Attempts to develop a xylitol production process using *Paraburkholderia sacchari*

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Biological Engineering

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Preface

The work presented in this thesis was performed at the Institute for Bioengineering and Biosciences of Instituto Superior Técnico (Lisbon, Portugal), during the period September 2019 - June 2020, under the supervision of Dr. Mª Teresa Cesário and Professor Mª Manuela Fonseca.

I declare that this document is an original work of my own authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the *Universidade de Lisboa*.

To my family and friends

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Abstract

Paraburkholderia sacchari is able to accumulate poly-3-hydroxybutyrate (P(3HB)) and produce xylitol and xylonic acid in the presence of *D*-xylose. Xylitol is a good alternative sweetener to sucrose as it is less caloric and has anti-cariogenic properties. Xylonic acid is a versatile chemical compound capable of replacing gluconic acid. Gluconic acid is a compound derived from glucose, an edible sugar whose price has increased in recent years.

The focus of this study was the production of xylitol from D-xylose by *P. sacchari*. Fed-batch cultivations in bench-scale stirred-tank bioreactors were carried out aiming to determine the operational conditions leading to high xylitol productivities. Cultivations carried out with xylose concentrations above 120 gL^{-1}, dissolved oxygen (DO) of 1% saturation and a stable pH of 6.8 promoted the production of xylonic acid, attaining 345 gL^{-1}, a yield of 0.99 g_{XylAc}/g_{Xyl} and a productivity of 2.10 $gL^{-1}h^{-1}$. Xylitol production in those conditions was practically inhibited, opposite to what was expected based on previous studies. Comparison of automatically acquired data from similar bioreactor cultivations with disparate results revealed that the pH has a huge impact on the productivities of xylitol. Cultivations carried out in the same conditions as described above but with oscillating pH values between 6.8 and 8 led to a xylitol concentration of 104 gL^{-1}, resulting in a xylitol yield of 0.38 g_{XyOH}/g_{Xyl} and a productivity of 0.55 $gL^{-1}h^{-1}$.

Keywords

Paraburkholderia sacchari; *Burkholderia sacchari*; *D*-xylose; *D*-xylitol; *D*-xylonic acid; Poly-3-hydroxybutyrate (P(3HB)); Biorefinery

Resumo

Paraburkholderia sacchari é capaz de acumular poly-3-hydroxybutyrate (P(3HB)) e produzir xilitol e ácido xilónico na presença de *D*-xilose. Xilitol é um bom adoçante alternativo à sacarose, visto ser menos calórico e ter propriedades anti-bacterianas. O ácido xilónico é um composto químico versátil capaz de substituir o ácido glucónico. O ácido glucónico é um composto derivado da glicose, um açúcar usado na alimentação cujo o preço aumentou nos últimos anos.

O foco deste trabalho foi a produção de xilitol a partir de D-xilose pela *P. sacchari*. Foram realizadas culturas em Fed-batch em biorreatores de tanque agitado em escala de bancada, com o objetivo de determinar as condições operacionais que levam a altas produtividades de xilitol. Culturas realizadas com concentrações de xilose acima dos 120 gL^{-1}, oxigénio dissolvido (DO) com 1% de saturação e pH a 6.8 promoveram a produção de ácido xilónico, atingindo 345 gL^{-1}, um rendimento de 0.99 g_{XylAc}/g_{Xyl} e uma produtividade de 2.10 $gL^{-1}h^{-1}$. A produção de xilitol nessas condições foi praticamente inibida, contrariamente ao esperado com base nos estudos anteriores. A comparação de dados adquiridos automaticamente durante a fermentação, entre fermentações semelhantes mas com resultados díspares mostraram que o pH tem um grande impacto na produtividade do xilitol. Culturas realizadas nas mesmas condições descritas anteriormente, mas com valores de pH oscilantes entre 6.8 a 8, levaram a uma concentração de xilitol de 104 gL^{-1}, resultando num rendimento de 0.38 g_{XyOH}/g_{Xyl} e uma produtividade de 0.55 $gL^{-1}h^{-1}$.

Palavras-chave

Paraburkholderia sacchari; *Burkholderia sacchari;* *D*-xilose; *D*-xilitol; ácido *D*-xilónico; Poly-3-hidroxibutirato (P(3HB)); Biorrefinaria

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List of Abbreviations

ABC	ATP-binding cassette
Acetoacetyl-CoA	Acetoacetyl-coenzyme A
Acetyl-CoA	Acetyl-coenzyme A
AOM	Acute otitis media
C. necator	Cupriavidus necator
CAGR	Compound annual growth rate
CCR	Carbon catabolite repression
CDW	Cell dry weight
DO	Dissolved oxygen
FDA	Food and Drug Administration
GC	Gas chromatography
GO	Glucose oxidase
HPLC	High Performance Liquid Chromatography
IS	Internal standard
KDY	2-keto-3-deoxyxylonate dehydratase
LB	Lignocellulosic biomass
MFS	Major facilitator superfamily
$NAD(P)^+$	Nicotinamide Adenine Dinucleotide Phosphate (oxidized form)
NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NAD^+	Nicotinamide Adenine Dinucleotide (oxidized form)
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
OD	Optical density
P	Product
P(3HB)	Poly-3-hydroxybutyrate
P. sacchari	*Paraburkholderia sacchari*
PHA	Poly-hydroxyalkanoate
RI	Refraction index
S	Substrate
SM	Seeding medium
STR	Stirred-tank reactor
TCA	Tricarboxylic acid cycle
UV	Ultraviolet
X	Biomass
XDH	Xylose dehydrogenase

XDY	Xylonate dehydratase
XI	Xylose isomerase
XK	Xylulokinase
XlyE	Low-affinity transporter
X_{OH}DH	Xylitol dehydrogenase
XR	Xylose reductase
Xyl	D-xylose
XylAc	D-xylonic acid
XylFGH	High-affinity transporter
XyOH	D-xylitol
YE	Yeast extract

List of Software

Microsoft Word 2019	Programmes Abstract
Microsoft Excel 2019	Calculation and graphical software
BioCommand	Batch control and data acquisition

Chapter 1

Introduction

This chapter gives a brief introduction to the thesis. In Section 1.1 an overview of the state of the current biorefinery concept and how this thesis is in the scope of this concept is given. Section 1.2 states the main purpose of the thesis.

1.1 Overview

Our society is currently experiencing serious environmental problems, which are aggravated by the over-populated state of our planet. The human population has become an undisputable force that has a deteriorating effect on both human and environmental health [SIN,2016]. Population growth, the rapid development of industries for economic growth, rapid urbanization, and the rise in living standards lead to continuous growth in global resource consumption, depleting the amount of natural resources available on the planet [SON,2015], [Z1,2009]. Moreover, the evolution of production processes has transformed them into complex systems that mainly use composite and hazardous materials, producing waste from mixed sources, which difficult sustainability management [ZAM,2015].

In the past few years, society is trying to embrace new measures in order to become more sustainable and confront the waste generation problem. In this context, new and sustainable raw resources for food, materials and energy production are being sought. The zero waste (ZW) concept has been highly embraced to stimulate sustainability regarding production and consumption as well as optimum recycling and resource recovery while restricting mass incineration and landfilling [ZER,2009]. Within this concept, the material flow is circular, which means no materials are wasted. If at their end of lives products cannot be reused or repaired, they can instead be recycled or recovered from the waste stream and used as inputs, substituting the demand for the extraction of natural resources [SON,2015].

One solution that has been developed, is to use biowaste as a new resource to produce valuable products. This concept can be included under the “biorefinery” umbrella, where biomass based wastes are transformed into useful bio-compounds. This biorefinery vision will contribute to sustainability not only by its inherent dependence on sustainable bioresources but also by recycling wastes. These compounds can range from bulk products (eg. bioenergy) to speciality chemicals and biodegradable polymers [VER,2019].

Lignocellulosic wastes, which are generated by agricultural and forestry processes, are abundant and cheap. Their use as feedstocks avoids their accumulation in the soil, which could cause serious environmental problems. Moreover, from an economic standpoint, the use of lignocellulosic biomass (LB) as raw material is also promising. In fact, LB is a nonedible feedstock and the worldwide most abundant renewable raw material in consequence of its renewable nature, since it is formed from available atmospheric CO_2, water and sunlight by photosynthesis. It comprises different fractions such as carbohydrates, proteins, and fats that can be converted, through the implementation of the biorefinery concept, to value-added products such as fuels and chemicals [CES,2015]. However, lignocellulosic materials have a resistance to enzymatic and chemical degradation, which hamper the transformation of these materials into sugar-rich hydrolysates. In order to hydrolyze LB, catalytic techniques are necessary, which increases the cost of these raw materials compared to the petroleum derivates. However, in the future, the use of LB raw materials will certainly be the most feasible alternative in our society [ISI,2015].

As mentioned by [RAP,2017], Para*burkholderia sacchari* was used to produce polyhydroxyalkanoate (PHA), xylonic acid and xylitol using lignocellulosic hydrolysate as substrate. Although the accumulation of PHA has already been studied by [CES,2015], the metabolism of xylonic acid and xylitol was discovered later, adding more potential value to these lignocellulosic wastes. This species, recently reclassified as *Paraburkholderia sacchari* [DOB,2016], is a non-model, Gram-negative polyhydroxyalkanoate-accumulating bacterium, isolated from the soil of sugarcane crops in Brazil [BRA,2001].

Paraburkholderia sacchari is an interesting bacterial option for industrial bioprocesses since it is capable of consuming several sugars like glucose, xylose, sucrose, arabinose and other simple sugars that are normally present in lignocellulosic hydrolysates. Additionally, and importantly, it is non-genetically modified and classified in “Safety Level 1”.

Poly-3-hydroxybutyrate (P(3HB) belongs to the PHA family, composed of four-carbon monomers of 3-hydroxybutyric acid. These insoluble energy-storage compounds are accumulated by many microbial strains under unbalanced conditions such as limitation of an essential nutrient like phosphorus or nitrogen, or the presence of excess carbon source [VER,2007]. The properties of these biopolymers are similar to conventional plastics such as polypropylene [TSZ,2005]. However, important characteristics of PHAs are their biodegradability, since there are micro-organisms that can degrade PHAs, and their biocompatibility as they have no toxic effect towards living organisms.

D-xylonic acid is a versatile chemical compound, one of the 30 most promising platform chemicals identified by the US Department of Energy [LIU,2012], capable of replacing gluconic acid in most of its applications. D-xylitol is a good alternative sweetener thanks to its beneficial properties concerning human health like its anti-cariogenic properties [TAN,1995]. It also contains fewer calories when compared to sucrose [MOH,2015].

As previously studied by [BON,2018], xylitol production by *P. sacchari* is enhanced after subjecting the bacteria to an inhibitory xylose concentration, after attaining the required growth. However, it is still not certain which metabolic pathway *P. sacchari* uses to metabolize xylose into xylitol, so the culture conditions to achieve optimal xylitol production conditions are currently under study.

1.2 Objective

This work aims at investigating the process operation conditions which channel the metabolism of xylose by Paraburkholderia sacchari towards xylitol.

Chapter 2

State of the Art

This chapter provides an explanation and overview of the current state of several concepts associated with this work. In section 2.1, a brief overview of the biorefinery concept and its current state is provided. In section 2.2 the different lignocellulosic biomass used as raw materials for biorefinery processes is explained. An in-depth analysis of the xylose metabolic network, as well as the specific *P. sacchari's* metabolic network, is provided in section 2.3. Section 2.4 contains a brief history of sweeteners and focuses on the physicochemical properties of xylitol, its applications, different production approaches and respective economical evaluation.

2.1 Biorefinery concept

A great fraction of worldwide energy and material products come from fossil fuel refinery. However, the feasibility of oil exploitation needs to decrease soon. The environmental concerns of fossil resources, due to excessive pollution and consequently global warming, their on-going price increase, and their uncertain availability, thanks to their non-sustainability, come as reasons to invest in alternative solutions able to mitigate climate change and reduce the consumption of fossil fuels. The replacement of oil with biomass as raw material for fuel and chemical production is an interesting option and the driving force for the development of biorefinery plants. This strategy needs a large investment in order to achieve the sustainability goals of our society. The biorefinery concept can be explained as the processing of renewable biomass into a spectrum of fuels and valuable products [CHE,2010].

Biomass can be described as a rich carbon renewable raw material that can substitute fossil based raw materials in the energy and chemical products industries. In biorefinery, almost all the types of biomass, that come from forest residues, waste food crops, food processing, animal farming or human wastes can be converted to different classes of biofuels and biochemicals through jointly applied conversion technologies. These products can be intermediates and/or final compounds in food, feed, materials, chemicals and the energy production results in fuels, power, heat, etc [MON,2016].

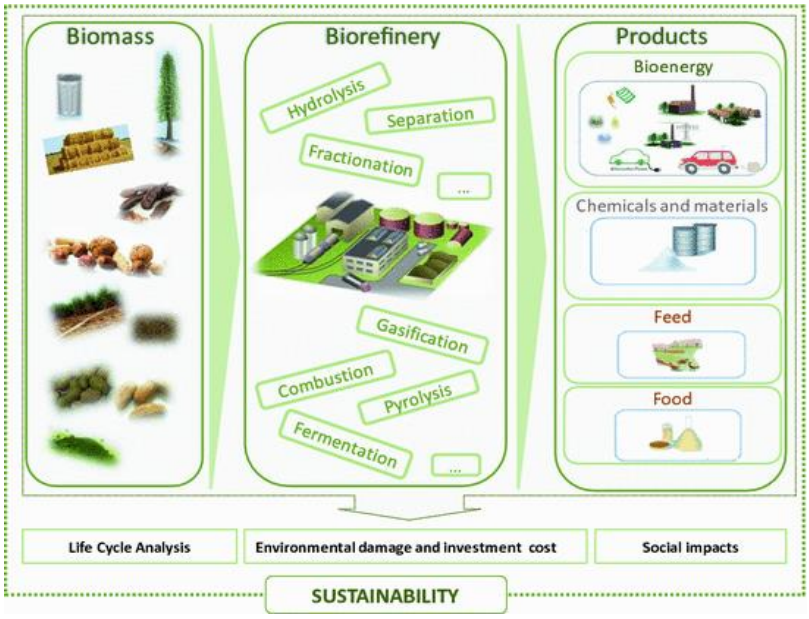

Figure 2.1: Biorefinery concept schematics based on [SUN,2002]

Lignocellulosic biomass (LB) is the most economical and highly renewable natural resource in the world. Besides wood, this raw material includes corn stover, straw, wheat stover, algae and others. The primary components in lignocellulosic biomass are cellulose, hemicellulose and lignin. Compositions differ for different types of biomass, however, cellulose is usually its major component. Lignocellulosic biomass is considered unfermentable because most microbes cannot degrade it. In fact, in LB the sugars are locked in a recalcitrant structure that requires a pretreatment step to release them, which, greatly increases the cost of these raw materials. Pretreatment methods include mechanical, hydrothermal, biological, chemical, ammonia or supercritical CO_2 explosion and ionic liquid extraction [ZHO,2014]. Nevertheless, LB raw materials will most probably be the most feasible alternative thanks to their unique eco-friendly nature, since they are formed from available atmospheric CO_2, water and sunlight through

biological photosynthesis. Moreover, they are carbon sources which are nonedible feedstocks.

Currently, more than 40 lignocellulosic biorefineries are operating across Europe, increasing the turnover of the total bioeconomy in this last few years [PIO,2016]. Examples of potential bio-based products include biofuels (e.g., bioethanol, biodiesel, and biogas), biochemicals (e.g., industrial enzymes and nutraceuticals), and biomaterials (e.g., biodegradable plastics). However, supported by specific EU policies, bioenergy and biofuels have received greater attention. By the year 2030, the EU aims to provide 25% of its transportation energy via biofuels derived from advanced biorefineries (second-generation biorefineries). By this time, it also intends to replace 30% of oil-based chemicals with bio-based chemicals and supplant nondegradable materials with degradable materials [HAS,2019].

While this industry faces significant challenges, such as limitations of conventional processing technologies, feedstock logistics, and uncertain market economics, ambitious policies from all over the world are being implemented in order to support this industry to achieve climate and bioenergy goals.

2.2 Lignocellulosic materials

One challenge that is common to all industries, and crucial to the success of the business, is to assure abundant and cheap raw materials, in order to produce products with a competitive market price. In the case of bioprocesses, the raw material in question is namely the carbon source. Biomass and biomass-derived materials have been pointed out to be one of the most promising alternatives. These materials are generated from atmospheric CO_2, water and sunlight through biological photosynthesis. Therefore, biomass has been considered to be the only sustainable source of organic carbon and the perfect equivalent to petroleum for the production of fuels and chemicals. Lignocellulosic biomass, which is the most abundant and bio-renewable biomass on earth, has an important role [ZHO,2011].

Forestry, agricultural and agro-industrial lignocellulosic wastes are accumulated every year in large quantities, causing serious environmental problems when they are disposed to the soil or landfill. Plus, they often fuel forest fires. However, they could be utilized for the production of several value-added products [ISI,2015]. Lignocellulosic wastes are an abundant and cheap raw material available to such bioprocesses, with the advantage that they are not a source of human food or animal feed, since they correspond to the non-edible part of the plant, resulting in a non-competitive raw material with the food industries [SUN,2002]. With this renewable raw material, production costs of bioprocesses are greatly reduced, as well as have a positive environmental impact supporting the sustainability ideals.

Figure 2.2: Examples of lignocellulosic feedstock, from left to right is corn stover, wood debris and sugarcane bagasse.

Lignocellulosic biomass is mainly composed of three polymers, cellulose, hemicellulose and lignin together with small amounts of other components like acetyl groups, minerals and phenolic substituents. Depending on the type of lignocellulosic biomass, these polymers are organized into complex non-uniform three-dimensional structures to different degrees and varying relative composition [ISI,2015].

The major component of lignocellulosic biomass is cellulose. Since about half of the organic carbon in the biosphere is present in the form of cellulose, the pre-treatment of LB for its depolymerization into glucose and posterior conversion into fuels and valuable chemicals has crucial importance [ZHO,2011].

Hemicellulose is the second most abundant polymer which is mainly arabinoxylans composed of *D*-xylose and *L*-arabinose, or other sugars [CHA,2018]. Hemicelluloses are embedded in the plant cell walls to form a complex network of bonds that provide structural strength by linking cellulose fibres into microfibrils and cross-linking with lignin

[AGB,2011]. Just like cellulose, hemicellulose pre-treatment is a difficult and crucial step thanks to the great resistance to enzymatic and chemical degradation offered by lignin.

The LB pre-treatment usually involves an aggressive treatment and expensive procedure which greatly increases the cost of these raw materials and consecutively the price of the final products. These pre-treatment methods are divided into different categories such as mechanical, chemical, physicochemical and biological methods or various combinations of these [BAR,2013]. Through research and development, pretreatment of lignocellulosic biomass has great potential for the improvement of efficiency and lowering the cost of production. The integration of various biomass pretreatment methods with other processes like enzymatic saccharification, detoxification, fermentation of the hydrolysates, and recovery of products will greatly reduce the overall cost of using lignocellulose for practical purposes [SAH,2005][ISI,2015].

Besides the difficulties associated with the LB hydrolysis, there is another factor associated with the use of sugar mixtures and thus with lignocellulosic hydrolysates. Many microbial strains have a regulatory mechanism, carbon catabolic repression (CCR), that prevents expression of the genes needed for catabolism of other carbon sources, usually pentoses, while the substrate that enables the fastest growth (normally glucose) is present [RAP,2017]. This behaviour is found in *E. coli,* where several exponential phases separated by lag phases can be observed when a sugar mix is supplied as a carbon

source to the cultivation, indicating a sequential sugar consumption [ROJ,2010]. Regarding *P.sacchari*, as observed by [CES,2014], when the cells are grown in a medium containing glucose, xylose and citric acid, the uptake of glucose and xylose only takes place when the citric acid has been completely consumed. *P. sacchari* can co-metabolize xylose and glucose, but the rate of xylose consumption decreases substantially in the presence of glucose [CES,2014],[RAP,2017].

2.3 Xylose metabolic network

Xylose is a very common sugar in residual lignocellulosic biomass being the second major sugar found in most lignocellulosic hydrolysates and the major sugar in hemicellulosic hydrolysates. For that reason, xylose is a very promising carbon source and it makes sense to understand the fundamentals of the mechanism used by *P. sacchari* to metabolise xylose into high-value by-products, such as xylitol. A review of the metabolic reactions involved in xylose metabolism by bacteria was carried out. The metabolic network for xylitol production will be discussed in detail since this metabolite is the focus of this work. However, to promote the xylitol metabolic pathway, the metabolic networks for P3HB and xylonic acid need to be addressed and inhibited to attain high xylitol yields. The xylose metabolic network scheme is represented in Figure 2.3. This section aims to provide a context for the various conditions chosen for the cultivation that will be discussed later in the text.

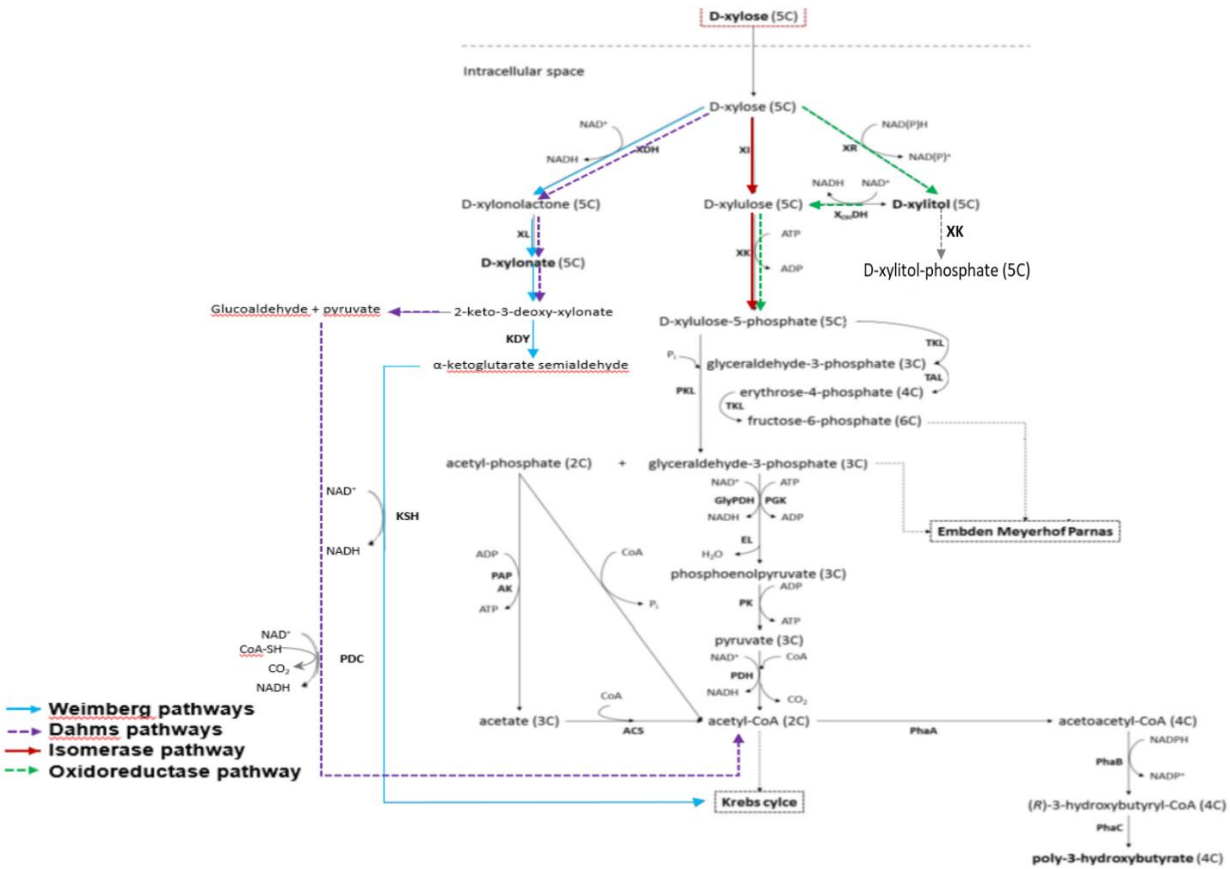

Figure 2.3: Four pathways of xylose metabolization by microorganisms: solid blue, Weinberg pathway; dashed violet, Dahms pathway; dashed green, oxidoreductase pathway; solid red, isomerase pathway. Partially taken from Master's thesis of [PAL,2016] and modified according to the literature review [MCC,2017]. The enzymes are abbreviated as follows: xylose isomerase (XI); xylulokinase (XK); xylose reductase (XR); xylitol dehydrogenase ($X_{OH}DH$); xylose dehydrogenase (XDH); xylonolactonase (XLS); transketolase (TKL); transaldolase (TAL); phosphoketolase (PKL); glyceraldehyde-3-phosphate dehydrogenase (GlyPDH); 3-phosphoglycerate kinase (PGK); enolase (EL); pyruvate kinase (PK); pyruvate dehydrogenase complex (PDH); acetate kinase (AK); pyrophosphate-acetate phosphotransferase (PAP); acetyl-CoA synthase (ACS); β-kethiolase (PhaA); acetoacetyl- CoA reductase (PhaB); PHA synthase (PhaC); xylonate dehydratase (XDY); 2-keto-3-deoxyxylonate dehydratase (KDY); α-ketoglutaric semialdehyde dehydrogenase (KSH); pyruvate dehydrogenase complex (PDC) [BON,2018].

2.3.1 Xylose transportation into the cell

In order to transport sugars and other nutrients in and out of the cells, various organisms can use different types of transport mechanisms. Regarding D-xylose, yeast and fungi can use facilitated diffusion or active transport, while bacteria tend to use active transport mechanisms [MCC,2017]. These types of mechanisms are enabled by carrier proteins, and hence exhibit the properties of specific inhibition, substrate specificity and saturability. These processes enable sugar transportation against a

concentration gradient at the expense of metabolic energy. The energy required can be provided by the hydrolysis of adenosine triphosphate (ATP), establishing a membrane potential as in the chemiosmotic energization mechanism, or by the transfer of phosphate from phosphoenolpyruvate (PEP) to the sugar substrate as in the group translocation mechanism [JEF,1983].

As reported by [MCC,2017], bacteria such as *E. coli, Clostridia*, *Lactococci*, and *Bacilli* use active transport for the uptake of xylose into the cell. Usually, there is a high and low-affinity transporter route. In E. coli, the most studied species, the high-affinity transporter (XylFGH) belongs to the ATP-binding cassette (ABC) family, while the low-affinity transporter (XlyE) belongs to the major facilitator superfamily (MFS), which acts as proton symporter making it pH-dependent [MCC,2017]. However, at least two mechanisms of repression were reported, including the CRP-dependent control of *xyl* genes, and the presence of arabinose, since the transporters allow the transportation of this sugar at lower efficiencies [DES,2010]. Both of these repression mechanisms lead to the preferential substrate being consumed first.

2.3.2 *P. sacchari's* xylose metabolic network

When xylose is consumed, there are four possible metabolization pathways. The isomerase pathway, which converts xylose into xylulose, typically used by prokaryotes. The oxidoreductase pathway, which is mostly present in eukaryotic microorganisms, where a reductase converts xylose into xylitol and a dehydrogenase converts xylitol into xylulose. Finally, the oxidative pathways, called Weimberg and Dahms pathways, also recognized as the non-phosphorylative way.

A couple of studies with the intent of encoding the *P. sacchari* genome were conducted. [CHE,2014] reported that the metabolic pathway used by *P. sacchari* is the isomerase pathway since genes present in its genome include those that encode for xylose isomerase (XI), xylulokinase (XK) and ABC xylose transporter. Regarding the Weimberg pathway, some of the genes involved in this pathway were found to have homologous genes in *P. sacchari*. However, the gene encoding for 2-keto-3-desoxy-D-xylonate dehydratase (KDY), responsible for converting 2-keto-3-desoxy-D-xylonate into α-ketoglutarate semialdehyde was not found. This could explain the accumulation of xylonic acid by *P. sacchari* reported by [RAP,2017]. The authors also reported that the genes of the oxidoreductase and Dahms routes were not found, suggesting that *P. sacchari* can not assimilate xylose by these pathways [CHE,2014].

Regarding the isomerase pathway, after the isomerization of xylose into xylulose catalysed by XI, xylulose is phosphorylated to xylulose-5-phosphate by XK. The degradation continues towards PPP pathway, which consists of several reversible transketolase (TKL) and transaldolase (TAL) reactions. The primary function is to provide NAD(P)H, which is used primarily for reducing power in biosynthetic reactions [MOA,2003]. The pathway has an oxidative and a non-oxidative phase which converts hexose phosphate to pentose phosphate and back to hexose phosphate [JEF,1983]. Carbon exits the sugar-phosphate pool by various routes. One of them is responsible for the formation of pyruvate. The pyruvate suffers an oxidative decarboxylation to form acetyl-coenzyme A (acetyl-CoA) which then suffers oxidation via the Krebs cycle (KC) to generate energy.

Instead of participating in the Krebs cycle, acetyl-CoA molecules have the option to go in a different route which leads to P(3HB) accumulation [JEF,1983]. The capacity of *P. sacchari* to accumulate P(3HB) led to numerous studies trying to optimize the production of this polymer, thanks to its biodegradability nature and its sustainable wise production.

2.3.3 Metabolic pathway to xylitol production

The most common way that organisms use to produce xylitol is through a single-step mechanism where xylose is reduced to xylitol by xylose reductase (XR) while the cofactors NADH/NADPH suffer oxidation. However, if there is a large amount of the cofactor NAD^+ in the medium, the enzyme xylitol dehydrogenase converts xylitol into xylulose, which then follows the path to PPP pathway. As stated by [QI,2016], and represented in Figure 2.4, the dehydrogenation reaction is reversible, and NADH oxidation can occur. Therefore, xylitol can be produced through two ways: through the reduction of xylose by the activity of XR, and through the isomerase pathway where $X_{OH}DH$ converts xylulose into xylitol.

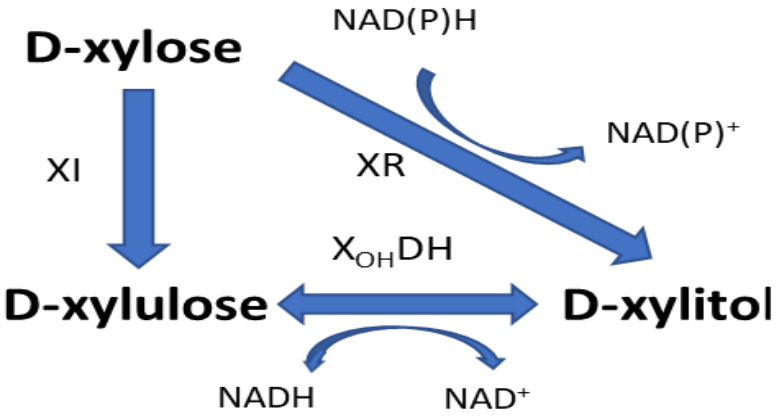

Figure 2.4: Xylose network metabolism related to xylitol production, the metabolite of interest of this work, based on literature review ([JEF,1983]). The enzymes are abbreviated as follows: xylose isomerase (XI); xylitol dehydrogenase ($X_{OH}DH$); xylose reductase (XR).

As reported by [WIN,1998], both XR and $X_{OH}DH$ enzyme activities are dependent on the concentration of xylose in the culture medium, since xylitol formation does not occur in the absence of this pentose. The influence of xylose concentration on xylitol production by *P. sacchari* was studied by [BON,2018], and it was verified that the emancipation of xylitol production only takes place under inhibitory xylose concentrations (above 120 gL^{-1}) in the medium. A possible explanation for this could be that the xylitol formation pathway is the quickest route for the cell to convert xylose and thus counteract the osmotic pressure of the extracellular medium, as can be seen in Figure 2.3.

Another key factor on the xylitol production is the aeration rate in the culture since this is directly related to the regeneration of the cofactors NAD^+/NADH or $NADP^+$/NADPH. Under aerobic conditions, the NADH formed during xylose metabolism can be reoxidized into NAD^+ in the electron transport system, and as a consequence, xylitol is not produced, since the high NAD^+/NADH ratio favours xylitol oxidation to xylulose. Under anaerobic conditions, microorganisms are unable to metabolize D-xylose because of redox imbalance between NAD^+ and NADH. Under oxygen-limited conditions, the electron transport system is unable to oxidize intracellular NADH completely, increasing the NADH concentrations, that subsequently favours the xylulose conversion into xylitol by the X_{OH}DH enzyme or the reduction of xylose into xylitol by the XR enzyme [RAF,2013], [MOH,2015].

However, other authors claim that an increase of the amount of dissolved oxygen (DO), also increases the quantity of xylitol produced, since the activity of the enzymes also depend on oxygen availability and not only on the regeneration of cofactors [GIR,1994].

2.4 Xylitol

2.4.1 History of sweeteners

The sensation of sweetness has undoubtedly been important to humanity throughout his entire existence. Since culinary practice appeared in our culture, and indeed continuing to the present day, “sweetness” and “sweetener” have for most people meant the respective taste and functional use of sucrose, which in turn has simply been referred to as “sugar.” However, there are numerous substances which have the property of sweetness and hence have the potential to be used as sweeteners. The various potential sweeteners can have different properties in addition to the sweetness which can have an important factor in their characteristics and functionality [HYV,1982].

However, from the nutritional and health point of view, there also exist important characteristics that can differ from different sweeteners, such as reducing the amount of energy which the sweetening component brings into the food system, avoidance of too rapidly absorbed carbohydrates, or reducing the exposure to types of food which are known to cause dental decay. Thanks to these other requirements, there has been an intensive search for suitable alternative sweeteners. The search led to the discovery of many sweeteners which hold promise in fulfilling some of the divergent special sweetening needs currently being developed and commercialized. One sweetener that has been highlighted from the special dietary applications standpoint, is xylitol, particularly in the areas of noncariogenic confections and disturbances of carbohydrate metabolism [HYV,1982].

2.4.2 The occurrence and history of xylitol

Xylitol is a five-carbon sugar alcohol that occurs widely in nature. It is found not only in fruits and vegetables but in seaweeds and some microorganisms as well. The content of xylitol in fruits and vegetables is usually low, and thus it is uneconomical to extract large amounts of xylitol from such sources [CHE,2010]. Xylitol was also found as a normal metabolic intermediate in mammalian carbohydrate metabolism, including that of human [HOL,1957][HYV,1982].

Xylitol was first prepared as a syrup almost simultaneously in the laboratories of Bertrand (1891) and Fischer and Stahel (1891). Years later, Wolfrom and Kohn (1942) were the first to succeed in obtaining crystalline xylitol upon hydrogenation of highly purified xylose. [CHI,1958] were the fists ones to obtain xylitol through a biological process, by using the fungi *Penicillium chrysogenum* to convert xylose into xylitol. Since xylitol was found to be a normal intermediate in carbohydrate metabolism, there has been an ever-increasing volume of knowledge about its metabolic behaviour as well as its use as a sweetener in current cuisine.

2.4.3 Physicochemical properties of xylitol

Xylitol is a sugar alcohol with the molecular formula $C_5H_{12}O_5$ with a molar mass of 152.15 g mol^{-1}. The structural formula of this polyol is represented in Figure 2.5. Xylitol is a meso compound completely lacking in optical activity in solution [HYV,1982]. In its solid state forms a white crystalline powder with no odour. It has a boiling point of 216 ºC and a melting point of 93.5 ºC. This compound is very soluble in water (642 mg/mL), pyridine and ethanol [LID,2007]. Since xylitol is not utilized by most microorganisms, it is usually safe from fermentation and microbial spoilage. It has a prolonged shelf life thanks to its heat stability, although caramelization can occur if it is subjected to temperatures near its melting point for a few minutes [ROW,2009]. The legitimacy for including polyols in the sugar field results from the fact that polyols are formed from sugars and can be converted to sugars (i.e. aldoses and ketoses) [MAK,1989]. Some chemical libraries outline sugars as crystalline, sweet carbohydrates, so sugar alcohols fall in this class [BAR,2016].

Figure 2.5: Structural formula of *D*-xylitol (left); Xylitol in its crystalline form (right)

2.4.4 Properties of xylitol vs. other alternative sweeteners

In the past few years, an increase in metabolic disorder cases provoked by unregulated sugar ingestion has been observed. This led to the search for alternative sweeteners with nutritional benefits for health.

Xylitol has been used since the 1960s as a food additive and sweetener. This sugar alcohol has the same physical appearance and the same sweetness level as the normal sugar (sucrose), allowing the substitution of sucrose on a weight-to-weight basis. Xylitol is highly endothermic provoking a cooling effect, known as “cold”, “fresh” or “minty”. This sugar is also considered a low caloric sweetener since has less 40% calories than sucrose (2.4 $calg^{-1}$ vs. 4.0 $calg^{-1}$) [MOH,2015].

A particularly relevant characteristic of this sweetener is its low glycemic index [ISL,2011]. This is explained thanks to the insulin-independent pathway that is used to metabolize xylitol into glucose-6-phosphate in the liver and red blood cells. Since xylitol undergoes a very slow metabolic process, blood glucose and the insulin concentration raise gently [KAL,1980]. Under conditions where insulin deficiency occurs, xylitol could be used to replace sucrose. With these characteristics, xylitol has been used as an excellent substitute in the regime of diet food and for non-insulin dependent diabetics [CHE,2010].

Xylitol does not participate in the Maillard reaction since does not react with amino acids. The Maillard reaction is a non-enzymatic browning reaction that reduces the nutritional value of proteins in foods and living organisms [BRU,2008].

One of the most important properties of xylitol, responsible for its growing demand in the market, is its anti-cariogenic properties. Xylitol promotes remineralization by increasing salivary flow and inhibits bacterial growth and metabolism in the plaque biofilm [RIT,2013]. As mentioned by [BAH,2012], regular xylitol consumption, at high enough doses, reduces *Streptococcus mutans* and *Streptococcus sobrinus* levels in both plaque and saliva. These microorganisms have a positive correlation between their presence in dental biofilm and saliva and the presence of caries. It is reported that the ability of xylitol to act as an anti-cariogenic agent is most likely due to its ability to be transported into caries-causing oral bacteria (like *S.mutans* and *S. sobrinus*), inducing the fructose phosphotransferase system (PTS) and inhibiting fermentation either by depleting the cell of high-energy phosphate or by poisoning the glycolytic system [TRA,1985].

Thanks to its low caloric and anti-cariogenic properties, xylitol has been used in many food products like sugar-free chocolate, chewing gum, hard candies, and other sweets for diabetics and dietary regimes [BAR,2016]. Furthermore, xylitol administered in chewing gum, lozenges, or syrup, can reduce the occurrence of acute otitis media (AOM) in children. Some studies in rats link xylitol to increased production of collagen (the most abundant protein in your body, found in large amounts in the skin and connective tissues), which may help counteract the effects of ageing on your skin [MAT,2005]. It has also been claimed that this compound has the capability of preventing osteoporosis, respiratory infections and colon disease [SIL,2012].

Figure 2.6: Common daily use products where xylitol is incorporated: chewing gums (left); syrup (center); lozenges (right).

It is important to mention that humans have generally tolerance relatively to this sugar alcohol, although xylitol could cause gastrointestinal effects if consumed in large quantities (above 60 g/day) since it can pull water into the intestine or get fermented by gut bacteria [MAK,2016]. This can lead to gas, bloating and diarrhoea. However, the body seems to adjust very well to xylitol, and so, if the intake is increased slowly, and the body has time to adjust, it won't cause any negative effects. Furthermore, long-term consumption of xylitol does appear to be completely safe [MAK,1976].

It is also noteworthy that the U.S. Food and Drug Administration (FDA) and the American Academy of Pediatric Dentistry approved the use of xylitol as a sweetener and substitute of normal sugar (sucrose) [AME,2010]. Xylitol has been strongly recommended to be used in the manufacture of baby food obtained by heat drying, since it does not participate in the Maillard reaction, avoiding the reduction of the nutritional value of proteins in those foods.

Xylitol has also the capacity of improving taste and colour of food when used as an ingredient, thus it has been broadly used as moisturizer, stabilizer, cryoprotectant, freezing point reducer and antioxidant.

There have been studies about the application of xylitol as a monomer to the synthesis of biodegradable polymers. Xylitol has been shown to be a great option as a monomer thanks to its capability of creating randomly crosslinked networks, nontoxic nature, the fact that is endogenous to the human metabolic system, FDA approval and its low cost.

Nowadays, two other sweeteners are found in the market that also serve as an alternative to sucrose, namely stevia and erythritol. Both of these compounds also occur widely in nature and can be extracted from fruits and plants. Regarding stevia, its active compounds are steviol glycosides (mainly stevioside and rebaudioside) that are extracted from *Stevia rebaudiana* [ASH,2015]. It was reported that stevia leaves a bitter taste when consumed in hot beverages and has no anti-cariogenic property, which is one of the disadvantages that stevia has over the other sweeteners[ASH,2015]. The limit daily consumption of high-purity stevia extracts permitted by FDA is only 12 milligrams per kilogram [ASH,2015]. In the case of erythritol, no negative effects have yet been reported [BOE,2015]. Although erythritol and xylitol are recognized as sugar alcohols due to their chemical structure, erythritol is less caloric and can be

consumed by dogs [SIL,2012]. It is important to mention that in xylitol production the carbon source used is xylose, while for erythritol production the carbon source is glucose. Currently, xylose production is more economical since the price of xylose is much lower than that of glucose. At present, the industrial xylitol production only occurs via chemical processes, while erythritol is already obtained by biotechnological routes. In fact, it seems not possible to synthesize erythritol by chemical means [BOE,2015],[SIL,2012].

2.4.5 Production

Regarding xylitol production, three main approaches have been proposed, namely, direct extraction, chemical process and biotechnological process.

As said in section 2.4.2, xylitol has been found in many natural sources such as fruits and vegetables. However, its direct extraction is uneconomical since the percentage of xylitol present in those sources is usually low, resulting in a small quantity of xylitol obtained.

Nowadays, the large scale production of xylitol is carried out by a chemical rout based on the catalytic hydrogenation of highly pure D-xylose in solution (obtained from hardwood hydrolysates) submitted to high temperature (140-200 ºC) and pressure (50-60 bar) conditions and in the presence of the toxic Raney nickel catalyst. The chemical process requires several purification steps because only pure xylose can be used in this chemical reduction [HAR,1979]. The conventional process (Figure 2.7) includes acid hydrolysis of the plant material, purification of the hydrolysate to either a pure xylose solution or a pure crystalline xylose, hydrogenation of xylose to xylitol, and crystallization of the xylitol [WIN,1998]. The conversion efficiency of the chemical process can be estimated from the raw material, xylan, or pure xylose: 8–15% from the initial raw material, 50–60% from xylan, and almost 98% from pure xylose [GRA,2007], [ARC,2018]. The obtained product is very expensive because of the extensive separation and purification procedures. In fact, the chemical method of xylitol manufacturing is laborious, energy-intensive, cost-intensive and with a hazardous environmental impact [RAF,2013].

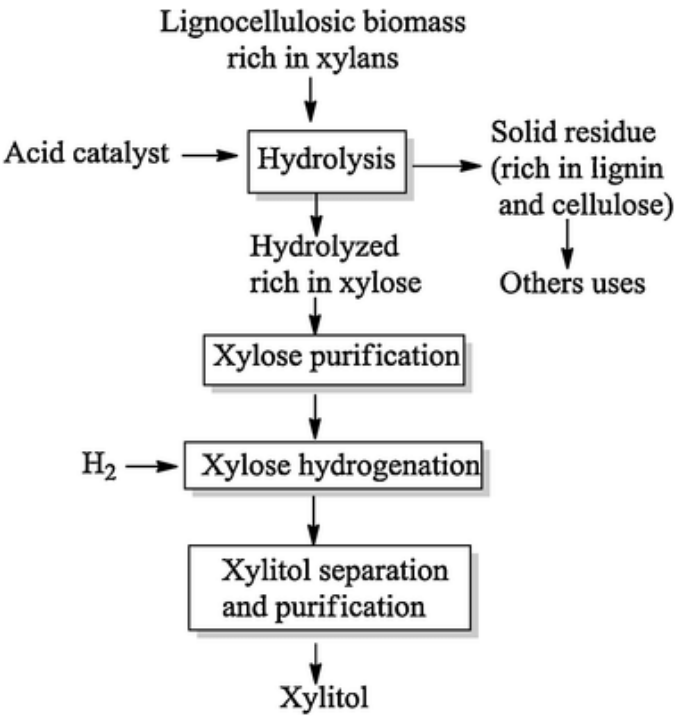

Figure 2.7: Main steps for xylitol production by chemical route [ARC,2018].

Over the last few years, many studies have been made trying to develop a more economical and

environmental-friendly production process, maintaining a high-quality product. Biotechnological methods are a promising choice that has been studied as a replacement of the conventional method since they involve much milder conditions of production. Another factor that enhances their replacement potential is that they can use mixtures of sugars, which alleviates the purification step of the lignocellulosic hydrolysates, resulting in a less energy-demanding and cheaper substrate purification step when compared to the conventional process [DAS,2017]. As alternatives to the conventional method, two biotechnological approaches seem promising, namely the microbial process and the enzymatic approach.

The xylitol process with the use of isolated enzymes enables high yields at lab scale, thus being considered as a good alternative option [PAR,2005]. However, and importantly, this process becomes less economical when upscaled because of the high, expensive cofactor requirement [DAS,2017].

Thus, this summary will focus on native microorganisms and recombinant strains, namely of filamentous fungi, yeast and bacteria, that have been under extensive study for the purpose of xylitol production. Many of these microorganisms have the advantage of being capable of utilizing xylose present in hydrolysates from lignocellulosic residues and convert it into xylitol, thus facilitating the purification step of the lignocellulosic hydrolysates.

Regarding microbial xylitol production, one needs to consider a number of cultivation parameters, namely the pH of the culture, temperature, aeration, carbon source concentration, nitrogen source, etc. With these many variables at play, the search for the optimal conditions for each microorganism has been an area extensively addressed. However, there is one variable responsible for high xylitol production, that seems to be much relevant throughout the wide range of tested microorganisms. This is the xylose concentration in the culture, which apparently needs to be higher than 100gL^{-1} [MOH,2015].

According to [DAS,2017], yeasts are preferred for xylitol production primarily due to their high pentose assimilation rates and xylitol productivity, due to stable expression levels of XR and X_{OH}DH. Currently, there are a couple of studies on xylitol productions by yeast (namely different species of C*andida*) with impressive results, achieving conversion efficiencies of nearly 86%, with a volumetric productivity of 4.88 gL^{-1}h^{-1}, which is relatively high when compared to other reported microorganisms. However, most of these yeasts are not considered as GRAS (Generally Recognized As Safe), by the FDA. Thus, there is a need to look for other microorganisms capable of producing xylitol.

Further microorganisms that were reported to be xylitol producers were filamentous fungi. However, their xylitol production is too low to be even competitive. This means that we need to direct our attention to bacterial species. A literature review about xylitol producers can be observed in Table 2.1.

Another approach that has been studied is to modify the wild strain into genetically modified strains, by adaptation, mutation and recombinant techniques. Many pentose fermenting microorganisms suffer from bottlenecks primarily in terms of low xylitol productivity. Selective gene manipulation to improve productivity has been targeted after gradual enrichment of the genetic database and increased knowledge about the metabolic mapping of many of these microorganisms. With that, it is possible to optimize selective xylose transporter systems to increase the xylose uptake rate, improvements have

include over-expression of XR to maximize the reduction of xylose into xylitol, deletion of the $X_{OH}DH$ gene to stop the oxidation of xylitol into xylulose and optimisation of the supply of cofactors involved in xylitol production (like NADH or NADPH) [DAS,2017].

In addition to the presented methods, there are several ways to improve the performance of microorganisms using different cultivation modes. For instance, in industry, the use of immobilized cells during a continuous fermentation mode is often attractive. This fermentation mode allows cell re-use giving high productivities during an extended period as compared to freely suspended cells. However, most bioreactor studies regarding xylitol production are reported to be operated under batch mode. Industrial production seems to be more favourable under batch mode operation. Xylitol fermentation suffers from the aforesaid problem due to the major drawback of reducing power requirement. Non-growing cells in a viable state ("resting cells"), require an additional co-substrate to carry out the reduction reaction. Hence, a fed-batch bioreactor system with intermediate co-substrate addition at repeated intervals may be a solution to improve productivity and thereby xylitol yield [DAS,2017].

One of the limiting steps in the biotechnological xylitol production is the downstream process. The recovery and purification of xylitol can be crucial to determine the viability of the process. Recovery of xylitol from fermentation broth with high purity has been targeted by many research groups [WEI,2010], [SAM,2006]. The most common downstream technique used to recover xylitol in the chemical industrial process is the crystallization technique, as it is less energy-consuming over distillation and other routes of purification to achieve stable and maximum product recovery [MAR,2007], [DAS,2017]. Many factors like the concentration of targeted product, salting out of product guided by temperature-dependent saturation pattern, seed crystal, crystallization time, etc. contribute to the efficiency of the crystallization process. The xylitol downstream process presents additional challenges as the broth contains many impurities, like microbial cells and cell debris, complex nutrients and other compounds like unreacted sugar leftovers and a mixture of sugar alcohols like arabinitol, sorbitol (which are very difficult to separate from xylitol) along with xylitol. Such undefined mixture requires several purification steps like ion-exchange chromatography, clarification by activated charcoal or liquid-liquid extraction process before crystallization, which might add to process cost. With that in mind, a lot of research is being done to discover new ways to produce good quality product maintaining the overall production costs to a minimum.

Table 2.1: A brief selection of microorganisms that transform biologically *D*-xylose into xylitol.

Strain	Recombinant strain	Risk group	$Y_{XyOH/Xyl}$ (gg^{-1}) or $[XyOH]_{max}$ (gL^{-1})	$Prod_{vol}$ ($gL^{-1}h^{-1}$)	t_{ferm} (h)	Operation mode	References
Corynebacterium spp.	-	1 or 2	0.69	0.21	336	Batch: xylose 150 gL^{-1}	[YOS,1971]
Enterobacter spp.	-	2	33.3	0.35	96	Batch: xylose 100 gL^{-1}	[YOS,1973]
Candida tropicallis ASM III	+	2	0.93	1.08	120	Batch: xylose 200 gL^{-1} (40% O_2 sat.)	[LOP,2004]
Candida tropicalis KCTC 10457	+	2	0.9	4.88	48	Fed batch:xylose 260 gL^{-1}	[KWO,2006]
Candida magnoliae TISTR 5663	i	1	0.72	1.15	270	Fed batch:xylose 234-284 gL^{-1}	[SIR,2013][SIR,2013][SIR,2013][SIR,2013]
Candida athensensis SB18	-	2	0.83	1.02	204	Batch: xylose 250 gL^{-1}	[ZHA,2012]
Candida tropicalis ATCC13803	i	1	0.75	3.9	48	Fed batch:xylose 100 gL^{-1}	[KIM,2002]
Debaryomyces nepalensis NCYC 3413	-	1	0.83	0.83	110	Batch: xylose 50 gL^{-1}; Fed-batch: xylose 50 gL^{-1}	[HIM,2015]
Burkholderia sacchari DSM 17165	-	1	17	0.39	43	Fed batch:xylose 600 gL^{-1}	[RAP,2017]

– Negative

+ Positive

i- Unknown.

2.4.6 Economic approach

The interest in xylitol has increased considerably during these last few years as the consumers are inclining towards sugar-free and low-calorie food products due to health and weight consciousness, and also because of its several commercial applications in different industrial sectors, such as food, dental-related products, cosmeceuticals and pharmaceuticals [DAS,2017].

Xylitol production has increased more than 40 times since 1978, which corresponds to approximately 6000 metric tons [ARC,2018]. According to [FEL,2019], the global xylitol market was estimated to be approximately US$ 823.6 million, and it is expected to generate more than US$ 1.15 billion by 2023, corresponding to a CAGR (compound annual growth rate) on the volume and value of approximately 5.7% between 2018 and 2023. Chewing gum manufactures are the major xylitol consumers as they will correspond to approximately 67% of the demand in 2020. According to a source from 2012, the prices of this compound are 4.5–5.5 $/kg for bulk purchase by pharma/chewing gum companies and 20 $ in supermarkets [GIR,2012]. As stated by [ARC,2018], the high price of xylitol in the market is directly related to the high cost of the chemical production process, as mentioned in section 2.4.5.

The biotechnological route to produce xylitol industrially is still costly and, as a consequence, presents a small share of the global market. However, the advantages aforementioned in section 2.4.5 may have higher potential impacts on the cost of this production process compared to the chemical process. It will depend on the efficiency and sustainability of the steps of the biotechnological route, as well as on the suitable integration of this bioprocess in a biorefinery. Much effort has been devoted to studying the use of lignocellulosic substrates to achieve a biotechnological process of xylitol production to be price competitive so that this process might be adopted. It is important to highlight that some commercial xylitol producers are already adopting biotechnological routes for xylitol production by taking into consideration the advantages of biotechnological methods [FEL,2019].

Chapter 3

Materials and Methods

This chapter describes the materials used in this thesis, such as the strain storage and inoculum preparation, the seeding medium and the culture conditions. A detailed explanation of the analytical methods used in this work is also given.

3.1 Microorganism

The microorganism used in this project was the *Paraburkholderia sacchari* DSM 17165. This strain has the ability to grow, accumulate PHAs, produce xylitol and xylonic acid on xylose.

3.2 Strain storage and inoculum preparation

P. sacchari was stored for long periods of time in cryovials. These were prepared by adding to sterile cryovials (2 mL) 300 µL of previously sterilised pure glycerol and 1500 µL of a *P. sacchari* liquid culture collected in the late exponential growth phase. This culture was prepared with seeding medium (described in Table 3.1), supplemented with 20 gL^{-1} of xylose and incubated in an orbital incubator (Aralab, AGITORB 200, Portugal) at 30ºC and 170 rpm for 24 h. The procedure described was prepared under aseptic conditions, achieved in a laminar flow chamber (BIOAIR Instruments aura 2000 MAC 4 NF, Italy), which was submitted to sterilization under UV light for 15 minutes and using sterile material. These cryovials were stored at -80 ºC.

3.3 Carbon sources

D-xylose (Danisco GmbH, Austria) was the carbon source used in the shake flask and bioreactor assays. All the sugar solutions were prepared with deionized water and then sterilized by autoclaving at 121°C for 20 minutes. The sugar solutions used as feed in the bioreactor assays consisted of a commercial xylose solution with a concentration of 600 gL^{-1}. When the feed solutions were not in use, they were stored at 4ºC.

3.4 Culture media

3.4.1 Seeding medium

The mineral medium [KIM,1994] used for growing the inoculum was prepared by mixing the right amount of the compounds described in Table 3.1 (with the exception of $MgSO_4.7H_2O$) with distilled water to achieve the concentration described. The pH was adjusted to 6.8 by adding the conjugated acid of the phosphate buffer, KH_2PO_4. The medium was sterilized by autoclaving at 121 ºC for 20 minutes. To avoid the formation of precipitates during the sterilization process, a 100 gL^{-1} $MgSO_4.7H_2O$ solution was prepared and autoclaved separately, and then added to the medium solution under sterile conditions.

Table 3.1**:** Seeding medium composition.

Compound	**Concentration (gL^{-1})[i]**	**Brand name**	**Purity (%)**
$Na_2HPO_4.2H_2O$	4.47	Panreac	99.0
KH_2PO_4	1.5	Panreac	99.0
$(NH_4)_2SO_4$	1.0	Panreac	99.0
Yeast Extract Powder	1.0	Himedia	_
$MgSO_4.7H_2O$	2 mLL^{-1}	Panreac	98.0-102.0
Oligo elements solution	1 mLL^{-1}	_	_

i Unless stated otherwise

3.4.2 Oligo elements solution

As mentioned in [KIM,1994], to prepare the oligo elements solutions, the compounds listed in Table 3.2 were dissolved with distilled water. The solution was autoclaved at 121 ºC for 20 minutes. To store this solution, it was kept at 4 ºC.

Table 3.2: Oligo elements solution composition.

Compound	**Concentration (gL^{-1})[i]**	**Brand name**	**Purity (%)**
$FeSO_4.7H_2O$	10	Sigma	>99.0
$ZnSO_4.7H_2O$	2.25	Sigma	>99.0
$CuSO_4.5H_2O$	1.00	Panreac	>99.0
$MnSO_4.H_2O$	0.379	Sigma	>99.0
$CaCl_2.2H_2O$	2.00	Merck	>99.5
$Na_2B_4O_7.10H_2O$	0.23	Merck	99.5-105.0
$(NH_4)MO_7O_{24}.4H_2O$	0.106	Merck	<99.0
HCL	10 mL 37%	Fisher Chemical	35

i Unless stated otherwise

3.4.3 Bioreactor cultivation medium

To prepare the bioreactor cultivation medium [KIM,1994], the compounds listed in Table 3.3 were mixed with distilled water. The final working volume was 1 L (including the inoculum and concentrated sugar solution) unless stated otherwise. The pH was adjusted to 6.8 with a 5 M KOH solution (Panreac). The mineral medium was sterilized inside the bioreactor for 20 minutes at a temperature of 121 ºC. As mentioned in the seeding medium preparation (Seeding medium), a 100 gL^{-1} $MgSO_4.7H_2O$ solution was prepared and autoclaved separately, so that the formation of precipitates could be avoided. The magnesium solution and a concentrated sugar solution (to ensure an initial xylose concentration of 30 gL^{-1}) were added later to the bioreactor aseptically. Sample collections and analyses were done.

Table 3.3: Mineral medium composition

Compound	Concentration (gL^{-1})[i]	Brand name	Purity (%)
KH_2PO_4	13.3	Panreac	99.0
$(NH_4)_2SO_4$	4.0	Panreac	99.0
Citric acid.H_2O	1.85	Panreac	99.5-102.0
$MgSO_4.7H_2O$	12 mLL^{-1}	Panreac	98.0-102.0
Oligo elements solution	10 mLL^{-1}	_	_
EDTA	40 mgL^{-1}	Fischer Scientific	99.5

3.5 Standards

To quantify the xylitol and xylonic acid produced by the cultures, xylitol (98%, Panreac) and D-xylonic acid calcium salt hydrate (97%, Carbosynth) were used as standards.

3.6 Culture conditions

3.6.1 Shake flask assays

A. Inoculum preparation

Regarding the shake flask assays, the inocula were prepared by transferring to a 500 mL shake flask the content of one cryovial (2 mL), a xylose solution to guarantee an initial 20 gL^{-1} xylose concentration, and the seeding medium solution (mentioned in section Seeding medium) to achieve a working volume of 100 ml. The shake flask containing the inocula was incubated at 30 ºC in an orbital incubator (Infors AG, Switzerland) at 170 rpm for 24 hours corresponding to the end of the exponential growth phase.

B. Cultivation

Shake flask assays were performed to test if the presence of xylitol in the growth medium could influence the *P. sacchari* metabolic activity. These assays were carried out by in 500 mL baffled Erlenmeyer flasks containing 100 mL of a liquid phase. The inoculum fraction was 2,6 % (v/v) and an initial xylose concentration of 20 gL^{-1}. Different initial xylitol concentrations (0, 10, 50 and 100 g/L) were used in order to analyse the possible xylitol inhibition. All the assays were performed in duplicate and the average value was considered.

C. Culture sampling

In order to analyse the bacterial growth, 5 culture samples were harvested during the exponential growth phase (between the 10º hour and the 20º hour according to). The bacterial growth was obtained by the measurement of the optical density of each sample.

3.6.2 Fed-Batch Bioreactor assays

A. Inoculum preparation

Inocula were prepared in shake flasks containing 50 ml seeding medium (unless stated otherwise) corresponding to 5 % (v/v) of the bioreactor volume.

B. Cultivation

Fed-batch cultivations were carried out in 2 L STR (New Brunswick Bioflo 115). To enable control, monitoring and data acquisition of the culture, the cultivation was operated using the BioCommand Batch Control software. The temperature of the culture was set to 30 ºC and the pH was controlled at 6,8 with a solution of 30% NH_4OH. The dissolved oxygen was set to 1% or 20% saturation and the agitation was set in cascade with the dissolved oxygen, that is, whenever the dissolved oxygen drops below the setpoint, the agitation rises in order to increase the dissolved oxygen until it reaches the set point again. The aeration rate used was 1 $L.min^{-1}$ or 2.6 $L.min^{-1}$.

Regarding the feeding strategy, either manual pulses or programmed pulses of a concentrated xylose solution (feed; 500 g/L, 1L) were added to the cultivation medium. Manual pulses were added periodically to guarantee the availability of xylose to attain a cell density of approximately 30 g/L. Xylitol production was promoted at this cell concentration by manually adding a pulse of the feeding solution to attain a xylose concentration higher than 120 g/L. The programmed automatic feeding, i.e. the timing and volume of feed addition, was calculated based on the rate of xylose consumption at a cell density of 30 g/L. For feed addition, the tubes were calibrated previously for the flow rate. For STR 1, the flow rate was $2.17x10^{-2}$ $L.min^{-1}$ and $2.20x10^{-2}$ $L.min^{-1}$ for STR 2. Figure 3.1 represents the flowchart of the main steps of the fermentation cycle.

C. Culture sampling

Culture samples were periodically harvested (three per day on average). Typically, each sample had an approximate volume of 5 mL, harvested with a syringe through a non-return valve in order to maintain the aseptic conditions. Biomass, sugar, P(3HB) and the metabolites xylitol and xylonic acid were analysed in each sample.

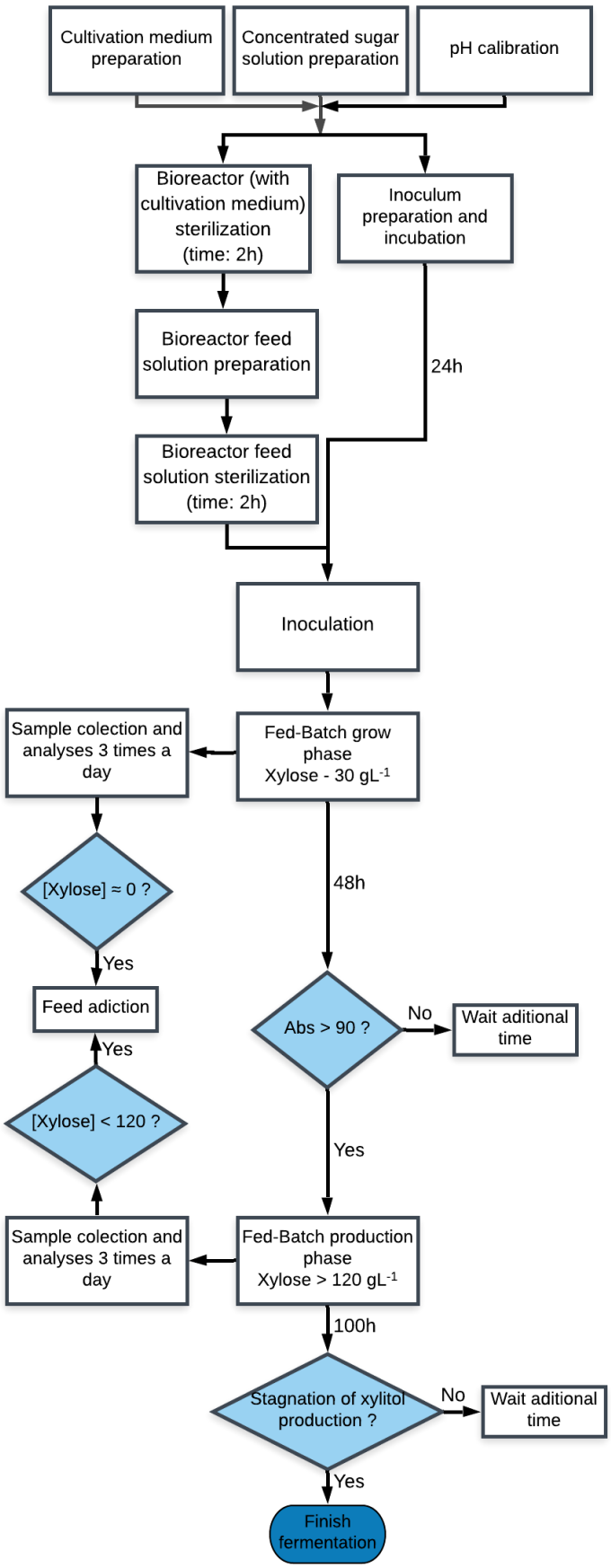

Figure 3.1: Flowchart of the main steps of a Fed-Batch fermentation cycle.

3.7 Analytical methods

3.7.1 Optical density measurements

In order to monitor the cellular growth during the cultivation, the optical density (OD) of samples was measured at 600 nm in a double beam spectrophotometer (Hitachi U-2000). An aliquot of the culture sample was diluted with deionized water so that the absorbance value red under the threshold (ca. 0.5-0.6). The solution used as reference was deionized water. Three mL glass cuvettes with an optical path length of 1 cm were used.

3.7.2 Cell dry weight determination

To determine the cell dry weight (CDW), 1.2 mL aliquots of culture samples were collected into dry and weighted microtubes. After the sample was centrifugated at 10000 rpm (9167 x g) for 5 minutes (in a Sigma 1-15 P microcentrifuge), the supernatant was rejected and the pellet was washed with deionized water. Then, the microtubes were dried at 60 ºC in a Mermet oven (Model 400) until constant weight. In order to determine the CDW, the weight difference of the microtubes after drying the pellets and the empty microtubes were divided by the volume of the collected aliquots.

3.7.3 Xylose, xylonic acid, xylitol and phosphate determinations

Xylose, xylonic acid, xylitol and phosphate concentrations were determined offline in a High Performance Liquid Chromatography (HPLC) apparatus (Hitachi LaChrom Elite). The HPLC is equipped with a Rezex ROA-Organic acid H+ 8% (300 mm _ 7.8 mm) column, an HPLC pump (Hitachi LaChrome Elite L-2130), an autosampler (Hitachi LaChrome Elite L-2200), a Hitachi L-2420 UV-Vis detector for organic acids and a Hitachi L-2490 refraction index (RI) detector for sugars and phosphate. For heating porpuses, it was connected externally to the HPLC system a column heater for large columns (Croco-CIL 100-040-220P, 40 cm _ 8 cm _ 8 cm, 30-99°C). The column was kept at 65 °C under a pressure of 26 bar, and the pump operated at a flow rate of 0.5 $mL.min^{-1}$. The injection volume was 20 µL and elution was achieved using a 5 mM solution of H_2SO_4 as the mobile phase.

A. Sample preparation

To prepare a sample to be analysed in HPLC, 300 µL of supernatant aliquots were mixed with 300 µL of a 50 mM solution of H_2SO_4 in a microtube. After vortexing, these solutions were centrifuged (in a Sigma 1-15 P microcentrifuge) at 10000 rpm (9167 x g) for 5 minutes. After the centrifugation, the sample for injection is prepared in an HPLC vial, consisting of 100 µL of the previous 1:2 diluted samples

plus 900 µL of the 50 mM H_2SO_4 solution, resulting in a final dilution of 1:20.

B. Calibration curves

Calibration curves were obtained for working ranges of 1 to 200 gL^{-1} for xylose, 4,9 to 97.5 gL^{-1} for xylonic acid, 0.5 to 100 gL^{-1} for xylitol and 0.1 to 20 gL^{-1} for phosphate (see equations B.1 to B.7 in appendix B.1).

Since xylose and xylonic acid had similar retention times in HPLC runs, an overlap of both peaks when using the RI detector was verified, which led to an incorrect determination of the xylose concentration. To solve this problem, the xylonic acid concentration was measured in the UV chromatograms (see Figure B.3) with an appropriate calibration curve. This value was used to calculate the area corresponding to xylonic acid when using the RI detector. This was computed using the calibration curve of xylonic acid in RI (see Figure B.2). Finally, to determine xylose concentration, the area of the overlapping peaks in RI chromatograms was computed and the area corresponding to the xylonic acid subtracted. Using the calibration curve for xylose measured with the RI detector, the concentration could then be calculated.

3.7.4 P(3HB) determination

P(3HB) concentration was determined offline by Gas Chromatography (GC). The apparatus used for the analyses was a GC (HP 5890 series II) equipped with a FID detector and a 7683B injector. The oven, injector, and detector were kept at constant temperatures of 60°C, 120°C, and 150°C, respectively. The capillary column was an HP-5 from Agilent J&W Scientific, 30 m in length and 0.32 mm of internal diameter. Data acquisition was performed by a Shimadzu CBM-102 communication Bus Module. Integration was performed by Shimadzu GC solution software (version 2.3). To identify the chromatographic peaks, it was used as standard 3-methyl hydroxybutyrate (Sigma).

A. Sample preparation: acidic methanolysis

To prepare the samples for the GC analyses, 1.2 mL aliquots of culture medium were harvested from the culture medium and consequently centrifuged at 10000 rpm (9167 x g) for 5 minutes. After the pellets were washed with deionized water, they were frozen for storage prior to acidic methanolysis. To perform the acidic methanolysis [CAV,2012] of the polymer, 1 mL of chloroform was added to the microtubes containing the cell pellet. The pellets were resuspended and transferred to Pyrex hermetic tubes with Teflon cases. Subsequently, 1 mL of an acidic methanol solution was added to each tube. This solution consists of 97 mL of methanol, 3 mL of H_2SO_4 (96%) and 330 µL of hexanoic acid as the internal standard (IS) per 100 mL of solution. These mixtures were vortexed for 1 minute and then incubated for 5 hours at 100 ºC in a Memmert GmbH oven (model 200). After cooling, 1 mL of NA_2CO_3 was added to

the tubes in order to neutralize the solution and stop the reaction. The samples were vortexed once again for 1 minute and then centrifuged at 4500 rpm (2263 x g) for 5 minutes in a Heraeus SEPATECH Labofuge centrifuge (model 200). Subsequently, 200 µL of the organic phase was withdrawn from each tube to appropriate vials and kept at -20ºC until GC analysis.

B. Calibration curve

To obtain the calibration curve for the P(3HB) quantification, P(3HB) samples previously produced and with a purity of 99.5% were used, which were submitted to acidic methanolysis, as described in the previous section. This calibration curve was obtained for a working range of 0.5 to 10 gL^{-1} and is described by equation B.8.

3.7.5 Overall yield and productivity calculations

In order to evaluate overall yields of the metabolites formed during the fed-batch cultivations of *P. sacchari*, the following equations listed below (3.1 and 3.2) were used. Summing up, these values were obtained dividing the total by-product mass obtained (mP), by the quantity of substrate consumed during the cultivation (mS_{cons}). To compute the product mass obtained (mP), it was multiplied the final product concentration ($[P]_f$) by the final volume (V_f). Regarding the substrate consumption (mS_{cons}), it was calculated by a mass balance, computing the difference between the initial substrate mass (mS_i), plus the mass of substrate added as feed during the fed-batch cultivation (mS_{feed}), and the final substrate mass in the culture broth (mS_f). Regarding the equation 3.2, it is necessary to multiply to the foregoing equation, the ratio between the molar mass of the substrate (MMsubs) and molar mass of the product (MMprod).

$$Y_{P/S}(gP/gS_{cons}) = \frac{[P]_f \times V_f}{mS_i + mS_{feed} - mS_f} = \frac{mP}{mS_{cons}} \tag{3.1}$$

$$Y_{P/S}(mol\ P/mol\ S_{cons}) = \frac{[P]_f \times V_f}{mS_i + mS_{feed} - mS_f} \times \frac{MM_{subs}}{MM_{prod}} = \frac{mP}{mS_{cons}} \times \frac{MM_{subs}}{MM_{prod}} \tag{3.2}$$

The volumetric productivity of the metabolites produced in the cultivation was also determined. These

values were calculated dividing the final product concentration ($[P]_f$) by the cultivation time (t_{cult}). The volumetric productivities referred exclusively to the production period, ignoring the time of the bacterial growth, i.e., dividing the final product concentration ($[P]_f$) by the production time (t_p). The production time was calculated by computing the difference between the final time of production (t_{pf}) and the initial time of production (t_{pi}). The initial time of production was considered the time when the biomass CDW achieved values between 25 and 30 gL^{-1}. Equations 3.3 and 3.4 described these calculations.

$$Prod_{vol}(gPL^{-1}h^{-1}) = \frac{[P]_f}{t_{cult}} \tag{3.3}$$

$$Prod_{vol(tp)}(gPL^{-1}h^{-1}) = \frac{[P]_f}{t_{pf} - t_{pi}} = \frac{[P]_f}{t_p} \tag{3.4}$$

Chapter 4

Results and Discussion

This chapter provides the context, description, analysis and discussion of the assays performed in this work. Alongside every assay, the discussion of the results is supplemented by the graphics that represent the time course of the growth, metabolite production and data obtained, as well a table showcasing overall yields and productivities of the metabolites.

As said in section 2.3.2, the genes of the oxidoreductase pathway were not found in *B sacchari* genome, however, that does not necessarily mean that they do not exist. As can be seen in Figure 2.4, the bacteria is only capable of producing xylitol if it has at least one of the genes that encode the enzymes responsible for the production of xylitol, namely XR or $X_{OH}DH$. Since xylitol production by *P. sacchari* does occur, one can conclude that at least one of these genes from the oxidoreductase pathway must be present.

For the sake of simplicity, for the rest of this work, it will be considered that *P. sacchari* does not have the gene that encodes the XR enzyme. Therefore, xylitol production mechanism would consist in two steps: the first one is the xylose conversion into xylulose by xylose isomerase (XI), and the second one consists in xylulose conversion into xylitol by xylitol dehydrogenase ($X_{OH}DH$) thanks to the oxidation of the cofactor NADH into NAD^+.

4.1 Shake flask assays

Fed-batch bioreactor assays done by [BON,2018] with the focus on xylitol production have shown a halt in metabolic activity of *P. sacchari* in the late phase of the fermentation when the xylitol concentration reaches values around 100 gL^{-1}.

To understand this, shake flask assays were carried out to test if the presence of xylitol in the cultivation medium could influence *P. sacchari's* metabolic activity. As described in section 3.6.1, *P. sacchari* was cultivated in seeding medium supplemented with 20 gL^{-1} of xylose to which different xylitol concentrations were added, namely 0, 10, 50 or 100 gL^{-1} to four different flasks. Different samples were harvested during cultivation. The bacterial growth was obtained by the measurement of the optical density of each sample.

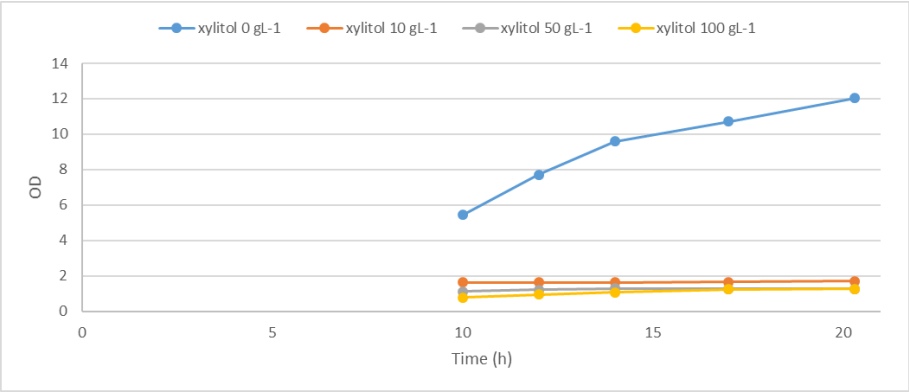

Figure 4.1: *P. sacchari's* growth in SM, supplemented with 20 gL^{-1} of xylose and 0, 10, 50 or 100 gL^{-1} of xylitol.

Figure 4.1 represents the growth of *P. sacchari* during the tenth and twentieth hour of cultivation, measured by the optical density.

As can be seen, the cell density of the control assay in the absence of xylitol is quite higher than when xylitol is present, showing that bacterial growth is completely inhibited by the presence of this compound in the medium already at low concentrations (10 gL^{-1}). Although the difference is not significant, the assays show a decrease in OD with the increase in xylitol concentration, showing that the inhibition is greater the higher the concentration of xylitol in the medium.

The inhibitory effect of xylitol to the cells as been reported before and can be explained by the formation of xylitol-phosphate which is toxic to the cells. As reported by [AKI,2009], the enzyme xylulokinase (XK), present in the metabolism of *P. sacchari,* can phosphorylate several sugars and polyols, including xylitol. This non-specific activity of xylulokinases results in the production and accumulation of toxic phosphorylated compounds that are not further metabolized. In this case, xylulokinase catalyses the phosphorylation of xylitol into xylitol-phosphate in the presence of xylitol (Figure 2.3), which is consequently accumulated having an inhibitory effect on the bacterial growth of *P. sacchari*.

4.2 Fed-Batch Bioreactor assays

The objective of these bioreactor assays was to find the best culture conditions so that the consumption of the carbon source was directed entirely to xylitol production. Having that in mind, one needed to find the conditions that favoured xylitol production while inhibiting xylonic acid and P(3HB) productions.

Regarding polymer production, as P(3HB) accumulation only occurs when the medium is limited by one of the essential nutrients, such as nitrogen or phosphate [SUD,2000], to avoid polymer formation the concentration of these nutrients was monitored during the fermentation and their exhaustion was circumvented. The initial phosphate concentration in the mineral medium was high enough to guarantee the presence of this compound until the end of the growth phase (when bacterial density attains values around 25 - 30 gL^{-1} CDW). Nitrogen was intermittently added to the medium as ammonium hydroxide (NH_4OH), the base chosen for pH control. It was used to neutralize the acid produced during growth and during xylonic acid production. This addition was monitored during the whole cultivation. Since these conditions were maintained in all fermentations and were effective in avoiding P(3HB) production as shown by [BON,2018], polymer accumulation was not determined throughout this work (except for one fermentation).

In most figures, a decrease of the dry weight of the culture after reaching the chosen biomass concentrations is observed. This is explained by the dilution observed upon the addition of a large feed pulse of about 200 mL. The addition of this large pulse is needed to reach high xylose concentrations and promote xylitol production. A decrease in the dry weight of the culture is verified until the end of the fermentation. This is due to the constant addition of feed and base solution throughout the fermentation. This decrease is aggravated when the biomass production stops, a consequence of the harsh conditions (like high xylose and xylitol concentrations) to which bacteria are submitted in the production phase.

4.2.1 Cultivation A

Based on the good results obtained by [BON,2018] in fed-batch cultivations, the same cultivation conditions were used throughout this work to attain high xylitol productivities.

The fermentation was performed in two steps. First, conditions were established to stimulate bacterial growth, i.e 20% of dissolved oxygen (DO), and an initial xylose concentration of 30 gL^{-1}. If necessary, pulses of a concentrated xylose solution (600 gL^{-1}) were added manually to avoid the limitation of carbon source. These conditions were kept until a concentration of 30 gL^{-1} cell dry weight (CDW) was attained.

As discussed in section 2.3.3, the promotion of xylitol production only takes place with inhibitory xylose concentration (around 120 gL^{-1}) in the medium, so in the second step, to promote the production of this metabolite, a manual pulse of the concentrated xylose solution was added to attain xylose concentrations higher than 120 gL^{-1}. During this production phase, manual or programmed pulses of the feed solution were added to maintain xylose concentration up to 120 gL^{-1} throughout the fermentation. The aeration has been reported as a crucial factor in xylitol fermentation using different yeasts strains

(Table 2.1). Low oxygen concentrations were shown to promote xylitol production. With that in mind, assays using low DO were performed by dropping the aeration rate down to 1 L.min^{-1} and setting the DO to 1% sat.

Figure 4.2 represents the time course of the growth and metabolite production, as well as data obtained in this fed-batch cultivation (cultivation A).

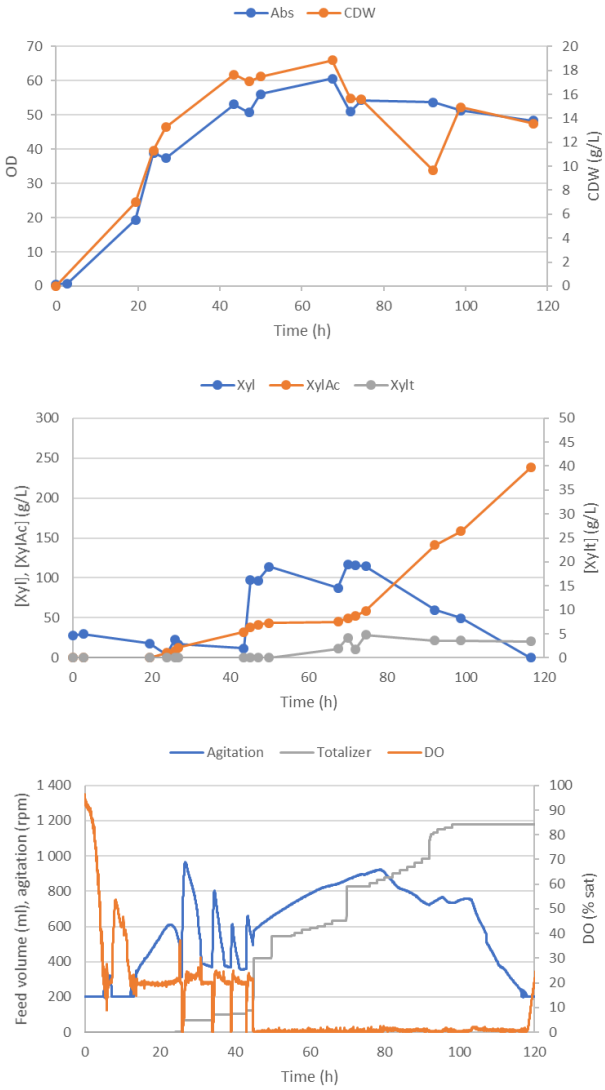

Figure 4.2: *P.sacchari* fed-batch cultivation A, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 1% sat of DO.

By the analysis of these graphics, it is noticeable that the goal of maintaining the xylose concentration above 120 gL^{-1} was not met, as the maximum value achieved was 117 gL^{-1}, spending most of the time at values around 100 gL^{-1}. At this point, it was assumed that this fact was the responsible factor for the lack of xylitol production, as it reached a value of just 3.4 gL^{-1} after 116.5 hours. On the other hand, xylonic acid production was way above expected, achieving a value of 238.1 gL^{-1} at the end of the fermentation. An interesting fact worth discussing is the sudden increase in xylonic acid concentration

verified around the 80th hour. A possible explanation is that xylonic acid was continuously being produced and accumulated intracellularly, being only excreted after 80 hours. This explanation is supported by the constant xylose concentration between the 50th and 70th hour, even though xylose was being added during that period. This implies that xylose was being metabolized towards a metabolite (in this case xylonic acid) during that period. It is still unknown the culture conditions that triggered the intracellular accumulation, as it did not happen again in the following fermentations analysed in this work.

It is important to mention that the very low final xylitol concentrations achieved using these cultivation conditions, opposite to the very high xylonic acid titres, rose many questions and doubts since these values completely differ from the results obtained previously by [BON,2018].

4.2.2 Cultivation B

For this next fermentation, the goal was to repeat the previous cultivation (Cultivation A) trying to correct what apparently went wrong, namely to maintain the xylose concentration at 120 gL^{-1} during the production phase of the fermentation. The remaining conditions for growth and production were kept constant. Figure 4.3 represents the time course of growth and metabolite production, as well as data obtained in the fed-batch cultivation B.

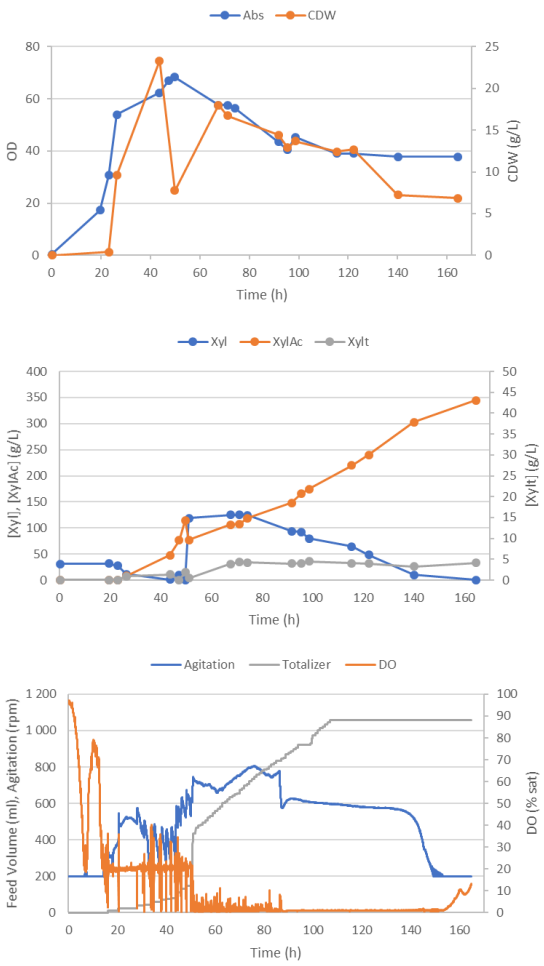

Figure 4.3: *P.sacchari* fed-batch cultivation B, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 1% of DO.

Analysing Figure 4.3, it is noticeable that xylitol production, just like in Cultivation A, continued to be negligible (approx. 4 g/L), reaching a yield of just 0.01 g_{XyOH}/g_{Xyl} and volumetric productivity of 0.03 $gL^{-1}h^{-1}$ (Table 4.1) even though the xylose concentration profile was achieved, i.e xylose concentration up to 120 gL^{-1} during the production phase. With this result, one can eliminate the hypothesis raised in the previous cultivation regarding the reason for the lack of xylitol production.

Just like in the previous cultivation, xylonic acid production was way above expected, even higher than Cultivation A, stabilizing at a concentration of 345 gL^{-1} after 164 hours. At this point, thanks to the high xylonic acid concentrations obtained in the last two cultivations, it was decided to analyse the fermentations not only from the xylitol production point of view but also from the xylonic acid production perspective.

The majority of microorganisms recognized as producers of xylonic acid are mostly recombinants, namely *Klebsiella pneumoniae* [WAN,2016], *Pichia kudriavzevii* [TOI,2013], *Escherichiacoli* EWX4 [LIU,2011], and *Pseudomona fragi* ATC

C4973 [BUC,1988]. Xylonic acid is also produced by native strains like *Gluconobacter oxydans*

[BUC,1988] and *Paraburkholderia sacchari* DSM 17165 (the one studied in this work as well as in [BON,2018]). So far, the best yields presented in the literature are around 1 g_{XylAc}/g_{Xyl} and volumetric productivities vary between 1.1 and 2.5 $gL^{-1}h^{-1}$. Except for the work by [BON,2018], where it achieved a yield of 1.15 g_{XylAc}/g_{Xyl} and volumetric productivity of 4.4 $gL^{-1}h^{-1}$, the yield and productivity achieved by this work keep up with the best results found in the literature, with a value of 0.99 g_{XylAc}/g_{Xyl} and 2.10 $gL^{-1}h^{-1}$ for yield and productivity respectively. It is important to mention that the work performed by [BON,2018], used also *P. sacchari* but different cultivation conditions.

Another important fact worth mentioning is the dissolved oxygen in the cultivation medium. In this fermentation, oxygen-limited conditions (1% of DO) were used, which difficult the reoxidation of the cofactor NADH into NAD^+ (essential for the xylonic acid production). This means that to have such high xylonic acid productivities, the culture conditions are stimulating the reoxidation of NADH through an alternative way. This topic will be discussed ahead.

At this point, it was still not certain what was the reason for the high xylonic acid production observed in this fermentation, but it is possible to state with certainty that this reason is also responsible for the lack of xylitol production obtained.

4.2.3 Cultivation C

Having in mind the poor results obtained in the previous cultivations regarding xylitol production, it was decided to change the culture conditions. Conditions similar to cultivations A and B were maintained except the DO %sat, which was kept at 20 % during the second phase. Similar assays have been carried out by [BON,2018] yielding high xylitol productions.

As discussed in section 2.3.3, oxygen-limited conditions (DO of 1%) promote xylitol production, since it prevents the reoxidation of the NADH (formed during the xylose metabolism) into NAD^+, which consequently would favoured xylitol dehydrogenation into xylulose thanks to the high NAD^+/NADH ratio [RAF,2013], [MOH,2015]. However, there is literature that supports the increase of xylitol production with the increase of the amount of dissolved oxygen in the medium (i.e DO of 20%), since the enzymatic activity of the enzyme responsible for xylitol production is also dependent on oxygen availability in the medium [GIR,1994].

Figure 4.4 represents the time course of growth and metabolite production, as well as data obtained in the fed-batch cultivation C.

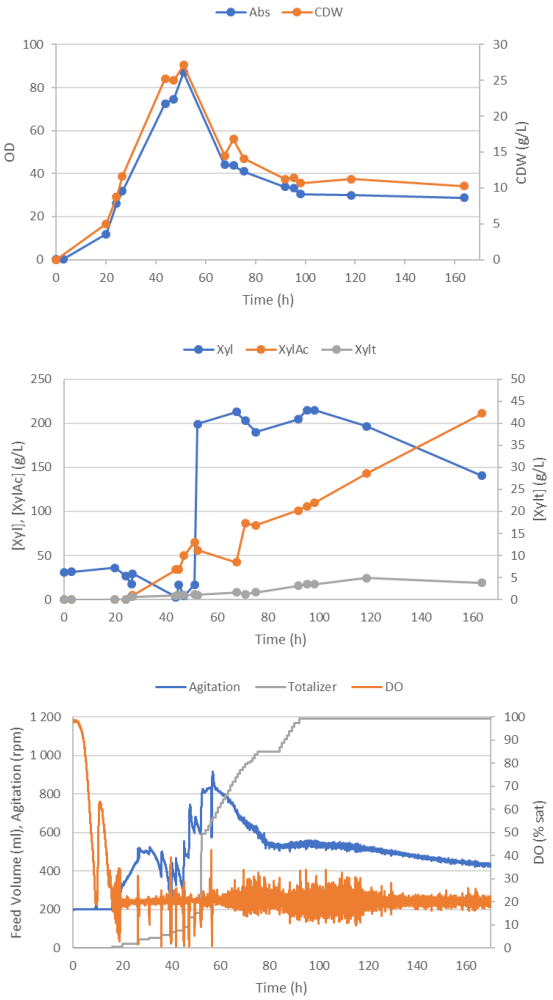

Figure 4.4: *P.sacchari* fed-batch cultivation C, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 20% of DO.

As can be seen in Figure 4.4, the production of xylitol did not increase compared to the previous cultivations, reaching a concentration value of just 3.8 gL^{-1} after 164 hours, corresponding to a yield of 0.02 g_{XyOH}/g_{Xyl} and volumetric productivity of 0.02 $gL^{-1}h^{-1}$ (Table 4.1). With this result, it can be assumed that the reason for the lack of xylitol production it is not associated with the aeration conditions and oxygen availability of the culture medium.

The xylonic acid concentration attained at the end of the cultivation was of 211 gL^{-1}, corresponding to the productivity of 1.29 $gL^{-1}h^{-1}$, which is 40% lower when compared to the Cultivation B. Since this cultivation was performed at a DO of 20%, higher xylonic acid productivities were expected. This result supports the hypothesis raised in the discussion of the results of the previous fermentation regarding the reoxidations of the cofactor NADH through an alternative way since the increase of the dissolved oxygen in the medium had no positive effect on the xylonic acid production.

The reduction of xylonic acid productivity could be explained by the higher xylose concentrations maintained during the second phase of the cultivation. Figure 4.4 shows that xylose concentration ended

up at around 200 gL^{-1}, way above the concentrations around 120 gL^{-1} achieved in the previous cultivations. This high xylose concentration resulted in a drastic biomass concentration reduction, as can be seen through the values of CDW and OD represented in Figure 4.4. Thanks to this reduction, the xylose uptake decreases and consequently, the xylonic acid productivity decreases as well. This result reflects on the value of the xylonic acid yield, which is practically the same as in Cultivation B, as it can be seen in Table 4.1.

At this point, it had been interesting to replicate this cultivation, but with a xylose concentration around 120 gL^{-1}, to test the effect that xylose concentration could have in xylonic acid productivities. However, as the objective was the production of xylitol, this option was not considered.

Table 4.1: Overall yields and productivities of xylonic acid (XylAc) and xylitol (XyOH) in the bioreactor fed-batch cultivations A, B and C.

Cultivation		**Xylitol**	**Xylonic Acid**	**Total**
A:	$\mathbf{Y_{P/S}}$ (g P/g Xyl)	0.01	0.68	0.69
30 gL^{-1} of	$\mathbf{Y_{P/S}}$ (mol g P/g Xyl)	0.01	0.61	0.62
Xylose	$\mathbf{Prod_{vol}}$ (g L^{-1} h^{-1})	0.03	2.04	
1% DO	$\mathbf{Prod_{vol\ (tp)}}$ (g L^{-1} h^{-1})	0.05	3.34	
B:	$\mathbf{Y_{P/S}}$ (g P/g Xyl)	0.01	0.99	1.00
30 gL^{-1} of	$\mathbf{Y_{P/S}}$ (mol g P/g Xyl)	0.01	0.89	0.90
Xylose	$\mathbf{Prod_{vol}}$ (g L^{-1} h^{-1})	0.03	2.10	
1% DO	$\mathbf{Prod_{vol\ (tp)}}$ (g L^{-1} h^{-1})	0.04	3.04	
C:	$\mathbf{Y_{P/S}}$ (g P/g Xyl)	0.02	1.01	1.03
30 gL^{-1} of	$\mathbf{Y_{P/S}}$ (mol g P/g Xyl)	0.02	0.91	0.93
Xylose	$\mathbf{Prod_{vol}}$ (g L^{-1} h^{-1})	0.02	1.29	
20% DO	$\mathbf{Prod_{vol\ (tp)}}$ (g L^{-1} h^{-1})	0.03	1.89	

4.2.4 Cultivation D

Since the Cultivation C did not show an increase in xylitol production, it was decided to lower the DO back to 1%, returning to the original conditions of Cultivation A and Cultivation B.

As mentioned in section 2.1 *P. sacchari* has the capability of accumulating P(3HB) in addition to the xylitol and xylonic acid production. The accumulation of this bioplastic was followed to confirm whether

the metabolism of this bacteria was being diverted from the production of xylitol to that of P(3HB). Figure 4.5 represents the time course of growth and metabolite production in cultivation D.

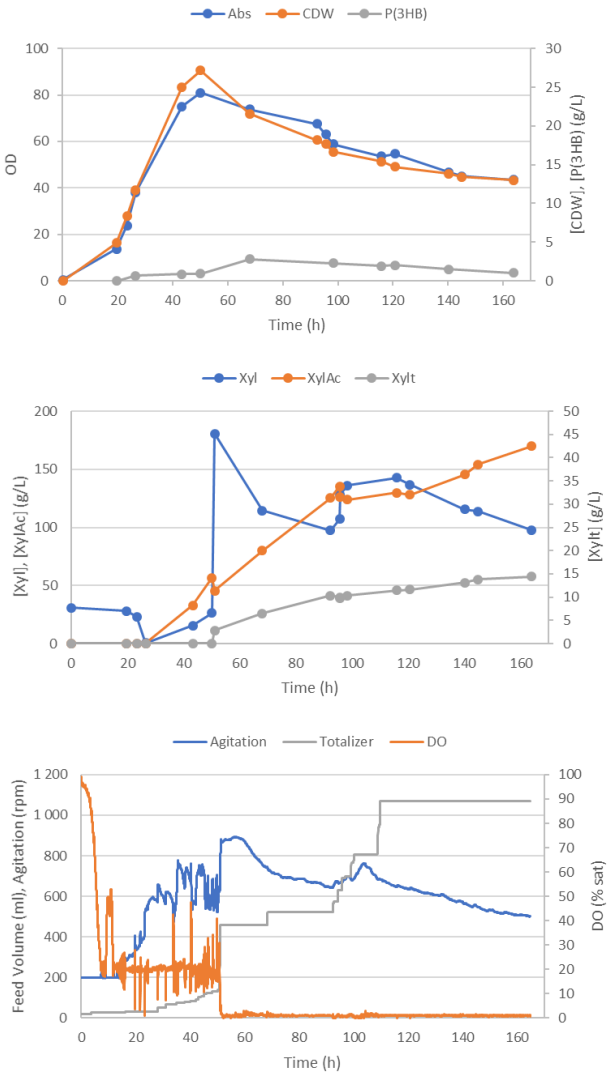

Figure 4.5: *P.sacchari* fed-batch cultivation D, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 1% of DO.

From the analysis of the first graphic of Figure 4.5, it is noticeable that the accumulation of P(3HB) is minimal, achieving a maximum value of 2.8 gL^{-1} at the 68th hour. This result was expected since it is already known that P(3HB) accumulation is promoted when the medium is subjected to the limitation of one of the essential nutrients, such as nitrogen, phosphate, among others (as stated by [SUD,2000], which was being avoided throughout these assays, as it has been explained at the beginning of section 4.2.

Regarding xylitol production, the concentration achieved after 163 hours was 15 gL^{-1}, which is three times higher than the previous cultivations A and B carried out in similar conditions. This corresponds to a yield of 0.06 g_{XyOH}/g_{Xyl} and a productivity of 0.09 $gL^{-1}h^{-1}$. However, these values remain well below

expectations when compared to the ones achieved with the fed-batch cultivations performed by [BON,2018].

On the other hand, xylonic acid production was much lower than the previous cultivations analysed. The yield obtained was 0.67 g_{XylAc}/g_{Xyl} and the productivity was 1.04 $gL^{-1}h^{-1}$ (half of what was obtained in Cultivation A and Cultivation B) as it can be observed in Table 4.2. At this point, it was still unknown the reason for the reduction of xylonic acid production as well as for the increase of the xylitol production. This direct relationship between the decrease of xylonic acid and increase of xylitol reinforce the idea mentioned in the discussion section of Cultivation B that the productivities of both byproducts are related to the same culture factor.

4.2.5 Cultivation E and F

In these two cultivations, the initial composition of the mineral medium was changed. Rather than start with a phosphate concentration of 13 gL^{-1}, it was decided to start with 3 gL^{-1} KH_2PO_4 and a subsequent pulse of a concentrated solution of KH_2PO_4 to attain 10 gL^{-1} was added whenever the initial phosphate was consumed, to avoid the limitation by this compound. This change was performed to simulate the conditions used by [BON,2018], where considerable xylitol concentrations were reached.

As studied by [AKI,2009], xylulokinase (XK), responsible for phosphorylation of xylulose into xylulose-5-phosphate (as seen in Figure 2.3) revealed an ability to phosphorylate several sugars and polyols (including xylitol) with low catalytic efficiencies. This non-specific activity of xylulokinase results in the production and accumulation of potentially toxic phosphorylated compounds that are not further metabolized. With that, one speculated that the high initial phosphate concentration could increase the xylulokinase activity, resulting in the phosphorylation of xylitol, thus compromising the productivities obtained of this compound.

The other conditions were kept the same as in previous cultivations. Cultivation E was performed with a DO of 20% sat. throughout the entire cultivation, while in cultivation F the DO was reduced to of 1% sat during the production phase.

The following figures (Figure 4.6, Figure 4.7) represent the time course of growth and metabolite production, as well as automatic data acquired during the bioreactor assay.

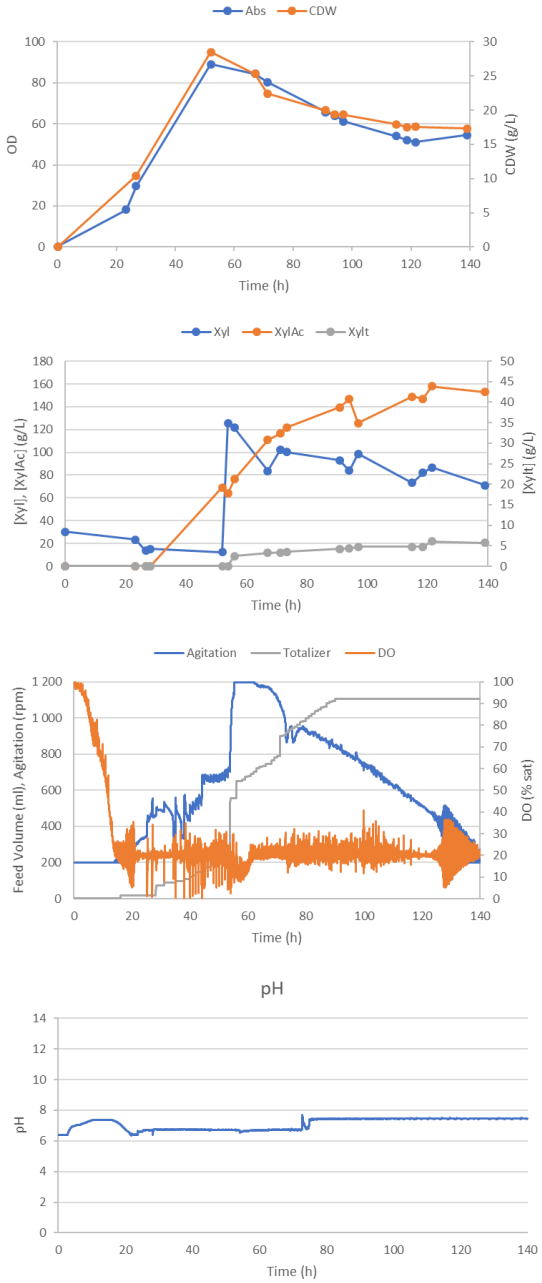

Figure 4.6: *P.sacchari* fed-batch cultivation E, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 20% of DO.

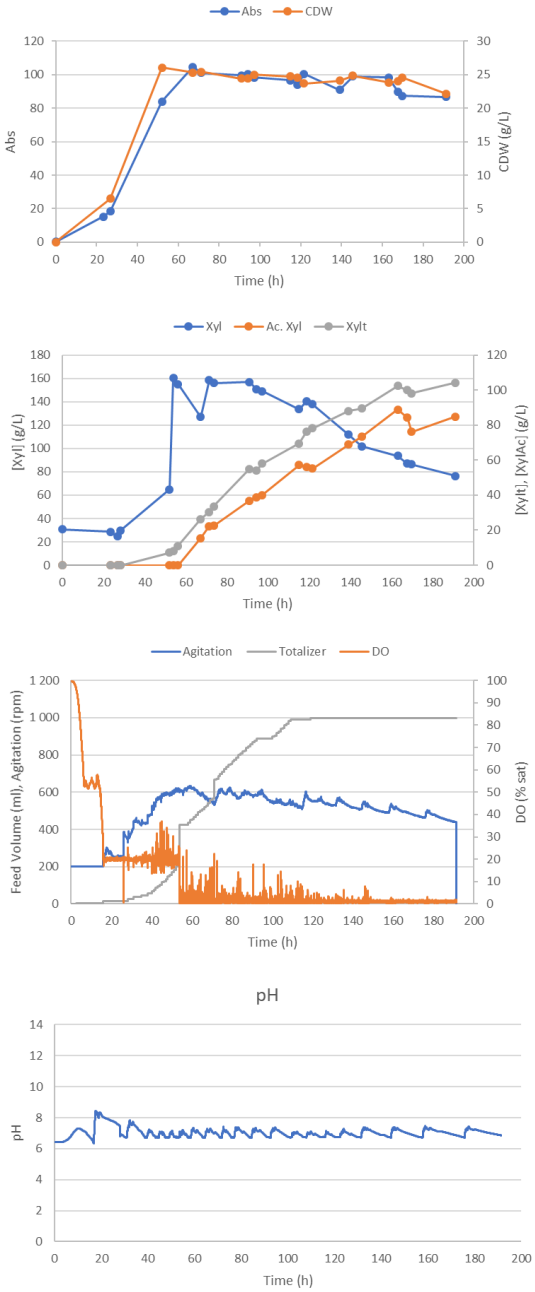

Figure 4.7: *P.sacchari* fed-batch cultivation F, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 1% of DO.

A substantial difference between cultivations E and F can be observed concerning xylitol production. While in cultivation E a xylitol concentration of 5 gL^{-1} after 139 hours is obtained, in cultivation F, a considerable amount of xylitol was produced for the first time in this work, achieving a xylitol

concentration of 104 gL^{-1} after 191 hours, resulting in a xylitol yield of 0.38 g_{XyOH}/g_{Xyl} with a productivity of 0.55 $gL^{-1}h^{-1}$ (Table 4.2). This value is six times higher than in Cultivation D, the one with the higher productivity so far. The results of cultivation F are similar to the ones achieved by [BON,2018], with an average yield and productivity of 0.34 g_{XyOH}/g_{Xyl} and 0.61 $gL^{-1}h^{-1}$ respectively.

With these results, the hypothesis proposed above regarding the xylitol production inhibition thanks to the initial high phosphate concentration is most likely invalid since both fermentations had the same phosphate concentration profile and different xylitol productivities. It is important to acknowledge that many variables were different between the two cultivations, namely, the pH profile and the DO sat %. So, to properly invalidate the hypothesis mentioned above, it would be necessary to perform more assays where just the phosphate concentration was changed.

Comparing Figure 4.6 and Figure 4.7, besides the set DO value which had already proven not to influence the final xylitol concentration, the pH profile differs considerably. Although the pH set-point was 6.8 in both fermentations, in cultivation F the pH suffers an oscillation throughout the cultivation reaching values close to 8. This oscillation occurred because a different type of tube was used for the addition of base aiming at pH control. The tube used in F had a higher diameter, thus higher flow rate, resulting in a greater amount of base added in each pulse, that caused the verified oscillation. It is important to mention that the oscillation in the pH profile verified at the beginning of fermentation E (during the first twenty hours) is normal, and it is due to the consumption of the citric acid presented in the mineral medium. While citric acid is being consumed the pH increases, decreasing after to 6.8 again when xylose starts being consumed and xylonic acid produced.

With this unexpected result, one can conclude that the optimum pH for *P. sacchari* growth (pH=6.8), may not be suitable for the production of xylitol, this being the reason why all previous cultivations did not produce xylitol. The pH profile in cultivation F shows that pH varies between 6.8 and 8, with an average of around 7.3. This means that the optimum pH for the xylitol production may be higher than the pH for bacterial growth (pH=6.8).

As discussed in Cultivation D, the key factor responsible for the lack of xylitol production seems to be directly related to the increase of xylonic acid production. In cultivation E (Figure 4.6), the xylonic acid concentration reached a value of 160 gL^{-1} after 160 hours, corresponding to a productivity of 1.1 $gL^{-1}h^{-1}$ while in cultivation F, 85 gL^{-1} were attained after 191 hours representing a productivity of 0.44 $gL^{-1}h^{-1}$ (Figure 4.7). In terms of yield, cultivation E reached a value of 0.55 g_{XylAc}/g_{Xyl}, which is also higher than the 0.31 g_{XylAc}/g_{Xyl} achieved in cultivation F.

It is important to mention that around the 70th hour, the pH set point of the cultivation E was changed to 7.5, to see if this change would influence the xylitol or xylonic acid productivities. This change caused no effect on xylitol production, however, a decrease in the rate of xylonic acid production is observed. This decrease in xylonic acid production reflects on the values of yield and productivity achieved, which, despite being higher than the ones in fermentation F, are lower than the values obtained in fermentation D (Table 4.2), thus supporting the idea that xylonic acid production is favoured at lower pH (probably around the ideal pH for *P. sacchari* growth at 6.8).

This pH-dependency on the productivities of xylitol and xylonic acid could explain the results obtained in the previous cultivations (Cultivation A, Cultivation B and Cultivation C), namely the negligible xylitol production and the high xylonic acid production. An analysis of the pH profiles regarding the previous cultivations was made at the end of this chapter.

As mentioned in the discussion of the results of Cultivation B, oxygen-limited conditions (1% of DO) was used, which difficult the reoxidation of the cofactor NADH into NAD^+ (essential for the xylonic acid production). However, the highest xylonic acid productivities were achieved in that cultivation, which means that the culture conditions are stimulating the reoxidation of NADH through an alternative way. With that result, one question arose: Does a lower pH promote re-oxidation of cofactors, even under oxygen-limiting conditions? Or, does lowering the pH increase the activity of the enzyme responsible for the oxidation of NADH?

A study performed with *Escherichia coli* by [DAN,1976] shows that the activity of the enzyme NADH dehydrogenase, responsible for the oxidation of NADH into NAD^+, suffers a drastic decrease with pH higher than 7. This decrease in enzymatic activity prevents the oxidation of NADH, promoting thus the conversion of xylulose into xylitol by the enzyme $X_{OH}DH$, or even the reduction of xylose into xylitol by the enzyme XR (in the case this is the pathway for xylitol production in *P. sacchari* and in the case NADH is the co-factor used), as can be seen in Figure 2.4.

At pH lower than 7, the xylitol pathway is inhibited and the deviation of D-xylulose (or D-xylose) for this route is reduced. With that, most of xylose is channelled to the xylonic acid production pathway thus increasing its productivity. On the other hand, the enzymatic activity of NADH dehydrogenase is not inhibited at this pH, and the essential reoxidation of NADH into NAD^+ for xylonic acid production takes place.

It is important to mention that this proposed explanation is just a theory. More work on this topic needs to be developed in order to validate this idea.

Table 4.2: Overall yields and productivities of xylonic acid (XylAc) and xylitol (XyOH) in the bioreactor fed-batch cultivations D, E and F.

Cultivation		Xylitol	Xylonic Acid	Total
D:	$Y_{P/S}$ (g P/g Xyl)	0.06	0.67	0.73
30 gL^{-1} of	$Y_{P/S}$ (mol g P/g Xyl)	0.06	0.61	0.67
Xylose	$Prod_{vol}$ (g L^{-1} h^{-1})	0.09	1.04	
1% DO	$Prod_{vol\ (tp)}$ (g L^{-1} h^{-1})	0.13	1.51	
E:	$Y_{P/S}$ (g P/g Xyl)	0.02	0.55	0.57
30 gL^{-1} of	$Y_{P/S}$ (mol g P/g Xyl)	0.02	0.50	0.52
Xylose	$Prod_{vol}$ (g L^{-1} h^{-1})	0.04	1.10	
20% DO	$Prod_{vol\ (tp)}$ (g L^{-1} h^{-1})	0.07	1.80	
F:	$Y_{P/S}$ (g P/g Xyl)	0.38	0.31	0.69
30 gL^{-1} of	$Y_{P/S}$ (mol g P/g Xyl)	0.38	0.28	0.66
Xylose	$Prod_{vol}$ (g L^{-1} h^{-1})	0.55	0.44	
1% DO	$Prod_{vol\ (tp)}$ (g L^{-1} h^{-1})	0.76	0.62	

4.2.6 Cultivation G

Having in mind the results obtained in Cultivation E and F, the influence of pH on the production of the xylitol was followed. The cultivation conditions during the growth phase were maintained similar to previous cultivations, with the pH set point at 6.8. When the bacterial density attained values around 25/30 gL^{-1} CDW the culture conditions were changed, namely xylose concentration was increased up to 120 gL^{-1}, the DO was set to 1% and the gas flow was set to 1 $L.min^{-1}$, as mentioned in Cultivation A. In addition to these changes, the pH was set to 8.5. Contrary to what was used in the fermentation F, the tube used for base addition was the same as in the other fermentations, i.e. with a smaller diameter, to avoid the oscillation on the pH profile verified in fermentation F. Figure 4.8 represents the time course of growth and metabolite production, as well as automatic data acquired during the fed-batch cultivation G.

From the analysis of the pH profile, it is noticeable that it did not stay at 6.8 during the growth phase, reaching values around 8 for almost 24 hours, stabilizing at 6.8 only after nearly 60 hours. This anomaly was responsible for the prolongation of the growth phase of the fermentation lasting almost 80 hours.

Despite the lag verified on the growth phase, there was a problem with the dissolved oxygen (DO) sensor, which explains why the values of DO did not stabilize at 1% during the production phase, having

oscillations which almost reached 40%. Consequently, the agitation did not increase (as usual), since it was set in cascade with the DO. Thanks to this low agitation value, the actual dissolved oxygen in the culture could have been even lower than the set point of 1%, imposing oxygen-limited conditions to the culture.

Analysing the CDW profile in Figure 4.8, an abrupt decrease in biomass concentration can be seen as soon as the production phase conditions were implemented. With that, one can conclude that the pH that was set in the production phase (pH=8.5) was too high for *P. sacchari* to survive. Consequently, the metabolic activity of the bacteria was too low, as it can be seen from the values of yields and productivities presented in Table 4.3. At the end of the fermentation, xylitol and xylonic acid concentrations stagnated at values of just 11 gL^{-1} and 25 gL^{-1} respectively, while the xylose concentration remained above 140 gL^{-1}. In this cultivation only 250 g of xylose was consumed instead of the average 500 g of previous fermentations, reflecting the detrimental effect of the high pH on the metabolic activity of the cells.

At this point, we could not prove if an increase in pH promotes xylitol production. This could still be true however the chosen pH value of 8.5 was too high resulting in a loss of metabolic activity from the bacteria. Having said that, the optimum pH for xylitol production might be between 7 and 8.

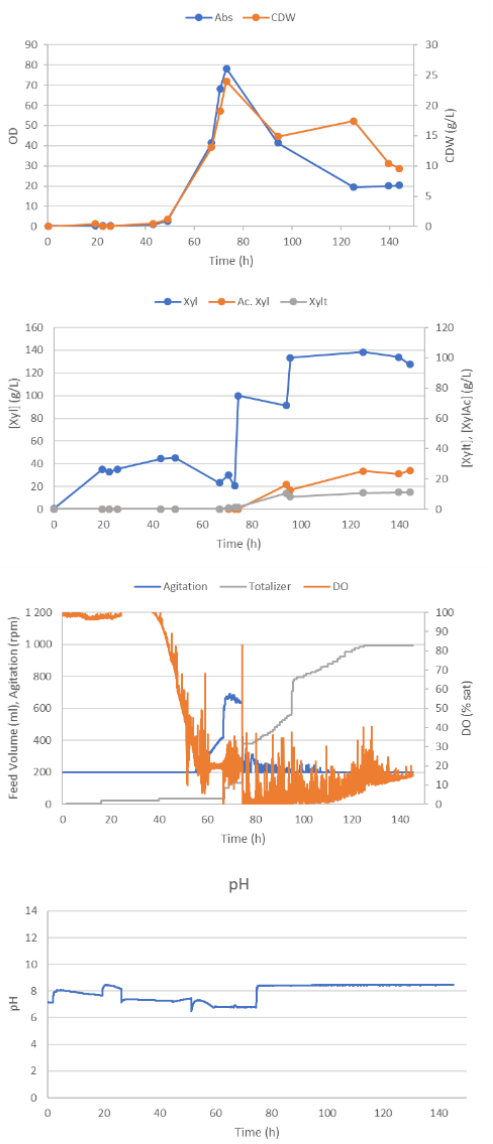

Figure 4.8: *P.sacchari* fed-batch cultivation G, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 8.5 and 1% of DO.

Table 4.3: Overall yields and productivities of xylonic acid (XylAc) and xylitol (XyOH) in the bioreactor fed-batch cultivations G.

Cultivation		**Xylitol**	**Xylonic Acid**	**Total**
G:	$\mathbf{Y_{P/S}}$ (g P/g Xyl)	0.06	0.15	0.21
30 gL^{-1} of	$\mathbf{Y_{P/S}}$ (mol g P/g Xyl)	0.06	0.13	0.20
Xylose	$\mathbf{Prod_{vol}}$ (g L^{-1} h^{-1})	0.08	0.18	
1% DO, pH=8.5	$\mathbf{Prod_{vol\ (tp)}}$ (g L^{-1} h^{-1})	0.16	0.37	

4.2.7 Xylitol concentration vs pH

After realising that the pH could have a great impact on xylitol production, the pH profile was retrieved for each cultivation and related to the xylitol concentration profile.

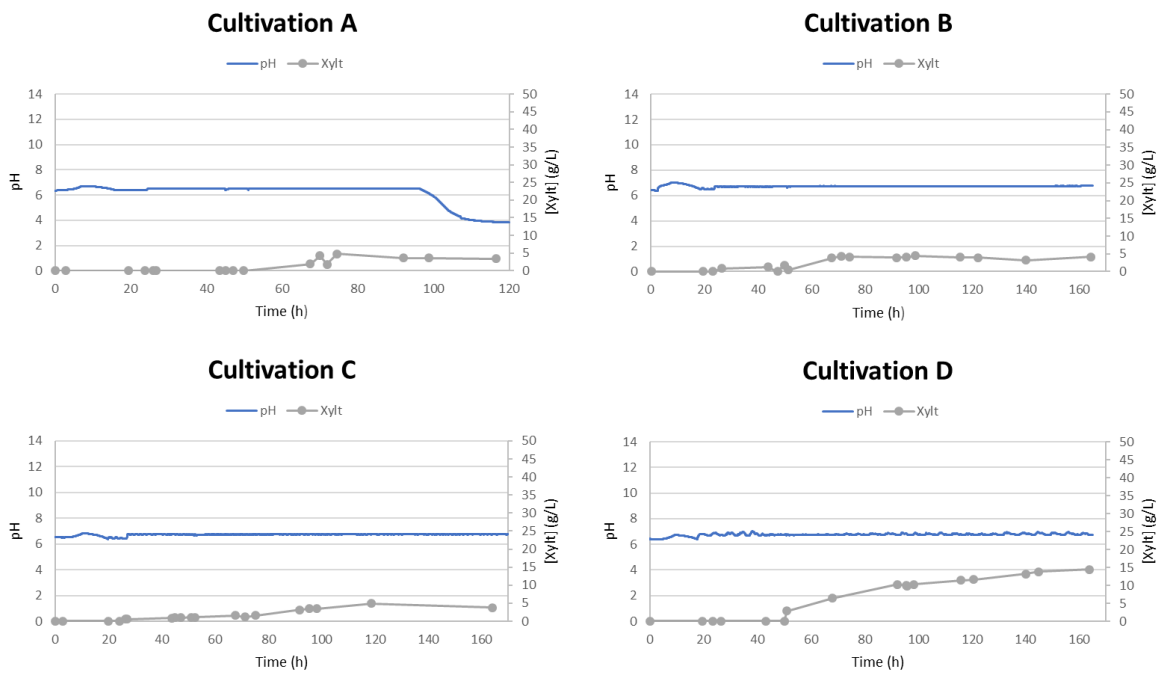

Figure 4.9: Data representing the pH profile and xylitol production of the cultivations A, B, C and D.

First of all, it is important to address the decrease in pH verified at the end of fermentation A. This decrease was due to the exhaustion of the base solution used to control the cultivation pH. Since there was no base to add to the culture, the pH decreased thanks to the constant xylonic acid production by *P. sacchari*. This decrease did not have a significant effect on the fermentation since it happened at the end of the production phase, around the 100th hour.

From the analysis of Figure 4.9, it can be seen that the pH profile of cultivations A, B and C is very similar (excluding the decrease at the end of cultivation A) as is the xylitol concentration attained with a maximum 5 gL^{-1}.

However, regarding cultivation D, a slight pH oscillation is observed which can be the reason for a higher xylitol concentration achieved of 15 gL^{-1}.

This result supports the data obtained in cultivation F, where the larger pH oscillation agrees with the higher xylitol concentration achieved of 104 gL^{-1}. The average pH of cultivation F is higher compared to cultivation D, supporting the idea of increasing xylitol productivity with the increase of the culture pH, as explained in section 4.2.5.

The difference in xylonic acid productivities verified in the first three cultivation (cultivation A, B and C) could not be explained with the pH profiles analysed above since all three cultivations have similar pH profiles and achieved different xylonic acid yields and productivities.

Chapter 5

Conclusions and prospects

This chapter finalises the dissertation, compiling the main conclusions of the study and suggesting future assays.

Due to an increased health and weight consciousness, the demand for sugar-free, low-calorie food products and for sugar substitutes has increased. Thanks to several applications in different industrial sectors, such as food, dental-related products, cosmeceuticals and pharmaceuticals, the interest in xylitol has increased considerably during these last few years.

Biological processes for xylitol production which are more economical and environmental-friendly comparing to conventional catalytic chemical processes are desirable. Moreover, biological processes that use xylose-rich lignocellulosic hydrolysates as carbon source may reduce drastically the cost of raw materials of the process.

The work developed in this dissertation aimed at finding the optimal operation conditions that channel the metabolism of xylose by *P. sacchari* towards xylitol production. The cultivation conditions were chosen based on the xylose metabolic pathways that lead to this extracellular metabolite. Due to the high xylonic acid concentrations obtained in several fermentations an analysis of the pathways leading to this metabolite was also carried out.

The toxic effect of xylitol towards *P. sacchari* was shown in a preliminary shake flask assay. This result shows that it is important to guarantee an effective xylitol recovery mechanism during xylitol production process, in order to attain the best xylitol productivities possible.

The major conclusion drawn from this work is that the pH seems to have a crucial impact on the productivities of xylitol and xylonic acid. The results indicate that the optimal pH for *P. sacchari* growth of 6.8 is too low for the production of xylitol. On the other hand, this pH seems to allow high xylonic acid productivities, even at oxygen-limited conditions (DO= 1 % sat).

One possible explanation is that the increase in pH lowers the enzymatic activity of NADH dehydrogenase. As a consequence, the oxidation of NADH into NAD^+ decreases, leading to a lower NAD^+/NADH ratio, which promotes the conversion of xylulose into xylitol.

The effect of pH on xylitol production could still not be demonstrated with the assays done in this work but is certainly a hot topic for future assays. Although xylitol production could increase with higher pH values, it was shown that the metabolism of *P. sacchari* is negatively affected by pH values near and above 8.5.

Regarding future work, it is still crucial to confirm the oxygen concentrations that benefit xylitol production. As discussed in section 2.3.3, there is literature supporting the increase of xylitol production with the increase of the amount of dissolved oxygen in the medium (i.e DO of 20% sat), since the enzymatic activity of the enzyme responsible for xylitol production, namely XR and $X_{OH}DH$ increases with the oxygen availability in the medium. Other studies support xylitol production through oxygen-limited conditions (DO of 1% sat) since it prevents the reoxidation of NADH (formed during xylose metabolism by the isomerase pathway) into NAD^+, which consequently would favour xylitol dehydrogenation into xylulose thanks to the high NAD^+/NADH ratio.

Other groups working with *P. sacchari* refer to the unexistence of the oxidoreductase metabolic route in *P. sacchari*. However, based on genome sequencing analysis, various putative dehydrogenase and oxidoreductases were identified. Confirmation of the metabolic route for xylitol production would facilitate

the manipulation of the conditions to improve xylitol productivity.

It is of utmost importance the necessity of reproducible assays to ascertain the influence of pH on xylitol and xylonic acid production. The conclusions discussed around this topic are mainly assumptions regarding the experimental results obtained and literature review. Therefore, further studies are required in order to confirm these findings and report them.

Because the production of xylonic acid or xylitol occurs after *P. sacchari* stops growing, it could be interesting to develop a bioprocess featuring cell re-use for the production of either metabolite. This could be done by immobilizing the cells or by using a perfusion bioreactor. This approach could be an efficient way to improve the overall yield and productivity of the bioprocess.

To conclude on the ability of *P. sacchari* to attain higher final xylitol and xylonic acid titres, a bioreactor with a larger volume would be worth testing. Also, online measurements by using sensors of xylose, phosphate and ammonia could facilitate monitoring of the optimal conditions in terms of substrate and nutrients supply.

Finally, several challenges were encountered throughout this study and not all the objectives proposed were fully met. Although many doubts and questions regarding the production of xylitol still remain to be clarified, significant progress was made towards better comprehend the production of this metabolite by *P. sacchari*. Once bioprocess conditions that promote xylitol production are found, the system should be tested with lignocellulosic hydrolysates, thus contributing to a more sustainable xylitol production.

Annex A

Medium composition for shake flask assays

Annex A

This Annex presents the medium composition of shake flask assays.

Figure A.1: Shake flask assays cultivation medium composition

Compound	Quantity (mL)	Concentration of Xylitol		Quantity (mL)	Concentration of Xylitol
Inoculum	2,6			2,6	
Xylose	4			4	
Concentrated medium	10	0 gL^{-1}		10	10 gL^{-1}
Xylitol	0			1,67	
H_2O	83,4			81,73	
Inoculum	2,6			2,6	
Xylose	4			4	
Concentrated medium	10	50 gL^{-1}		10	100 gL^{-1}
Xylitol	8,33			16,67	
H_2O	75,07			66,73	

Annex B

Substrate and by-products of xylose metabolism quantification

This Annex presents the equations that describe the calibration curves used to quantify xylose and its metabolites through HPLC runs.

B.1 Xylose, xylonic acid, xylitol and phosphate determination

Equations represented below (B.1 to B.4) describe the calibration curves obtained, as well as their correlation factor, for xylose, xylonic acid and xylitol in working ranges of 15 to 180 gL-1 (xylose), 4.9 to 97.5 gL-1 (xylonic acid) and 0.5 to 100 gL-1 (xylitol).

$$[Xylose]\ (gL^{-1}) = 5.86 \times 10^{-6} \times A_{peak} + 7.79 \times 10^{-1}\ (r^2 = 0.9995) \quad \text{(B.1)}$$

$$[Xylonic\ Acid]^{UV}\ (gL^{-1}) = 5.53 \times 10^{-6} \times A_{peak} - 2.71 \times 10^{-2}\ (r^2 = 0.9984) \quad \text{(B.2)}$$

$$[Xylonic\ Acid]^{RI}\ (gL^{-1}) = 9.87 \times 10^{-6} \times A_{peak} + 1.62 \times 10^{-1}\ (r^2 = 0.9984) \quad \text{(B.3)}$$

$$[Xylitol]\ (gL^{-1}) = 5.89 \times 10^{-6} \times A_{peak} + 3.93 \times 10^{-1}\ (r^2 = 0.9997) \quad \text{(B.4)}$$

Equations represented below (B.5 to B.7) describe the calibration curves obtained, as well as their correlation factor, for the second HPLC column (used from the Cultivation B), for xylose, xylitol and phosphate in working ranges of 1 to 200 gL^{-1} (xylose), 0.5 to 100 gL^{-1} (xylitol) and 0.1 to 20 gL^{-1} (phosphate). It is important to mention that the xylonic acid calibration curve was not done for the new HPLC column, thanks to the absence of this reagent in the laboratory. Regarding the phosphate calibration curve, it was only done for the new HPLC column since phosphate was only determined in the fermentation with the new column.

$$[Xylose]\ (gL^{-1}) = 5.90 \times 10^{-6} \times A_{peak} - 4.24 \times 10^{-1}\ (r^2 = 0.9996) \quad \text{(B.5)}$$

$$[Xylitol]\ (gL^{-1}) = 6.10 \times 10^{-6} \times A_{peak} - 3.93 \times 10^{-1}\ (r^2 = 0.9997) \quad \text{(B.6)}$$

$$[Phosphate]\ (gL^{-1}) = 1.06 \times 10^{-5} \times A_{peak} + 0.87 \times 10^{-2}\ (r^2 = 0.9998) \quad \text{(B.7)}$$

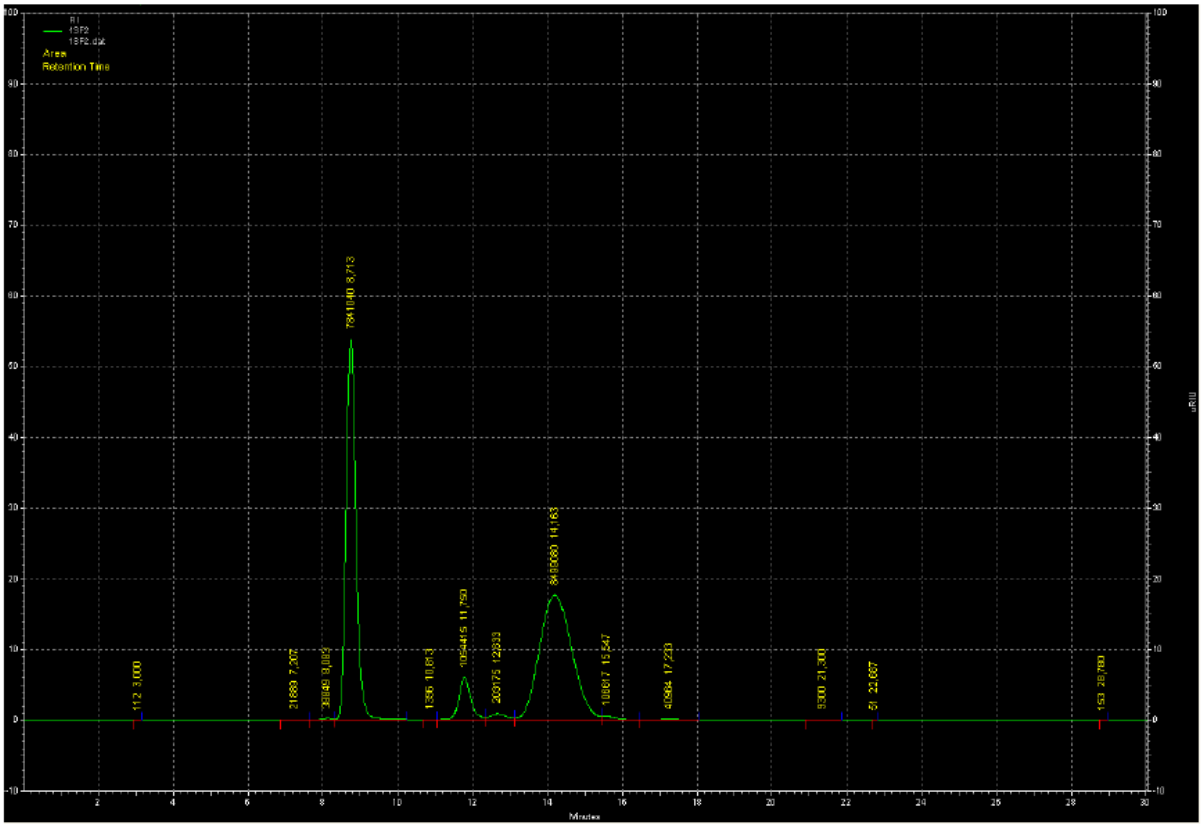

Figure B.2: HPLC chromatogram for RI detector.

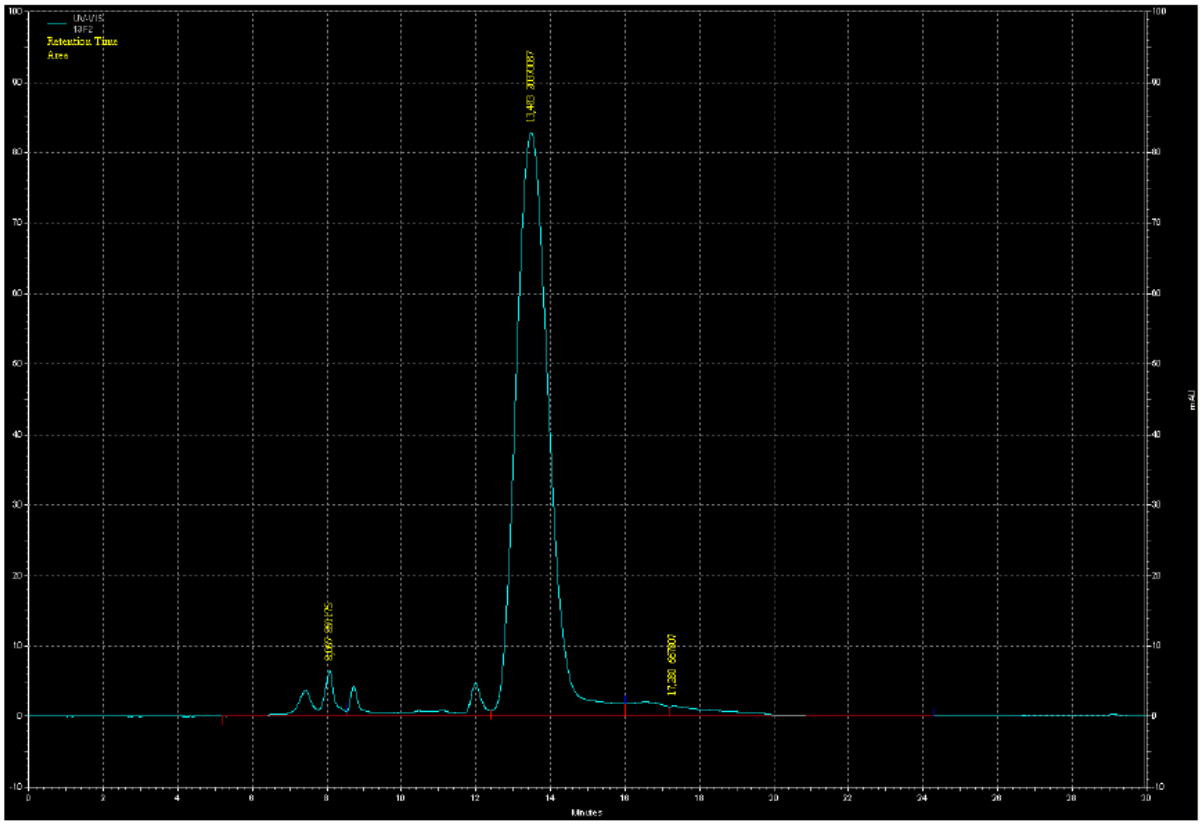

Figure B.3: HPLC chromatogram for UV-visible detector.

B.2 P(3HB) determination

The equation below B.8, describes the calibration curve obtained, as well as its correlation factor, for P(3HB) in the working range of 0.5 to 10 gL^{-1}.

$$[P(3HB)]\ (gL^{-1}) = 7.92 \times 10^{0} \times \left(\frac{A_{P(3HB)peak}}{A_{IS\ peak}}\right) + 4.00 \times 10^{-1}\ (r^{2} = 0.9939) \qquad \text{(B.8)}$$

Annex C

Results of other cultivations

Annex C.

This Annex presents the results of other cultivations that were not worth discussing.

C.1 Cultivation H & I

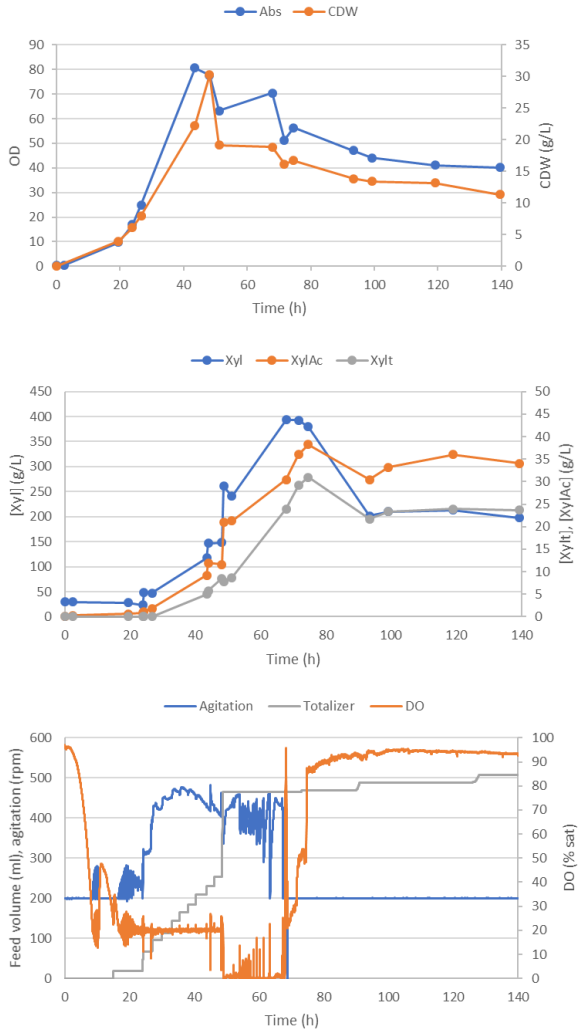

Figure C.4: Data obtained in *P.sacchari* fed-batch cultivation H, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 1% of DO.

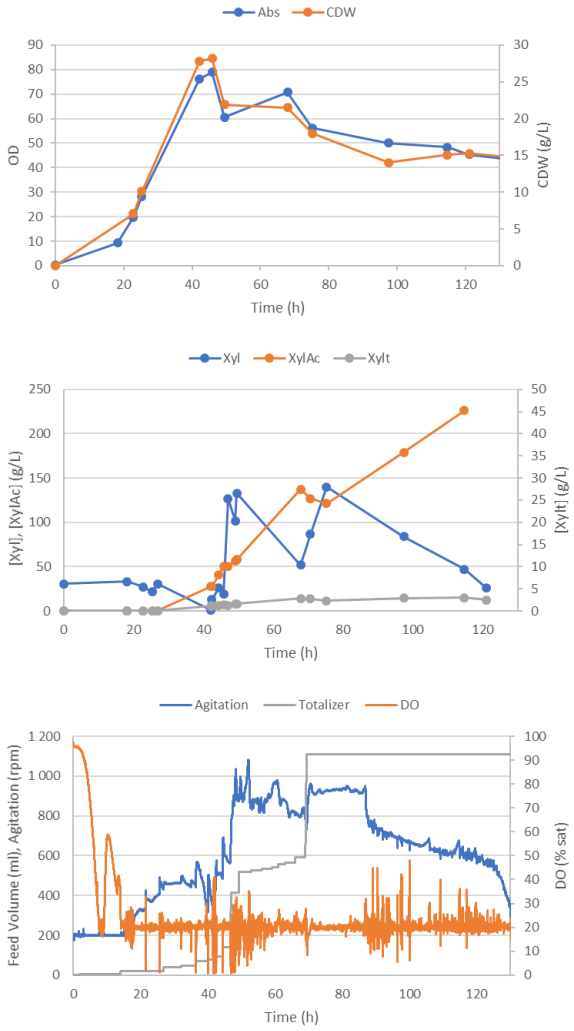

Figure C.5: Data obtained in *P.sacchari* fed-batch cultivation I, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 20% of DO.

C.2 Cultivation J & K

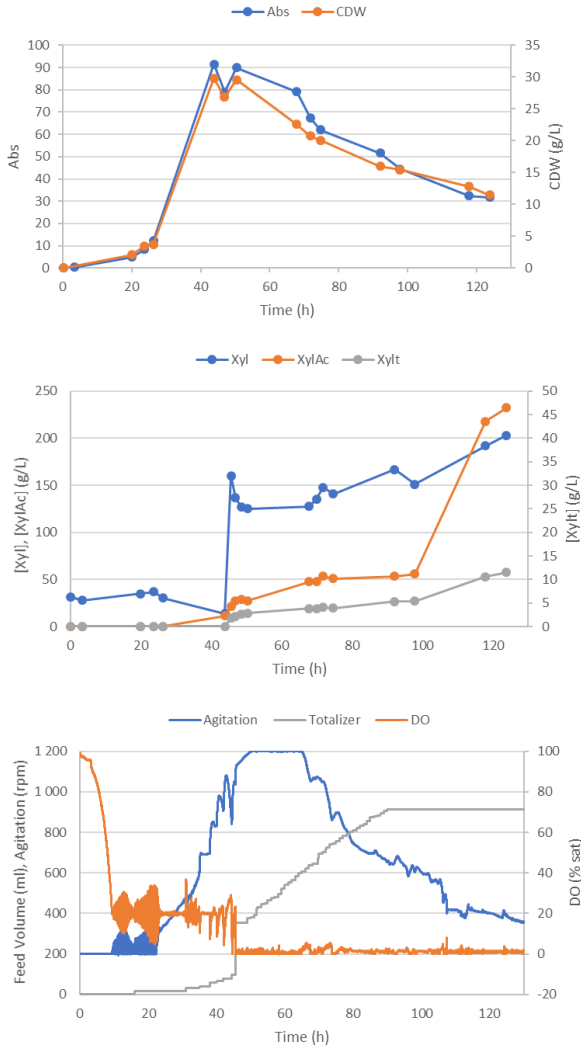

Figure C.6: Data obtained in *P.sacchari* fed-batch cultivation J, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 1% of DO.

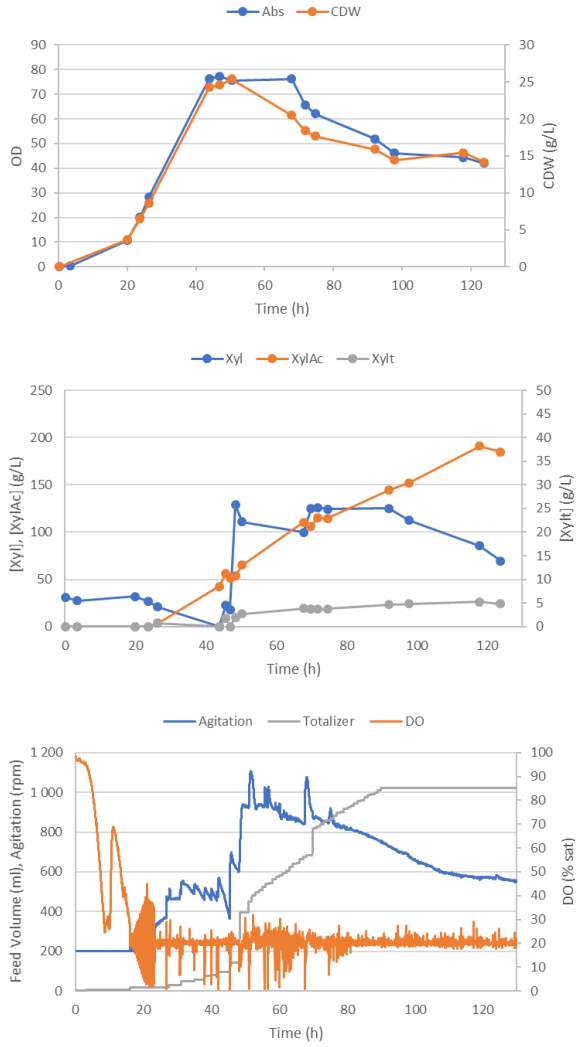

Figure C.7: Data obtained in *P.sacchari* fed-batch cultivation K, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 20% of DO.

Table C.1: Overall yields and productivities of xylonic acid (XylAc) and xylitol (XyOH) in the bioreactor fed-batch cultivations H, I, J and K.

Cultivation		Xylitol	Xylonic Acid	Total
H:	$\mathbf{Y_{P/S}}$ (g P/g Xyl)	0.06	0.08	0.14
30 gL^{-1} of	$\mathbf{Y_{P/S}}$ (mol g P/g Xyl)	0.06	0.07	0.13
Xylose	$\mathbf{Prod_{vol}}$ (g L^{-1} h^{-1})	0.17	0.24	
1% DO	$\mathbf{Prod_{vol\ (tp)}}$ (g L^{-1} h^{-1})	0.25	0.36	
I:	$\mathbf{Y_{P/S}}$ (g P/g Xyl)	0.01	0.79	0.80
30 gL^{-1} of	$\mathbf{Y_{P/S}}$ (mol g P/g Xyl)	0.01	0.72	0.72
Xylose	$\mathbf{Prod_{vol}}$ (g L^{-1} h^{-1})	0.02	2.12	
20% DO	$\mathbf{Prod_{vol\ (tp)}}$ (g L^{-1} h^{-1})	0.03	3.47	
J:	$\mathbf{Y_{P/S}}$ (g P/g Xyl)	0.03	0.54	0.57
30 gL^{-1} of	$\mathbf{Y_{P/S}}$ (mol g P/g Xyl)	0.03	0.49	0.52
Xylose	$\mathbf{Prod_{vol}}$ (g L^{-1} h^{-1})	0.09	1.88	
1% DO	$\mathbf{Prod_{vol\ (tp)}}$ (g L^{-1} h^{-1})	0.15	2.98	
K:	$\mathbf{Y_{P/S}}$ (g P/g Xyl)	0.02	0.66	0.68
30 gL^{-1} of	$\mathbf{Y_{P/S}}$ (mol g P/g Xyl)	0.02	0.60	0.61
Xylose	$\mathbf{Prod_{vol}}$ (g L^{-1} h^{-1})	0.04	1.49	
20% DO	$\mathbf{Prod_{vol\ (tp)}}$ (g L^{-1} h^{-1})	0.06	2.46	

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