TÉCNICO LISBOA

Master Thesis in Biological Engineering

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

José Rafael Proença Domingues

Thesis to obtain the Master of Science Degree in Biological Engineering

Instituto Superior Técnico / BERG, June 2020

ABSTRACT

Paraburkholderia sacchari, previously called *Burkholderia sacchari* is able to accumulate poly-3-hydroxybutyrate (P(3HB)) and produce two extracellular compounds namely 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 in most of its applications.

The focus of this study was the production of xylitol from D-xylose by *B. 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 were carried out with conditions tested previously that promoted xylitol production. Xylose concentrations above 120 gL^{-1}, a dissolved oxygen (DO) of 1% saturation and a stable pH value of 6.8 were tested. These conditions promoted instead the production of xylonic acid and values of 345 gL^{-1}, a yield of 0.99 g_{XylAc}/g_{Xyl} and a productivity of 2.10 $gL^{-1}h^{-1}$ were attained. 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} after 191 hours, resulting in a xylitol yield of 0.38 g_{XyOH}/g_{Xyl} and a productivity of 0.55 $gL^{-1}h^{-1}$. These results show that pH has a very important role on the productivity of xylitol and might also influence the production of xylonic acid.

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

I. INTRODUCTION

Our society is currently experiencing serious environmental problems, which are aggravated by the over-populated state of our planet. 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 [1][2].

In the past few years, society is trying to embrace new measures in order to become more sustainable and confront the waste generation problem. One solution that has been developed, is to use biowaste as a new resource to produce valuable products. These compounds can range from bulk products (eg. bioethanol) to speciality chemicals and biodegradable polymers [3]. This will contribute to sustainable processes not only because sustainable bioresources are used but also because wastes are recycled.

The use of lignocellulosic biomass (LB) as raw material is a promising option from an economic and environmental standpoint. In fact, LB is a nonedible feedstock and the worldwide most abundant renewable raw material. It can be converted into value-added products such as fuels and chemicals through the implementation of the biorefinery concept [4]. Besides wood, this raw material includes agricultural residues such as corn stover, straw, wheat stover and others. The primary components in lignocellulosic biomass are cellulose, hemicellulose and lignin. Cellulose and hemicellulose polymers are potential sugar platforms for biological processes. LB sugars are released by applying a combined treatment consisting of chemical and enzymatic hydrolysis. This will ultimately increase the cost of the raw materials in the bioprocesses. Nevertheless, LB raw materials are most probably the most feasible alternative to fossil fuels thanks to their unique eco-friendly nature.

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 biochemical to be applied both in chemical, enzymatic or biological processes.

Xylitol is a five-carbon sugar alcohol that occurs widely in nature. It 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. It is highly endothermic (provoking a cooling effect), has less 40% calories than sucrose [9] and has a low glycemic index, thus being an excellent sugar substitute for non-insulin dependent diabetics [18]. Besides, xylitol has anti-cariogenic properties and can reduce the occurrence of acute otitis media (AOM) in children. Thanks to that, it has been used in many food products like chewing gum, lozenges or syrup [19].

Nowadays, the large scale production of xylitol is carried out by a chemical route based on the catalytic hydrogenation of highly pure D-xylose in the presence of the toxic Raney nickel catalyst. This method is laborious, energy-intensive, cost-intensive and with a hazardous environmental impact [20].

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 and can use mixtures of sugars, which alleviates the purification step of the lignocellulosic hydrolysates [21].

According to Dasgupta *et al*, 2017 [21], yeasts are preferred for xylitol production primarily due to their high pentose assimilation rates and xylitol productivity and the stable expression levels of the enzymes in the xylitol metabolic pathway. However, most of these yeasts are not considered as GRAS by the FDA. Thus, there is a need to look for other microorganisms capable of producing xylitol.

Paraburkholderia sacchari is a non-model, Gram-negative polyhydroxyalkanoate-accumulating bacterium, isolated from the soil of sugarcane crops in Brazil [5]. It is an interesting microorganism for industrial bioprocesses since it is capable of consuming several sugars like glucose, xylose, sucrose and arabinose and is capable of producing polyhydroxyalkanoates (PHA), xylonic acid and xylitol using the sugars in lignocellulosic hydrolysates as substrate [6]. Additionally and importantly, it is non-genetically modified and classified in “Safety Level 1”.

Poly-3-hydroxybutyrate (P(3HB) belongs to the PHA family. 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 and excess carbon [7]. The properties of these biopolymers are similar to conventional plastics, however important characteristics such as their biodegradability and biocompatibility contribute to its great potential from an environmental standpoint.

D-xylonic acid is a versatile chemical compound, one of the 30 most promising platform chemicals identified by the US Department of Energy [8], capable of replacing gluconic acid in most of its applications.

Xylose might be metabolised by four different pathways that are depicted in Figure *I.1*. The isomerase pathway converts xylose into xylulose (typically used by prokaryotes), the oxidoreductase pathway (mostly present in eukaryotic microorganisms), where a reductase converts xylose into xylitol and a dehydrogenase converts xylitol into xylulose, and finally, the oxidative pathways, Weimberg and Dahms pathways, also recognized as the non-phosphorylative pathways.

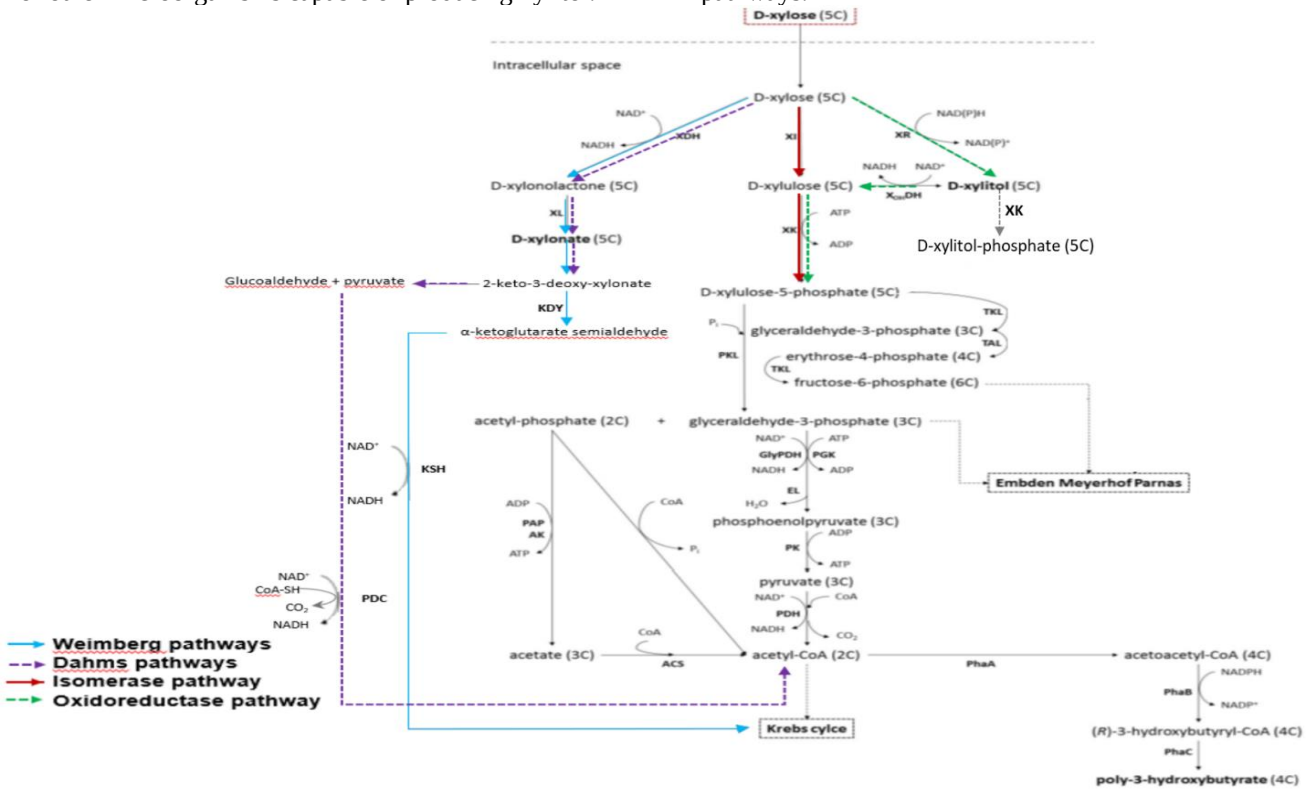

Figure I.1: Four pathways of xylose metabolization by microorganisms: solid blue, Weinberg pathway; dashed violet, Dahms pathway; dashed green, oxidoreductase pathway; solid red, isomerase pathway. Partially taken from Master's thesis of [26] and modified according to the literature review [10]. The 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) [23]

Cherix *et al*, 2014 [11] reported that the xylose metabolic pathway used by B. 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, which are all present in this pathway. Regarding the Weimberg pathway, some of the genes involved in this pathway were found to have homologous genes in *B. 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 *B. sacchari* reported by Raposo *et al*, 2017 [6]. The authors also reported that the genes of the oxidoreductase and Dahms routes were not found, suggesting that *B. sacchari* can not assimilate xylose by these pathways [11].

Regarding the isomerase pathway, after the isomerization of xylose into xylulose catalysed by XI, xylulose is phosphorylated and then continues towards the PPP pathway, where the formation of NAD(P)H occurs. One of the compounds formed during this route, acetyl-CoA, can suffer oxidation via the Krebs cycle to generate energy, or upon limitation of an essential nutrient, go in a different route which leads to P(3HB) accumulation [12].

The most common way that microorganisms 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 *et al*, 2016 [13], and represented in Figure *I.2*, 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.

Previous studies carried out in our group showed the ability of *B. sacchari* to produce xylitol [23][6]. Because Cherix *et al* 2014 [11] reported that the genes encoding for the enzymes of the oxidoreductase pathway were not found in *B sacchari* genome it was assumed that the missing gene in this pathway was the gene encoding for the XR enzyme [23]. The most probable mechanism for xylitol production in *B. sacchari* was thus considered to be in two steps: first the conversion of xylose to xylulose by xylose isomerase (XI), and then the conversion of xylulose into xylitol by $X_{OH}DH$ thanks to the oxidation of the cofactor NADH into NAD^+. This was the mechanism considered in this work.

In this work, the ability of *B. sacchari* to produce xylitol from pure xylose was studied. Fed-batch cultivations in bioreactors were developed aiming to attain high xylitol productivities. The cultivation conditions were chosen based on the metabolic pathway leading to xylitol production and on previous assays carried out by our group.

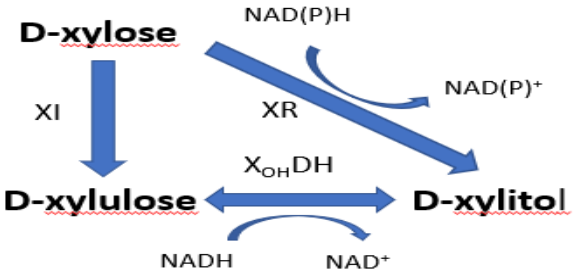

Figure I.2: Xylose network metabolism related to xylitol production, the metabolite of interest of this work, based on literature review [12]. The enzymes are abbreviated as follows: xylose isomerase (XI); xylitol dehydrogenase ($X_{OH}DH$); xylose reductase (XR).

II. Materials and Methods

A. *Microorganism and inoculum preparation*

The microorganism used in this project was the *Paraburkholderia sacchari* DSM 17165. To store cultures of *B. sacchari*, it was added to 2 mL sterile cryovials, containing 300 µL of pure glycerol, 1500 µL of a previous grown liquid culture collected in the late exponential growth phase, prepared with seeding medium (described in Table *II.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.

B. *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.

C. Culture media

1) Seeding medium

The mineral medium [22] used for growing the inoculum was prepared by mixing the compounds described in Table *II.1* (with the exception of $MgSO_4.7H_2O$) with distilled water. 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 II.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

2) Oligo elements solution

To prepare the oligo elements solutions [22], the compounds listed in Table *II.2* were dissolved in distilled water. The solution was autoclaved at 121 ºC for 20 minutes. This solution was stored at 4 ºC.

Table II.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) Bioreactor cultivation medium

To prepare the bioreactor cultivation medium [22], the compounds listed in Table *II.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. A 100 gL^{-1} $MgSO_4.7H_2O$ solution was prepared and autoclaved separately. 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.

Table II.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

D. 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 to prepare the standard solutions to be quantified by HPLC.

E. Culture conditions

1) Shake flask assays

The inoculum was prepared by transferring to a 500 mL shake flask the content of one cryovial (2 mL), a xylose solution to guarantee a 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 was incubated at 30 ºC in an orbital incubator (Infors AG, Switzerland) at 170 rpm for 24 hours i.e. until the end of the exponential growth phase.

Shake flask assays were performed to test if the presence of xylitol in the growth medium could influence the *B. sacchari* metabolic activity. These assays were carried out 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 were used in order to analyse the possible xylitol inhibition. For precision purposes, all the assays were performed in duplicate and the average value was considered.

In order to analyse bacterial growth, 5 culture samples were harvested along the cultivation time. The bacterial growth was obtained by the measurement of the optical density of each sample.

2) Fed-Batch Bioreactor assays

The inocula were prepared as described in section Shake flask assays, with a volume corresponding to 5 % of the

bioreactor initial working volume (50 ml, unless state otherwise).

Fed-batch cultivations were carried out in 2 L STR (New Brunswick Bioflo 115). 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. The aeration rate used was 1 $L.min^{-1}$ or 2.6 $L.min^{-1}$.

Samples were harvested periodically and ran in the HPLC to know the exact xylose concentration of the culture. Pulses of a concentrated xylose solution were added (manual or programmed pulses) to maintain the desired xylose concentration. The tubes for feed addition were previously calibrated.

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

F. Analytical methods

1) Optical density measurements

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 could stay under the threshold (ca. 0.5-0.6). The solution used as reference was deionized water. 3 mL glass cuvettes with an optical path length of 1 cm were used.

2) Cell dry weight determination

It was collected 1.2 mL aliquots of culture samples 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. The microtubes were dried at 60 ºC in a Mermet oven (Model 400) until constant weight. To determine the CDW, the ration between the weight difference of the microtubes after drying the pellets and the empty microtubes and the volume of the collected aliquots was considered.

3) Xylose, xylonic acid, xylitol, and phosphate determination

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.

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 was 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.

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

Since xylose and xylonic acid had similar retention times in HPLC runs, an overlap of both peaks when using the RI detector was detected. To avoid that, the xylonic acid concentration was measured in the UV chromatograms with an appropriate calibration curve. The area of xylonic acid in RI was computed through the concentration previously calculated using another calibration curve determined for the RI chromatograms. To determine the exact xylose peak area, it was subtracted the xylonic acid peak area to the total peak area measured with the RI detector.

4) P(3HB) determination

P(3HB) concentration was determined offline by Gas Chromatography (GC). The apparatus used for the analyses was a GC (Agilent Technologies 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, a standard 3-methyl hydroxybutyrate (Sigma) was used.

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 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. Then 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 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). It was withdrawn 200 µL of the organic phase from each tube to appropriate vials and kept at -20ºC until GC analysis.

The calibration curve for P(3HB) was obtained for a working range of 0.5 to 10 gL^{-1}.

III. Results and Discussion

A. *Shake flask assays*

Shake flask assays were carried out to test if the presence of xylitol in the cultivation medium could influence *B. sacchari's* metabolic activity. *B. 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.

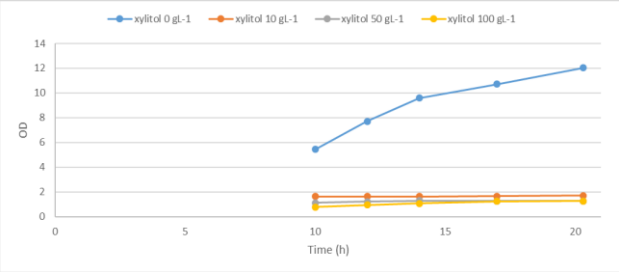

Figure III.1: B. sacchari's growth in SM, supplemented with 20 gL^{-1} of xylose and 0, 10, 50 or 100 gL^{-1} of xylitol.

The assay with 0 gL^{-1} of xylitol shows a gradual increase in the OD, indicating bacterial growth during the 20 hours. This is not observed in the assays with xylitol, where the bacterial growth is completely inhibited by the presence of this compound in the medium. Although the difference is not very significant, the assays show a decrease in OD with the increase in xylitol concentration, concluding 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 [25].

B. *Fed-Batch Bioreactor assays*

The objective of these bioreactor assays was to find the best culture conditions so that in the production stage 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 productions while inhibiting xylonic acid and P(3HB) production.

To avoid polymer formation, nitrogen and phosphate were supplied in excess. 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. Nitrogen was intermittently added to the medium during pH control by adding as base ammonium hydroxide.

Since these conditions were maintained in all fermentations, and having in mind the results obtained by Bondar 2018 [23], which use the same approach and successfully avoided the polymer formation, polymer accumulation was not determined throughout this work.

As reported by Winkelhausen, E and Kuzmanova, S [14], both XR and X_{OH}DH enzyme activities are dependent on the concentration of xylose in the culture medium. The influence of xylose concentration on xylitol production by B. sacchari was studied by Bondar 2018 [23], and it was verified that the emancipation of xylitol production only takes place under inhibitory xylose concentrations (above 120 g/L) 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.

Moreover, based on xylitol metabolic pathways, a low oxygen concentration during the production phase is also expected to improve xylitol production. Fed-batch cultivations aiming at high xylitol concentrations were performed in two steps. First, it was established the perfect conditions to stimulate bacterial growth, i.e 20% of dissolved oxygen (DO), with an aeration rate of 2.6 $L.min^{-1}$ and with an initial xylose concentration of 30 gL^{-1} until a concentration of 30 gL^{-1} CDW was attained.

In the second step, xylose concentration was kept above 120 gL^{-1} while the aeration rate was decreased to 1 $L.min^{-1}$ and the DO was set to 1% sat.

1) *Cultivation A*

Cultivation A was carried out as described above and the results are shown in Figure *III.2*. It is noticeable that xylitol production was 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 III.1)even though the xylose concentration profile was achieved, i.e xylose concentration up to 120 gL^{-1} during the production phase and the DO was lowered to 1% sat.

Xylonic acid production instead was way above expected, stabilizing at a concentration of 345 gL^{-1} after 164 hours. At this point, thanks to the high xylonic acid concentrations obtained, it was decided to analyse the fermentations not only from the xylitol production point of view but also from the xylonic acid production perspective.

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 must be stimulating the reoxidation of NADH through an alternative way. This topic will be discussed ahead.

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 Bondar 2018 [23].

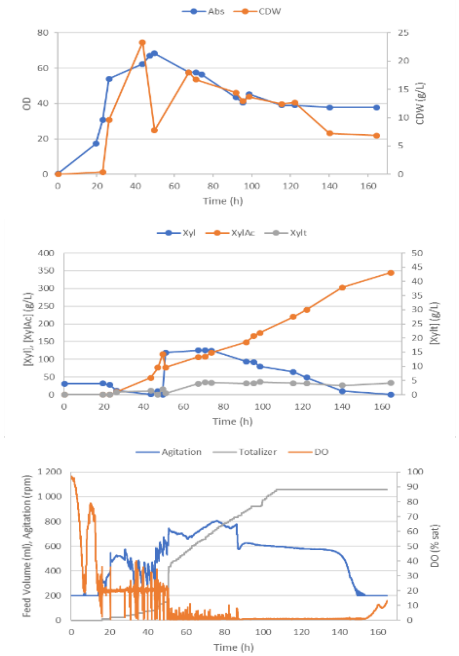

Figure III.2: B.sacchari fed-batch cultivation A, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 1% of DO

2) Effect of Dissolved Oxygen (Cultivation B)

Another key factor in xylitol production is the aeration rate in the culture. Based on the metabolic pathways to xylitol production it is expected that under aerobic conditions, the NADH formed during xylose metabolism can be reoxidized into NAD^+, and as a consequence, xylitol is not produced since the high NAD^+/NADH ratio favours xylitol oxidation to xylulose. Under oxygen-limited conditions, the electron transport system is unable to oxidize intracellular NADH completely, increasing the NADH concentrations, that subsequently favour the xylulose conversion into xylitol by the X_{OH}DH enzyme or the reduction of xylose into xylitol by the XR enzyme [15][16]. However, as reported by Girio et al, 1994 [17], an increase in 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 To test this, during the production phase, the DO was set to 20% sat in this cultivation.

As can be seen in Figure *III.3*, the production of xylitol did not increase compared to cultivation A. With this result, it can be assumed that the lack of xylitol production is not associated with the aeration conditions and oxygen availability of the culture medium.

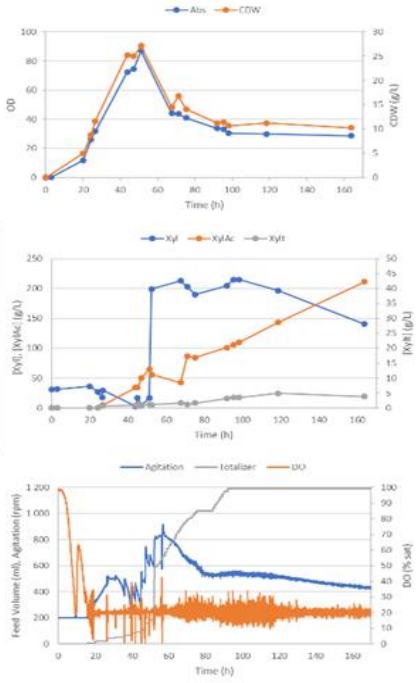

Figure III.3: B.sacchari fed-batch cultivation B, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 20% of DO

A reduction of about 40% in the concentration of xylonic acid at the end of the fermentation is noticed when compared to cultivation A. Since the increase of the dissolved oxygen in the medium had no positive effect on the xylonic acid production, the hypothesis that there is an alternative mechanism for the reoxidations of the cofactor NADH is supported.

Table III.1: Overall yields and productivities of xylonic acid (XylAc) and xylitol (XyOH) in the bioreactor fed-batch cultivations A and B.

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

3) P(3HB) production confirmation (Cultivation C)

The accumulation of P(3HB) was followed to confirm whether the metabolism of this bacteria was being diverted from the production of xylitol to this bioplastic.

From the analysis of Figure *III.4*, it is noticeable that the accumulation of P(3HB) is minimal. This result was expected since the conditions to promote the production of this bioplastic were being avoided.

The concentration of xylitol achieved at the end of the fermentation was three times higher than the previous cultivations. However, these values remain well below expectations when compared to the ones achieved by Bon, 2018 [23].

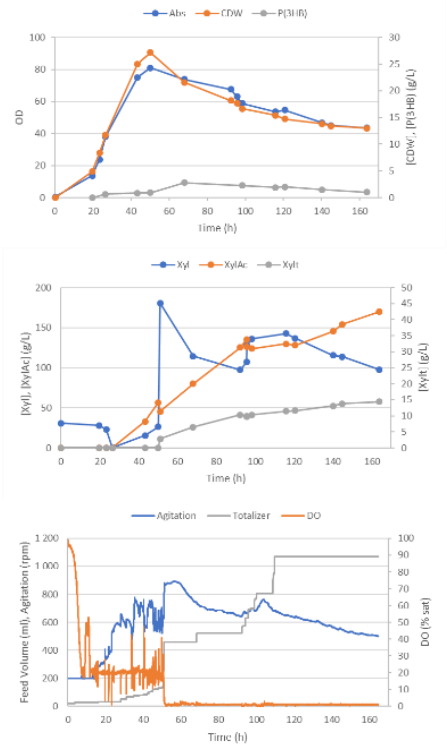

Figure III.4: B.sacchari fed-batch cultivation C, mineral medium supplemented with 30 gL-1 of xylose as the main carbon source, at pH 6.8 and 1% of DO

On the other hand, xylonic acid production was much lower than the previous cultivations analysed (half of what was obtained in Cultivation A and Cultivation B). This direct relationship between the decrease of xylonic acid and increase of xylitol reinforce the idea that the productivities of both metabolites are related to the same culture factor.

4) Effect of the initial phosphate concentration (Cultivation D & E)

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 due to the suspicion that the high phosphate concentration, to which the fermentation was initially submitted, could increase the xylulokinase activity, resulting in the phosphorylation of xylitol.

A substantial difference between cultivations D and E can be observed concerning xylitol production. While in cultivation D a xylitol concentration of 5 gL^{-1} after 139 hours was obtained, in cultivation E, 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 (Table *III.2*).

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 %.

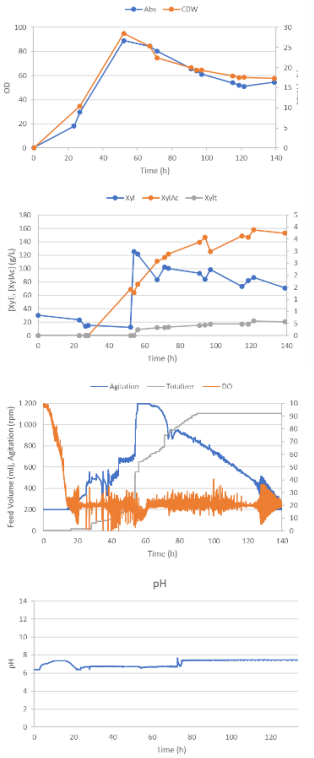

Figure III.5: B.sacchari fed-batch cultivation D, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 20% of DO.

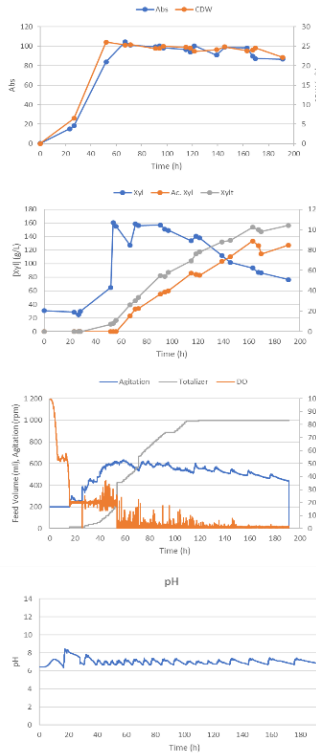

Figure III.6: B.sacchari fed-batch cultivation E, mineral medium supplemented with 30 gL^{-1} of xylose as the main carbon source, at pH 6.8 and 1% of DO.

Comparing Figure *III.5* and Figure *III.6*, 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 E the pH suffers an oscillation throughout the cultivation reaching values close to 8.

With this unexpected result, one can conclude that the optimum pH for *B. 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 Figure *III.6* 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).

A study performed by Dancey *et al*, 1976 [24] 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 *B. sacchari* and in the case NADH is the co-factor used), as can be seen in Figure *I.2*.

At pH lower than 7 the enzymatic activity of NADH dehydrogenase is not inhibited and the essential reoxidation of NADH into NAD^+ takes place, stimulating xylonic acid production. The xylitol pathway is in these conditions 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.

Table *III.2*: Overall yields and productivities of xylonic acid (XylAc) and xylitol (XyOH) in the bioreactor fed-batch cultivations *C*, D *and* E.

Cultivation		**Xylitol**	**Xylonic Acid**	**Total**
C:	**$Y_{P/S}$** (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	
D:	**$Y_{P/S}$** (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	
E:	**$Y_{P/S}$** (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	

5) Effect of changing the pH to 8.5 during the production phase (Cultivation F)

In this cultivation, the pH was set to 8.5 to test its influence on xylitol production.

It is noticeable that the pH 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 pH anomaly was responsible for a long lag phase that has not been observed before and caused the prolongation of the growth phase of the fermentation for almost 80 hours.

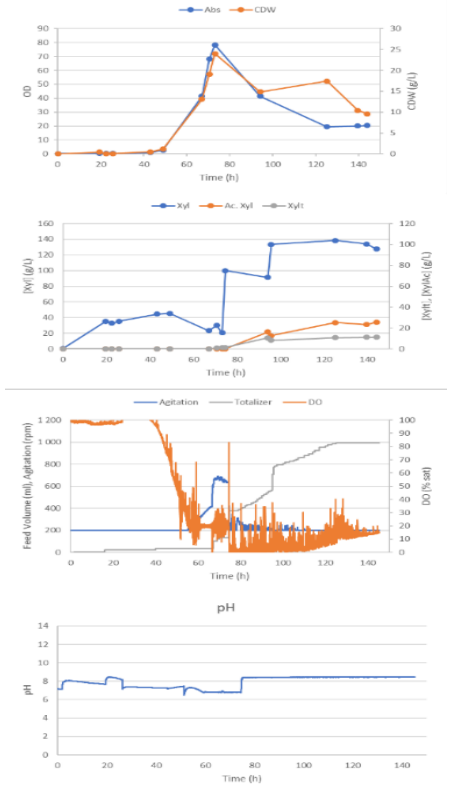

Figure III.7B.sacchari fed-batch cultivation F, mineral medium supplemented with 30 gL-1 of xylose as the main carbon source, at pH 8.5 and 1% of DO.

Analysing the CDW profile in Figure *III.7*, 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 *B. sacchari* to survive

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

Table III.3: Overall yields and productivities of xylonic acid (XylAc) and xylitol (XyOH) in the bioreactor fed-batch cultivations F.

IV. Conclusion and Prospects

The toxic effect of xylitol towards *B. 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 *B. 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).

Although xylitol production could increase with increasing pH, it should not exceed 8.5 as it was shown that the metabolism of *B. sacchari* is negatively affected at this pH.

Regarding future work, it is still crucial to confirm the oxygen concentrations and pH values that benefit xylitol production. Moreover, confirmation of the metabolic pathway for xylitol formation in *B. sacchari* is needed. This would facilitate the manipulation of the conditions to improve xylitol productivity.

Because the production of xylonic acid or xylitol occurs after *B. 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 *B. 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.

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.

REFERENCES

[1] Qingbin Song, J. L. (2015). *Minimizing the increasing solid waste through zero waste strategy*. Beijing, China: Journal of Cleaner Production.

[2] Minghua Z1, X. F.. (2009). *Municipal solid waste management in Pudong New Area, China.* Shanghai: Waste Manag.29(3):1227-33.

[3] Vernès, L., Li, Y., Chemat, F., & Abert-Vian, M. (2019). Biorefinery Concept as a Key for Sustainable Future to Green. *Plant Based "Green Chemistry 2.0": Moving from Evolutionary to Revolutionary*, 15.

[4] Cesário, M. T. F. and de Almeida, M. C. M. D. (2015). Lignocellulosic Hydrolysates for the Production of Polyhydroxyalkanoates. In Kamm, B., editor, Microorg. Biorefineries, volume 26, chapter 4, pages 79–104. Springer-Verlag Berlin Heidelberg, Berlin, first edition.

[5] Brämer, C. O., Vandamme, P., da Silva, L. F., Gomez, J. G., & Steinbüchel, A. (2001). Polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar-cane plantation in Brazil. *International journal of systematic and evolutionary microbiology*, *51*(5), 1709-1713.

[6] Raposo, R. S., de Almeida, M. C. M., de Oliveira, M. D. C. M., da Fonseca, M. M., & Cesário, M. T. (2017). A Burkholderia sacchari cell factory: production of poly-3-hydroxybutyrate, xylitol and xylonic acid from xylose-rich sugar mixtures. *New biotechnology*, *34*, 12-22.

[7] Verlinden, R. A., Hill, D. J., Kenward, M. A., Williams, C. D., & Radecka, I. (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. *Journal of applied microbiology*, *102*(6), 1437-1449.

[8] Liu, H., Valdehuesa, K. N. G., Nisola, G. M., Ramos, K. R. M., & Chung, W. J. (2012). High yield production of D-xylonic acid from D-xylose using engineered Escherichia coli. *Bioresource technology*, *115*, 244-248.

[9] Mohamad, N. L., Mustapa Kamal, S. M., & Mokhtar, M. N. (2015). Xylitol biological production: a review of recent studies. *Food reviews international*, *31*(1), 74-89.

[10] McClintock, M. K., & Zhang, K. (2017). Xylose Metabolism and Its Metabolic Engineering Applications. *Engineering Microbial Metabolism For Chemical Synthesis: Reviews And Perspectives*, 209.

[11] Cherix, J., Guaman, L., Gomez, J., Taciro, M., and Silva, L. (2014). Analysis of xylose catabolism in Burkholderia sacchari for the production of polyhydroxyalkanoates. 14th Int. Symp. Biopolym.

[12] Jeffries, T. W. (1983). Utilization of xylose by bacteria, yeasts, and fungi. In *Pentoses and Lignin* (pp. 1-32). Springer, Berlin, Heidelberg.

[13] Qi, X. H., Zhu, J. F., Yun, J. H., Lin, J., Qi, Y. L., Guo, Q., & Xu, H. (2016). Enhanced xylitol production: Expression of xylitol dehydrogenase from Gluconobacter oxydans and mixed culture of resting cell. *Journal of bioscience and bioengineering*, *122*(3), 257-262.

[14] Winkelhausen, E., & Kuzmanova, S. (1998). Microbial conversion of D-xylose to xylitol. *Journal of Fermentation and Bioengineering*, *86*(1), 1-14.

[15] Rafiqul, I. S. M., & Sakinah, A. M. (2013). Processes for the production of xylitol—a review. Food Reviews International, 29(2), 127-156.

[16] Mohamad, N. L., Mustapa Kamal, S. M., & Mokhtar, M. N. (2015). Xylitol biological production: a review of recent studies. *Food reviews international*, *31*(1), 74-89.

[17] Girio, F. M., Roseiro, J. C., Sá-Machado, P., Duarte-Reis, A. R., & Amaral-Collaço, M. T. (1994). Effect of oxygen transfer rate on levels of key enzymes of xylose metabolism in Debaryomyces hansenii. *Enzyme and microbial technology*, *16*(12), 1074-1078.

[18] Chen, X., Jiang, Z. H., Chen, S., & Qin, W. (2010). Microbial and bioconversion production of D-xylitol and its detection and application. *International Journal of Biological Sciences*, *6*(7), 834.

[19] Barathikannan, K., & Agastian, P. (2016). Xylitol: Production, Optimization and Industrial Application. *Int. J. Curr. Microbiol. Appl. Sci*, *5*(9), 324-339.

[20] Arcaño, Y. D., García, O. D. V., Mandelli, D., Carvalho, W. A., & Pontes, L. A. M. (2018). Xylitol: A review on the progress and challenges of its production by chemical route. *Catalysis Today*.

[21] Dasgupta, D., Bandhu, S., Adhikari, D. K., & Ghosh, D. (2017). Challenges and prospects of xylitol production with whole cell bio-catalysis: A review. *Microbiological research*, *197*, 9-21.

[22] B. S . Kim, S. C. Lee, S. Y. Lee, H. N. Chang, Y. K. Chang, and S. I. Woo. Production of poly(3-hydroxybutyric acid) by fed-batch culture of *Alcaligenes eutrophus* with glucose concentration control. *Biotechnol. Bioeng.*, 43(9):892–898, 1994.

[23] M. Bondar. Exploiting Bioprocess Routes for Xylonic Acid and Xylitol using *Burkholderia sacchari.* Masters thesis, Instituto Superior Técnico, 2018.

[24] Dancey, G. F., Levine, A. E., & Shapiro, B. M. (1976). The NADH dehydrogenase of the respiratory chain of Escherichia coli. I. Properties of the membrane-bound enzyme, its solubilization, and purification to near homogeneity. *Journal of Biological Chemistry*, *251*(19), 5911-5920.

[25] Akinterinwa, O., & Cirino, P. C. (2009). Heterologous expression of D-xylulokinase from Pichia stipitis enables high levels of xylitol production by engineered Escherichia coli growing on xylose. *Metabolic engineering*, *11*(1), 48-55.

[26] I. Palolo. Xylose upgrade directing the metabolism of xylose towards P(3HB), xylitol and xylonic acid in *Burkholderia sacchari*. Masters thesis, Instituto Superior Técnico, 2016.