Universidade do Minho
Escola de Engenharia

Sara Isabel Leite Baptista

Agro-food residues and engineered yeast platforms for natural sweeteners production

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Agro-food residues and engineered yeast platforms for natural sweeteners production

Doctoral Thesis
Doctoral Program in Chemical and Biological Engineering

Work developed under supervision of
Prof. Doctor Lucília Maria Alves Ribeiro Domingues
Doctor Aloia Romaní Pérez

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DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

Resíduos agroalimentares e plataformas de leveduras modificadas para produção de adoçantes naturais

O açúcar é um componente prevalente nas dietas modernas, sendo valorizado pela sua capacidade singular de adoçar os alimentos. No entanto, o consumo excessivo de açúcar refinado tem sido associado a doenças, como obesidade e diabetes. Consequentemente, há um crescente interesse em substitutos naturais do açúcar. Além disso, a promoção de uma economia circular tem recebido destaque na agenda política de muitos países, com planos de ação que incluem a implementação de biorrefinarias para produzir energia e produtos de elevado valor. Nos últimos anos, a produção de biocombustíveis e outros produtos a partir de biomassas renováveis tem surgido como uma alternativa à economia baseada em petróleo. O desenvolvimento de processos inovadores que sejam rentáveis e ambientalmente responsáveis requer a utilização de fábricas celulares robustas. A levedura *Saccharomyces cerevisiae* tem sido amplamente estudada como uma plataforma para a produção de biocombustíveis e produtos naturais de alto valor a partir de biomassas renováveis. Este estudo visa explorar as propriedades atrativas das estirpes industriais de *S. cerevisiae*, como sua elevada capacidade fermentativa e habilidade de tolerar condições de processamento desafiadoras, para desenvolver estirpes recombinantes capazes de produzir adoçantes naturais. Face a este enquadramento, a presente tese resultou (1) na construção de uma estirpe de levedura capaz de alcançar títulos e rendimentos notáveis de xilitol através da expressão de uma aldose redutase endógena com atividade de xilose redutase, (2) num processo sustentável baseado em tecnologias verdes para a produção de xilitol a partir da fração sólida e liquida resultante do pré-tratamento do caroço do milho (3) numa abordagem integrada de valorização para converter múltiplos resíduos da indústria do vinho em xilitol, etanol e biomassa de levedura, (4) no estabelecimento da levedura *S. cerevisiae* como biocatalisador para a conversão de arabinose da polpa de beterraba em arabitol, aproveitando a atividade promíscua da aldose redutase anteriormente expressa, juntamente com a sobre expressão de um transportador de galactose, e (5) numa estratégia de produção sustentável de tagatose utilizando algas vermelhas, e os seus resíduos de processamento subvalorizados, como fonte de galactose numa isomerização mediada pela enzima L-arabinose isomerase. Estes resultados suportam o desenvolvimento de uma biorefinaria focada na produção de adoçantes naturais e evidenciam o potencial da *S. cerevisiae* como plataforma celular, contribuindo para o estabelecimento de uma economia circular e sustentável baseada em recursos biológicos.

Palavras-chave: Adoçantes naturais, Arabitol, Biorrefinaria; Engenharia genética; *Saccharomyces cerevisiae* industriais; Xilitol, Tagatose.

Abstract

Agro-food residues and engineered yeast platforms for natural sweeteners production

Sugar is a prevalent component of modern diets, valued for its special ability to sweeten food. However, consuming excessive amounts of refined sugar has been associated with non-communicable diseases and health issues such as obesity and diabetes. As a result, there has been growing interest in natural sugar substitutes. On the other hand, the promotion of a circular economy is ranked high on the political agenda of many countries and their action plan includes the implementation of cost-effective and sustainable biorefineries for energy with high-value chemicals production. In recent years, the production of biofuels and other chemicals from renewable biomasses using biotechnology has emerged as a viable alternative to the traditional petroleum-based economy. The development of sustainable and commercially viable processes relies heavily on the use of robust cell factories. The yeast *Saccharomyces cerevisiae* has gained significant attention as a cell factory to produce biofuels and high-value natural products from renewable biomasses. This study aims to utilize the attractive properties of industrial *S. cerevisiae* strains, including their high fermentative capacity and ability to tolerate harsh process conditions, to develop recombinant strains for natural sweeteners production. With this in mind, the present thesis resulted in: (1) a xylitol producing strain capable of attaining remarkable xylitol titers and yield, resulting from the expression of an endogenous aldose reductase with xylose reductase activity, (2) a sustainable bioprocess based on green technologies for xylitol production from corn cob whole slurry using the above mentioned strain (3) an integrated multi-feedstock valorization approach for converting a range of winery wastes into xylitol, ethanol and yeast biomass, (4) the establishment of *S. cerevisiae* as a biocatalyst for the conversion of arabinose from sugar beet pulp into arabitol, by harnessing the promiscuous activity of the endogenous aldose reductase previously expresses, together with the overexpression of a galactose transporter, and (5) sustainable tagatose production strategy by using red seaweed and its undervalued processing residues as source of galactose in a L-arabinose isomerase mediated-isomerization. Collectively, these findings support the development of a cost-effective biorefinery that focuses on natural sweetener production by tailoring *S. cerevisiae* as a cell factory platform, contributing to the establishment of a bio-based economy.

Keywords: Agro-food wastes, Arabitol, Biorefinery; Genetic Engineering; Industrial *Saccharomyces cerevisiae*; Natural sweeteners, Xylitol, Tagatose.

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List of abbreviations and acronyms

ANOVA	Analysis Of Variance
ACK	Acesulfame potassium
AR	Aldose Reductase
ArOS	Arabinooligosaccharides
ASs	Artificial sweeteners
BGL1	ß-Glucosidase 1
CBP	Consolidated Bioprocessing
CoA	Coenzyme A
CRISPR	Clustered Regularly Interspaced Palindromic Repeats
DCW	Dry Cell Weight
DNS	Dinitrosalicylic Acid
EFSA	European Food Safety Authority
ESR	Enzyme to Substrate Ratio
FAO	Food and Agriculture Organization of the United Nations
FIT	Feed-In-Time
FDA	Food and Drug Administration
FPU	Filter Paper Units
GM	Grape Must
GRAS	Generally Regarded As Safe
gRNA	Guide RNA
GOS	Glucooligosaccharides
G6PD	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
HMF	5-Hydroxymethylfurfural
HPLC	High-Performance Liquid Chromatography
Kcal	Kilocalorie
LAD	L-Arabitol dehydrogenase
LB	Lysogeny Broth
LSR	Liquid Solid Ratio
L-AI	L-Arabinose isomerase
NAD^+	Oxidized Nicotinamide Adenine Dinucleotide

NADH	Reduced Nicotinamide Adenine Dinucleotide
$NADP^+$	Oxidised Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NREL	National Renewable Energy Laboratory
OIV	International Organisation of Vine and Wine
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PGI	Phosphoglucose isomerase
PPP	Pentose Phosphate Pathway
P-SSF	Pre-Saccharification and Simultaneous and Fermentation
RNA	Ribonucleic Acid
S_0	Severity
SBP	Sugar Beet Pulp
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SY	Solid Yield
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
USER	Uracil-Specific Excision Reagent
UV	Ultraviolet
VS	Vine Shoots
WHO	World Health Organization
WL	Wine Lees
XDH	Xylitol Dehydrogenase
XI	Xylose Isomerase
XK	Xylulokinase
XOS	Xylooligosaccharides
XR	Xylose Reductase
YE	Yeast Extract
TCA	Tricarboxylic acid
YP	Yeast extract, Peptone
YPD	Yeast extract, Peptone, Dextrose
Y-PER	Yeast Protein Extraction Reagent

Scientific outputs

The scientific outputs of this thesis are listed below. The results presented in this thesis have been partially published elsewhere.

Book chapters:

Baptista SL, Romaní A, Domingues L. 2021. Biotechnological Advancements, Innovations and Challenges for Sustainable Xylitol Production by Yeast, p. 420–427. In Zaragoza, Ó, Casadevall, A (eds.), Encyclopedia of Mycology. Elsevier, Oxford.

Peer-reviewed journal articles:

Baptista SL, Cunha JT, Romaní A, Domingues L. 2018. Xylitol production from lignocellulosic whole slurry corn cob by engineered industrial *Saccharomyces cerevisiae* PE-2. Bioresour. Technol. 267:481-491. DOI:10.1016/j.biortech.2018.07.068.

Baptista SL, Carvalho LC, Romaní A, Domingues L. 2020. Development of a sustainable bioprocess based on green technologies for xylitol production from corn cob. Ind Crops Prod 156:112867.. DOI:10.1016/j.indcrop.2020.112867

Cunha JT, Soares PO, Baptista SL, Costa CE, Domingues L. 2020. Engineered *Saccharomyces cerevisiae* for lignocellulosic valorization: a review and perspectives on bioethanol production. Bioengineered. 11 (1). 883–903. DOI: 10.1080/21655979.2020.1801178.

Baptista SL, Romaní A, Oliveira C, Ferreira S, Rocha CMR, Domingues L. 2021. Galactose to tagatose isomerization by the L-arabinose isomerase from *Bacillus subtilis*: a biorefinery approach for *Gelidium sesquipedale* valorisation LWT 151:112199. DOI:10.1016/j.lwt.2021.112199

Baptista SL*, Costa CE*, Cunha JT, Soares PO, Domingues L. 2021. Metabolic engineering of *Saccharomyces cerevisiae* for the production of top value chemicals from biorefinery carbohydrates. Biotechnol. Adv. 47. 107697. DOI: 10.1016/j.biotechadv.2021.107697.

Baptista SL, Romaní A, Cunha JT, Domingues L. 2023. Multi-feedstock biorefinery concept: Valorization of winery wastes by engineered yeast. J. Environ. Manage. 326:116623. DOI:10.1016/j.jenvman.2022.

***** *Authors contributed equally to this work*

Submitted manuscripts:

Baptista SL, Soares PO, Romaní A, Domingues L. Arabitol production from sugar beet pulp: recombinant industrial *Saccharomyces cerevisiae* as whole cell biocatalysts.

Posters in conferences:

Baptista SL, Oliveira C, Romaní A, Domingues L. 2019. The L-arabinose isomerase from the food grade *Bacillus subtilis* for the production of tagatose: a natural sweetener. p.394 Book of Abstracts MicroBiotec 19 - Congress of Microbiology and Biotechnology, Coimbra, Portugal.

Soares PO, Baptista SL, Romaní A, Domingues L. 2019 Arabitol production from lignocellulosic biomass through GRE3-overexpressing industrial *Saccharomyces cerevisiae* strains. p.392 Book of Abstracts Congress of Microbiology and Biotechnology, Coimbra, Portugal.

Baptista SL, Cunha JT, Romaní A, Domingues L. 2021. Wine industry residues for biotechnological production of xylitol p.310 Abstracts Book Microbiotec 21 - Congress of Microbiology and Biotechnology,

Baptista SL, Cunha JT, Romaní A, Domingues L. 2022. Integrated approach for the valorisation of wine industry residues: production of xylitol and bioethanol. p. 292 Book of Abstracts 3rd IberoAmerican Congress on Biotechnology. Braga, Portugal

Baptista SL, Soares PO, Domingues L. 2022. Engineering *Saccharomyces cerevisiae* for the production of sugar alcohols. p. 28 Book of Abstracts 3rd IberoAmerican Congress on Biotechnology. Braga, Portugal.

Oral communications in conferences:

Baptista SL, Romaní A, Cunha JT, Domingues, L. 2018. Biotechnological production of xylitol: engineering industrial Saccharomyces cerevisiae for valorization of lignocellulosic biomass. Vol. 48 4-CIAB - Proceedings of the 4th Iberoamerican Congress on Biorefineries. Jaén, Spain. ISBN: 978-84-9159-144-3

Baptista SL, Aguiar TQ, Romaní A, Johansson B, Domingues L. 2018. Engineering of robust yeast for valorisation of coffee industry wastes. ESBES 2018 - 12th European Symposium on Biochemical Engineering Sciences. Lisbon, Portugal.

Thesis outline

The research activities resulting in this doctoral thesis were mainly developed at CEB – Centre of Biological Engineering, University of Minho (Braga, Portugal) under the supervision of Professor Lucília Domingues and Doctor Aloia Romaní. As part of their academic background and research experience, the candidate completed a short stay at University of Vigo under supervision of Professor Gil Garrote, which provided a unique opportunity to delve deeper into the Hydrothermal Pretreatment of Lignocellulosic Biomass. To further develop their expertise, the candidate completed the specialization courses: Genome Editing for Cell Factories at the Novo Nordisk Foundation Center for Biosustainability, DTU Biosustain and Industrial Biotechnology for Lignocellulose Based Processes at University of Chalmers. This doctoral thesis is divided into seven chapters, five of them describing experimental research published or submitted to international peer-reviewed journals.

Chapter I encompasses a comprehensive review focusing on sugar alternatives, namely natural sweeteners and their biotechnological production. Additionally, the state of the art of the development of a biorefinery based on agro-food wastes and its application to sweeteners biosynthesis using the yeast *Saccharomyces cerevisiae*.

Chapter II focuses on the genetic engineering of the industrial *Saccharomyces cerevisiae* PE-2 strain, for increased xylitol production by expression of different enzymes with xylose reductase activity. Furthermore, an integrated strategy was developed for the production of xylitol from whole slurry corn cob in a presaccharification and simultaneous saccharification and fermentation process.

Chapter III explores xylitol production using corn cob as a renewable source by the previously constructed PE-2 yeast strain. Lignocellulose-to-xylitol process was optimized to increase xylitol titres included exploring the use of high solid loadings in the hydrothermal pre-treatment of corn cob for an efficient solubilization of xylan in xylooligosaccharides and xylose, enzyme saccharification of pre-treated corn cob and simultaneous saccharification and fermentation using different enzyme and substrate loadings.

Chapter IV focuses on the valorization of multiple agro-industrial wastes (grape must, vine shoots and wine lees) from the wine industry. Grape must was used as a low-cost source for yeast propagation. Xylitol production from xylose-rich hemicellulosic fraction of pretreated vine shoots was demonstrated and

optimized in an experimental design, and the cellulosic fraction was used for the production of ethanol. The addition of glucan-rich cellulosic fraction to grape must and/or wine lees proved effective in increasing ethanol titers.

Chapter V focuses on the development of *S. cerevisiae* strains capable of arabitol production, through the expression of an endogenous aldose reductase that catalyzes the conversion of arabinose to arabitol, and a transmembrane transporter to improve arabinose uptake and therefore obtain increased arabitol titers, while using sugar beet pulp as a renewable carbon source.

Chapter VI describes the molecular cloning and expression of the L-arabinose isomerase from food-grade *Bacillus su*btilis in the *Escherichia coli* expression system to assess its efficiency in the production of tagatose. Furthermore, a sustainable tagatose production strategy was developed by using seaweed biomass and waste-derived as substrates.

Chapter VII provides the main findings of this thesis, highlighting its relevance for the advancement of biotechnological production of sweeteners within a biorefinery scheme and outlining potential directions for further investigation.

Chapter I
General Introduction

This chapter is partially based on the following review articles:

Baptista SL*, Costa CE*, Cunha JT, Soares PO, Domingues L. 2021. Metabolic engineering of *Saccharomyces cerevisiae* for the production of top value chemicals from biorefinery carbohydrates. Biotechnol. Adv. 47. 107697. DOI: 10.1016/j.biotechadv.2021.107697.

Cunha JT, Soares PO, Baptista SL, Costa CE, Domingues L. 2020. Engineered *Saccharomyces cerevisiae* for lignocellulosic valorization: a review and perspectives on bioethanol production. Bioengineered. 11 (1). 883–903. DOI: 10.1080/21655979.2020.1801178.

Baptista SL, Romaní A, Domingues L. 2021. Biotechnological Advancements, Innovations and Challenges for Sustainable Xylitol Production by Yeast, p. 420–427. In Zaragoza, Ó, Casadevall, A (eds.), Encyclopedia of Mycology. Elsevier, Oxford.

** Authors contributed equally to this work*

1.1. Sugar consumption

The term "sugar" is difficult to define, possibly due to its dual function as an ingredient and nutrient (1). Chemically, sugar is a simple form of carbohydrates that comprise monosaccharides (e.g., glucose, galactose and fructose), and disaccharides (sucrose, lactose and maltose). These mono and disaccharides can be naturally found in fruits, vegetables and milk, and they can be added as an ingredient to foods to add sweetness, provide functional properties (texture, viscosity, color, etc.) and preserve food (2). Both World Health Organization (WHO) and European Food Safety Authority (EFSA) define free sugars as all naturally present in honey, syrups and fruit juice as well as those added to a food or beverage by the manufacturer, cook or consumer (3, 4) (Figure 1.1).

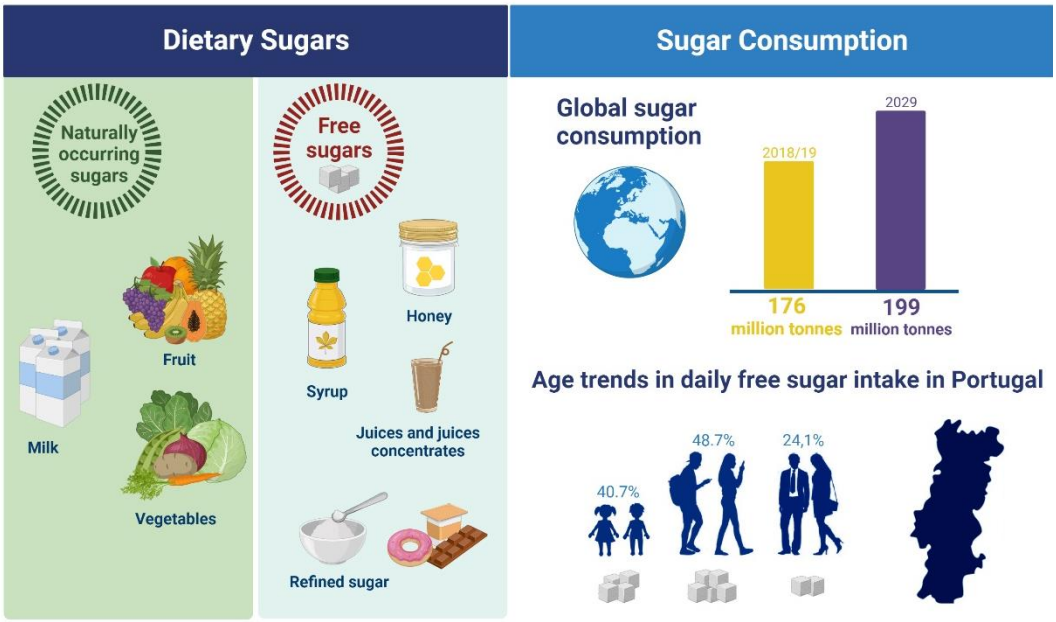


Figure 1.1. Schematic representation of the dietary sugars and their classification, including naturally occurring sugars found in fruit, vegetables and milk, and free sugars that include sugars in honey, syrup, fruit juices and fruit juice concentrates as well as sugars added to foods and beverages. Global sugar consumption and trends in daily free sugar intake for children, adolescents, and adults in Portugal.

Sugars are an energy source, required for the correct functioning of organs such as the brain and heart. As glucose is the main source of energy for the brain, humans have developed several neural pathways to seek out and motivate its intake. The sweet taste of sugar is one of the most basic sensory perceptions for humans. Our natural predisposition to consume sweet foods when they are readily available derives from our affinity for sweet flavors (5). It acts at the level of our conscious awareness to provide a pleasant sensation experience when consuming sugar-rich foods (6). Population and economic growth are expected to increase global sugar consumption to 199 Mt by 2029 (Figure 1.1). Over the projected

period, the average world level of consumption is predicted to rise from 22.5 to 23.5 kg per capita, with significant variations between countries and regions (7). However, excessive sugar intake impairs the nutritional content of diets since it provides considerable energy without essential nutrients (4). The rising obesity rate in the world has been partially attributed to excessive consumption of added sugars (8). Overconsumption of free sugars contributes to unhealthy weight gain, increasing the risk of diseases such as, obesity, type 2 diabetes, hypertension, liver and cardiovascular disease, dyslipidemia, hyperuricemia and dental caries (Figure 1.2) (9).

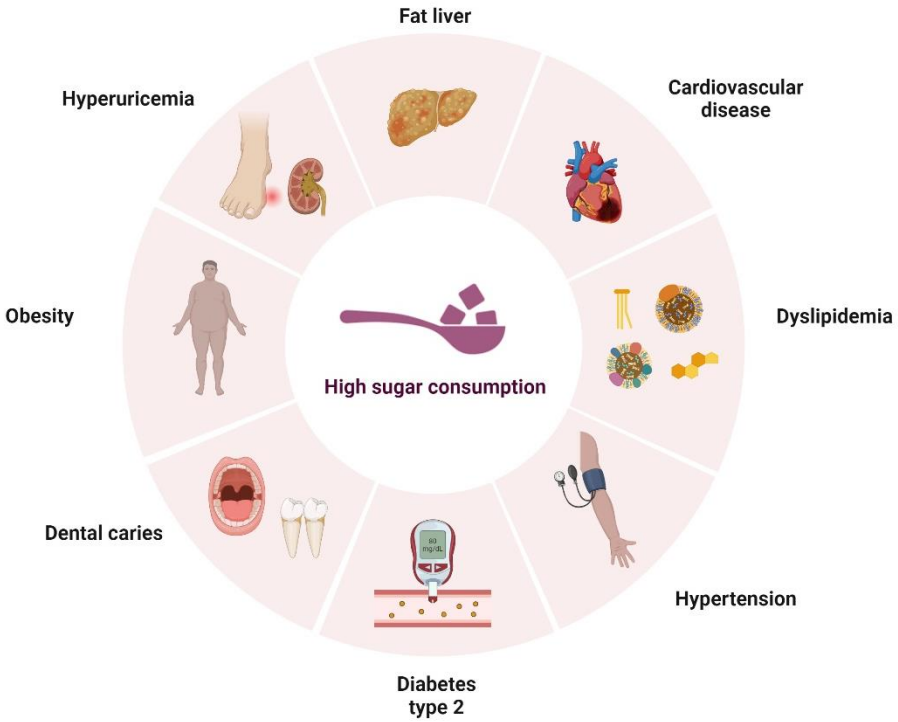


Figure 1.2. Overview of the adverse health effects related with increased sugar intake.

Nowadays, there is a strong emphasis on the relationship between dietary sugar intake and these multiple health implications, with some countries implementing sugar reduction associated recommendations and regulation (9–11). WHO recommends adults and children to reduce daily intake of free sugars to less than 10% of total energy intake (4). Significant efforts to quantify dietary sugar intakes have been made to implement policies that will change the food environment into one that does not promote the development of obesity and metabolic disease. Only few countries worldwide report any form of dietary data in sugar intake. The most available data on sugar intake is available for countries in the greater Europe region, followed by Australia and New Zealand, the United States, and some of the Americas. However, the lack of data for developing countries led to significant gaps in examining sugar intakes around the world, reflecting gaps in nutritional monitoring in general. Notwithstanding, the available data

shows that free sugar intakes as a percentage of total energy intake (%) are the highest for children and adolescents and the lowest for adults (10). This trend has also been observed in Portugal, where more than 24.3% of the Portuguese population consume free sugar above the limit recommended by WHO, with greater consumption in adolescents (48.7%) and children (40.7%) (Figure 1.1) (12).

1.2. Sugar substitutes

Over the next 10 years, increases in worldwide sugar consumption are expected to come mainly from developing countries. In contrast, it is projected that the level of sugar consumption per capita in most developed countries will decline due to rising concerns about the negative health effects of sugar overconsumption (Figure 1.2) and the implementation of taxes on caloric sugary products in an effort to reduce sugar consumption. With the rising consumer interest in healthy lifestyles and the reduction of sugar, salt, and fat intake, several food producers have replaced sugar with sweeteners in order to provide the feeling of sweetness in their products (7). According to a recent analysis, artificial sweeteners are currently used in more than 6000 products worldwide, including foods/beverages, animal feeds, and personal care items (13). Substituting added sugars with low- and/or no-calorie sweeteners has the potential to improve health by reducing daily caloric intake and therefore lowering the risk of excessive weight gain (14)

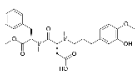
Sweeteners can be synthesized or extracted from plant sources, and the number of sugar alternatives is growing as a result of research and development in food science and technology (14). Each individual sweetener is distinct in terms of sweetness intensity, structure, metabolism, and absorption and excretion profiles (15, 16). The definitions and terminology for sweeteners vary in the literature. They can be categorized according to their nutritional value (nutritive and non-nutritive) or sweetness intensity (high and low intensity) or source (natural and artificial). In this review, sweeteners are classified according to their source: artificial and natural.

1.2.1. Artificial sweeteners

Artificial sweeteners (ASs), also known as synthetic, non-nutritive, or high-intensity sweeteners, are chemically synthesized compounds with sweetening potencies multiple times higher than sucrose (Table 1). This implies that, when sugar is replaced by ASs, much smaller quantities of ASs are needed to offer the same sweetness as sugar, and therefore ASs are only found in trace amounts in food or beverages (15). These low- or no-calories sweeteners include aspartame, acesulfame potassium (ACK), saccharin,

sucralose, neotame and advantame (Table 1.1). Some of these ASs undergo practically no metabolism after minimal absorption (sucralose) or extensive absorption (ACK and saccharin). Others, like aspartame, neotame and advantame, are firstly digested in the intestinal tract before absorption, and only their digestion breakdown products are absorbed and metabolized. In all cases, ASs and their metabolites are eliminated quickly, with no bioaccumulation in the body (15, 17, 18). Although ASs are approved and regulated by government and international food safety authorities, like EFSA and Food and Drug Administration (FDA), their safety is a topic of public debate (19). Their metabolism, absorption profiles and excretion pathways are well documented, and despite numerous research and reviews demonstrating their safety (20, 21), some research has associated ASs with adverse health conditions, such as cancers, Alzheimer's disease, multiple sclerosis, and others (22, 23). Initially, the main concern was their carcinogenic potential, but more recently, the attention has switched to their possible negative impact on metabolic health (24). These observations raise new concerns about the use of artificial sweeteners and confer unfavorable consumer perception of these sugar substitutes.

Table 1.1 Comparison of artificial sweeteners in terms of structure, sweetness intensity and amount of acceptable daily intake defined by regulatory bodies European Food Safety Authority (EFSA) and US Food and Drug Administration (FDA).

			Acceptable daily intake[3]	
Sweetener (E number)[1]	**Chemical formula**	**Sweetness intensity[2]**	**EFSA**	**US FDA**
Aspartame (E951)	$C_{14}H_{18}N_2O_5$	200 times	40	50
Acesulfame K (E950)	$C_4H_4KNO_4S$	200 times	9	15
Saccharin (E954)	$C_7H_5NO_3S$	300 times	5	15
Sucralose (E955)	$C_{12}H_{19}Cl_3O_8$	600 times	15	5
Neotame (E961)	$C_{20}H_{30}N_2O_5$	13 000 times	0-2	0.30
Advantame (E969)		20 000 times	5	32.8

[1] E. number: code for substances used as food additives; [2] Compared with sucrose (gram-for-gram basis); [3] mg intake per kg of body weight

1.2.2. Natural sweeteners

Since naturalness is seen as an advantage by consumers, the demand for naturally derived sweeteners has increased in recent times (25, 26), offering a new and significant commercial opportunity for food producers (27). The need for naturally occurring sugar substitutes in the food industry has

intensified their extraction from plants. Nonetheless, future expansion in the natural sweeteners market may be constrained by agricultural sustainability and the high cost of extraction from plants. Biotechnological production of natural sweeteners may reduce the supply scalability constraints associated with plant-based manufacturing while enhancing the overall sustainability of the process.

Natural sweeteners that occur in minor amounts in nature, can be further partitioned into steviol glycosides and rebaudiosides, rare sugars, sugar alcohols, and sweet proteins. This comprehensive review will focus on sugar alcohols, namely xylitol and arabitol and rare sugars, in particular tagatose.

1.2.2.1. Sugar alcohols

Sugar alcohols, also known as polyols, are attractive substitutes to sucrose since they are minimally absorbed and metabolized, providing less calories (Table 1.2).

Table 1.2 Caloric value, relative sweetness, and main applications of polyols.

Sweetener (E number) [1]	Kcal/g	Sweetness intensity [2]	Uses
Sorbitol (E420)	2.6	0.6	Reduced calorie foods and beverages, pharmaceuticals such as cough syrups
Xylitol (E967)	2.4	1.0	Dietary supplements, confectionary, toothpaste, and chewing gum.
Mannitol (E421)	1.6	0.5	Diabetic foods. Used in combination with other sweeteners.
Erythritol (E968)	0.2	0.8	Calorie-reduced food, confectionary and bakery products.
Isomalt (E953)	2.1	0.5	Sugar-free confectionary
Lactitol (E966)	2.0	0.4	Foods, especially baked products and processed foods.
Maltitol (E965)	2.1	0.9	Foods, especially baked products and processed foods.
Arabitol (-)	0.2	0.7	-

[1] E. number: code for substances used as food additives in UE; [2] Compared with sucrose (sucrose =1);

These naturally occurring compounds are formed by replacing the aldehyde or ketone group in sugar with a hydroxyl group (–OH) (28). Sugar alcohols have a comparable taste to sucrose and are applied in a comparable amount when used as replacements, which make them bulk sweeteners (29). The structures of sugar alcohols are very similar to sugars and therefore have similar functional properties,

which are crucial to confer body and sweetness to foods as well as sucrose does (24, 29, 30). However, the osmotic effects of unabsorbed polyols reaching the colon, may cause a laxative effect in humans and animals, when consumed in excessive doses (19, 31). Despite the possible laxative effects of polyols, their usage as bulk sweeteners has been linked to low glycemic index, oral health and prebiotic activity (29).

Currently, the most used polyols are sorbitol, xylitol, erythritol, isomalt, lactitol, maltitol and mannitol. Arabitol, which is also relevant, is not yet approved in the European Union (EU) despite being considered a GRAS food additive and an approved ingredient in the United States (32). Moreover, some sugar alcohols, such as xylitol, sorbitol, and arabitol, have the potential to act as building block chemicals, as they can be transformed into higher-value derivative compounds (33).

1.2.2.1.1. Xylitol

Xylitol is a five-carbon naturally present in small amounts in some fruits and vegetables (34, 35). It was discovered by Emil Fisher, in 1891, through D-xylose hydrogenating experiments and approved by FDA as safe for human consumption in 1986 (36, 37). This sugar alcohol was used as a sugar substitute before its benefits were properly understood. During World War II, widespread consumption of xylitol sought to address the sugar shortage (38). Xylitol presents a sweetness profile similar to sucrose with a low caloric value (Table 1.2), being partially absorbed (50%) in the small intestine and metabolized in the liver with no effect on blood glucose or insulin levels. The unabsorbed fraction that reaches the distal parts of the gastrointestinal tract is degraded by intestinal microbiota leading to the formation of short-chain fatty acids (29, 37, 39). Due to the low-caloric content and the insulin independent-metabolism, xylitol is widely used as sweetener in candy, bakery, and dairy products, and also as additive in functional foods especially applied in diabetes management. Consequently, xylitol market is expanding and is expected to reach $1.37 thousand million by 2025 with a price of $4000-5000 per ton (40).

Among polyols, xylitol has the highest sweetness and the lowest heat of solution, i.e., the heat absorbed when a substance dissolves. This provides the cooling sensation and rapid dissolution, which are advantageous for use in throat medication, mint-flavored lozenges, and sugar-free chewing gum (41). Additionally, xylitol exhibits a unique role in caries prevention. It is not metabolically used by oral flora, reducing the growth of biofilm-forming bacteria, responsible for dental caries formation. In this sense, the substitution of sugar by xylitol in food along with its incorporation in dental care products (toothpaste and mouthwash) has considerable impact on dental health (42, 43). Besides its use as a sweetener anticariogenic agent, xylitol finds applications as a food emulsifier, humectant, stabilizer, and thickener (29) and exhibits antifungal and antibacterial properties (44). In addition to these advantageous

properties, xylitol has been identified as one of the 12 top value-added compounds to be attained from biomass and can be also used in the chemical industry as an intermediate for the synthesis of ethylene glycol, propylene glycol, glycerol, xylaric acid, and polymers (33, 45, 46). This platform chemical can be obtained from xylose, the second most abundant sugar in lignocellulosic biomass after glucose.

The industrial production of xylitol started in 1975 by Finnish Sugar Co. Ltd (Finland), through chemical hydrolysis and catalytic hydrogenation of xylan. Xylan is a polysaccharide composed by ß-1,4-linked xylose, abundantly present in agro-industrial residues such as corn cob. The procedure involves acid pretreatment of these lignocellulosic materials for xylan decomposition into monomeric xylose. The resulting xylose-enriched hydrolysates are purified using exchange chromatography and activated carbon prior to the catalytic hydrogenation of xylose to xylitol since the chemical conversion requires pure xylose (47). Due to the high working temperature, this chemically based method involves high energy consumption, particularly during the hydrogenation and purification steps. It also requires the use of high-sensitivity catalysts, as well as the addition of hydrogen and pressure during the hydrogenation of xylose, which increase the cost of production and consequently the price of the final product (48). Therefore, to minimize manufacturing costs and make the process more environmental friendly, the conversion steps involved in the conversion of lignocellulosic materials to xylitol need to be reduced.

1.2.2.1.2. Arabitol

Arabitol, also known as arabinitol, is a five-carbon sugar alcohol polyol that exists in two forms: D-arabitol and L-arabitol (49). It is a stereoisomer of xylitol, with substantially fewer calories and 70% of the sweetness of sucrose (Table 1.2) (49, 50). In contrast to its counterpart xylitol, arabitol has received less research attention, nonetheless, it is known that it has a comparable inhibitory effect on caries-associated oral bacteria, and the combination of both arabitol and xylitol may be beneficial to promote dental health. However, their synergistic action needs more investigation (51). Due to the overall similarity of these two sugar alcohols, arabitol has been recognized as a potential contender to xylitol (52). Furthermore, D-arabitol can be utilized as a starting material for the manufacture of xylitol (53–55).

Arabitol, along with sorbitol and xylitol, was identified among the twelve building block chemicals that may be produced from renewable sugars and then converted into new valuable compounds such as arabinoic acid, xylonic acids, propylene, and ethylene glycol (33). Significant interest in novel products such as arabitol in the ever-expanding market for alternative sweeteners and its attractive properties highlight the significance of industrial production of arabitol.

D-Arabitol is chemically synthesized by the oxidative decarboxylation of glucose to arabinonic acid and the corresponding arabinonolactones, followed by the catalytic hydrogenation. The selectivity of the hydrogenation step can be compromised by decarboxylation and epimerization reactions that result in other products (56). L-arabitol in turn is formed by chemical hydrogenation of L-arabinose, a constituent of plant cell walls widely found in nature. This hydrogenation is normally carried out with Ni or, more recently, Ru catalysts requiring high energy (57).

1.2.2.2. Rare sugars

Rare sugars are defined as "monosaccharides and their derivatives that are present in limited quantities in nature" (58). They are found in a variety of foods, including honey, cherries and mushrooms but it is difficult to estimate how many structurally distinct monosaccharides exist in nature. To date, 42 naturally occurring monosaccharides have been identified, but the real number is probably even higher (59). Among these monosaccharides, the ones that have been more studied include D-psicose (or D-allulose), D-tagatose, D-sorbose and D-allose.

These naturally occurring sugars have recently gained attention as possible alternatives to sucrose (8, 30, 59). They are characterized by their good palatability, lack of unpleasant aftertaste, and comparable texture and bulk qualities to sucrose (8, 59). Rare sugars, like other alternative sweeteners, are either not metabolized by the human body or metabolized to a lesser extent than conventional sugars and therefore provide few calories and have a reduced glycemic index (8, 30).

1.2.2.2.1. Tagatose

Tagatose, or D-Tagatose, is a ketohexose that occurs naturally in gum exudate of the tropical tree *Sterculia Setigera* (60) and dairy products (61, 62), but not in the required amount for commercialization (63). Tagatose is an isomer of D-galactose and an epimer of D-fructose isomerized at C4 that has been recognized as GRAS by JECFA, the Joint FAO/WHO Expert Committee on Food Additives, since 2001 and authorized for use in food and beverages by the European Union in 2005, following approvals by regulatory agencies in the United States, Australia, New Zealand and South Korea (64). This rare sugar can be used as a sugar substitute since it has good bulk properties, 92% of the sweetness of sucrose and low caloric value (1.5 kcal/g) (8, 64). D-tagatose is malabsorbed in the small intestine, most of the ingested tagatose reaches the large intestine for fermentation, producing short chain fatty acids that are subsequently absorbed (8, 64, 65). Preclinical research revealed that tagatose decreased glucose levels through interfering with the absorption of carbohydrates via inhibition of intestinal disaccharidases and

glucose transport (8, 65, 66). In contrast to fructose, which has a high glycosylation capacity and promotes lipogenesis, tagatose has a low glycosylation index (67). In addition to its antioxidant properties (68), the consumption of tagatose has been associated with multiple health benefits: (i) antidiabetic and antiobesity potential because it promotes glycogen synthesis, decreases glycogen utilization, and also attenuates intestinal glucose absorption; (ii) an increase in HDL cholesterol, which may prevent heart attack; (iii) a prebiotic effect, as D-tagatose in the colon promotes the growth of beneficial bacteria and (i) dental health since it is nonfermentable by oral flora and it does not promote caries or tooth decay (69–71).

Tagatose was discovered in 1897 by Lobry de Bruyn and Van Elenstein, while studying the alkaline modification of D-galactose (72) and its manufacture and application in food products were patented in 1989 by the American company Biospherics Inc (69–71). Currently, tagatose is used as a sweetener in a wide range of foods and beverages including breakfast cereals, diet and non-diet soft drinks, ice cream, yogurt and formula diets for meal replacement (69). Owning to its unique features and ability to compete with sugar-substituting sugar alcohols markets, tagatose is expected to create a new market in the coming years, with a commercial price comparable to that of sorbitol and other sugar alcohols (73, 74).

To meet increasing commercial demand, tagatose is currently produced through isomerization of galactose using chemicals and/or enzymes. The chemical conversion of galactose into tagatose occurs under alkaline conditions (pH 12), with a metal hydroxide and an inorganic salt acting as a catalyst to increase the isomerization rate. At high pH, metal hydroxide forms an insoluble complex with tagatose, which is subsequently neutralized with an acid to yield tagatose and a salt. In addition to high energy consumption and chemical waste disposal requirements, the chemical method is nonspecific and results in the formation of undesirable byproducts, requiring additional neutralization and purifying procedures that may raise production costs (69). For these reasons, commercial tagatose production by enzymatic conversion of galactose has been proposed since the 2000s (75) The industrial galactose isomerization process using arabinose isomerase (AI) has several advantages over the chemical process, including milder pH and temperature conditions, a specific reaction, a shorter reaction time, less energy, and the potential for catalyst improvement through molecular evolution. Although enzymatic techniques for the isomerization of an aldose to ketose are extensively used on a commercial scale like in the case of the conversion of glucose from starch to fructose, the enzymatic method for tagatose has only recently been established at industrial level (69, 76).

1.3. Sustainable production of sweeteners

1.3.1. The biorefinery concept

With the depletion of fossil fuel reserves, the world faces a demand for renewable energy sources for the production of biofuels, chemical and value-added products. The growing consumption of fossil fuels not only anticipated the depletion of reserves for the next 40-50 years (77), but also intensified the emission of greenhouse gas and all the climate changes promoted by global warming (78). Therefore, the production of chemicals and energy using renewable resources is becoming increasingly important to address these limited fossil resources, the climatic crisis and other environmental problems (79). A key step for the development of sustainable processes is the shift from petroleum- to bio-based processes in a biorefinery context. In a broad sense, biorefining is described as the sustainable processing of biomass into a range of marketable biobased products and bioenergy (80). The biorefinery concept comprehends the use of a wide range of technologies to convert renewable resources, such as agro-industrial wastes into a spectrum of building blocks that can be used for the production of biofuels, chemicals or other value-added compounds (81). Contrary to the petroleum-based refinery, where natural resources are largely exploited with tremendous waste generation, biorefinery embodies a major shift by integrating systems that enable the sustainable processing of renewable materials contributing to circular economy (81).

The establishment of a biorefinery fulfills two main purposes: an energy goal, which is driven by the need for renewable energy sources; and an economic goal, focusing on the development of a biobased industry capable of generating profit and competing with fossil fuels (82). The biofuel industry has made significant progress in meeting energy goals. However, fuel is often considered a low-value product, and despite high-volume production, the returns on investment required to establish a biorefinery can be limited, creating a barrier to achieving economic goals (83). Therefore, a biorefinery able to complement biofuel production with high-value biobased products can effectively aid in the reduction of non-renewable fuel consumption and simultaneously deliver the economic incentive to expand the biorefining industry (84).

Considering the diverse range of available biomass that can be utilized through diverse conversion routes, leading to a wide array of biorefinery schemes, biorefineries can be classified based on the type of biomass utilized:

(1) First generation (1G): These biorefineries primarily utilize biomass with readily available sugars or starches, which are often edible food sources including corn, palm oil, sugarcane, rice, potato sugar beet, soybean oil, etc. Due to their high sugar or starch content, these materials are ideal for the

production of biofuels. However, there are social, economic, and environmental issues associated with the use of first-generation feedstock, such as resource depletion or an increase in demand and price (85).

(2) Second generation (2G): This biorefinery concept addresses the major drawback of the first generation by using lignocellulosic biomass, which includes a wide range of non-food crops, forest, agricultural and food processing residues. This alternative feedstock is distinguished by its ubiquity, sustainability, availability, and high production capacity, it reduces the concern over the sustainability of food while keeping the low price (85, 86). However, the complex internal structures of these lignocellulosic materials require additional procedures to valorize their components in different streams, which presents a relevant challenge (86, 87).

(3) Third generation (3G): these biorefineries are based on algae biomass, including both microalgae and macroalgae. This aquatic biomass offers several advantages, such as (i) high ocean-growth productivity that exceed terrestrial crops with no competition for land; (ii) widespread distribution; (iii) low rate of biomass fluctuation and (iv) higher photosynthetic efficiency and higher oxygen production in comparison to terrestrial biomass. Due to these characteristics, algae biomass is seen as promising renewable resource for improving the sustainability of future bio-based fuels and chemicals (85, 88–90).

In light of this, the utilization of second and third generation biomasses presents an attractive opportunity to serve as a sustainable source of sugars for sweetener production, aligning with the principles of the biorefinery philosophy. However, the fractionation process of these biomasses may pose significant challenges due to their complex structural composition, which may require targeted solutions to address. Despite these challenges, leveraging these biomasses as a sugar source holds the promise of enabling more efficient and environmentally friendly production processes, which may offer significant benefits for sustainable industrial practices.

1.3.1.1. Second generation biomass: Agro-food wastes

Second generation biomasses also known as lignocellulosic materials are among the most abundant renewable biomass sources available on Earth, offering the advantage of not competing with land needed for food production (91, 92). Lignocellulosic biomass can be obtained from energy crops, forest biomass cardboard municipal residues and agro-food wastes (93, 94). In this context, residues generated in the food and agricultural activities are considered an important source of lignocellulosic biomass. Food and Agriculture Organization (FAO) estimates that the total amount of food waste may be closer to 1300 million tons, which roughly corresponds to one-third of the food produced annually for human

consumption (95–99). In developed countries, it is estimated that the amount of food waste generated is about 12 times higher than in developing countries. Moreover, in developing countries, the highest percentage of food losses occurs during the post-harvest and processing stages, where the impact and losses are greater due to the lack of adequate and optimal economic infrastructure. Conversely, in developed countries, food waste stems from consumer and suppliers, resulting in the disposal of edible products that are still suitable for consumption (96–99).

The complex and recalcitrant structure of lignocellulosic biomass comprises cellulose, hemicellulose and lignin. The content of each fraction and the structural arrangement between those fractions may vary with the source of the biomass, and with that, the accessibility to monomer sugars will also differ (100). Lignin is a complex and highly branched polyphenolic polymer mainly present in the cell wall of lignocellulosic biomass, providing rigidity to the plants. Cellulose is a homopolymer of D-glucose and can represent up to 70% of the total lignocellulosic biomass (101). Its crystalline matrix structure, due to the extensive hydrogen bonds between glucose molecules, makes it resistant to de-polymerization and insoluble in water (102). Hemicellulose is a heteropolymer consisting of short, linear and branched chains of several monomers, including hexoses (such as glucose and galactose) and pentoses (such as xylose and arabinose), whose composition depends on the source of the lignocellulosic biomass. Due to the diverse and high sugar content, hemicellulose represents a valuable source for the production of platform chemicals. However, the recalcitrance of the lignocellulosic materials poses a significant challenge as it requires a pretreatment to break the recalcitrant structure of lignocellulose, followed by a saccharification of polysaccharides to produce fermentable sugars (103, 104).

Generally, the pretreatment step of lignocellulosic biomass is required to disrupt lignin-cellulose-hemicellulose complexes (Figure 1.3), which results in the removal of lignin, decrease of the cellulose crystallinity and increase of the surface area and porosity of the biomass for accessibility of the hydrolytic enzymes. This step is considered the first stage of a biorefinery (105, 106) and includes acid-based, hydrothermal, chemical and oxidative methods or the use of solvents. Hydrothermal treatment, also referred as autohydrolysis or liquid hot water, is usually employed to solubilize hemicellulose into oligosaccharides. The process yields a residual solid phase mainly composed of cellulose and lignin. After the pretreatment, different sugars are obtained, which can serve as building blocks for the bioproduction of a wide range of products of interest (including sweeteners such as xylitol, arabitol and tagatose) using a given microorganism. However, during the pretreatment occurs the formation of degradation compounds like weak acids, furans and phenolic compounds (107, 108). These

lignocellulosic-derived by-products generated in pretreatment process act as inhibitors for enzymes and microorganisms when their concentration is above a critical threshold.

In order to obtain fermentable monosaccharides, such as xylose, glucose and arabinose, cellulose and hemicellulose resulting from pretreatment should be submitted to a hydrolysis process, normally performed by the addition of acid catalysts or enzymes. Although acid hydrolysis is a commonly used method, it presents significant drawbacks, including the production of inhibitory compounds resulting from the degradation of sugars, as well as the requirement for recovery or neutralization of the acids prior to the fermentation process (100, 110). Enzyme specificity to the substrate, low temperatures and generation of minimum inhibitors are the key aspects of enzymatic hydrolysis that render this process as the most promising and effective. On the other hand, enzymes costs and yields lower than theoretical values are the main holdups associated with enzymatic hydrolysis (100, 111).

The enzymatic hydrolysis of hemicellulose and cellulose can be carried out in different configurations, including separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). SHF allows for optimal conditions for both the enzyme and microorganism to be selected, as both stages are carried out separately. Alternatively, enzymatic hydrolysis can be performed simultaneously with the fermentation step in an SSF process (Figure 1.3). This approach offers several advantages over SHF. Firstly, SSF removes glucose and cellobiose through fermentation, reducing end-product inhibition of the hydrolysis process. Secondly, it eliminates the need to separate glucose from the lignin fraction, preventing potential sugar loss. Lastly, SSF reduces the number of vessels required, resulting in lower investment costs. It is estimated that capital investment required for SSF could be reduced by over 20% compared to SHF (112).

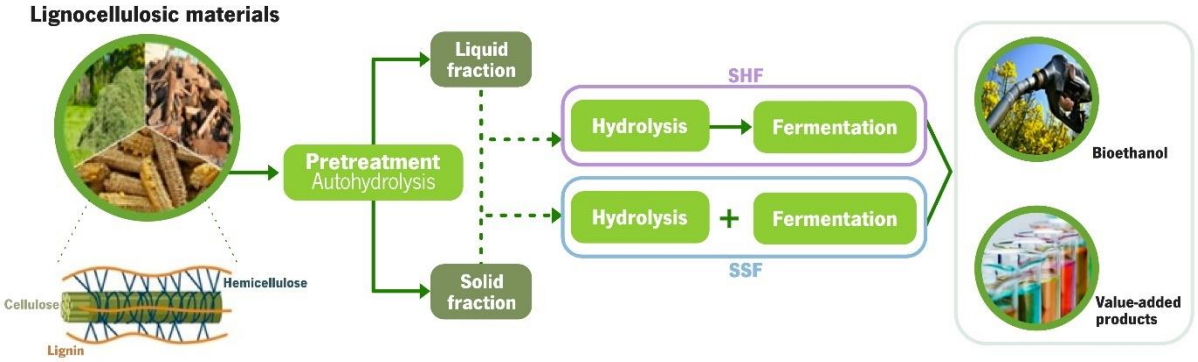


Figure 1.3. Overview of the key processes for the production of biofuels and value-added chemicals from lignocellulosic biomass. The recalcitrant structure of lignocellulose is broken down by the pretreatment, followed by separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF).

Due to the high cost associated with using refined sugars, such as glucose, xylose, arabinose, galactose, and mannose as feedstock for natural sweeteners production, the hemicellulosic fraction of lignocellulosic materials (namely, industrial agro-food wastes) presents a promising alternative by providing these monosaccharides. However, the successful production of high-value natural sweeteners like xylitol, arabitol, and tagatose, requires careful consideration of their specific building-block sugar requirements. To ensure that the necessary sugar components are extracted to meet the specific requirements of each desired end product, the selection of sugary feedstocks for this thesis was based on the chemical composition of a wide range of renewable resources, with the following feedstocks being considered:

a) Corn cob

Corn or maize (*Zea Mays*) is an important cereal for both human and animal consumption, being one of the most widely cultivated cereal crops in the world (113). During corn processing, 0.3 Mt of corncobs are generated from 1 Mt of corn, and owing to the extensive corn production, more than 200 million tons (Mt) of corncobs are generated each year, ranking them first on the list of agro-wastes produced worldwide (114, 115).

Corn cob is typically composed of 38.9% cellulose, 28.5% hemicellulose 28.5%, and 20.5% lignin (116). Due to its high cellulose and hemicellulose-derived xylan content, corncob is an attractive feedstock for a variety of biorefinery schemes. The economic profitability of biorefinery concept for biofuels and biochemicals production, in particular, will benefit from channeling the cellulosic glucose for ethanol and xylan into the production of economically attractive products such as XOS and xylitol (116).

b) Sugar beet pulp

Sugar (sucrose) is usually obtained from two crops, cane sugar and sugar beet (117). Sugar beet (*Beta vulgaris*) is an important agricultural crop and a nutrient-dense vegetable. It is cultivated in temperate zones and accounts for ~13% of the world's table sugar production. Sugar beet is also used to produce ethanol and a wide range of bio-based products, including pharmaceuticals, plastics, textiles, and chemicals. Sugar beet world production is expected to slightly expand and reach 302 Mt by 2030, (118) which will be accompanied by the generation of large amounts of sugar beet pulp, a byproduct of sugar beet processing. This leftover is a suitable raw material for biorefinery due to its low cost and accessible availability, as well as its attractive composition of cellulose (20–25%) and hemicellulose (22–

30%), including galactan and araban (24%–32%), and pectin (24–32%). Moreover, their low lignin content (1–3%) facilitates the biomass fractionation procedures (Joanna et al., 2018; Martínez et al., 2018).

c) Winery wastes and by-products

Wine has been consumed by people all around the world for hundreds of years. As a result, the winery industry generates significant economic value and is one of the major agri-food sectors (121). With a predicted vineyard area of 7.3 million hectares in 2021, grapes are one of the most widely grown fruit crops worldwide. Approximately 56% of the world's agricultural land is devoted to cultivating grapes, with Europe countries Spain and France being the main grape producers (122).

As a result of intensive viticulture and winemaking processes, a variety of wastes (vine shoots, grape pomace and wine lees) are largely produced along with a significant volume of wastewater (121, 123–125).

Among these residues, vine shoots (VS), resulting from the agronomic practice of pruning, represent up to 93% of winery leftovers and their production is estimated to be 1 to 3 tons per hectare of grape cultivation (121, 126). The most common waste management procedures are combusting or disposing for decomposition, with both having negative environmental impacts. In addition to the fact that each disposal option provides no valuable product, burning releases carbon dioxide that contributes to global warming, and landfill decomposition requires land space and the transportation of waste. Since VS are lignocellulosic materials composed of cellulose, hemicellulose, and lignin, they have vast potential as a source of energy and value-added products. Thus, VS have been used as a substrate for the manufacture of many other types of biobased products including oligosaccharides, proteins, lactic acid, bioactive compounds, biosurfactants, xylitol, and biofuels such as ethanol and biogas (127–134).

Wine lees (WL) are another winery waste product, formed at the bottom of wine containers after fermentation, consisting of a liquid phase rich in ethanol and organic acids (lactic acid or acetic acid), and a solid phase rich in microorganisms (yeast and bacteria) from wine fermentation, proteins, metals, organic salts, lignin, and insoluble polysaccharides (135). It is collected after the fermentation and ageing processes, and accounts for 6% of wine volume (136–138). WL have not been fully valorized despite their promise as a source of added value chemicals. To comply with European Regulation No 479/2008, residual ethanol is recovered by distillation and the leftovers are disposed of with wastewater (136). The extraction of phenolic compounds and tartaric acid has been suggested and it is occasionally applied on large scale (139, 140). However, these recovery solutions do not completely valorize this waste product. For example, WL has the potential to be used as a nitrogen supplement to support cell growth in

biotechnological processes, thus reducing or even eliminating the need for standard commercial media supplements such as yeast extract (141–143)

Grape must (GM) is a sugar-enriched grape juice composed of glucose and fructose, with a total sugar content fluctuating between 18 and 22%, depending on cultivar varieties and climate conditions (143). Grape musts with a low sugar content (17%) are considered low-quality grape musts since they produce inferior table wine with insufficient alcoholic content to fulfill regulatory standards and are not used for wine production (144). As the wine sector in Europe is strictly regulated with controlling measures to combat the persistent production of wine surpluses over recent decades. The distillation of remaining wine for the manufacturing of cognac, vinegar, and other consumable foods and drinks is one of the various measures taken by the European Community (145–147). This excess can be managed by repurposing large amounts of low-quality grape musts and grape must surplus, namely by using it as a substrate for biotechnological processes, which could reduce process costs (143, 148).

1.3.1.2. Third generation Biomass: Seaweed and seaweed waste biomass

Macroalgae, also known as seaweed, are multicellular organisms recognized by their high carbohydrate content and cell walls with low hemicellulose content and absence of lignin, which facilitates carbohydrate recovery (85, 149). Seaweed is divided into three groups: red algae (*Rhodophyta*), green algae (*Chlorophyta*), and brown algae (*Phaeophyceae*) (150). Red macroalgae, the most abundant and widely distributed seaweed, are mostly composed of carbohydrates (40-70% of their dry weight), particularly polysaccharides such as carrageenan, agar, and cellulose (151, 152). According to their predominant carbohydrate type: agar and carrageenans, red algae can be further divided in agarophytes and carrageenophytes, respectively. Agar, the main carbohydrate in agarophytes, is mainly composed by agarose, an heteropolysaccharide formed by D-galactose and 3,6-anhydro-L-galactose units. Carrageenan, the main carbohydrate in carrageenophytes, is a polysaccharide formed of sulfated galactans (composed by galactose units) linked by -1,4- and -1,3-glycosidic bonds (85).

The annual global production of seaweed is estimated at about 34.7 million tonnes in 2019, with Asian countries being the largest producers (97.4% of world production) (153). Nowadays, the most common applications for seaweed include food, fertilizers, phycocolloids (alginates, agars, and carrageenans), and cosmetics (150). Seaweed industries, including the phycolloid extraction and pharmaceutical and cosmetic sectors, generate a significant amount of biomass waste. To prevent competing with seaweed biomass for food sector, the manufacture of biofuels and high value chemicals

from marine algae, the third-generation feedstocks, should concentrate on the utilization of residual and waste biomass (154).

Considering that algae biomass contains little or no lignin, the pretreatments conditions are less severe than for lignocellulosic biomass, which result in shorter reaction times, lower temperatures, and lower reagent concentrations (85, 90). To obtain fermentable sugars from marine macroalgae, the selection of appropriate pretreatment that allows an efficient fractionation of whole seaweed is essential for the manufacture of biofuels and other valuable products. These fractionation approaches have been recently reviewed and well documented by Del Río et al (85). Dilute acid pretreatment is the most efficient and cost-effective process to access the high carbohydrate content in this biomass. Nevertheless, there is a need for more environmental friendly fractionating technology based on green solvents, such as water – hydrothermal processing or deep eutectic solvents.

1.3.2. Biotechnological production of sweeteners

The scarcity of natural sweeteners in nature limits their application in the food industry since the isolation of these compounds, if achievable, has a very low yield and requires the utilization of environmental pollutants. Therefore, natural sweeteners need to be produced by (bio)chemical methods using inexpensive and readily available substrates (155). In fact, the use of renewable feedstocks is already implemented for the industrial production of xylitol and tagatose (69, 156). For xylitol, the xylose is recovered from corn cob feedstocks by acid pre-treatment, whereas for tagatose, the disaccharide lactose in cheese whey is hydrolyzed (by acid or enzymes), yielding a combination of glucose and galactose, with the latter being utilized for tagatose synthesis. Despite the use of renewable raw materials, these manufacturing methods are far from environmental friendly, requiring significant energy and polluting chemical usage (157).

Multiple organisms, including bacteria, yeast, and filamentous fungi, produce secondary metabolites that are considered natural products (158). The discovery of penicillin from the fungus *Penicillium notatum,* by Alexander Fleming in 1928, was a major step toward using chemicals produced from microorganisms in medicine, agriculture, the food industry, and scientific research (159). Microbial fermentation has been shown to be a viable alternative to traditional chemical synthesis since it includes lower temperature and pressure conditions and does not involve polluting solvents and catalysts. Nowadays, a large portfolio of chemicals is being produced utilizing microbial cell factories due to the continuous international effort of research groups and companies (79, 160). Moreover, microorganisms can be exploited to produce a variety of compounds and materials using renewable resources.

Unfortunately, microbial metabolism has not evolved to meet the practical needs of humanity, and consequently, when microorganisms are isolated from nature, their efficiency in producing a particular chemical is rather low. In this sense, metabolic engineering is used to transform these microorganisms into highly efficient cell factories capable of producing large amounts of a desired chemical (161).

Considering the bottlenecks associated with pretreatment and hydrolysis processes, cost-efficient exploitation of agro-food wastes for biofuels and value-added products is dependent on a robust microorganism to perform the fermentation process. The yeast *Saccharomyces cerevisiae*, generally regarded as safe (GRAS), has been broadly used in the biotechnology industry. It is handled in large-scale operations and is a model eukaryotic system, with an in-depth studied molecular and cellular biology and a variety of genetic tools available. As eukaryotic organism, it comprises multiple organelles that could be used as compartments for the biosynthesis of different compounds (162–165). Furthermore, industrial environments have been recognized as a source of *S. cerevisiae* strains with higher robustness, fermentation capacity and resistance to stress factors found in harsh industrial processes when compared with laboratory strains (92, 166, 167). Acetic acid, furfural, and 5-hydroxymethyl-2-furaldehyde (HMF) are the most common inhibitors that accumulate in the hemicellulosic (liquid) fraction of lignocellulose during pretreatment and/or hydrolysis, affecting yeast growth and reducing product yield and productivity (168, 169). Industrial *S. cerevisiae* isolates can better cope with this inhibitory challenge, as these robust strains display higher tolerance against stress factors (166, 170, 171). Moreover, thermotolerance is one of the traits presented by some industrial yeast strains that can be desirable for a simultaneous saccharification and fermentation (SSF) of lignocellulose due to the higher optimal temperatures of hydrolytic enzymes in comparison with the optimal temperature for *S. cerevisiae* fermentation (108, 172, 173). Industrial isolates may also possess intrinsic capabilities and specificities to respond to genetic engineering, either not only for tolerance or but also for pentose metabolism, which reveals the necessity of a personalized genetic engineering to the selected yeast chassis and lignocellulosic biomass used in the fermentation process (172, 174).

1.3.2.1. Biotechnological production of xylitol

Biotechnological approaches for xylitol production aim to reduce the chemical inputs of the conventional processes and have the inherent advantage of directly using hemicellulose hydrolysates, since microbial conversion does not require isolated and purified xylose, eliminating the need for the costly purification steps prior to xylose reduction (82).

Considerable research efforts have focused on native xylose-utilizing yeasts (*Candida, Debaromyces, Kluveromyces, Pichia* sp). Xylose assimilation capacity is conferred by the oxidoreductase pathway in which two enzymes, xylose reductase (XR) and xylose dehydrogenase (XDH), are used to convert D-xylose to D-xylulose (Figure 1.4). Xylitol is the first intermediate of this pathway and its accumulation is caused by the cofactor imbalance between xylose reductase (XR) and xylose dehydrogenase (XDH) enzymes. XR presents dual cofactor dependence but uses NADPH over NADH, whereas XDH is NAD^+ dependent. The differences in cofactor specificity in the XR and XDH reactions and the generation of NADPH by the pentose phosphate pathway (PPP) limit the availability of NAD^+ for the oxidation of xylitol to xylulose, resulting in xylitol secretion (175, 176). Among different xylose-utilizing yeasts, *Candida* species present the best xylitol production capacity (47, 177) and some process and metabolic engineering approaches have already proven to be successful in enhancing overall titers and productivities (178–180). However, xylitol yields are limited by the use of xylose as the carbon source for cell growth and maintenance energy. Given this context, the use of *S. cerevisiae*, which is naturally incapable of metabolizing xylose, is an appealing approach to enhance xylitol yields. The expression of genes coding for enzymes with XR activity allows the direction of xylose flux only for bioconversion and the xylitol produced is not further oxidized to xylulose and metabolized by this yeast. Consequently, engineered strains require a co-substrate for cell growth and metabolism but also to regenerate cofactors, essential for the NAD(P)H-dependent XR enzyme catalysis.

Initial attempts to produce xylitol in *S. cerevisiae* relied on the expression of the *XYL1* gene from the xylose-consuming *Scheffersomyces stipitis* (formerly known as *Pichia stipitis*) coding for XR, which increased the yields near to the theoretical maximum (1 g of xylitol per gram of xylose) (181). The recombinant *S. cerevisiae* expressing the *XYL1* gene was also evaluated for xylitol production using different co-substrates (glucose, ethanol, acetic acid, and glycerol), and only glucose and ethanol were efficiently used (182). Similarly, the use of glucose improved the xylitol production by the recombinant *S. cerevisiae* expressing the *XYL1* gene from *Candida shahatae*, in comparison with the results obtained using galactose and maltose as co-substrate (183). Nevertheless, the use of glucose as a co-substrate inhibits the transport of xylose into the cells (184) decreasing xylitol productivity. A commonly used strategy for improving the xylose uptake in the presence of glucose is using a high molar ratio of xylose to glucose during the bioconversion phase. This glucose-limited fed-batch fermentation strategy has already proven to be successful for cofactor regeneration and to generate maintenance energy without glucose repression, resulting in high productivities and yields of xylitol (Table 1.3) (185–187). Another approach to bypass glucose repression and improve xylose transport relied on the expression of the *B.*

subtilis araE gene coding for an arabinose:H^+ symporter, which has been previously proven to enhance xylose transport capacity in *S. cerevisiae* (188). The expression of *araE* together with the expression of *XYL1* gene from *S. stipitis*, increased the xylitol productivity to 2.47 g/(L·h) (189). Alternatively, (190) efficiently produced xylitol without glucose repression through the utilization of cellobiose, a dimer of glucose. For this, a recombinant *S. cerevisiae* strain expressing a xylose reductase from *S. stipitis* was further engineered for cellobiose utilization by the expression of the *CDT-1* and *GH1-1* genes (from the filamentous fungus *Neurospora crassa*) coding for a cellodextrin transporter and intracellular ß-glucosidase, respectively. The resulting strain showed cellobiose and xylose co-consumption and higher xylitol productivity, compared to sequential utilization of glucose and xylose (190). More recently, adaptive evolution followed by genome sequencing of the evolved strains coupled with reverse engineering strategies showed that reduced glucose phosphorylation rates led to simultaneous glucose and xylose utilization, improving the xylitol production (191). As afore mentioned, XR enzymes show a preference for NADPH over NADH and the main source of NADPH in yeast cells is the oxidative PPP. To overcome the preference for NADPH and allow the simultaneous utilization of NADPH and NADH cofactors, the co-expression of wild and mutant *S. stipitis XYL1* genes enhanced the xylitol yield and productivity. This strain was further engineered to increase the intracellular concentrations of NADPH and NADH cofactors by the overexpression of both *ZWF1* and *ACS1* genes encoding for glucose-6-phosphate dehydrogenase (G6PDH) and acetyl-CoA synthetase. The overproduction of the G6PDH increases the flux through PPP, responsible for NADPH production, and acetyl-CoA synthetase can contribute for cofactor regeneration since it is responsible for the conversion of acetate to acetyl-CoA, which is further metabolized in the TCA cycle, generating NADH. This engineered strain produced 196.2 g/L of xylitol with remarkable productivity of 4.27 g/(L·h) (192). Another metabolic engineering approach to further increase the NADPH availability focused on the redirection of the carbon flux to the PPP, limiting the carbon flux into glycolysis. The downregulation of the *PGI1* gene, coding for phosphoglucose isomerase (PGI) reduces the conversion of glucose-6-phosphate to fructose-6-phosphate in glycolysis first step, resulting in glucose-6-phosphate accumulation, which can be used by G6PDH in the PPP. The reduction of PGI activity alone was not successful, except with the simultaneous overexpression of the *ZWF1* gene that improved the xylitol productivity by 1.9-fold when compared with the parental strain expressing only the *XYL1* gene (193).

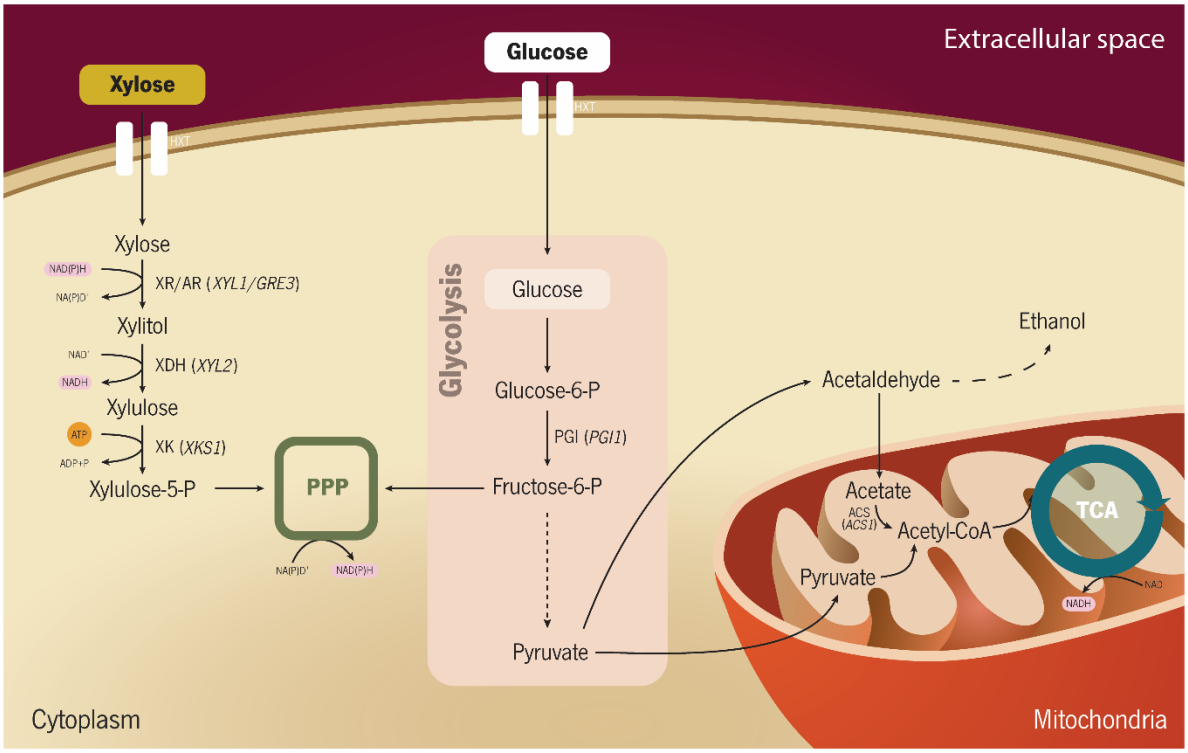


Figure 1.4. Metabolic pathways involved in xylitol production by yeast and introduced genetic modifications. Dashed arrows indicate multiple step reactions. Abbreviations: XR, xylose reductase; AR, aldose reductase; XDH, xylitol dehydrogenase; XK, xylose kinase; PPP, pentose phosphate pathway; PGI, phosphoglucose isomerase.

Much progress has been made in the production of xylitol from lignocellulosic-derived xylose. Kogje and Ghosalkar (194) engineered *S. cerevisiae* to produce xylitol from a non-detoxified corn cob hydrolysate supplemented with synthetic glucose in fed-batch mode. The recombinant ScpGT strain expressing the *SUT1* gene, coding for a specific xylose transporter and overexpressing the *GRE3* gene produced 22.4 g/L of xylitol. The dilution of the hemicellulosic hydrolysate decreased the xylose concentration from 65 g/L to 40 g/L also enabling the mitigation of negative effects caused by the presence of lignocellulose-derived inhibitors (194). The same authors expressed both *GRE3* and *SUT1* genes in an industrial strain to produce xylitol from a detoxified corn cob hydrolysate, using glycerol as co-substrate. The recombinant *S. cerevisiae* XP-RTK efficiently produced 47 g/L of xylitol with a maximal productivity of 0.37 g/(L·h) (195). In fact, the bioconversion process might profit from the replacement of glucose by glycerol, which prevents catabolite repression and ethanol fermentation, favoring the biomass production. Cell surface engineering of *S. cerevisiae* has been proposed to produce xylitol directly from pretreated lignocellulosic biomass. Guirimand et al. (196) engineered a *S. cerevisiae* strain to express *XYL1* from *S. stipitis* and co-display three different hydrolases on its cell surface: β-glucosidase (from *Aspergillus aculeatus*), xylosidase (from *Aspergillus oryzae*) and xylanase (from *Trichoderma*

reesei). For the cell surface attachment, the target proteins were fused to the anchoring domain of yeast cell wall proteins, SED1 or SAG1. The recombinant strain produced 5.8 g/L of xylitol directly from the xylooligosaccharides-enriched liquid fraction of pretreated rice straw, representing 79.5% of the theoretical yield from the xylose contained in the hydrolysate. To improve xylitol titer, the rice straw hydrolysate was submitted to a membrane separation step (nanofiltration) to increase xylose concentration that also removed fermentation inhibitors, resulting in the production of 37.9 g/L of xylitol. Nevertheless, both rice straw hydrolysates (unfiltered and membrane separated) showed an incomplete xylose conversion. In this sense, the recombinant strain was further improved in terms of promoters, secretion signal and anchoring domain sequences, achieving maximal xylitol concentrations of 6.97 g/L and 4.2 g/L from rice straw hydrolysate and Kraft pulp residue, respectively (197). Furthermore, this cell surface strategy was combined with the expression of different sugar transports to improve the xylose uptake, and the expression of *MAL11* gene, encoding for a maltose transporter, resulted in a 30% increase in xylitol production (197).

A considerable number of metabolic engineering strategies have been successfully applied to convert *S. cerevisiae* into an efficient xylitol producer. The recent research development has greatly expanded the understanding of mechanisms involved in xylitol synthesis by yeast, which is essential to develop sustainable xylitol production systems based on renewable raw material. However, the full potential of the technology for production at large scale, needs to be improved to reach industrial applications.

Table 1.3. Metabolic engineering strategies for xylitol production in *S. cerevisiae*

Strain	Genetic modification	Substrate/ cultivation strategy	Titer (g/L)	Xylitol Productivity (g/L-h)	Yield (g/g)	Reference
GPY55-15Bα + pMA91	Expression of *XYL1* (coding for xylose reductase) from *Scheffersomyces stipitis*	Xylose + Glucose, Batch	19	NR	> 0.95	(181)
H475		Xylose + Glucose, ethanol, acetate and glycerol, Batch	NR	NR	≈ 1	(182)
EH13.15:pY2XR		Xylose + Glucose, Fed-batch	105	1.69	> 0.95	(187)
BJ3505/δXR		Xylose + Glucose, Fed-batch	78	1.1	0.9	(186)
		Xylose + Glucose, Fed-batch and cell-recycling fermentation	116	2.34	0.9	(185)
Y294:pRG16	Expression of *XYL1* from *Candida shehatae*	Xylose + Glucose, Batch	15	NR	0.86	(183)
DXXA	*XYL1* gene from *Scheffersomyces stipitis*; *AraE* (arabinose: H^+ symporter) from *Bacillus subtilis*	Xylose + Glucose, Fed-batch	178	2.47	≈ 1	(189)
D-10-BT	*XYL1* (xylose reductase) from *Scheffersomyces Stipitis; CDT-1* (cellodextrin transporter); *GDH1-1*(ß-glucosidase) from the *Neurospora crassa*	Xylose + Cellobiose, Fed-batch	93	1.50	0.98	(190)
SR8#22	Evolved strain mutated in *GLK1* (glucokinase), *HXK1* (hexokinase 1) and *HXK2* (hexokinase 2) genes, expressing *XYL* (xylose reductase)		21	NR	≈ 1	(191)
ScpGT	*GRE3* (endogenous aldose reductase) and *SUT1* (xylose specific transporter)	Xylose + Glucose, Fed-batch	21	0.34	≈ 1	(194)
		Corn cob hydrolysate + Glucose, Fed-batch	22	NR	≈ 1	
DWM-ZWF1-ACS1	*XYL1* (xylose reductase, NADPH-dependent) from *Scheffersomyces stipitis; ΔXYL* (mutant xylose reductase, NADH-preferring); *ZWF1* (glucose-6-phosphate dehydrogenase); *ACS1* (acetyl-CoA synthetase)	Xylose + Glucose, Fed-batch	196	4.27	≈ 1	(192)
XP-RTK	*XYL1* (xylose reductase) from *Scheffersomyces stipitis, Candida Tropicalis, Neurospora crassa* and *SUT1* (xylose specific transporter)	Glycerol, Corn cob hydrolysate Fed-batch	47	0.37	≈ 1	(195)
YPH499-XR-BGL-XYL-XYN	*XYL1 (xylose reductase) from Scheffersomyces stipitis, BGL (β-glucosidase) from Aspergillus aculeatus, XylA (β-xylosidase) from Aspergillus oryzae and XYN (endoxylanase II) from Trichoderma reesei*	Rice straw hydrolysate, CBP	6	NR	≈ 1	(196)
		Rice straw hydrolysate (membrane-filtrated), CBP	38	NR		
YPH499-XR-BGL-XYLsss-XYNsss	*XYL1* (xylose reductase) from *Scheffersomyces stipitis, BGL* (β-glucosidase) from *Aspergillus aculeatus, XylA* (β-xylosidase) from *Aspergillus oryzae* and *XYN* (endoxylanase II) from *Trichoderma reesei, SED1* promoter, secretion signal and *anchoring domain*	Rice straw hydrolysate, CBP	7	NR	≈ 1	(197)
		Kraft Pulp, CBP	4	NR		

1.3.2.2. Biotechnological production of arabitol

The production of arabitol through chemical synthesis has become problematic due to the high number of by-products generated and the environmental impact of chemical waste management (Fabre et al., 2002). As a result, there has been a growing interest in using biotechnological methods to produce arabitol.

The first description of the isolation of microorganisms capable of producing arabitol by fermentation was reported in 1956. Arabitol was produced along with other sugar alcohols (glycerol, erythritol, D-arabitol, and mannitol) from glucose by a plethora of osmophilic yeast isolated from different sources (198). Various species of yeast, including Zygosaccharomyces, Debaryomyces, Candida, Pichia, and Metschnikowia, have been found to naturally produce arabitol as part of their metabolic processes (Table 1.4). This polyol serves as both an osmoprotectant and a carbohydrate storehouse (199). Furthermore, arabitol plays a vital role in the defence mechanism of yeast in response to environmental stress and has potential as a redox regulator and virulence factor in host-pathogen interactions (200, 201).

The production of arabitol from glucose is linked to the pentose phosphate pathway (PPP). Glucose is firstly phosphorylated to glucose-6-phosphate, which enters in the PPP. At the glucose-6-phosphate branching point (the starting product of oxidative phase of PPP), glucose-6-phosphate can follow two different metabolic fates depending on the yeast species (202). In some organisms, glucose-6-phosphate is converted to ribulose-5-phosphate, which is further dephosphorylated to D-ribulose and then a NADP-dependent dehydrogenase converts D-ribulose to D-arabitol. Alternatively, glucose-6-phosphate is converted into xylulose-5-phosphate and dephosphorylated to D-xylulose. Subsequently, NAD-dependent arabitol dehydrogenase reduces D-xylulose to D-arabitol (203, 204).

Significant efforts have been made to produce D-arabitol using glucose as the sole carbon source. *Candida fannata* R28, isolated from soy sauce mash, produced 50 g/L arabitol from 100 g/L of glucose in 36h of cultivation, demonstrating that only 50% of glucose was utilized for arabitol synthesis while the other 50% was used by the cells for growth and maintenance (205). The highest reported titer of arabitol by microbial fermentation was achieved by *Metschnikowia reukaufii* AJ14787. After optimizing temperature and pH parameters, a fed-batch fermentation of 700 g/L glucose (supplemented with ammonium sulfate and yeast extract) resulted in 206 g/L of arabitol (0.29 g/g yield) and more than 20 g/L of glycerol as a byproduct after 100 hours of cultivation (206). Despite these fermentation optimization efforts, the production yield is still considerably lower than the theoretical yield. The yeast *Zygosaccharomyces rouxii,* industrially used on the production of soy sauce and fermented foods, is

considered an attractive arabitol producer, due to its osmotolerance ability and food safety. Saha et al 2007 described the production of 83.4 g/L of arabitol with a corresponding yield of 0.48 g/g using an isolated *Z. rouxii* NRRL Y-27624 (204). In another study, a repeated-batch fermentation process resulted in 93.5 g/L of arabitol from 200 g/L of glucose using the *Z. rouxii* JM-C46 (207). More recently, the same strain was genetically engineered to overexpress key genes (arab and ardh2 encoding ribulokinase and d-arabinitol 2-dehydrogenase) of arabitol synthesis pathway, which starts from ribulose-5P. In addition, G6PD and 6PGDH coding genes were overexpressed for enhancing the supply of d-ribulose-5P, and two transhydrogenases encoded by *pntAB* and *sthA* heterologous genes were expressed to ensure the cofactor supply required for d-arabinitol 2-dehydrogenase-mediated hydrogenation reduction of D-ribulose to D-arabitol. These metabolic engineering strategies, together with the optimization of fermentation conditions resulted in a remarkable concentration of 149 g/L of arabitol with a yield of 0.75 g/g, after 144 hours of cultivation (208). Despite attempts to optimize the fermentation conditions and increase the yield, the production of arabitol remains a challenge, with low production yields and long incubation periods. Additionally, the co-production of other products, such as glycerol, presents further hurdles to the industrial use of native arabitol-producing yeasts (199).

The US Department of Energy has classified arabitol as one of the top twelve energy building blocks for biorefinery, further emphasizing the need for efficient and sustainable methods of producing this important polyol. In recent years, research has focused on finding yeast strains that can produce arabitol from alternative carbon sources, such as glycerol and arabinose, with the aim of utilizing a broad range of waste products.

Given that glycerol is a by-product of the biodiesel industry, various methods for its utilization as a substrate for arabitol production have been proposed (Table 1.4). In an early screening study, species from different genera produced different polyols or polyol mixtures from glycerol. Among the cultures screened, strains belonging to the genera Debaryomyces and Geotrichum produced arabitol (concentrations ≥ 5 g/L) after 3 days of cultivation (209). Later, a study to improve the arabitol production from glycerol by optimizing the operating parameters such as pH, dissolved oxygen concentration and medium composition, resulted in 40 g/L of arabitol (yield of 55%) after 5 days of *Debaryomyces hansenii* NRRL Y-7843 cultivation (210). The ability to produce arabitol from crude glycerol was demonstrated for the first time by *Candida quercitrusa* NBRC1022, which produced 85.1 g/L after 10 days, corresponding to a yield of 0.40 g/g. Another study using crude glycerol reported a production of 119 g/L of arabitol by an osmotolerant mutant *Yarrowia lipolytica* ARA9 under nitrogen-excess conditions (211). The maximum D-arabitol production reached 118.5 g/L at 108 h with the yield of 0.49 g/g. More recently,

the non-conventional *Wickerhamomyces anomalus* was able to produce 265 g/L of arabitol from glycerol given with a pulse of 200 g/L and continuously fed at the rate of 1.75 g/L/h for 129 h. This arabitol titer obtained from glycerol required tightly controlled production conditions of both growth and production phases (212).

Arabitol can also be synthesized from arabinose by bacteria and yeast species. In bacteria, the set of genes for arabinose metabolism is organized in araBAD operon, which contains three structural genes *ara*A, *araB* and *araD* coding for L-arabinose isomerase, L-ribolukinase and L-ribulose 5-phosphate 4-epimerase. These genes catalyse conversion of L-arabinose to L-ribulose 5-phosphate that enters the PPP pathway. In yeast, arabinose is first converted by an aldose reductase (AR) to L-arabitol. The next step in the pathway is the conversion of L-arabitol to L-xylulose, which is facilitated by the enzyme L-arabitol dehydrogenase (LAD). L-xylulose is then reduced to xylitol by L-xylulose reductase (LXR). Finally, xylitol is converted to D-xylulose and D-xylulose-5-phosphate by xylitol dehydrogenase (XDH) and D-Xylulokinase (Figure 1.5).

Research into arabitol-producing yeasts dates back to the 1970s, when studies were conducted on the handling and utilization of lignocellulosic materials. Barnett et al., (213) in 1976 identified 100 out of 400 yeast strains capable of growing aerobically on L-arabinose. Table 1.4 highlights the native yeast strains that are best suited for producing arabitol from arabinose. Saha and Bothast (214) evaluated 49 yeast strains from the genera Candida, Pichia, Debaryomyces, Kluyveromyces, and Saccharomycopsis that were capable of utilizing L-arabinose. Based on their superior rates of substrate conversion to arabitol, *Candida entomaea* NRR Y-7785 and *Pichia guilliermondii* NRRL Y-2075 were selected to grown in 50 g/L of L-arabinose at different pH and temperatures. Results showed a maximal production of 35 g/L of arabitol for *C. entomaea* (yield of 0.7g/g) and 36 g/L for P*. guilliermondii* (yield of 0.71 g/g). Another study screened 116 different yeast species for the ability to ferment arabinose and found two strains (*Candida auringiensis* and *Candida succiphila*) that produced arabitol as the main polyol under the tested conditions (Table 1.4) (215). Later investigations screened over 1600 yeast strains to identify yeasts that ferment arabinose and xylose. More recently, another study highlights the potential of newly isolated yeast strains from seawater and sugarcane plantation soil for arabitol production. The seawater isolate, *Pichia manchurica*, was found to have the highest arabitol production titter (12.8 g/L) but with a limited conversion yield of 0.10 g/g (216).

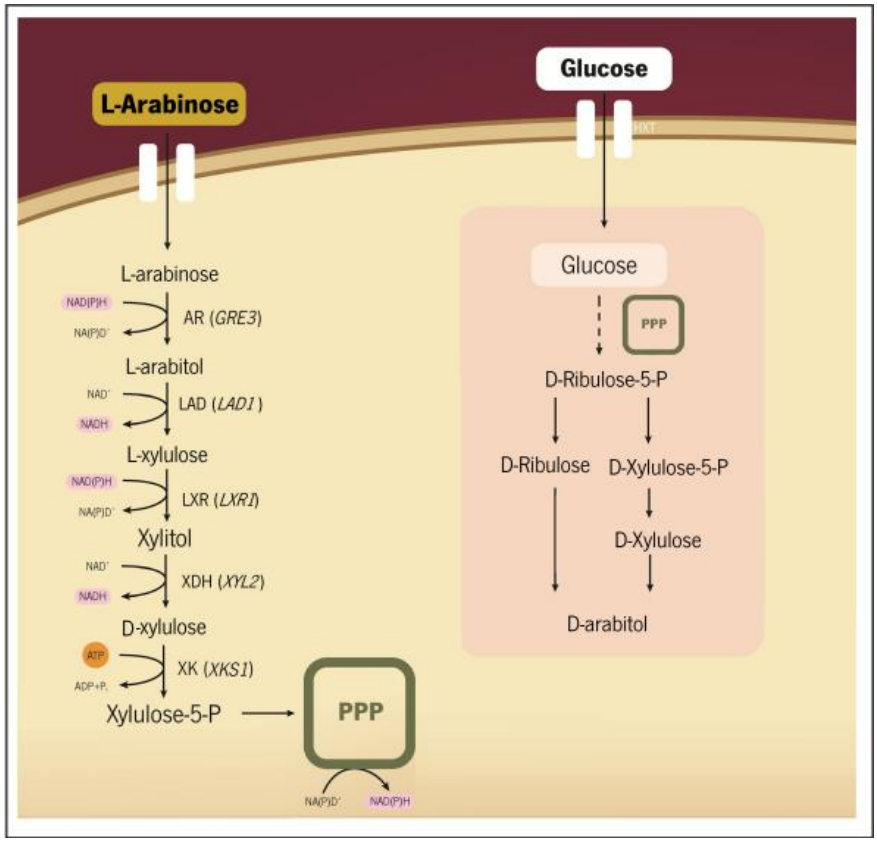


Figure 1.5. Different metabolic pathways involved in arabitol production, using arabinose and glucose by yeast. Dashed arrows indicate multiple step reactions. Abbreviations: AR, aldose reductase; LAD, L-arabitol dehydrogenase; LXR, L-xylulose reductase; XDH, xylose dehydrogenase; XK, xylose kinase; PPP, pentose phosphate pathway.

Although *S. cerevisiae* is widely used in the food industry and has potential for producing arabitol, the research efforts have been focused on the arabinose fermentation, with ethanol as target product. Since arabitol is an intermediate compound of arabinose utilization pathway, these strategies have resulted on the production of arabitol as by-product. In 2001, Sedlak and Ho (217) utilized strong promoters from glycolytic genes to express the *E. coli ara*BAD operon in a *S. cerevisiae* strain, with the goal of producing ethanol. However, instead of ethanol, the heterologous expression resulted in the production of 13-15 g/L of arabitol. Similarly, a recombinant *S. cerevisiae* strain 424A(LNH-ST), able to efficiently ferment xylose to ethanol, was further modified for arabinose utilization by overexpression of two additional fungal genes: *LAD1* from *Trichoderma reesei* and *LXR1* from *Ambrosiozyma monospora*. The resulting new strain exhibited production of ethanol from L-arabinose, with a yield higher than 40% and co-produced 15 g/L of arabitol (218). Considering that *S. cerevisiae* is a well-established microorganism with a long history of use in biotechnological processes, owing to its robustness and GRAS status, it represents a relevant cell platform for genetic engineering aimed at producing arabitol.

Table 1.4. Studies on yeast production of arabitol using different carbon sources.

Substrate	Yeast strain	Operation conditions	Arabitol		Reference
			Titer (g/L)	Yield (g/g)	
Glucose	*Zygosaccharomyces rouxii* NRRL Y-27,624	Batch, 30 °C, 350 rpm, pH 5	83.4	0.48	(204)
	Zygosaccharomyces rouxii JM-C46	Repeated Fed-batch, 30 °C, 300 rpm, 0.8 vvm	93.5	0.47	(207)
	Zygosaccharomyces rouxii JM-C46 – ZR5A	Fed-batch, 30 °C, 600 rpm, 1 vvm	149	0.74	(208)
	Candida famata R28	Batch, 30 °C, 280 rpm, pH 3.6	50	0.50	(205)
	Metschnikowia reukaufii AJ14787	Fed-batch, 30 °C, 600 rpm, pH 5	206	0.29	(206)
Glycerol	*Candida quercitrusa* NBRC1022	Fed-batch, 28 °C, 800 rpm, pH 6	85.1	0.40	(219)
	Wickerhamomyces anomalus WC 1501	Fed-batch, 32.5 °C, 0.5 vvm, pH 5.9	265	0.74	(212)
	Yarrowia lipolytica	Fed-batch, 30 °C, 600 rpm, pH 5, 1 vvm	119	0.49	(211)
	Debaryomyces hansenii NRRL Y-7843	Batch, 30 °C, 250 rpm, pH 3.5	40	0.02	(210)
Arabinose	*Candida entomaea* NRRL Y-7785	Batch, 34 °C, 200 rpm, pH 5	35	0.70	(214)
	Pichia guilliermondii NRRL Y-2075	Batch, 34 °C, 200 rpm, pH 4	35.5	0.71	(214)
	Candida auringiensis NRRL Y-11848	Batch, 25 °C, 140 rpm	73	0.73	(215)
	Candida succiphila Y-1199	Batch, 25 °C, 140 rpm	81	0.81	(215)
	Pichia manchurica	Batch, 30 °C, 180 rpm, pH 6	12.8	0.10	(216)

1.3.2.3. Biotechnological production of tagatose

The first report of the microbial production tagatose dated from 1984. The strain *Arthrobacter globiformis* ST48, isolated from soil samples, produced 14 g of tagatose using galactitol as carbon source (220). The production of tagatose from galactitol using an oxidoreductase enzyme (galactitol 2-dehydrogenase) was studied in other bacteria such as, *Mycobacterium smegmatis* (221), *Enterobacter agglomerans* (222) and *Gluconobacter oxydans* (223), but despite the high galactitol oxidation yields (85 - 92%), this substrate was not further exploited owing to its high cost and limited availability (224, 225). Tagatose can be also produced from sorbose using D-tagatose-3-epimerase (226), but considering that sorbose is a rare sugar, its commercial application seems to be limited. Although it has been demonstrated that epimerases and oxidoreductases enzymes can be used to produce tagatose, the most common route is isomerization of galactose by L-arabinose isomerase (227).

The enzyme L-arabinose isomerase (L-AI) is responsible for the reversible isomerization of arabinose to ribulose and is also capable of isomerize galactose to tagatose. Currently, few commercial enzyme-based bioprocesses are implemented, and extensive research efforts have been devoted to find L-Ais with high D-galactose isomerization activity. Table 1.5 summarizes the enzymatic properties of a variety of L-Ais isolated from different microorganisms.

Tagatose production utilizing LAI has shown some limitations that may be impeding commercial viability: (i) unfavorable enzymatic kinetics because galactose is not the native substrate of L-AI; (ii) low enzyme stability, especially in the absence of divalent metal ions; and (iii) thermodynamic equilibrium that leads to an incomplete conversion of galactose, generating a mixture of galactose and tagatose. To improve the catalytic activity of L-AI toward galactose, several research groups have used enzyme engineering methods such as the creation of chimeras by homologous recombination of homologous L-AIs (228), site-saturation (229) and site-directed mutagenesis (229–232). To address the low stability of L-AIs, cell surface display (233) and enzyme encapsulation approaches (234–238) have shown improvements in L-AI stability and conversion yield. The L-AI from *Lactobacillus brevis* was displayed on the surface of *Bacillus subtilis* endospores and used as an immobilized biocatalyst for producing tagatose, achieving a remarkable conversion of 80% from 125 g/L of galactose (239). Finally, the thermodynamic equilibrium is the most challenging aspect of galactose isomerization to tagatose. This limitation is inherent to enzymatic reactions catalyzed by isomerases, in which the concentrations of substrates and products reach an equilibrium that do not change with time. The application thermophilic L-AI enzymes can achieve higher conversions since the equilibrium shifts toward tagatose at high temperatures (240–

242). However, reaction temperatures above 80 °C induce tagatose degradation, which results in the formation of an undesirable caramel-like aroma compound and a brown pigment (243).

Table 1.5. Sources and properties of L-arabinose isomerases with specific activity towards galactose.

Microbial source	Optimal conditions		Metal ion requirement	Conversion yield (%)	Reference
	pH	temperature			
Acidothermus cellulolyticus	7.5	75	Mn^{2+}, Co^{2+}	50	(244)
Alicylobacillus hesperidum	7.0	70	Mn^{2+}, Co^{2+}	43	(245)
Anoxybacillus flavithermus	9.5–10.5	95	Ni^{2+}	60	(246)
Arthrobacter sp. 22c	5.0–9.0	47–52	No requirement	30	(224)
Bacillus stearothermophilus IAM11001	7.5	65	Mn^{2+}	36	(247)
Bacillus stearothermophilus US100	7.5	80	Mn^{2+}, Co^{2+}	48	(248)
Bacillus thermoglucosidasius	7.0	40	Mn^{2+}	46	(249)
Bifidobacterium adolescentis	6.5	55	Mn^{2+}	57	(250)
Bifidobacterium longum	6.0–6.5	55	Mg^{2+}, Mn^{2+}	35	(251)
Klebsiella pneumoniae	8.0	40	Mn^{2+}	34	(252)
Lactobacillus fermentum CGMCC2921	6.5	65	Mn^{2+}, Co^{2+}	55	(253)
Lactobacillus plantarum NC8	7.5	60	Mn^{2+}, Co^{2+}	30	(254)
Lactobacillus plantarum SK-2	7.0	50	Mn^{2+}	25	(255)
Lactobacillus sakei 23K	5.0–7.0	30–40	Mn^{2+}, Mg^{2+}	36	(256)
Lactobacillus reuteri 100-23	6.0	65	Mn^{2+}, Co^{2+}	35	(257)
Lactococcus lactis	8.0	50	Co^{2+}	42	(258)
Lactobacillus parabuchneri CICC 6004	6.7	45	Ca^{2+}	39	(259)
Lactobacillus brevis	7.0	65	Co^{2+}	43	(260)
Pediococcus pentosaceus PC-5	6.0	50	Mn^{2+}, Co^{2+}	52	(261)
Shewanella sp. ANA-3	5.5–6.5	15–35	Mn^{2+}	34	(262)
Thermotoga neapolitana	7.0	85	Mn^{2+}, Co^{2+}	68	(263)
Thermotoga maritima	7.0–7.5	90	Mn^{2+}, Co^{2+}	56	(240)
Thermus sp.	8.5	60	Mn^{2+}	54	(264)
Thermoanaerobacterium saccharolyticum NTOU1	7.0–7.5	70	Mn^{2+}, Co^{2+}	41	(265)

Otherwise, it was shown that adding borate improved galactose to tagatose isomerization (266). Borate forms compounds with hexoses and ketones that do not participate in the reaction. The higher affinity of borate for ketoses than hexoses cause a significant shift in the equilibrium toward tagatose, but also increases the reaction temperature (267). The addition of borate to the isomerization catalyzed by *Geobacillus thermodenitrificans* L-AI resulted in 370 g/L tagatose from 500 g/L galactose, corresponding to a conversion yield of 74% (268). Although significant improvements to conversion may be achieved by introducing borate to the reaction buffer, the removal of this salt from product would also increase purification costs. Alternatively, the permeabilization of whole-cell biocatalysts by chemical agents (detergents or solvents) has been shown to be useful to circumvent this thermodynamic limitation. Bober et al. (238), used Triton X-100 and SDS as permeabilization surfactants to permeabilize the food-safe probiotic bacterium *Lactobacillus plantarum* and observed an increase in tagatose production after treatment with SDS, greater than that of treatment Triton X-100. *L. plantarum* whole-cell biocatalyst achieved a maximal conversion of ~85%, at 50 °C in 48 h batch process, enhancing the equilibrium conversion compared to free enzyme system (238). Recently, a disruptive strategy has been suggested to circumvent thermodynamic equilibrium by substituting isomerization for in vivo oxidoreaductase reactions. A recombinant *S. cerevisiae* strain capable of fermenting cellobiose and lactose (269), was engineered to produce tagatose from lactose through the deletion of the *GAL1* gene (encoding a galactose kinase) to eliminate galactose utilization, and the expression of the heterologous xylose reductase and the galactitol dehydrogenase. The engineered yeast produced 38 g/L of tagatose from lactose at a 9:1 ratio of tagatose to galactose (270).

Tagatose has been the topic of intense research over the past two decades, which has led to a significant expansion of enzyme-based technologies for its biosynthesis. The use of purified enzymes, whole cells, crude enzyme extracts, and the combination of whole-cell systems with purified or crude enzymes are among the biosynthesis approaches explored. Wanarska and Kur (224) isolated the *araA* gene from psychrotolerant *Arthrobacter* sp. 22c for cloning and expression in *Escherichia coli*, and the purified L-AI was used to isomerize the galactose resulting from the cultivation of a recombinant *Pichia pastoris* on lactose. The yeast, engineered to secret the ß-galactosidase from *Arthrobacter chlorophenolicus,* hydrolyzed 90% of lactose into glucose and galactose. The former was readily metabolized by the yeast and the later was accumulated in the medium since the yeast lacks all genes for galactose metabolism. Even though this strategy can prevent the formation of byproducts, resulted in a poor galactose conversion yield (30%). In another study, the hydrolysis of lactose and galactose isomerization for tagatose production was established by using crude enzymes of recombinant

Escherichia coli BL21 expressing a heterologous L-AI. Taking advantage of the thermostability of the native E. coli β-galactosidase, which was capable of hydrolysing lactose at 50 °C (the optimal temperature for galactose isomerization by L-AI), no exogenous β-galactosidase addition was required. In addition, the remaining sugars (glucose and unconverted galactose) were fermented by a *S. cerevisiae* strain, yielding a final production of 26 g/L of ethanol and 44 g/L of tagatose (271). The crude enzyme preparations are an inexpensive option, and the presence of extra enzyme activities may affect the quality and yield of the final product.

Currently, large scale production of tagatose from synthetic galactose has not proven economically viable. However, this monomeric sugar is widely present in natural sources (marine algae, lignocellulosic biomass and dairy wastes) and the utilization of galactose derived from these materials is under research (272). Cheese whey, the main dairy by-product (273), is increasingly recognized as an inexpensive and largely available source of tagatose. A variety of strategies have been proposed for the biosynthesis of tagatose from lactose-derived galactose sugars, using a combination of β-galactosidase and L-AI enzymes (274–276). Nevertheless, a key problem in handling lactose-containing industrial wastes is the pH factor. Considering that the optimum pH and temperature of each reaction are substantially different, the enzymatic hydrolysis using β-galactosidase followed by enzymatic isomerization by L-AI requires an optimized design of experimental conditions for each step, which increases the process complexity and costs (274, 277). Alternately, marine algae may be seen as an attractive source of galactose since they are easily obtainable and abundant in nature. Presently, few biotechnological processes using these feedstocks have been explored for tagatose production (278, 279).

Despite biotechnological advances to establish efficient tagatose production processes, the shift to an exclusive microbial-based system based on the valorization of agro-food wastes is still dependent on additional improvements focusing on the use of low-cost raw materials.

1.4. Aim of the thesis

Meeting the urgent need for sustainable natural sweeteners has proven challenging, with limited progress made to date. The predominant focus of studies in the scientific literature has been on bioprocesses that rely on chemical catalytic processes or bioprocesses based on synthetic media using non-Saccharomyces microorganisms, which are not well-suited to the complexities of fermentation using lignocellulosic hydrolysates. These approaches have still fallen short of achieving sustainable and economically viable biorefineries. To address these challenges, a shift towards the use of renewable carbon sources and the reintroduction of agro-industrial waste into the supply chain is necessary. The use of such materials can increase the sustainability of biorefineries by reducing operational costs and promoting the concept of a circular bioeconomy.

This thesis aims to develop industrial yeast-based processes for producing natural sweeteners such as xylitol, arabitol, and tagatose from 2nd and 3rd generation biomasses in an integrated process. To achieve this goal this thesis is mainly focused on:

1) Identify industrial yeast strains that are capable of producing natural sweeteners under industrial process-like conditions.
2) Engineer yeast strains to convert carbon sources such as xylose, arabinose, and galactose into xylitol, arabitol and tagatose, valorizing agricultural and industrial derived residues like corn cob, vine shoots, sugar beet pulp, and red seaweed biomass.
3) Integrate the engineered yeast into the biomass-to-sweeteners process using various agro-food wastes and evaluate different configurations for more efficient processes.
4) Optimize fermentation conditions to increase titers and yields through a combination of different conditions and carbon sources and assess alternative fermentation configurations.

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Chapter II.

Xylitol production from lignocellulosic whole slurry corn cob by engineered industrial *Saccharomyces cerevisiae* PE-2

This chapter is based on the following original research article:

Baptista SL, Cunha JT, Romaní A, Domingues L. 2018. Xylitol production from lignocellulosic whole slurry corn cob by engineered industrial *Saccharomyces cerevisiae* PE-2. Bioresour. Technol. 267:481-491. DOI:10.1016/j.biortech.2018.07.068.

Abstract

In this work, the industrial *Saccharomyces cerevisiae* PE-2 strain, presenting innate capacity for xylitol accumulation, was engineered for xylitol production by overexpression of the endogenous GRE3 gene and expression of different xylose reductases from *Pichia stipitis*. The best-performing GRE3-overexpressing strain was capable to produce 148.5 g/L of xylitol from high xylose-containing media, with a 0.95 g/g yield, and maintained close to maximum theoretical yields (0.89 g/g) when tested in non-detoxified corn cob hydrolysates. Furthermore, a successful integrated strategy was developed for the production of xylitol from whole slurry corn cob in a pre-saccharification and simultaneous saccharification and fermentation process (15% solid loading and 36 FPU) reaching xylitol yield of 0.93 g/g and a productivity of 0.54 g/L·h. This novel approach results in an intensified valorization of lignocellulosic biomass for xylitol production in a fully integrated process and represents an advance towards a circular economy.

Keywords: Xylitol production, Industrial *Saccharomyces cerevisiae* PE-2, GRE3 aldose reductase, Corn cob hemicellulosic hydrolysate, Pre-saccharification and simultaneous saccharification and fermentation (PSSF)

2.1. Introduction

Concerns regarding the progressive exhaustion of the fossil resources, and the resultant environmental and economic problems, have created the necessity to replace the current petroleum-based economy. Lignocellulosic biomass, being the most abundant and renewable biomass available on earth, has been receiving growing attention as a substitute for the fossil fuels (1). Lignocellulose presents a recalcitrant structure, mainly composed of cellulose (glucose monomers), hemicellulose (hexose and pentose sugars) and lignin; and the attainment of fermentable sugars from these biomasses requires pre-treatment and hydrolysis steps that also result in the release of microbial inhibitory compounds (2). In order to efficiently replace the petroleum based industry, the lignocellulose potential as a substrate for biofuels and value added chemicals must be fully exploited. In fact, the cellulosic fraction has already been extensively studied for the production of biofuels, nevertheless the attainment of a sustainable lignocellulose-based bioeconomy should include the valorization of the hemicellulose fraction for the production of value added products (1).

Xylitol, a sugar alcohol, has been identified as one of the 12-top value added compounds to be attained from biomass (3). It is a natural sweetener used as a sugar substitute in food and pharmaceutical industries, as it presents advantageous properties, such as, low energy content, insulin-independent metabolism, anticariogenecity, among other pharmacological properties (4). Furthermore, xylitol can also be used in the chemical industry as an intermediate for the synthesis of polymers (5). Currently, xylitol is industrially produced through chemical hydrolysis and hydrogenation of xylan, an expensive and laborious process. Furthermore, even using lignocellulose as a raw material, it is not an environmental-friendly process, resulting in a growing interest in the microbiological production of xylitol (6).

Several yeast, such as *P. stipitis* (*Scheffersomyces stipitis*), *Debaryomyces hansenii*, *Kluyveromyces marxianus* and Candida spp, are naturally capable of consuming xylose through a xylose reductase/xylitol dehydrogenase (XR/XDH) pathway, which converts xylose into xylitol, and subsequently to xylulose. Xylitol is a common by-product of this pathway, mainly resultant of the co-factor imbalance between the reaction catalyzed by the NAD(P)H-dependent xylose reductase (normally with preference for NADPH) and the NAD^+-dependent xylitol dehydrogenase (4). Considering these, yeast that are naturally capable of xylose consumption have been extensively studied for xylitol production (7–9), nevertheless the yields of xylitol from xylose are limited by the use of xylose for yeast growth and maintenance energy. In this sense, the use of *S. cerevisiae*, (a

GRAS microorganism naturally incapable of xylose metabolism) with increased expression of enzymes with xylose reductase activity has emerged as a solution to increase xylitol yields from xylose (10–16), since it allows an easier control of the sugars directed for yeast metabolism. Furthermore, the use of *S. cerevisiae* strains isolated from industrial harsh conditions presents another advantage in terms of increased tolerance towards the presence of lignocellulosic-derived inhibitors, in comparison to laboratorial strains and non-Saccharomyces yeast (17). In recent studies, the industrial *S. cerevisiae* PE-2, isolated from a first generation bioethanol plant, was found to be naturally prone to xylitol accumulation when expressing the XR/XDH pathway from *S. stipitis* (even using a NADH-preferable XR mutant). This accumulation was partially reverted by the deletion of *GRE3* (18, 19), a gene that codifies an unspecific aldose reductase (using NADPH as co-factor), previously reported to be responsible for xylitol accumulation in *S. cerevisiae* (20).

Despite the promising results already obtained with recombinant *S. cerevisiae* for xylitol production (13–15, 21), only few work focus on lignocellulosic hydrolysates (14, 22) and there are no studies focusing on the valorization of both cellulosic and hemicellulosic fractions of the lignocellulosic biomass. Considering this, and the *S. cerevisiae* PE-2 potential for xylitol accumulation, this strain was used as chassis in this study to express the xylose reductase from *P. stipitis*, both the wild type and a mutant with preference for NADH, and to overexpress the endogenous *GRE3* gene, to: (1) evaluate xylitol production in terms of enzyme and co-factor preference, (2) develop an efficient strategy for xylitol production through the valorization of corn cob whole slurry.

2.2. Materials and Methods

2.2.1. Strains and plasmid construction

The yeast strains used in this study are listed in Table 2.1 Escherichia coli NZy5 (NZYTech) was used for plasmid propagation and maintenance. Yeast DNA transformation was carried out using the LiAC/SS carrier DNA/PEG method (23). Yeast strains were maintained at 4 °C on YPD plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar). For recombinant yeast strains, liquid and solid YPD media were supplemented with 150 mg/L and 200 mg/L of geneticin (G418), respectively.

For the construction of *S. cerevisiae* PE-2 strains expressing a native *P. spititis* xylose reductase (XR) and a NADH-preferable xylose reductase mutant (XR-N272D) from *P. spititis*, the *URA3* marker of the p417 and p418 vectors (Table 2.1) was replaced by the kanMX marker by in vivo homologous recombination: the kanMX geneticin resistance cassette was amplified from plasmid pUG6 (24) with the primers KanMX4_FW and KanMX4_RV and co-transformed with the p417 and p418 vectors digested in the NheI restriction site (Figure 2.1). Transformants were selected on YPD plates containing 200 µg/mL of G418. The resulting vectors were named p417-kan and p418-kan and the *S. cerevisiae* PE-2 strains carrying them were given the name PE-2-XRwt and PE-2-XRmut, respectively (Table 2.1). Additionally, to overexpress the *GRE3* gene, a plasmid containing this gene was constructed by homologous recombination from the described above p417-kan. Briefly, the p417-kan plasmid was digested with AatII, to remove most of the XR_wt gene sequence, and co-transformed with the *GRE3* gene, amplified with primers GRE3-TEF1-FW and GRE3-TDH3-RV from chromosomal DNA of PE-2 strain, originating the plasmid p417-kan-GRE3 (Figure 2.1). *S. cerevisiae* PE-2 and CENPK.113-5D transformants containing this plasmid were selected in YPD plates containing 200 µg/mL of G418 and were named PE-2-GRE3 and CENPK.113-5D-GRE3, respectively. The correct recombination between the DNA molecules was confirmed by colony PCR.

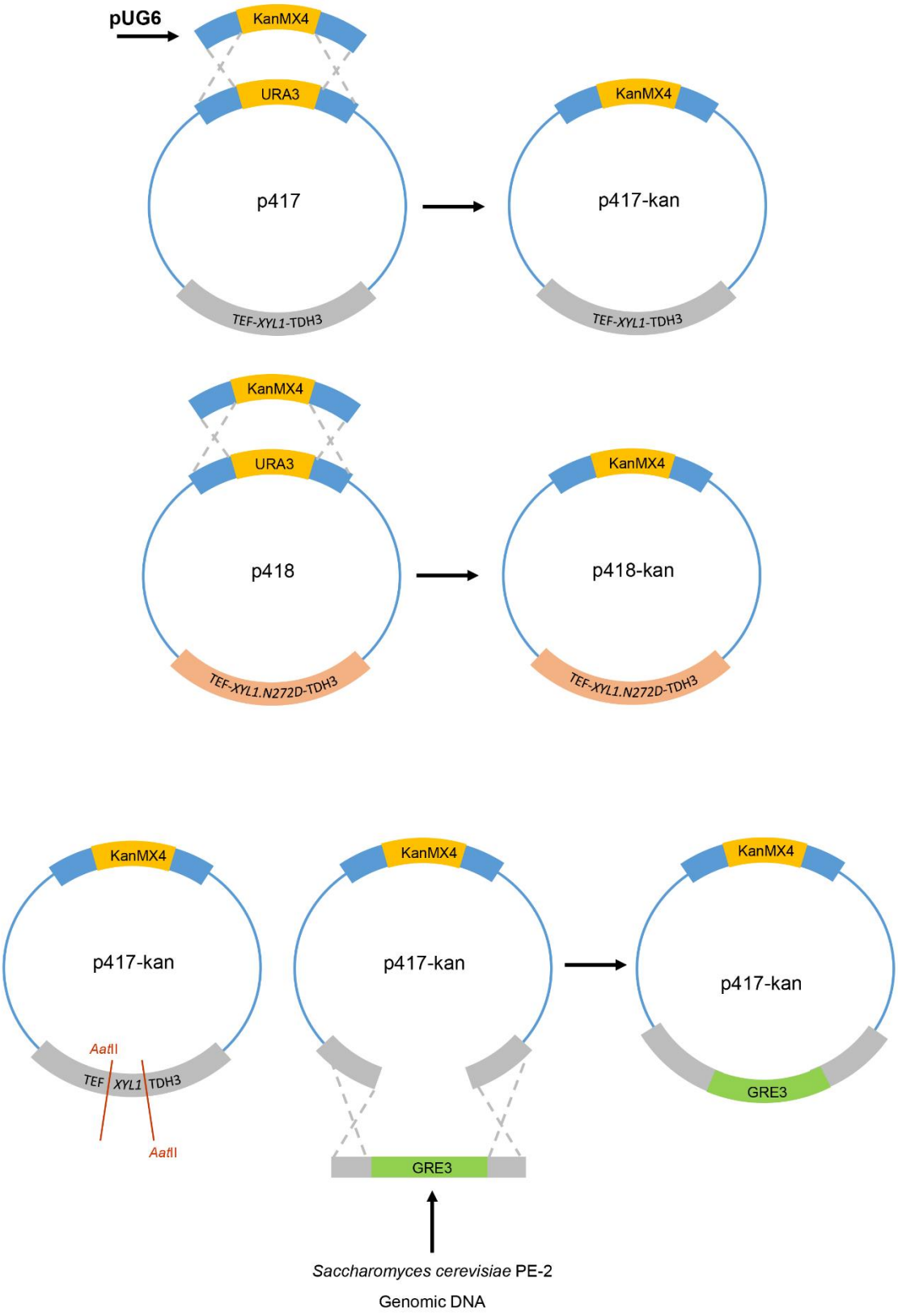

Figure 2.1 Schematic representation of plasmids construction.

Table 2.1. Yeast strains, plasmids and primers used in this work. Upper-case sequences correspond to sequences complementary to the template, and lower-case sequences correspond to homologous recombination sites with the desired digested vectors.

***S. cerevisiae* strains**	**Relevant Genotype**	**Reference**
CEN.PK 113-5D	MATa; ura3-52	(25)
PE-2	Diploid; Isolated from bio-ethanol plants	(26)
PE-2-XR_{wt}	PE-2, p417-kan	This work
PE-2-XR_{mut}	PE-2, p418-kan	This work
PE-2-GRE3	PE-2, p417-kan-GRE3	This work
CEN.PK 113-5D	CEN.PK 113-5D, p417-kan-GRE3	This work

Plasmids	**Relevant Features**	
p417	pYPK0_TEF1p_PsXYL1_TDH3t, URA3	(27)
p418	pYPK0_TEF1p_PsXYL1(N272D)_TDH3t, URA3	(27)
p417-kan	pYPK0_TEF1p_PsXYL1_TDH3t, KanMX4	This work
p418-kan	pYPK0_TEF1p_PsXYL1(N272D)_TDH3, tKanMX4	This work
p417-kan-GRE3	pYPK0_TEF1p_ScGRE3_TDH3t, KanMX4	This work

Primers	**Sequence**
KanMX4_FW	ctcacgttaagggattttggtcatgagCACATACGATTTAGGTGACACTATAGAAC
KanMX4_RV	catctttgacagcttatcatcgataagctCGACTCACTATAGGGAGACC
GRE3_TEF1_FW	ggaacgccaggttgcccactttctcactagtgaaaATGTCTTCACTGGTTAC
GRE3_TDH3_RV	taaatcctgatgcgtttgtctgcacagatggcgcgTCAGGCAAAAGTGGGG

2.2.2. Inoculum

Yeast cells for inoculation were grown overnight at 30 °C and 200 rpm in Erlenmeyer flasks filled with YPD medium to 40% of their total volume. The cell suspension was collected by centrifugation for 5 min at 3000 rpm, 4 °C and suspended in 0.9% (w/v) sodium chloride solution, in order to achieve a final concentration of 400 g fresh yeast/L. The fermentation experiments were conducted with the concentrated cell suspension, with a cellular concentration from 9 to 11 g fresh yeast/ L (corresponding to 3 to 5 g of dry yeast/L).

2.2.3. Fermentation experiments

Batch experiments were carried out in complete YP medium (20 g/L peptone and 10 g/L yeast extract) with 30 g/L of xylose and 20 g/L of glucose as carbon source, in 250 mL Erlenmeyer flasks (working volume 40 mL) at 30 °C and 200 rpm.

Fed-batch fermentations were performed in complete YP medium with 30 g/L of glucose and different concentrations of xylose: 64.6 g/L, 126 g/L and 159 g/L and in corn cob hydrolysate medium (supplemented with 20 g/L of peptone and 10 g/L of yeast extract). These experiments were conducted in a 3.7 L Bioengineering's RALF bioreactor (working volume 2 L for synthetic media and 1.5 L for corn cob hydrolysate) at 30 °C, 400 rpm and 2 vvm aeration rate (15, 21). The bioreactor was equipped with a condenser cooled with water to prevent evaporation. The pH-value of 5 was automatically adjusted by addition of NaOH (5M) or HCl (5M) solutions. After glucose in the medium was completely depleted, a glucose stock solution of 300 g/L was continuously fed at a flow rate of 4.8 mL/h. Samples were withdrawn at desired times and stored at 4°C after centrifugation for further analysis of sugars (glucose and xylose) and xylitol and ethanol. Biomass concentration in the media was measured by dry cell weight.

2.2.4. Corn cob hydrolysate: Autohydrolysis pretreatment

Corn cob was collected, milled and submitted to hydrothermal treatment (autohydrolysis) under non-isothermal conditions (T_{max} of 205 °C, corresponding to a severity of 3.85) based of previous works (28, 29) in a 2 L stainless steel reactor (Parr Instruments Company) equipped with Parr PDI temperature controller (model 4848) at liquid to solid ratio of 8 g distilled water/1 g of corn cob oven dry. After treatment, the resulting solid and liquid phases (whole slurry) were separated by filtration. Solid phase (pretreated corn cob) was recovered and washed for Solid Yield (SY) determination. Corn cob and pretreated corn cob were analysed for chemical composition following standard methods described by NREL protocols (NREL/TP-510-42618-42622-4218). Composition of hydrolysates (sugars, acetic acid and furan compounds) was analysed by HPLC.

2.2.5. Enzymatic saccharification of pretreated corn cob

Enzymatic saccharification of pretreated corn cob was carried out at 30 °C, 200 rpm in an orbital shaker using a percentage of solids of 5 and 10% at different enzyme loadings (6, 12 and 24 FPU/g). Enzyme used in these assays was Cellic CTec2 (kindly supplied by Novozymes, Bagsvaerd, Denmark).

Cellulase and hemicellulase activities of Cellic CTec2 were 122 FPU/mL and 9764 U/mL, determined following the procedure described by (30) and (31), respectively. Samples were withdrawn and analysed by HPLC to determine the glucose and xylose concentration and glucose yield from experiments. Glucose yield was calculated following the equation:

$$Glucose\ yield\ (\%) = \frac{G}{\frac{180}{162} f\ B} 100 \qquad \text{equation 1}$$

where, G is glucose concentration (g/L), B is dry corn cob biomass concentration (g/L), f is glucan fraction in dry biomass (g per g), 180/162 is the stoichiometric factor that converts glucan to equivalent glucose.

2.2.6. Pre-saccharification and Simultaneous Saccharification and Fermentation (PSSF and SSF) assays of corn cob whole slurry

Simultaneous saccharification and fermentation (SSF) assays were carried out in 250 mL Erlenmeyer flasks (working volume 40 mL) at 30 °C and 200 rpm in an orbital shaker, using both solid and liquid phases (whole slurry) from corn cob autohydrolysis as substrates. Autohydrolyzed solid phase (pretreated corn cob) was sterilized at 121 °C for 20 min, whereas the liquid phase (corn cob hydrolysate) was subjected to a second step of acid hydrolysis with 0.5% (w/w) H_2SO_4 for 165 min at 125 °C (29). The obtained hydrolysates, enriched in xylose, were neutralized with $CaCO_3$ until pH 5 and sterilized by filtration (0.2 µm) and aseptically added to autoclaved solid fraction. SSF assays were carried out using 5% solids at 6 and 12 FPU/g. Pre-saccharification and simultaneous saccharification and fermentation (PSSF) assays were carried out in 250 mL Erlenmeyer flasks (40 mL of working volume) and in a 3.7 L Bioengineering's RALF bioreactor (1.5 L of working volume). Enzymatic saccharification stage of whole slurry (solid and liquid phases) was carried out for 24 h using 5 or 10% solids at 12 or 24 FPU/g. During these PSSF experiments, fed-batch of 5% solids (supplemented with respective enzyme loading) was conducted to further feed glucose. Figure 2.2 shows a schematic representation of corn cob processing for xylitol production carried out in this work.

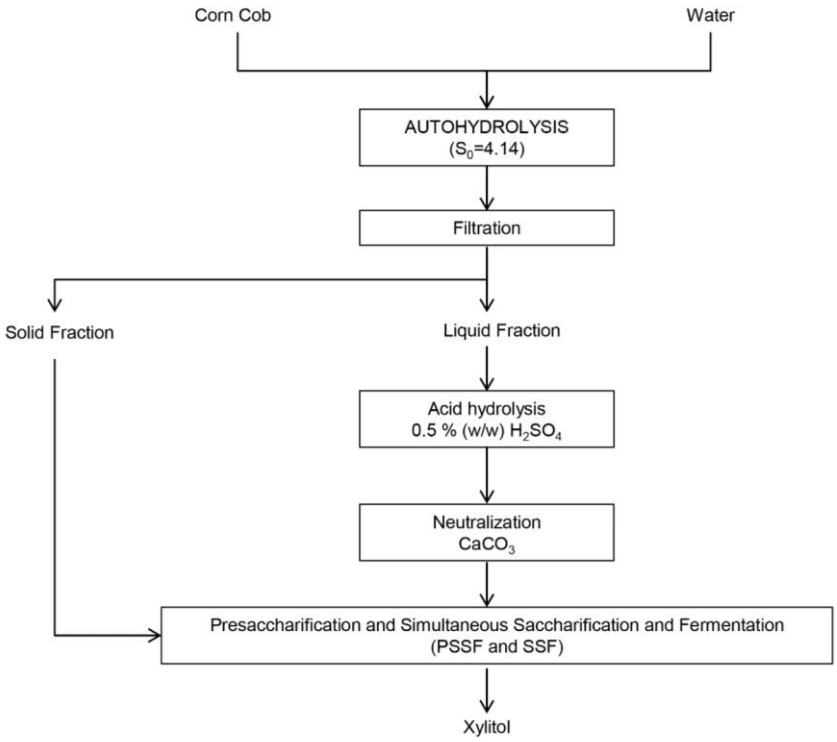

Figure 2.2. Flowchart of main steps involved in the corn cob to xylitol process.

2.2.7. Analytical methods

Samples from corn cob analysis, autohydrolysis treatment of corn cob (hydrolysate and pretreated corn cob) and from fermentation assays were analysed for quantification of sugars (glucose, xylose, arabinose), acetic acid, xylitol, furfural, hydroxymethylfurfural (HMF) and ethanol by HPLC utilizing a BioRad Aminex HPX-87H (300 x 7.8 mm) column, at 60 °C, using 0.005 M sulfuric acid as eluent in a flow rate 0.6 mL/min. The peaks corresponding to sugars, acetic acid, xylitol and ethanol were detected using a Knauer-IR intelligent refractive index detector, whereas furfural and HMF were detected using a Knauer-UV detector set at 280 nm.

2.2.8. Determination of fermentation parameters

Metabolic yield of xylitol from xylose ($Y_{XL/Xy}$) was defined as g of xylitol produced/g of xylose consumed. Biomass yield ($Y_{X/S}$,) was expressed as g of dry cell/g of glucose consumed. The xylitol productivity (Qp_t, g/L h) was calculated as follows:

$$Qp_t = \frac{[XL]_t}{t} \qquad \text{equation 2}$$

where [XL] is xylitol concentration at time t divided by time t.

2.3. Results and discussion

2.3.1. Evaluation of strains for xylitol production in batch fermentation

Several factors interfering with the xylitol production have already been identified, such as specific XR activity, transport of xylose into the cell and generation of reduced cofactors (32). In this sense, the recombinant strains PE-2-XR_{mut}, PE-2-XR_{wt} and PE-2-GRE3, expressing different xylose/aldose reductases, were compared in terms of xylitol production (Figure 2.3, Table 2.2).

Considering that xylose is not naturally consumed by *S. cerevisiae*, the recombinant strains need a carbon source for cell growth and co-factors regeneration (12). Therefore, to evaluate the xylose consumption and the xylitol production patterns for the different strains, aerobic batch fermentations were performed on medium containing glucose and xylose (Figure 2.3, Table 2.2). In yeast, xylose uptake occurs by facilitated diffusion through the hexose uptake systems (33), being competitively inhibited by glucose (34). Accordingly, all strains showed xylose uptake only after a considerable decrease in glucose concentration for all tested strains (Figure 2.3). After glucose depletion, the ethanol produced during the cultivations was re-assimilated and used as co-substrate for co-factors regeneration by yeast, allowing further xylitol production. Although the cell mass production was similar in all experiments (~20 g/L at 48 h), xylose was converted into xylitol at different rates by the strains: PE-2-XR_{mut} produced 12.7 g/L of xylitol, with16.3 g/L of xylose remaining in the medium (Figure 2.3a); while the PE-2-XR_{wt} and PE-2-GRE3 strains produced 24.2 and 27.8 g/L of xylitol, respectively, by conversion of almost all xylose present in the media (Figure 2.3b and c). The xylitol yield attained by PE-2-XR_{wt} and PE-2-GRE3 was close to the theoretical (Table 2.2), however PE-2-GRE3 consumed xylose considerably faster and produced higher amounts of xylitol (28 g/L) with a superior productivity of 0.54 g/L·h at 48h. Both strains PE-2-XR_{wt} and PE-2-GRE3 express an enzyme with higher specificity for NADPH (35, 36), while the PE-2-XR_{mut} express an NADH-preferable xylose reductase enzyme. It is well known that the pentose phosphate pathway (PPP), an essential metabolic pathway in the glucose metabolism, is a major source of NADPH (37), and in this case, the use of glucose as co-substrate may result in a superior performance of the strains expressing enzymes with NADPH preference. Furthermore, the fact that XR_{wt} is capable of using both NADH and NADPH, while the aldose reductase encoded by *GRE3* uses solely NADPH, may explain the higher production of xylitol by the PE-2-GRE3 strain. Accordingly, the xylitol production from a *GRE3*-overexpressing *S. cerevisiae* strain, was already described as a better strategy for xylitol production, in comparison with the expression of XR of *P. stipitis*, in the presence of glucose as co-substrate (14). In fact, the use of different co-substrates

could result in different mechanisms of co-factors regeneration and consequently different responses by the enzymes. Another study of comparison of *S. cerevisiae* strains harboring *XYL1* gene of *P. stipitis* or overexpressing *GRE3* gene, using ethanol as co-substrate, reported a superior xylitol production by the recombinant strain expressing the XR, justified by a higher regeneration of NADH during ethanol oxidation to acetate and subsequent metabolization in the TCA cycle (38).

CEN.PK 113-5D-GRE3 was used as control to confirm the advantage of using as chassis a natural xylitol accumulating background (18, 19), and despite having consumed more than 90% of xylose in the medium, only produced 19.1 g/L of xylitol. Besides and as expected, this laboratorial strain exhibits a slower carbon source consumption (glucose and ethanol) and consequently, a lower yield (0.68 g/g) and xylitol productivity (0.22 g/L·h) when comparing to the high-rate glucose-consuming industrial PE-2 strain (39). Moreover, additional process advantages are expected when using PE-2 as chassis, namely the tolerance to inhibitors present in hydrolysates (17) that enable the use of non-detoxified hydrolysates simplifying significantly the overall process.

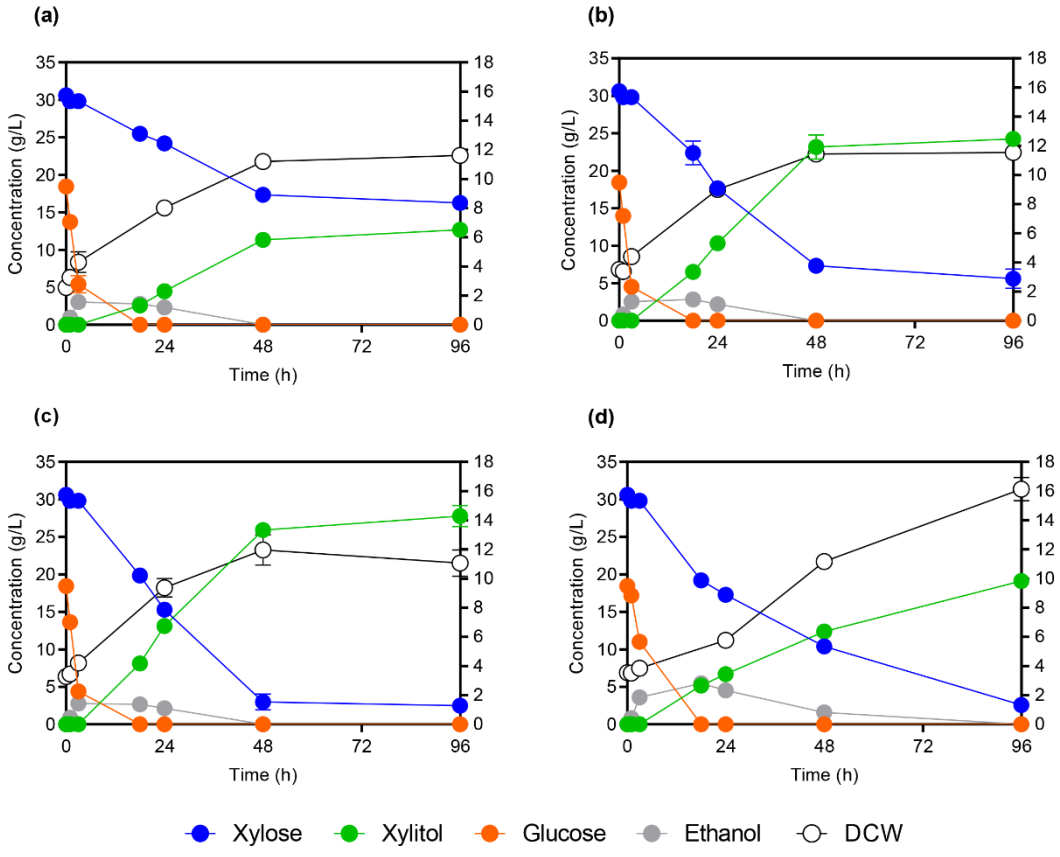

Figure 2.3. Performance of the *S. cerevisiae* (a) PE-2-XR$_{mut}$, (b) PE-2-XR$_{wt}$, (c) PE-2-GRE3 and (d) CEN.PK 113-5D-GRE3 by the time course of xylose and glucose consumption as well as xylitol and ethanol production.

2.3.2. Fed-batch fermentations

As observed in the batch fermentations (section 3.1.), bioconversion stops when the carbon source is depleted, probably due to the lack of reducing power. In this way, to maintain co-factor regeneration and yeast metabolism without catabolite repression of xylose uptake, a glucose-limited fed-batch fermentation strategy has been previously applied for enhancement of xylitol productivity (10, 11, 14–16, 32, 40). Considering that high xylose concentrations may pose as another limitation for xylitol production, the limited-glucose fed-batch strategy was used in bioreactor to evaluate PE-2-GRE3 xylitol productivity from increasing concentrations of initial xylose (64.6, 126 and 159 g/L) (Figure 2.4, Table 2.2).

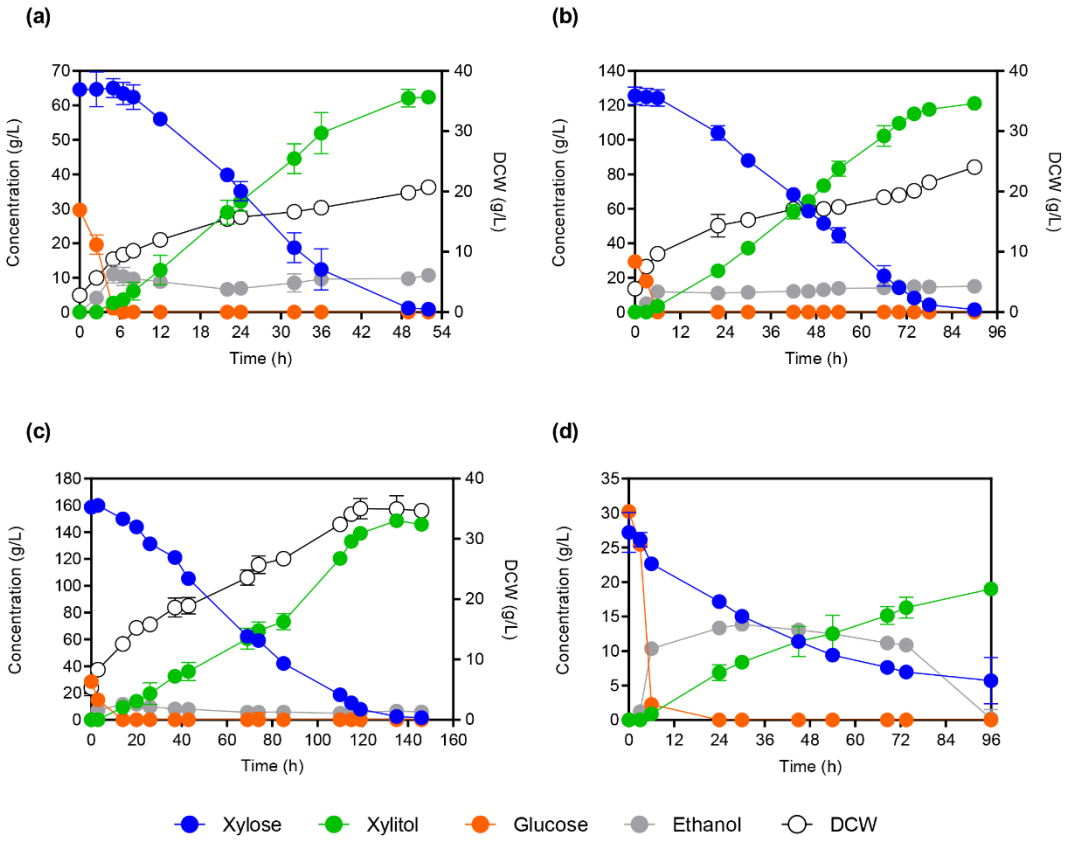

Figure 2.4. Performance of *S. cerevisiae* PE-2-GRE3 in glucose-limited fed-batch fermentations in YP medium with (a) 65 g/L, (b) 126 g/L and (c) 159 g/L xylose; in (d) corn cob hydrolysate. Xylose and glucose consumption as well as xylitol and ethanol production. The dotted line denoted dry cell weight (DCW). The experimental results were revised for the effects of dilution and sampling on the broth volume.

To attain higher yeast biomass and increase xylitol productivity all fermentations were firstly conducted in batch mode until depletion of the initial 30 g/L of glucose; which was followed by a continuous feeding of low glucose concentrations. The ethanol formed in the batch growth phase

remained constant during the fed-batch xylitol production phase while biomass growth was observed even though at a slower rate. In these fed-batch assays, the maximum dry cell mass achieved was of 34.7 g/L (Figure 2.4c). In addition, no glucose was detected in the broth during this phase, indicating that glucose gradually supplied was instantly consumed by the yeast. After starting the glucose feed, xylose uptake occurred at constant rate (reaching nearly xylose depletion) and xylitol was produced close to theoretical yields in all the experiments, with 62.4, 121.1 and 148.5 g/L of xylitol being produced from 64.6, 126 and 159 g/L of initial xylose, respectively (Figure 2.4). Furthermore, and despite some variation, xylitol productivity does not seem to be diminished by high initial xylose concentrations (Table 2.2), indicating that there are no saturation of xylose transporter system and no substrate inhibition of the xylose reductase activity.

Table 2.2. Main results of batch and fed-batch fermentations of the recombinant *Saccharomyces cerevisiae* strains in glucose and xylose synthetic media and corn cob lignocellulose hydrolysate (Fed-Batch 4).

Culture	*S. cerevisiae* strains	Xy_{t0} (g/L)	Xy_{tf} (g/L)	$Xyol_{tf}$ (g/L)	$Y_{Xyol/Xy}$ (g/g)	Q_{pmax} (g/L·h)
Batch						
1	PE-2-XR_{mut}	30.6 ± 0.8	16.3 ± 0.4	12.7 ± 0.1	0.88 ± 0.03	0.24 ± 0.01
2	PE-2-XR_{wt}	30.6 ± 0.8	5.62 ± 1.30	24.2 ± 0.6	0.97 ± 0.03	0.48 ± 0.03
3	PE-2-GRE3	30.6 ± 0.8	2.57 ± 0.01	27.8 ± 1.4	0.99 ± 0.04	0.54 ± 0.00
4	CEN.PK 113-5D-GRE3	30.6 ± 0.8	2.60 ± 0.55	19.1 ± 0.4	0.68 ± 0.00	0.22 ± 0.01
Fed-Batch						
1	PE-2-GRE3	64.6 ± 0.92	0.86 ± 0.73	62.4 ± 0.02	0.98 ± 0.00	1.44 ± 0.23
2	PE-2-GRE3	126 ± 5.1	1.51 ± 0.09	121.1 ± 3.59	0.98 ± 0.01	1.56 ± 0.15
3	PE-2-GRE3	159 ± 2.08	2.52 ± 0.13	148.5 ± 4.02	0.95 ± 0.01	1.16 ± 0.08
4	PE-2-GRE3	27.2 ± 0.01	5.71 ± 0.01	19.0 ± 0.01	0.89± 0.00	0.39 ± 0.01

Xy_{t0} is the xylose concentration at time t0 = 0 h; Xy_{tf} is the xylose concentration at final time; $Xyol_{max}$ is the maximum xylitol concentration; $Y_{Xyol/Xy}$ is the xylitol yield from xylose consumed; Q_{pmax} is the maximum productivity achieved in the assays.

The xylitol production obtained with this strategy is in the upper range of previous works using recombinant *S. cerevisiae* strains. Oh et al. (21) with an engineered *S. cerevisiae* for co-utilization of xylose and cellobiose attained 93 g/L of xylitol. Some studies describe a gradual addition of xylose to the culture medium in order to increase xylitol productivity. In fact, Lee et al. (15), with a recombinant *S. cerevisiae* expressing a xylose reductase from *P. stipitis*, obtained 105 g/L of xylitol with a productivity of 1.69 g/L·h, maintaining a low substrate concentration using a fed-batch strategy with simultaneous addition of xylose and glucose during the bioconversion phase. More recently, Kim et al. (13) achieved high xylitol productivity in a glucose-limited fed-batch culture with pulsed addition of xylose, producing a maximum of 178 g/L of xylitol. Nevertheless, it should be

noted that the strain used expresses the arabinose H+ symporter (AraE) from *Bacillus subtilis*, in addition to the *XYL1* gene from *Scheffersomyces stipitis*, which substantially increases xylose uptake (13). In comparison, the PE-2 strain presented in this work, with the sole overexpression of *GRE3*, withstands high substrate loading, efficiently converting high values of initial xylose into xylitol, and presents similar productivities to the ones attained with lower xylose concentrations or with modifications in the yeast xylose uptake system (13, 15, 21).

2.3.3. Hydrothermal treatment of corn cob: hemicellulosic hydrolysate

The use of renewable and low cost raw materials as lignocellulosic biomass (including agricultural and forest residues) is mandatory to develop a sustainable bioprocess for xylitol production (7, 41). In this sense, corn cob was selected for its high xylan content (41–44). The chemical composition of corn cob (expressed in g/100 g wood in oven-dry basis ± standard deviation based on three replicate determinations) was: 30.89% ± 0.45 of xylan, 27.32% ± 0.24 of glucan, 22.92% ± 0.84 of Klason lignin, 3.52% ± 0.22 of arabinan, 2.15% ± 0.03 of acetyl groups and 6.05% ± 0.22 of extractives. The complex and recalcitrant structure of lignocellulosic materials hinder the access to monomeric sugars (45). Hydrothermal treatment (also known as autohydrolysis or liquid hot water) followed by dilute acid treatment has been extensively used for the enhancement of cellulose saccharification (28, 46) and to obtain hemicellulosic hydrolysate enriched in xylose (19, 43, 47). The operational conditions of corn cob processing were selected based on literature (28, 43). The solid phase was recovered for the solid yield (56.2 ± 0.02) determination. Chemical composition of solid phase (expressed in g/100 g of pretreated corn cob in oven-dry basis ± standard deviation based on three replicate determinations) was: 39.63% ± 0.75 of glucan, 15.05% ± 0.45 of xylan, 38.33% ± 0.88 of Klason lignin and 0.31% ± 0.01 of arabinan. After autohydrolysis treatment, 81.5% of cellulose (measured as glucan) and 27.2% of xylan were recovered in the solid phase. On the other hand, composition of hemicellulosic hydrolysate was as follow: 2.25 ± 0.00 g/L of glucose, 27.82 ± 0.38 g/L of xylose, 3.11 ± 0.00 g/L of arabinose, 2.83 ± 0.00 g/L of acetic acid, 1.09 ± 0.27 g/L of furfural and 0.04 ± 0.03 g/L of HMF. Therefore, 68.2% of xylan was solubilized and recovered in the liquid phase (or hemicellulosic hydrolysate) as xylose, after acid hydrolysis of the autohydrolysis liquor. The hemicellulosic hydrolysate was composed mainly by xylose (28 g/L) and acetic acid (2.88 g/L), and its composition was similar to the one reported by Rivas et al. (43) for sequential stages of autohydrolysis and dilute acid posthydrolysis of corn cob autohydrolysis liquor.

Hemicellulosic hydrolysates obtained from different lignocellulosic biomass (such corn cob, wheat straw, rapeseed straw, brewer´s spent grain) are the most commonly renewable substrates used for xylitol production (8, 9, 41). Generally, hemicellulosic hydrolysates for xylitol production are submitted to costly steps of detoxification to improve the fermentation process since most yeast have low tolerance towards inhibitory compounds, such as acetic acid, furfural and HMF (9, 17). In this work, non-detoxified corn cob hydrolysate was used as substrate for xylitol production by the inhibitor-tolerant engineered PE-2-GRE3 strain, under conditions described for the bioreactor assays using synthetic media (Figure 2.4a-c). Initial glucose was consumed within 10 h of fermentation and 15 g/L of ethanol was produced from glucose consumed (Figure 2.4d). After glucose depletion, hemicellulosic hydrolysate was supplemented with glucose in fed-batch mode. As expected, the xylitol productivity in hemicellulosic hydrolysate (Table 2.2, fed-batch 4) was lower in comparison with productivities obtained in synthetic media**,** probably due to the presence of inhibitors such as furfural, HMF and acetic acid that have a negative effect on yeast growth (48). As seen in Figure 2.4d, 74% of xylose was consumed at 72 h of fermentation with a maximal xylitol production of 17.5 g/L, corresponding to a xylitol yield of 0.89 g/g. These results can be positively compared with the one obtained by a natural xylitol-producing *Candida tropicalis* strain from a non-detoxified hydrolysate, added in fed-batch mode, with a reported yield of 0.7 g/g (49). A fed-batch strategy similar to the one used in this work was also proposed by Kogje and Ghosalkar (22) for xylitol production using a recombinant *S. cerevisiae* XP-RTK strain (overexpressing *GRE3* and a xylose specific transporter from *P. stipitis*), which produced 16 g/L of xylitol from non-detoxified but diluted corn cob hydrolysate with a maximal productivity of 0.21 g/L·h.

2.3.4. Simultaneous saccharification and fermentation assays of pretreated corn cob for xylitol production

In order to develop an integrated and sustainable process, the solid phase obtained from the autohydrolysis pretreatment and mainly composed by glucan (39.6 %) was proposed as co-substrate to supplement glucose for xylose bioconversion into xylitol. Cellulose saccharification of pretreated corn cob for glucose release aims to mimic glucose supplementation in fed-batch mode to keep a basal level of glucose supply. For that, different percentage of solids (5 and 10 %) and enzyme loadings (6, 12 and 24 FPU/g) were assayed to evaluate the glucose release by enzymatic hydrolysis. Figure 2.5a presents the glucose profile obtained from saccharification of pretreated corn cob biomass. As seen, glucose concentration varied in the range of 6.9-32.9 g/L,

corresponding to experiments at 6 FPU/g and 5% of solids and 24 FPU/g and 10% of solids, respectively. Xylose released from the solid by enzymatic saccharification was also quantified (data no shown) achieving concentrations in the range of 3.36-6.25 g/L. Glucose yields of enzymatic saccharification assays are shown in Figure 2.5b and varied from 32.4 to 78.8 %. The glucose concentration and glucose yield were significantly influenced by the increase of enzyme loading. Considering that glucose at high concentration limits xylose uptake by yeast (21), the use of 5% of solid (Figure 2.5a) seemed more suitable to maintain a low level of glucose thus to and improve xylitol yield and productivity. Moreover, a previous study using pretreated corn cob (only the solid fraction resultant from alkali pretreatment) for xylitol production by SSF revealed higher yields when using a 5% solid loading (50).

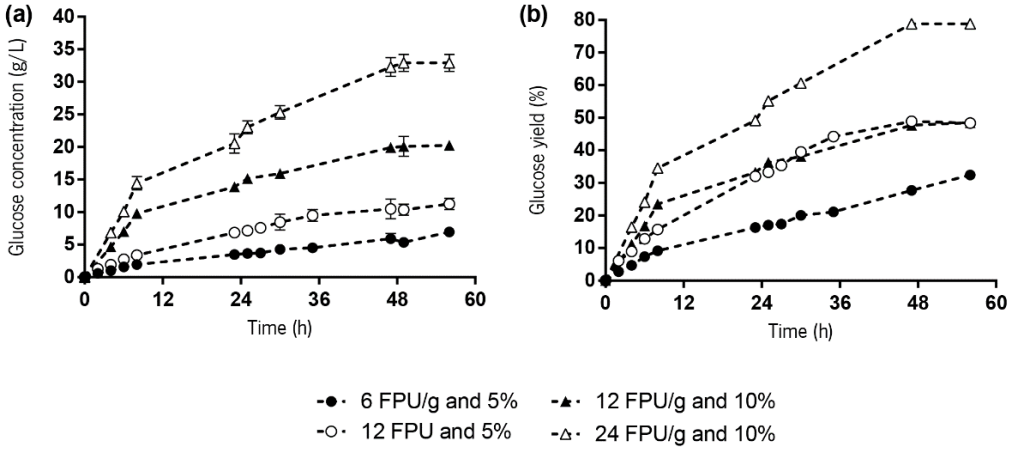

Figure 2.5. Time course of: (a) glucose concentration (g/L) and (b) glucose yield (%) in enzymatic saccharification experiments.

Figure 2.6a-b shows the SSF assays carried out for xylose bioconversion into xylitol using 6 and 12 FPU/g. As seen, SSF carried out with 6 FPU/g showed an incomplete xylose consumption (54.19%) and a xylitol production of 14.13 g/L (Table 2.3). Under this condition, the glucose released during enzymatic hydrolysis was not sufficient to allow the complete conversion of xylose into xylitol. On the other hand, the SSF assay at 12 FPU/g and 5% of pretreated corn cob (Figure 2.6b) showed a xylose consumption of 89.9% with a xylitol production of 23.2 g/L which corresponded to 0.91 g/g of xylitol yield (Table 2.3). The increase of enzyme loading allowed a higher cellulose saccharification which improved the xylitol production (1.64 fold higher than xylitol produced by SSF at 6 FPU/g). Xylitol yield in this condition was also considerably higher than others reported for an SSF of the solid fraction of an alkali pretreated corn cob using *S. cerevisiae* and *C. tropicalis* strains (0.71 g/g by *C. tropicalis,* 0.52 g/g by *S. cerevisiae* and 0.69 g/g in co-culture) (50).

A saccharification step before the SSF process, also known as Pre-saccharification and simultaneous saccharification and fermentation (PSSF), is proposed as an alternative strategy for xylitol production from whole slurry of corn cob. This strategy aims to mimic the conditions used for xylitol production from synthetic media (section 3.2), where the initial glucose concentration is used for yeast biomass growth aiming an increase in xylitol productivity. Figure 2.6c shows glucose production during the pre-saccharification step (9 g/L). After this stage, yeast cells were added and glucose was rapidly consumed (<3h) resulting in the maximal ethanol production of 4.8 g/L. At 30 h and 54 h, PSSF assay was supplemented with 5% of solid loading (pretreated corn cob) in order to maintain a glucose feed during the bioconversion process of xylose into xylitol. In this experiment, 79.9% of xylose was consumed at 96 h of PSSF process and maximal xylitol concentration of 29.6 g/L with a xylitol yield of 0.93 g/g was achieved (Table 2.3). Xylitol productivity (not considering the 24 h of pre-saccharification) achieved a maximal value of 0.54 g/Lh, being slightly lower than the maximal productivity obtained in SSF-2 (0.74 g/Lh). Nevertheless, xylitol production was increased compared to SSF-2 experiment (Table 2.3), reaching 27% higher xylitol concentration. This increase is caused by the addition of xylan-containing solid at different times of PSSF, resulting in a proportional increase of available xylose for conversion.

As promising results were obtained from this strategy, the laboratory scale-up to a 3.7 L bioreactor was evaluated (PSSF-2, Figure 2.6d). The initial percentage of pretreated corn cob was increased to 10% (Table 2.3), to achieve a higher concentration of initial glucose and consequently increase biomass growth, which resulted in the production of 25 g/L of glucose in the 24 h of pre-saccharification step. After yeast addition, glucose was fermented into ethanol achieving a maximal concentration of 16.4 g/L. This unexpected high ethanol yield, which limits glucose use for yeast growth, was probably caused by the bioreactor design that may not be appropriate to work with a moderate-high solid loading (15%), hindering the oxygen mass transfer. Nevertheless, bioconversion of xylose into xylitol started at 28h. It should be noted that while xylitol concentration progressively increases after 28h, there seems to be a stabilization of xylose concentration in the medium up until 96 h, which is explained by the gradual release of xylose (ca. 9.39 g/L in total) from the xylan-containing solid loads. Even with the non-optimal conditions of solid load and aeration, more than 76% of available xylose was consumed in 96 h, resulting in the production of 24.3 g/L of xylitol with a yield of 0.88 g/g. Despite the need of additional optimization regarding bioreactor design, air flow and solid loading these results pave the way for the possibility of scaling-up xylitol production from lignocellulosic whole slurry envisioning an industrial scale.

Table 2.3. Operational conditions and main results obtained from simultaneous saccharification and fermentation (SSF) and pre-saccharifcation and saccharification and fermentation (PSSF) of corn cob whole slurry.

	Operational conditions						Results				
	Solid loading (%)			Enzyme load (FPU/g)							
Experiment	**0 h**	**30 h**	**54 h**	**0 h**	**30 h**	**54 h**	**Xpot* (g/L)**	**Xf (g/L)**	**Xylitol max (g/L)**	**Yp/s (g/g)**	**Qpmax (g/L·h)**
Batch											
SSF-1	5	-	-	6	-	-	30.58 ± 0.87	14.01 ± 1.21	14.13± 0.87	0.85 ± 0.08	0.30 ± 0.01
SSF-2	5	-	-	12	-	-	28.63 ± 0.83	3.14 ± 0.67	23.24± 2.14	0.91 ± 0.11	0.74 ± 0.01
Fed-batch											
PSSF-1	5	5	5	12	12	12	39.81 ± 0.97	8.07 ± 0.44	29.61 ± 1.43	0.93 ± 0.04	0.54 ± 0.01
PSSF-2	10	5	-	24	12	-	36.41± 0.90	8.63 ± 0.98	24.32 ± 2.37	0.88 ± 0.12	0.30 ± 0.02

*__Xpot__ was calculated considering the sum of xylose concentration in the t0 of SSF or PSSF with the xylose produced from xylan saccharification

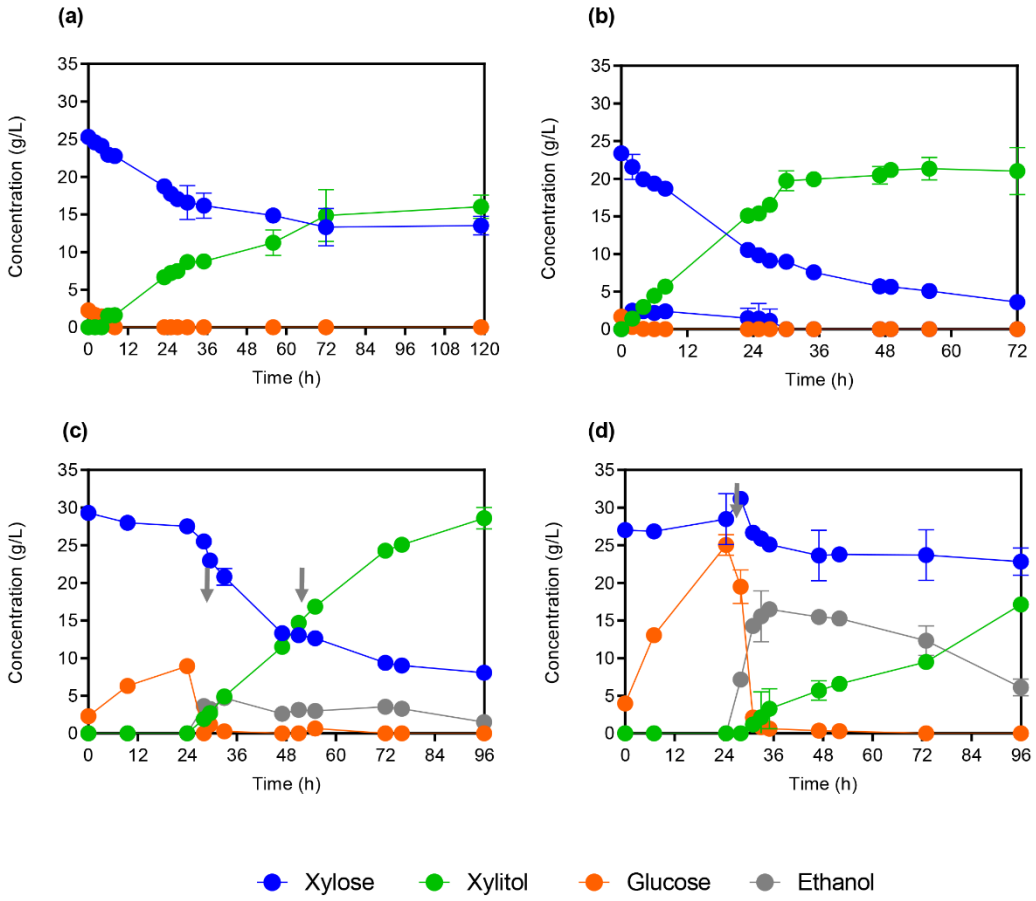

Figure 2.6. Strategies for xylitol production from corn cob whole slurry by simultaneous saccharification and fermentation (SSF) process: (a) using 5% of solid and 6 FPU/g and (b) 5% of solid and 12 FPU/g and by presaccharification and simultaneous saccharification and fermentation (PSSF) process (added solid in fed-batch): (c) using 15 % of solids and 12 FPU/g and (d) 15 % and 20 FPU/g in bioreactor. Xylose and glucose consumption as well as xylitol and ethanol production. The arrows indicate the addition of solid.

2.4. Conclusions

This work shows that overexpressing the *GRE3* endogenous gene in the robust and innate xylitol accumulation *S. cerevisiae* PE-2 strain enhances xylitol productivity when compared with expression of different xylose reductases in the same or different yeast chassis. In fed-batch fermentations, with limited-glucose feeding, the PE-2-GRE3 strain efficiently produce xylitol from remarkable high xylose concentrations. In addition, high yields of xylitol from non-detoxified corn cob hydrolysates were attained, in spite of the presence of inhibitory compounds. Furthermore, this work shows, for the first time, the feasibility of using whole slurry corn cob for xylitol production in a simultaneous saccharification process.

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Chapter III.

Development of a sustainable bioprocess based on green technologies for xylitol production from corn cob

This chapter is based on the following original research article:

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Abstract

In this work, a sustainable and environmental friendly strategy for the biotechnological production of xylitol was proposed and optimized. For this purpose, corn cob was hydrothermally pretreated at high solid loadings (25%) for an efficient solubilization of xylan in hemicellulose derived compounds, xylooligosaccharides and xylose. Xylose enriched streams were obtained from the enzymatic saccharification of the whole slurry (solid and liquid fraction) resulting from the autohydrolysis pretreatment. The xylitol production in a simultaneous saccharification and fermentation (SSF) process, by the recombinant *Saccharomyces cerevisiae* PE-2-GRE3 strain, was optimized using different enzyme and substrate (pretreated corn cob solid) loadings by an experimental design. This study demonstrated a significant effect of substrate loading on the production process achieving a maximal concentration of 47 g/L with 6.7 % of pretreated corn cob and 24 FPU/g of enzyme loading, with partial detoxification of the hydrolysate. Furthermore, the 1.42-fold increase in xylitol titer and 1.56-fold increase in productivity achieved in a SSF using an acetic acid free-hydrolysate evidenced the negative effect of acetic acid on the yeast-based xylitol production process. The combination of these green technologies and the optimization of the proposed strategy enhanced the overall xylitol production through the valorization of corn cob.

Keywords: autohydrolysis; added-value chemical; corn cob whole-slurry; industrial Saccharomyces cerevisiae; simultaneous saccharification and fermentation; xylitol.

3.1. Introduction

The excessive dependence of non-renewable fossil resources and the need for climate change mitigation are the main driving forces for the development of novel technologies to produce high value chemicals from renewable resources. Lignocellulosic biomass, which includes plant-derived materials, from wood and grass to agro-industrial residues, is the most abundant renewable feedstock and appears to be the most promising starting material for high value chemicals production (1, 2).

Xylitol is included within the twelve building blocks that can be produced from lignocellulosic sugars and subsequently converted to a number of high-value bio-based chemicals or materials for food, pharmaceutical and chemistry industries (3–5). It is a naturally occurring sugar alcohol that presents a sweetness profile similar to sucrose but with 40% less calories. In addition to its low-caloric content, exhibits other benefits especially anti-diabetic and anti-cariogenic properties (6). Currently, xylitol is commercially produced by hydrogenation of xylose extracted from lignocellulosic biomass. In this production process, xylose-enriched hydrolysates are obtained through acid hydrolysis of hemicellulose and subsequent concentration. In spite of using an inexpensive and renewable raw material, is not environmental-friendly and requires large energy requirements. In this sense, the production of xylitol through microbial fermentation of sugars from renewable feedstocks has gained increasing interest (7).

There is a wide range of xylose-fermenting yeasts able to produce xylitol as a by-product of xylose utilization pathway (8). However, xylitol yields are limited by the use of xylose as carbon source for yeast growth and maintenance energy. To overcome this limitation, the expression of enzymes with xylose reductase activity in *Saccharomyces cerevisiae*, naturally incapable of xylose utilization, has shown to increase the conversion of xylose into xylitol close to the maximum theoretical yield (~100%), since the produced xylitol is not further metabolized (9–12). Moreover, the possibility of using robust *S. cerevisiae* strains, isolated from harsh environmental industrial conditions, with higher tolerance to the lignocellulosic-derived inhibitors represents another advantage for xylitol production in lignocellulose-based processes (13, 14). Considering this, the *S. cerevisiae* PE-2 industrial strain presenting innate capacity for xylitol accumulation (15), was recently engineered to overexpress an endogenous aldose reductase with xylose reductase activity (encoded by *GRE3* gene) and efficiently used as whole-cell biocatalyst for xylitol production (12).

Among lignocellulosic biomass, corn cob is potentially the most favorable feedstock for xylitol production due its high xylan content (16). Nevertheless, the main challenge of corn cob

processing, like other lignocellulosic materials, is the requirement of pretreatment technologies to break down its recalcitrant structure and to obtain xylose enriched hemicellulosic hydrolysates (17). Hydrothermal pretreatment (also known as autohydrolysis) represents an environmental friendly alternative to dilute acid hydrolysis, the most common pretreatment to solubilize the hemicellulosic fraction in lignocellulose-based xylitol production processes (8, 18). The autohydrolysis method, using water as reaction media, yields a liquid fraction mainly composed by xylooligosaccharides (XOS) and increases cellulose accessibility to enzymatic hydrolysis (19–21). The hemicellulosic derived compounds (oligosaccharides) can be hydrolyzed by acid or enzymes. Enzymatic hydrolysis of XOS offers several advantages compared to acid hydrolysis since it occurs at milder operational conditions with less inhibitory compounds formation and does not require neutralization procedures before fermentation (22). Given the limited research on enzymatic hydrolysis of XOS and also in the valorization of whole-slurry (containing both cellulose and xylooligosaccharides polysaccharides in solid and liquid fractions, respectively) in presence of lignocellulose-derived inhibitors, (22, 23) the main goal of this work is the development a high effective strategy using green technologies (autohydrolysis, enzymatic saccharification and fermentation) for xylitol production from corn cob.

3.2. Materials and Methods

3.2.1. Raw material and autohydrolysis pretreatment

Corn cob was milled to a particle size less than 8 mm, homogenized, and submitted to autohydrolysis. The raw material was mixed with water at different solid loadings: 20, 25 and 30 g of corn cob solid dry weight per 100 g of water and heated to temperatures of 205 °C in a 2 L stainless steel reactor (Parr Instruments Company) equipped with Parr PDI temperature controller. Temperature and time of autohydrolysis was correlated using the following equation, which allows the determination of severity factor (R_0) expressed as severity (S_0= log R_0) as follows:

$$S_0 = \log\left[\int_0^{t_{max}} \frac{T(t)-T_{ref}}{\omega}\cdot dt + \int_{t_{max}}^{t_f} \frac{T'(t)-T_{ref}}{\omega}\cdot dt\right] \quad (1)$$

where, t_{max} and t_f refers to the time (min) required to achieve the maximum temperature and the t_0 is referred to time of the heating-cooling profiles (limited by T_{ref}), respectively, while T(t) and T'(t) correspond to the temperature profiles for the stages of heating and cooling. T_{ref} is the reference temperature (373.15 K) and ω is an empirical parameter related to the activation energy, set to 14.75 K for corn cob.

After treatment, liquid and solid fractions were separated by filtration and solid fraction (pretreated corn cob) was recovered and washed for Solid Yield (SY) determination. Chemical composition of corn cob and pretreated corn cob were analyzed following NREL protocols (NREL/TP-510-42618-42622-4218). The concentrations of sugars, acetic acid and furan compounds were measured by HPLC. For determination of oligosaccharides and acetyl groups, one aliquot of hydrolysate was submitted to an analytical hydrolysis (4 % w/w H_2SO_4 at 121 °C for 20 min).

3.2.2. Enzymatic saccharification of pretreated corn cob

Enzymatic saccharification of pretreated corn cob was carried out at 45 °C, 150 rpm in an orbital shaker using 5% of pretreated corn cob and enzyme loadings of 24 FPU/g for 96 h. Commercial enzyme preparation used in these assays was Cellic CTec2 (kindly supplied by Novozymes, Bagsvaerd, Denmark). Cellulase and hemicellulase activities of Cellic CTec2 were 122 FPU/mL and 9764 U/mL, determined following the procedures previously described (24, 25). Enzymatic saccharifications were carried out using pretreated corn cob as substrate in water (named slurry) and pretreated corn cob in hydrolysate (named whole slurry) as shown in Figure

3.1a. Glucose and xylose concentrations were analyzed by HPLC. Glucose (GY) and xylose yield (XY) were calculated following the equations:

$$GY\ (\%) = \frac{G_t - G_{t0}}{G_{POT}} 100 \qquad (2)$$

where, Gt is the glucose concentration (g/L) achieved at time t and Gt_0 is the glucose concentration at the beginning of the experiments; whereas G_{POT} represents the potential glucose concentration that was calculated as:

$$G_{POT} = Bf\frac{180}{162} \qquad (3)$$

where, B is dry corn cob biomass concentration (g/L), f is glucan fraction in dry biomass (g per g) and 180/162 is the stoichiometric factor that converts glucan to equivalent glucose.

$$XY\ (\%) = \frac{X_t - X_{t0}}{X_{POT}} 100 \qquad (4)$$

where, Xt is the xylose concentration (g/L) achieved at time t and Xt_0 is the xylose concentration at the beginning of the experiments, whereas X_{POT} represents the potential xylose concentration that was calculated as:

$$X_{POT} = Bf\frac{150}{132} + XOS \qquad (5)$$

where, B is dry corn cob concentration (g/L), f is xylan fraction in dry biomass (g per g) 150/132 is the stoichiometric factor that converts xylan to equivalent xylose and XOS is xylooligosaccharides concentration measured as xylose equivalent in g/L present in the hydrolysate (XOS were only considered for potential xylose for the enzymatic saccharification of whole slurry).

3.2.3. Yeast strain and inoculum

The yeast strain used in this work was the yeast strain *Saccharomyces cerevisiae* PE-2, isolated from 1st generation bioethanol plants in Brazil, (14, 26–28) overexpressing the endogenous *GRE3* gene, *S. cerevisiae* PE-2-GRE3 (12). Yeast strain was maintained at 4 °C on YPD plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar) supplemented with 200 mg/L of geneticin (G418). Yeast cells for inoculation were grown overnight at 30 °C and 200 rpm in YPD medium supplemented with 150 mg/L of G418. The cell suspension was collected by centrifugation for 5 min at 3000 rpm, 4 °C and suspended in 0.9% (w/v) sodium chloride solution. The fermentation experiments were conducted with a cellular concentration of 11 g and 22 g fresh yeast/L corresponding to 5 g and 10 g of dry yeast/L, respectively.

3.2.4. Preparation of corn cob hydrolysate: detoxification, neutralization and sterilization

Corn cob hydrolysate and corn cob hydrolysate after dilute acid hydrolysis (0.5% w/w of H_2SO_4 for 165 min at 125 °C) (29) were submitted to ion exchange detoxification to remove acetic acid, as previously described (30). Briefly, corn cob hydrolysates were mixed with Amberlite IR-120 cationic resin (in H^+ form) at a mass ratio of 10 g cationic resin per gram of hydrolysate for 1 h with agitation. Cationic resin was recovered by filtration and the hydrolysate was treated for 2 h under agitation with Mto-Dowex M43 anionic resin (in OH^- form) at a mass ratio of 20 g anionic resin per gram of acetic acid present in the hydrolysate. The resulted acid-hydrolyzed corn cob hydrolysate was neutralized with $CaCO_3$ until pH 5 and the pH of corn cob hydrolysate was adjusted with NaOH or HCl solutions. Both hydrolysates were sterilized by filtration (0.2 μm) and added to solid fraction (sterilized at 121 °C for 20 min) to obtain the whole-slurry used for xylitol production.

3.2.5. Pre-saccharification and Simultaneous Saccharification and Fermentation (PSSF and SSF) assays of corn cob whole slurry

Simultaneous saccharification and fermentation (SSF) and Pre-saccharification and simultaneous saccharification and fermentation (PSSF) assays of whole-slurry were carried out in Erlenmeyer flasks at 30 °C and/or 35 °C in an orbital shaker at 200 rpm. For PSSF, an enzymatic saccharification step of whole slurry was carried out for 24 h using 5 or 8 % solids at 8 or 24 FPU/g at 45 °C and 200 rpm. After this step, temperature was decreased up to 35 °C for cell inoculation. SSF assays at optimal conditions, were carried out using corn cob hydrolysate with or without diluted acid post hydrolysis. Corn cob hydrolysate medium was supplemented with 20 g/L of peptone and 10 g/L of yeast extract.

3.2.6. Experimental design of Simultaneous Saccharification and Fermentation of whole slurry

Simultaneous saccharification and fermentation (SSF) process conditions were evaluated and optimized following a full factorial design (2 factors with two replicates of the central point, 10 total experiments). The independent variables evaluated were solid loading of pretreated corn cob or x_1 (ranged between 4-12 % w/w) and enzyme to substrate ratio (ESR) or x_2 (ranged between 8-24 FPU/g). Dependent variables were correlated with the independent variables by empirical models, following the equation:

$$y_j = b_{0j} + \sum_{i=1}^{2} b_{ij} x_i + \sum_{i=1}^{2} \ldots \sum_{k \geq i}^{2} b_{ikj}\, x_i x_k \qquad (6)$$

where y_j (j=1 to 3) is the dependent variable; x_i or x_k (i or k: 1 to 2, k ≥ i) are the normalized, independent variables and b_{0j}...b_{ikj} are regression coefficients calculated from experimental data by multiple regression using the least-squares method. The experimental data were fitted to the proposed models using commercial software (Microsoft Excel, Microsoft Office 365 ProPlus).

3.2.7. Determination of fermentation parameters

Xylitol yield (Y_{XL}) and productivity (Q_{pt}) were calculated as follows:

$$Y_{XL} = \frac{[XL]_t}{[X_{POT}]} \cdot 100 \qquad (7)$$

where, XL is the concentration of xylitol at time *t*, X_{POT} is the potential xylitol considering the xylose, xylooligosaccharides and the xylan present in the SSF.

$$Qp_t = \frac{[XL]_t}{t} \qquad (8)$$

where [XL] is xylitol concentration at time *t* divided by time *t*.

3.2.8. Analytical methods

Samples from saccharification and fermentation assays, chemical characterization and autohydrolysis treatment (including solid and hydrolysate) of corn cob were analyzed for quantification of sugars (glucose, xylose, arabinose), acetic acid, xylitol, furfural, hydroxymethylfurfural (HMF) and ethanol by HPLC using a BioRad Aminex HPX-87H (300 x 7.8 mm) column, at 60 °C, and 0.005 M sulfuric acid as eluent in a flow rate 0.6 mL/min. The peaks corresponding to sugars, acetic acid, xylitol and ethanol were detected using a Knauer-IR intelligent refractive index detector, whereas furfural and HMF were detected using a Knauer-UV detector set at 280 nm.

3.3. Results and discussion

3.3.1. Autohydrolysis pretreatment for corn cob processing: effect of solid loading

The xylitol production from lignocellulosic biomass depends on the fractionation pretreatment used to obtain xylose for the bioconversion process (7, 18). In this study, the hydrothermal pretreatment of corn cob at high solid content (between 20% and 30%) was evaluated in order to maximize xylan solubilization and recovery the hemicellulose derived compounds, especially xylose and xylooligosaccharides. The biomass processing strategy proposed for xylitol production is shown in Figure 3.1.

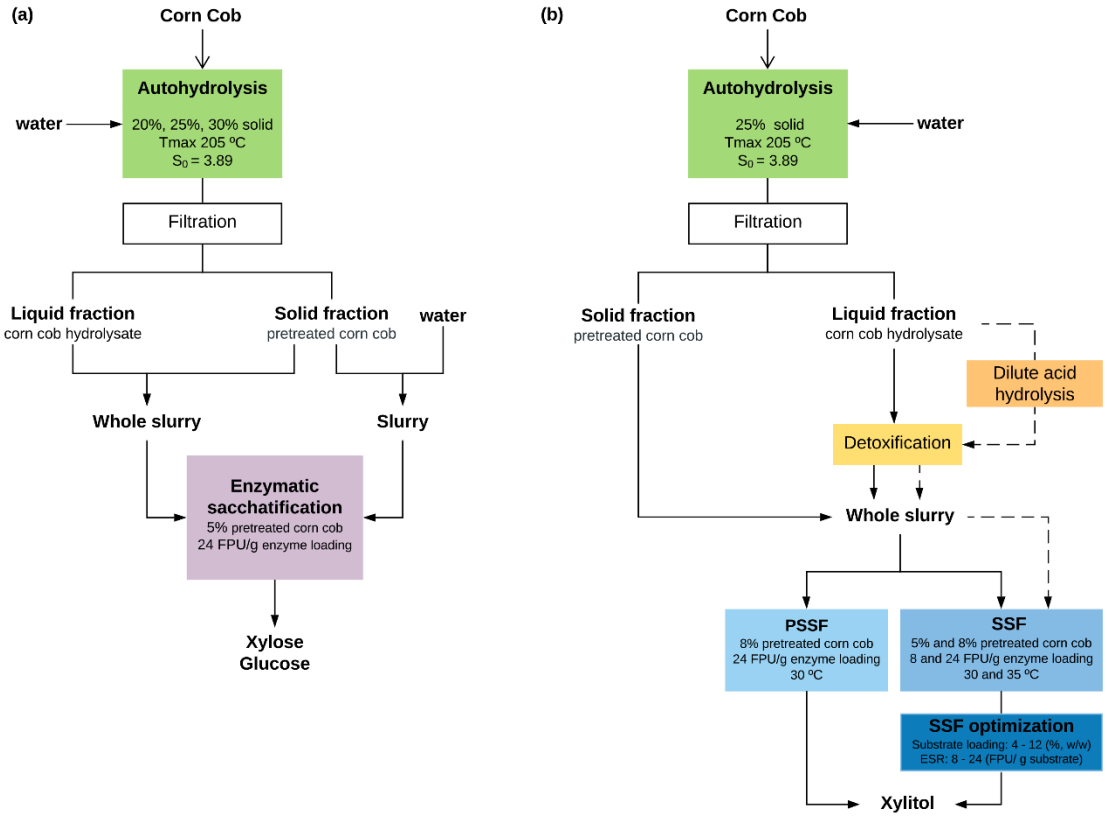

Figure 3.1. Flowchart of experimental procedure for (a) evaluation of the effect of autohydrolysis pretreatment at different solid loadings on enzymatic saccharification and (b) optimization of operational conditions and process configuration for xylitol production using whole slurry corn cob. Dotted lines refer to an optional strategy for xylitol production by the complete removal of acetic acid from hemicellulosic hydrolysate.

Corn cob was chemically analyzed and its composition (based on three replicates) was: 28.8% ± 1.63 of glucan, 29.6% ± 1.88 g of xylan, 22.9% ± 0.30 g of Klason lignin, 3.4% g ± 0.83 of arabinan and 2.0% ± 0.04 g of acetyl groups per 100 g of dry weight.

The pretreatment severity was based on previous works that have shown that the use of 12 g of corn cob per 100 g of water lead to maximum concentration of xylooligosaccharides (29, 31). In order to reduce the water consumption in the process and increase the xylose concentration in the liquid fraction (hydrolysate), the solid loading of corn cob was evaluated in the range of 20 to 30 g of corn cob per 100 g of water at T_{max} of 205 ° C (S_0=3.89) (12). The use of high-solid loadings in the pretreatment minimizes the water consumption and reduces the energy required for heating, improving the economic and environmental sustainability of the process (32, 33). Nevertheless, increased solid concentrations could negatively affect the process efficiency by insufficient mixing, limitations of heat and mass transfer and also by increasing the concentration of inhibitor compounds in the hydrolysate. Chemical composition of solid and liquid fractions after pretreatment is shown in Table 3.1.

Table 3.1. Chemical composition of solid and liquid fractions obtained from corn cob processing by autohydrolysis at Severity of 3.89 using high solid loading

Solid loading (g of corn cob per 100 g of water)	**20**	**25**	**30**
Solid yield (g of autohydrolyzed corn cob/100 g of corn cob)	57.7	60.0	57.7
Autohydrolyzed corn cob composition (g of component/100 g of pretreated corn cob)			
Glucan	48.6 ± 0.6	43.8 ± 0.6	44.4 ± 0.1
Xylan	16.8 ± 0.1	17.0 ± 0.2	18.3 ± 0.3
Arabinan	1.21 ± 0.10	1.29 ± 0.11	1.21 ± 0.02
Acetyl groups	0.53 ± 0.01	ND[a]	ND
Klason Lignin	19.4 ± 0.6	22.2 ± 0.2	21.2 ± 0.1
Liquid phase composition (g/L)			
Glucose	0.73 ± 0.04	0.75 ± 0.06	0.71 ± 0.04
Xylose	2.87 ± 0.14	7.52 ± 0.32	8.80 ± 0.44
Arabinose	1.31 ± 0.07	2.28 ± 0.04	2.09 ± 0.1
Acetic acid	1.56 ± 0.08	2.26 ± 0.15	3.65 ± 0.18
Hydroxymethylfurfural (HMF)	0.44 ± 0.02	0.26 ± 0.01	0.75 ± 0.04
Furfural (F)	1.34 ± 0.07	1.29 ± 0.06	4.30 ± 0.21
Glucooligosaccharides (GOS)	1.28 ± 0.3	3.15 ± 0.05	2.31 ± 0.01
Xylooligosaccharides (XOS)	25.1 ± 1.4	31.75 ± 1.10	31.9 ± 0.08
Arabinooligosaccharides (ArOS)	1.05 ± 0.22	Not detected	0.50 ± 0.03

The recovery of glucan and lignin in the solid phase varied in the range of 88.9-97.4 g of glucan/100 g of glucan and 60.2-71.7 g of lignin/100 g of lignin in raw material, respectively. Chemical composition of liquid fraction (Table 3.1) showed that the increase of solid loading up to 25% in the pretreatment resulted in the highest concentration of released xylooligosaccharides (38.1 g/L). Therefore, under this condition, 65 % of xylan (measured as sum of xylose and xylooligosaccharides) was recovered in the liquid fraction, corresponding to 46.9 g/L of potential xylose that may be used as substrate for xylitol production. For a solid loading of 30%, the concentration of XOS was lower (31.9 g/L) due to dehydration of xylose to furfural (4.3 g/L). Furthermore, this condition resulted in higher concentration (3.65 g/L) of acetic acid, a degradation compound generated *in situ* during pretreatment that acts as catalyst for the hemicellulose hydrolysis (34), which could be directly related to a higher degradation of xylose into furfural comparing to the conditions using 20 and 25 % of solid loading. The effectiveness of pretreatments at high solid loadings (> 15 %) has been demonstrated in several strategies, such as the process developed by Inbicon AS (Denmark) using hydrated wheat straw with recycled condensate or the wet explosion pretreatment of lobelly pine (35). Similar solid loadings (20 and 25 %) were also tested for hydrothermal treatment of brewer´s spent grain, generating higher oligosaccharides concentration using 25 % of solid loading in the pretreatment (36). In addition, presoaked wheat straw was maintained at temperatures between 195-205 °C and residence time in the range of 6-12 min by injection of stream, resulting in a concentration of solids in the reactor between 23% and 28% (w/w), with the correspondent whole slurries being used for ethanol production by SSF (37).

Despite being an attractive strategy to obtain higher sugar concentration, the use of high solid loading in the pretreatment generates higher amounts of degradation compounds, such as furfural, hydroxymethylfurfural and acetic acid (37). Among these compounds that have inhibitory effects on enzymatic and fermentation processes, acetic acid (measured as sum of acetic acid and acetyl groups) was the major product in the hydrolysates, varying in the range of 4.57 to 9.02 g/L, raising with the increase of solid loading (39).

3.3.2. Enzymatic hydrolysis of corn cob slurry and whole slurry

Considering that hemicellulose and lignin derived compounds present in the hydrolysate (i.e., xylooligosaccharides and phenolic compounds) could inhibit the enzyme activities, reducing the saccharification yield (23, 40, 41), the enzymatic hydrolysis of both fractions resulting from

biomass pretreatment were evaluated by enzymatic saccharification of slurry (pretreated corn cob and water) (Figure 3.2) and the whole-slurry (pretreated corn cob and hydrolysate) (Figure 3.3).

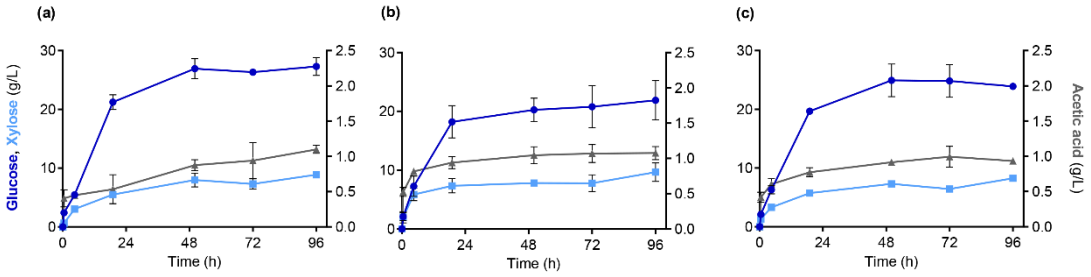

Figure 3.2. Enzymatic saccharification of slurry using 5% of pretreated corn cob from autohydrolysis at (a) 20%, (b) 25% (c) 30% of solid loading. Profiles of glucose, xylose and acetic acid concentrations.

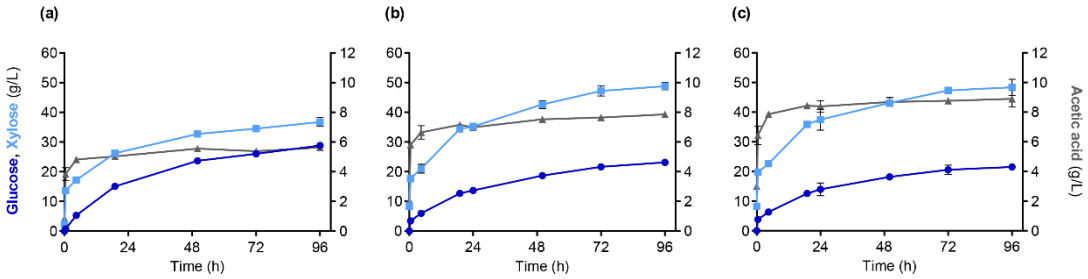

Figure 3.3. Enzymatic saccharification of whole-slurry using 5 % of pretreated corn cob from autohydrolysis at (a) 20%, (b) 25% (c) 30% of solid loading. Profiles of glucose, xylose and acetic acid concentrations.

As expected, the glucose concentration and glucose yield were higher in the enzymatic saccharification assays of slurries (Figure 3.2, Table 3.2) comparing with the results obtained from whole-slurries (Figure 3.3, Table 3.2), showing a clear effect of hemicellulosic hydrolysates on cellulose saccharification. The negative effect of oligosaccharides on cellulose saccharification was also demonstrated by the high glucose yield (99%) achieved with the hydrolysate containing lower oligosaccharides content (pretreatment with 20% of solid loading) in comparison with the glucose yields (< 76%) achieved in the saccharifications of whole-slurries obtained from autohydrolysis using higher solid loadings (25 and 30%). This effect was also described by Oliveira and co-workers (2018), which reported 25% less glucose production for the enzymatic saccharification of eucalyptus whole-slurry.

In terms of xylose yield, the xylose concentration was inferior in the hydrolysate containing lower amount of xylooligosaccharides, resulting in a final xylose concentration of 36.8 g/L (98% of xylose yield). The enzymatic saccharification of whole slurries obtained from the autohydrolysis with 25% and 30% of solid loading resulted in equivalent xylose concentrations (48.8 and 48.4 g/L,

respectively) but the highest xylose yields (> 95%) were achieved using whole slurries obtained from the autohydrolysis with 20% and 25% of solid loading. A similar result (21 g/L of xylose corresponding to 93% of xylose yield) was observed with a hydrolysate obtained from industrial wheat straw processing (Ibicon and Beta-Renewable)(22)

Table 3.2. Operational conditions used in the enzymatic saccharification of 5% pretreated corn cob using 24 FPU/g and main results (glucose concentration and yield and xylose concentration and yield) obtained at 96h.

Operational Conditions		**Main Results**			
Substrate	Solid loading in autohydrolysis (%, w/w)	Glucose concentration (g/L)	Glucose Yield (%)	Xylose concentration (g/L)	Xylose Yield (%)
Slurry	20	27.3	101.1	8.9	92.9
Whole-Slurry		28.8	99.4	36.8	97.8
Slurry	25	21.9	90.0	9.7	100.0
Whole Slurry		23.1	72.3	48.8	99.2
Slurry	30	23.9	97.0	8.3	79.4
Whole Slurry		21.5	75.0	48.4	93.5

The enzymatic cocktail used in the whole slurries assays (Figure 3.3) also hydrolyzed acetyl groups present in the hemicellulosic hydrolysate and pretreated solid corn cob, achieving a maximal concentration of acetic acid of 8.9 g/L (Figure 3.3c). In fact, weak acids such as acetic acid may inhibit the cell growth or increase the fermentation lag phase, affecting the fermentation performance in the subsequent step of xylose to xylitol bioconversion (13, 43, 44). Considering the results obtained from the enzymatic saccharification of whole-slurries, the autohydrolysis with 25% of corn cob showed to be more advantageous in terms of xylooligosaccharide conversion and xylose concentration. Therefore, this operational condition was selected for the xylose to xylitol bioconversion process. In addition, the hydrolysate was detoxified by anion exchange for a complete removal of free acid acetic, reducing the final concentration after enzymatic saccharification from 7.9 g/L to 5.6 g/L.

3.3.3. Determination of operational conditions for xylitol production using whole slurry corn cob

The whole-cell bioconversion process for the production of xylitol involves the xylose transportation into the yeast cell and the conversion into xylitol by the aldose reductase encoded

by the *GRE3* gene. The yeast *S. cerevisiae* is a non-xylose-utilizing organism and therefore the recombinant PE-2-GRE3 strain need to be supplied with a carbon source to regenerate co-factors and ensure maintenance energy generation (11). The glucan-enriched solid phase (obtained from corn cob autohydrolysis) can be efficiently hydrolyzed by enzymes, providing glucose for cell metabolism during the bioconversion of xylose (from corn cob hydrolysate) into xylitol (12)

Considering that SSF process efficiency is strongly affected by the temperature, preliminary SSF experiments were performed at 30 and 35 °C in order to evaluate its influence on xylitol production process (Table 3.3). As seen in Figure 3.4, the use of 5% of pretreated corn cob and 24 FPU/g of enzyme loading allows the release of both xylose and glucose from XOS and cellulose hydrolysis and subsequent utilization of xylose for xylitol production and glucose for cell metabolism. The increasing concentration of xylitol and the simultaneous accumulation of xylose in medium suggest that enzymatic hydrolysis occurs faster than the bioconversion process. After glucose depletion, the yeast started to utilize the earlier produced ethanol by switching metabolism from glycolysis to aerobic utilization of ethanol, which prevents the competitive inhibition of xylose uptake by glucose and might be involved with the increased xylitol conversion rate, observed during the ethanol consumption phase. Despite the similar trends in fermentation profiles, the SSF performed at 35 °C resulted in a higher xylitol concentration (51.7 g/L) and the maximal productivity was about 1.3-fold higher (0.52 g/L·h at 70 h) compared to 30 °C (0.39 g/L·h at 70 h). In this sense, the subsequent SSF experiments were performed at 35 °C.

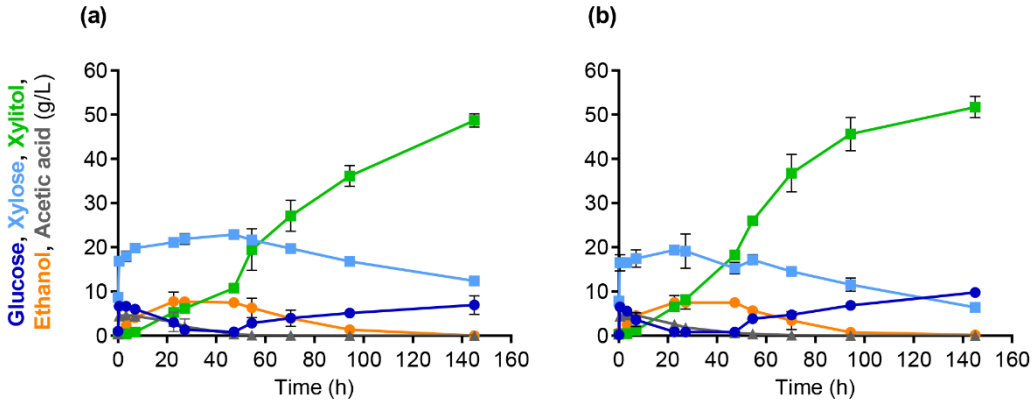

Figure 3.4. Xylitol production from corn cob whole slurry by simultaneous saccharification and fermentation (SSF) process using 5% of solid and 24 FPU/g at (a) 30°C and (b) 35°C.

In addition, as SSF processes require an equilibrium between the optimum temperature for enzymatic hydrolysis and for yeast fermentation (45, 46), the effect on xylitol production of a saccharification before the SSF process (PSSF – pre-saccharification and simultaneous saccharification and fermentation) was investigated (Figure 3.5). The pre-saccharification step was performed at optimum temperature for enzymatic hydrolysis, using 8 FPU/g ($PSSF_1$) and 24 FPU/g ($PSSF_2$) of enzyme loading and 8% of pretreated corn cob. SSF assays without pre-saccharification were performed for comparison (Table 3.3).

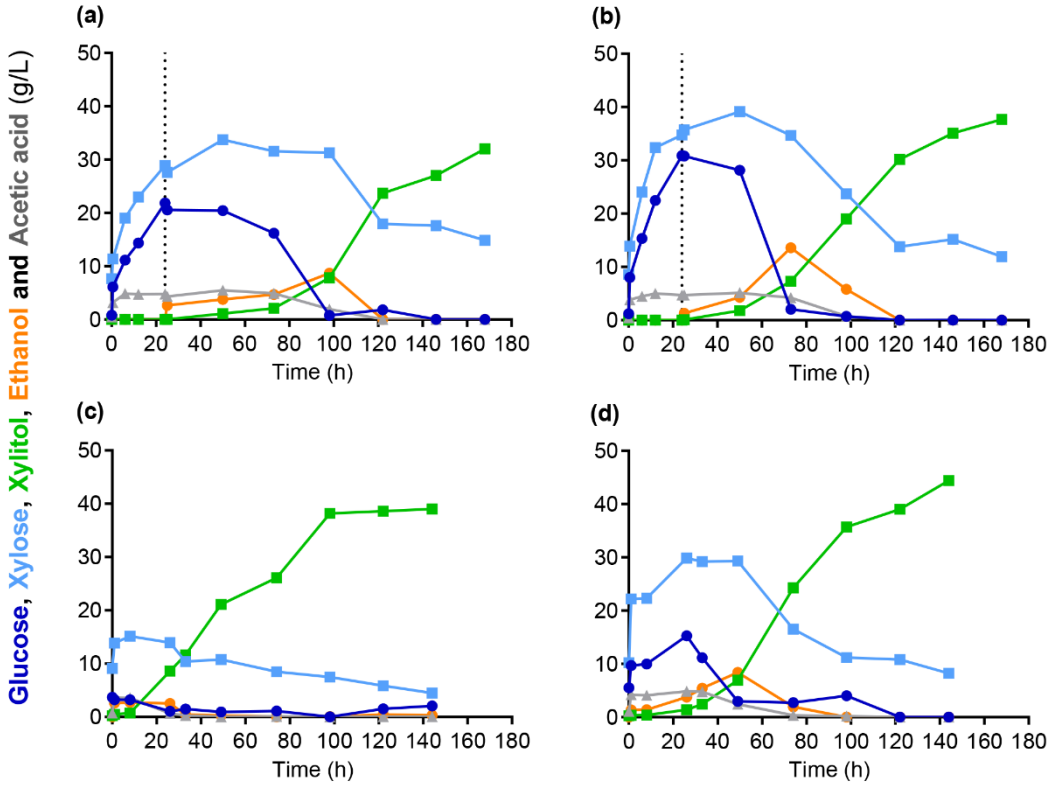

Figure 3.5. Xylitol production from corn cob whole slurry by pre-saccharification simultaneous saccharification and fermentation (PSSF) and simultaneous saccharification and fermentation (SSF) using 8% of solid. (a) $PSSF_1$ with 8 FPU/g and (b) $PSSF_2$ with 24 FPU/g of enzyme loading. (c) SSF_1 with 8 FPU/g and (d) SSF_2 with 24 FPU/g of enzyme loading. The dotted lines indicate the yeast inoculation time.

As seen in Figure 3.5A, in the first 24 h of saccharification 28.9 g/L of xylose and 21.8 g/L of glucose were released from the whole slurry by using 8 FPU /g ($PSSF_1$). After yeast inoculation, the glucose released from hydrolysis was entirely consumed and the ethanol produced was subsequently re-assimilated. However, the xylitol concentration (32 g/L) and xylitol productivity

(0.19 g/L·h) achieved in $PSSF_1$ were lower compared to SSF_1 (Figure 3.5C) that resulted in 39 g/L of xylitol and 0.27 g/L·h of productivity. In $PSSF_2$, the utilization of 24 FPU/g increased the initial concentration of xylose and glucose to 34.7 and 30.9 g/L, respectively (Figure 3.5B). This higher initial availability of sugars did not lead to higher xylitol production in comparison to the SSF_2 (Figure 3.5D) that was conducted without pre-saccharification (37.9 g/L, 0.22 g/L·h vs 44 g/L, 0.30 g/L·h).

In fact, the catabolite repression caused by high glucose concentrations have been for long recognized as the main factor for xylose transport inhibition in yeast, since glucose and xylose uptake occur by facilitated diffusion through the same transport system that present low affinity for xylose (47, 48). As the pre-saccharification, under the evaluated conditions, was found to have a negative effect on the maximal xylitol concentration and productivity, the following experiments were performed under SSF conditions.

Table 3.3. Operational conditions (temperature, substrate, and enzyme loading) of simultaneous saccharification and fermentation (SSF) and pre-saccharification and simultaneous saccharification and fermentation (PSSF) and main results obtained (xylitol concentration, yield and productivity).

Run	Operational conditions			Results				
	Temperature (°C)	Substrate loading (%, w/w)	Enzyme loading (FPU/g)	X_{pot} [a] (g/L)	Xf [b] (g/L)	$Xylitol_{max}$ (g/L)	Xylitol Yield (%)	Qp_{max} [c] (g/L·h)
SSF_{30}	30	5	24	52.86	11.92	48.69	92.11	0.336
SSF_{35}	35	5	24	52.86	6.41	51.73	97.87	0.357
$PSSF_1$	45°C; 35°C	8	8	58.65	14.84	32.36	55.17	0.193
$PSSF_2$	45°C; 35°C	8	24	58.65	11.94	37.96	64.72	0.226
SSF_1	35	8	8	58.65	4.46	39.00	66.49	0.271
SSF_2	35	8	24	58.65	8.22	44.38	75.66	0.308

[a] X_{pot} potencial xylose, calculated considering the sum of xylose concentration in the t_0 of SSF or PSSF with the xylose produced from xylan and XOS saccharification.

[b] X_f xylose concentration in the t_f

[c] Q_{max} maximal productivity, calculated when xylitol was maximum

3.3.4. Optimization of Xylitol production by SSF process: Experimental design

Considering the results obtained in preliminary assays, the SSF strategy at 35 °C was selected for optimization of xylitol production using an experimental design. For that, pretreated corn cob loading (x_1) and enzyme to substrate ratio-ESR (x_2) were selected as independent variables and the

dependent variables were xylitol production at the end of SSF process (y_1), xylitol yield (y_2) and productivity (y_3). Table 3.4 includes the experimental matrix (dimensional and normalized, dimensionless independent variables) and dependent variables. Time course of SSF experiments (run 1-10) can be seen in Figure A3.1 included in Appendix.

Table 3.4. Operational conditions (expressed in terms of dimensional and dimensionless independent variables) of simultaneous saccharification and fermentation (SSF) assays and experimental results obtained (xylose concentration, yield and productivity) for dependent variables y_1 to y_3.

Run	x_1	x_2	Substrate loading (%, w/w)	ESR (FPU/ g substrate)	Final xylitol concentration (g/L) or y_1	Xylitol yield (%) or y_2	Productivity (g/L·h) or y_3
1	-1	-1	4	8	22.8	45.6	0.19
2	0	-1	8	8	39.0	66.9	0.27
3	1	-1	12	8	24.6	37.0	0.17
4	-1	0	4	16	16.0	32.0	0.11
5	0	0	8	16	40.3	69.0	0.28
6	0	0	8	16	43.7	75.02	0.31
7	1	0	12	16	11.1	16.7	0.08
8	-1	1	4	24	40.4	80.6	0.28
9	0	1	8	24	40.0	68.6	0.28
10	1	1	12	24	12.3	18.6	0.09

In spite of the removal of acetic acid from the pretreated corn cob hydrolysate by ion exchange, the detoxification process only removes the acetic acid released from the autohydrolysis pretreatment and during the subsequent whole slurry enzymatic saccharification, more acetic acid is produced as a result of hydrolysis of acetyl groups linked to xylooligosaccharides. In the first hours of SSF experiments the concentrations of this compound achieved an average concentration of 4.3 g/L, which could explain the longer lag phases, affecting the overall productivity (Appendix: Figure A3.1). The maximal xylitol concentrations (>40 g/L) were attained with the high enzyme loading (run 5, 6, 8 and 9) and substrate loadings (pretreated corn cob) lower than 8% (Table 3.4). Whereas the highest xylitol yield (81%) was obtained with 4% of solid and 24 FPU/g of ESR (run 8). For a correct interpretation of the results, the experimental variables were correlated according to Equation (4). The fitting parameters were included in Table 3.5. The regression coefficients, the

correspondent statistical significance (based in the Student´s t test) and the significance of the model (based on Fisher´s F parameter) measure the correlation and significance of the developed model for xylitol production by SSF. As seen in Table 3.5, linear and quadratic terms for variable x_1 (substrate loading) and combination of substrate loading and ESR (x_2) were significant ($P < 0.05$; $P < 0.1$). The coefficient R^2 of model was >0.9 for xylitol concentration and yield, and only 0.88 for xylitol productivity.

Table 3.5. Regression coefficients, values and significance (based on a t-test).

Response variable	Xylitol concentration		Xylitol yield		Xylitol productivity	
	coefficient	P value	coefficient	P value	coefficient	P value
b_0	38.63	0.0003	64.76	0.001	0.26	0.0007
b_1	-5.20	0.073	14.33	0.039	0.04	0.091
b_2	1.78	0.455	3.05	0.553	0.002	0.924
b_{12}	-7.45	0.047	-13.37	0.082	-0.04	0.129
b_{11}	-21.71	0.003	-33.20	0.012	-0.14	0.010
b_{22}	6.44	0.135	10.27	0.247	0.04	0.227
R^2	0.93		0.94		0.88	
Adjusted-R^2	0.85		0.77		0.79	
F	11.0		6.9		6.0	
Significance level	98 %		96 %		95 %	

The representation of the effect of independent variables on response variables were evaluated using a response surface model (Figure 3.6). Although the use of high ESR improved xylitol production, this variable was not significant in the proposed model. Substrate loading was the variable with the more significant impact on xylitol production, yield and productivity, showing a clear optimum with a substrate loading of 6.8% at highest ESR (24 FPU/g). Under these conditions, xylitol yield was higher than 80%. On the other hand, productivities were lower than 0.32 g/L·h, showing that the enzymatic saccharification of XOS and glucan could be limiting step of the process. The influence of solid loading on xylitol production could be related with the glucose catabolite repression, indicating that lower glucan concentration in the SSF is advantageous for xylitol production. According to the model, the optimum condition to maximize xylitol yield and productivity was 6.76% of substrate loading (w/w) and ESR of 24 FPU/g. In order to validate this prediction, an additional SSF experiment was carried out under these conditions (Figure 3.7A), resulting in a concentration of xylitol of 42.9 g/L (at 144 h) and xylitol productivity of 0.30 g/L·h,

with a corresponding error of 7.66 and 6.66 %, respectively. These results verified the suitability of the model for predicting the experimental observations.

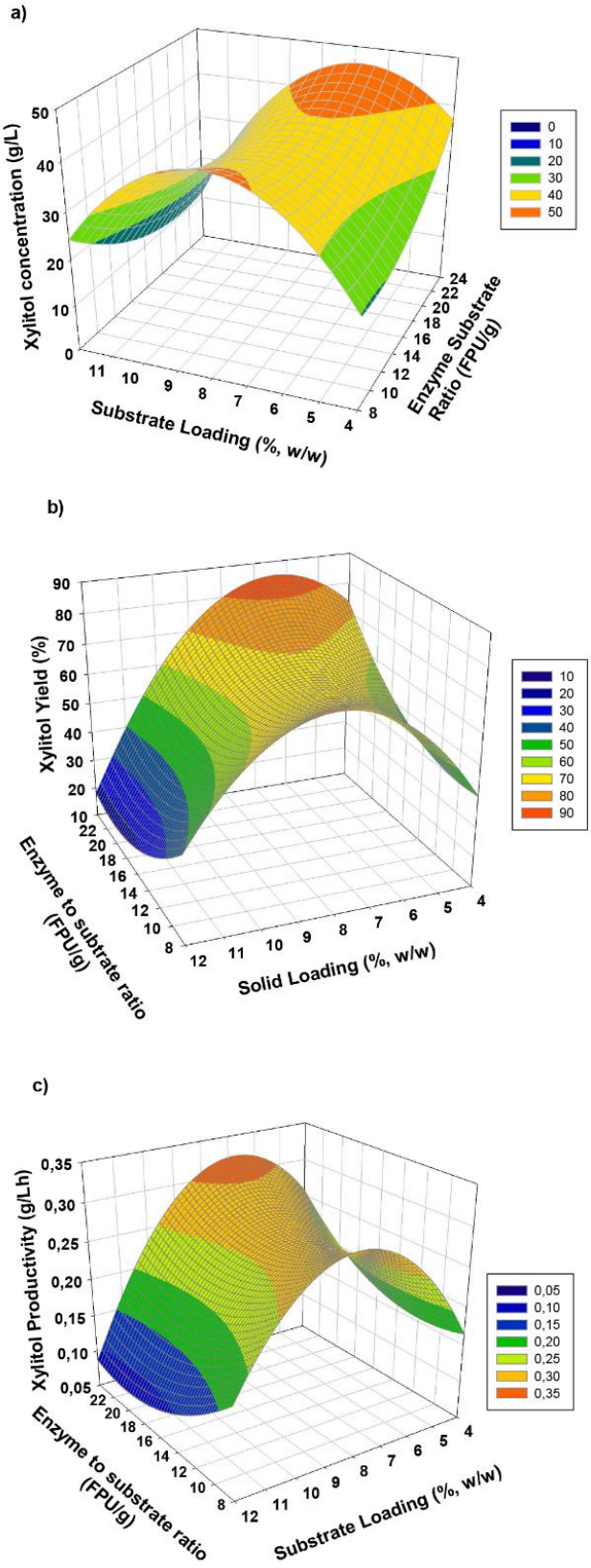

Figure 3.6. Response surface for fitted for (a) xylitol concentration (g/L), (b) xylitol yield (%) and (c) xylitol productivity (g/L·h).

3.3.5. Acid hydrolysis of hydrolysate for xylitol production by SSF

As mentioned before, acetic acid can severely affect the fermentation performance of the yeast and decrease its xylitol production capacity. Considering this negative effect, the corn cob hydrolysate, composed by xylooligosaccharides linked to acetyl groups, was submitted to an acid post hydrolysis for depolymerization and deacetylation of xylooligosaccharides to yield free xylose and acetic acid. The acetic acid was completely removed from the resulting acid-hydrolyzed hydrolysate by ion exchange detoxification and used in a SSF, under the previously optimized conditions. The recombinant strain tested in detoxified acid-hydrolyzed liquor showed a superior fermentative capacity (Figure 3.7B) converting xylose to xylitol considerably faster and producing 1.56-fold more xylitol (67.03 g/L) in comparing to SSF using enzymatic-hydrolyzed autohydrolysis liquor (Figure 3.7A). Additionally, as this process uses the yeast cells as whole-cell biocatalysts, the inoculum was increased up to 22 g wet cells/L to maximize the bioconversion of whole-slurry corn cob into xylitol (Figure 3.7C). In fact, the increase of biocatalyst concentration resulted in higher xylitol concentration (71.7 g/L), clearly improving the volumetric productivity at 48h (0.83 g/L·h compared with 0.65 g/L·h obtained with 11g/L of inoculum) and xylitol yield (94.6% in comparison with 84.4 %).

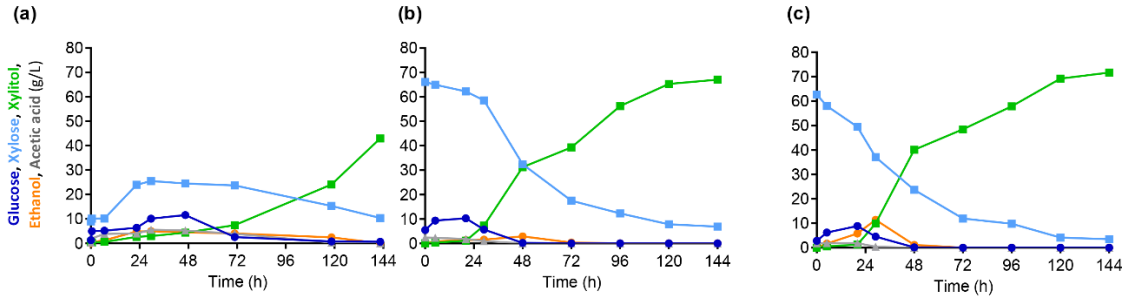

Figure 3.7. Simultaneous saccharification and fermentation of corn cob whole slurry under optimal conditions (6.76 % of substrate loading and 24 FPU/g) using 11 g/L of inoculum and (a) enzymatic hydrolyzed hydrolysate; (b) acid-hydrolyzed hydrolysate and (c) 22 g/L of inoculum and acid-hydrolyzed hydrolysate.

This evaluation showed a strong negative effect of acetic acid on yeast performance and revealed a clear advantage in using an acetic-acid deprived hydrolysate for an improved xylitol production. The inhibitory effect of this compound can be in part overcome by removing acid from

the broth during the fermentation or through the development of metabolic engineering strategies, such as the overexpression of genes involved in acetic acid tolerance (43, 49). Moreover, the highest xylitol concentration obtained in this work (Figure 3.7C) can be favorably compared to xylitol production reported in literature using concentrated hemicellulosic hydrolysates (50–52). Therefore, the use of high solid loadings in the pretreatment, approach followed in this work, is effective and avoids hydrolysate concentration steps, reducing time and cost of operation.

3.4. Conclusions

This work showed the feasibility of using high solid loadings in the hydrothermal pretreatment to obtain hydrolysates highly enriched in hemicellulose derived compounds (mainly xylooligosaccharides and xylose) suitable for the enzymatic hydrolysis and xylitol bioconversion, avoiding the need for costly evaporation steps. An experimental design was conducted to optimize the xylitol production by the recombinant *S. cerevisiae* strain. In addition, the absence of acetic acid resulted in the corn cob hydrolysate led to a further improved xylitol productivity and resulted in 72 g/L of xylitol, which represent the highest titer reported in *S. cerevisiae* using lignocellulosic biomass. The results obtained in here demonstrate an efficient and sustainable xylitol production, applying green technologies (autohydrolysis and saccharification and fermentation) for an integrated valorization of lignocellulosic biomass.

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Chapter IV.

Multi-feedstock biorefinery concept: valorization of winery wastes by engineered yeast

This chapter is based on the following original research article:

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Abstract

The wine industry produces significant amounts of by-products and residues that are not properly managed, posing an environmental problem. Grape must surplus, vine shoots, and wine lees have the potential to be used as renewable resources for the production of energy and chemicals. Metabolic engineering efforts have established *Saccharomyces cerevisiae* as an efficient microbial cell factory for biorefineries. Current biorefineries designed for producing multiple products often rely on just one feedstock, but the bioeconomy would clearly benefit if these biorefineries could efficiently convert multiple feedstocks. Moreover, to reduce the environmental impact of fossil fuel consumption and maximize production economics, a biorefinery should be capable to supplement the manufacture of biofuel with the production of high-value products. This study proposes an integrated approach for the valorization of diverse wastes resulting from winemaking processes through the biosynthesis of xylitol and ethanol. Using genetically modified *S. cerevisiae* strains, the xylose-rich hemicellulosic fraction of hydrothermally pretreated vine shoots was converted into xylitol, and the cellulosic fraction was used to produce bioethanol. In addition, grape must, enriched in sugars, was efficiently used as a low-cost source for yeast propagation. The production of xylitol was optimized, in a Simultaneous Saccharification and Fermentation process configuration, by adjusting the inoculum size and enzyme loading. Furthermore, a yeast strain displaying cellulases in the cell surface was applied for the production of bioethanol from the glucan-rich cellulosic. With the addition of grape must and/or wine lees, high ethanol concentrations were reached, which are crucial for the economic feasibility of distillation. This integrated multi-feedstock valorization provides a synergistic alternative for converting a range of winery wastes and by-products into biofuel and an added-value chemical while decreasing waste released to the environment.

Keywords: Winery residues, Bioethanol, Xylitol, Engineered *Saccharomyces cerevisiae*, Integrated biorefinery

4.1. Introduction

The wine industry is culturally and commercially important, constituting a large sector of global agriculture (1). Grapes are one of the most commonly cultivated fruit crops worldwide, with an estimated vineyard area of 7.3 million hectares in 2021 (2). Spain (13%), France (11%), China (11%), Italy (10%), Turkey (6%) and the USA (5%) are the top 6 vine-growing countries (Figure 4.1a), representing 56% of the total area planted with vines. World wine production has been relatively stable over the past years (Figure 4.1b). In 2021, it is estimated to be 260 million hectolitres (mhL), a decline of around 3 mhL (-1%) from 2020 (2).

By-products of the viticulture and winemaking processes include vine shoots, grape pomace (including seeds, stalks, and skins), and wine lees. In addition to these waste products, wineries produce a significant volume of wastewater as well as an excess of grape must (Figure 4.1c) (3–6). Vine shoots (VS) from the agronomic practice of pruning account for up to 93% of winery leftovers (7). VS are composed of cellulose, hemicellulose, and lignin and can be considered as a platform synthesis of a plethora of biobased products such as oligosaccharides, proteins, lactic acid, bioactive compounds, biosurfactants, xylitol, and biofuels such as ethanol and biogas (8–15). In this sense, using VS as a source of energy and value-added products rather than combusting or disposing on the ground to decompose is a more cost-effective and environmentally friendly option. Wine lees (WL) are a common winery waste that forms at the bottom of wine containers after fermentation. It is composed of settled yeast cells and residual ethanol, representing 6% of wine the volume (16, 17). WL have been used to recover ethanol by distillation, extract tartaric acid and phenolic compounds, and produce biogas and medium-chain carboxylates (18–22). Furthermore, WL have been evaluated for culture media supplementation in biotechnological processes due to their high protein and nitrogen content, as well as the presence of vitamins and essential amino acids (23–25). The wine sector in Europe is strictly regulated with control measures to address the recurrent overproduction of wine in recent decades. One of the several measures adopted by the European community is the distillation of remaining wine for the production of cognac, vinegar, and other consumable foods and beverages (26–28). The use of grape must surplus (GM) for the biosynthesis of polyols, mannitol and erythritol was recently proposed as a business opportunity for producers to get rid of excess wine production (25, 29). Given the high sugar content of GM, namely fructose and glucose, using this low-cost by-product as a substrate for cell growth could contribute to the circular economy and lower the overall cost of the process. The elimination of the various residues produced at different stages of grape and wine production is becoming not only

an environmental concern but also an economic issue, which may reduce the sector's competitiveness (30).

Biorefining is considered one of the enabling technologies of the circular economy, aiming at the valorization of a wide variety of biomass, including agriculture residues, into a broad range of products and energy (31). The establishment of biorefineries producing high-value chemicals in addition to low-value biofuels may reduce the use of non-renewable fuels and provide the required financial incentive to drive the development of biorefining technologies (32, 33).

With the remarkable progression of synthetic biology and metabolic engineering, the yeast *Saccharomyces cerevisiae* has become a reliable cell factory for sustainable production processes like second-generation ethanol and several bio-based chemicals (32, 34). It is crucial to select the appropriate yeast chassis to engineer, taking into account the target compound as well as the feedstock and process, in order to achieve high titers, yields, and productivity (35, 36). Accordingly Cunha et al., (37), engineered a selected robust *S. cerevisiae* industrial strain to display cellulolytic enzymes on the cell surface. This strain was then used in consolidated bioprocessing to produce ethanol directly from lignocellulose. Additionally, recent advances in xylitol production by Simultaneous Saccharification and Fermentation (SSF) might be an attractive strategy for valorizing winery-derived wastes. An industrial *S. cerevisiae* strain, with an innate ability to accumulate xylitol (38), was previously engineered for xylitol production by the overexpression of the endogenous aldose reductase (*GRE3*), which converts xylose into xylitol (39), resulting in high xylitol titers from pretreated corn cob (40) or hardwood xylan extracted with aqueous solutions of deep eutectic solvents (41).

Taking all together and considering that a successful biorefinery deployment relies on the efficient conversion of a broad range of feedstocks into marketable products, here we propose an integrated biorefinery approach (Figure 4.1d) that utilizes both liquid and solid winery waste streams to efficiently co-produce ethanol and xylitol. The product ethanol fulfills the global energy demand while xylitol increases the biorefinery profitability.

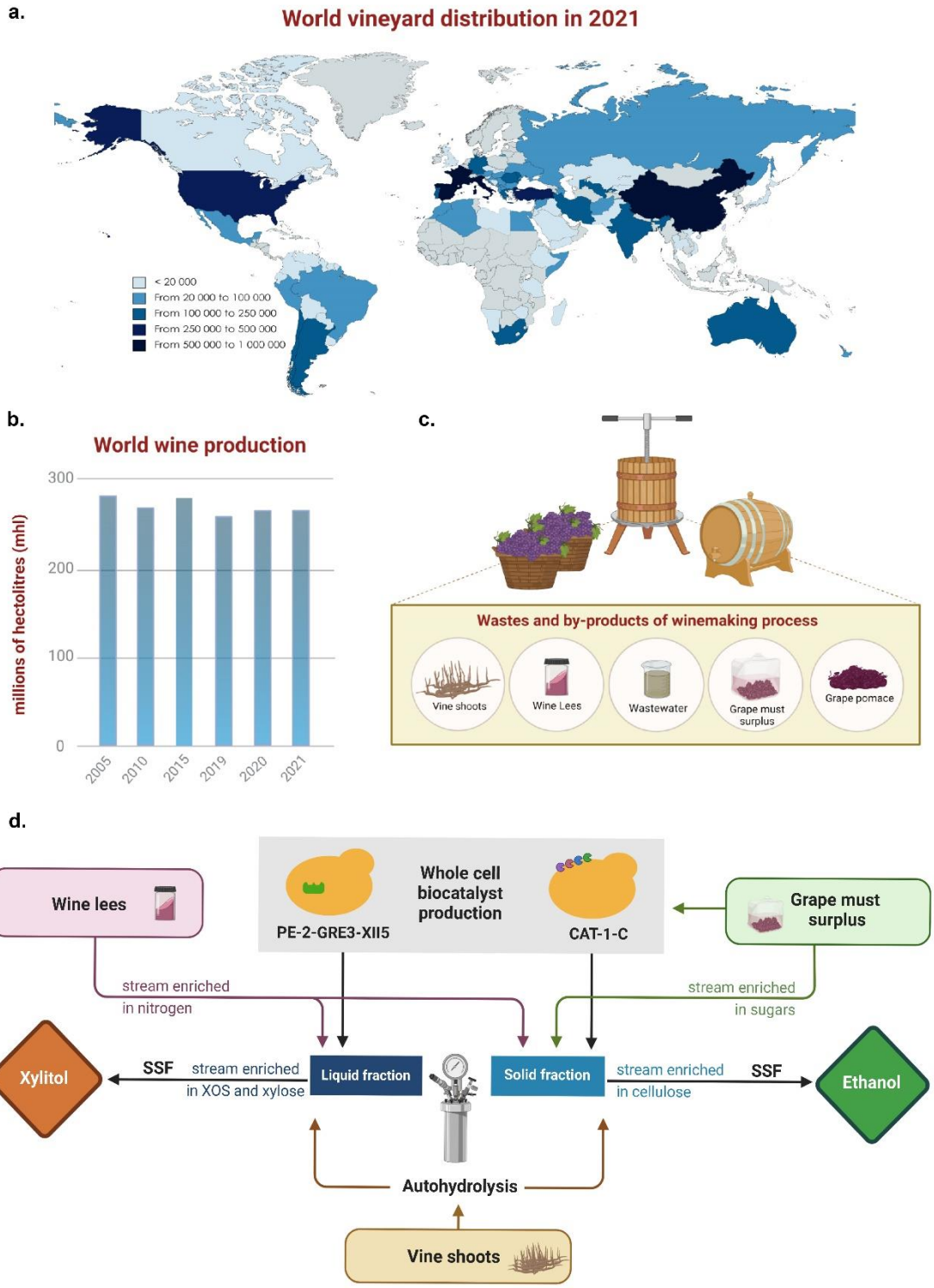

Fig 4.1. (a) The world vineyard surface area in 2021, (b) global wine production in 2005, 2010, 2015, 2019, 2020 and 2021, (c) representation of wastes resulting from wine industry. Based on International Organisation of Vine and Wine (OIV) data 2005–2021. (d) A graphical representation of the integrated process

4.2. Materials and Methods

4.2.1. Strains, plasmids and construction of engineered strains

Escherichia coli DH5α/NZY5α (Nzytech, Portugal) was used for cloning work and plasmid maintenance. *E. coli* cells were grown at 37 °C, in Lysogeny Broth medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.0) supplemented with 100 μg/mL of ampicillin for transformant selection. All plasmids and primers used in this work are listed in Appendix: Table A4.1 and A4.2. Plasmid construction was performed by USER cloning, described by Jensen et al. (42). The integrative plasmid (p2909_TEF-1_GRE3) was constructed by inserting the *GRE3* gene from pGRE3 (39) into pCfB2909, an EasyClone-MarkerFree integrative vector without any selection marker (43). Sanger sequencing, to confirm correct cloning, was purchased from Eurofins Genomics. Yeast transformation was performed following the lithium acetate method (44). The industrial *S. cerevisiae* PE-2 and CAT-1 strains, isolated from a first-generation bioethanol plant (45), were used as chassis strains in this work. The PE-2 strain was transformed with a Cas9-expressing plasmid before further modifications. The transformants were selected in YPD media (10 g/L yeast extract, 20 g/L peptone, 20 g/L of glucose) supplemented with agar (20 g/L) and geneticin G418 (200 μg/mL). Subsequently, the guide RNA (gRNA) helper vector targeting the XII-5 integration site (pCFB3050) was transformed along with the constructed integrative vector (p2909_TEF-1_GRE3) in the strain expressing the Cas9 protein, generating the strain PE-2-GRE3-XII5. Gene integration was confirmed by colony PCR.

CAT-1 was previously engineered to display *Aspergillus aculeatus* β-glucosidase 1 (EC 3.2.1.21; BGL1), *Trichoderma reesei* endoglucanase II (EC 3.2.1.4; EGII), *Talaromyces emersonii* cellobiohydrolase I (EC 3.2.1.91; CBH1) and *Chrysosporium lucknowense* cellobiohydrolase II (EC 3.2.1.91; CBH2) resulting in a robust strain (CAT-1-C) with hydrolytic activity for the direct ethanol production from cellulose (37).

4.2.2. Raw materials

Vine shoots (*Vitis vinifera* L.), grape must (variety White Verdejo) and wine lees (variety Red) were kindly provided by the Center of Biofuels and Bioproducts, Agrarian Technological Institute of Castilla and León (ITACyL). Grape must (GM) and wine lees (WL) were kept at – 20 ˚C until use.

Vine shoots (VS) were analysed for extractives, carbohydrates and lignin following the NREL (National Renewable Energy Laboratory) procedures, as described in Romaní et al. (46). GM and

WL were previously analysed elsewhere following the procedures described by Hijosa-Valsero and co-workers (2021).

4.2.3. Autohydrolysis pretreatment of vine shoots

Vine shoots (VS) were submitted to a hydrothermal treatment also known as autohydrolysis. The raw material was mixed with water at a liquid-solid ratio (LSR) of 4 and 6 (kg water per kg of oven-dry vine shoots) and heated to a maximal temperature (T_{max}) of 210-220 °C in a 2 L stainless steel reactor (Parr Instruments Company). The pretreatment severity (S_0) was calculated according to (40). After pretreatment, solid-liquid separation was performed by filtration and the solid fraction (pretreated VS) was recovered and washed for Solid Yield (SY) determination. The chemical composition of raw material and pretreated vine shoots was analysed according to NREL protocols (NREL/TP-510-42618-42622-4218). Acetyl groups and oligosaccharides in the liquid fraction were determined by acid post-hydrolysis of one aliquot of liquor (4 % w/w H_2SO_4 at 121 °C for 20 min). Sugars, furans and acetic acid were quantified by HPLC (analytical conditions described in section 2.7). The hemicellulosic hydrolysate was concentrated by a vacuum rotatory evaporator following the conditions reported by Domínguez et al. (47).

4.2.4. Enzymatic saccharification of vine shoots hydrolysate

For enzymatic saccharification of the liquid fraction obtained from the hydrothermal treatment (vine shoots hydrolysate), 30 mL of hydrolysate were mixed with 75-600 uL of cellulase cocktail (cellulase, enzyme blend [Cellic CTec2], Sigma-Aldrich) to achieve an Enzyme to Substrate Ratio (ESR) in the range of 88-350 U/g. The mixture was incubated at 30 °C and 200 rpm.

Glucose (GY) and xylose (XY) yields can be calculated via Eq.1 and Eq.2:

$$GY\ (\%) = \frac{G}{\frac{180}{162} f\ B} 100 \qquad (1)$$

where G is the concentration of glucose (g/L), B is dry vine shoots biomass concentration (g/L), f is glucan fraction in dry biomass (g/g) and 180/162 (1.11) is the stoichiometric factor that converts glucan to equivalent glucose.

$$XY\ (\%) = \frac{X}{\frac{150}{132}\cdot(f\cdot B)+XOS+X} 100 \qquad (2)$$

where X is the concentration of xylose (g/L), B is dry vine shoots biomass concentration (g/L), f is the sum of xylan fraction in dry biomass (g/g), XOS (xylooligosaccharides), and X (xylose)

measured as xylose equivalent (g/L), 150/132 (1.13) is the stoichiometric factor that converts xylan to equivalent xylose.

4.2.4.1. Enzymatic activity determination

Cellulase and hemicellulase activities of Cellic CTec2 were 143 FPU/mL and 626 U/mL, respectively. For the measurement of xylanase activity in Cellic CTec2, the enzymatic cocktail was incubated with 10 g/L of xylan from beechwood (Sigma, ≥ 90% purity) in 50 mM sodium citrate buffer (pH 5.0) for 10 min at 250 rpm orbital agitation at 30 °C. The amount of reducing sugar released from the substrate was measured by the DNS method (48). One unit of xylanase activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute. Cellulase activity of the enzymatic cocktail was determined by the filter paper assay following the NREL protocol (NERL/TP-510–42,628) (49).

4.2.5. Media and culture conditions

4.2.5.1. Pre-culture conditions

To evaluate the effect of grape must on yeast propagation, strains were grown in YPD and grape must-based media, at 300 rpm, 30 °C in 250 mL baffled shaker flasks with a working volume of 75 mL. Grape must-based media consisted of dilute solutions of grape must (GM), prepared by the addition of distilled water to a final concentration of 10% - 50% of GM. GM was paper-filtered and sterilized in an autoclave (121 ˚C, 20 min) before dilution. For subsequent assays, yeast cells were grown on 50% white grape must (pH 5) for 24 h, and cell growth was scaled up to 1 L baffled shaker flasks with a working volume of 0.3 L. After propagation, cells were harvested by centrifugation (5 min, 3000 rpm, 4 °C), washed in 0.9% (w/v) NaCl solution and used to inoculate the culture medium with a concentration of 30-100 g of wet yeast/L. For the SSF experiment using cellulase-displaying CAT-1-C strain, the yeast was grown for 72 h to ensure an efficient cellulase expression (37).

4.2.5.2. Batch cultivation for xylitol production

Production of xylitol by PE-2-GRE3-XII5 was performed in YP medium (10 g/L yeast extract, 20 g/L peptone) with 15 g/L of glucose and 15 g/L of xylose and in vine shoots hydrolysate, previously saccharified by hemicellulase enzymes (section 2.4.), in 100 mL shake flasks (working volume 30 mL) at 30 °C and 200 rpm. For vine shoots hydrolysate experiments, the initial pH of the medium

was set to 5.0 and $CaCO_3$ was added in stoichiometric proportion considering the concentration of potential acetic acid. Samples were periodically collected for sugar and xylitol determination by HPLC.

4.2.5.3. Simultaneous Saccharification and Fermentation of vine shoots hydrolysate for xylitol production – experimental design

The *S. cerevisiae* PE-2 strain genome-engineered to overexpress the *GRE3* gene (section 2.1, PE-2-GRE3-XII5 strain) was used for xylitol production from xylose and xylooligosaccharides of hydrolysate in a Simultaneous Saccharification and Fermentation (SSF) process. The experiments were performed using concentrated vine shoots hydrolysate, in 100 mL shake flasks (working volume of 30 mL), at 200 rpm and 30 °C. The pH was adjusted as described above.

The full factorial design evaluated the independent variables: inoculum size and hemicellulase enzyme loading. The inoculum size (or x_1) ranged between 30-100 g of wet yeast/L and Enzyme to Substrate Ratio (ESR) or x_2 ranged between 159-477 U/g. The dependent variables, xylitol concentration and yield, were correlated with the independent variables by empirical models, following the polynomial expression:

$$y_j = b_{0j} + \sum_{i=1}^{2} b_{ij} x_i + \sum_{i=1}^{2} \sum_{k \geq i}^{2} b_{ikj}\, x_i x_k \qquad (3)$$

where y_j (j=1 to 2) is the dependent variable; x_i or x_k (i or k: 1 to 2, k ≥ i) are the normalized, independent variables and b_{0j}...b_{ikj} are regression coefficients calculated from experimental data by multiple regression using the least-squares method. The experimental data were fitted to the proposed models using commercial software (Microsoft Excel, Microsoft Office 365 ProPlus).

4.2.5.4. Simultaneous Saccharification and Fermentation of pretreated vine shoots for ethanol production

The assays were performed in 100 mL shake flasks (working volume of 30 mL) with a glycerol lock to prevent oxygen entry and create oxygen-depleted conditions. Ethanol production was followed by the weight difference of Erlenmeyer flasks (associated with the release of carbon dioxide) as previously described (37, 50–52). The media consisted of 15% pretreated vine shoots

(dry weight basis) supplemented with (i) 9% WL, (ii) 9% WL and 33 % GM, and (iii) 42 % of GM (wet weight basis). Lees and must were sterilized by autoclave (20 min, 121 ˚C) before addition. All media were adjusted to pH 5.0, as described above. Cellic CTec2 was added in a final concentration of 6 FPU/g of solids. The assays were performed in an orbital shaker at 150 rpm and 40 °C.

4.2.6. Determination of fermentation parameters

Xylitol yield from xylose (YXyOL/Xy) was calculated by the ratio between produced xylitol and consumed xylose. Xylitol yield from xylan (YXyOL/XyAN) was calculated by the ratio between xylitol concentration (g/L) at the end of the SSF assays, and the potential xylose concentration (g/L) in the used media, multiplied by 100. The potential xylose was calculated by multiplying the xylan concentration by the stoichiometric factor that converts xylan to equivalent xylose (150/132) and by adding the XOS concentration in the liquor.

Ethanol yield (YetOH/gluAN) was calculated by the ratio between the ethanol concentration (g/L) at the end of the SSF assays, and the potential glucose concentration (g/L) in the used media, multiplied by the theoretical stoichiometric yield (0.511g of ethanol that is produced per gram of glucose), and the final ratio value was multiplied by 100. The potential glucose was calculated by multiplying the glucan concentration by the stoichiometric factor that converts glucan to equivalent glucose (180/162). Xylitol (QXt) and ethanol (QEt) productivity (g/L·h) at the time (t) was calculated as the ratio between the xylitol and ethanol concentration (g/L), at the time (t), divided by that time (t).

4.2.7. Analytical methods

Samples from the different assays were analysed for quantification of glucose, xylose, xylitol, acetic acid, ethanol, HMF and furfural by HPLC using a Bio-Rad Aminex HPX-87H column, operating at 60 °C, with 0.005 M H2SO4 and at a flow rate of 0.6 mL/min. For fructose and xylose separation and quantification, when simultaneously present, the samples were analysed by HPLC using a Bio-Rad Aminex HPX-87P column, at 85 °C, with H2O at 0.5 mL/min.

4.3. Results and discussion

4.3.1. Biorefinery scheme and chemical composition of winery wastes

To establish a sugar platform based on the use of by-products generated during winemaking processing and viticulture practices, vine shoots (VS), the surplus of grape must (GM), and wine lees (WL) were the waste resources considered as renewable carbon-based feedstocks. Figure 4.1d displays an overview of the design proposed in this study for the integral valorization of these winery wastes into bio-based products (xylitol, ethanol, and biocatalysts), using the wide range of sugars (xylose, fructose, and glucose) present in these feedstocks**.** GM, mainly composed of fructose and glucose (Appendix: Table A4.3), was evaluated as an inexpensive carbon source for whole-cell biocatalysts production. These biocatalysts are required for subsequent conversion steps involving the native aldose reductase (39) and the heterologous cellulases (51), to produce xylitol and ethanol, respectively. WL, on the other hand, are nitrogen-rich residues (Appendix: Table A4.3) that may be employed as a low-cost alternative to peptone and yeast extract to minimize total production costs. Thus, GM and WL having accessible carbon and nitrogen sources could be applied to ethanol and xylitol production processes without further processing. Despite the interesting composition of VS (Appendix: Table A4.3), its recalcitrant lignocellulosic structure requires a pretreatment to extract fermentable sugars. VS has a polysaccharide content of 46% (Appendix: Table A4.3), with glucan and xylan being the main sources of glucose and xylose, respectively. This chemical composition is consistent with the literature (8, 11).

Most research studies have focused on the valorization of a particular winery waste (5, 53, 54). Comparatively, limited research focuses on multi-waste valorization for the synthesis of more than one product (55). Therefore, with the ultimate goal of enhancing the feasibility and sustainability of an integrated biorefinery while protecting the environment and promoting the circular economy, this study proposes a novel configuration process (Figure 4.1d) to integrate multiple waste streams for the production of multiple products.

4.3.2. Grape must surplus as feedstock for whole-cell biocatalysts production

Industrial production of yeast cell biomass requires the efficient replication of cells to maximize yields and productivity, along with an optimum yeast product performance in subsequent industrial applications. Low-cost substrates that are easily accessible in large quantities are required to ensure good process economics. These substrates should have high concentrations of carbon

compounds capable of being completely converted to cell biomass (56). Sugar-rich grape must (GM) fits these requirements to become a preferred substrate for the production of industrial yeast biomass. However, conditions of propagation have been shown to affect the cellular performance of *S. cerevisiae* (57). To evaluate the feasibility of utilizing GM as a carbon source, yeast cells were grown in aerated cultures with 10, 25, and 50% of grape must (Figure 4.2). The data provided in Figure 4.2a clearly shows that the growth kinetics was similar between the three tested conditions, and the biomass yield increased with increasing grape must concentration. Regardless of GM sugar consumption, in 42 hours of cultivation, yeast cells consumed around 80% of the sugar present in the GM-based media (Appendix: Figure A4.1). Using 50% of GM, the maximal biomass concentration of 5.2 g/L was attained after 24 hours of cultivation, indicating that high substrate concentrations did not negatively affect microbial growth. Despite the efficient utilization of GM to produce yeast biomass, the viability of these cells as biocatalysts in bioproduction processes must be verified.

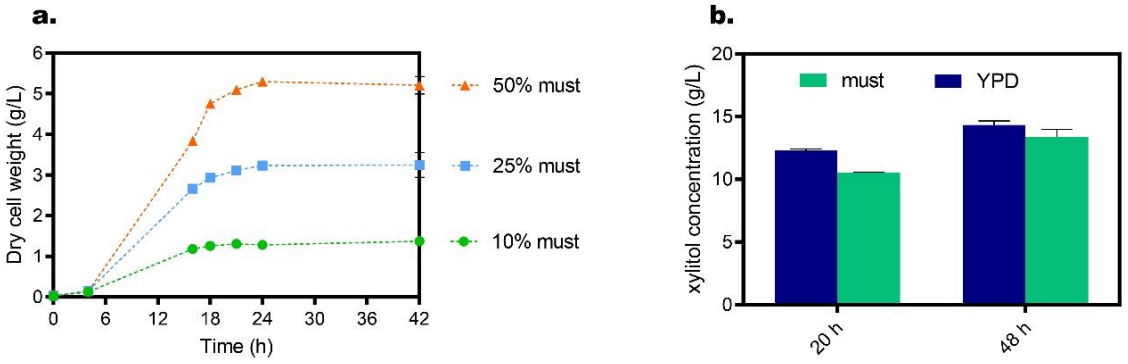

Figure 4.2. Evaluation of grape must as a raw material for yeast growth. (a) Yeast biomass production using (●) 10%, (■) 25% and (▲) 50% of grape must. (b) Xylitol production at 24h and 48h by yeast previously grown in grape must-based media (green) and YPD media (blue). When the error bar is not shown, it is smaller than the corresponding data symbol.

The robust PE-2 strain, when genetically engineered with a xylose consumption pathway (XR/XHD from *Scheffersomyces stipitis*), was found to have a natural propensity to accumulate xylitol (35, 38) and has been previously used as chassis to overexpress the *GRE3* gene in an episomal expression vector (39). Chromosomal gene integration provides advantages over plasmid-based expression (such as higher strain stability and reduced population variability) and the use of antibiotic-based selection markers in industrial strains is unfavorable due to the possibility of drug resistance spread (43). Thus, in this work, a novel PE-2-GRE3-XII5 recombinant strain was

constructed by the integration of the *GRE3* gene in the yeast genome. Single-copy chromosomal expression is often weaker than multicopy plasmid expression. Nevertheless, gene expression is highly dependent on several components of the genetic construction, including promoters, ribosome-binding sites, and enhancers or activators (58). The single integration in a diploid strain, with a strong promoter (*TEF1*), resulted in an equivalent xylitol conversion phenotype when compared with the plasmid-based construct (Appendix: Figure A4.2).

Thus, the capacity of the genome-engineered PE-2-GRE3-XII5 strain, pre-grown in synthetic (YPD) and in GM-based media, was compared in batch cultivations using xylose-containing medium, supplemented with glucose. This carbon source supplementation is needed to create cofactors and maintain energy since *S. cerevisiae* does not natively metabolize xylose. Glucose was consumed in less than 2 hours by the YPD and GM grown cells (data not shown). After 20h and 48h of cultivation, cells propagated in YPD produced 12.3 and 14.3 g/L of xylitol, and yeast cells grown in GM-based media produced 10.7 g/L in 20h and 13.4 g/L, respectively (Figure 4.2b). No clear difference in xylitol production was observed between cells grown in different substrate sources, indicating that non-supplemented GM is a suitable substrate to be used for cell propagation and could be implemented as a renewable raw material.

In yeast production plants, the utilization of by-products to produce biomass has already been adopted. Molasses, derived from beet or cane processing, is the substrate of choice for biomass production at a reduced cost. However, molasses need to be supplemented with essential elements for yeast growth, such as nitrogen, magnesium, phosphate, and vitamins. In addition, the employment of molasses in a variety of different industrial uses, such as bioethanol and lipids production (59–61) has also contributed to increasing the price of the commodity, which promotes the search for novel substrates for yeast biomass propagation (56, 62). Considering this, a possible outlet for surplus grape products is the use of GM as a replacement for molasses to yield yeast biomass in future integrated biorefineries that need to be able to expand and continuously diversify their feedstocks. As 50% of GM allows the highest biomass yield while maintaining cell function, 50% was chosen as the optimum must concentration for yeast propagation experiments.

4.3.3. Vine Shoots (VS) for ethanol and xylitol production: VS pre-treatment

Hydrothermal processing, also known as autohydrolysis, is commonly used for the solubilization of hemicellulose into oligosaccharides as the first step in a biorefinery (63), generating a residual

biomass mostly constituted of cellulose and lignin (64). The severity range of hydrothermal treatment was selected based on the literature (8). The operational conditions and main results obtained (liquid and solid phases compositions) were listed in Table 4.1.

Table 4.1. Chemical composition of solid and liquid fractions resulting from the hydrothermal pre-treatment of vine shoots (VS).

T_{max} (°C) or S_0(-)	210 or 3.70	215 or 3.89	220 or 3.99	215 or 3.89	
LSR (g/g)	6	6	6	4	
Solid yield (g/100 g of VS)	60.2	58.5	60.2	59.8	
a) Solid fraction (g of component/100 g of pretreated VS)					
Glucan	33.3 ± 0.01	39.7 ± 0.05	40.6 ± 0.07	37.4 ± 0.18	
Xylan	8.13 ± 0.10	6.89 ± 0.06	4.89 ± 0.01	9.56 ± 0.12	
Acetyl groups	1.19 ± 0.02	0.91 ± 0.01	0.78 ± 0.00	1.89 ± 0.02	
Klason Lignin	45.0 ± 0.90	43.4 ± 1.06	47.3 ± 0.04	43.9 ± 1.21	
b) Liquid fraction (g/L)					
					C.H.
Glucose	0.81 ± 0.04	0.76 ± 0.05	1.0 ± 0.02	0.84	2.21
Xylose	1.35 ± 0.01	2.21 ± 0.04	2.64 ± 0.01	1.60	3.55
Acetic acid	1.98 ± 0.08	2.76 ± 0.11	3.87 ± 0.18	0.64	1.02
Hydroxymethylfurfural (HMF)	0.34 ± 0.00	0.52 ± 0.01	0.81 ± 0.00	n.d.	0.19
Furfural (F)	0.39 ± 0.01	0.96 ± 0.00	1.85 ± 0.02	n.d.	0.17
Glucooligosaccharides (GOS)	16.82 ± 0.3	16.13 ± 0.5	13.51 ± 0.22	26.40	58.81
Xylooligosaccharides (XOS)	15.41 ± 0.3	15.56 ±0.6	12.81 ± 0.1	25.07	57.15
Acetyl groups	4.15 ± 0.02	4.04 ± 0.01	3.59 ± 0.02	6.29	13.66

S_0: Severity

LSR: liquid-solid ratio

C.H.: concentrated hydrolysate, composition after evaporation

n.d.: not detected

According to the xylan content in the raw material, xylan was the highest solubilized polymer, with up to 78.5 % of solubilization. The recovery of glucan after pretreatment ranged from 62.6 to 76.3 g glucan/100 g glucan in raw material. These glucan recovery values were lower than those of other hardwoods (such as Eucalyptus and Paulownia woods subjected to autohydrolysis pretreatment) (64, 65). This indicates that some glucan was solubilized as glucooligosaccharides, as verified by chemical analysis of the liquid fraction (Table 4.1). As a result, hydrothermal treatment conditions were favorable for obtaining a hemicellulosic hydrolysate enriched in oligosaccharides (26.3-32.2 g/L, calculated as the sum of glucooligosaccharide,

xylooligosaccharide, and acetyl groups). The maximal concentration of potential xylose (17.7 g/L, calculated as the sum of xylose and xylooligosaccharides) was achieved at 215 °C (S_0=3.89). The temperature increase to 220 °C led to the reduction of oligosaccharides in the hydrolysate and an increase in sugar degradation compounds, such as furfural and hydroxymethylfurfural (HMF). Among the tested conditions, the hydrolysate obtained at 215 °C is more suitable for the production of xylitol. Furthermore, it is important to consider the glucose, in the form of glucooligosaccharides, that may be derived from hemicellulosic hydrolysate since the engineered strain requires a co-substrate for the xylose to xylitol conversion process (39, 40).

4.3.3.1. Ethanol production from pretreated VS

The cellulolytic *S. cerevisiae* CAT-1-C strain, displaying the *Aspergillus aculeatus* β-glucosidase 1 (BGL1), *Trichoderma reesei* endoglucanase II (EGII), *Talaromyces emersonii* cellobiohydrolase I (CBH1) and *Chrysosporium lucknowense* cellobiohydrolase II (CBH2) on the cell surface (37), was evaluated for ethanol production in an SSF assay using pretreated VS as substrate. To achieve high ethanol yields, SSF experiments with the recombinant strain CAT-1-C requires supplementation with Cellic Ctec2 at low concentration of 6 FPU/g of solids (37).

Previous research on ethanol production from hydrothermally treated vine shoots produced 19.1 g/L (from a solid obtained at S_0 of 4.60, using 16% of solids and 15 FPU/g) (66) and 13.3 g/L (S_0=4.65, using 10% of solids and 20 FPU/g) (67). Considering the hydrolytic capability of the recombinant strain, here a small dosage of commercial Cellic Ctec2 (6 FPU/g of solids) was added to the SSF experiments.

Hydrothermal treatment of hardwood biomass (such as eucalyptus, paulownia or vine shoots) is remarkable for its high solubilization selectivity for hemicelluloses. Nonetheless, the appropriate conditions for XOS production are insufficient for high cellulose-to-glucose conversion. To achieve high glucose concentrations and, subsequently, high ethanol yields from hardwood, a delignification process is usually applied to increase the glucan content and improve the enzymatic convertibility of glucan into glucose (68, 69). As an alternative to this costly and hazardous delignification process, GM-derived sugars may be used to boost the sugar concentration and, consequently, final ethanol concentration. This would follow the same line of thought as cheese whey incorporation in lignocellulosic-to-ethanol valorization processes (37, 70, 71), with the advantage of being composed of sugars that are readily consumed by the yeast. Thus, to examine the effect of GM and WL incorporation in the SSF for ethanol fermentation, different mixture

combinations of 15% of pretreated VS with grape must, wine lees, and grape must plus wine lees were evaluated (Figure 4.3).

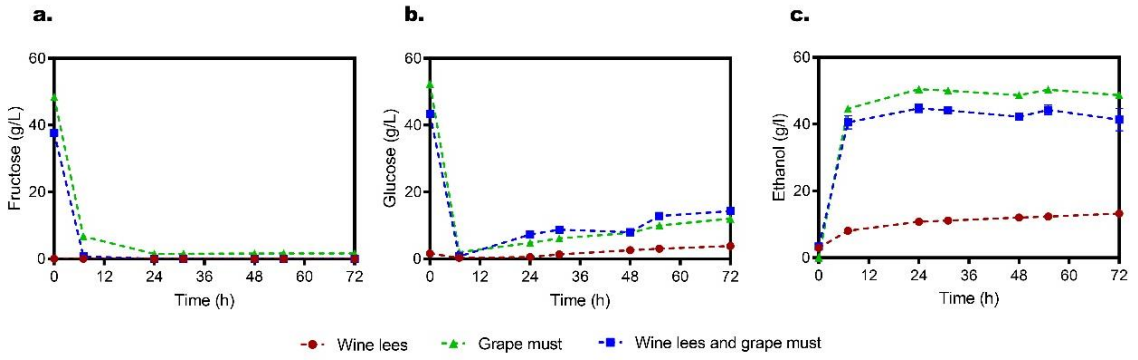

Figure 4.3. Simultaneous saccharification and fermentation of vine shoots solid residue (15%) supplemented with wine lees (WL) (●), wine lees (WL) and grape must (GM) (■), and grape must (GM) (▲) by the CAT-1-C strain. Profiles of (a) fructose, (b) glucose and (c) ethanol concentrations. When the error bar is not shown, it is smaller than the corresponding data symbol.

The addition of GM, as well as GM in combination with WL, showed similar results. As expected, glucose and fructose from GM were consumed in less than 8h in both experiments (Figures 4.3a and 3b). The glucose accumulation after 24h of SSF (Figure 4.3b) indicates a loss of glucose uptake by the yeast cells, probably due to loss of fermentative capacity, limiting further ethanol production. A maximal concentration of 50.5 g/L of ethanol was obtained when GM has integrated alone with pretreated VS in the SSF process (corresponding to an ethanol yield of 0.32 g/g). The incorporation of WL together with GM also yielded more than 4% w/w, the critical threshold for the economic feasibility of the distillation process (72). However, the additional supplementation with WL does not significantly improve the fermentation performance. Compared to the SSF of VS supplemented with WL, which yielded 10 g/L of ethanol (0.16 g/g), the integration of GM led to a 3.7-fold increase in ethanol in SSF supplemented with GM and WL, and a 4.9-fold increase in SSF supplemented with GM.

4.3.3.2. Xylitol production from VS hydrolysate

- **Enzymatic saccharification and fermentation of Vine Shoots hydrolysates**

Hydrothermal treatment yielded a liquid fraction enriched in glucooligosaccharides (GOS) and xylooligosaccharides (XOS), which may be broken down into fermentable sugars by the action of enzymes. Enzymatic hydrolysis of these oligosaccharides offers multiple advantages over acid

hydrolysis, including softer conditions, fewer inhibiting chemicals produced, and no neutralization before fermentation (73). Moreover, enzymatic approaches using commercial enzyme preparations have been used to deconstruct hemicellulose-derived oligomers to pure xylose and glucose (40, 73, 74).

Saccharification experiments were performed on VS hydrolysate obtained at S_0 of 3.89. The release of glucose and xylose using a commercial enzymatic cocktail, Cellic Ctec2 (626 U/mL of hemicellulose activity) was monitored over 48 h. This cellulase mixture was selected due to its high hemicellulose activity, tolerance to inhibitory compounds (oligosaccharides and phenolic compounds) and higher thermostability (73). Although the vine shoot hydrolysate contained similar amounts of potential xylose (17.8 g/L) and glucose (16.9 g/L), the hydrolysis of xylooligomers was more effective in all tested conditions (Figure 4.4).

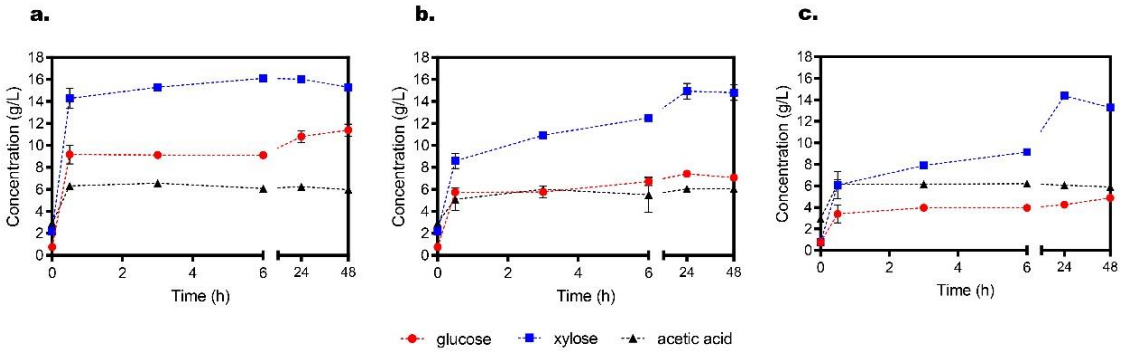

Figure 4.4. Enzymatic saccharification of vine shoots hydrolysate from autohydrolysis at 215 ˚C using the enzyme to substrate ratio (ESR) fixed at (a) 350 U/g, (b) 175 U/g and (c) 88 U/g. Profiles of glucose (●), xylose (■) and acetic acid (▲) concentrations. When the error bar is not shown, it is smaller than the corresponding data symbol.

In addition to xylose and glucose monomers, the hydrolysis of acetyl groups attached to XOS releases acetic acid, an inhibitory product that negatively affects yeast growth (75–77). Using a high ESR (350 U/g of oligomers), the highest xylose yield was achieved after 6 hours of enzymatic saccharification (90%, based on the potential xylose concentration) (Figure 4.4a). It is possible to achieve more than 80% XOS hydrolysis after 48 hours with lower ESR (175 U/g and 88 U/g), but industrial processes benefit from a rapid and efficient conversion. The use of high enzyme loadings clearly affected the glucose production, ESR of 175 and 350 U/g led to a 1.5 and 3.9-fold increase in glucose concentration after 24 hours of saccharification, respectively.

As above mentioned, the genome-engineered PE-2-GRE3-XII5 yeast strain is a non-xylose-consuming organism and therefore needs to be supplied with a carbon source. Considering this,

the VS hydrolysate attained at S_0 of 3.89 and saccharified using 350 U/g of enzyme is an appropriate renewable substrate for xylitol production since it can provide xylose for bioconversion as well as glucose for cell growth. Figure 4.5 shows the evaluation of VS hydrolysate for xylitol production by the novel genome-engineered PE-2-GRE3-XII5 strain.

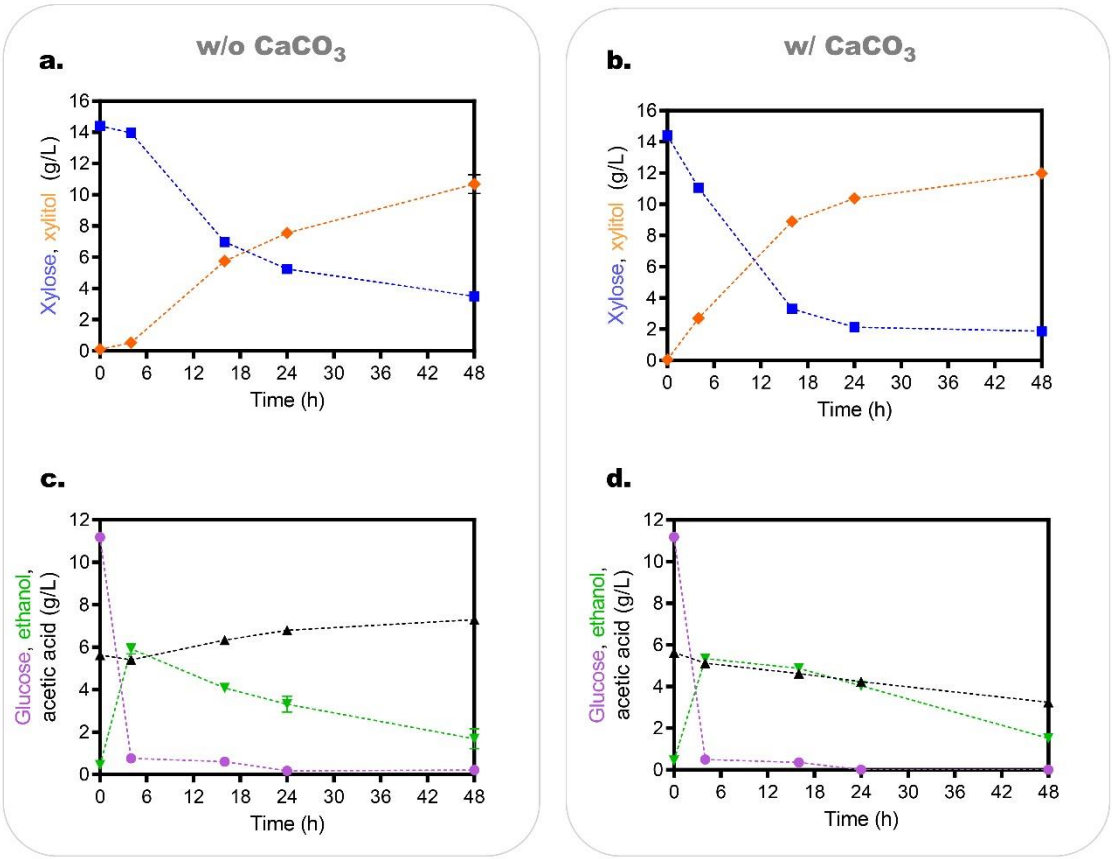

Figure 4.5. Time course of xylose (■) and xylitol (◆) (**a,b**), and glucose (●), ethanol (▼) and acetic acid (▲) (**c,d**) concentrations in the enzymatically hydrolysed vine shoots hydrolysate with (**a,c**) and without (**b,d**) $CaCO_3$ for the novel genome-engineered PE-2-GRE3-XII5 strain. Each data point represents the average ± standard deviation of biological duplicates. When the error bar is not shown, it is smaller than the corresponding data symbol.

Despite the initial concentration of 5.6 g/L acetic acid in the medium (Figure 4.5a), the strain could convert 75% of xylose to xylitol with a corresponding yield of 0.98 g/g. Acetic acid is the main inhibitor found in VS hydrolysate. Previous research has shown that acetic acid may completely inhibit cell growth of xylose-consuming yeasts, including *Candida guilliermondii*, *C. tropicalis* and *C. boidinii* (78–80). The yeast *S. cerevisiae*, on the other hand, has an innate metabolic ability to overcome acetic acid-mediated inhibition of lignocellulosic hydrolysate fermentation (81). A recent study developed a biological treatment with *S. cerevisiae* for detoxifying acetic acid produced during the processing of lignocellulosic olive stone in order to increase the productivity of *C. boidinii*, which

was employed to convert lignocellulosic-derived xylose to xylitol (80). In addition, robust *S. cerevisiae* isolates, such as the chassis strain used in this work, have shown increased tolerance to inhibitors (35, 45). Still, robust yeast strains were shown to differentially express tolerance genes (for instance, *PRS3*, *RPB4* and *ZWF1*) in response to different lignocellulosic inhibitor loads (82). In spite of the robustness of the yeast strain used as host strain, the acetic acid produced during the enzymatic hydrolysis of corn cob hydrolysate, if higher than 4.3 g/L, reduced xylitol productivity (40). Taking into account our previous findings, as well as the fact that yeast cells can adapt to weak acids in a glucose-containing medium with a pH of 4.5 and grow after a lag phase (83), the addition of calcium-carbonate ($CaCO_3$) in the hydrolysate to buffer the medium was also investigated.

As observed in Figure 4.5b, the recombinant strain when cultured in media supplemented with $CaCO_3$ was able to convert almost all the sugar in 24 hours, producing 12 g/L of xylitol at a yield of 0.96 g/g. This methodology led to an increase in xylitol productivity (maximal 0.56 g/(L·h)), a 1.5-fold improvement over cultures without $CaCO_3$ supplementation.

In contrast to the accumulation of acetic acid observed in the medium without $CaCO_3$ (Figure 4.5c), it was possible to observe a slight consumption of acetate during cultivation with $CaCO_3$ (Figure 4.5d). In fact, it has been suggested that the toxic effects of acetic acid accumulated in the culture medium have a role in the process of chronological ageing in yeast (84), and buffering the medium can extend the chronological life span (85). Related results were obtained in lactic acid fermentation of brewer's spent grain hydrolysate, where $CaCO_3$ addition increased volumetric productivity using *Lactobacillus rhamnosus* (86).

- **Experimental design for xylitol production from vine shoots hydrolysate by SSF**

Considering the results obtained from hydrothermal treatment, an additional experiment at 215 °C reducing the LSR to 4 g/g was carried out to increase the potential xylose (xylooligosaccharides and xylose) for further conversion into xylitol (Table 4.1). The hemicellulosic hydrolysate was enriched up to 26.7 g/L of potential xylose and 27.3 g/L of potential glucose, resulting in a 1.6-fold increase in sugar concentration when compared to the hydrolysate generated at LSR of 6 g/g. Acetic acid and acetyl groups were also increased up to 6.3 g/L. To increase the sugar content and remove volatile components, vacuum evaporation was applied to VS hydrolysate, as previously proposed by Dominguez and co-workers (2021) for *Paulownia* wood-derived hydrolysate. Following

processing, the concentrated hydrolysate contained 58.8 g/L of GOS, 57.2 g/L of XOS, and 13.7 g/L of acetyl groups (Table 4.1).

To assess the capacity of the novel genome-engineered PE-2-GRE3-XII5 strain to produce xylitol from a non-supplemented and concentrated VS hydrolysate in an SSF process, a complete factorial design was proposed to study the variables: i) inoculum size and ii) enzyme loading. The concentrated VS hydrolysate (66%), corresponding to 40 g/L of potential xylose, was used as fermentation media. The experimental matrix and main results obtained (xylitol concentration and xylitol yield) are listed in Table 4.2.

Table 4.2. Operational conditions of Simultaneous Saccharification of non-supplemented and concentrated Vine Shoots hydrolysate, and results obtained for dependent variables y_1 to y_3. Regression coefficients, values and significance (based on a t-test).

Run	x_1	x_2	Inoculum (g of wet yeast/L)	Enzyme loading (U/g substrate)	Final xylitol concentration (g/L) or y_1	Productivity (g/(L·h)) or y_2	Xylitol yield (g/g) or y_3
1	-1	-1	30	159	18.2	0.13	0.300
2	0	-1	65	159	16.9	0.12	0.279
3	1	-1	100	159	15.6	0.11	0.257
4	-1	0	30	318	18.5	0.13	0.305
5	0	0	65	318	21.0	0.15	0.347
6	0	0	65	318	21.0	0.15	0.346
7	0	0	65	318	21.9	0.15	0.361
8	1	0	100	318	22.5	0.16	0.370
9	-1	1	30	477	19.7	0.14	0.324
10	0	1	65	477	24.1	0.17	0.396
11	1	1	100	477	24.3	0.17	0.400
coefficient			Xylitol concentration at 144 h		Xylitol productivity		Xylitol yield
b_{0j}			21.363		0.1505		0.3519
b_{1j}			0.995[c]		0.0067[c]		0.0164[c]
b_{2j}			2.868[a]		0.02[b]		0.0473[a]
b_{12j}			1.8025[b]		0.0125[a]		0.0297[b]
b_{11j}			-0.9428		-0.0063		-0.0155
b_{22j}			-0.9428		-0.0063		-0.0155
R^2			0.937		0.925		0.937
F_{exp}			14.97		12.42		14.97
Significance level (%)			>99		>9		>99

[a]Coefficients significant at the 99% [b] 95% and [c]90% confidence level.

During the enzymatic saccharification of VS hydrolysate, the hydrolysis of acetyl groups linked to XOS released quantities of acetic acid ranging from 24.8 to 30.1 g/L (Appendix: Figure A4.3). High enzyme loading (runs 10 and 11) and a large inoculum size produced the highest xylitol concentrations (24.1 and 24.3 g/L). This supports the already reported correlation between high initial cell biomass and improved tolerance to the stress induced by hydrolysate-derived inhibitors (87, 88). Since experimental design is a useful tool for determining the influence of independent

variables on dependent variables as well as optimizing conditions, a correlation analysis was performed on the experimental variables using equation 3. The regression coefficients, model significance, and statistical significance for xylitol concentration, productivity, and yield are included in Table 4.2. As seen, linear terms for variable x_1 (inoculum size) and x_2 (hemicellulose loading-ESR) and the combination of inoculum size and hemicellulase loading were significant at 90, 99, and 95% of confidence levels. Both independent variables studied had a positive effect on xylitol production, yield, and productivity. Despite the positive results, the xylitol yields and productivities achieved in these experiments (Table 4.2) are far from the theoretical, probably due to the loss of fermentative capacity by the recombinant yeast. To test whether nutritional supplementation could support yeast physiology maintenance, the concentrated VS hydrolysate was supplemented with commercial peptone (20 g/L) and yeast extract (10 g/L). Additionally, the addition of WL as a low-cost nitrogen source was also evaluated (Figure 4.6).

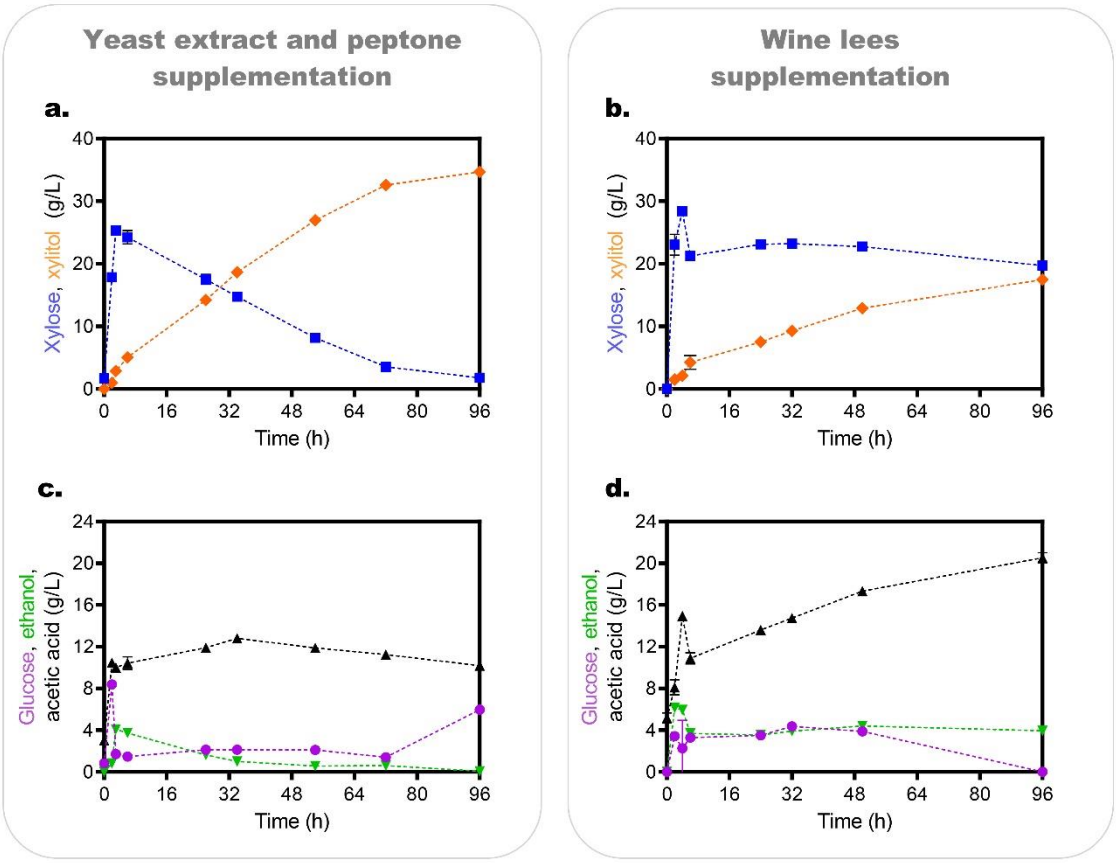

Figure 4.6. Simultaneous saccharification and fermentation of concentrated vine shoots hydrolysate under optimal conditions (100 g of wet yeast/L of inoculum and 170 U of Cellic Ctec2) supplemented with (a) yeast with commercial yeast extract and peptone and (b) wine lees. Profiles of xylose (■) and xylitol (◆), glucose (●), acetic acid (▲) and ethanol (▼) concentrations. When the error bar is not shown, it is smaller than the corresponding data symbol.

As can be observed, the addition of commercial nutrients (YP supplementation) permitted a faster and nearly complete conversion of xylose to xylitol, producing 37 g/L of xylitol (0.93 g/g of yield) (Figure 4.6a). In contrast, WL supplementation had no positive influence on yeast performance, resulting in a similar production to those obtained without nutritional supplementation (run 11, Table 4.2). It should be noted that the release of acetic acid during the SSF supplemented with WL reached 20.5 g/L. (Figure 4.6d). Since the maximal acetic acid concentration that could be formed during VS saccharification is 14.7 g/L, the extra acetic acid appears as a by-product of yeast metabolism. The slower rate of conversion and lower xylitol production may have resulted from the release of this carboxylic acid, which was previously related to longer lag phases (40). According to another study, the presence of components such as yeast extract provides some protection against the adverse stress effects of acetic and lactic acids (89). However, no systematic investigation has been conducted to determine if and which complex-medium components may protect yeast from these inhibitory effects.
Apart from being often used for the fermentation of cellulosic ethanol, SSF may be efficiently applied for xylitol production. Other studies that should not be compared to this study have reported the application of this process configuration for the valorization of corn cob (39, 40, 90, 91) and corn stover (92).

4.3.4. Integrated Biorefinery from wine waste

Winery leftovers account for 20% of total wine production (93, 94). The high organic content of these wastes not only implies ecological concerns if discharged without further treatment but also provides a reservoir of carbon sources for chemicals and energy production. In comparison to the first-generation bioethanol industry, their integration into a second-generation biorefinery model would reduce the environmental impact of the wine industry through cost-effective waste valorization (95, 96). Furthermore, multi-product biorefineries are being promoted (32) rather than traditional biorefineries that focus on the production of a single product (32, 97). Therefore, the selection of the most appropriate high-value products to complement the production of low-value biofuel in a biorefinery is crucial for achieving high profitability. The sugar-alcohol xylitol is included in the US Department of Energy list of high-value compounds that can be produced from biorefinery carbohydrates (98) with a current market price of 7.95 €/kg (99). A recent techno-economic analysis of a lignocellulosic biorefinery for the production of xylitol coupled with second-generation ethanol revealed that integrating the biorefinery is more cost-effective than producing ethanol alone

in terms of overall investment cost (100). Overall, the biorefining perspective proposed in this study allowed to obtain 37 g/L of xylitol and 50 g/L of ethanol in two separated streams through a valorization of multiple types of waste from the wine industry (Figure 4.1). These results can be positively compared to those obtained from using winemaking wastes to produce one single product. In fact, the ethanol concentrations attained with this approach are higher than those previously reported for the fermentation of autoclaved grape must (10 g/L) (101) and hydrothermally treated and delignified vine shoots (20 g/L) (102). Furthermore, the xylitol titer is similar to the one previously reported from the sequential fermentation of grape shoots by *Lactobacillus acidophilus* and *Debaryomyces hansenii*, which yielded 31.3 g/L of xylitol but with a previous detoxification step (103). Annually, the required trimming of grape vines creates around 5 tonnes per hectare of grape cultivation (104). Taking into account the results hereby obtained, besides ethanol approximately 67 kg of xylitol could be produced from 1 ton of vine shoots, signifying a worthy co-valorization route. Other strategies using olive pruning (105), olive stones (106), and corn cob (107) for co-producing xylitol and bioethanol have been recently developed, but winery wastes have not been evaluated before. Considering the environmentally friendly methodologies employed, which did not include harmful chemicals addition, as well as the superior concentrations achieved, the results obtained here can be compared favorably to those that have already been published in the literature.

4.4. Conclusions

This work provides an integrated novel biorefinery concept which aims at valorizing winery wastes. The utilization of grape must, produced in excess, showed to be feasible for yeast propagation and may lower overall process costs. To maximize the conversion of all polysaccharides in vine shoots lignocellulosic biomass, hemicellulosic and cellulosic fractions resulting from vine shoots hydrothermal processing were converted into xylitol and ethanol, respectively. In the hemicellulosic-to-xylitol conversion process, a robust industrial isolate was genome-engineered for enhanced xylitol production and the experimental design demonstrated a significant effect of enzyme loading and inoculum size on the production process. Moreover, nutrient supplementation was shown to further overcome acetic acid inhibition. In the cellulosic-to-ethanol conversion process, the combination of vine shoots with surplus grape must enabled a sustainable production of ethanol, with an economically feasible distillation process. Furthermore, by applying a robust yeast chassis with cell surface display of cellulases, the enzyme added to the

process was reduced. Still, the xylitol and ethanol titers attained in two different streams, 37 and 50 g/L, respectively, compare quite favorably with single-product wine wastes valorization processes. Collectively, this strategy establishes the solid basis for the implementation of an integrated biorefinery by expanding the scope of winery multi-waste effective utilization.

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Chapter V.

Arabitol production from sugar beet pulp: recombinant industrial *Saccharomyces cerevisiae* as whole cell biocatalysts

This chapter is based on the following original research article:

Baptista SL, Soares PO, Romaní A, Domingues L. Arabitol production from sugar beet pulp: recombinant industrial *Saccharomyces cerevisiae* as whole cell biocatalysts. (*Submitted*).

Abstract

Arabitol is a polyol that holds great promise in the food sector as a low-calorie sweetener. The current chemical methods for arabitol production are expensive and harmful to the environment, and the biological approaches utilizing native arabitol-producing yeasts generate a variety of by-products and low arabitol yields.

The *Saccharomyces cerevisiae GRE3* gene encodes an NADPH-dependent aldose reductase that converts aldoses to their respective alcohols. Taking advantage of the broad substrate specificity of this enzyme, here we demonstrate the feasibility of using engineered industrial yeast strains overexpressing the *GRE3* gene for the single-step conversion of arabinose to arabitol. The resultant strains were able to simultaneously convert xylose and arabinose to their corresponding sugar alcohols, xylitol and arabitol. The best-performing strain was further modified to improve its arabinose transport ability, enhancing the arabinose to arabitol conversion yield and productivity. Lastly, this strain was utilized to produce arabitol from arabinose-rich sugar-beet pulp non-detoxified hydrolysate, achieving an arabitol titer of 15 g/L with a yield of 1 g/g. This strategy enabled the simultaneous production of sugar alcohols, advancing the development of a yeast production platform capable of converting bulk sugars from agro-food wastes.

Keywords: Arabitol, xylitol, *Saccharomyces cerevisiae*, sugar beet-pulp, CRISPR/Cas9

5.1. Introduction

Arabitol is a five-carbon polyol with potential applications in the food and pharmaceutical industries (1, 2). Polyols, or sugar alcohols, are low-calorie sweeteners derived from the hydrogenation of their sugar source that may be employed as additives to enhance the flavor and texture of foods and drinks (3). Among polyols, arabitol has a very low glycemic response and the lowest caloric content (0.2 kcal/g vs. 4 kcal/g sucrose), providing 70% of the sweetness of sucrose (4). It is a stereoisomer of xylitol, a well-known and fully established sugar alternative with beneficial anti-cariogenic characteristics (5–7). Similarly, arabitol exhibits a similar inhibition effect on caries-associated oral bacteria (8). Due to the growing popularity of sugar-free and low-calorie foods and beverages, as well as the desire of modern consumers to maintain a healthy weight, the market for sugar substitutes is expanding. As a result, there is an undeniable demand for additional products, such as arabitol. In addition, arabitol and xylitol are two of the twelve building blocks that can be produced from renewable sugars and then converted into new, useful chemicals such as arabinoic and xylonic acids, propylene, and ethylene glycol (9, 10).

Arabitol can be chemically produced in a two-step process involving the oxidative decarboxylation of glucose to arabinonic acid and the corresponding γ-lactone and δ-lactone, followed by the catalytic hydrogenation (11) or by hydrogenation of arabinose using Ni or Ru-based catalysts (12, 13). Since industrial-scale chemical synthesis involves costly and hazardous catalysts, high energy needs, and separation stages to extract H_2 gas from an aqueous solution of arabitol, there is a rising interest in the microbiological production of this sweetener (2, 14).

Several yeast species are naturally capable to produce arabitol from glucose (14–16), and glycerol (17–19). However, poor production yields and substantial by-product formation (ethanol and/or glycerol) are barriers to using these yeasts to synthesize arabitol. On the other hand, arabitol can be synthesized from arabinose-fermenting yeasts (20–30). It is a common by-product of the arabinose-utilization pathway, resulting mostly from a co-factor imbalance between the conversion of arabinose to arabitol by aldose reductase (AR) and the subsequent conversion of arabitol to xylulose by arabitol dehydrogenase (LAD). Due to the strong affinity of AR for NADPH, less NADH is recycled in this reaction, resulting in an insufficient supply of NAD+ for the NAD+-dependent LAD activity and an accumulation of arabitol (31). Although native arabinose fermenting yeasts have been considered for sugar-alcohol synthesis, it is difficult to obtain high arabitol yields since the arabitol production by these yeasts is coupled with cell growth (Table 5.1).

Table 5.1. Comparison of native arabitol-producing yeasts for arabinose conversion, arabitol titer, and yield.

Strain	Arabinose conversion (%)	Arabitol (g/L)	$Y_{arabinose/\ arabitol}$ (g/g)	Reference
Debaryomyces nepalensis	78	22.7	0.26	(30)
Candida sp. NY7122	99.5	10.7	0.53	(29)
Debaryomyces hansenii	100	2.86	0.10	(28)
Candida auringiensis NRRL Y-11848	100	73	0.73	(27)
Candida succiphila Y-1199	100	81	0.81	(27)
Pichia guilliermondii NRRL Y-2075	100	35.5	0.71	(26)
Candida entomaea NRRL Y-7785	100	35	0.70	(26)
Candida parapsilosis DSM 70125	100	17.9	0.55	(25)
Pichia manchurica	100	12.8	0.10	(24)

The yeast *Saccharomyces cerevisiae* is a workhorse for the large-scale production of first- and second-generation bioethanol (32), as well as for the synthesis of a variety of valuable chemicals from various carbon sources, including lignocellulosic materials (33). The GRAS classification of this yeast and its widespread use in the food industry are important factors that improve its potential for arabitol production. *S. cerevisiae* strains engineered to ferment arabinose (34, 35) and both xylose and arabinose (36, 37) to ethanol produced arabitol as a by-product. However, the polyol production was still low. Considering that *S. cerevisiae* produces an unspecific AR highly, encoded by the *GRE3* gene (38), which can reduce xylose and arabinose to xylitol and arabitol (39), we devised a strategy based on the direct conversion of arabinose into arabitol through the overexpression of this native gene. Additionally, the galactose permease (Gal2p) coding gene (*GAL2*) was overexpressed to enhance the production of arabitol. In addition, we demonstrate the feasibility of converting arabinose, in sugar beet pulp hydrolysate to arabitol. This is the first demonstration of the production of arabitol from lignocellulosic waste using *S. cerevisiae* as a robust and adaptable host.

5.2. Materials and Methods

5.2.1. Strains and media

Escherichia coli DH5/NZY5 (Nzytech, Portugal) was used for plasmid construction and propagation. *E. coli* cells were cultivated at 37 °C in Lysogeny Broth medium (0.5% yeast extract, 1% tryptone, 1% NaCl, pH 7.0) and 100 μg/mL ampicillin was supplied for transformant selection when required. Yeast strains were propagated at 30 °C and maintained at 4 °C on YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar). For plasmid-carrying yeast strains, YPD media was supplemented with 200 ug/mL of geneticin G418.

5.2.2. Plasmids and strains construction

All strain, plasmids and primers used are listed in Appendix: Table A5.1 lists strains and plasmids, whereas Table A5.2 contains a list of primers. Yeast strains were transformed using the lithium acetate technique (40). The recombinant strains were confirmed by colony PCR. The genomic integration of *GRE3* into the industrial strains (PE-2, CAT-1 and CA11) was achieved via CRISPR–Cas9-based genome editing. The integrative plasmid (p2909 TEF-1 GRE3) was created by introducing the *GRE3* gene from pGRE3 (41) into pCfB2909 (42) using USER cloning method (43). The correct cloning was verified using Sanger sequencing by Eurofins Genomics. Before further modifications, yeast strains were transformed using a Cas9-expressing plasmid. Following that, the guide RNA plasmid (pCFB3050 targeting for XXI-5 integration site) was transformed into the Cas9-expressing strains together with the constructed integrative vector (p2909 TEF-1 GRE3) generating the newly engineered strains (Table A5.1). For the construction of *S. cerevisiae* PE-2-GRE3-XII5 strain expressing the Gal2p transporter, the *GRE3* gene of the pGRE3 vector was replaced by the *GAL2* gene, amplified from genomic DNA of CENPK.113-7D. Plasmid assembling was performed with the In-Fusion HD Cloning Kit (Clontech, Mountain View, USA).

5.2.3. Sugar Beet Pulp characterization and processing

Sugar beet pulp (SBP), kindly provided by Azucarera Ebro (local pulp factory, Spain), was the raw material employed in this work. SBP was chemically analyzed followed the procedures described elsewhere (49). Quantitative hydrolysis with 72% sulfuric acid was performed in triplicate to determine polymers content (expressed per 100 g of SBP): 25.99 ± 0.49 g of galacturanan, 26.18 ± 0.53 g of glucan, 17.47 ± 0.53 g of arabinan, 7.67 g ± 0.39 of galactan, 1.27 ± 0.03 g

of rhamnosyl moieties, 3.79 ± 0.2 g of acetyl groups and 4.21 ± 0.7 of acid-insoluble soluble residue. To obtain the hydrolysate enriched in arabinose, SBP was submitted to hydrothermal treatment using a liquid-to-solid ratio of 4 kg/kg oven-dry basis in a 1.6 L pressurized reactor (Büchiglasuster versoclave, Switzerland) equipped with temperature control, external fabric mantel as heater and internal water flow for cooling purposes. Hydrothermal treatment was carried out under non-isothermal conditions, heated until 170 °C (corresponding to the severity of 2.77). After that, SBP hydrolysate was separated from pretreated biomass by centrifugation. SBP hydrolysate was acid hydrolyzed (0.5% H_2SO_4 at 120 °C for 165 min) and analyzed by HPLC after neutralization for the determination of its chemical composition, containing 3.25 g/L of xylose, 20.17 g/L of arabinose, 0.96 g/L of formic acid, 2.41 g/L of acetic acid, 0.15 g/L of hydroxymethylfurfural (HMF) and 0.19 g/L of furfural.

5.2.4. Enzymatic activities determination

Cells were collected for enzyme assays after 24 hours of fermentation in YPD medium at 30 °C. Y-PER reagent (Thermo Fisher Scientific) was used to prepare crude cells extracts. The protein content of crude cell extracts was measured using the Bradford assay (Bio-Rad) (REF). AR enzymatic activities were measured (in triplicate) in each cell extract by detecting the decrease of NADPH in a reaction mixture at 30 °C using a microplate reader spectrophotometer, at 340 nm. The reaction compositions (including adequate dilutions of cell crude extract) were adapted from previously published experiments and included the following components: triethanolamine (100 mM, pH 7.0), NADPH (0.2 mM), and xylose or arabinose (350 mM) for AR (REF). Specific activity is defined in units per milligram of protein (U/mg protein), where one unit (U) represents one micromol NADPH reduced or oxidized per minute.

5.2.5. Culture conditions

Yeast cells for inoculation were cultivated on overnight at 30 °C and 200 rpm in Erlenmeyer flasks filled with YPD medium to 30% of their entire capacity. To reach a final concentration of 300 g of fresh yeast per liter of culture, the cell suspension was centrifuged for 5 minutes at 3000 rpm, 4 °C, and suspended in 0.9% (w/v) sodium chloride solution. The cultivation assays were performed using a concentrated cell suspensions of 10 and 70 g fresh yeast/L.

The engineered strains were grown in Sugar beet pulp (SBP) hydrolysate and FIT (Feed-In-Time) medium based on EnPump200 (Enpresso GmbH, Germany) to simulate fed-batch cultivations with

continuous glucose release. YPD media containing arabinose or arabinose and xylose were supplemented with a polysaccharide substrate and an enzyme mix (Reagent A, Enspresso GmbH, Germany) according to the instructions supplied with the product.

Bioreactor cultivation was performed in YP medium (2% peptone, 1% yeast extract) with 30 g/L of glucose and 30 g/L of arabinose. The experiment was conducted in a 3.7 L Bioengineering's RALF bioreactor (working volume 2 L) at 30 °C, 400 rpm and 2 vvm aeration rate (Lee et al., 2000; Oh et al., 2013). To avoid evaporation, the bioreactor was equipped with a condenser cooled with water. The pH value was automatically adjusted by the addition of 5M solutions of NaOH or HCl. After the initial glucose in the medium was completely exhausted, a 300 g/L glucose stock solution was supplied continuously at a rate of 4.8 mL/h.

5.2.6. HPLC analysis

Samples from pretreatment of sugar beet pulp and culture experiments were analysed for the concentrations of glucose, xylose, arabinose, xylitol, arabitol, and ethanol by HPLC using a Bio-Rad Aminex HPX-87P column (85 °C, H2O, 0.5 mL/min) and a refractive index detector (Agilent).

5.3. Results and Discussion

5.3.1. Conversion of arabinose and xylose into arabitol and xylitol by CENPK.133-5D *S. cerevisiae* strain overproducing aldose reductase

The yeast *S. cerevisiae* cannot ferment arabinose and xylose, however, is able to reduce these pentose sugars to the corresponding sugar alcohols by an unspecific aldose reductase (AR) via an NADPH-dependent process. The *S. cerevisiae* AR, encoded by the *GRE3* gene, is closely related to the xylose reductase from other yeasts but exhibits no activity with NADH. This NADPH-specific enzyme catalyzes the reduction of a variety of aldehydes and has been found to have affinity for both xylose and arabinose (39). The overexpression of the native *GRE3* gene in *S. cerevisiae* strains has proven to be effective in the conversion of xylose to xylitol (41, 50–52).

Considering the structural and metabolic similarity of these pentose sugars, we started our investigation by analyzing the capacity of a laboratory CEN.PK 113-5D-GRE3 strain, previously engineered to overexpress the *GRE3* gene, for the conversion of arabinose into arabitol (41). The recombinant strain was evaluated in arabinose (Figure 5.1a), and arabinose plus xylose (Figure 5.1b) containing media. Both media were supplemented with glucose for cell grow and co-factor regeneration, and therefore arabinose/xylose were exclusively used to produce the target chemical (arabitol and xylitol), resulting in high arabitol/xylitol production yields. An initial concentration of glucose (20 g/L) was supplied to promote the initial growth of cell biomass. To ensure the cell maintenance during the bioconversion, the cultivations were performed in FIT (Feed-In-Time) medium, in which glucose was gradually released from a polysaccharide substrate allowing a small-scale simulation of fed-batch culture.

As shown in Figure 5.1a, glucose was readily consumed and completely exhausted in less than 2 hours. In presence of glucose, arabinose was not taken up by the cells. After glucose depletion, arabinose uptake occurred at a constant rate and arabitol was produced by the recombinant strain. After glucose uptake, the yeast consumed the ethanol produced and no residual ethanol was detected at the end of cultivation (96h). Approximately 51% of available arabinose was taken up by the cells and converted into arabitol (9.6 g/L) (Table 5.2). Our results confirm that after arabinose reduction, arabitol was not further metabolized and there was an accumulation of this sugar-alcohol. This is in accordance with previous findings reporting the very low or null activities of different polyol dehydrogenases of *S. cerevisiae* (53). In comparison to maximal yields obtained with native arabitol-producing yeast *Candida succiphila* which do not exceed 0.81 g/g in optimized production conditions (25 °C with moderate aeration) (27), the recombinant strain demonstrated

a superior capacity to convert arabinose to arabitol with a corresponding yield of 0.98 g/g (Table 5.2), close to the maximum theoretical yield of 1 g/g.

Regarding the evaluation of the recombinant strain in a medium containing arabinose and xylose (Figure 5.1b), glucose and xylose were assimilated simultaneously, and arabinose uptake occurred only after a considerable decrease in glucose concentration. Whereas all available xylose was converted into xylitol, only 50% of arabinose was converted into arabitol. Xylitol and arabitol maximal productivities were 0.27 g/(L·h) and 0.06 g/(L·h), respectively. These different substrate conversion levels suggest an enzyme preference for xylose rather than arabinose. Thus, to confirm the successful overexpression of the endogenous AR and to better understand its role in pentose reduction, the level of activity of this enzyme was verified by measuring the individual enzyme activities using arabinose and xylose as substrates (Figure 5.1c). Consistent with the conversion yields obtained for xylose and arabinose (Figure 5.1b), the AR activity with xylose as substrate was 0.92 ± 0.05 U/mg of total protein, compared with 0.39 ± 0.05/mg of total protein when arabinose was used as substrate.

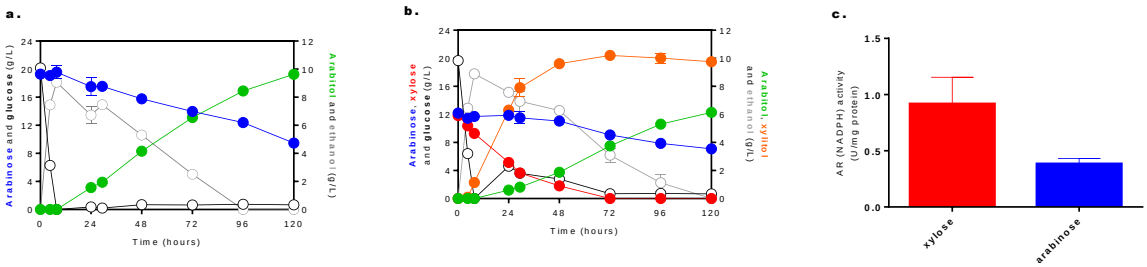

Figure 5.1. Performance of *S. cerevisiae* CENPK.113-5D strain overexpressing *GRE3* gene in medium containing (a) arabinose and (b) arabinose and xylose and enzymatic activities of AR using xylose and arabinose as substrates.

5.3.2. Engineering industrial *S. cerevisiae* strains for arabitol production

A platform strain may prove useful not only by producing a desirable chemical, but also by offering high performance under industrial fermentation conditions (54). Compared to laboratory strains, industrial *S. cerevisiae* strains demonstrated to have intrinsic resistance to severe conditions found in industrial environments, such as high temperatures and the presence of chemicals that hinder fermentation (46, 55, 56). There is also evidence that industrial isolates might respond differently to genetic engineering for pentose metabolism (57), and it is essential to select the appropriate yeast chassis to obtain high product titer, yield, and productivity (58). PE-2, CAT-1, and CA11 strains, isolated from bioethanol plants, were demonstrated to have increased resistance to fermentation inhibitors in lignocellulose hydrolysates (56) and greater robustness to

large-scale stressful fermentation conditions (46). Considering this and taking to account the results obtained with the CENPK.113-5D laboratory strain, we engineered these three industrial *S. cerevisiae* strains to overproduce the endogenous AR by overexpression of the *GRE3* gene.

In comparison with expression from plasmids, chromosomal integration of genes is preferable due to its higher stability and lower cell population variability (59). Moreover, the utilization of antibiotic resistance markers for selecting and maintaining recombinant plasmids in industrial strains is undesirable due to the possibility of drug resistance spread (42). However, single-copy expression from the chromosome can result in lower gene expression levels than the same construct from a multicopy plasmid. Hence, we constructed genome-integrated and plasmid-based strains to compare arabitol production efficiencies (Figure 5.2). The conversion profiles are shown in Figures 5.2a (PE-2-GRE3-XII5 and PE-2-pGRE3), b (CA11-GRE3-XII5 and CA11-pGRE3) and c (CAT-1-GRE3-XII5 and CAT-1-pGRE3).

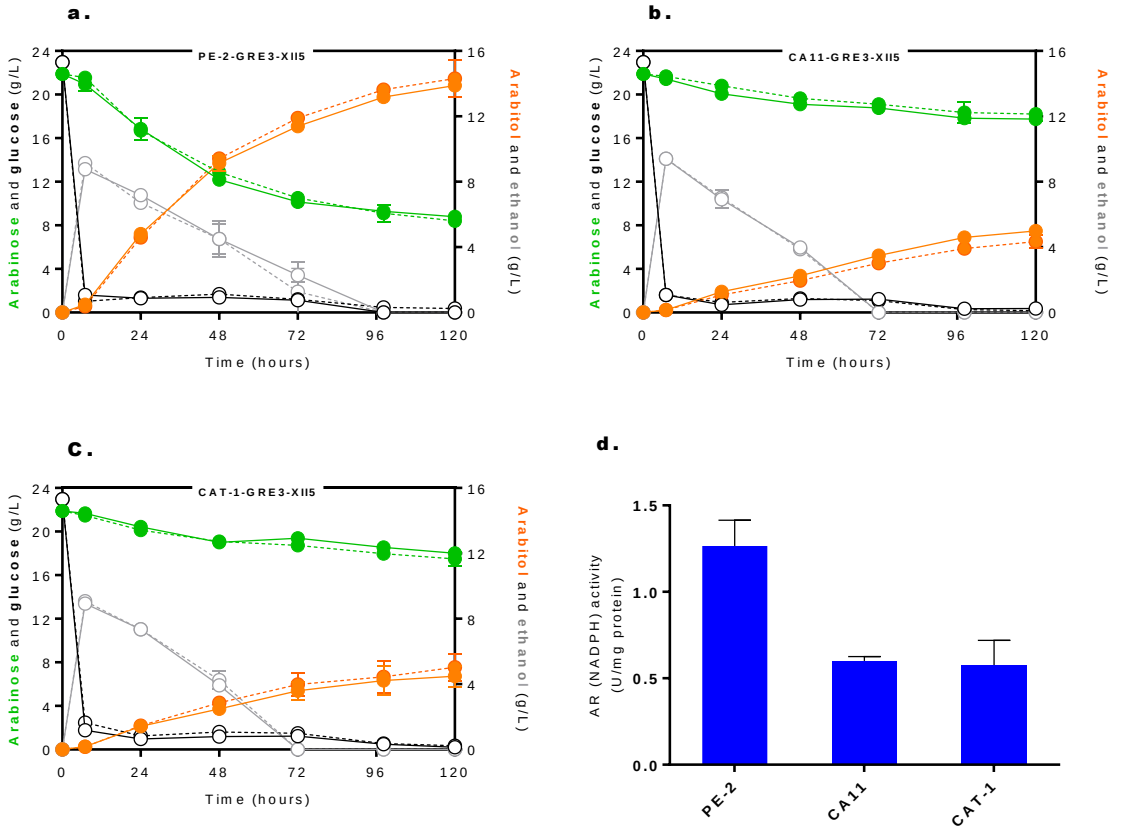

Figure 5.2. Arabitol production after the overexpression of *GRE3* gene in the industrial (a) PE-2, (b) CA11 and (c) CAT-1 strains. Profiles for arabinose, arabitol, glucose and ethanol concentrations by genome-integrated strains (filled lines) and plasmid-based strains (dotted lines). Data are presented as mean value and standard deviations of two independent biological replicates.

Table 5.2. Main results of Feed-In-Time (FIT), bioreactor and batch cultivations of the recombinant *S. cerevisiae* strains in arabinose and glucose synthetic media and Sugar Beet Pulp (SBP) hydrolysate.

Culture/media	Strain	Ara_{t0} (g/L)	Ara_{tf} (g/L)	$Araol_{tf}$ (g/L)	$Y_{Ara/Araol}$ (g/g)	Q_{pmax} (g/L·h)
FIT arabinose + glucose	CENPK.113-5D-GRE3	19.3 ± 0.31	9.46 ± 0.01	9.64 ± 0.01	0.98 ± 0.02	0.09 ± 0.00
	PE-2-pGRE3	22.9 ± 0.00	8.41 ± 0.07	14.3 ± 1.15	1.00 ± 0.09	0.20 ± 0.02
	PE-2-GRE3-XII5	21.9 ± 0.00	8.02 ± 0.02	14.0 ± 0.06	0.94 ± 0.01	0.20 ± 0.00
	CA11-pGRE3	22.9 ± 0.00	17.8 ± 0.02	5.00 ± 0.01	0.97 ± 0.04	0.05 ± 0.00
	CA11-GRE3-XII5	21.9 ± 0.00	17.5 ± 0.66	4.34 ± 0.42	0.94 ± 0.02	0.04 ± 0.01
	CAT-1–pGRE3	22.9 ± 0.00	18.0 ± 0.48	4.48 ± 0.65	0.98 ± 0.03	0.06 ± 0.00
	CAT-1-GRE3-XII5	21.9 ± 0.00	17.0 ± 0.67	4.8 ± 0.30	1.00 ± 0.01	0.06 ± 0.00
Bioreactor arabinose + glucose	PE-2-GRE3-XII5	30.4 ± 0.90	9.68 ± 0.04	20.8 ± 1.02	1.00 ± 0.00	0.31 ± 0.07
FIT Arabinose + glucose (10 g wet cells/L)	PE-2-GRE3-XII5-GAL2	11.4 ± 0.17	0.00 ± 0.00	11.0 ± 0.05	0.96 ± 0.01	0.39 ± 0.02
FIT Arabinose + glucose (70 g wet cells/L)	PE-2-GRE3-XII5-GAL2	11.4 ± 0.39	0.00 ± 0.00	11.3 ± 0.20	0.99 ± 0.01	0.20 ± 0.05
Batch SBP hydrolysate	PE-2-GRE3-XII5-GAL2	15.4 ± 0.82	0.84 ± 0.35	14.5 ± 0.66	1.00 ± 0.01	0.35 ± 0.15

All recombinant strains studied showed the ability to convert arabinose into arabitol as shown in results displayed in Table 5.2 and Figure 5.2. It is noteworthy that chromosomally integrated (filled lines) and plasmid-based (dotted lines) strains exhibited similar conversion profiles, probably due to the constitutive expression of the AR coding gene under the control of the pTDH3 promoter, which is a promoter of the key glycolytic gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase protein) that exhibits high levels of transcription in *S. cerevisiae,* and it is considered strong promoter in literature (60). Yet, the position of gene integration is an additional factor that

significantly affects the level of chromosomal expression (59). Mikkelsen and co-workers (61) identified XII-5 as an integration site that ensures a high level of gene expression, located in intergenic regions that do not inhibits cellular growth.

Although the glucose consumption and ethanol production were similar in all experiments, a clear difference was observed among strains in terms of arabitol production. PE-2-GRE3-XII5 and PE-2-pGRE3 were able to convert 63% of arabinose, producing 14.0 and 14.3 g/L of arabitol, respectively. After 120h of cultivation, CA11 and CAT-1 derived strains converted around 20% of arabinose, with arabitol titters not exceeding 5 g/L (Table 5.2) (Figure 5.2b and c). Accordingly, the PE-2-GRE3-XII5 showed the highest activity of AR enzyme (1.27 ± 0.15/mg total protein) and both CA11-GRE3-XII5 and CAT-1-GRE3-XII5 were found to possess similar levels of arabinose reductase activity (0.60 ± 0.03/mg total protein and 0.58 ± 0.14/mg total protein, respectively) (Figure 5.2c). As expected, strains without overexpression of the AR coding gene displayed residual enzyme activities (data not shown). Arabitol production by strains with PE-2 background was significantly higher than the other strains, exhibiting higher substrate conversion and productivity (0.2 g/(L·h)). Previous research observed that PE-2 expressing xylose oxidoreductase pathway has a natural propensity to accumulate the sugar alcohol xylitol in comparison to other industrial isolates (58, 62). According to these findings, the PE-2 background showed to have superior conversion performance and the genome-integrated strain PE-2-GRE3-XII5 was selected for the subsequent assays.

5.3.3. Boosting the arabitol production in engineered yeast

To increase arabitol titter and evaluate the viability of large-scale arabitol production from arabinose, the PE-2-GRE3-XII5 exhibiting the highest conversion in shake flask culture was tested in bioreactor (Figure 5.3). Despite lacking a specific L-arabinose transporter, *S. cerevisiae* can transport this sugar into the cell by the HXT hexose transporters (63). However, this sugar uptake system has a low affinity for arabinose and its transport is inhibited by the presence of high glucose concentrations (64). Thus, in order to facilitate simultaneous glucose consumption and arabinose conversion without uptake inhibition, the cultivations were performed in a glucose-limited fed-batch fermentation, in which glucose was slowly released to the medium to avoid high glucose concentrations**.** After the consumption of the initial glucose concentration (30 g/L), the assays were conducted in fed-batch mode to maintain glucose concentrations around 0.72 g/L. There was no detectable glucose in the broth, indicating that the glucose that was gradually supplied was

immediately metabolized by the yeast. After fed-batch cultivation, 68% of arabinose was converted to arabitol with a product yield of 1g/g. This methodology led to an increase in arabinose conversion (68%), arabitol titter (20.7 g/L) and maximal productivity (0.31 g/L/h), which corresponds to an improvement of 8, 45 and 55%, respectively, compared to the results from shake flask cultivations (Figure 5.2a).

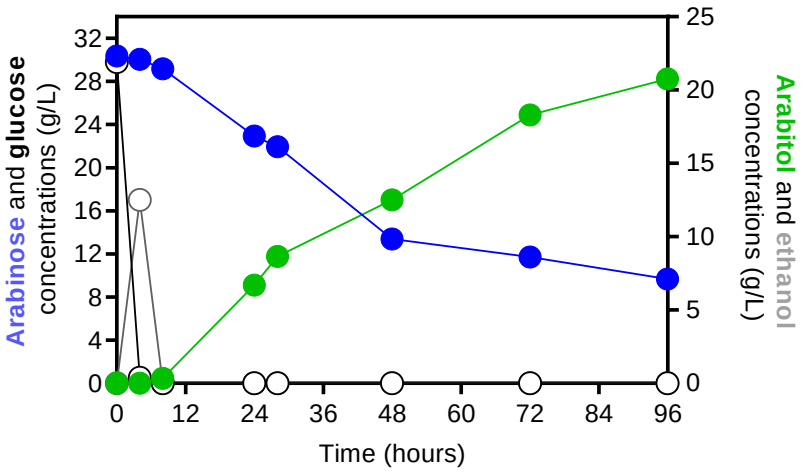

Figure 5.3. Arabitol production by the PE-2-GRE3-XII5 strain in a 2 L bioreactor. Profiles of arabinose, glucose, arabitol and ethanol concentrations during the fed-batch fermentation.

Despite the fact that the recombinant strain demonstrated improved arabitol production, arabinose utilization during fermentation remains incomplete. The inefficient transport of arabinose may be one of the major drawbacks of effective arabinose-to-arabitol conversion. In addition to HXT transporters, it was demonstrated that the yeast galactose permease (Gal2p) is able to transport arabinose (63). In order to increase arabinose uptake and, ultimately, arabitol production, we overexpressed the *GAL2* galactose permease coding gene in the recombinant PE-2-GRE3-XII5. Then, the capacity of the strain was evaluated with different inoculum concentrations (Figure 5.4). The recombinant strain expressing the *GAL2* was able to convert all the arabinose present in the medium in 72h. In contrast to the previous experiments (Figure 5.2a and Figure 5.3), the produced ethanol was not readily re-assimilated, resulting in an accumulation. Additionally, increasing the inoculum from 10 to 70 g wet cells/L improved the overall process (Figure 5.4b) and maximal productivity reached the highest value of 0.4 g/(L·h). Recently, a genetic improvement approach was performed in *Candida parapsilosis* DSM 70125 to create fusants with enhanced arabitol synthesis capacity by genome shuffling. The best-producing fusant, when grown on optimal medium, produced 12 g/L of arabitol with a corresponding yield of 0.49 g/g (65) .

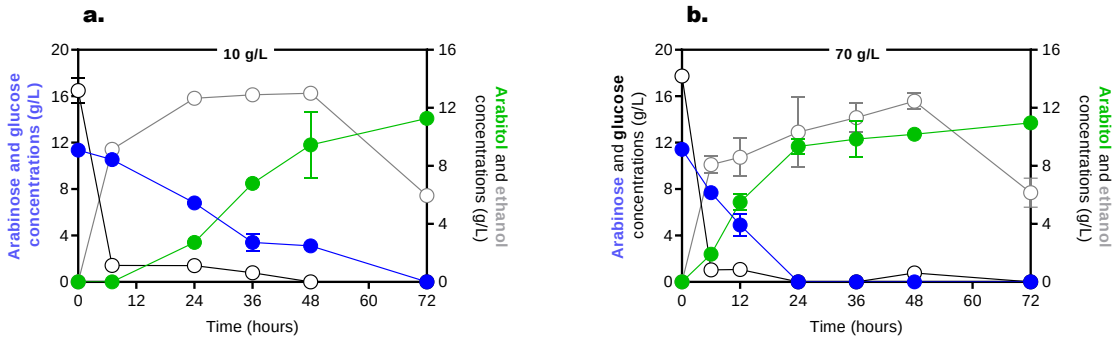

Figure 5.4. Cultivation of the recombinant PE-2-GRE3-XII5 overexpressing the *GAL2* gene in FIT medium with arabinose using (a) 10 g/L and (b) 70 g/L of wet cells for inoculation. Profiles for arabinose, arabitol glucose and ethanol concentrations. Data are presented as mean values and standard deviations of two independent biological replicates.

5.3.4. Sugar beet pulp processing for arabitol production

After establishing complete utilization of arabinose to produce arabitol in defined media through Gal2p expression, we used this recombinant strain for direct production of arabitol from renewable by-product derived from Sugar Beet Pulp (SBP) processing. SBP was selected based on literature, as a renewable source of arabinose, due to its high content in arabinan (49). In order to obtain a hydrolysate enriched in this monosaccharide, SBP was submitted to autohydrolysis followed by H_2SO_4 treatment for the extraction of hemicellulose in form of arabinooligosaccharides and subsequent hydrolysis into arabinose. These sequential treatments resulted in a hydrolysate containing 20.2 g/L of arabinose, representing 40% of arabinan extraction regarding raw material. These results can be compared with previous results using SBP (49).

Based on the findings obtained with different inoculum sizes (Figure 5.4), SBP hydrolysate was inoculated with 70 g/L wet cells and supplemented with glucose, following the previous process strategy (Figure 5.5). The bioconversion for arabitol production using the PE2-GRE3-XII5-GAL2 strain resulted in 15 g/l of arabitol, corresponding to a yield of 1 g/g and productivity of 0.29 g/(L·h). Notably, the recombinant strain cultivated on non-detoxified hemicellulosic hydrolysate exhibited fermentation performance comparable to that observed in synthetic medium (Table 5.2).

The use of renewable feedstocks to produce arabitol is important from both an environmental and economic perspective. Recently, a preliminary assessment indicated the potential economic viability of a SBP through the valorization of arabinose rich stream to arabitol, coupled with the production of levulinic and mucic acids (66). However, there has been limited research into the application of wastes and residues. While the production of arabitol from crude glycerol and

glucose-rich raw materials is more studied (67–73), there are no studies focusing on the valorization of arabinose-rich SBP hydrolysate for arabitol synthesis. Exploring the utilization of SPB hydrolysate to produce arabitol expands the scope of renewable feedstocks eligible for arabitol production.

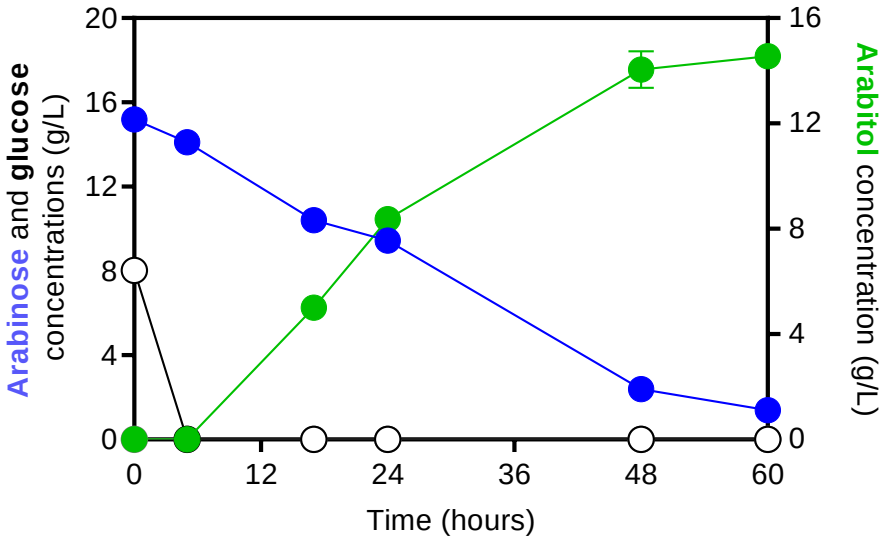

Figure 5.5. Performance of *S. cerevisiae* PE-2-GRE3-XII5-GAL2 recombinant strain in sugar beet pulp hydrolysate. Profiles for arabinose, arabitol glucose and ethanol concentrations. Data are presented as mean value and standard deviations of two independent biological replicates.

5.4. Conclusions

This study demonstrates the use of *S. cerevisiae* strains as whole-cell biocatalysts for the production of the sugar alcohol arabitol. Overexpression of the *GRE3* endogenous gene allowed for the direct and efficient conversion of arabinose to arabitol, reaching the theoretical maximum yield. In addition to this high yield, bioreactor culture increased overall productivity. The overexpression of the Gal2p transporter in PE-2-GRE3-XII5 strain enabled the full arabinose utilization, improving the arabinose conversion. Moreover, this recombinant strain PE-2-GRE3-XII5-GAL2 was able to efficiently convert arabinose from a non-detoxified SBP hydrolysate, producing 15 g/L of arabitol. This work also demonstrates for the first time that an arabinose-rich hemicellulosic hydrolysate may be converted directly into arabitol using an *S. cerevisiae* platform strain capable of converting other pentose pathway-derived chemicals such as xylitol. Overall, the findings show innovation in the effective utilization of carbon compounds in biomass resources, as well as a novel approach

for engineering *S. cerevisiae* as a platform yeast for the synthesis of commercially important chemicals.

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Chapter VI.

Galactose to tagatose isomerization by the L-arabinose isomerase from *Bacillus subtilis*: a biorefinery approach for *Gelidium sesquipedale* valorization

This chapter is based on the following original research article:

Baptista SL, Romaní A, Oliveira C, Ferreira S, Rocha CMR, Domingues L. 2021. Galactose to tagatose isomerization by the L-arabinose isomerase from *Bacillus subtilis*: a biorefinery approach for *Gelidium sesquipedale* valorization LWT 151:112199. DOI:10.1016/j.lwt.2021.112199

Abstract

Tagatose is a rare sugar with increasing commercial interest as sweetener. Biotechnological production of D-tagatose by enzymatic isomerization of D-galactose provides an alternative to chemical processes. In the last years, L-arabinose isomerases (L-AIs) from different origins have been studied to increase the effectiveness of tagatose production. In this work, the L-AI from *Bacillus subtilis,* previously reported to have unique substrate specificity for L-arabinose, was expressed in *Escherichia coli* and studied for isomerization of D-galactose to D-tagatose. The recombinant enzyme demonstrated, for the first time, tagatose bioconversion capacity, reaching ~ 59% conversion. Furthermore, a sustainable tagatose production strategy was developed by using *Gelidium sesquipedale* red seaweed and its undervalued processing residues as source of galactose. L-AI successfully converted the galactose-rich hydrolysate, obtained from direct acid hydrolysis of seaweed to tagatose (50.9% conversion). Additionally, the process combining autohydrolysis of *G. sesquipedale* and acid hydrolysis of the remaining residue allowed a full integral valorization of polysaccharides: 13.33 g of agar, an important hydrocolloid, coupled with the production of 5.97 g of tagatose. These results confirmed that seaweed biomass and waste-derived are promising substrates for tagatose production by L-AI*,* contributing to the advancement of circular economy and to the actual needs of food industry.

Keywords: Tagatose, L-arabinose isomerase, red macroalgae, *Gelidium sesquipedale*, biorefinery approach

6.1. Introduction

D-Tagatose is a rare hexose monosaccharide that occurs naturally in the tropical tree *Sterculia setigera* and is present in small amounts in dairy products (1, 2). This sugar is 92% as sweet as sucrose but has a lower glycemic index and caloric value (1.5-2.5 kcal/g vs. 4 kcal/g sucrose), which may help to prevent or manage diabetes and promote weight loss by reducing the calorific intake control in beverages and food (3–5). In fact, the rising concern about the health impacts of excessive sugar consumption has created interest on low-calorie sweeteners (6), such as tagatose. Over the past few years, a variety of low-calorie foods have been developed by replacing sugar with tagatose. The replacement of sucrose by tagatose showed little or no effect on desirable sensory characteristics and acceptability of food products (7–9) and has proven to be effective in reduction of HMF formation during food thermal processing (10). Besides, tagatose possesses various important health-promoting properties, such as anti-cariogenic, antioxidant, cytoprotective, prebiotic, and antihyperglycemic activities, making it an attractive GRAS (generally regarded as safe) ingredient in drug manufacture (11, 12). It has also been reported that tagatose could be potentially used to prevent colon cancer since it is partially absorbed in the small intestine and mostly degraded by intestinal microbiota, leading to the formation of short-chain fatty acids, in particular butyrate that inhibits the proliferation of colorectal cancer cells (13–15). Risk management, safety and quality of food products represent fundamental concerns of the European institutions and structures (16). D-tagatose was authorized as a novel food ingredient in the European Union (EU) in 2005 (17). Tagatose currently comprises a significant portion of the total sugar substitutes market share, valued at 16.5 billion in 2020 and projected to continue to experience growth, reaching USD 20.6 billion by 2025 (18).

Tagatose can be produced through chemical or enzymatic isomerization of galactose. The chemical synthesis, which involves a calcium/sodium chloride as a catalyst, presents several disadvantages such as by-product formation, complex purification steps, high energy consumption and chemical waste disposal needs (19). Biotechnological production of tagatose by enzymatic isomerization emerged as green alternative to the chemical route. L-arabinose isomerase (L-AI, EC 5.3.1.4) that is responsible for the reversible isomerization of L-arabinose to L-ribulose can also isomerize D-galactose to D-tagatose (20). A variety of L-AIs from different microbial sources, including mesophilic, thermophilic, and hyperthermophilic bacteria, have been used for tagatose biocatalysis (21).

Marine macroalgae, commonly known as seaweeds, are considered a promising renewable resource for improving the sustainability of future bio-based fuels and chemicals production due to their high ocean-growth productivities that exceed terrestrial crops, with no competition for land. In addition, seaweeds are in general characterized by their high carbohydrate composition and cell walls containing low amounts of hemicellulose and no lignin, which positively influence the carbohydrates recovery (22–24). Red macroalgae, the most abundant marine algae, present higher carbohydrate content and a variety of compounds with bioactive properties (antioxidant, anti-inflammatory, immunostimulatory and anti-viral effects) (25, 26). In particular, *Gelidium* is mainly composed by agar (a linear galactan composed by galactose and 3,6-anhydro-L-galactose linked by α-1,3- and β-1,4-glycosidic bonds) (27) and contains a small cellulose fraction (composed by glucose units). This red seaweed is commercially explored for extraction of agar due to its high-quality for food and pharmaceutical applications (28), generating hundred thousand tons of processing residues annually (29). These residues, which remain after the conventional methods of agar extraction, contain cellulose and agar leftovers, representing an undervalued carbohydrate-rich waste material suitable for use in the production of added-value chemicals (29).

Despite the significant potential of red seaweed as source of galactose for tagatose production, its research exploitation remains limited (30) and there are no studies focusing on the valorization route of *Gelidium* residues to the high-value sweetener, tagatose. Considering this, the aim of this study was the recombinant production of L-AI enzyme in *Escherichia coli* by expression of the *araA* gene from the food grade bacterium *Bacillus subtilis* for characterization of its isomerization activity for D-galactose into D-tagatose. Furthermore, the red algae *Gelidium sesquipedale* was exploited as galactose source to produce tagatose by using recombinant L-AI. For that, red algae *G. sesquipedale* was submitted either to a full hydrolysis process to obtain galactose or to a fractionation process for the co-production of galactose and agar in an integrated valorization process.

6.2. Materials and Methods

6.2.1. Reagents and enzymes

The enzymes for cloning were obtained from New England Biolabs Inc. (NEB). DNA primers and DNA sequencing were purchased from Eurofins Genomics. Growth media and high-purity D-galactose and D-tagatose were obtained from Liofilchem, Acros Organics and Sigma-Aldrich, respectively. Plasmid extraction was performed using GenElute Plasmid Miniprep Kit (Sigma-Aldrich).

6.2.2. Strains and media

Escherichia coli NZY5α (NZYTech) was used for plasmid construction and maintenance and NZYBL21 (DE3) (NZYTech) was used for recombinant protein production. *E. coli* strains were grown at 37°C and 225 rpm in lysogeny broth (LB) medium supplemented with 50 μg/mL kanamycin for selection. Solid medium was prepared by adding 2% (w/v) agar to the medium.

6.2.3. Recombinant L-arabinose isomerase production and purification

The *B. subtilis* DSM-92 *araA* gene was amplified by polymerase chain reaction (PCR) (forward (FW) primer: CTCCCATGGGACTTCAGACAAAGGATTATGAATTC and reverse (RV) primer: GGGTACCCTACTTTTGAAGCCGGTAAAG) from the carrying plasmid pMEC8000 (31) and cloned between *Nco*I and *Kpn*I restriction sites of the plasmid pETM10 (EMBL), in fusion with the N-terminal His6 tags. The resulting vector (pETM-10_araA) was transformed into chemically competent *E. coli* NZY5α cells (NZYTech). For protein expression, pETM-10_araA was transformed into the *E. coli* NZYBL21 (DE3) strain. A transformant colony was grown at 37 °C in 500 mL of LB medium supplemented with 50 μg/mL kanamicin to an OD_{600nm} of 0.6. At this point, L-AI expression was induced by adding 0.5 mmol/L of Isopropyl β-d-1-thiogalactopyranoside (IPTG) to the bacterial culture. Bacterial cells were collected, 16 h after IPTG-induction, by centrifugation at 4 °C for 20 min at 15,317 × g. Cells were lysed with NZY Bacterial Cell Lysis Buffer (NZYTech) containing 1 mmol/L of PMSF, following the manufacturer's instructions. Purification of the recombinant L-AI was performed by affinity chromatography using the 5 mL HisTrapTM column (GE Healthcare, Chicago, IL, USA) using 20 mmol/L sodium phosphate pH 7.4, 500 mmol/L NaCl with 40 mmol/L imidazole as equilibration and washing buffer. To elute the recombinant protein, 20 mmol/L sodium phosphate pH 7.4, 500 mmol/L NaCl with 300 mmol/L imidazole was used. After purification, elution buffer was exchanged with 50 mmol/L phosphate, having 0.5 mmol/L of Mn^{2+} using PD10 columns (GE Healthcare). Purified protein was analyzed by SDS-PAGE using 15% (w/v)

acrylamide gels, followed by BlueSafe staining (NZYTech). The protein concentration was estimated from the absorbance at 280 nm using the respective molar extinction coefficient (ε = 94560 L/(mol·cm)). Purified L-AI was stored at 4 °C until use. The insert sequences were verified by a commercial sequencing service (Eurofins MWG Operon).

6.2.4. Enzyme activity assays

Enzyme activity was determined by measuring the formation of D-tagatose from D-galactose. The reactions were performed in a final volume of 1 mL as follows: 400 µg of purified enzyme were incubated in 20 mmol/L phosphate buffer, pH 7.0 with 0.5 mmol/L of MnCl2 and 100 mmol/L of galactose. Reactions were initiated by adding purified enzyme to prewarmed reaction mixtures in a water bath at 37 °C. Reactions were conducted for 20 min before being stopped by cooling on ice. Enzyme activity was determined by measuring colorimetrically the accumulation of tagatose, using the cysteine carbazole-sulfuric-acid method (32). The absorbance of three technical replicates of each reaction were measured at 560 nm. One unit of isomerase activity was defined as the amount of L-AI that produced 1 mol of product per min under the assay conditions. Tagatose production was confirmed via high performance liquid chromatography (HPLC) analysis (conditions described in section 2.6). The temperature effect on galactose isomerization was analyzed by incubating L-AI at temperatures ranging from 20 °C to 65 °C in a water bath. The pH effect was measured by incubating the enzyme in different pH buffers (50 mmol/L): Tris hydrochloride (pH 8.0 to 9.0), sodium phosphate buffer (pH 6.0 to 7.5), sodium acetate buffer (pH 4.5 to 5.5) and sodium citrate buffer (pH 3.0 to 4.0). Enzyme stability was measured by incubating the enzyme at different temperature and pH values and the reaction was sampled at defined time intervals.

6.2.5. Bioconversion assays

To determine efficiency of recombinant L-AI to convert D-galactose into D-tagatose, the bioconversion experiments were performed in phosphate buffer (50 mmol/L), 0.5 mmol/L of $MnCl_2$ and/or *G. sesquipedale* hydrolysates at optimum pH and temperature during 24h in an orbital shaker. The effect of enzyme dosage and substrate concentration was studied by using different galactose concentrations (10, 55, 100 g/L) and enzyme loadings (0.5, 3.5 and 7 mg/mL). Kinetic conversion was performed under optimal conditions with samples taken periodically. Galactose and tagatose concentrations were determined by HPLC.

6.2.6. Raw material

G. sesquipedale was provided by Iberagar, S.A. (Sociedade LusoEspanhola de Coloides Marinhos). The raw material was cut into smaller pieces, washed with distilled water and oven dried before use. *G. sesquipedale* was chemically analyzed following standard methods described by National Renewable Energy Laboratory (NREL) protocols (NREL/TP-510-42618-42622-4218), and its composition (expressed in g/100 g of raw material in oven-dry basis ± standard deviation based on three replicate determinations) was: 14.78 ± 0.73 of ash, 14.52 ± 0.09 of crude protein, 28.38 ± 4.20 of water extractives, (composed by 0.35 ± 0.01 of glucan, 19.17 ± 3.72 of galactan). The extractive free residue was: 14.64 ± 1.88 of glucan, 18.71 ± 5.59 of galactan and 1.59 ± 0.11 of acid-insoluble residue (28).

6.2.7. Processing of *Gelidium sesquipedale*

Acid hydrolysis and autohydrolysis of *G. sesquipedale* were performed in a stainless-steel reactor (Parr Instruments Company) equipped with Parr PDI temperature controller.

For acid hydrolysis pretreatment, 12 g/100 g of *G. sesquipedale* biomass was mixed with 1.5 % (w/w) of sulfuric acid solution at 150 °C for 10 min. The resulting solid and liquid fractions were separated by filtration, and the composition of hydrolysate (sugars, acetic acid and furanic compounds) was analyzed by HPLC.

For the autohydrolysis pretreatment, the seaweed samples were mixed with water (30 g of *G. sesquipedale* solid dry weight per 100 g of water) and heated to 150, 160 and 170 °C under non-isothermal conditions. After autohydrolysis pretreatment, the samples were cloth filtered at 80 °C to avoid the gelling of the medium (28). The pretreated *Gelidium r*esidues (solid fraction remaining after autohydrolysis) were recovered and washed for Solid Yield (SY) and 6% of this residue was subjected to acid hydrolysis with 1.5 g/100 g of sulfuric acid solution at 120 °C for 30 min. The liquid fraction was subjected to a freeze-thaw cycle to recover agar from the liquor. For agar weight determination, the resulting samples were dehydrated with ethanol (96%) and dried at 60 °C (28). The composition of remaining liquor (sugars and furanic compounds) was analyzed by HPLC.

To remove inhibitors, *G. sesquipedale* hydrolysate was detoxified by mixing with activated charcoal (liquor to solid ratio of 10 g of hydrolysate per gram of activated charcoal) for 1 h (33).

6.2.8. Analytical methods

Sugars (galactose and glucose) and 5-hydroxymethyl furfural (HMF) obtained from chemical analysis of *Gelidium sesquipedale* and solid and liquid phases resulting from autohydrolysis and

acid treatments were quantified by HPLC using a BioRad Aminex HPX-87H column (300 × 7.8 mm) with a gel particle size of 9 µm, at 60 °C and 5 mmol/L sulfuric acid as eluent in a flow rate of 0.6 mL/min. The peaks corresponding to sugars were detected using a Knauer-IR intelligent refractive index detector, whereas HMF were detected using a Knauer-UV detector set at 280 nm.

Samples obtained from isomerization assays were analysed to determine the content of galactose and tagatose by HPLC using a Bio-Rad Aminex Carbohydrate HPX-87P column (300 x 7.8 mm) column with a gel particle size of 9 µm, which was kept at 85 °C and pure water used as mobile phase with a flow rate of 0.5 mL/min, and detected by refractive index detector (RI-2031).

6.2.9. Statistical analysis

The software GraphPad version 6.0 for Windows was used to perform the statistical analysis. Data are presented as means ± standard deviation of triplicate determination. Differences among the different conditions of auto hydrolysis pre-treatment were verified using one-way ANOVA test, with subsequent Tukey's test as a post hoc comparison of means. Statistical significance was established at $p< 0.05$ for the comparison and assembled in homogenous groups represented by letters.

6.3. Results and discussion

6.3.1. L-AI recombinant production and purification

L-arabinose isomerase is a 496 amino acids-long protein encoded by the gene *araA* involved in arabinose utilization (34). The enzymatic activity of *B. subtilis* L-AI regarding isomerization of L-arabinose has already been fully described (35), but its isomerization activity of D-galactose remains to be demonstrated. The *araA* gene from *B. subtilis* was cloned into the pETM10 expression plasmid and subsequently expressed in *E. coli* BL21 (DE3). SDS–PAGE analysis of the extracts of *E. coli* BL21 cells, harboring pETM10_araA induced at 37 °C with IPTG, revealed the presence of protein with a molecular weight around ≈ 56 kDa, which is consistent with the previously reported molecular weight for this protein, 56 kDa (Figure 6.1A) (35). The L-AI protein was purified in a single Ni^{2+} affinity chromatography step and over 50 mg of purified L-AI protein was obtained per g of wet cell mass (Figure 6.1A). The purified recombinant L-AI was enzymatically active, catalyzing the isomerization of galactose to tagatose, with an activity of 8.35 U/mg at standard conditions described in 2.3 section. Tagatose production was confirmed by HPLC analysis (Figure 6.1B) which supported the L-AI activity towards the galactose substrate. In contrast to the work by Kim et al. (35) that reported L-AI activity only towards D-arabinose, the enzymatic study conducted in this work with the same enzyme did not show limitations in its isomerase activity towards galactose.

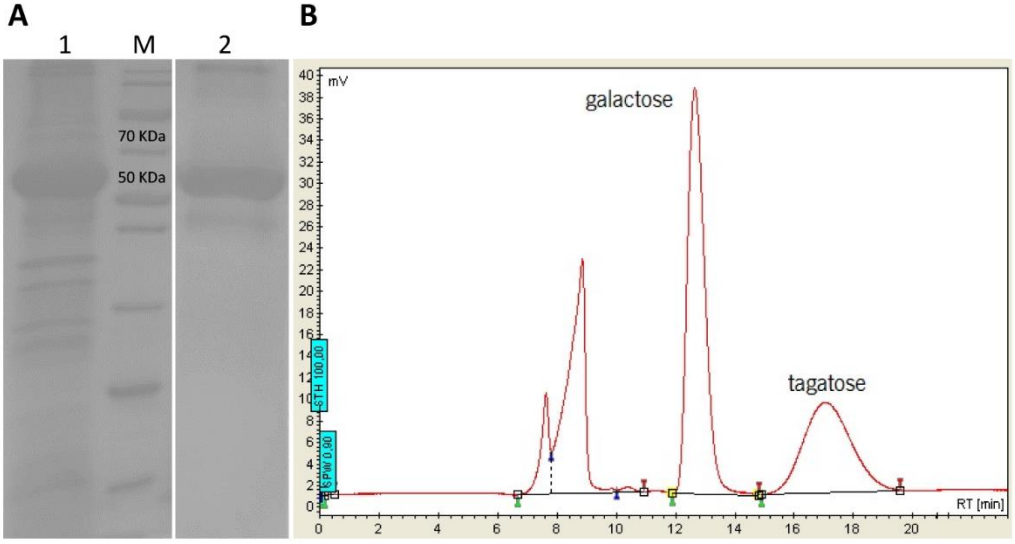

Figure 6.1. Analysis of production and activity of recombinant L-AI. (A) SDS-PAGE analysis of the production and purification of L-AI from E. coli: Lane 1, crude extract of cells producing L-AI; Lane M, molecular weight marker; Lane 2, IMAC purified L-AI. (B) HPLC analysis of galactose isomerization by L-AI into tagatose product. The retention times of galactose and tagatose were 12.63 min and 17.03 min, respectively.

6.3.2. The effect of temperature and pH on L-AI activity

To determine the optimal temperature for D-galactose to D-tagatose isomerization, the enzyme activity was measured by carrying out standard assays at different temperatures. As shown in Figure 6.2A, the L-AI achieved maximal activity at 42 °C temperature, which is higher than the optimal temperature of *B. subtilis* L-AI using arabinose as substrate (35). The thermostability profile of L-AI (Figure 6.2C) demonstrated that the enzyme is highly stable at 32 °C. At 37 °C only ~50% of enzyme activity is kept after 6 h of incubation and the increase of the reaction temperature to 42 °C is accompanied by rapid enzyme inactivation (6h). Considering that tagatose decomposes and caramelizes at high temperatures, isomerization reactions performed at lower temperatures can prevent changes in color (browning) and flavor. Furthermore, the mild reaction conditions require less energy consumption, contributing positively to the environmental sustainability and process economics.

The effect of pH on L-AI activity was tested by measuring the enzyme activity at pH values ranging from 4.0 to 10.0 (Figure 6.2B). The optimal pH for L-AI isomerase is 7.5, which is consistent with previous reported pH profile of this enzyme (35). L-AI maintained its high-level activity at pH 7 and 7.5 with over 80% of its maximum activity retained for 24h (Figure 6.2D). Like other L-AI enzymes from other microbial sources, L-AI from *B. subtilis* displays maximal activity at neutral pH (21).

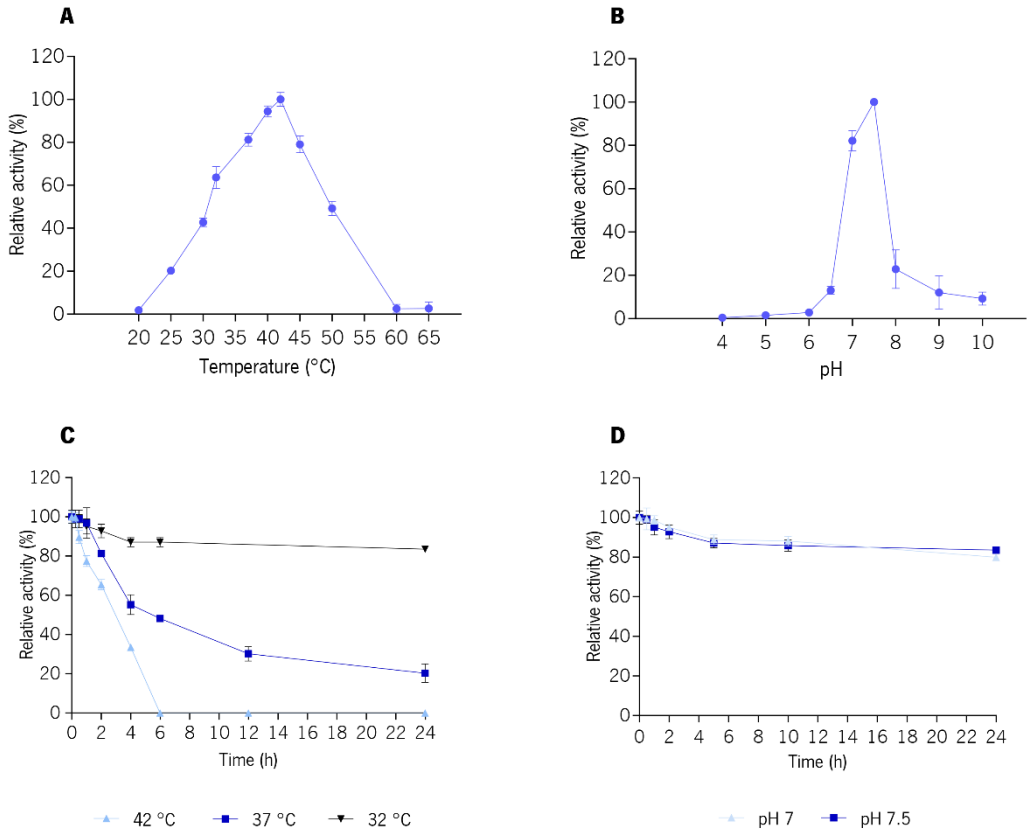

Figure 6.2. Characterization of L-AI for tagatose production: effect of temperature (A), pH (B) and stability analysis at different temperatures (C) and pH values (D). Activity was measured using the standard assay and the data are means from three independent biological replicates.

6.3.3. Tagatose production by B. subtilis L-AI

To evaluate the effect of galactose and purified L-AI concentrations in the biotransformation, the production of tagatose was evaluated during 24 h using 10 g/L of galactose and 0.5, 3.75 and 7 g/L of purified L-AI (Figure 6.3A). The highest conversion (59.1 %) was obtained by increasing the enzyme loading up to 7 g/L, which led to a final concentration of 5.9 g/L of tagatose. The bioconversion was also evaluated using 7 g/L of L-AI and different concentrations of galactose: 15, 50 and 90 g/L (Figure 6.3B). Tagatose production increases with increasing galactose concentrations, achieving a maximal concentration of 30.7 g/L, but the conversion decreases from 59% to 34.8 %. Similar results were reported by testing immobilized *Lactobacillus plantarum* cells producing L-AI, in which high concentrations of galactose resulted in reduced initial reaction velocity and lower conversion (36). Figure 6.3C shows the conversion profile during 24 h. During the first 6 h, 49% of galactose was isomerized to tagatose, attaining a conversion of 58%. Like others isomerases, isomerization of D-galactose to D-tagatose results in a mixture of the substrate and product due to thermodynamic equilibrium, leading to conversions lower than 60 % (21). Considering that at higher temperatures, the reaction equilibrium shifts toward tagatose, L-AIs from thermophilic microorganisms show higher conversion ratios: 60% and 68% obtained from *Anoxybacillus flavithermus* and *Thermotoga neapolitana* 5608, respectively. However, the high reaction temperatures (≥80 °C) required by these thermophilic enzymes, also result in undesirable reaction of caramelization (21, 37). The LAI mesophilic enzyme used in this work achieved, at lower temperatures, conversions similar to their thermophilic counterparts, avoiding product caramelization.

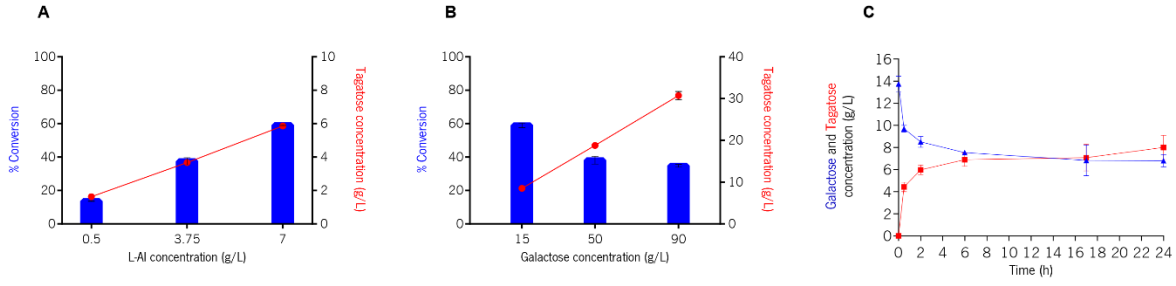

Figure 6.3. Effect of the L-AI (A) and galactose concentration (B) on bioconversion of galactose into tagatose, and time course of tagatose production during L-AI isomerization (C).

6.3.4. Seaweed *Gelidium sesquipedale* processing for tagatose production

Marine macroalgae *G. sesquipedale* present high galactose content and can be exploited as renewable substrate in the manufacturing of tagatose. To obtain a hydrolysate rich in galactose, two approaches were considered for the hydrolysis of the galactan present in the red seaweed: (a) acid hydrolysis to recover galactose from galactan in the liquid fraction and (b) autohydrolysis, using water as reaction medium, to recover galactan as agar in liquid phase, followed by acid hydrolysis of solids remaining from autohydrolysis (residue) to obtain a liquor enriched in galactose (Figure 6.4).

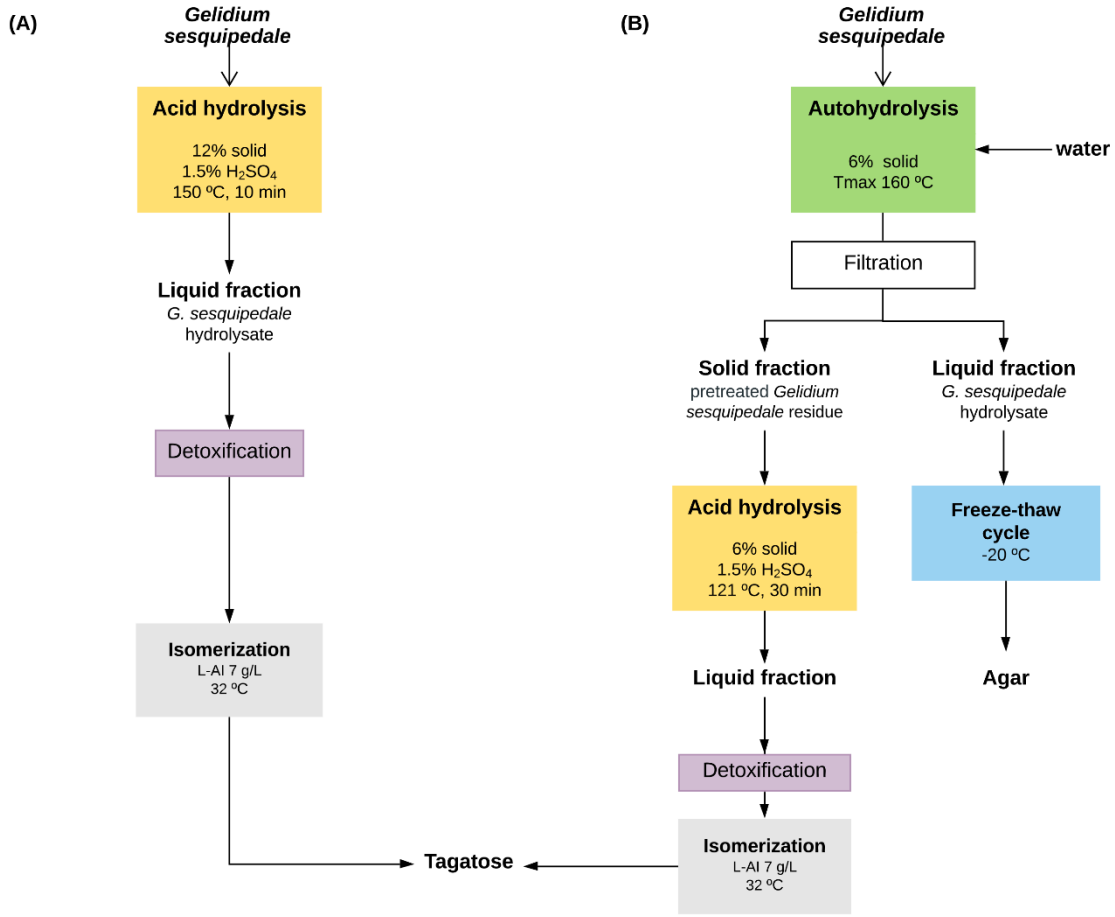

Figure 6.4. Flowchart of experimental procedure for tagatose production from galactose derived from *G. sesquipedale*.

The direct acid hydrolysis of seaweed (Figure 6.4A) was carried out with sulfuric acid (1.5%, 10 min, 150° C). These conditions were selected based on a previous evaluation of the effects of temperature, sulfuric acid concentration and red seaweed load (data not shown), in which maximal galactose concentration was achieved in the hydrolysate. Direct acid hydrolysis of red seaweed

(Figure 6.4A) resulted in a hydrolysate composed of 22.28 g/L of galactose and 6.56 g/L of HMF, corresponding to an extraction of 44.13 g of galactan/100g of galactan in the raw material. The presence of HMF in the liquor also indicates that 18.7 % of hexoses were dehydrated to HMF, a furanic compound recognized as inhibitor of bioconversion processes (38). Similar concentration of galactose and HMF were obtained by HCl hydrolysis of *Gelidium amansii* (39).

Though the traditional agar extraction process could be used to recover the first valuable fraction, autohydrolysis pretreatment may also be considered, with advantages in the extraction yield and extraction times (40). As previously reported, 50.6 % of galactan present in *G. sesquipedale* is easily extracted with water and solid biomass obtained after extraction (residues) still contain 18.71 ± 5.59 g of galactan per 100 g of raw material (28). In this sense, sequential processing of *G. sesquipedale* by autohydrolysis pretreatment and acid hydrolysis of biomass residues was performed to obtain valuable products in two separate streams: agar from autohydrolysis and a galactose-rich hydrolysate from the hydrolysis of *G. sesquipedale* residue (Figure 6.4B). To evaluate the temperature effect on red seaweed fractionation using autohydrolysis, three temperatures namely 150, 160 and 170° C were tested. Heating profiles are shown in Appendix, Figure A6.1. Table 6.1 presents the solid yield (g of solid residue after autohydrolysis per 100 g of raw material) and chemical composition of solid and liquid fractions obtained after pre-treatments.

Table 6.1. Chemical composition of liquid and solid fractions resulting from *G. sesquipedale* autohydrolysis at 150, 160 and 170 °C. Different letters indicate significant differences ($p < 0.05$).

	150 °C	160 °C	170 °C
a) Liquid fraction composition (g/100 g of raw material) *			
Galactose	1.00 ± 0.02 ^{b}	1.46 ± 0.09 ^{a}	1.79 ± 0.01 ^{a}
Glucose	0.20 ± 0.00 ^{b}	0.27 ± 0.00 ^{a}	0.26 ± 0.00 ^{a}
Agar	6.47 ± 1.01 ^{c}	13.33 ± 0.92 ^{b}	19.75 ± 0.89 ^{a}
b) Solid fraction composition (g/100 g of pretreated raw material)			
Solid Yield (SY)	69.6	72.0	68.4
Galactan	25.86 ± 0.64 ^{b}	34.52 ± 0.53 ^{a}	28.74 ± 2.15 ^{b}
Glucan	19.83 ± 0.36 ^{a}	20.36 ± 0.18 ^{a}	15.94 ± 1.22 ^{b}
Acid insoluble residue	6.90 ± 0.37 ^{a}	6.89 ± 0.31 ^{ab}	5.83 ± 0.46 ^{b}

* before freeze-drying.

Considering that part of the galactan can be solubilized in water, agar was quantified from the liquid fraction after treatment. The agar extraction was dependent on the increase of temperature

(Table 6.1). At temperatures higher than 160 °C, autohydrolysis allowed to obtain more than 13 g of agar per 100 g of *G. sesquipedale,* which confirms that autohydrolysis may be used to extract agar as alternative to conventional methods that use lower temperatures (95 °C) and longer times (41). Low concentrations of free sugars were quantified in the liquid fraction, values ranging between from 0.57 to 1.06 g/L of galactose. In the pretreated biomass (*G. sesquipedale* residues) galactan was the main polysaccharide, followed by glucan and acid insoluble residue (Table 6.1). Under the tested conditions, autohydrolysis at 160 °C resulted in a recovery of 35.19 % of galactan as agar (13.33 g/100g of raw material), remaining 24.85 g of galactan in 72g solid residue. In this condition, the galactan content was maximal in the pretreated solid (34.52 g/100g of pretreated raw material) showing statistically significant differences ($p < 0.05$) when comparing with the other tested conditions (150 and 170 °C). Considering this, the solid residue remaining from the autohydrolysis at 160 °C was selected for acid hydrolysis to obtain a hydrolysate composed by galactose. Hydrolysis conditions were selected by a previous evaluation of acid concentration, solid loading and temperature and time (data not shown). The acid hydrolysis of the *Gelidium* residues resulted in a liquor containing 11.93 g/L of galactose with a corresponding galactan to galactose yield of 57.60 g of galactose/100g of galactan in the raw material, which was higher than the yield obtained by direct acid hydrolysis of seaweed.

To evaluate the potential of recombinant L-AI to convert galactose derived from *G. sesquipedale* into tagatose, the hydrolysates produced from the *G. sesquipedale* algae and residues were tested as galactose source. For that, these liquors were detoxified to avoid inhibition of the enzyme due to presence of furan and phenolic compounds. Considering that at elevated temperatures (42 °C) the enzyme activity is maximal, but the enzyme quickly inactivates, the experiments with *G. sesquipedale* hydrolysates were performed at 32 °C, temperature at which enzyme stability is ensured. L-AI enzyme successfully converted galactose present in both liquid fractions into tagatose, reaching a maximum conversion of 50.9% from the detoxified liquor obtained from direct acid hydrolysis of seaweed. This conversion of galactose to tagatose is considerably higher than the one obtained (34.5%) by using as substrate galactose from the green algae *Caulerpa racemosa* and L-AI purified from *Lactobacillus plantarum* (42). The utilization of the liquor derived from acid hydrolysis of autohydrolysed *G. sesquipedale* residue achieved a conversion of 42.0%. This conversion was similar to those obtained with a trienzymatic complex, for simultaneous agar hydrolysis and galactose isomerization into tagatose, in red seaweed biomass with conversions of

33.8 %, 44.0%, 43.1% and 32.7% from *Gelidium amansii, Gracilaria verrucosa, Chondrus ocellatus, and Grateloupia asiatica*, respectively (30).

The overall balance of the two approaches of *G. sesquipedale* processing for tagatose production (Figure 6.5) shows that direct acid hydrolysis of 100g of seaweed allowed the recovery of higher concentration of galactose (18.57 g) and consequently, tagatose (9.45 g) but with no agar recovery. On the other hand, autohydrolysis of red seaweed followed by an acid hydrolysis of remaining residue resulted in 13.33 g of agar, 8.34 g of galactose and 5.97 g of tagatose per 100 g of raw material, which entails a revalorization of 73 % of the galactan present in *G. sesquipedale*. This clearly shows the advantage of using sequential pre-treatments to obtain valuable products in separate streams for an integral valorization of this resource in a biorefinery concept. Despite the benefits of using a renewable carbon source to produce tagatose, scaling-up from laboratory to large-scale production is a challenging requisite for commercial manufacturing, and depends on several factors, such as, water, materials and energy consumption, downstream technologies, among others. The complexity of large-scale process implementation highlights the necessity of a complete analysis of the process, from an environmental and economic perspective, to assess its realistic potential.

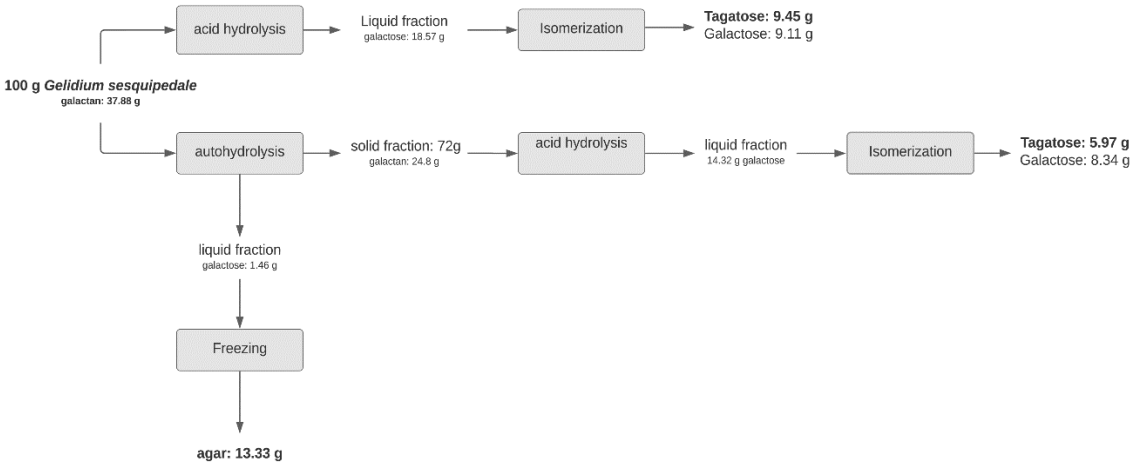

Figure 6.5. Overall mass balance of the different strategies for tagatose production from *G. sesquipedale.*

6.4. Conclusions

L-AI enzyme was overproduced in *E. coli* by expressing the *araA* gene from the food grade bacterium *B. subtilis.* The enzymatic activity of purified recombinant L-AI was characterized in respect to D-galactose isomerization, showing an optimal activity at 42°C and pH 7.5. The recombinant enzyme was also characterized in terms of its efficiency for D-tagatose production

from D-galactose, exhibiting a conversion of 59%, which is comparable to the one reported for thermophilic enzymes. Furthermore, this work shows the feasibility of the enzymatic process by using red algae *Gelidium* as a substrate for tagatose biocatalysis in a biorefinery concept.

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Chapter VII

Conclusions and Future Perspectives

The world is facing an urgent need to reduce its reliance on fossil resources, which are not only finite but also responsible for a range of environmental problems. One promising solution is the production of biobased compounds from renewable sources. The biorefinery concept, which aims to transform biomass into a range of products, is at the forefront of this shift. Although biofuels such as bioethanol have been the main focus of biorefineries, there is an increasing interest in exploring new processes and products that can be integrated into the circular economy. Moreover, the increasing demand for sugar substitutes reflects a broader shift towards healthier, more natural diets and a growing awareness of the negative health effects of excessive sugar consumption. Therefore, natural sweeteners are of interest to a range of industries. However, most research studies on sweeteners have focused on the use of synthetic media and laboratory yeast strains. While this approach is necessary for optimizing the yield of sweetener microbial biosynthesis and unraveling novel pathways, it does not necessarily address the economic goals of a biorefinery or the need to reduce our reliance on fossil resources. To establish a successful biorefinery, it is important to incorporate high-value low-volume chemicals, such as natural sweeteners. This requires the searching for renewable resources by means of the valorization of agro-industrial wastes and repurposing them to produce compounds of interest. The main objective of this thesis was to contribute to this goal by exploring the potential of agro-industrial waste streams for the microbial production of natural sweeteners.

In light of these, the main purpose of this thesis was to contribute to the development of biorefineries using *Saccharomyces cerevisiae* as a platform for the production of natural sweeteners. Following this, the main outcomes of this research are presented below:

1) the role of different enzymes with xylose reductase activity was evaluated, demonstrating that increasing the expression of a specific endogenous aldose reductase (*GRE3* gene) in the yeast strain *S. cerevisiae* PE-2, which naturally accumulates xylitol, improves the xylitol yield and productivity compared to using different types of xylose reductases in the same or other types of yeast chassis. Also, the resulting GRE3-overpressing strain is capable of attaining remarkable xylitol titers from very high levels of synthetic xylose and efficiently produce xylitol from corn cob hydrolysates without detoxification.
2) The feasibility of using whole slurry corn cob for xylitol production in a simultaneous saccharification process was demonstrated. The using of high solid loadings (25%) in the hydrothermal pretreatment to obtain hydrolysates highly enriched in xylooligosaccharides, and xylose, was found to be a greener approach, avoiding the need for costly evaporation steps. These hydrolysates showed to be suitable for enzymatic hydrolysis and xylitol bioconversion using the

recombinant *S. cerevisiae* strain, previously constructed. Also, the absence of acetic acid in the corn cob hydrolysate led to even higher xylitol productivity, resulting in the highest titter reported for *S. cerevisiae* using lignocellulosic biomass (42 g/L). This enabled a significant contribution towards the development of sustainable and efficient processes for xylitol production, using green technologies such as autohydrolysis and saccharification and fermentation while valorizing lignocellulosic biomass.

3) utilization of this recombinant xylitol-producing strain allowed the valorization of a wider range of feedstocks (vine shoots, wine lees and the grape must surplus). By exploring multiple wastes from winery industry, the co-production of xylitol together with ethanol and yeast biomass was achieved. The proposed biorefinery provided a sustainable solution for producing high-value chemicals and biofuels from underutilized biomass, simultaneously addressing the issue of wine waste management.
4) The possibility of using the GRE3-overpressing *S. cerevisiae* strain for a direct and efficient conversion of arabinose to arabitol was proven for the first time. As the endogenous aldose reductase is reported to have promiscuous activity, its role in arabinose to arabitol conversion was evaluated in a laboratorial strain. By confirming the capability of the endogenous aldose reductase for arabitol production, the expression of the *GRE* gene in different industrial yeast chassis led to the selection of the PE2 background as best arabitol producer. Also, the further modification of this strain to overexpress the *GAL2* transporter gene led to the full conversion of arabinose from a non-detoxified Sugar Beet Pulp hydrolysate.
5) The effectiveness of using L-arabinose isomerase from *Bacillus subtilis* (Bs L-AI) in the biotechnological production of tagatose was proved. By cloning and expressing the recombinant Bs L-AI in *Escherichia coli*, the capacity of converting galactose to tagatose was newly reported. As galactose is highly present in red seaweed, the application of green pretreatment technologies (autohydrolysis) lead to the production of tagatose and other valuable products (agar).

Taken together, the findings of this thesis open up the possibility of developing a biorefinery that utilizes 2nd and 3rd generation feedstocks as a renewable and sustainable source for the production of natural sweeteners by biocatalytic processes.

This study encompassed various strategies to produce and enhance the titers and yields of some natural sweeteners. These strategies included the expression of endogenous and heterologous genes, investigation of the catalysts involved in bioconversion steps, and integration of agro-food wastes to

increase the economic viability and contribute to sustainability. By employing these approaches, the study aimed to develop a more efficient and eco-friendly process for natural sweetener production.

The integration of agro-industrial wastes into bioprocesses for natural sweetener production has proven to be a feasible approach for future implementation. Furthermore, given the diversity of sugars required for sweetener production, the development of yeast strains with expanded sugar utilization abilities offers new possibilities in this field. Also, the findings of this thesis showed the versatility of the yeast *S. cerevisiae*, in particular, industrial isolates, in handling a wide range of biomass feedstocks. Its versatility as robust microbial platform in promoting sustainable processes make it a valuable contributor to the development of a circular bioeconomy, which is essential for creating a greener future.

The rising demand for natural sweeteners and the growing concerns over the use of fossil resources have accelerated the adoption and establishment of biobased processes. Although there are still many challenges to overcome in incorporating agro-industrial residues into these industrial bioprocesses.

For example, although most enzymes may be produced at industrial scale using genetically engineered organisms, bioconversions using whole cells provide various advantages (1). The presence of the protective cellular membrane contributes to the stabilization of enzymes, facilitating its use under severe reaction conditions. Whole cells control pH ionic strength and are capable of producing and regenerating cofactors, which eliminate the need for external cofactor supply. Furthermore, the utilization of whole cells eliminates the expensive and time-consuming purifying methods, reducing the total cost of the process (2). In this context, there is still ample room for improvement in developing a strategy that simplifies product separation and purification, resulting in an overall reduction in production costs. Therefore, future studies should focus on devising a more efficient and cost-effective tagatose production strategy based on direct yeast conversion of galactose derived from algae biomass in a whole-cell conversion process.

Despite the fact of L-arabinose isomerases have been the catalyst of choice for tagatose production, there are some limitations that prevent it from becoming commercially viable. These limitations include unfavorable enzymatic kinetics because galactose is not the native substrate, low enzyme stability, especially in the absence of divalent metal ions, and a low equilibrium constant for galactose to tagatose isomerization (3). Future studies should focus on enhancing the enzymatic properties of L-AI for industrial application. In fact, the stability of L-AI from *Lactobacillus fermentum* CGMCC2921 displayed on the surface of spore of *Bacillus subtilis* 168 was significantly improved, converting 75% galactose (100 g/L) into tagatose after 24h and the conversion rate remained at 56% at the third cycle (4). In this sense, cell

surface-display of whole cells biocatalysts with GRAS status (as *S. cerevisiae*) or encapsulation in particles, can stabilize enzymes and remarkably enhance the production of tagatose.

Moreover, obtaining high-purity tagatose from a galactose-tagatose mixture is challenging due to the structural similarities. Given this, implementing adsorption-assisted purification strategies based on specific absorbent with a superior binding affinity for tagatose over galactose need to be explored to improve the downstream processing and thermodynamic limitations between the two sugars. Recently, a polymeric derivative of phenylboronic acid (PBA) was effective for specific *in situ* product removal of ketoses from aldose-containing reaction mixtures (5–7). In this sense, the development of affinity-based technique designed to galactose to tagatose isomerization is highly desired.

The results presented in this thesis reached the target yield of 1g/g both for xylitol and arabitol production, but the productivity was hindered by the presence of acetic acid. Due to the acetylation of hemicellulose and lignin in the plant cell wall, the hydrolysates inevitably contain significant amounts of acetate, toxic to fermenting microorganisms that negatively affected the bioconversion efficiency of hemicellulose-derived sugars. While not explored in this thesis, the overexpression of genes such as *HAA1*, *PRS3* (8) and *RCK1* (9), or gene deletion such as *ZRT3* (10) can be devoted to engineer acetate tolerance in the GRE3-overexpressing yeast for alleviating or even eliminate the inhibitory and toxic effects of acetate.

Moreover, by combining the findings of this thesis with existing literature, there is potential to further maximize the value of lignocellulosic biomass. For instance, the solid fraction that remains after hydrothermal pretreatment could be subjected to organosolv treatments (11) to recover lignin, which is a valuable product that contains aromatic compounds and can be used to produce various chemicals. This approach not only enables the utilization of all components in the biomass, but also enhances the susceptibility of cellulose to enzymatic degradation, which increases the efficiency of the subsequent simultaneous saccharification and fermentation (SSF) process.

In summary, the envisioned biorefinery would enable the production of both natural sweeteners and ethanol from a diverse range of renewable resources, and its commercial viability would be secured by a number of strategies outlined in this thesis, including the valorization of both hemicellulosic and cellulosic fractions of biomass, the attainment of high ethanol titers which would reduce distillation-related costs, the optimization of equipment usage through integrated processes such as SSF, the reduction of expenses associated with nutritional supplementation, and the production of non-biofuel high-value compounds. As a final observation, the findings presented in this thesis reinforce the pivotal role that industrial *S. cerevisiae* plays in the attainment of a seamlessly integrated production of biofuels and other

bioproducts. This contribution is expected to have a great impact on the expansion and sustainable development of the biorefining industry.

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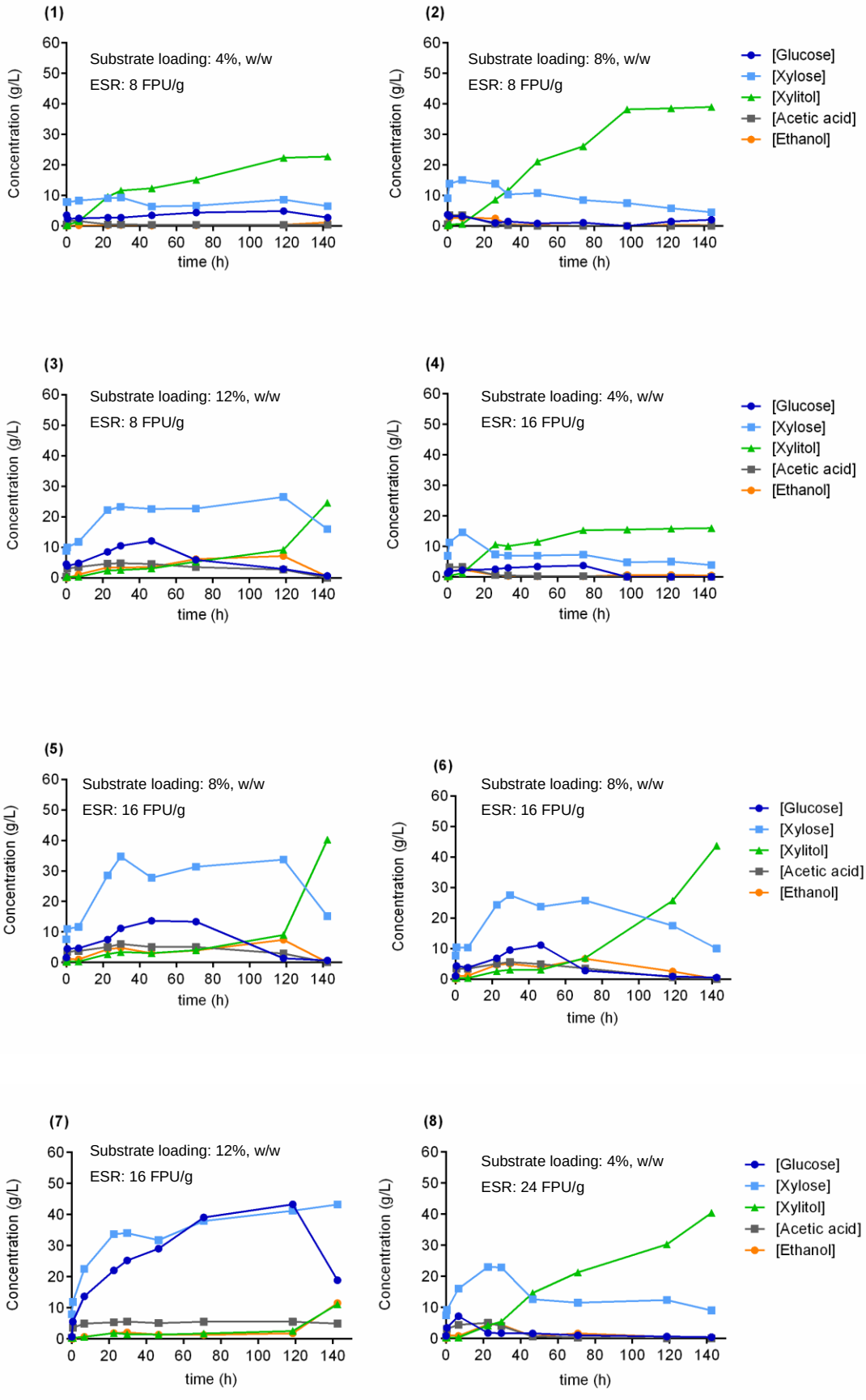
Appendix

Chapter III


(1)
Substrate loading: 4%, w/w
ESR: 8 FPU/g
(2)
Substrate loading: 8%, w/w
ESR: 8 FPU/g
(3)
Substrate loading: 12%, w/w
ESR: 8 FPU/g
(4)
Substrate loading: 4%, w/w
ESR: 16 FPU/g
(5)
Substrate loading: 8%, w/w
ESR: 16 FPU/g
(6)
Substrate loading: 8%, w/w
ESR: 16 FPU/g
(7)
Substrate loading: 12%, w/w
ESR: 16 FPU/g
(8)
Substrate loading: 4%, w/w
ESR: 24 FPU/g
Concentration (g/L)
time (h)
[Glucose]
[Xylose]
[Xylitol]
[Acetic acid]
[Ethanol]

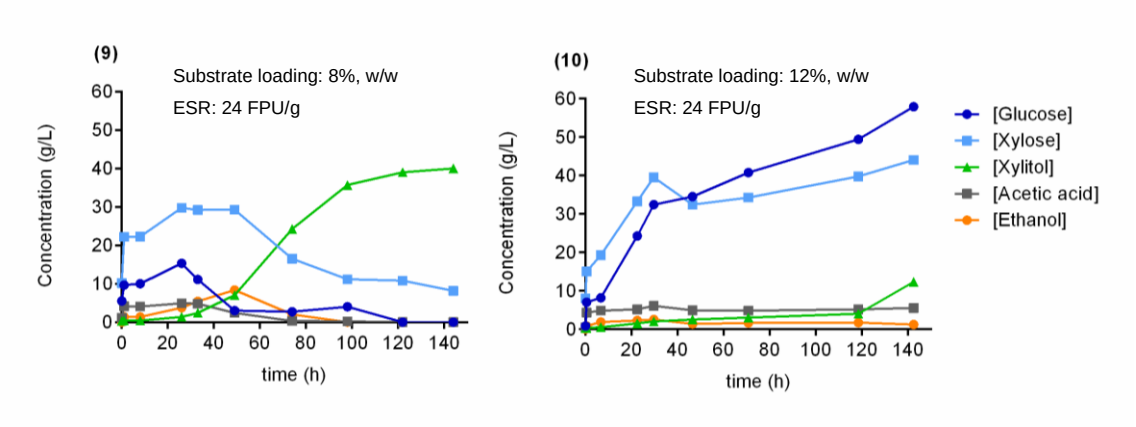


Figure A3.1. Time course of glucose, xylose, xylitol, ethanol and acetic acid concentration from simultaneous saccharification and fermentation (SSF) assays of the experimental design.

Chapter IV

Table A4.1. List of plasmids used in this work.

Plasmid name	Description	Reference
pGRE3	Episomal plasmid for *GRE3* overexpression under *TEF1* promoter, *KanMX* selection marker	(39)
pCfB2312	Episomal plasmid for Cas9 expression under *TEF1* promoter, *KanMX* selection marker	(108)
pCfB2909	EasyClone-MarkerFree Integrative Vector for XII-5 insertion site (Chr XII: 839226..840357)	(43)
pCfB3050	gRNA helper vector targeting XII-5 insertion site, *NatMX* selection marker	(43)
p2909_TEF-1_GRE3	MarkerFree integrative vector, XII-5, P_{TEF1}-*GRE3*	This study

Table A4.2. List of primers used in this work.

Primer name	Sequence	Application
TEF1_GRE3_FW	AGTGCAGGUAAAACATTAAATAACAATGCATAC	amplification of the *GRE3* gene for cloning into integrative vector
TEF1_GRE3_RV	CACGCGAUTCAGGCAAAAGTGGGG	
PR-899	CCACCGAAGTTGATTTGCTT	verification of site XII-5 chromosomal integration
PR-900	GTGGGAGTAAGGGATCCTGT	
PR-2220	CCTGCAGGACTAGTGCTGAG	
PR-2221	GTTGACACTTCTAAATAAGCGAATTTC	

Overhangs used for USER cloning are underlined.

Table A4.3. Chemical composition of the winery wastes used in this work.

a. Chemical composition of grape must and wine lees[1]		
	Grape must (GM)	**Wine lees (WL)**
Component (g/L)		
Sucrose	n.d.	n.d.
Glucose	111	n.d.
Fructose	116	n.d.
Protein	4.3	76.3
Total nitrogen - Kjeldahl	0.69	12.2
Phenolic compounds	0.15	1.51
Ethanol	n.d.	99.3
Density (g/mL)	1.09	1.05
b. Chemical composition of vine shoots (g of component/100 g of vine shoot)		
Glucan	32.02 ± 0.13	
Xylan	13.72 ± 0.06	
Arabinan	0.15 ± 0.06	
Acetyl groups	3.55 ± 0.04	
Klason Lignin	22.12 ± 0.07	
Extractives	10.24 ± 0.06	

n.d.: not detected.

[1] Data reported by (Hijosa-Valsero et al., 2021a)

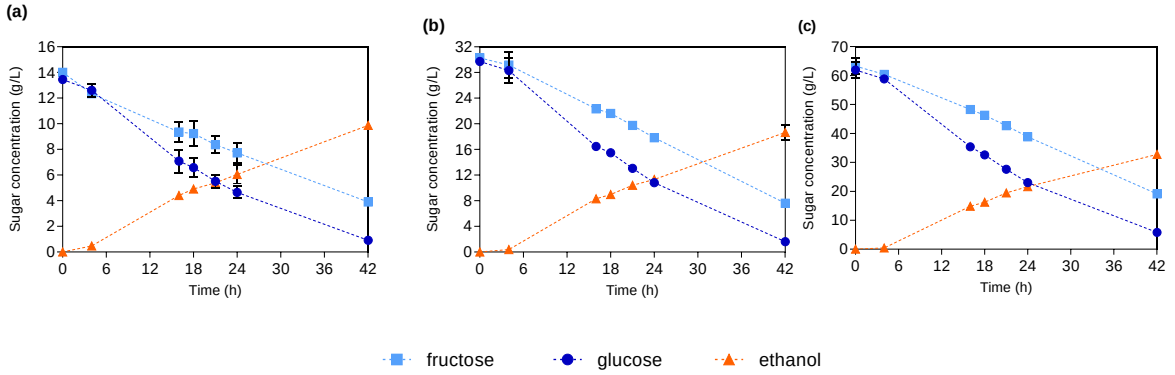


Figure A4.1. Sugar consumption of the *S. cerevisiae* PE-2-GRE3-XII-5 with (a) 10% (b) 25% and (c) 50% of grape must.

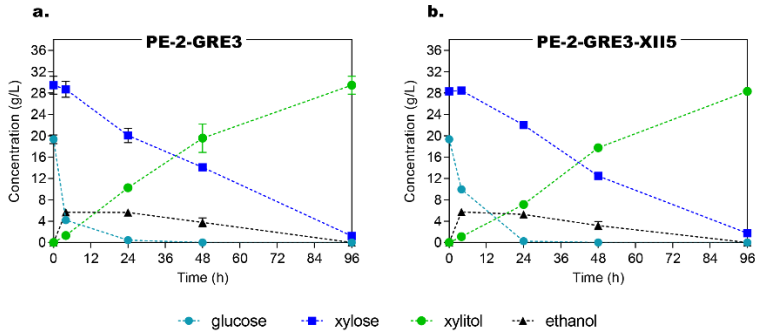


Figure A4.2. Performance of the *S. cerevisiae* (a) PE-2-GRE3 overexpressing the *GRE3* gene in an episomal vector and (b) the genome engineered PE-2-GRE3-XII5 by the time course of xylose and glucose consumption as well as xylitol and ethanol production.

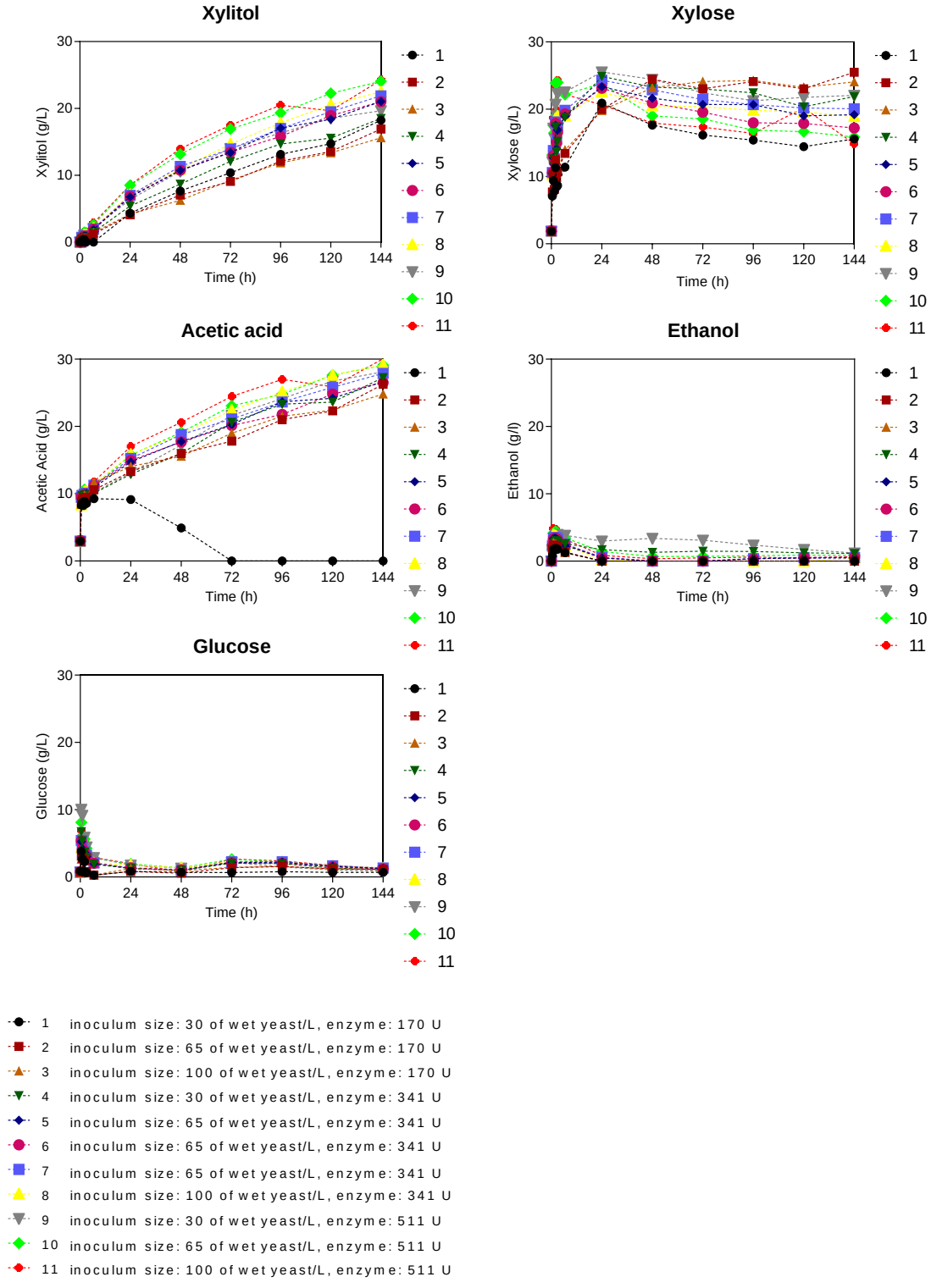


Figure A4.3. Time course of xylitol, xylose, ethanol and glucose concentrations from simultaneous saccharification and fermentation (SSF) assays of the experimental design.

Chapter V

Table A5.1. Strains and plasmids used in this work.

***S. cerevisiae* strains**	**Relevant Genotype/features**	**Reference**
CEN.PK 113-7D	MATa;	(44)
PE-2	Diploid; Isolated from Brazilian bio-ethanol plants	(45)
CAT-1	Diploid; Isolated from Brazilian bio-ethanol plants	(46)
CA11	Diploid; isolated from Brazilian "cachaça" fermentation processes	(46)
CEN.PK-pGRE3	CEN.PK 113-5D, pGRE3	(41)
PE-2-pGRE3	PE-2, pGRE3	(41)
CA11-pGRE3	CA11, pGRE3	This work
CAT-1-Pgre3	CAT-1, pGRE3	This work
PE-2-GRE3-XII5	PE-2, p2909_TEF-1_GRE3	(47)
PE-2-GRE3-XII5-GAL2	PE-2, p2909_TEF-1_GRE3, pGAL2	This work
CA11-GRE3-XII5	CA11, p2909_TEF-1_GRE3	This work
CAT-1-GRE3-XII5	CAT-1, p2909_TEF-1_GRE3	This work
Plasmids		
pGRE3	Episomal plasmid for *GRE3* overexpression under *TEF1* promoter, *KanMX* selection marker	(41)
pCfB2312	Episomal plasmid for Cas9 expression under *TEF1* promoter, *KanMX* selection marker	(48)
pCfB2909	EasyClone-MarkerFree Integrative Vector for XII-5 insertion site (Chr XII: 839226.840357)	(42)
pCfB3050	gRNA helper vector targeting XII-5 insertion site, *NatMX* selection marker	(42)
p2909_TEF-1_GRE3	MarkerFree integrative vector, XII-5, P_{TEF1}-*GRE3*	(47)
pGAL2	Episomal plasmid for *GAL2* overexpression under *TEF1* promoter, *KanMX* selection marker	This work

Table A5.2. Primers used for cloning steps in this study. For primers sequences, underlined lower case indicate addition of homologous recombination for plasmid assembling.

Primers	Sequence	Amplification
TEF1_GRE3_FW	agtgcaggu**AAAACATTAAATAACAATGCATAC**	amplification of the *GRE3* gene for cloning into integrative vector
TEF1_GRE3_RV	cacgcgau**TCAGGCAAAAGTGGGG**	
PR-899	CCACCGAAGTTGATTTGCTT	verification of site XII-5 chromosomal integration
PR-900	GTGGGAGTAAGGGATCCTGT	
PR-2220	CCTGCAGGACTAGTGCTGAG	
PR-2221	GTTGACACTTCTAAATAAGCGAATTTC	
SB-6	CGCGCCATCTGTGCAGACAA	Amplification of pGRE3 backbone for construction of pGAL2
SB-7	TTTCACTAGTGAGAAAGTGGGCAACCTG	
gal2_IF_rv	TTGTCTGCACAGATGGCGCGttattctagcatggccttgtacca	Amplification of GAL2 from CENPK.113-5D for construction of pGAL2
GAL2_IF_fw	CCACTTTCTCACTAGTGAAAatggcagttgaggagaacaatatgcctg	

For primers sequences, underlined lower case indicate addition of homologous recombination for plasmid assembling.

Chapter VI

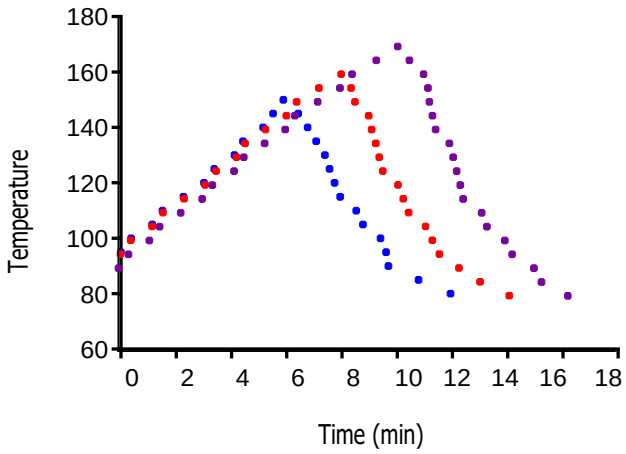


Figure A6.1. Temperature profiles of autohydrolysis of *G. sesquipedale* performed at the 150 °C (blue) 160 °C (red) and 170 °C (purple).