital shaker at 30 °C, during 120 h. The raw hydrolysates obtained after dilute sulphuric acid hydrolysis and pH adjustment to 5.0 with $Ca(OH)_2$, from all selected raw materials, were directly used as feedstocks and the results are expressed in Table 9.

Table 9 – Effect of different raw hemicellulosic hydrolysates on polyols production by *K. pastoris*, in shake flask assays.

Feedstock	Glucose initial (g/L)	Glucose final (g/L)	Xylose initial (g/L)	Xylose final (g/L)	Arabinose initial (g/L)	Arabinose final (g/L)	Biomass produced (g/L)	$Y_{X/S}$ ($g_{biomass}$/ $g_{glucose}$)	Xylitol (g/L)	$Y_{P/S}$ ($g_{xylitol}$/ g_{xylose})	Arabitol (g/L)	$Y_{P/S}$ ($g_{arabitol}$/ $g_{arabinose}$)
BSG hydrolysate	5.14	0.28	8.19	5.89	5.07	4.69	5.70±0.94	1.17	1.51±0.07	0.39	0.90±0.09	2.38
Grape stalks hydrolysate	6.77	0.09	5.21	2.11	1.77	1.41	14.58±0.19	2.18	0.78±0.04	0.25	0.34±0.04	0.98
Grape pomace hydrolysate	1.71	0.04	2.10	0.38	0.85	0.67	7.08±0.17	4.24	0.36±0.07	0.21	0.21±0.02	1.21
Corncobs hydrolysate	2.27	1.54	8.24	6.03	0.94	0.88	13.70±0.15	18.77	0.81±0.05	0.36	0.21±0.03	3.02
Sawdust hydrolysate	1.39	1.30	8.95	8.41	0.43	0.43	0.92±0.09	10.22	0.02±0.01	0.00	0.07±0.01	0.00
Banana peels hydrolysate	8.20	0.00	1.15	0.11	1.41	1.02	15.18±0.33	1.88	0.06±0.01	0.05	0.02±0.01	0.06

By observing the results expressed in Table 9, it is possible to establish a relation between the initial concentration of glucose and the amount of biomass produced. The hydrolysates having the highest glucose concentrations, namely, banana peels (8.20 g/L) and grape stalks (6.77 g/L), generated more biomass, around 15.18 and 14.58 g/L, respectively (Table 9). These results are in accordance with the work performed by Araújo et al. (2017) using the same *K. pastoris* strain and mixtures of synthetic glucose and xylose as carbon source. In that experiments, an increase in biomass production was obtained with the decrease in xylose:glucose ratios. Comparing cultivations with xylose:glucose ratios of 75:15 and 15:75 there was a significant increase in biomass production from 11.1 to 30.2 g/L, respectively [33].

Analysing the sugar uptake from Table 9, it is possible to observe that after 120 h, *K. pastoris* had been consumed a global percentage of 95 % of glucose present in each hydrolysate, with the exception for corncobs and sawdust hydrolysates, in which the high amount of acetic acid (2.90 and 2.75 g/L, respectively (Table 7), alongside with other toxic compounds, namely, furfural (0.11 and 0.32 g/L, respectively (Table 7) and phenols (0.19 and 2.08 g/L, respectively (Table 7), probably induced inhibitory effects. The considerable amounts of acetic acid may have toxic effects due its ability to get inside the cells, acidifying their interior and, consequently, leading to the inhibition of growth and xylose metabolism [99]. BSG stands out as the most

promising substrate for polyols production, reaching the highest amounts of xylitol and arabitol, 1.51 and 0.90 g/L, respectively (Table 9). Among other hypothesis, the preference for BSG hydrolysate may be sustained by the fact that it has great potential to fulfill the requirement for a proper culture media. For example, Cooray et al. (2017) evaluated BSG as a novel media for yeast growth and discovered a rich BSG media, with several metabolites and amino acids, having a great potential to replace the conventional yeast extract-peptone-dextrose (YPD) media. BSG media was capable to sustain normal metabolomics activity in *Rhodosporidium toruloides* [49].

In the cultivations with BSG raw hydrolysates, around 97 % of glucose was consumed, but no more than 40 % of xylose was uptake by the yeast. Therefore, increasing the cultivation time may be an interesting strategy to check if the yeast would be able to assimilate more xylose and, theoretically, lead to a higher xylitol production.

Grape stalks and corncobs were the following hydrolysates to present promising results, with the production of 0.78 and 0.81 g/L of xylitol, respectively, but with lower amounts of arabitol (0.34 and 0.21 g/L, respectively).

In a study conducted by Serrano et al. (2018), the effect of commercial xylose concentration was evaluated using the same *K. pastoris* strain. For an initial concentration of 10 g/L xylose, it was registered a maximum xylitol production of 0.54 g/L, with a yield of 0.05 $g_{xylitol}/g_{xylose}$ [37]. Apparently, these results demonstrate that BSG, grape stalks and corncobs are good carbon sources for polyols production by *K. pastoris*.

In the tests with grape pomace and banana peels very low amounts of xylitol and arabitol were produced, which was expected in consequence of the low initial concentrations of xylose and arabinose (less than 2.10 g/L) in both hydrolysates. Even though sawdust hydrolysate was a xylose-rich substrate, its employment directly as feedstock did not result in the production of xylitol neither arabitol. As already mentioned above, this may be explained by the high amounts of acetic acid present in sawdust hydrolysates (around 2.75 g/L – Table 7).

In addition, liquors obtained after hydrolysis only with water, avoiding the addition of sulphuric acid, were also conducted in similar experimental conditions for xylitol and/or arabitol production. However, water was not efficient in the depolymerization of hemicellulose into its monomeric sugars, releasing only a small percentage of glucose, whereas xylose and arabinose were not detected in none of the liquors (Table 10). In consequence, it was possible to verify that there was no production of xylitol and/or arabitol when the selected raw materials were subjected to hydrolysis in the absence of the acid. These results reinforce the need for a proper choice of hemicellulosic biomass pretreatment and allowed to conclude that, in this work, the addition of sulphuric acid is an important step for the production of polyols.

Table 10 – Effect of BSG, grape pomace, sawdust and banana peels hydrolysis using only water (without H_2SO_4) on biomass and polyols production by *K. pastoris*, in shake flask assays.

Feedstock	Glucose initial (g/L)	Glucose final (g/L)	Xylose initial (g/L)	Xylose final (g/L)	Arabinose initial (g/L)	Arabinose final (g/L)	Biomass produced (g/L)	Xylitol (g/L)	Arabitol (g/L)
BSG (hydrolysis without H_2SO_4)	0.06	0.01	n.d.	n.d.	0.14	0.09	0.51	n.d.	0.04
Grape pomace (hydrolysis without H_2SO_4)	0.52	0.01	n.d.	n.d.	0.06	0.06	1.02	0.01	0.01
Sawdust (hydrolysis without H_2SO_4)	0.15	0.00	n.d.	n.d.	0.01	0.01	0.12	n.d.	n.d.
Banana peels (hydrolysis without H_2SO_4)	9.86	0.10	n.d.	n.d.	n.d.	n.d.	10.60	n.d.	n.d.

n.d., not detected

The optimization of culture conditions, such as temperature and pH, using commercial xylose as carbon source and the same strain of *K. Pastoris* studied in the present work, was performed by Serrano et al. (2018) [37]. In that work, it was demonstrated that cultivations at 37 °C and with an initial pH of 7 resulted in the highest xylitol production obtained (12 g/L), with a yield of 0.35 $g_{xylitol}/g_{xylose}$ [37]. In the present work, direct fermentation of BSG and corncobs raw hydrolysates led to higher yields of xylose conversion into xylitol, namely, 0.39 and 0.36 $g_{xylitol}/g_{xylose}$, respectively.

3.3.2. Bioreactor cultivations with BSG hydrolysates

Given the promising results of xylitol production from BSG raw hydrolysate in shake flask, the process was scaled-up to a bioreactor cultivation. The bioreactor cultivation was carried out in batch mode, the pH was maintained at 5.0 by automatic addition of 5 M NaOH or 2 M HCl and the air flow rate was constant and equal to 1 L/min.

The results, present in Figure 8, demonstrate the yeast preference for sugars, as it consumed all glucose available for growth, expressed by the increase of CDW, from 0.9 to 5.5 g/L, during the first 15 h. A significant uptake of xylose and arabinose was only observed afterwards, until their exhaustion at 45 h of cultivation, approximately (Figure 8). This sequential sugar uptake is in accordance with the data reported for most yeasts. For example, Araújo et al. (2017) found out that *K. pastoris* preferred glucose as substrate for growth and, only after glucose exhaustion, there was xylose uptake by yeast with the concomitant production of xylitol [33]. In a different study, Kamat et al. (2013) evaluated the ability of the yeast *Cyberlindnera saturnus* to produce xylitol from corncobs hydrolysate. A total glucose assimilation was completed by 24 h of incubation, followed by xylose utilization that began at 24 h, but xylitol production was only noticed at 48 h and increased till 144 h [103]. This order of preference has been attributed to the competition for transport systems and glucose repression of the enzymatic machinery for pentose assimilation. [7]. Preziosi-Belloy et al. (1997) evaluated the sugar assimilation by the yeast

Candida parapisilosis from aspenwood hemicellulosic hydrolysate and observed low enzyme activities during glucose uptake [104].

Under the described conditions, xylitol and arabitol productions reached a maximum value of 0.94 g/L and 0.61 g/L, respectively, after 73 h of cultivation (Figure 8). In fact, the highest xylitol and arabitol concentrations were detected approximately 24 h after pentoses exhaustion. It may take some time for yeast to produce and excrete the polyols or there is a minimum concentration of polyols inside the cells required to trigger their release. Additionally, some the polyols produced could be being converted into xylulose, that integrates the central metabolism of carbon, leading to biomass production, justifying the increase of CDW observed in the absence of glucose.

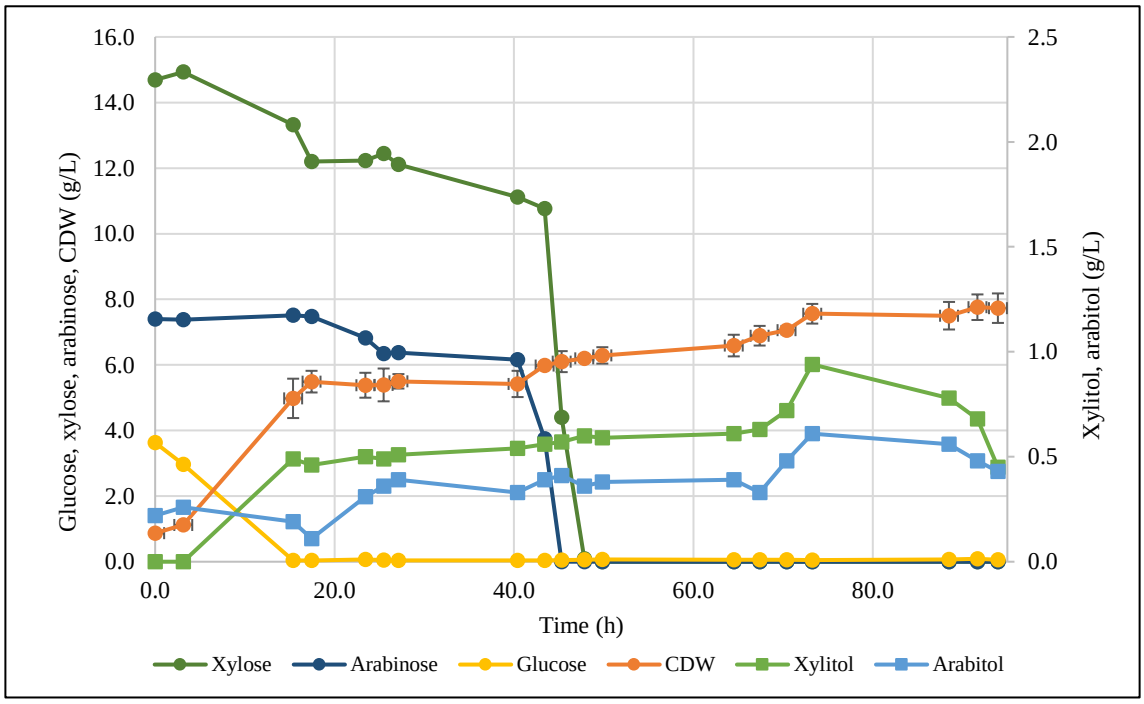


Figure 8 – Cultivation profile and production of polyols by *K. pastoris* using raw BSG hydrolysate as the sole carbon source.

The bioreactor production of polyols revealed to be slightly lower than the obtained in the shake flask assays (1.51 g/L of xylitol and 0.90 g/L of arabitol) for the same raw hydrolysate (Table 9). However, xylitol productivities were similar in shake flask (0.30 g/(L.d)) and bioreactor (0.31 g/(L.d)) cultivations.

In an attempt to increase xylitol and arabitol fermentation from the BSG raw hydrolysates in bioreactor cultivations, the effect of pH was investigated. The bioreactor cultivation was performed using the conditions described above, however, after the exponential phase of growth, pH control was turned off. In other words, after the first 24 h of cultivation, there was no pH control, in order to have culture conditions closer to the shake flask assays.

In this experiment, lower productions were achieved: 0.35 g/L of xylitol and 0.15 g/L of arabitol (Figure 9). By observing the growth profile in Figure 9, there was a significant increase of CDW after 55 h of cultivation, with the simultaneous pH increase up to 7.0. This finding suggests that, probably, the BSG raw hydrolysate have complex sugars in its composition and, at pH 5, the yeast is not able to produce the enzymatic machinery needed to convert those complex sugars into monomers, which can thus be assimilated for cell growth.

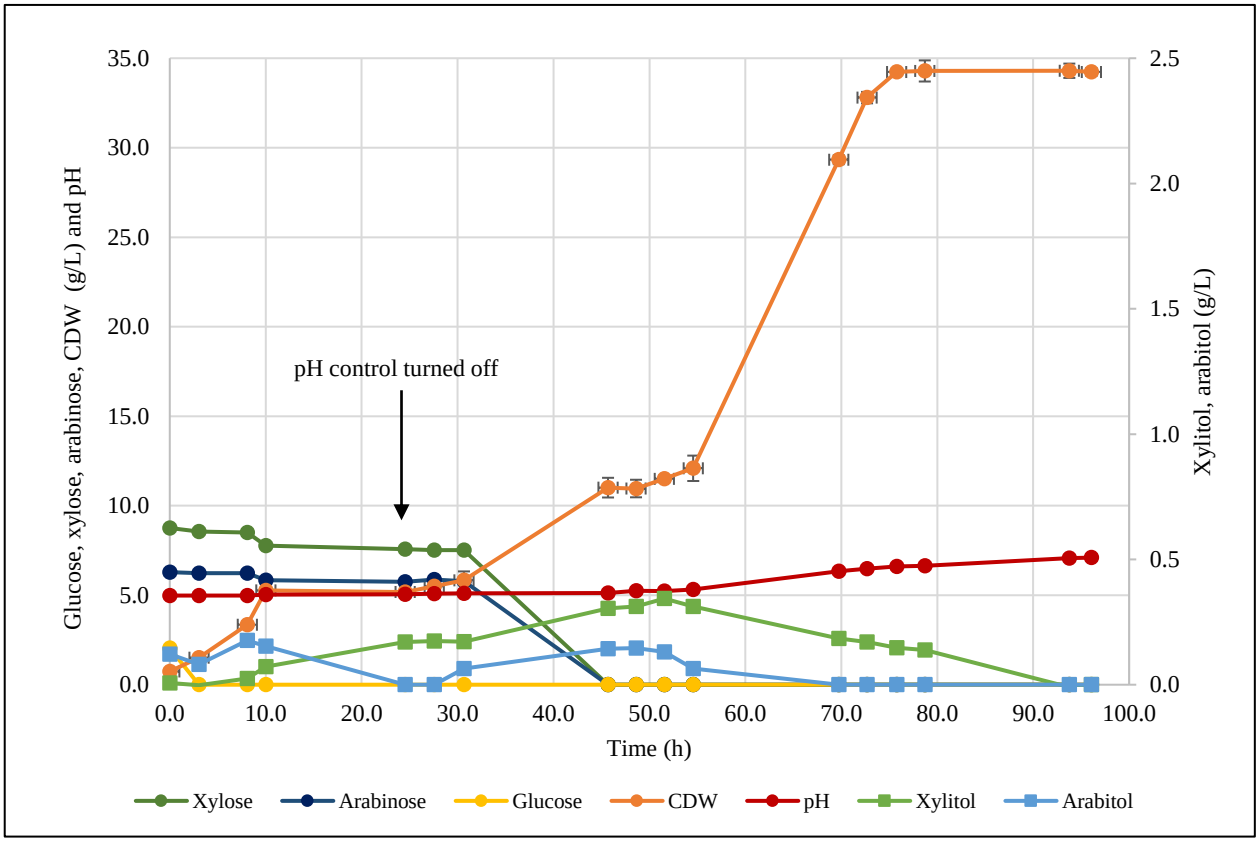


Figure 9 – Effect of pH on the cultivation profile and the production of polyols by K. pastoris, using raw BSG hydrolysate as the sole carbon source.

Interestingly, as in the previous bioreactor assay, there was a complete consumption of xylose and glucose, but in this case, it was not reflected in a considerable increase of polyols production, but translated into a preference for growth. This may have happened because under fully aerobic conditions, NADPH is rapidly oxidized to NAD^+, which favors oxidation of xylitol to xylulose by XDH. Xylulose is the integration point with the pentoses phosphate pathway and the central metabolism of carbon, leading to the production of energy, biomass and certain intermediates, namely ethanol [7]. In fact, ethanol was detected by HPLC in several cultivation samples, however, the complexity of the hemicellulosic hydrolysates hindered a proper quantification, since some peaks were partially overlapped.

Oxygen availability regulates the carbon consumption balance for growth and bioconversion. Thus, aeration plays an important role in polyols production, interfering with the redox imbalance of $NADPH/NAD^+$, which is crucial for the two-step conversion mechanism of

xylose into xylitol and xylitol into xylulose [4]. For example, Fonseca el al. (2007) conducted a study using mixtures of arabinose and xylose for assays with the yeast *P. guilliermondii* [131]. It was discovered that under oxygen limited conditions, *P. guilliermondii* accumulated more xylitol from xylose (0.63 g/g) and, a considerable amount of arabitol was also produced from arabinose (0.47 g/g) [131]. It has been reported in several studies that restriction of the oxygen availability increases the $NADPH/NAD^+$ ratio, reducing the enzymatic activity of XDH, and consequently, the conversion of xylitol into xylulose is inhibited, favoring xylitol accumulation [7].

The effect of aeration was also investigated in bioreactor cultivations using the BSG raw hydrolysate. Thus, the air flow rate was set to 1 L/min for the first 24 h (growth phase) and, then, decreased to 0.4 L/min until the end of cultivation. When the airflow rate was changed to 0.4 L/min, the pO_2 available in the culture medium was 70 % and, within 24 h, a decreased to 47 % was observed. After that 48 h of cultivation, the pO_2 available dropped to 5 % and the oxygen limitation was observed until 68 h of cultivation. Finally, an increase in pO_2 to 85 % was noticed at 72 h of cultivation, which remained constant until the end. In addition, the pH was adjusted to 5.0 prior to the cultivation, but the assay was performed with no pH control.

Once again, the changes in cultivation conditions did not reflect increased production efficiency (0.45 g/L of xylitol and 0.17 g/L arabitol) but revealed interesting findings (Figure 10).

The absence of pH control did not result in severe pH fluctuations, a decrease from pH 5 to 4.2 was noticed at the first 24 h and then a slight increase to pH 4.3 was observed, which remained practically constant until the end of cultivation. Dasgupta et al. (2017) reported that a bioreactor without pH-stat could successfully provide an effective production xylitol, because contrary to organic acids production, in general, polyols production does not lead to great variations in the reactor proton concentration [9]. In the cultivation described above (Figure 9), there was a pH increase to 7, but polyols production was negligible, however, the production of organic acids may have occurred. Here, even though the use of the same carbon source, that pH fluctuation was not noticed, probably due to the oxygen limitation, which interferes with yeast metabolism.

Glucose uptake was similar to the cultivations with a constant airflow rate of 1L/min (Figure 8 and 9), because the first 24 h were not limited by oxygen. It has been reported that transport of glucose requires aerobic conditions [105]. By opposition, xylose assimilation was slower under limited oxygen conditions and its exhaustion only occurs at 70 h, more than 20 h later when compared to fully aerobic conditions. The delay in xylose assimilation could be expected, because under oxygen limitations the sugar transportation faces up catabolic repression.

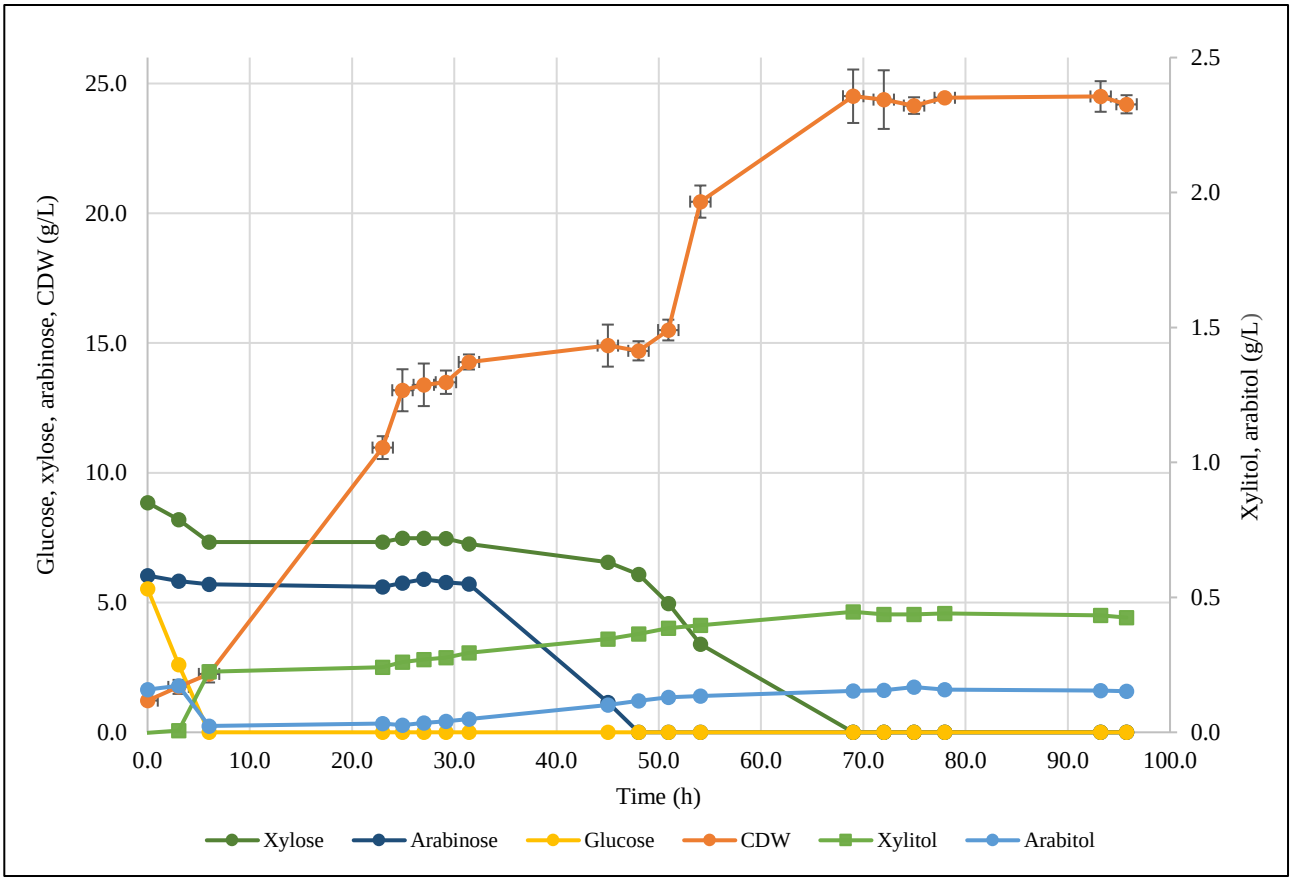


Figure 10 – Effect of pH and limited oxygen conditions on the cultivation profile and the production of polyols by *K. pastoris*, using raw BSG hydrolysate as the sole carbon source.

Of particular interest is the maintenance of polyols produced under restricted oxygen conditions, because after reaching the maximum value, the polyols concentration in the medium remains constant by the end of cultivation (Figure 10). By opposition, when the yeast was cultivated with a constant airflow rate of 1L/min, the quantity of polyols produced started to disappear after the maximum value is reached (Figure 8 and 9). This polyols consumption may be used to satisfy the yeast energy demand. Kumdam et al. (2014) discovered the ability of *D. nepalensis* to produce arabitol during the stationary phase and, then, observed arabitol assimilation for the yeast survival [6].

3.3.3. Effect of concentration of raw hydrolysates

Initial sugars concentration has been reported to have a significant influence on polyols productivity improvement. It depends on the microorganism and the carbon source used (synthetic solutions or hydrolysates) [7]. For example, in a study conducted by Tamburini et al. (2015) for the optimization of xylitol production by using *Candida tropicalis*, it was evident that an increase in xylose concentration from 40 to 80 g/L led to a productivity improvement from 0.20 to 0.60 g/L/h [106]. Besides that, concentrations higher than 80 g/L had inhibitory effects, for example 100 g/L of xylose led to a 60 % decrease in productivity in comparison with the

values obtained for 80 g/L of xylose [106]. Similarly, Serrano et al. (2018) found out an increase in xylitol production from 0.54 g/L to 12.00 g/L, by increasing initial xylose concentration from 10 to 60 g/L [37].

The raw hydrolysates employed in this work did not have very high sugars concentrations, with a total sugars content (glucose+xylose+arabinose) bellow 25.7 g/L. Especially, in the bioreactor cultivations described above (section 3.3.1), glucose, xylose and arabinose were completely exhausted during cultivations. Bearing this in mind, different experiments were performed using concentrated hydrolysates. If there are more sugars available in the culture medium, it would be expected that yeast growth and polyols production might also increase.

The first experiments were carried out in shake flask, using the same pH, aeration, temperature, agitation and cultivation time, described for the screening assays present in section 3.3.1. However, in this case, BSG raw hydrolysates were prior concentrated in a drying oven and then used as feedstock. Concentrated banana peels hydrolysate was also included in these assays because, despite not being the most interesting carbon source for direct polyols production, it is very promising for yeast growth, due to its high glucose concentration (8.20 g/L – Table 9). This may open the possibility to use different hydrolysates in the same cultivation assay for distinct purposes, for example, one directed for growth (rich in xylose) and other for polyols production (rich in xylose and arabinose).

The results presented in Table 11 revealed that the cultivation with twofold concentrated BSG had similar results in comparison with BSG raw hydrolysate cultivation (Table 9), attaining a similar production of 1.56 g/L of xylitol. Once again there was only a xylose uptake of 40 %, which may explain the similarity in xylitol production. As expected, twofold concentration BSG contained more glucose (12 g/L) than the raw hydrolysate (5 g/L), resulting in a higher amount of biomass produced, 13.73 g/L. Increase cultivation time it would be critical to understand the real effect of twofold concentrated BSG in the xylose bioconversion into xylitol, to evaluate if the yeast would be able to assimilate the 60 % of xylose that was still in the culture medium to produce xylitol and, thus, justify the addition of a concentration step to the process.

Table 11 – Effect of concentration of raw BSG and banana peels hydrolysates on polyols production by *K. pastoris*, in shake flask assays.

Feedstock	Glucose initial (g/L)	Glucose final (g/L)	Xylose initial (g/L)	Xylose final (g/L)	Arabinose initial (g/L)	Arabinose final (g/L)	5-HMF initial (g/L)	Acetic acid initial (g/L)	Biomass produced (g/L)	Xylitol (g/L)	Arabitol (g/L)
BSG hydrolysate concentrated 2 x	12.09	0.00	13.14	7.68	9.44	5.63	0.18±0.05	0.91±0.04	13.73±0.25	1.56±0.07	0.43±0.05
BSG hydrolysate concentrated 3 x	9.06	8.73	19.75	18.02	9.52	9.52	0.79±0.07	2.92±0.12	0.87±0.04	0.03±0.01	0.01±0.00
Banana peels hydrolysate concentrated 2 x	15.52	13.66	3.26	3.88	3.53	3.75	1.31±0.09	0.97±0.03	2.45±0.12	0.00±0.00	0.02±0.01

Threefold concentration of BSG and twofold concentration of banana peels were not effective strategies, since production of polyols was not noticed, neither considerable amounts of biomass. It becomes evident that sugar uptake was inhibited, because neither glucose, the preferential sugar, was assimilated. The most probable explanation for this inhibition is the consequent concentration of toxic compounds, such as, acetic acid and 5-HMF as a result of drying oven process, namely, 2.92 and 0.97 g/L of acetic acid and 0.79 and 1.31 g/L of 5-HMF, respectively.

For hemicellulosic hydrolysates, it has been reported that the concentration of toxic compounds as a result of the concentration of sugars is also a major determinant in polyols production [99]. For example, in similar conditions, using rice straw and eucalyptus hydrolysates (both with 60 g/L of xylose) for xylitol production by *C. guilliermondii FTI 20037*, different xylitol yields were obtained, namely, 0.65 and 0.20 $g_{xylitol}/g_{xylose}$, respectively [107, 108]. The high concentration of acetic acid (6.33 g/L) in eucalyptus hydrolysate is the most probable explanation for a lower yield [108].

A number of studies have been focused in understanding the effect of toxic compounds present in hemicellulosic hydrolysates in cultivations with different yeast. Yeast strains exhibited differences in inhibitor tolerance, some of them have been reported the yeast ability to convert these inhibitory by-products into less toxic compounds [109].

3.3.4. Bioreactor cultivation with banana peel hydrolysate

In order to comprehend the *K. pastoris* behaviour in the presence of acetic acid and 5-HMF, twofold concentrated banana peel hydrolysate was used as feedstock in a bioreactor cultivation with an airflow rate of 1 L/min. As in the previous bioreactor assay, there was no pH control (initial pH of 5).

Interestingly, Figure 11 shows that *K. pastoris* used the toxic compounds first and sugar assimilation started at 40 h of cultivation, when there was no acetic acid and 5-HMF detected in the culture medium. These findings suggested that probably *K. pastoris* transformed the toxic compounds into less toxic metabolites, as evidenced by the rapid yeast sugar uptake and a concomitant increase in biomass production from 2.83 g/L to 13.89 g/L after exhaustion of acetic acid and 5-HMF in the cultivation medium. Similar data have been reported in literature. For example, Taherzadeh et al. (2000) studied the physiologic effects of 5-HMF on the yeast *S. cerevisiae*, using 50 g/L of glucose as carbon source and 4 g/L of 5-HMF [110].

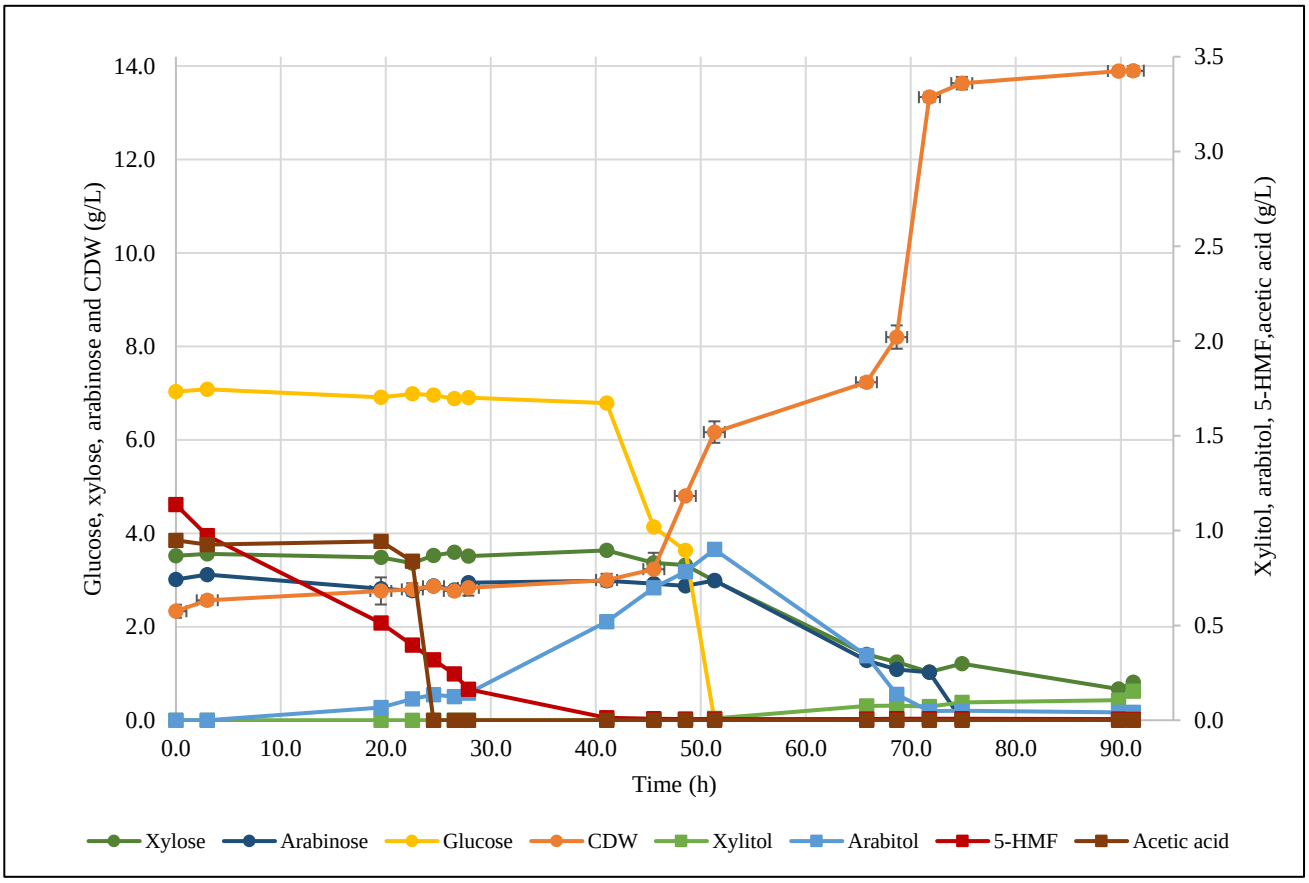


Figure 11 – Effect of twofold concentration raw banana peels hydrolysate on the cultivation profile and the production of polyols by *K. pastoris.*

The yeast was able to convert 5-HMF into 5-hydroxymethylfurfuryl. Moreover, when furfural and 5-HMF were both added, yeast growth was totally inhibited while both compounds were still present in the medium [110]. Akillioglu et al. (2011) also observed that 5-HMF decreased exponentially, attaining high yields (79-84 %) of 5-HMF conversion into its furfuryl alcohol [111].

The results also expressed Figure 11 also indicated that there was no xylose bioconversion into xylitol, whereas arabitol was produced, achieving a concentration of 0.90 g/L at 51 h of cultivation, but arabinose consumption only started afterwards. Thus, it is evident that there was no arabinose bioconversion into arabitol. The presence of arabitol in the culture medium may be explained as having a cell protection role against the environmental stress, caused by the high levels of toxic compounds. This assumption is supported, for example, by a study conducted by Dragosits et al. (2010), to evaluate the effect of different osmolarities on *K. pastoris* [112]. Surprisingly, arabitol was the main compound released to compensate the osmotic stress, instead of glycerol. In medium and high osmolarities conditions were observed 3- and 4-fold increases in arabitol concentration, respectively, in comparison with low osmolarities [112]. In another study, Kayingo et al. (2005) observed arabitol production by *C. albicans* in response to oxidative stress [113]. In addition, osmophilic yeasts are characterized by their ability to accumulate arabitol in

response to stress induced by high/low salt and sugar concentration, preventing the dehydration or the swelling, respectively [6].

Interestingly, after reaching the maximum concentration, arabitol was assimilated by the yeast and a concomitant biomass production was noticed. This indicates that arabitol can be possibly used as substrate by *K. pastoris*.

3.3.5. Detoxification of BSG hydrolysates

In this work is perceptible that dilute sulphuric acid hydrolysis is an important step to convert hemicellulose into monomeric sugars. However, it also generates toxic compounds which, in most cases, slow down or inhibit yeast growth and/or production of polyols [18].

Commonly, in order to increase the initial sugars concentration, the hemicellulosic hydrolysates are subjected to concentration processes and, consequently, the liquors obtained are potentially more toxic to microorganisms [7]. Generally, to overcome the toxicity problem, the hydrolysates are exposed to detoxification treatments, aiming the removal of the toxic compounds.

In section 3.3.2., the negative effect of toxic compounds present in concentrated hydrolysates on yeast growth and polyols production was evident. For that reason, a detoxification treatment using activated charcoal was applied to some hemicellulosic hydrolysates. In this way, BSG raw hydrolysates were subjected to concentration processes followed by activated charcoal treatments. The hydrolysates obtained were used as feedstock in shake flask assays, in the same conditions employed in the previous cultivations (section 3.3.2), but with longer duration (168 h).

Figure 12 gives an overview of the processes from the lignocellulosic matter until the bioproduction assays.

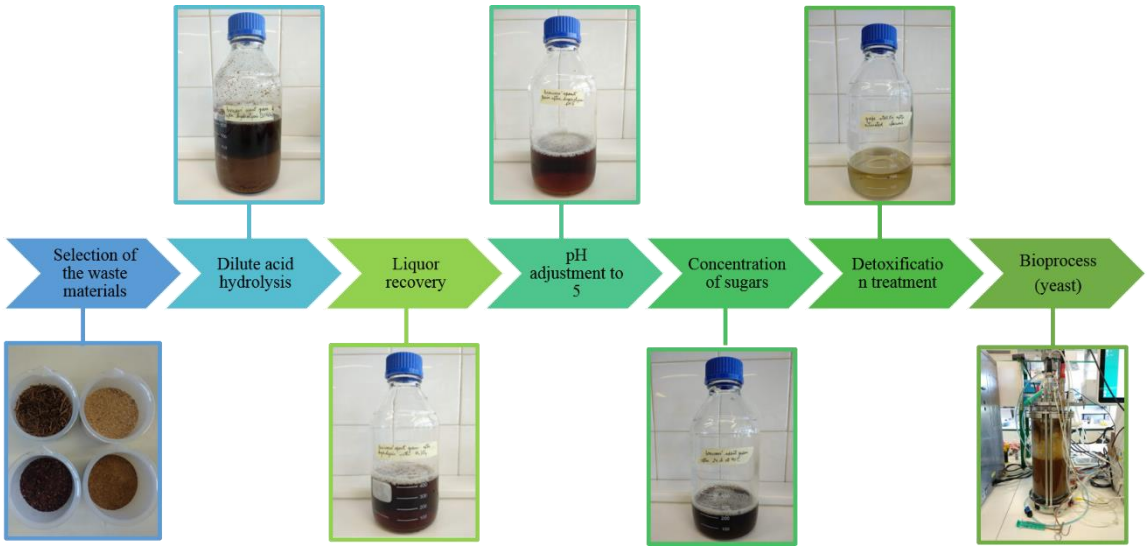


Figure 12 – Schematic representation of the steps involved in polyols production, from the lignocellulosic waste materials until de bioproduction assays, including the transformation of the raw materials occurred in each step.

According to the results present in Table 12, threefold concentrated BSG subjected to a detoxification treatment with activated charcoal revealed a great improvement in the production of biomass and xylitol, in comparison with non-detoxified threefold concentrated BSG, where no production of biomass or polyols was observed (Table 11). The decreased concentration of toxic compounds (33 % for acetic acid and 72 % for 5-HMF) is the most probable explanation for the production of 20.26 g/L of biomass and 3.97 g/L of xylitol, reaching a yield of 0.47 $g_{xylitol}/g_{xylose}$ (Table 12). The final threefold concentrated hydrolysate revealed a decrease in the content of acetic acid (1.96 g/L) and 5-HMF (0.22 g/L) in comparison with the non-detoxified hydrolysate (2.92 and 0.79 g/L, respectively). This reduction was not expected, since the results of Chapter 2 showed that activated charcoal treatment was not effective to remove acetic acid. Therefore, the removal of acetic acid may be explained by an additional step used to sterilize the hydrolysates, autoclaving them (121 °C and 1 bar) for 20 min. Due to the high temperature applied in the sterilization step, there was probably a partial evaporation of the acetic acid. The concentration of the hydrolysates by evaporation at high temperatures have been reported as a detoxification method, leading to a complete removal of furfural and partial elimination of acetic acid [114]. In addition, as expected in accordance with the results presented in Chapter 2, there was an efficient removal of 5-HMF during the detoxification treatment of threefold and six-fold concentrated BSG hydrolysates.

Table 12 – Effect of detoxification treatment applied to concentrated BSG hydrolysates on polyols production by *K. pastoris*, in shake flask assays.

Feedstock	Glucose initial (g/L)	Glucose final (g/L)	Xylose initial (g/L)	Xylose final (g/L)	Arabinose initial (g/L)	Arabinose final (g/L)	5-HMF initial (g/L)	Acetic acid initial (g/L)	Biomass produced (g/L)	Xylitol (g/L)	Arabitol (g/L)
BSG hydrolysate concentrated 3 x	13.78	0.29	18.29	9.95	10.56	9.07	0.22	1.96	20.26±0.25	3.97±0.10	0.82±0.05
BSG hydrolysate concentrated 6 x	27.56	25.08	36.58	35.41	21.11	21.11	0.24	3.92	2.03±0.17	0.01±0.01	0.05±0.02

The improvement in the polyols production due to activated charcoal treatment is in accordance some studies reported in literature. In et al. (2001), for example, was able to improve the production of xylitol by the yeast *C. guilliermondii* by 22 %, using activated charcoal to detoxified concentrated rice straw hydrolysates used as feedstock, leading to an increase in the xylitol/xylose yield from 0.59 g/g to 0.72 g/g [115].

The six-fold increased concentration of BSG hydrolysate led to a sugar-rich hydrolysate, containing 27.56 g/L of glucose, 36.58 g/L of xylose and 21.11 g/L of arabinose. However, the sugars concentration did not favored the bioprocess performance, because there was no production of biomass neither polyols. Once again, the high amount of acetic acid seems to have a negative impact on the process. In this case, the detoxification treatment was not a successful strategy. Additional detoxification processes should be performed in order to remove the acetic

acid, for example, the use of ion exchange resins that have proven to be efficient in the removal of that compound [116].

In order to evaluate the effect of the detoxification treatment in scale-up cultivations, a bioreactor assay was performed using twofold concentrated BSG hydrolysate treated with activated charcoal as feedstock. The initial pH was adjusted to 5.0, there was no pH control during the assay and the airflow rate was maintained at 0.5 L/min.

Interestingly, some differences in the results obtained stand out (Figure 13), namely, the increase in glucose consumption rate and the earlier assimilation of xylose and arabinose by yeast. These findings suggest that a slight inhibition occurred when BSG hydrolysates were not detoxified, because even when BSG raw hydrolysates were used in bioreactor cultivations (Figures X8, X9 and X10), the glucose was assimilated at a lower rate. For example, Carvalheiro at al. (2005) conducted an experiment for xylitol production by yeast *D. hansenii*, using concentrated BSG hydrolysate and concentrated BSG hydrolysate subjected anion-exchange resins treatment. A reduction of the lag phase from 5 to 3 h was noticed after detoxification treatment, probably due to the reduction of toxic compounds [117].

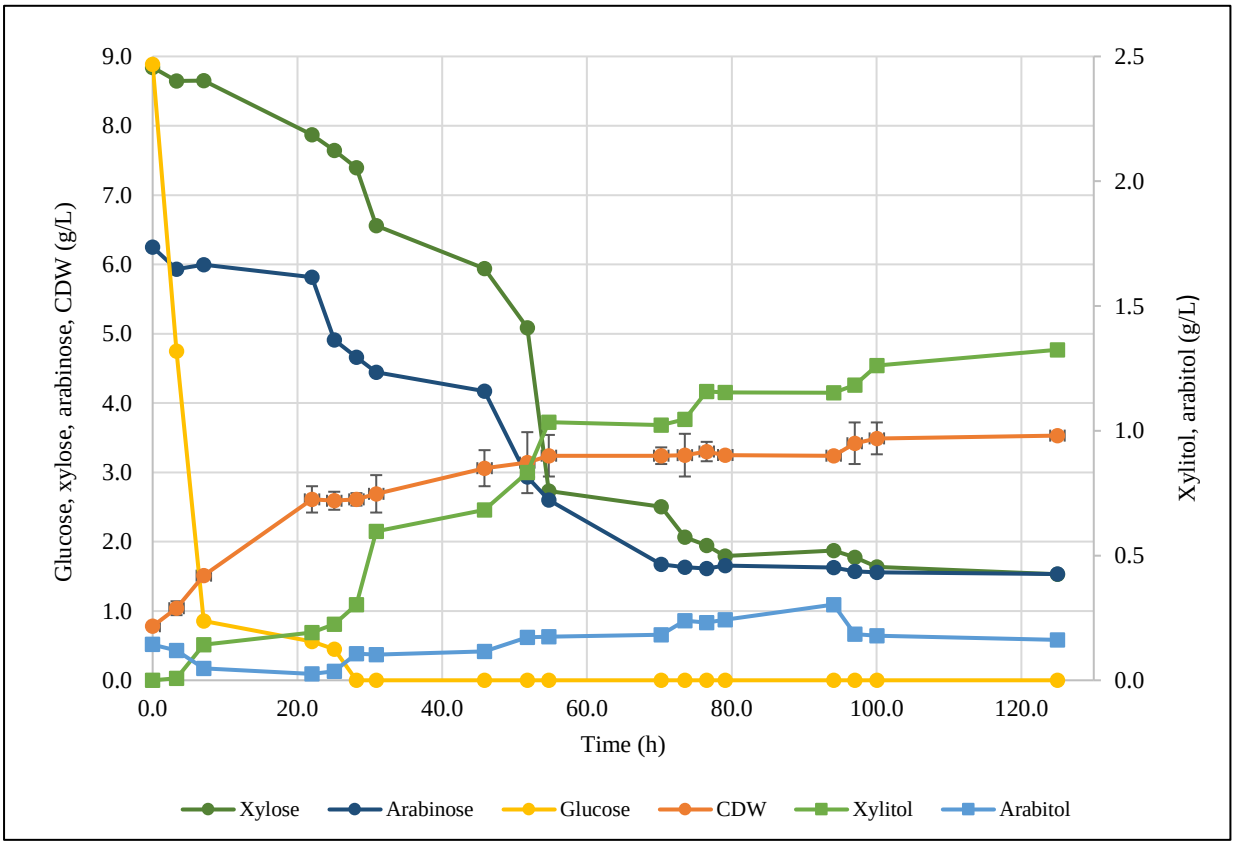


Figure 13 – Effect of hydrolysate's detoxification treatment on the cultivation profile and the production of polyols by *K. pastoris*, using twofold concentrated BSG hydrolysate as the sole carbon source.

Glucose consumption did not lead to the expected growth, achieving only 3.06 g/L of biomass. The reduction in the airflow rate from 1 L/min to 0.5 L/min was perhaps the reason for that, because *K. pastoris* requires considerable amounts of oxygen to achieve high cell densities [118]. In addition, the decrease in the airflow rate alongside with some limitations in the transportation of xylose and arabinose into the cells, possibly contributed for the non-consumption of all xylose and arabinose available. Nevertheless, the yeast achieved a maximum xylitol production of 1.33 g/L, a higher value than those obtained in the previous bioreactor assays. A relatively low xylitol/xylose yield of 0.18 g/g was obtained, because the maximum xylitol production occurred after 125 h of cultivation and some of xylose was possibly consumed for yeast survival, not being exclusively used for bioconversion into xylitol.

As already mentioned, experiments with different lignocellulosic wastes and microorganism have been vastly investigated in literature for polyols production. Table 13 reviews some that wastes, as well as, some yeasts that have been studied mainly for xylitol production, comparing them with the results obtained in this section of this work. *Candida sp.* have a strong presence in the Table 13, since they have been reported as the best xylitol producers, attracting a lot of interest.

Table 13 – Production of xylitol and/or arabitol from different lignocellulosic wastes by yeasts.

Lignocellulosic waste	Microorganism	Fermentation Mode	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	CDW (g/L)	Xylitol (g/L)	$Y_{P/S}$ ($g_{xylitol}$ /g_{xylose})	Arabitol (g/L)	$Y_{P/S}$ ($g_{arabitol}$ /$g_{arabinose}$)	Reference
BSG	*K. pastoris* DSM 70877	Batch (shake flask)	13.78	18.29	10.56	20.26	3.97	0.47	0.82	0.52	This work
BSG	*K. pastoris* DSM 70877	Batch (bioreactor)	8.89	8.84	6.25	3.06	1.33	0.18	0.30	0.05	This work
BSG	*D. hansenii* CCMI 941	Batch (shake flask)	21.60	60.60	24.80	n.a.	n.a.	0.39	n.d.	-	[117]
Corncobs	*C. tropicalis* As 2.1776	Fed-batch (bioreactor)	3.24	31.25	3.91	11.30	96.50	0.83	n.d.	-	[69]
Corncobs	*C. tropicalis* W103	Fed-batch (bioreactor)	5.40	28.70	3.70	5.12	68.40	0.70	n.d.	-	[59]
Corncobs	*D. nepalensis* NCYC 3413	Batch (shake flask)	1.80	51.70	3.40	n.a.	14.60	0.3	n.d.	-	[119]
Corn stover	*P. stipitis* NRRL Y-30785	Batch (shake flask)	2.39	21.5	1.60	n.a.	12.50	0.61	n.d.	-	[120]
Corn fiber	*Candida entomaea NRRL* Y-7785	Batch (shake flask)	n.a.	6.50	4.90	n.a.	0.65	0.10	2.69	0.64	[121]
Corn fiber	*Pichia guilliermondii NRRL* Y-2075	Batch (shake flask)	n.a.	6.50	4.90	n.a.	0.62	0.10	2.58	0.63	[121]
Sugarcane bagasse	*C. guilliermondii* FTI20037	Batch (shake flask)	1.82	15.73	1.45	9.78	50.50	0.81	n.d.	-	[116]
Sugarcane bagasse	*C. tropicalis* CLQCA-24F-125	Batch (shake flask)	5.80	61.70	7.60	11.07	27.12	0.67	n.d.	-	[122]
Horticultural waste	*C. athensensis* SB18	Batch (bioreactor)	8.58	36.47	2.87	12.07	100.10	0.81	n.d.	-	[123]
Bamboo culm	*C. magnoliae*	Batch (bioreactor)	3.60	19.00	1.40	7.90	10.50	0.59	n.d.	-	[124]
Wood sawdust	*C. tropicalis* BCRC 20520	Batch (shake flask)	4.63	9.51	0.91	n.a.	41.4	0.70	n.d.	-	[125]

n.a. data not available; n.d. not detected

Contrary to *K. pastoris*, the presented yeasts use xylose as a carbon source for growth. Thus, *K. pastoris* may use all xylose available in hemicellulosic hydrolysates in a more efficient way, converting it only into xylitol, which represents an advantage.

Evidently, *Candida sp.* reach great productions of xylitol with high yields (Table 13). For example, a bioreactor assay using the yeast *C. athensensis* and agricultural waste as carbon source (200 g/L xylose), resulted in a xylitol production of 100 g/L, with a yield of 0.81 g/g. In this study, the horticultural waste was prior subjected to a pretreatment in the presence of 1% H_2SO_4 and detoxified using activated charcoal [123].

In the present work the maximum xylitol/xylose yield obtained was 0.47 g/g, in erlenmeyer cultivations, using threefold concentrated BSG detoxified with activated charcoal. However, some other xylitol producer yeasts revealed lowers yields (Table 13). For example, Carvalheiro et al. 2005, investigated the effect of detoxification of BSG hydrolysate in xylitol production by *D. hansenii*, using anion exchange resins at pH 5.5, and achieved a yield of 0.39 $g_{xylitol}/g_{xylose}$ [117]. In another case, Paidimuddala et al. (2014) evaluated the ability of *D. nepalensis* to convert xylose-rich hydrolysates obtained from corncobs and found out a production of 14.6 g/L of xylitol, with a xylitol/xylose yield of 0.3 g/g [119]. Considering the pathogenic nature of *Candida sp.* and that the process of xylitol production from BSG hydrolysates using *K. pastoris* is not optimized, the results obtained in this section are promising.

3.3.6. Fed-batch shake flask cultivations

The depolymerization of hemicellulosic waste materials leads to sugar-rich hydrolysates, representing suitable fermentation media for polyols production. However, the composition of the hydrolysates influences the success and effectiveness of the bioproduction.

As noticeable by the results regarding the composition of the different hemicellulosic hydrolysates used in this work (Chapter 2), there are significant differences between them, namely, in sugars content, total phenols, toxic compounds and amino acids composition. In addition, these hydrolysates also contain other compounds that may affect the bioconversion process. For example, some vitamins (biotin, folic acid, niacin, choline, riboflavin, thiamine, pantothenic acid and pyridoxine), together with some minerals have been found in BSG hydrolysates, as well as, oligosaccharides and polysaccharides [49].

In order to evaluate a possible synergistic effect, resulting from the mixture of different hydrolysates, in polyols production by *K. pastoris*, some experiments were performed. In these studies, a fed-batch strategy was also implemented, aiming a theoretically increase in xylitol productivity. Concentrated sawdust hydrolysates subjected to activated charcoal detoxification were used as feed solutions after the yeast exponential growth, in the stationary phase where the

production of polyols occurs, since sawdust hydrolysates have the highest xylose content of all tested raw materials.

Initially, shake flask assays were carried out using BSG raw hydrolysates and grape stalks raw hydrolysates as feedstocks. After 72 h of cultivation, when the yeast reached the maximum growth, a pulse with threefold detoxified concentrated sawdust hydrolysate (25 mL) was performed in all cultivations. Aeration was an additional parameter evaluated, i.e., some cultivations were fully aerobic, whereas, other were fully aerobic during the growth phase (the first 72 h) and anaerobic afterwards until 168 h.

Curiously, regardless of the different conditions applied in the shake flask assays, similar results were achieved, as indicated in Table 14. Similar biomass productions (4.17 – 5.67 g/L) were expected, because the concentration of glucose in BSG and grape stalks raw hydrolysates had the close values (around 3.9 g/L) and fully aerobic conditions were set during growth phase for all assays.

Table 14 – Effect of the mixture of two hemicellulosic hydrolysates, including a pulse feed at 72h of cultivation, on polyols production by *K. pastoris*, in shake flask assays.

Feedstock	Pulse feed	Aeration conditions	Glucose initial (g/L)	Glucose final (g/L)	Xylose initial (g/L)	Xylose final (g/L)	Arabinose initial (g/L)	Arabinose final (g/L)	Biomass produced (g/L)	Xylitol (g/L)	Arabitol (g/L)
BSG raw hydrolysate	Threefold detoxified concentrated sawdust hydrolysate	Fully aerobic	3.89	0.00	7.41	0.48	3.59	0.00	5.55±0.23	1.95±0.05	0.18±0.02
BSG raw hydrolysate		Fully aerobic until the pulse feed and anaerobic afterwards	3.83	0.00	7.37	0.37	3.53	0.00	4.17±0.12	2.10±0.09	0.19±0.08
Grape stalks raw hydrolysate		Fully aerobic	3.98	0.02	3.75	0.52	1.20	1.25	5.67±0.16	2.21±0.12	0.18±0.09
Grape stalks raw hydrolysate		Fully aerobic until the pulse feed and anaerobic afterwards	3.92	0.00	3.65	0.79	1.15	1.17	5.11±0.08	1.96±0.0.07	0.19±0.04

BSG hydrolysates had higher xylose content than grape stalks hydrolysates (7.4 g/L and 3.6 g/L, respectively). More than 95 % was consumed in both cultivations in fully aerobic conditions and, around 80 % in limited oxygen conditions, but similar xylitol productions were reached in all cultivations, although the higher consumption of xylose in BSG assays. Limited regeneration of $NADPH^+$, an important cofactor required by XR to convert xylose into xylitol may be a plausible explanation for that [28].

In order to increase biomass production, a final set of shake flask experiments was designed. Concentrated banana peels hydrolysates were added to concentrated BSG hydrolysates and grape stalks hydrolysates, due to its advantageous glucose richness. Thus, half of feedstock solution had twofold concentrated banana peels hydrolysate and the other half twofold concentrated BSG or grape stalks hydrolysate. A pulse feed was also performed after 72 h, but in

these experiments, there was a six-fold concentration of sawdust hydrolysates, because in the previous cultivation, xylose has been almost totally exhausted. Once again, different aeration conditions were evaluated.

By observing Table 15, the addition of banana peels hydrolysates to the feedstock solutions had the intended effect. There was an increase of more than 50 % in cell density, reaching biomass concentration between 9.98 and 12.52 g/L.

Table 15 – Effect of the mixture of three hemicellulosic hydrolysates, including a pulse feed at 72h of cultivation, on polyols production by *K. pastoris*, in shake flask assays.

Feedstock	Pulse feed	Aeration conditions	Biomass produced (g/L)	Xylitol (g/L)	Arabitol (g/L)
Twofold concentrated BSG + banana peels hydrolysates	Six-fold detoxified concentrated sawdust hydrolysate	Fully aerobic	12.52±0.19	1.77±0.05	0.74±0.07
Twofold concentrated BSG + banana peels hydrolysates		Fully aerobic until the pulse feed and anaerobic afterwards	11.68±0.21	3.17±0.11	0.91±0.03
Twofold concentrated grape stalks + banana peels hydrolysates		Fully aerobic	10.30±0.12	0.79±0.09	0.13±0.09
Twofold concentrated grape stalks + banana peels hydrolysates		Fully aerobic until the pulse feed and anaerobic afterwards	9.98±0.28	0.91±0.04	0.11±0.02

Here, different xylitol productions were achieved in different culture conditions. A mixture of concentrated BSG and banana peels as the main feedstock, alongside with a concentrated pulse feed of sawdust hydrolysate, in anaerobic conditions during stationary phase, were the only cultivations to reveal an improvement in both polyols production, achieving 3.17 g/L of xylitol and 0.91 g/L of arabitol (Table 15). Curiously, despite presenting higher polyols productions, less xylose was consumed by the yeast under these limited oxygen conditions, in comparison with the correspondent fully aerobic assays (4.54 and 2.26 g/L of xylose left in the culture mediums after 168 h, respectively), which is in agreement with some results previously discussed in this work.

Advantages in polyols productions resorting to fed-batch fermentations have also been reported in literature, especially when hemicellulosic hydrolytes are used as feedstock, due to an attenuation of the inhibitory effects of toxic compounds. For example, Li et al. (2011) evaluated batch and two-stage fed-batch fermentation processes from corncob hydrolysates, using *Candida tropicalis* As 2.1776. Corncob hydrolysates were obtained by dilute acid hydrolysis (1.0 % H2SO4 (v/v), 121 °C, 40 min) and then subjected to vacuum concentration, overtitration and activated charcoal treatment, removing most of toxic compounds. Batch fermentation revealed a production of 58.3 g/L of xylitol. However, the results of two-stage fed-batch fermentation indicated a higher xylitol production of 96.5 g/L after 120 h, with a yield of 0.83 g/g [69]**.**

The combination of concentrated banana peels, grape stalks and sawdust hydrolysates was not a successful strategy, either under fully aerobic or semi anaerobic conditions, attaining 0.79 and 0.91 g/L of xylitol (Table 15), respectively, which did not even reach 50 % of the

production obtained in the mixture of grape stalks and concentrated sawdust hydrolysates (2.21 and 1.96 g/L of xylitol – Table 14).

3.3.7. Fed-batch bioreactor cultivations

Some of these assays using mixtures of hydrolysates from different raw materials and fed-bath strategies were also scaled-up to bioreactor cultivations.

As in shake flask assays, raw BSG hydrolysates were employed in two different fed-batch bioreactor cultivations, in which threefold concentrated and detoxified sawdust hydrolysate was selected as feed solution (500 mL for each assay). Aeration was the main difference between the cultivations, in one of the assays the airflow rate was 1 L/min during the growth phase and was decreased to 0.4 L/min afterwards and, in the other scenario, the airflow rate was maintained at 0.5 L/min all time.

By opposition to the shake flask assays, the results from bioreactor cultivations showed significant differences (Figures 14 and 15).

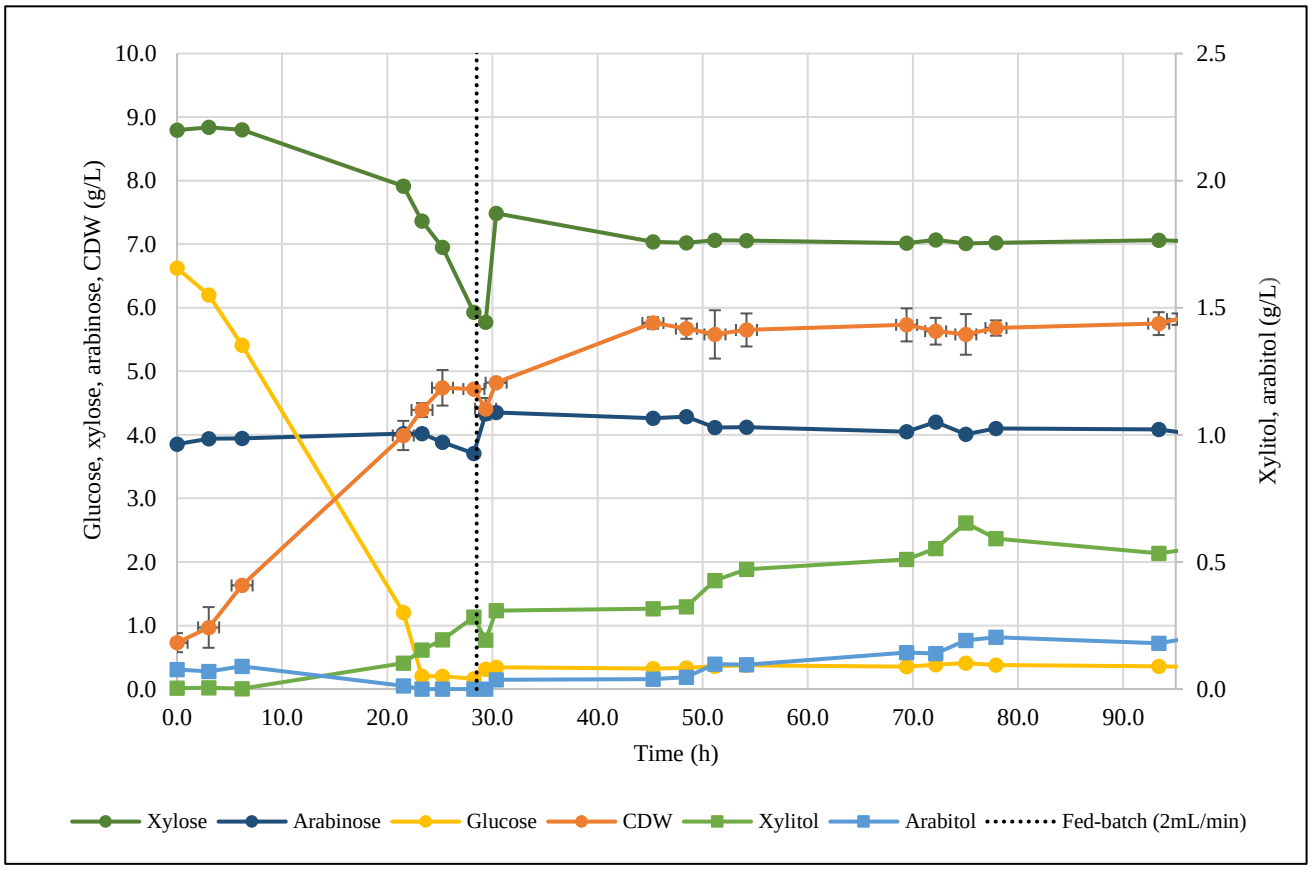


Figure 14 – Effect of fed-batch and air supply variations on the cultivation profile and the production of polyols by *K. pastoris*, using raw BSG hydrolysate as the initial feedstock and feeding of threefold concentrated and detoxified sawdust hydrolysate.

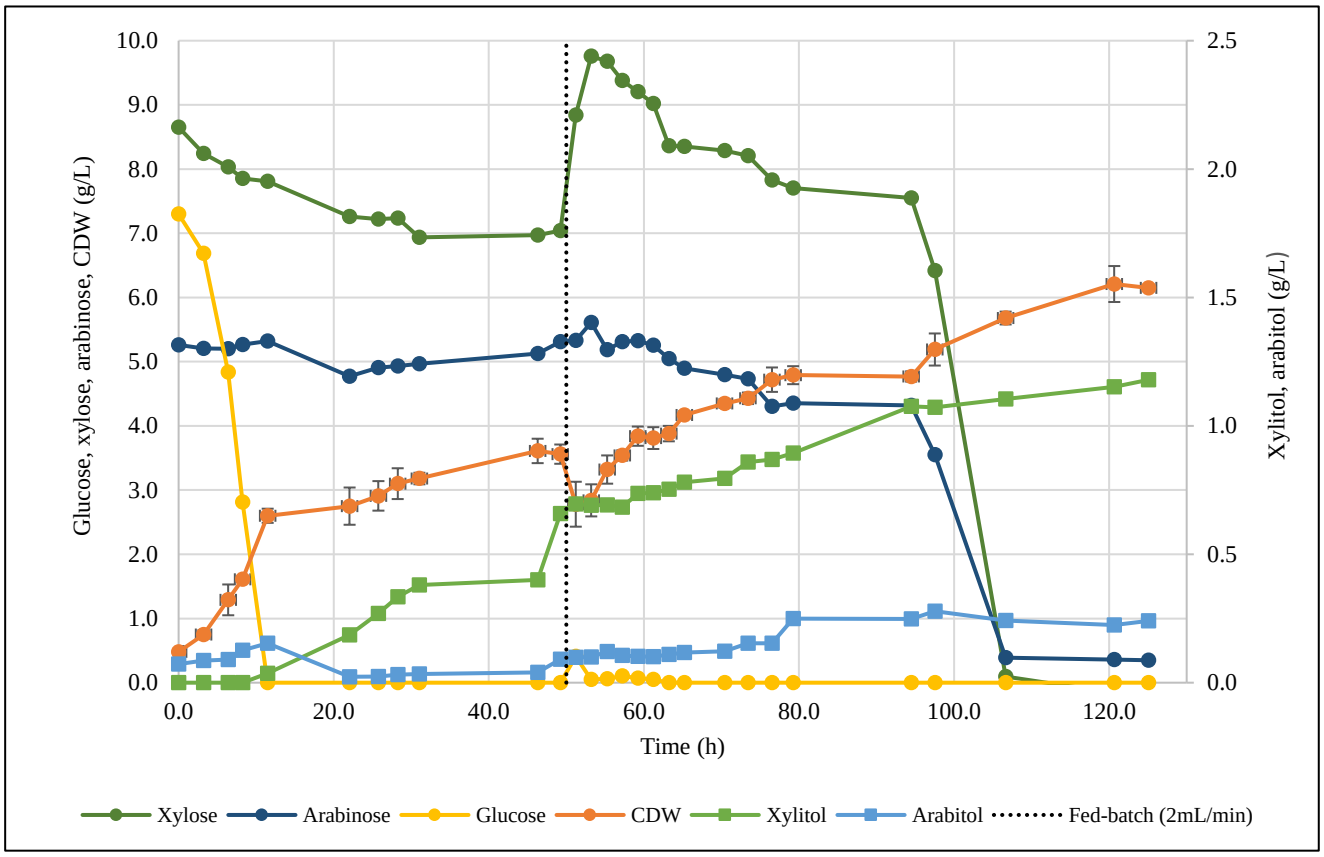


Figure 15 – Effect of fed-batch and a constant airflow rate on the cultivation profile and the production of polyols by *K. pastoris*, using raw BSG hydrolysate as the initial feedstock and feeding of threefold concentrated and detoxified sawdust hydrolysate.

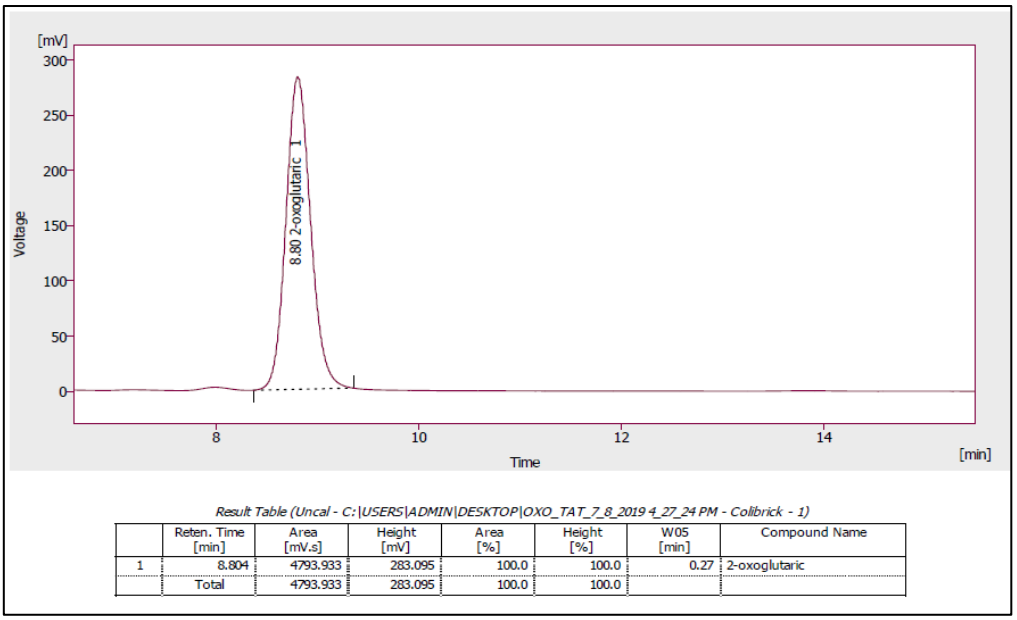
In the cultivation conditions selected, a constant airflow rate of 0.5 L/min has a greater boost on xylose bioconversion into xylitol, with a maximum production of 1.18 g/L (Figure 15), twice higher than the amount obtained in cultivation with changes in the airflow rate (0.65 g/L of xylitol), represented in Figure 14. Additionally, using an airflow rate of 1 L/min during growth phase led to a slower glucose uptake rate, but higher biomass production, in comparison with the use of 0.5 L/min. These findings are in agreement with the data reported in Figure 13, suggesting that a constant airflow rate of 0.5 L/min is profitable for xylitol production by *K. pastoris*.

Xylose and arabinose have been depleted after 105 h when 0.5 L/min of air was continuously supplied, whereas, there was no significant assimilation of the same sugars when the airflow rate was changed from 1 L/min to 0.4 L/min. The yeast adaptation to the new oxygen conditions may cause a lag phase, which may have conditioned the mechanism of sugars uptake.

3.3.8. Production of by-products

Despite the improvements in xylose bioconversion into xylitol by *K. pastoris* as a result of different strategies adopted, the production of xylitol is still below its theoretical potential.

Considering sugars exhaustion during some cultivations, the possibility of production of organic acids or other polyols was evaluated in some cultivation samples. The presence of sorbitol, mannitol and erythritol was not detected, neither significant amounts of succinic and lactic acids. However, during HPLC analysis, a peak with a significant subjacent area was detected at 210 nm in several samples, having a retention time around 38.5 min. Interestingly, an increase in peak area was observed with a concomitant disappearance of the toxic compounds present in the culture media. Thereby, there was a great hypothesis of that peak represent the less toxic byproduct resulting from the bioconversion of the toxic fermentation inhibitors. For example, the metabolic pathway of 5-HMF and furfural in *Cupriavidus basilensis* have been reported as containing several steps, ending in production of 2-oxoglutaric acid, which is detected at 210 nm [126]. Thus, a solution of 2-oxoglutaric acid was analyzed by HPLC. Despite its detection at the same wavelength, there was no match in the retention time, since 2-oxoglutaric acid was detected at 8.8 min (Figure 16).

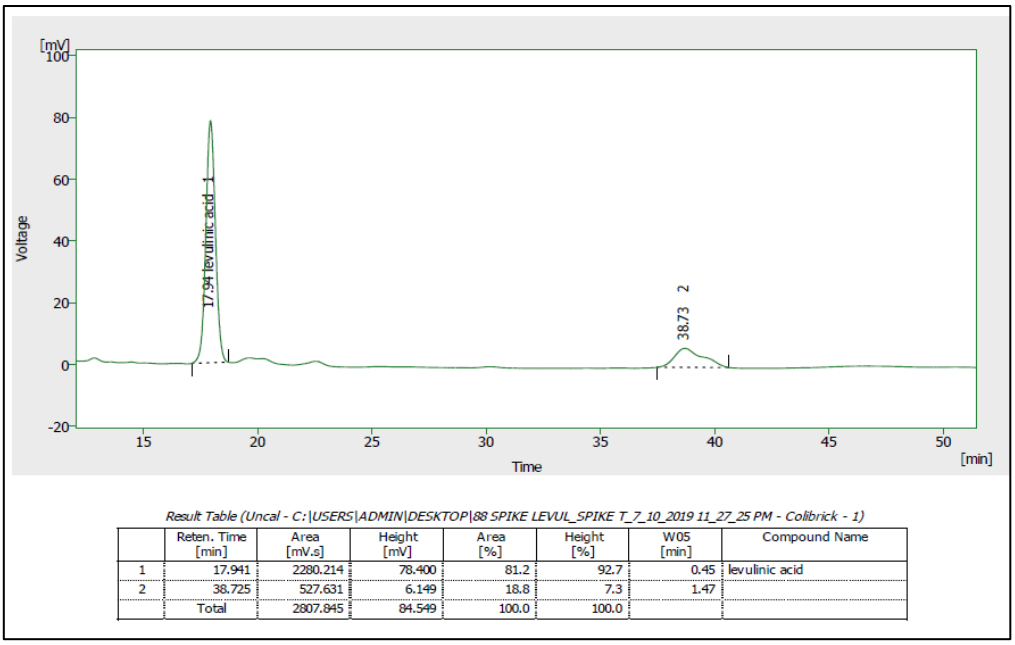


Result Table (Uncal - C:|USERS|ADMIN|DESKTOP|OXO_TAT_7_8_2019 4_27_24 PM - Colibrick - 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	8.804	4793.933	283.095	100.0	100.0	0.27	2-oxoglutaric
	Total	4793.933	283.095	100.0	100.0		

Figure 16 – HPLC spectra of a standard solution of 2-oxoglutaric acid.

For instance, levulinic acid could also represent a valid possibility, because it is one of the organic acids synthetized from renewable carbon sources, such as, lignocellulosic waste materials, through the bioconversion of 5-HMF, for example [127]. Once again, there was no match in the retention times during HPLC analysis, since levulinic acid had a lower retention time and two distinct peaks were observed (Figure 17).



Result Table (Uncal - C:\USERS\ADMIN\DESKTOP\88 SPIKE LEVUL_SPIKE T_7_10_2019 11_27_25 PM - Colibrick - 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	17.941	2280.214	78.400	81.2	92.7	0.45	levulinic acid
2	38.725	527.631	6.149	18.8	7.3	1.47	
	Total	2807.845	84.549	100.0	100.0		

Figure 17 – HPLC spectra of a standard solution of levulinic acid added to a cultivation sample.

Additionally, a different idea came up. Literature data has reported the synthesis of ethyl levilunate by esterification of levulinic (or levulinate) acid in the presence of ethanol. Beyond that route, ethyl levulinate can be obtained from furfural or directly produced from sugars, always in the presence of ethanol (Figure 18) [128]. As already mentioned, ethanol was detected in several cultivation samples, supporting this hypothesis.

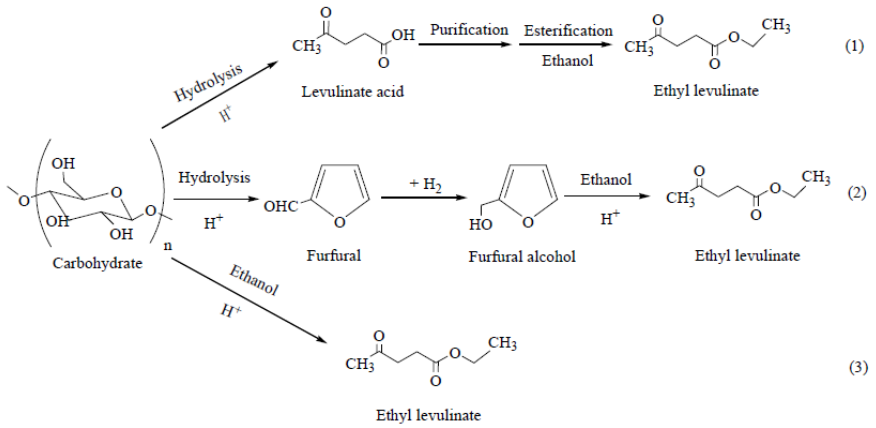
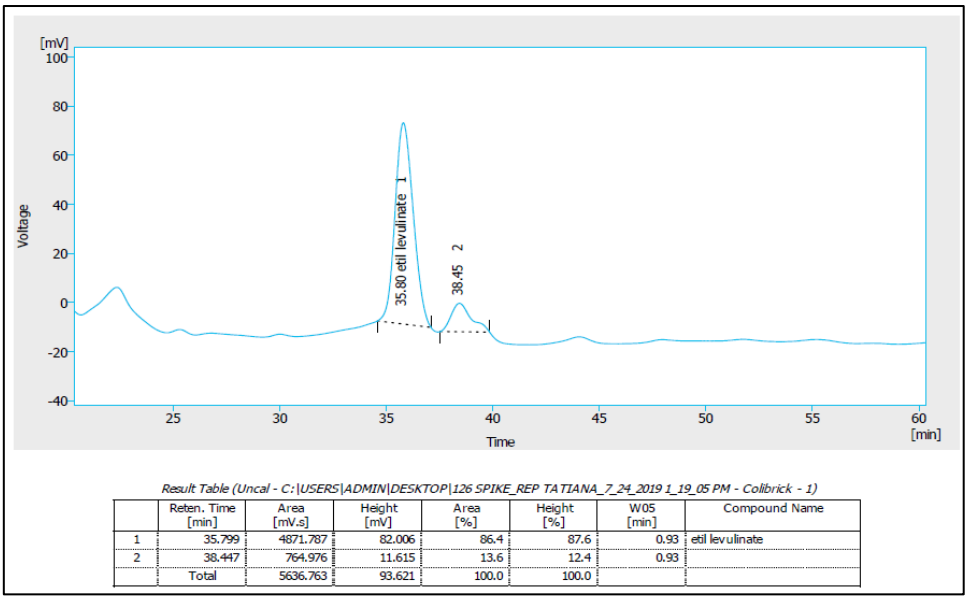


Figure 18 – Synthesis of ethyl levulinate from distinct routes. (Adapted from [128])

Thereby, a standard solution of ethyl levulinate was subjected to HPLC analysis. As represented in Figure 19, the retention times were close, but two peaks were obtained, indicated the ethyl levulinate was not the compound. Additional analysis should be performed in order to identify and characterize that compound, which may eventually have some economic value.



Result Table (Uncal - C:\USERS\ADMIN\DESKTOP\126 SPIKE_REP TATIANA_7_24_2019 1_19_05 PM - Colibrick - 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	35.799	4871.787	82.006	86.4	87.6	0.93	etil levulinate
2	38.447	764.976	11.615	13.6	12.4	0.93	
	Total	5636.763	93.621	100.0	100.0		

Figure 19 – HPLC spectra of a standard solution of ethyl levulinate added to a cultivation sample.

3.3.9. CGC production

The copolymer CGC, composed of chitin and glucan moieties, is found as a component of the cell wall in most fungi and yeasts [31]. Due to the great properties of its two bioactive molecular components, it has a plenty of uses in diverse areas, including pharmaceutical, cosmetics and food [33].

The focus of this work was the polyols production, however, the production of CGC by *K. pastoris* was also briefly evaluated, by extracting the polymer from the biomass produced in all bioreactor experiments described above. The main purpose was to observe the impact of those different lignocellulosic hydrolysates used as feedstocks in the production and composition of CGC. This polymer represents a high value product, which may be interesting for the economical sustainability of the bioproduction of xylitol.

By observing the Table 16, low amounts of CGC, with concentrations from 0.08 to 0.39 g/L, were obtained, in comparison with some values reported in literature. For example, 6.25, 3.12 and 0.52 g/L of CGC were produced by *K. pastoris* using waste glycerol from biodiesel process, sugarcane molasses and spent coffee grounds as feedstock, respectively [118].

Although the lower CGC concentration in cultivation broths, an interesting finding was made. In the cultivation owning the largest CGC production, a high CGC content on *K. pastoris'* cells was found, achieving a value of 51.5 %. This percentage of CGC in biomass is significatively higher than some values reported in many studies. For example, Araújo et al. (2017) attained lower polymer content, 18, 12 and 12% in the cell wall of *K. pastoris*, for glucose/xylose mixtures containing 15, 50 and 75 % of xylose, respectively. Moreover, Araújo et al. (2013) reported a

CGC content of 15.18, 17.53 and 15.04 % in the biomass, using waste glycerol from the biodiesel process, sugarcane molasses and spent coffee grounds for the cultivation of *K. pastoris*, respectively [118]. Similarly, Roca et al. (2012) reported a content of 16 % of CGC present in *K. pastoris* biomass, using waste glycerol from biodiesel process as feedstock [31]. In a different study, Feofilova et al. (2006) found out CGC levels from 8 % to 25 % in the cell wall of *Aspergillus niger*, depending on the fungus developmental stage and increased by the medium acidification [129].

Among all bioreactor cultivations performed, the bioreactor assay with the highest production of biomass (41.68 g/L) revealed the largest production of CGC (0.39 g/L), in which raw BSG hydrolysate was the sole carbon source used, there was no pH control in stationary phase and an airflow rate of 1 L/min was kept constant. Followed by the cultivation with the same feedstock, but a decrease in the airflow rate to 0.4 L/min in stationary phase, which revealed a biomass concentration of 22.98 g/L and the second highest amount of CGC (0.25 g/L) (Table 16). These findings are in line with the results expected, since CGC production is associated with cell growth [33].

Table 16 – Comparison of production and composition of CGC in the different bioreactor assays performed for polyols production by *K. pastoris*.

Experiment		Glucose/xylose/ arabinose ratio (g/g/g)	CDW (g/L)	Yield ($g_{CDW}/g_{glucose}$)	% CGC in biomass	CGC (g/L)	% Chitin	Xylitol (g/L)
Feedstock	**Culture conditions**							
Raw BSG hydrolysate	Airflow rate of 1 L/min; pH 5	4:15:7	6.86	1.89	13.8±0.3	0.08±0.01	27.6±0.2	0.94
Raw BSG hydrolysate	Airflow rate of 1 L/min; no pH control in stationary phase	2:9:6	41.68	20.42	51.5±0.2	0.39±0.12	24.1±0.1	0.35
Raw BSG hydrolysate	Airflow rate of 1 L/min in growth phase and 0.4 L/min afterwards; no pH control	6:9:6	22.98	4.15	10.0±0.2	0.25±0.09	28.3±0.4	0.45
Twofold concentrated banana peels hydrolysate	Airflow rate of 1 L/min; no pH control	7:4:3	11.57	1.64	24.4±0.4	0.10±0.01	10.6±0.3	0.15
Twofold concentrated and detoxified BSG hydrolysate	Airflow rate of 0.5 L/min: no pH control	9:9:6	3.06	0.33	28.2±0.6	0.14±0.02	16.8±0.6	1.33
Raw BSG hydrolysate + feed of sawdust hydrolysate	Airflow rate of 1 L/min in growth phase and 0.4 L/min afterwards; no pH control	7:9:4	5.39	0.80	19.8±0.2	0.08±0.01	18.6±0.4	0.65
Raw BSG hydrolysate + feed of sawdust hydrolysate	Airflow rate of 0.5 L/min: no pH control	7:9:5	5.67	0.78	23.1±0.3	0.13±0.03	18.5±0.9	1.18

Differences in CGC composition were also found in the different cultivations, as shown by the polymers' chitin content. The CGC polymers more enriched with chitin, presenting contents of 27.6, 24.1 and 28.3 %, were the ones produced in the presence of higher xylose/glucose ratios, namely, 15:4, 9:2 and 9:6 g/g (Table 16). These results are in accordance with a study performed by Araújo et al. (2017), for the evaluation of CGC production in bioreactor cultivations of *K. pastoris*, using glucose and xylose mixtures. It was observed that the addition of xylose had impact on CGC composition, leading to an increase in chitin content. The biopolymer presented higher chitin:glucan ratios, namely, 22:78, 27:73 and 25:75, for the experiments with 15, 50 and 75 % of xylose, respectively, by opposition to a lower ratio of 14:86

when glucose was the sole carbon source [33]. In that study, xylitol production was noticed, and an apparent relation between chitin content and xylitol concentration was established. An increase of chitin content was concomitant with a higher xylitol production. However, this trend is difficult to examine in the present study due to the low productions attained. It was only possible to observe that the highest bioreactor xylitol production achieved was 1.33 g/L, with a concomitant production of CGC containing 16.8 % of chitin and, the highest chitin content in CGC was observed with a concomitant production of 0.45 g/L of xylitol (Table 16).

3.4. Conclusions

Among the hemicellulosic hydrolysates tested, BSG hydrolysate stood out as the most suitable feedstock for xylose bioconversion into xylitol. Concentration and detoxification of BSG hydrolysate carried out in shake flask assays led to the highest xylitol production of 3.97 g/L, with a xylitol/xylose yield of 0.47 g/g. It is evident the positive impact of the additional treatment steps in comparison with cultivations using raw hydrolysate as the sole carbon source, in which 1.51 g/L of xylitol were obtained (yield of 0.39 $g_{xylitol}/g_{xylose}$).

Bioreactor cultivations attained lower xylitol productions. The yeast achieved a maximum xylitol production of 1.33 g/L (yield of 0.18 $g_{xylitol}/g_{xylose}$) in a batch cultivation with an airflow rate of 0.5 L/min, using twofold concentrated and detoxified BSG hydrolysate as the sole carbon source. Although the different aeration conditions tested in the bioreactor assays, the constant airflow rate of 0.5 L/min seemed to lead to higher productions, which is an advantage in having the association of growth and fermentation, instead of two discrete processes.

On the other hand, a very low arabinose bioconversion into arabitol by *K. pastoris* was found. However, arabitol release was noticed under stressful conditions, induced by the presence of large amounts of toxic compounds.

Interestingly, the HPLC analysis demonstrated that the culture was channeling part of the carbon provided by the hemicellulosic hydrolysates into other metabolic pathways and/or products, which explains the low polyols productions attained in spite of the different strategies employed, namely, concentration, detoxification and mixture of hydrolysates, batch and fed-batch cultivations with different amounts of oxygen provided. The unidentified compounds produced by *K. pastoris* may be of great interest, having a high commercial value.

Additionally, *K. pastoris* showed low production of the copolymer CGC (bellow 0.39 g/L), but a high CGC content of 51.5% was found on *K. pastoris'* cells. A CGC content of 50 % is significantly higher than the value found in literature data (8-25 %), which may be attractive at large-scale productions.

Chapter 4

General conclusions and future work

4. General conclusions and future work

The polyols production from lignocellulosic hydrolysates achieved in this work was low in comparison to the best xylitol producers belonging to *Candida* sp. (pathogenic). However, it is noteworthy that *K. pastoris* owns a GRAS status and this work demonstrated the yeast's potential to produce xylitol from hemicellulosic hydrolysates, which supports further research to develop and optimize the bioprocess.

The search for an alternative lignocellulosic wastes pretreatment , in particular for BSG, would be interesting in order to reduce (recycle) or eliminate the addition of sulphuric acid and, thus, its environmental impact. Additionally, increase *K. pastoris* tolerance to inhibitory compounds would be a potential strategy to skip the detoxification step, in order to get a simpler process and reduce costs. For example, yeasts can be adapted to hydrolysates by successive batch cultures using cells from the previous assays [9]. In a study conducted by Misra et al. (2013), the adaptation of *C. tropicalis* to corncobs hydrolysates lead to a 1.22-fold increase in xylitol production when compared to the parent strain [130].

The biotechnological production of xylitol is still far to replace the current chemical process. To tackler the major bottlenecks, efficient technologies for biomass pretreatment, potentiation of microbial fermentation and xylitol recovery with high level of purity are required.

Considering that hemicellulose corresponds to 20-30% of the total lignocellulosic biomass and 35-40% of total fermentable sugars, exclusive production of xylitol might not be economically feasible.

Cellulose and lignin remain in the solid fraction during acid hydrolysis and, in this work, only the liquor was used. Nowadays, there is an interesting concept of zero waste biorefineries for waste valorisation, designed to use all by-products generated, transforming them into different value-added compounds. Thereby, exploration of the reuse of cellulose and lignin for different purposes would be important to enhance the valorization of these wastes.

Taking this into account, *K. pastoris* represents a suitable microorganism to integrate a zero waste biorefinery aiming to the efficient and cos-effective processing of lignocellulosic wastes, transforming them into different value-added compounds.

References

1. **Grembecka**, M. (2015). Sugar alcohols—their role in the modern world of sweeteners: a review. *European Food Research and Technology*, 241(1), 1-14.
2. **Barbieri**, G., Barone, C., Bhagat, A., Caruso, G., Conley, R., Parisi, S. (2014). Sweet compounds in foods: sugar alcohols. *The Influence of Chemistry on New Foods and Traditional Products*, 15(3), 51-59.
3. **Livesey**, G. (2003). Health potential of polyols as sugar replacers, with emphasis on low glycaemic properties. *Nutrition Research Reviews*, 16(2), 163-191.
4. **de Albuquerque**, L., da Silva Jr, J., de Macedo, R., Rocha, P. (2014). Biotechnological production of xylitol from lignocellulosic wastes: a review. *Process Biochemistry*, 49 (11), 1779-1789.
5. **Nabors**, O., Gelardi, R. (2001). Alternative sweeteners: An overview. *Food Science and Technology-New York Marcel Dekker*, 12(3), 1-12.
6. **Kumdam**, H., Murthy, N., GummadiN. (2014). Arabitol production by microbial fermentation-biosynthesis and future applications. *International Journal of Sciences & Applied Research*, 1, 1-12.
7. **Felipe Hernández-Pérez**, A., de Arruda, V., Sene, L., da Silva, S., Kumar Chandel, A., de Almeida Felipe, G. (2019). Xylitol bioproduction: state-of-the-art, industrial paradigm shift, and opportunities for integrated biorefineries. *Critical Reviews in Biotechnology*, 39(7), 924-943.
8. **Arcaño**, D., García,. V., Mandelli, D., Carvalho, A., Pontes, M. (2018). Xylitol: A review on the progress and challenges of its production by chemical route. *Catalysis Today*, 28 (1), 1–12.
9. **Dasgupta**, D., Bandhu, S., Adhikari, K., Ghosh, D. (2017). Challenges and prospects of xylitol production with whole cell bio-catalysis: a review. *Microbiological Research*, 197, 9-21.
10. **Kordowska-Wiater**, M. (2015). Production of arabitol by yeasts: current status and future prospects. *Journal of Applied Microbiology*, 119(2), 303-314.
11. **Research and Markets** – Xylitol Market: Global Industry Trends, Share, Size, Growth, Opportunity and Forecast 2018-2023.
    https://www.researchandmarkets.com/research/2wbg5g/global_xylitol?w=4 (Published: Jun 2017 and accessed May 2019).
12. **Grand View Research**. Xylitol Market Analysis By Application (Chewing Gum, Confectionery, Bakery, Oral Care), By Region (North America, Europe, Asia Pacific, Latin America, MEA), And Segment Forecast, 2014–2025.
    https://www.grandviewresearch.com/industry–analysis/xylitol–market (Published: May 2017 and accessed May 2019).
13. **Granström**, B., Izumori, K., Leisola, M. (2007). A rare sugar xylitol. Part I: the biochemistry and biosynthesis of xylitol. *Applied Microbiology and Biotechnology*, 74(2), 277-281.
14. **Rao**, V., Goli, K., Gentela, J., Koti, S. (2016). Bioconversion of lignocellulosic biomass to xylitol: an overview. *Bioresource Technology*, 213, 299-310.
15. **Chiang**, C., Sih, J., Knight, G. (1958). The conversion of D-xylose to xylitol by *Penicillum chrysogenum*. *Biochimica et Biophysica Acta*, 29(3), 664.
16. **Ōnishi**, H., Suzuki, T. (1966). The production of xylitol, L-arabinitol and ribitol by yeasts. *Agricultural and Biological Chemistry*, 30(11), 1139-1144.
17. **Ōnishi**, H., Perry, B. (1965). The production of meso-glycero-ido-heptitol and D-glycero-D-ido-heptitol by *Pichia miso*. *Canadian Journal of Microbiology*, 11(6), 929-934.

18. **Rafiqul**, M., Sakinah, M. (2013). Processes for the production of xylitol—a review. *Food Reviews International*, 29(2), 127-156.
19. **Shrotri**, A., Kobayashi, H., Fukuoka, A. (2017). Catalytic conversion of structural carbohydrates and lignin to chemicals. *Advances in Catalysis*, 60, 59-123.
20. **Zamani**, A. (2015). Introduction to lignocellulose-based products. *Lignocellulose-Based Bioproducts*, 1-36.
21. **Baruah**, J., Nath, K., Sharma, R., Kumar, S., Deka, C., Baruah, C., Kalita, E. (2018). Recent trends in the pretreatment of lignocellulosic biomass for value-added products. *Energy Resource*, 6, 141.
22. **Kumar**, K., Sharma, S. (2017). Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review. *Bioresources and Bioprocessing*, 4(1), 7.
23. **de Jong**, E., Gosselink, J. (2014). Lignocellulose-based chemical products. *Bioenergy Research: Advances and Applications*, 277-313.
24. **Carvalheiro**, F., Duarte, C., Gírio, F. M. (2008). Hemicellulose biorefineries: a review on biomass pretreatments. *Journal of Scientific & Industrial Research*, 849-864.
25. **Palmqvist**, E., Hahn-Hägerdal, B. (2000). Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresource Technology*, 74(1), 25-33.
26. **Chandel**, A. K., Da Silva, S., Singh, V. (2013). Detoxification of lignocellulose hydrolysates: biochemical and metabolic engineering toward white biotechnology. *BioEnergy Research*, 6(1), 388-401.
27. **Mussatto**, S. I., Santos, C., Roberto, C. (2004). Effect of pH and activated charcoal adsorption on hemicellulosic hydrolysate detoxification for xylitol production. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology*, 79(6), 590-596.
28. **Pal**, S., Choudhary, V., Kumar, A., Biswas, D., Mondal, K., Sahoo, K. (2013). Studies on xylitol production by metabolic pathway engineered *Debaryomyces hansenii*. *Bioresource Technology*, 147, 449-455.
29. **Wei**, J., Yuan, Q., Wang, T., Wang, L. (2010). Purification and crystallization of xylitol from fermentation broth of corncob hydrolysates. *Frontiers of Chemical Engineering in China*, 4(1), 57-64.
30. **Chagas**, B., Farinha, I., Galinha, F., Freitas, F. Reis, M. A. (2014). Chitin–glucan complex production by *Komagataella (Pichia) pastoris*: impact of cultivation pH and temperature on polymer content and composition. *New Biotechnology*, 31(5), 468-474.
31. **Roca**, C., Chagas, B., Farinha, I., Freitas, F., Mafra, L., Aguiar, F., Oliveira, R., Reis, M. A. (2012). Production of yeast chitin–glucan complex from biodiesel industry by product. *Process Biochemistry*, 47(11), 1670-1675.
32. **Ahmad**, M., Hirz, M., Pichler, H., Schwab, H. (2014). Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. *Applied Microbiology and Biotechnology*, 98(12), 5301-5317.
33. **Araújo**, D., Freitas, F., Sevrin, C., Grandfils, C., Reis, M. A. (2017). Co-production of chitin-glucan complex and xylitol by *Komagataella pastoris* using glucose and xylose mixtures as carbon source. *Carbohydrate Polymers*, 166, 24-30.
34. **Farinha**, I., Duarte, P., Pimentel, A., Plotnikova, E., Chagas, B., Mafra, L., Grandfils, C., Freitas, F., Fortunato, E., Reis, M. A. (2015). Chitin–glucan complex production by *Komagataella pastoris*: downstream optimization and product characterization. *Carbohydrate polymers*, 130, 455-464.

35. **Baumann**, K., Carnicer, M., Dragosits, M., Graf, A. B., Stadlmann, J., Jouhten, P., Maaheimo, H., Gasser, B., Albiol, J., Mattanovich, D., Ferrer, P. (2010). A multi-level study of recombinant Pichia pastoris in different oxygen conditions. *BMC Systems Biology*, 4(1), 141.
36. **Cheng**, H., Lv, J., Wang, H., Wang, B., Li, Z., Deng, Z. (2014). Genetically engineered *Pichia pastoris* yeast for conversion of glucose to xylitol by a single-fermentation process. *Applied Microbiology and Biotechnology*, 98(8), 3539-3552.
37. **Serrano**, P. (2018). Production of xylitol by the yeast *Komagataella pastoris* (Master dissertation in Biotechnology by FCT/UNL).
38. **Ravella**, S. R., Gallagher, J., Fish, S., Prakasham, R. S. (2012). Overview on commercial production of xylitol, economic analysis and market trends. *d-Xylitol Springer*, 291-306.
39. **Abubakar**, U. S., Yusuf, K. M., Safiyanu, I., Abdullahi, S., Saidu, S. R., Abdu, T., Indee, A. M. (2016). Proximate and mineral composition of corn cob, banana and plantain peels. *International Journal of Food Science and Nutrition*, 1(6), 25-27.
40. **Padam**, B. S., Tin, S., Chye, F. Y., Abdullah, I. (2014). Banana by-products: an under-utilized renewable food biomass with great potential. *Journal of Food Science and Technology*, 51(12), 3527-3545.
41. **Ahmad**, T., Danish, M. (2018). Prospects of banana waste utilization in wastewater treatment: A review. *Journal of Environmental Management*, 206, 330-348.
42. **Mardawati**, E., Andoyo, R., Syukra, A., Kresnowati, M., Bindar, Y. (2018). Production of xylitol from corn cob hydrolysate through acid and enzymatic hydrolysis by yeast. *IOP Conference Series: Earth and Environmental Science*, 141, 12-79.
43. **Nagarajan**, A. (2016). Mini review on corncob biomass: a potential resource for value-added metabolites. *European Journal of Experimental Biology*, 6(5), 9-13.
44. **Prozil**, O., Evtuguin, D. V., Silva, M., Lopes, P. (2014). Structural characterization of lignin from grape stalks (Vitis vinifera L.). *Journal of Agricultural and Food Chemistry*, 62(24), 5420-5428.
45. **Semitela**, S., Pirra, A., & Braga, F. G. (2019). Impact of mesophilic co-composting conditions on the quality of substrates produced from winery waste activated sludge and grape stalks: Lab-scale and pilot-scale studies. *Bioresource Technology*, 12, 16-22.
46. **Beres**, C., Costa,. N., Cabezudo, I., da Silva-James, K., Teles, S., Cruz, P., Freitas, P. (2017). Towards integral utilization of grape pomace from winemaking process: A review. *Waste Management*, 68, 581-594.
47. **Muhlack**, R. A., Potumarthi, R., Jeffery, W. (2018). Sustainable wineries through waste valorisation: A review of grape marc utilisation for value-added products. *Waste Management*, 72, 99-118.
48. **Mussatto**, S. I., Roberto, I. C. (2006). Chemical characterization and liberation of pentose sugars from brewer's spent grain. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology*, 81(3), 268-274.
49. **Cooray**, T., Lee, J., Chen, W. N. (2017). Evaluation of brewers' spent grain as a novel media for yeast growth. *Amb Express*, 7(1), 117.
50. **Holdings**, K. (2017). Kirin beer university report: global beer production by country in 2012.
51. **Rafiqul**, M., Sakinah, A., Karim, R. (2014). Production of xylose from Meranti wood sawdust by dilute acid hydrolysis. *Applied Biochemistry and Biotechnology*, 174(2), 542-555.

52. **Eliche-Quesada**, D., Corpas-Iglesias, F. A., Pérez-Villarejo, L., Iglesias-Godino, F. (2012). Recycling of sawdust, spent earth from oil filtration, compost and marble residues for brick manufacturing. *Construction and Building Materials*, 34, 275-284.
53. **Den**, W., Sharma, K., Lee, M., Nadadur, G., Varma, S. (2018). Lignocellulosic biomass transformations via greener oxidative pretreatment processes: access to energy and value-added chemicals. *Frontiers in chemistry*, 6, 23-78.
54. **Kucharska**, K., Rybarczyk, P., Hołowacz, I., Łukajtis, R., Glinka, M., Kamiński, M. (2018). Pretreatment of lignocellulosic materials as substrates for fermentation processes. *Molecules*, 23(11), 29-37.
55. **Dominguez**, M., Gong, S., Tsao, T. (1996). Pretreatment of sugar cane bagasse hemicellulose hydrolysate for xylitol production by yeast. *Seventeenth Symposium on Biotechnology for Fuels and Chemicals*, 24(3), 49-56.
56. **Baadhe**, R., Potumarthi, R., Mekala, K. (2014). Influence of dilute acid and alkali pretreatment on reducing sugar production from corncobs by crude enzymatic method: a comparative study. *Bioresource Technology*, 162, 213-217.
57. **Zheng**, A., Zhao, Z., Chang, S., Huang, Z., Zhao, K., Wei, G., Li, H. (2015). Comparison of the effect of wet and dry torrefaction on chemical structure and pyrolysis behavior of corncobs. *Bioresource Technology*, 176, 15-22.
58. **Pointner**, M., Kuttner, P., Obrlik, T., Jager, A., Kahr, H. (2014). Composition of corncobs as a substrate for fermentation of biofuels. *Agronomy Research*, 12(2), 391-396.
59. **Cheng**, K., Zhang, A., Ling, Z., Ping, X., Huang, W., Ge, P., Xu, M. (2009). Optimization of pH and acetic acid concentration for bioconversion of hemicellulose from corncobs to xylitol by *Candida tropicalis*. *Biochemical Engineering Journal*, 43(2), 203-207.
60. **Anukam**, I., Goso, B., Okoh, O., Mamphweli, N. (2017). Studies on characterization of corn cob for application in a gasification process for energy production. *Journal of Chemistry*, 21, 48-72.
61. **Mariotti**, F., Tomé, D., Mirand, P. (2008). Converting nitrogen into protein beyond 6.25 and Jones' factors. *Critical Reviews in Food Science and Nutrition*, 48(2), 177-184.
62. **Sluiter**, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Wolfe, J. (2008). Determination of total solids in biomass and total dissolved solids in liquid process samples. *National Renewable Energy Laboratory, Golden, CO, NREL Technical Report No. NREL/TP-510-42621*, 1-6.
63. **Chagas**, B. F. (2015). Production and characterization of Chitin-Glucan Complex by *Komagataella pastoris*. (PhD dissertation by FCT/UNL).
64. **Rivera**, P., Moldes, A. B., Torrado, M., Domínguez, M. (2007). Lactic acid and biosurfactants production from hydrolyzed distilled grape marc. *Process Biochemistry*, 42(6), 1010-1020.
65. **Salgado**, M., Rodríguez, N., Cortés, S., Domínguez, M. (2012). Effect of nutrient supplementation of crude or detoxified concentrated distilled grape marc hemicellulosic hydrolysates on the xylitol production by *Debaryomyces hansenii*. *Preparative Biochemistry and Biotechnology*, 42(1), 1-14.
66. **Rivas**, B., Torrado, A., Rivas, S., Moldes, B., Domínguez, M. (2007). Simultaneous lactic acid and xylitol production from vine trimming wastes. *Journal of the Science of Food and Agriculture*, 87(8), 1603-1612.

67. **Carvalheiro**, F., Duarte, C., Medeiros, R., Gírio, M. (2007). Xylitol production *by Debaryomyces hansenii* in brewery spent grain dilute-acid hydrolysate: effect of supplementation. *Biotechnology Letters*, 29(12), 1887-1891.
68. **Rafiqul**, M., Sakinah, M. (2012). Kinetic studies on acid hydrolysis of Meranti wood sawdust for xylose production. *Chemical Engineering Science*, 71, 431-437.
69. **Li**, M., Meng, X., Diao, E., Du, F. (2012). Xylitol production by Candida tropicalis from corn cob hemicellulose hydrolysate in a two-stage fed-batch fermentation process. *Journal of Chemical Technology & Biotechnology*, 87(3), 387-392.
70. **Ur-Rehman**, S., Mushtaq, Z., Zahoor, T., Jamil, A., Murtaza, A. (2015). Xylitol: a review on bioproduction, application, health benefits, and related safety issues. *Critical Reviews in Food Science and Nutrition*, 55(11), 1514-1528.
71. **Kamal**, M., Mohamad, L., Abdullah, L., Abdullah, N. (2011). Detoxification of sago trunk hydrolysate using activated charcoal for xylitol production. *Procedia Food Science*, 1, 908-913.
72. **Mussatto**, I., Carneiro, M., Silva, P., Roberto, C., Teixeira, J. A. (2011). A study on chemical constituents and sugars extraction from spent coffee grounds. *Carbohydrate Polymers*, 83(2), 368-374.
73. **Carvalheiro**, F., Esteves, P., Parajó, C., Pereira, H., Gırio, M. (2004b). Production of oligosaccharides by autohydrolysis of brewery's spent grain. *Bioresource Technology*, 91(1), 93-100.
74. **Zhao**, X., Zhang, L., & Liu, D. (2012). Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. *Biofuels, Bioproducts and Biorefining*, 6(4), 465-482.
75. **Bardiya**, N., Somayaji, D., Khanna, S. (1996). Biomethanation of banana peel and pineapple waste. *Bioresource Technology*, 58(1), 73-76.
76. **Palacios**, S., Ruiz, A., Ramos-Gonzalez, R., Martínez, J., Segura, E., Aguilar, M., Ilyina, A. (2017). Comparison of physicochemical pretreatments of banana peels for bioethanol production. *Food Science and Biotechnology*, 26(4), 993-1001.
77. **Mussatto**, S. I., Dragone, G., Roberto, I. C. (2006b). Brewers' spent grain: generation, characteristics and potential applications. *Journal of Cereal Science*, 43(1), 1-14.
78. **Xiros**, C., & Christakopoulos, P. (2012). Biotechnological potential of brewers spent grain and its recent applications. *Waste and Biomass Valorization*, *3*(2), 213-232.
79. **Tišma**, M., Jurić, A., Bucić-Kojić, A., Panjičko, M., Planinić, M. (2018). Biovalorization of brewers' spent grain for the production of laccase and polyphenols. *Journal of the Institute of Brewing*, 124(2), 182-186.
80. **Sousa**, C., Uchôa-Thomaz, A., Carioca, B., Morais, S., Lima, A. D., Martins, G., Silva, J. (2014). Chemical composition and bioactive compounds of grape pomace (Vitis vinifera L.), Benitaka variety, grown in the semiarid region of Northeast Brazil. *Food Science and Technology*, 34(1), 135-142.
81. **Spigno**, G., Pizzorno, T., De Faveri, M. (2008). Cellulose and hemicelluloses recovery from grape stalks. *Bioresource Technology*, 99(10), 4329-4337.
82. **Amendola**, D., De Faveri, M., Egües, I., Serrano, L., Labidi, J., Spigno, G. (2012). Autohydrolysis and organosolv process for recovery of hemicelluloses, phenolic compounds and lignin from grape stalks. *Bioresource Technology*, 107, 267-274.

83. **Egüés**, I., Serrano, L., Amendola, D., De Faveri, M., Spigno, G., Labidi, J. (2013). Fermentable sugars recovery from grape stalks for bioethanol production. *Renewable Energy*, 60, 553-558.
84. **Ko**, H., Chiang, N., Chiu, C., Liu, C., Yang, L., Shiau, L. (2008). Integrated xylitol production by fermentation of hardwood wastes. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology*, 83(4), 534-540.
85. **Steiner**, J., Procopio, S., Becker, T. (2015). Brewer's spent grain: source of value-added polysaccharides for the food industry in reference to the health claims. *European Food Research and Technology*, 241(3), 303-315.
86. **Barakat**, A., de Vries, H., & Rouau, X. (2013). Dry fractionation process as an important step in current and future lignocellulose biorefineries: a review. *Bioresource Technology*, 134, 362-373.
87. **Carvalheiro**, F., Duarte, L. C., Medeiros, R., Gírio, F. M. (2004). Optimization of brewery's spent grain dilute-acid hydrolysis for the production of pentose-rich culture media. *Proceedings of the Twenty-Fifth Symposium on Biotechnology for Fuels and Chemicals Held May 2003, in Breckenridge,* 1059-1072.
88. **Mussatto**, S. I., & Roberto, I. C. (2004). Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. *Bioresource Technology*, 93(1), 1-10.
89. **Duarte**, C., Carvalheiro, F., Lopes, S., Marques, S., Parajo, C., Gírio, M. (2004). Comparison of two posthydrolysis processes of brewery's spent grain autohydrolysis liquor to produce a pentose-containing culture medium. *Proceedings of the Twenty-Fifth Symposium on Biotechnology for Fuels and Chemicals Held May 2003, in Breckenridge*, 1041-1058.
90. **Moldes**, B., Bustos, G., Torrado, A., Domínguez, J. M. (2007). Comparison between different hydrolysis processes of vine-trimming waste to obtain hemicellulosic sugars for further lactic acid conversion. *Applied Biochemistry and Biotechnology*, 143(3), 244-256.
91. **Ping**, Y., Ling, Z., Song, G., Ge, P. (2013). Xylitol production from non-detoxified corncob hemicellulose acid hydrolysate by *Candida tropicalis*. *Biochemical Engineering Journal*, 75, 86-91.
92. **Emaga**, H., Bindelle, J., Agneesens, R., Buldgen, A., Wathelet, B., Paquot, M. (2011). Ripening influences banana and plantain peels composition and energy content. *Tropical Animal Health and Production*, 43(1), 171-177.
93. **Villarreal**, M., Prata, R., Felipe, A., Silva, J. (2006). Detoxification procedures of eucalyptus hemicellulose hydrolysate for xylitol production by *Candida guilliermondii*. *Enzyme and Microbial Technology*, 40(1), 17-24.
94. **Mattos**, N., Tonon, V., Furtado, A., Cabral, M. (2017). Grape by-product extracts against microbial proliferation and lipid oxidation: a review. *Journal of the Science of Food and Agriculture*, 97(4), 1055-1064.
95. **Vu**, H., Scarlett, J., Vuong, V. (2018). Phenolic compounds within banana peel and their potential uses: a review. *Journal of Functional Foods*, 40, 238-248.
96. **Berson**, E., Young, S., Kamer, N., Hanley, R. (2005). Detoxification of actual pretreated corn stover hydrolysate using activated carbon powder. *Applied Biochemistry and Biotechnology*, 124(1-3), 923-934.
97. **Barathikannan**, K., Agastian, P. (2016). Xylitol: Production, Optimization and Industrial Application. *Int. J. Curr. Microbiol. Appl. Sci*, 5(9), 324-339.

98. **Srivani**, K., Pydi Setty, Y. (2012). Parametric optimization of xylitol production from xylose by fermentation. *Asia-Pacific Journal of Chemical Engineering*, 7, S280-S284.
99. **Felipe**, M. (2004). Biotechnological production of xylitol from lignocellulosic materials. *ACS Symposium Series*, 889, 300-315.
100. **Dasgupta**, D., Ghosh, D., Bandhu, S., Agrawal, D., Suman, S., Adhikari, D. (2016). Purification, characterization and molecular docking study of NADPH dependent xylose reductase from thermotolerant *Kluyveromyces sp.* IIPE453. *Process Biochemistry*, 51(1), 124-133.
101. **Antunes**, F., dos Santos, J., da Cunha, A., Brumano, P., dos Santos Milessi, S., Terán-Hilares, R., Gaikwad, S. (2017). Biotechnological production of xylitol from biomass. *Production of Platform Chemicals from Sustainable Resources*, 12, 311-342.
102. **Gong**, S., Chen, F., Flickinger, C., Tsao, T. (1981). Conversion of hemicellulose carbohydrates. *Bioenergy*, 3, 93-118.
103. **Kamat**, S., Gaikwad, S., Ravi Kumar, A., Gade, N. (2013). Xylitol production by *Cyberlindnera (Williopsis) saturnus*, a tropical mangrove yeast from xylose and corn cob hydrolysate. *Journal of Applied Microbiology*, 115(6), 1357-1367.
104. **Preziosi-Belloy**, L., Nolleau, V., Navarro, J. (1997). Fermentation of hemicellulosic sugars and sugar mixtures to xylitol by *Candida parapsilosis*. *Enzyme and Microbial Technology*, 21(2), 124-129.
105. **Barnett**, A., & Sims, A. (1982). The requirement of oxygen for the active transport of sugars into yeasts. *Microbiology*, 128(10), 2303-2312.
106. **Tamburini**, E., Costa, S., Marchetti, M., Pedrini, P. (2015). Optimized production of xylitol from xylose using a hyper-acidophilic *Candida tropicalis*. *Biomolecules*, 5(3), 1979-1989.
107. **Silva**, C. J., Mussatto, S. I., Roberto, I. C. (2006). Study of xylitol production by *Candida guilliermondii* on a bench bioreactor. *Journal of Food Engineering*, 75(1), 115-119.
108. **Canettieri**, E., Silva, J., Felipe, M. (2002). Xylitol production from eucalyptus chips by biotechnological means. *Brazilian Journal of Pharmaceutical Sciences*, 38(3), 323-331.
109. **Liu**, Z., Slininger, J., Dien, S., Berhow, A., Kurtzman, P., Gorsich, W. (2004). Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2, 5-bis-hydroxymethylfuran. *Journal of Industrial Microbiology and Biotechnology*, 31(8), 345-352.
110. **Taherzadeh**, J., Gustafsson, L., Niklasson, C., Lidén, G. (2000). Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, 53(6), 701-708.
111. **Akıllıoglu**, G., Mogol, B. A., Gökmen, V. (2011). Degradation of 5-hydroxymethylfurfural during yeast fermentation. *Food Additives & Contaminants: Part A*, 28(12), 1629-1635.
112. **Dragosits**, M., Stadlmann, J., Graf, A., Gasser, B., Maurer, M., Sauer, M., Mattanovich, D. (2010). The response to unfolded protein is involved in osmotolerance of *Pichia pastoris*. *Bmc Genomics*, 11(1), 207.
113. **Kayingo**, G., Wong, B. (2005). The MAP kinase Hog1p differentially regulates stress-induced production and accumulation of glycerol and D-arabitol in *Candida albicans*. *Microbiology*, 151(9), 2987-2999.
114. **Wilson**, J., Deschatelets, L., Nishikawa, K. (1989). Comparative fermentability of enzymatic and acid hydrolysates of steam-pretreated aspenwood hemicellulose by *Pichia stipitis* CBS 5776. *Applied Microbiology and Biotechnology*, 31(5-6), 592-596.

115. **In, S**. (2001). Hydrolysate detoxification with activated charcoal for xylitol production by *Candida guilliermondii*. *Biotechnology Letters*, 23(20), 1681-1684.
116. **de Arruda**, V., Rodrigues, B., da Silva, V., de Almeida Felipe, G. (2011). Evaluation of hexose and pentose in pre-cultivation of *Candida guilliermondii* on the key enzymes for xylitol production in sugarcane hemicellulosic hydrolysate. *Biodegradation*, 22(4), 815-822.
117. **Carvalheiro**, F., Duarte, C., Lopes, S., Parajó, C., Pereira, H., Gırio, F. M. (2005). Evaluation of the detoxification of brewery's spent grain hydrolysate for xylitol production by *Debaryomyces hansenii* CCMI 941. *Process Biochemistry*, 40(3-4), 1215-1223.
118. **Araújo**, D. (2013). Production of chitin-glucan complex by *Pichia pastoris* (Master dissertation in biotechnology by FCT/UNL).
119. **Paidimuddala**, B., Gummadi, S. N. (2014). Bioconversion of non-detoxified hemicellulose hydrolysates to xylitol by halotolerant yeast *Debaryomyces nepalensis* NCYC 3413. J. *Microb. Biochem. Technol*, 6(6), 327-33.
120. **Rodrigues**,. C., Kenealy, R., Jeffries,W. (2011). Xylitol production from DEO hydrolysate of corn stover by *Pichia stipitis* YS-30. *Journal of Industrial Microbiology & Biotechnology*, 38(10), 1649-1655.
121. **Saha**, B. C., & Bothast, R. J. (1996). Production of L-arabitol from L-arabinose by *Candida entomaea* and *Pichia guilliermondii*. *Applied Microbiology and Biotechnology*, 45(3), 299-306.
122. **Guamán-Burneo**, C., Dussán, J., Cadete, M., Cheab, A., Portero, P., Carvajal-Barriga, E., Rosa, A. (2015). Xylitol production by yeasts isolated from rotting wood in the Galápagos Islands, Ecuador, and description of *Cyberlindnera galapagoensis* fa, sp. *Antonie Van Leeuwenhoek*, 108(4), 919-931.
123. **Zhang**, J., Geng, A., Yao, C., Lu, Y., Li, Q. (2012). Xylitol production from D-xylose and horticultural waste hemicellulosic hydrolysate by a new isolate of *Candida athensensis* SB18. *Bioresource Technology*, 105, 134-141.
124. **Miura**, M., Watanabe, I., Shimotori, Y., Aoyama, M., Kojima, Y., Kato, Y. (2013). Microbial conversion of bamboo hemicellulose hydrolysate to xylitol. *Wood Science and Technology*, 47(3), 515-522.
125. **Ko**, S., Kim, J., Kim, J. H. (2006). Production of xylitol from D-xylose by a xylitol dehydrogenase gene-disrupted mutant of *Candida tropicalis*. *Appl. Environ. Microbiol.*, 72(6), 4207-4213.
126. **Koopman**, F., Wierckx, N., de Winde, H., & Ruijssenaars, J. (2010). Identification and characterization of the furfural and 5-(hydroxymethyl) furfural degradation pathways of *Cupriavidus basilensis* HMF14. *Proceedings of the National Academy of Sciences*, 107(11), 4919-4924.
127. **Deng**, W., Wang, Y., & Yan, N. (2016). Production of organic acids from biomass resources. *Current Opinion in Green and Sustainable Chemistry*, 2, 54-58.
128. **Zhao**, S., Xu, G., Chang, C., Fang, S., Liu, Z., Du, F. (2015). Direct conversion of carbohydrates into ethyl levulinate with potassium phosphotungstate as an efficient catalyst. *Catalysts*, 5(4), 1897-1910.
129. **Feofilova**, P., Nemtsev, V., Tereshina, M., Memorskaya, S. (2006). Developmental change of the composition and content of the chitin-glucan complex in the fungus *Aspergillus niger*. *Applied Biochemistry and Microbiology*, 42(6), 545-549.
130. **Misra**, S., Raghuwanshi, S., Saxena, K. (2013). Evaluation of corncob hemicellulosic hydrolysate for xylitol production by adapted strain of *Candida tropicalis*. *Carbohydrate Polymers*, 92(2), 1596-1601.

131. **Fonseca**, C., Spencer-Martins, I., Hahn-Hägerdal, B. (2007). L-arabinose metabolism in *Candida arabinofermentans* PYCC 5603 T and *Pichia guilliermondii* PYCC 3012: influence of sugar and oxygen on product formation. *Applied Microbiology and Biotechnology*, 75(2), 303-310.