Sustainable Polyhydroxybutyrate Production in Integrated Forest Biorefineries

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November 2019

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy

Abstract

Biopolymers can provide environmentally friendly alternatives to the use of fossil fuel derived polymers. Among others, their production can be net carbon negative and their disposal can help fertilizing soil by composting. However, the environmental benefits of these biopolymers are largely untapped as their overall production capacity is marginal. This low production is primarily caused by their high production cost and selling price, which hinder most biopolymers to compete on the market. One of the most promising, yet expensive biopolymers are the microbiologically produced polyhydroxyalkanoates (PHAs), since they are compostable and also biodegrade in the marine environment. Furthermore, the currently used raw materials for their production (corn starch and vegetable oils) are not only expensive, but also compete with food production. Alternatively, in Canada, wood is abundantly available through the pulp and paper infrastructure, with residues which can be fed to modern biorefineries. This work explores the feasibility of integrating the most well-known PHA, polyhydroxybutyrate (PHB), into forest biorefineries using the bacterium *Paraburkholderia sacchari*.

For this work, the first forest biorefinery scheme was based on the hydrolysis of softwood hemicellulose as a feedstock for PHB production. Softwood cellulose and lignin were recovered to be further converted into other products. Softwood hemicellulose has a favourable composition as compared to most other hemicelluloses, as it has a high share of the six carbon sugars mannose, glucose and galactose. Mannose and galactose were tested as carbon sources for *P. sacchari* for the first time and showed maximum specific growth rates of 97% and 60% relative to glucose, respectively. However, the presence of inhibitory compounds (acetate, 5-hydroxymethylfurfural, furfural and phenols) inhibited all bacterial growth. It was found that the inhibition comes from strong synergistic effects when the inhibitors are present in mixtures, and the magnitude of the effects was quantified with a mixture design model. Albeit the initial inhibition could be overcome by a high initial cell density (optical density ≥ 5.6) when using a simulated softwood hemicellulose hydrolysate, this approach was not successful for real softwood hemicellulose hydrolysate. Nevertheless, when the hydrolysate was added as a feed solution after an initial growth phase of 24 h, the sugars were all consumed. In comparison with

an inhibitor-free hardwood hydrolysate, the growth rate and PHB yield were lower with softwood hemicellulose hydrolysate. While the sugar composition of the hydrolysate was therefore promising for PHB production, the inhibitory effect currently makes it unsuitable as the carbon source.

The second biorefinery scheme studied was based on a pilot plant process developed by FPInnovations using hardwood biomass. The process converts hardwood cellulose and hemicellulose into a holocellulose hydrolysate, which was used as carbon source for PHB production. Using this hardwood hydrolysate in shake flask fermentations increased the maximum specific growth rate and PHB accumulation of the bacterium as compared to simulated hydrolysates. In high-cell density bioreactor fermentations, the wood hydrolysate afforded one of the highest PHB concentrations to date from lignocellulosic biomass. The chemical composition, thermal transitions and viscoelastic properties were similar to literature values of PHB. The number average molecular mass was 246.4 kDa with a PDI of 3.29. These results make PHB from hardwood hydrolysate, as *co*-product with H-lignin, a sustainable production scheme for industrial PHB production.

Résumé

Les biopolymères peuvent offrir des alternatives respectueuses de l'environnement à l'utilisation de polymères dérivés de combustibles fossiles. Entre autres, leur production peut être négative en carbone et leur biodégradation peut aider à fertiliser le sol par le compostage. Cependant, les avantages environnementaux de ces biopolymères sont largement inexploités car leur capacité de production globale est marginale. Cette faible production est principalement due en raison de leur coût de production et de leur prix de vente élevés, ne pouvant ainsi compétitionner avec la concurrence sur le marché. Les polyhydroxyalkanoates (PHA), produits par fermentation, sont des biopolymères coûteux mais très prometteurs puisqu'ils sont compostables et biodégradables en milieu marin. En outre, les matières premières actuellement utilisées pour leur production (amidon de maïs et huiles végétales) sont non seulement coûteuses, mais aussi en concurrence avec la production alimentaire. Au Canada, le bois est disponible de façon durable et abondante dans l'infrastructure des pâtes et papiers, et les résidus peuvent être alimentés aux bioraffineries modernes. Ces travaux explorent la faisabilité d'intégrer le type de PHA le plus connu, le polyhydroxybutyrate (PHB), dans les bioraffineries forestières à l'aide de la bactérie *Paraburkholderia sacchari*.

Lors de ces travaux, le premier système de bioraffinerie forestière investigué était basé sur l'hydrolyse de l'hémicellulose de bois résineux comme matière première pour la production de PHB. La cellulose de bois résineux et la lignine ont été récupérées pour être converties en d'autres produits. L'hémicellulose de résineux a une composition exceptionnelle par rapport à la plupart des autres hemicelluloses puisque sa composition est élevée en mannose, glucose et galactose. Pour la première fois, le mannose et le galactose furent testés comme sources de carbone pour *P. sacchari* et ont montré des taux de croissance spécifiques maximum de 97% et 60% par rapport au glucose, respectivement. Cependant, la présence de composés inhibiteurs (acétate, 5-hydroxyméthylfurfural, furfural et phénols) a inhibé toute croissance bactérienne. Il a été constaté que l'inhibition provient d'effets synergiques forts lorsque les inhibiteurs sont présents dans les mélanges et l'ampleur des effets a été quantifiée avec un modèle de conception de mélange. Bien que l'inhibition initiale ait pu être surmontée par une densité cellulaire initiale élevée (densité optique $\geq$ 5.6) lors de l'utilisation d'un hydrolysat d'hémicellulose de bois résineux simulé, cette approche n'a pas été couronnée de succès pour l'hydrolysat d'hémicellulose

de résineux réel. Néanmoins, lorsque l'hydrolysat a été ajouté comme solution d'alimentation après une phase initiale de croissance de 24 h, les sucres ont tous été consommés. En comparaison avec un hydrolysate de bois de feuillus sans inhibiteur, le taux de croissance et le rendement de PHB étaient plus bas avec l'hydrolysat d'hémicellulose de bois résineux. Alors que la composition en sucre de l'hydrolysat était prometteuse pour la production de PHB, l'effet inhibiteur le rend actuellement inadapté comme source de carbone.

Le deuxième système de bioraffinerie étudié était basé sur un procédé pilote développé par FPInnovations à partir de biomasse de bois de feuillus. Le procédé convertit la cellulose du bois ainsi que l'hémicellulose en hydrolysat d'holocellulose, qui fut employé comme source de carbone pour la production de PHB. L'utilisation de l'hydrolysat de bois de feuillus en fermentation a augmenté le taux de croissance spécifique maximum et l'accumulation de PHB dans la bactérie par rapport aux hydrolysats simulés. Par la suite, les essais dans des bioréacteurs à haute densité cellulaire a permis d'obtenir l'une des concentrations de PHB les plus élevées à ce jour à partir de biomasse lignocellulosique. La composition chimique, les transitions thermiques et les propriétés viscoélastiques étaient semblables aux valeurs trouvées dans la littérature. La masse molaire moyenne en nombre 246.4 kDa avec une dispersité de 3.29. Ces résultats démontrent que le PHB synthétisé en utilisant l'hydrolysat de bois, un sous-produit, comme source de carbone permet un système de production durable pour la production industrielle de PHB.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ΔH^0 – melting enthalpy for 100% crystalline PHB

ΔH^* – melting enthalpy for PHB sample

$\%\mu_{max,glu}$ – maximum specific growth rate per maximum specific growth rate of glucose

ΔS – sugar consumption (difference between initial sugar concentration and final sugar concentration)

A – acetate

a.u. – arbitrary units

ABE – acetone–butanol–ethanol

Acetyl-CoA – Acetyl-Coenzyme A

ANOVA – analysis of variance

ATP – adenosine triphosphate

BIC – Bayesian information criterion

BUE – Biomass Utilization Efficiency

CDM – cell dry mass

CDM_{24h} – cell dry mass after 24h

CHP – combined heat and power

CS – combined severity

DMA – dynamic mechanical analysis

DSC – differential scanning calorimetry

E' – storage modulus

E'' – loss modulus

ED – Entner-Doudoroff

EMP – Embden-Meyerhof-Parnas

F – furfural

FTIR – Fourier transform infrared spectroscopy

GHG – greenhouse gas

GPC – gel permeation chromatography

GWP_{100} – global warming potential over a 100 years' time horizon

H – HMF

HHH – hardwood holocellulose hydrolysate from thermomechanical pulping

HMF – 5-hydroxymethylfurfural

IC – ion chromatography

LCA – life cycle analysis

mcl – medium chain length

MHG – Meredian Holding Group

MI – material index

M_n – molecular number average

M_w – molecular mass average
n.d. – not determined
NADH – Nicotinamide adenine dinucleotide
NADPH – Nicotinamide adenine dinucleotide phosphate
NMR – Nuclear magnetic resonance
NREL – National Renewable Energy Laboratory
NREU – non-renewable energy use
OD – optical density
OD_{600} – optical density at 600 nm
OECD – Organization for Economic Co-Operation and Development
PDI – polydispersity index
PEF – product environmental footprint
PHAs – polyhydroxyalkanoates
PHB – poly[(*R*)-3-hydroxybutyrate]
PHB_{24h} – PHB concentration after 24h
PHB4HB – poly[(*R*)-3-hydroxybutyrate-*co*-4-hydroxybutyrate]
PHBHHx – poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate]
PHBV – poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate]
PHBVP – poly(*(R)*-3-hydroxybutyrate-*co*-*(R)*-3-hydroxyvalerate-*co*-3-*(R)*-hydroxypropionate)
PLA – polylactic acid
PPS – Pentose-Phosphate Shunt
PTS – phosphotransferase system
scl – short chain length
SG – PHB from Sigma Aldrich
SH – PHB from simulated hydrolysate
SHH – softwood hemicellulose hydrolysate from dilute acid hydrolysis
tan δ – ratio of loss modulus per storage modulus
T_c – crystallization temperature
T_g – glass transition temperature
TGA – Thermogravimetric analysis
T_m – melting temperature
TMP-Bio – thermomechanical pulping-based process
UDP – uridine diphosphate
US – United States of America
V – vanillin
VFA – volatile fatty acids
WH – PHB from wood hydrolysate
WWF – World Wildlife Fund
*X*r – residual biomass

XRD – X-ray diffraction

$Y_{CDM/S}$ – cell dry mass yield per sugar consumption

Y_e – extraction yield

$Y_{PHB/CDM}$ – PHB yield per cell dry mass

$Y_{PHB/S}$ – PHB yield per sugar consumption

μ_{max} – maximum specific growth rate

Acknowledgements

Starting the PhD in Montréal was a leap of faith to be more independent, use science for the common good and learn in an intercultural environment. I am most grateful for the people who have been part of this journey and helped me navigate the challenges along the way. They have enriched my life in countless ways.

I am most indebted to my parents, Gabriele Mook and Wolf Dietrich, for their unconditional love and support, without which I would have gotten nowhere in life. My sister Lara and my brother Vini have steadily cheered for me. Furthermore, I would like to thank my wonderful cousin Henrike, my nephew Caspar, my stepfather Dirk and the extended family for their companionship. In Montréal, my "adopted parents" Delphine and Robert Bonfils were my anchor and most trusted advisors. The time spent together with their family, Joseph, Léonard and Suzanne, was a highlight of my stay.

Throughout the PhD, the guidance and advice of my supervisors Prof. Marie-Josée Dumont, Dr. Luis Del Rio and Prof. Valérie Orsat were invaluable. Prof. Dumont has been a most dedicated and helpful supervisor, and I look up to her brilliance and work ethics. Dr. Del Rio enabled me to learn many things at FPInnovations and has been a knowledgeable and kind mentor. Prof. Orsat has always been there when needed with wise solutions. I have been lucky to have had their supervision.

In addition to my supervisors, I would like to thank Prof. Driscoll for his insightful feedback at my comprehensive exam. At McGill, I am grateful for Prof. Driscoll and Prof. Raghavan for allowing the use their laboratory equipment. Furthermore, I am very thankful for Prof. Ian Strachan, who advocated for me as a student. Our collaborators at University of São Paulo, Edmar R. Oliveira-Filho, José G. C. Gomez, Marilda K. Taciro, Luiziana F. da Silva allowed me to perform experiments in their lab and I am much indebted to the collaboration. Prior to starting the thesis, my previous supervisors Prof. Jörg Eppinger and Prof. N. Raveendran Shiju shaped my scientific training and I am thankful for what they taught me.

Regarding the experiments, I am indebted to several employees. Among many people whom I am grateful for at FPInnovations, Sylvie Renaud has always assisted with her expertise and I am tremendously grateful to have learned from her experience. Furthermore, I would like to thank Dr. Waleed Wafa Al Dajani, Jonathan Langlois, Alain Thibault, Brent Thomas, Beth Ambayec, Daniela Velasco and Simon Gandrieau for operating equipment needed for the experiments. At McGill, Yvan Gariépy has given countless advice, especially with the GC-MS, and I am also grateful for Dr. Darwin Lyew, who always knew where to find what. Furthermore, I would like to thank Robin Stein and Petr Fiurasek from the McGill Chemistry Material Characterization facility for assistance and use of the facility. While their work was not directly related, I am grateful for Roma Trottier, Saafa Bolboul, Cheryl Deveau, Suzanne Meilleur and César Caman, as they performed their essential work in a kind manner.

I am grateful for the generous financial support from the Richard H. Tomlinson Doctoral Fellowship and the National Science and Engineering Research Council of Canada (NSERC).

Furthermore, the Graduate Mobility Award (McGill) kindly provided funding for the research visit in Brazil.

My labmates have been both colleagues and friends, and I thank them for the shared moments. Agneev and Daihui were the first to graduate and have given helpful advice. Na, Shrestha, Zhanghu, Daniel, Guillermo, Derek, Surabhi and Emmanuel have had a great humour and engaged in stimulating discussions, both about science and social justice. I would like to thank Derek for his useful advice about fermentation strategies. I would also like to thank Jacob and Anthony, whose warmth and insight enlightened our shared office, the "Bunker".

With their truthfulness and courage to seek, I would not be the same researcher without the Meaningful Science team. I am thankful for Jaaved for being a partner in embracing responsible research. The reflections with Kimberly, Alex, Mi Lin and Ashlee were eye-opening. Finally, Erin, Deasy, Hamed and Aurélie became beloved colleagues in the attempt of being mindful in science.

Having friends to count on was the most precious gift during this project. I am highly indebted to Aidan, Jesse, Sam, Krittika, Tanya, Jason and Tatiana for making Montréal a home. "The hikers", Daniela, Steve, Ernest, Gary, Enrique, Jorge and Simon, have been like a family. While geographically distant, Malin, Simon, Sebastian, Seba, Ahmed B., Michael and Bharath have always been close to my heart and I love them with every fiber of my being. Finally, the connection with Brenda and Daniel, who I only met in the last year of the thesis, was a great joy.

Contribution of Authors

The PhD thesis is submitted in the form of six articles, which are published, accepted or submitted for publication to international and peer-reviewed scientific journals. Karolin Dietrich (the candidate), created the research proposal, designed and conducted the experiments, analyzed the data, and wrote the articles and the thesis. Prof. Marie-Josée Dumont (supervisor and Professor in the Department of Bioresource Engineering of McGill University), Dr. Luis F. del Rio (co-supervisor and Manager at FPInnovations), and Prof. Valérie Orsat (co-supervisor and Professor in the Department of Bioresource Engineering of McGill University), contributed to all aspects of this research work. They provided direction for the experiments, gave advice, and edited the thesis and the manuscripts before submission for publication.

Dr. Timothy Schwinghamer conducted part of the statistical analysis of the third publication (Chapter 3) and is listed as a co-author on the publication. Edmar R. Oliveira-Filho, a doctoral student under Prof. Luiziana F. da Silva, participated in the experiments and the data analysis of the fifth publication (Chapter 4). Edmar's supervisors, Prof. Luiziana F. da Silva, José G. C. Gomez, and Marilda K. Taciro provided advice on the experimental design and the experiments were conducted in their laboratory. They are all listed as co-authors on the publication. The list of articles that have been published, accepted for publication or submitted for publication is provided below:

1. Dietrich, K.; Dumont, M.-J.; Del Rio, L. F.; Orsat, V., Producing PHAs in the bioeconomy — Towards a sustainable bioplastic. *Sustain. Prod. Consum.* **2017,** *9* (Supplement C), 58-70.
2. Dietrich, K.; Dumont, M.-J.; Del Rio, L. F.; Orsat, V., Sustainable PHA production in integrated lignocellulose biorefineries. *New Biotechnol.* **2019,** *49*, 161-168.
3. Dietrich, K.; Dumont, M.-J.; Schwinghamer, T.; Orsat, V.; Del Rio, L. F., Model Study To Assess Softwood Hemicellulose Hydrolysates as the Carbon Source for PHB Production in Paraburkholderia sacchari IPT 101. *Biomacromolecules* **2018,** *19* (1), 188-200.
4. Dietrich, K.; Dumont, M.-J.; Orsat, V.; Del Rio, L. F., Consumption of sugars and inhibitors of softwood hemicellulose hydrolysates as carbon sources for

polyhydroxybutyrate (PHB) production with Paraburkholderia sacchari IPT 101. *Cellulose* **2019**.

5. Dietrich, K.; Oliveira-Filho, E. R.; Dumont, M.-J.; Orsat, V.; Gomez, J. G. C.; Taciro, M. K.; da Silva, L. F.; Del Rio, L. F., Increasing PHB production using wood: The fermentation of an industrially scalable hardwood hydrolysate to PHB. **2019,** to be submitted.
6. Dietrich, K.; Dumont, M.-J.; Orsat, V.; Del Rio, L. F., In-Depth Material Characterization of Polyhydroxybutyrate (PHB) from a Forest Biorefinery. **2019,** to be submitted.

Chapter 1: Introduction

1.1 General introduction

Globally, many countries are pursuing a transition to a bioeconomy to ensure the sustainable supply of raw materials [1]. In the transition, biobased products are in increasing competition with petroleum-based products. Currently, petroleum-based products are inexpensive because they are efficiently produced in integrated petroleum refineries. For economic competitiveness and to meet sustainability targets, biobased products require biorefineries to process biomass into products using environmentally friendly conversion technologies [2]. Biorefineries ideally produce various products such as fuels, chemicals and materials from agricultural, forest and municipal waste residues using multiple conversion processes [3-4].

Among biobased products, bioplastics have key advantages that address sustainability targets. Global efforts to facilitate innovation in the plastic lifecycle were initiated by the Canadian government [5]. The main motivation stated was to reduce plastic waste, which is increasingly accumulating on land and in the oceans. The current linear lifecycle of plastics leads to an estimated economic loss of $100-150 billion dollars representing 95% of the lost material value in single used plastic packaging. The “Canadian Action Plan to Reduce Plastic Waste” has the goal to accelerate innovation to move towards a circular and low carbon economy [6]. Generally, to make plastics part of the circular economy refers to either using renewable feedstock, such as Canadian biomass residues or recycled resin, and their decomposition properties, such as certified compostable (home or industrial), or ensuring they are fully recyclable [7]. Among the alternatives, polyhydroxyalkanoates (PHAs) have been highlighted due to their versatile decomposition behaviour, which enables their degradation within six weeks in home composts, anaerobic digesters, and in the ocean [8].

PHAs are compostable biobased polyesters that are valuable potential biorefinery co-products, yet they currently make up less than 0.1% of the global polymer production [9]. Their comparable physical properties to common petro-polymers make them promising

substitution candidates [10]. They are produced by bacteria as carbon storage, when exposed to conditions that limit the availability of other nutrients such as phosphorus or nitrogen [11]. PHAs are a diverse family of different hydroxyalkanoates. Polyhydroxybutyrate (PHB) was the first PHA to be discovered and has since been studied as a reference polymer. While industrial PHA production has been rapidly growing, obtaining a cheap and sustainable carbon source is still a challenge [12]. Compared to the current feedstocks (glucose from corn starch or sugar beets, and sucrose from sugarcane) [13], lignocellulose is potentially cheaper and more sustainable [14].

Lignocellulose is the fibrous material that constitutes the plant cell wall and has the highest potential to ensure long-term sustainable carbon supply as it is abundant and underutilized [2]. Lignocellulosic feedstocks for industrial biorefinery plants are mainly wood residues, agricultural by-products, grasses and municipal solid waste (MSW) [15]. Chemically, lignocelluloses consist of cellulose (40-80 %), hemicelluloses (10-40 %) and lignin (5-25 %) (Table 1-1) [16]. Woody biomass is an attractive lignocellulose feedstock for Canada as it is abundant, sustainably harvested and already valorized into multiple products (e.g. timber, wood panels, and paper). Among its constituents, cellulose and hemicelluloses are polymers of the sugars glucose, mannose, galactose, xylose, arabinose and rhamnose, while lignin consists of aromatic subunits. The sugars are fermentable to biofuels (e.g. bioethanol), biochemicals (e.g. succinic acid, lactic acid, xylitol) and biomaterials (e.g. polyhydroxyalkanoates) [17]. This study focuses on the use of woody biomass from integrated biorefinery schemes.

Table 10-1: Main components of wood used in pulp and paper processing. The values are average values of normal wood in percent dry wood mass.[a]

	Softwood	Hardwood
Cellulose	37-43	39-40
Hemicelluloses:		
Galactoglucomannans	15-20	
Arabinoglucuronoxylan	5-10	
Glucuronoxylan		15-30
Glucomannan		2-5
Lignin	25-33	20-25
Extractives	2-5	2-4

[a] Adapted from [18].

Generally, PHA production from lignocellulose hydrolysates has multiple challenges. For example, the hydrolysates often contain degradation products from sugar and lignin that inhibit fermentation processes. Nevertheless, recent research has shown promising improvements of PHA production from lignocellulose [16, 19-21]. Yet, lignocellulose conversion processes to sugar-rich hydrolysates have not been proven on an industrial scale, which increases the risks for scaling up. Furthermore, the production of PHA must ideally produce other bioproducts simultaneously for an optimal biomass valorization. So far, little is known about PHA production in such integrated biorefineries [22]. The goal of this research project is to use wood resources produced or compatible with integrated processes as substrates for the co-production of PHB (Figure 1-1). Expected outcomes are reduced PHA production costs, an increased product portfolio of forest biorefineries, valorization of a waste stream, and easy upscaling.

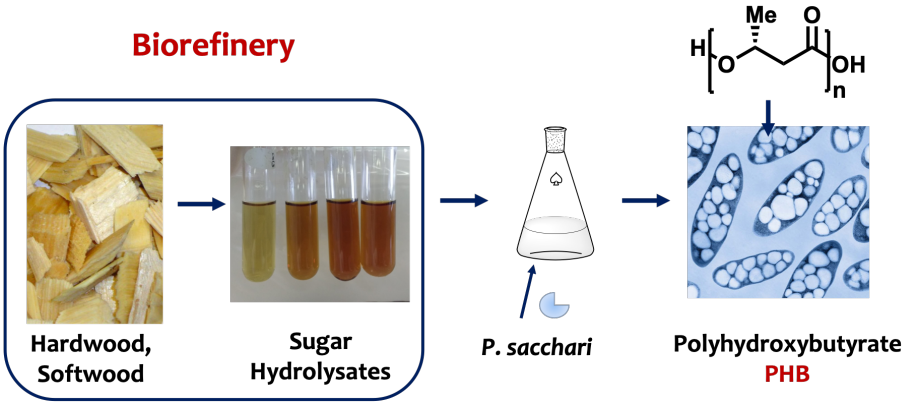


Figure 1-1: PHB production scheme from woody biomass.

1.2 Objective

The overall objective of this study was to produce PHB from Canadian softwood and hardwood resources by integrating PHB production in forest biorefinery schemes using *Paraburkholderia sacchari,* a natural and non-harmful bacterial strain.

1.3 Hypotheses

a) The softwood hemicellulose hydrolysate composition, namely the high share of the C6 sugars mannose and galactose, is favourable for bacterial growth and PHB accumulation.

The inhibitory effect of other components present can be predicted by inhibitor thresholds and mixture effects.

b) The inhibitory effect of softwood hemicellulose hydrolysate can be alleviated by dilutions with an inhibitor-free hardwood hydrolysate. Most of the inhibitory compounds will be metabolized by the bacteria when they are present below the inhibitory threshold.
c) Inhibitor-free hardwood hydrolysates containing the C5 sugar xylose produce a higher concentration of PHB than synthetic hydrolysates with the same carbon composition.
d) PHB from hardwood hydrolysate has the same material properties as compared to PHB from synthetic hydrolysates and commercial PHB.

1.4 Study objectives

The specific study objectives to reach the overall goal and considering the hypotheses were:

1. To review the current and anticipated industrial production of PHB, evaluate data from existing lifecycle analyses, and study the PHB production from forest resources to select most promising processes for wood conversion to PHB (Chapter 2).
2. To assess softwood hemicellulose hydrolysate as a potential carbon source for PHB production in shake flask fermentations, specifically the fermentability of the different types of sugars present and the inhibitory effect of other components (Chapter 3).
3. To study the metabolic fate of the heterogeneous compounds in softwood hemicellulose hydrolysate as compared to a less complex hardwood hydrolysate in shake flask fermentations to PHB (Chapter 4).
4. To produce PHB in high-cell density cultures, fed with hardwood hydrolysates that are a co-product alongside with a lignin product, in a fed-batch bioreactor and compare the hardwood hydrolysate with a synthetic hydrolysate (Chapter 5).
5. To isolate the PHB from the hardwood hydrolysate and from the synthetic hydrolysate and characterize the composition and the thermal and mechanical properties of the polymers (Chapter 6).

Chapter 2: Literature Review

2.1 Abstract

Biodegradable polymers such as polyhydroxyalkanoates (PHAs) can reduce pollution caused by the increasing global polymer demand. Although industrial production of PHAs grew rapidly in the past years, their total market share is still marginal. While this is often attributed to their higher price, which is mainly caused by high production costs, the industrial success of PHAs can also depend on policy framework. Environmental assessment tools such as life cycle analysis and the product environmental footprint showed that PHAs can contribute to greenhouse gas emission reduction targets, waste reduction as well as green jobs and innovation in the biotechnology sector. As many countries aspire to these targets under the umbrella of bioeconomy concepts, inclusion into the respective policies can stimulate industrial PHA production. With a high variability in the industrial production of PHAs in terms of feedstock, energy source, polymer properties etc., the choice of optimization criteria influences the design of new production processes. Considering the political targets for bioeconomy products is therefore useful to direct the technical design of sustainable PHA production, for example in integrated lignocellulose biorefineries.

2.2 Introduction

Polyhydroxyalkanoates (PHAs) are microbiologically produced polyesters that combine high functionality (tunable mechanical and physical properties) with low environmental impact (biodegradability and non-toxicity), making them promising candidates for sustainable polymer production. Their properties range from brittle thermoplastics to gummy elastomers and can be controlled by the choice of substrate, bacteria and fermentation conditions. With flexibility in their properties, PHAs can potentially substitute polypropylene, polyethylene and polystyrene, which are the three main polymers of the global polymer market [23].

Global polymer production has continuously risen for fifty years to 299 million tonnes in 2013 [24]. This is equivalent to the weight of 45 pyramids of Giza per year that need to be disposed at the end of their use. Between 22-43% of polymers end up in landfills, thus wasting the carbon feedstock and potentially leading to groundwater pollution by the leaching of toxic

additives. Only 9% of post-consumer polymers are recycled in the United States as compared to 26% in Europe, where another 36% is incinerated for energy generation. But even with efficient collection systems in place, the recovered polymers are often shipped to countries with lower environmental regulations, where they are reprocessed at low-tech, family-run facilities that often lack proper procedures for disposal of contaminants, wastewater and air pollution control [24]. Finally, polymer wastes accumulate in the natural environment where they can remain for up to two thousand years [25]. The pollution caused by polymers is especially harmful in the marine environments, where an estimated 100 million tonnes of polymers cause an ecosystem service damage of approximately US$ 13 billion per year [26].

In contrast, PHAs are both compostable and biodegradable in marine environments by ASTM standards. This is an important difference to other bio-based polymers such as polylactic acid (PLA), which is compostable, but may remain in marine environments for up to a thousand years [25]. Naturally occurring prokaryotes such as Archaea and bacteria (e.g. *Cupriavidus necator, Alcaligenes latus, Aeromonas hydrophila, Pseudomonas oleovorans, Haloferax mediterranei*) decompose PHAs into carbon dioxide and water, which are consumed during plant growth. Moreover the plant-derived biomass serves as feedstock for PHA production, thereby closing the carbon cycle [27]. Replacing fossil-based polymers with PHAs can potentially reduce greenhouse gas emission by 200% and fossil energy use by 95% [25]. In addition, PHAs naturally occur in human blood and tissues and are non-toxic. This biocompatibility enables new applications to be developed from PHAs to for the medical field [10, 28].

Besides economic considerations, the industrial production of PHAs can depend on a governmental decision for sustainable development. The sustainable industries and consumption goals in the sustainable development targets of the United Nations (Global Goals for 2030) emphasize the transition from current industrial patterns [29]. This could provide an incentive to national governments to promote changes towards more sustainable practices. It is widely recognized that to meet the material needs of a growing world population while maintaining functioning ecosystems, societies need to switch to plant-based resources that are renewable on a short time scale and whose conversion and consumption must be environmentally friendly [30]. As a biodegradable, non-toxic, carbon neutral and bio-based material, PHAs have the potential to contribute to sustainable industrial growth [31].

Within the global context, policies for sustainable production of biobased products are framed through concepts of the bioeconomy. The bioeconomy is a term for a politically desired transition from fossil feedstock to renewable resources that requires a systematic change of the entire energy and chemical industry [30]. In this review, we discuss PHAs as promising biomaterials for current bioeconomy strategies and the process and product design criteria required to maximize the benefits of their implementation.

2.3 The global bioeconomy & biomaterials

2.3.1 Bioeconomy, biobased economy and knowledge-based bioeconomy

The bioeconomy is still a new notion and is mainly a series of strategic or vision-like concepts from public and governmental institutions [32-33]. The term 'biobased economy' first appeared in 2000 and governmental strategies to develop a bioeconomy have been published since 2004 [33]. In 2009, a landmark publication of the Organization for Economic Co-Operation and Development (OECD) named 'The Bioeconomy to 2030' facilitated the development and implementation of bioeconomy policies in many states [34-35]. Currently, more than 30 countries have defined goals to reach a biobased economy and more countries are developing distinct national bioeconomy strategies.

Within the political context, the bioeconomy has multiple definitions and goals, but they share a common direction [32]. The OECD used the term 'bioeconomy' and defined it *via* its technology, namely an economy where biotechnology creates a large share of outputs [35]. The European Commission adopted a broader definition and used the term 'knowledge-based bioeconomy' as 'the sustainable production and conversion of biomass, for a range of food, health, fiber and industrial products and energy' [36]. Other publications stressed sustainability as part of the bioeconomy. For example, the World Wildlife Fund (WWF) defined the bioeconomy as 'production paradigms that rely on biological processes and, as with natural ecosystems, use natural inputs, expend minimum amounts of energy and do not produce waste as all materials discarded by one process are inputs for another process and are reused in ecosystems [37]. Bioeconomy concepts therefore represent a transition from fossil to bio-based commodities that incorporate the promise of sustainable industrial production [34].

While the bioeconomy is considered still in its infancy regarding biomass conversion technologies, it is also described as rapidly growing [33]. In many strategies, the development

and application of biotechnology are key tools [33, 38-40]. European bioeconomy strategies at the national, regional and industrial levels for example focused on industrial biotechnology development to convert a range of biomass feedstock and extract the maximum possible value [32]. The bioeconomy therefore relies on research and innovation, with small and medium-sized enterprises accounting for ca. 80% of bioeconomy-compatible companies in most developed countries [41].

However, apart from technology development, socioeconomic factors influence the success of the anticipated transition. First, the bioeconomy challenges the industrial resource base and established value chains. Instead of the petrochemical production routes, it envisions an agro-ecological supply system, where stakeholders of the agri-food chain cooperate with the chemical and energy industry. In Europe, the adoption and diffusion of new technologies were hesitant at the business to business level. High switching costs, lack of quality standards and uncertain customer responses were barriers for the industrial change [33].

In addition, societal concerns are an important hindrance of the market adoption of new products. End-consumers were skeptical about the benefits of the new products. Genetically modified feedstock, biodiversity loss through monocultures and rainforest depletion by palm oil imports are examples of a loss of trust in bioproducts. On the other hand, acceptance typically increases with knowledge of the perceived usefulness of bioproducts. This indicates that the successful market introduction of biobased products requires better communication with the end user, which could be achieved through certifications, quality labels and education campaigns [33].

The main societal challenges addressed by the bioeconomy concept are energy security and climate change. With the result of the Conference of Parties21 in Paris, all state signatories agreed on national efforts to mitigate climate change to go into effect in 2020 [42]. The main anticipated drivers for increased emissions and environmental stresses are the expected population growth and increased income, which leads to increased [30, 34, 37, 40]. The bioeconomy is seen as a tool for decoupling consumption from resource depletion and environmental pollution, thereby providing green industrial [30, 39].

With the focus to secure energy demand and reduce greenhouse gas (GHG) emissions, bioeconomy strategies were first based on promoting energy and biofuels from biomass. The policies included feed-in tariffs and mandated production targets for biofuels, sometimes

accompanied by targets for GHG savings per unit of biofuel [34]. However, in petrorefineries, fuel production is synergistically coupled to chemical production. While fuels are high volume and low value products, chemicals are low volume but high value products [43]. Similarly, bioethanol is required in high volumes but has a low price. The development of profitable biochemicals and biomaterials has therefore received much attention in research [44].

Despite the successes in biochemical and biomaterial process development, as of 2015 few national strategies include them in their policy framework [32-34]. Moreover, the existing support for bio-based products is mainly limited to subsidies for research and development. This is surprising, since several studies have highlighted the economic and environmental benefits of biomaterials. The next section gives an overview of these benefits and how PHA production relates to them.

2.3.2 Biomaterials in the bioeconomy

Biobased chemicals and materials can impart multiple environmental and economic benefits to the bioeconomy. The chemical sector accounts for 10% of global final energy use, which makes it the largest industrial energy user. On average, 50-85% of production costs of bulk chemicals are energy costs. Furthermore, fossil resources are the current material base of chemicals [34].

The technical greenhouse gas reduction potential of biobased chemicals by 2030 was estimated at 1.3-1.4 Gt CO_2/a as compared to 3.2-3.7 Gt CO_2/a for biofuels [34]. An assessment in Europe called 'Medium and long-term opportunities and risks of the biotechnological production of bulk chemicals from renewable resources' [45] showed similar results. Comparing the non-renewable energy savings of white biotechnology chemicals with petrochemicals, up to 75% of savings were possible with future use of lignocellulosic feedstock and up to 85% with sugarcane.

With limited land available, the efficiency of climate impact per land required is measured with non-renewable energy use and greenhouse gas emission savings per hectare. Compared to bioethanol, white biotechnology chemicals also score better in non-renewable energy savings per unit of agricultural land used (GJ/ha). The total land used for biochemicals ranged from 1.0 to 38.1 Mio ha with either corn starch, sugarcane or lignocellulosic biomass as feedstock, compared

to a total agricultural area in the EU-25 of about 180 Mio ha in 2002. For lignocellulosic feedstocks, this represented merely a range of 0.2-9% of agricultural land use [14].

Furthermore, the longer and more complex supply chain for biomaterials was estimated to create 4-9 times the added value as compared to energy uses and support 5-10 times more employment. As an example, in the United States of America (US) a shift of 20% of conventional plastic production to bioplastics could create a net of 104,000 jobs [41].

Polymers are the most important group of organic chemicals [45]. The OECD estimated that in 2100, 25% of oil production would be needed for a predicted annual polymer demand of 1 billion tonnes [40]. Biopolymers have a total technical polymer substitution potential of 33-90% for petropolymers. However, global biopolymer consumption was merely 0.4% in 2011. Furthermore, by 2020 the expected share of biopolymers in the European polymer market is only 4.4-6.7% with 2-3 million tonnes production [45]. Therefore, the anticipated total GHG emission savings from biobased polymers are small – for example for the chemical sector, biobased polymer production is expected to account for merely 0.2-5% of the savings. Nevertheless, improvements in biotechnological processes and synergies with biofuel production could increase the projected share of biomaterials and their associated environmental benefits.

One of the reasons for the slow market entry of biobased polymers is the low efficiency of biotechnological production processes [34, 45]. The economic and environmental benefits of biobased materials are therefore coupled to biotechnology innovation, particularly in the production (titer (product in g/L)), yield (product per substrate in g/g), productivity (product in g/(L·h)) and downstream processing (product separation and purification) steps. Furthermore, processes to efficiently convert lignocellulosic biomass and agricultural waste streams are still immature [45]. As these processes mature, integrated biorefineries can be built that produce a wide range of products from different feedstock [37]. In contrast to specialized, single-product biorefineries, multiple-product biorefineries can be more stable to market fluctuations. Consequently, biomaterials as *co*-products in integrated biorefineries have the potential to increase the environmental and economic benefits of biomass conversion.

The final advantage of biomaterials is waste reduction. The concept of biorefineries based on waste conversion to products that re-enter the production cycle upon their disposal closes the loop of material consumption cycles [37]. Recent developments in the EU include the approach of a ‘circular economy’ [46-47]. In this model, products are designed to be reused or recycled,

thereby becoming a feedstock for a subsequent process instead of waste. In the case of PHAs, the biodegradability allows the polymers to be composted, where it is mineralized to CO_2, which is reabsorbed during photosynthesis by the plants that serve as feedstock for PHA production. This closes the carbon cycle and makes PHAs eligible as a circular economy product [48].

In order to harvest the potential benefits of biomaterials for a bioeconomy, several studies suggest including biomaterials in bioeconomy policy goals [34, 40-41, 49-50]. Philp [34] suggested a policy design that measures biobased chemicals in terms of ethanol equivalents to reduce the complexity from the diverse product portfolio. Mandated production goals can then be set according to defined percentage GHG savings as compared to the petrochemical equivalent, a production volume factor that stimulates the replacement of bulk materials with higher GHG emission savings and a production efficiency factor that encourages innovation in yield, titer and productivity. The European Policy Centre [47] argued that 'green labels' should be introduced for goods that have a proven positive impact on the environment, rather than a reduced negative impact. Following this approach would require defining and quantifying the impacts, for example by using lifecycle analysis.

The potential policy support would stimulate the development of industrial PHA production and also define the innovation direction for PHAs. While substantial research efforts have already targeted process optimization and reduction of feedstock costs [13, 51-53], PHA production in the bioeconomy would also require proven GHG emission savings, reduced land use per kg product, reduced fertilizer use, careful consideration of the use of genetically modified organisms, low toxicity in the production process caused by chlorinated extraction solvents, reduced human toxicity and proof of compostability by ASTM standards. For successful and long-term growth of PHA production, it is therefore beneficial to improve both its technoeconomic and environmental performance. While the next section gives an overview of current bioeconomy policies related to biobased materials, the sustainability assessment of PHAs is further discussed in section 4.

2.3.3 International & national strategies of the bioeconomy

A supportive and cohesive policy framework is considered a key issue for the development of the bioeconomy, and the market success of biobased polymers such as PHAs will depend on their inclusion into these frameworks [32].

After the introduction of a vision for bioeconomy policies by the OECD, the US and the European Commission adopted detailed national strategies with defined targets in bio-based products. The US published its bioeconomy blueprint in 2012, which included funding of biomass R&D, industrial biotechnology and biorefinery roadmaps [39]. The European Commission first published a strategy for a European bioeconomy by innovation in 2012 [54]. The strategy used policy instruments such as the research funding program Horizon 2020 [32], Public-Private Partnerships such as the €3.7 billion Bio-based Industries Joint Undertaking between the EU and the Biobased Industries Consortium [38], a common agricultural policy [47] and the European Commission Expert Group for Bio-Based Products [55].

Recently, several proposals included concepts of a circular economy, among them The 7th Environment Action Programme 'Living well, within the limits of our planet', which informs EU environmental policies until 2020 [47], and several directives of the European Commission [56]. Among the targets until 2030 are recycling of 65% of municipal waste, 75% of packaging waste, reduction to a maximum 10% of all waste to landfills and economic incentives for producers of greener products, e.g. of packaging, electronic equipment and vehicles. In Canada, BIOTECanada published the initiative 'The Canadian Blueprint: Beyond Moose and Mountains' in 2010 [57].

Biomass rich countries such as China, India, Brazil and Russia have partial strategies to create a local, higher value production of their natural resources that are currently exported for low value [34]. Malaysia for example has committed to an ambitious bioeconomy strategy, the Biotechnology Transformation Programme, which targets to contribute Malaysian Ringgit 48 billion of the Gross National Income in 2020 and create 170,000 jobs [58].

2.4 Current industrial PHA production

2.4.1 Global PHA producers

PHAs have been commercially produced since the 1980s but their application stagnated due to low oil prices. In the beginning of 2003, the increase of oil prices to over US $100 per barrel led to a revived industrial interest for PHAs [59]. Since then, new plants opened in China, the US, Italy and Brazil [13]. PHA production is conducted in industrial fermentation reactors. The processes vary from substrate choice, bacterial strains, integration in existing bio-processing plants and type of PHA.

In 2009, Metabolix and ADM opened the biggest plant with a production capacity of 50,000 t/a in Iowa, USA (Table 2-1). A wet corn milling plant belonging to ADM and adjacent to the microbial fermentation facility separates corn into grains, starches and sugars. The sugar stream is then used as carbon feedstock for the PHA-producing bacteria [25].

Tianjin Green Bioscience in joint venture with DSM in China, Bio-on in Italy and Meredian in the USA each built facilities of 10,000 t/a. Tianjin has gradually scaled up their fermentation from a 30 L vessel to eight pots of 150,000 L fermenter volume which required large-scale reactor design to overcome oxygen transfer limitation. In their commercialization strategy, the company started with strain development followed by equipment design and included development of downstream processing conditions to facilitate the application of their product [60].

In Italy, starting from local sugar beet juice, Bio-on developed a range of PHA polymers with melting points ranging from 40° C to 180° C [61].

Meredian now operates under the name Meredian Holding Group (MHG) and based their production on locally grown canola oil [62].

Smaller facilities by Yikeman, Shandong and Zhejiang Tian An in China produce 3,000 t/a and 2,000 t/a by using dextrose from local corn and cassava [63].

In Brazil, industrial PHA production has been integrated into existing sugarcane mills [59, 64-66]. The company Copersucar in joint venture with the two research institutions Technological Research Institute of São Paulo and Biomedical Institute of University of São Paulo developed the initial pilot plant process funded by a government research grant in 1991. In the process, both hydrolyzed bagasse and purified sucrose are used as feedstock for PHA production with simultaneous production of ethanol and molasses. Higher alcohols (butanol, pentanols) obtained as side products from bioethanol fermentation serve as PHA extraction solvents and bagasse combustion provides energy to run the entire plant on biomass. Successful pilot plant operation led to the creation of PHB Industrial S/A that currently produces 10,000 t/a using 17% of the sugars produced in the mill for PHB.

The USA based company Newlight Technologies took a different approach and chose methane as the carbon source for fermentation with a planned capacity of 43,000 t/a within the next 20 years [67].

Compared to global petroleum-based polymer production, biopolymers still represent only 1% of all polymers in use, but the industry is rapidly growing. For example, the global average annual growth rate between 2003 and 2007 was 38% [68]. Furthermore, the newly emerged PHA production already leads to an existing industrial value chain that is likely to expand with increased availability of commercial PHAs.

2.4.2 Commercial PHA products

Initially, PHAs have been used for everyday articles and packaging with a diverse range of applications such as shampoo bottles (Wella AG), plastic bags, razors, feminine hygiene products, medical surgical garments, carpets and upholstery (P&G, Biomers, Metabolix and several other companies) [13]. Most current large-scale PHA producers sell PHA as raw material in the form of pellets and powder that can be processed by conventional extrusion, injection moulding, thermoforming and film blowing.

Table 2-1: Commercial PHA products in 2015.

Company	Brand Name	PHA type	Class	Product
Metabolix, USA (www.metabolix.com)	Mirel®	PHB	raw materials	injection moulding
			cosmetics	micropowders
			packaging	coating for paper and cardboard
			water treatment	denitrification for Aquariums
			plasticizers	additives for PVC and PLA
MHG Bio, USA (www.mhgbio.com)	Nodax™	mcl PHA (unclear)	raw materials (resins)	bags, bottles, hygiene, mulch
Biocycles, Brazil (www.biocycle.com.br)	Biocycle®	PHB	raw materials (pellets)	plastic sheet extrusions, injection, coating paper
Bio-On, Italy (www.bio-on.it)	MINERV-PHA™	PHA (unclear)	raw materials	automotive, electronics, packaging..
TianAn Biopolymer, China (www.tianan-enmat.com)	ENMAT	PHB, PHBV	raw materials (powder, pellets)	thermoplastics: injection moulding, extrusion, thermoforming, blown films; Fiber & Nonwovens; Denitrification: water treatment
		Ethyl 3-HB	fine chemicals	
Tianjin GreenBio (+DSM) (www.tjgreenbio.com)	Sogreen™	PHB, P4HB	raw materials (resin, pellets)	
Tepha, US (www.tepha.com)	TephaFLEX ®	P4HB	medical materials	suture, mesh, surgical film
Newlight Technologies (newlight.com)	AirCarbon™	PHA (unclear)	raw materials	extrusion, blown film, cast film, thermoforming, fiber spinning, and injection moulding applications
			cosmetic packaging	R&D partnership with the Body Shop
Polyferm Canada (www.polyfermcanada.com)	VersaMer ™	PHOHHx, PHNHHp, PHNHHpHN: HUD:	raw materials (pellets, latex)	thermoelastomers

On the other hand, some companies market their PHA directly as the end-product with a defined application. Among them, Metabolix developed a product portfolio of additives for PVC and PLA, micropowder for cosmetics, paper and cardboard coatings and denitrification agents for aquariums. Enmat included the biochemical 3-hydroxybutyrate from polyhydroxybutyrate (PHB) depolymerization in their products. TephaFLEX uniquely produces sutures, mesh and films for medical applications. Newlight Technologies signed a contract with The Body Shop to replace their cosmetic packaging.

At the research level, potential PHA applications extend to biofuels in form of the hydroxyalkanoate methyl ester, drug delivery carriers, fine chemical precursors in form of

enantiomerically pure monomers in (*R*)-configurations, health food additives and therapeutic drugs in form of PHA monomers [13].

2.4.3 Industrial fermentation conditions

Current industrial and pilot fermentation plants use both wild type and recombinant bacteria and produce both short chain length (scl) and medium chain length (mcl) PHAs. The main scl-PHAs are poly[(*R*)-3-hydroxybutyrate] (PHB) and the *co*-polymers poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate] (PHBV) and poly[(*R*)-3-hydroxybutyrate-*co*-4-hydroxybutyrate] (PHB4HB). On the other hand, the chemical composition of medium chain length PHAs such as poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] (PHBHHx) is more heterogeneous [59]. The main carbon sources are glucose, fructose and fatty acids such as lauric acid. *Ralstonia eutropha* has been the most commonly employed wild type strain for scl PHAs in industry due to high cell density and PHA content (100-200 g/L, and 75-80% respectively), simplicity of growth and well-known metabolism. For mcl-PHA, the common bacteria are *Aeromonas hydrophila* and *Pseudomonas oleovorans*. The most prominent recombinant bacteria are recombinant *E. coli* due to their convenience for genetic manipulation, fast growth, high final cell density and ability to utilize inexpensive carbon sources. For PHB production, a recombinant *E. coli* has been reported to grow to a cell dry mass of 206 g/L containing 73% PHB with a productivity of 3.4 g/(L h) and has also been used to produce the mcl PHBVHHx [59].

2.4.4 PHA production costs

In 2004, the price for commercial PHAs was 15-17 times higher than the major petroleum-based polymers and 4-6 times higher than commercial polylactic acid from Cargill [69]. Metabolic engineering, improved fermentation conditions, and higher production capacities were able to reduce the cost to around US$ 5 per kg in 2009, which was still three times higher than the price for polypropylene [25]. PHAs therefore still have a limited market, despite their potential to substitute 33% of commercial polymers [69].

Several obstacles hinder cheaper PHA production beginning with historically low petroleum prices due to shale gas exploitation. Additionally, glucose from corn starch as a feedstock increased in price and generally, fermentation processes have higher process costs related to lower yields as compared to processes in chemical reactors. Further obstacles include

difficulties controlling PHA structures and properties, challenges extracting PHA with existing downstream technologies and lack of high-value applications [70]. Therefore, much of the PHA research focused on reducing production costs.

Overall, the most important factors contributing to the final PHA price are productivity (g per L per h), yield per substrate, cost of raw materials and the recovery method [71]. Furthermore, total manufacturing costs decrease with process scale-up, which also increases the fraction of raw material costs, dominated by the carbon source, on the final PHA price. However, low substrate costs were only effective at similar process efficiencies [71]. Consequently, reducing PHA production costs at a large scale depends on overall process optimization rather than improvement of individual indicators [28].

2.4.5 Technoeconomic assessment

In order to evaluate the industrial feasibility of process design, technoeconomic assessments are important tools that combine the profitability of the plant with optimal process kinetics [72-73]. Given an existing fermentation process, technoeconomic assessments can be conducted with process planning software (e.g. BioPro Designer®, Aspen Plus©). They feed a knowledge input of products and bacterial properties both upstream and downstream into a process model for different reactor types that yield energy and material balances, size and cost of equipment as well as an overall economic analysis [74]. The process design conveniently works with literature data. Furthermore the recovery step can be included as a separate step allowing for individual optimization [75]. The outcomes of the simulations identify the factors that influence the final PHA price, whose relation and impact can be measured by sensitivity analysis. As an example, for short chain length PHA production, an increase of the PHA content was found to be the most important because it had multiple reduction effects on equipment and recovery costs, while PHA productivity, PHA yield per substrate and substrate costs only decreased one cost factor [71].

2.5 Sustainability assessment

As the requirements for market entry can differ from the business versus the policy perspective, research and development can choose different PHA optimization directions. The approaches can be separated into a technoeconomic optimization for optimum economic

performance and a sustainability optimization for best environmental and social performance [27]. However, it is evident that the interest in economic and sustainability performance overlap to some degree for both business and policy, given that the policies aim is economic growth and that businesses benefit from green premium prices and an image of sustainability [76]. This section discusses sustainability assessments of PHAs. We specifically ask how the results of previous assessments can inform the development of PHAs to sustainable biopolymers that fulfill the demands of bioeconomy and circular economy visions.

2.5.1 Sustainability assessment tools

Many studies investigated the sustainability of PHAs. Among the possible sustainability assessment tools are Life Cycle Analysis (LCA), Carbon Footprint, Carbon Efficiency, Sustainable Process Index, health and safety Score Cards and more recently the Biomass Utilization Efficiency (BUE) [27, 77-78]. Life cycle analysis is the most common tool. Briefly, it quantifies the environmental impact of the entire production chain from biomass collection to either factory gate or PHA disposal in defined impact categories such as greenhouse gas emissions, energy use, acidification and land use change [79]. The sustainable process index measures the whole product to service chain of PHA production in area units [64]. The score card analysis employs an 'environmental health and safety' perspective and compares polymers in a grading scheme [77]. The Biomass Utilization Efficiency is a measure of the amount of biomass ending up in the biobased product as a comparison of different material efficiencies [78]. Therefore, different tools focus on different aspects of sustainability assessment.

The environmental burden of PHAs include high overall energy consumption, high acidification and eutrophication potential due to fertilizers and pesticides, health hazards due to extraction with chlorinated solvents, high water use and wastewater production without recycling. Furthermore, the use of genetically modified organisms was discussed as a health hazard by Alvarez-Chávez et al. [77]. Since the results spanned a range of possible outcomes depending on the feedstock type, energy use [12] and product application [80], the design of more sustainable PHA production processes by careful choice of parameters is possible.

Comprehensive life cycle analyses for large-scale PHA applications are still rare due to a lack of commercial data and the focus of isolated aspects of PHA production such as CO_2 emissions [81]. They can also predict environmental hot-spots of new processes.

2.5.2 LCA for feedstock

The choice of feedstock and *co*-use of feedstock for energy production can lower the environmental impact of PHA production and even make the material a net carbon sink. Hermann et al. [14] compared the non-renewable energy use, renewable energy use, greenhouse gas emissions and land use. They tested three feedstocks: glucose from corn starch, sucrose from sugarcane including bagasse for energy generation and C5/C6 sugars from corn stover. The greenhouse gas emissions from biomass feedstock can be CO_2, CH_4 or N_2O from fertilizer use. The production of 1 kg of fermentable sugars resulted in negative GHG emissions only for sugarcane (-0.54 kg CO_2-eq/t), the corn stover had low emissions (0.16 kg CO_2-eq/kg) and corn starch had the highest emissions (0.40 kg CO_2-eq/kg). Both corn stover and sugarcane showed negative non-renewable energy use (NREU) of -4.4 GJ/t and -12.8 GJ/t respectively, while corn starch was at 6.2 GJ/kg. Sugarcane had a higher renewable energy use (41.8 GJ/t) than corn stover (29.2 GJ/t). However, the corn stover had the lowest land use (0.05 ha/t) as compared to sugarcane and corn starch (both 0.13 ha/t). A prior analysis of the European BREW project had the same findings [45]. Miscanthus and poplar were also considered, but their yields were not high enough to cover the demand for the European market. The sugarcane performance mainly differed from corn stover due to energy *co*-production from bagasse.

Sugars from sugarcane also had the lowest prices [45]. In the past years, the world sugar prices were highly volatile, partly due to the ethanol production policies of Brazil. In general Brazil is the largest sugar exporter and usually ranks first or second in the lowest sugar production costs. Good climate, low wages and land availability enable low sugar production costs of an average of US$ 235/t compared to a world average of US$ 353/t in 2008 [82]. Sugars from starch crops in the EU and US were between € 200-300/t, while sugars from beets in the EU were more than double at € 700/t but were subsidized. Since the subsidies were planned to be gradually removed and sugarcane does not grow in the EU, the report concluded that C5/C6 sugars from corn stover will be the best option for the EU once suitable lignocellulose conversion technologies are available on the market [45].

2.5.3 PHA production

Cristóbal et al. [12] recently reviewed LCA studies of PHAs and compared them to a harmonized product environmental footprint (PEF). They found twelve LCA studies on PHA,

among which three consider disposal options (cradle-to-grave). The majority limited the impact categories, with climate change as the most common impact category chosen. As an alternative, the PEF analyzed fourteen impact categories including human toxicity (cancer and non-cancer effects), ecotoxicity for aquatic fresh water and water resource depletion, which they estimate important for specific value chains of PHAs. They considered local PHA production from corn starch in Europe and from sugarcane in Brazil.

The impact on climate change in kg CO_2-eq/kg PHA in the reviewed LCA studies ranged from –2.3-6.9 as compared to 2.72 with sugarcane feedstock and to 4.26 for corn starch in the PEF (Table 2-2). Values below 0.49 kg CO_2-eq/kg PHA were from studies that accounted for carbon storage in the polymer, where the temporarily captured atmospheric CO_2 in the material is considered as carbon sequestration. Burning residual biomass for energy production lowered the GHG emissions. For example, in a prior review of LCA studies by Chen and Patel [83], burning of residual corn stover reduced GHG emissions from 1.7 kg CO_2-eq/kg PHA to −1.2 kg CO_2-eq/kg PHA. They also reported that fermentation is an energy intensive process and is therefore the highest contributor to climate change potential [83]. Furthermore, different downstream processes gave a range of NREUs from less than 40 GJ/t to more than 110 GJ/t, indicating the potential savings for improved downstream techniques [45]. Finally, feedstock with low productivities increased GHG emissions, for example rapeseed oil had emissions of 5-6.9 kg CO_2-eq/kg PHA. In the PEF, 66% of the climate change impact from sugarcane resulted from energy consumption, while the main contribution from corn came from the starch production.

Another aspect of climate change mitigation is the potential of carbon capture in biobased materials. Pawelzik et al. [84] discussed two options for biogenic carbon for LCAs of biobased materials studies. One option is to consider biogenic carbon as CO_2 neutral and exclude it from the inventory analysis, while the alternative is to account for the biogenic carbon content as temporary carbon storage that offsets anthropogenic emissions. Since life cycle standards account for emissions in a time frame of 100 years (International Reference Life Cycle Data System, British Standards Institution), even a temporary storage of 10 years in biobased materials cannot be considered sequestration. However, it can delay radiative forcing and current emissions. They suggest a separation of the material carbon footprint and the process carbon footprint. The material footprint for biobased materials is zero when they are fully oxidized upon

disposal, either by incineration or composting. Consequently, disposal which leads to methane release would create material GHG emissions and should be avoided.

The carbon footprint of PHAs without the use of agricultural leftovers for process energy (1.7-4.2 kg CO_2-eq/kg PHA) was higher than for PLA and biobased polyethylene (0.4-1.3 kg CO_2-eq/kg polymer) [83]. Nevertheless, compared to high density polyethylene as petrochemical counterparts, the GHG emission savings from current PHA production are already at 85% with sugarcane and 20% with corn starch. Future use of lignocellulose and sugarcane can increase these savings to 90 and 100% respectively [85]. For a given amount of land, the GHG emission savings per hectare are similar for PHA and PLA and are above 12 tonnes/ha with sugarcane feedstock, as compared to fuel ethanol from sugarcane of 10-16 t CO_2-eq/ha. Lignocellulose use from corn stover is projected to save over 25 t CO_2-eq/ha for 1 tonne of PHA. Therefore, PHA as a biomaterial has a high potential to contribute towards reducing GHG emissions.

The feedstock growth phase had the highest impact on several categories [12]. For example, sugar production from sugarcane and corn starch accounted for more than two third of the value in aquatic marine eutrophication, terrestrial eutrophication, human toxicity and particulate matter. Phosphorus consumption, nitrogen consumption and soil organic matter loss were all higher for corn starch due to the lower agricultural efficiency of corn. Weiss et al. [86] argued that conventional farming practices are the key contributors for high eutrophication and stratospheric ozone depletion, even though the impact of agricultural systems highly differs according to the geography, climate and farming practices.

Additionally, the use of genetically modified crops and microorganisms has potential risks when human and environmental toxicity has not yet been fully assessed. The sustainable biomaterials collaborative recommended not to use genetically modified feedstock when designing sustainable biopolymers [77]. Furthermore, customers showed low acceptance of new agricultural materials [33] and the willingness to pay green premium prices for genetically modified crops as well as food crops in general were lower than for non-genetically modified and non-food crops [76]. Nevertheless, The BREW project assessed risks associated with GMO use for biomaterials and concluded that this issue is larger for genetically modified agricultural feedstock (Green Biotechnology) than for genetically modified bacteria (White Biotechnology)

since the bacteria stay within the factory and are therefore kept separate from ecosystem interactions [45].

The lower agricultural efficiency for corn is a result of a lower sugar yield per hectare (4.8 tonnes/ha) compared to sugarcane (11.1 tonnes/ha). This impacts the land use, which can cause high GHG emissions [86]. Both biomass and soils can be carbon sinks, but the assessment involves many interrelated factors (e.g. temperature, precipitation, pH) and has a high uncertainty [84]. Land use further involves the soil organic carbon content, nutrient and water use, and impacts biodiversity. For corn starch as feedstock, removal of 50% of corn stover from the field for energy production was estimated to decrease soil organic carbon levels by 40 kg carbon per tonne of PHB at the current tillage practices [83]. This needs to be considered for long-term soil fertility. For example, in Canada, changing from tillage to non-tillage has increased the soil carbon [84].

Direct land use change is the intentional transition of a current use of land for feedstock growth and can be positive in the case of restoring degraded land or negative, for example cutting down a forest. Indirect land use is an unintentional change caused by the growth of biomass feedstock and is most often not assessed due to a lack of data. For corn starch, PHA land use was at 0.3-0.4 ha/t as compared to 0.18-0.28 ha/t for PLA [45]. The impacts of land use can also be reduced by the use of non-food biomass such as lignocellulose, higher product yields, useful *co*-products from one feedstock and sustainable resource management [86].

The high process water demand is a potential concern for biobased chemicals and can impact the environment such as fresh water stored in lakes as well as human health by a lack of freshwater for hygiene and ingestion as well as water shortages for irrigation [84]. The LCA standard of the International Reference Life Cycle Data System distinguishes between net water extractions from the environment from different sources (e.g. surface fresh water, renewable ground water, sea water, and internally recycled water). Cristóbal et al. [12] accounted for water resource depletion in the product environmental footprint analysis and found a lower depletion for sugarcane (2.35 m^3 water-eq) than for corn starch (3.91 m^3 water-eq).

The best waste disposal options were considered to be anaerobic digestion with energy recovery and composting, where the compost can be used as fertilizer [12] or to replenish carbon stocks in soil [86]. However, this will depend on the correct disposal of PHAs, since they could also end in landfills through municipal waste systems.

Table 2-2: Climate Change impact for PHAs and counterparts for different stages of life cycle.

	GHG emissions [kg CO_2-eq/FU]	Notes	Reference
Feedstock [FU=tonne biomass]			
Corn Starch	0.40		
Sugarcane	−0.54		[14]
Corn Stover	0.16		
PHA Production [FU=kg PHA]			
Overview of LCAs	−2.3-6.9		
PEF with sugarcane	2.72	Harmonized Product environmental footprint	[12]
PEF with corn starch	4.26	Harmonized Product environmental footprint	
Savings to petrochemical	2.9	Corn starch compared to high density polyethylene	[70]
Products [FU=one product]			
Monitor housing PHA [One housing, 2.2 kg]	0.1-8.8	PHB composites	[80]
Monitor housing petropolymer	15.1	High-impact polystyrene	
Internal car panel PHA [All int. car panels, 20 kg]	552.1-722.8	PHB composites	
Internal car panel petropolymer	569.9	Glass-fibers-filled polypropylene	
Downstream Processing [FU=h]			
Sodium hydroxide	4.08	Alkaline digestion, 2.5% w/v NaOH (0.25-4.0 N), 4 h, 37 °C, 500 rpm	[87]
Sodium hypochlorite	28.46-29.46	Alkaline digestion, 13% v/v NaOCl (2.5-7.5% w/v), 4 h, 37 °C, 500 rpm, (+ DCM)	
Sulfuric acid	6.27	Acid treatment, 2.5-10% v/v H_2SO_4, 5% w/v biomass, 1-30 h, 37-100 °C. Bleaching with NaOCl, 3%, 1 h	

2.5.4 LCA for downstream processing

The downstream processing of PHAs is considered a bottleneck in their production due to the use of hazardous solvents such as chloroform and diethyl ether or chemicals such as sodium hypochlorite that have a high indirect energy consumption as well as a high process energy [77, 83]. López-Abelairas et al. [87] compared different recovery methods based on their environmental performance. They assessed the recovery efficiency (purity, percent of recovery and PHB properties) as well as cost and environmental impacts of acid treatments (sulfuric acid followed by bleaching) and alkaline treatments (sodium hypochlorite in dichloromethane, sodium hydroxide and a combination).

The highest GHG emissions resulted from the use of sodium hypochlorite (13,728 kg CO_2-eq/kg), therefore sodium hypochlorite digestion had the highest carbon footprint (28.71-29.46 kg CO_2-eq/h), whereas treatment with sodium hydroxide or sulfuric acid had the

lowest emissions (4.08 kg CO_2-eq/h and 6.27 kg CO_2-eq/h respectively). These treatments also had the lowest recovery costs with 1.14 US$/kg PHB for sodium hydroxide and 1.24 US$/kg PHB for sulfuric acid as compared to 5.86-7.41 US$/kg PHB for sodium hypochlorite. All treatments caused a reduction of the polymer's molecular mass compared to the classic chloroform extraction. The sulfuric acid treatment resulted in a very high crystallinity of 84% as compared to 52-60% for the other treatments. The mass-average molar mass, melting temperature and crystallinity with exception of the sulfuric acid treated PHB were in the range of the commercialized PHBs by Biomer and Copersucar.

Due to the low cost, high purity (98%) and recovery (79%) as well as low GHG emissions and low polymer degradation, the sulfuric acid treatment was identified as a viable downstream processing alternative to chloroform extraction and sodium hypochlorite digestion.

2.5.5 LCA for PHA products

Besides PHA production, the final product use has an impact on the environmental benefits of PHAs. Pietrini et al. [80] compared PHB composites used as a monitor housing and as internal panels of an average car with their petrochemical counterparts (high-impact polystyrene and glass-fibers-filled polypropylene respectively) with respect to NREU and global warming potential over a 100 years' time horizon (GWP_{100}). While the PHB production from sugarcane until the factory gate was a net NREU saver (-22.7 GJ/t PHB) and carbon sink (-3100 kg CO2 eq/t), further processing and use impacted the benefits. As a monitor housing, all PHB composites tested scored better than their conventional counterpart and the best composite reduced NREU and GWP_{100} by about 99%. In contrast, for the internal car panels, only one composite reduced NREU by 5% and GWP_{100} by 3% and the other composites showed no relevant environmental savings.

The poor environmental performance for PHBs in internal car panels was ascribed to their higher weight and the resulting higher fuel consumption. The higher weight was due to a lower Young's modulus and higher density, which required more material to provide the same performance. This was illustrated by the material index (MI) which is a measure of the functional performance of a material for a given application. For the internal car panels, the performance features are limited by the stiffness requirement for a panel with specified width and length and with free thickness. The resulting material index is a function of the Young's modulus E and the

density ρ (Equation 2-1). A large Young's modulus and low density give a high MI, which means that less material is required to fulfil the function. As a result, the relative NREU was a function of the Young modulus of the different composites and reduced from ca. 1.1-1.3 for a Young's modulus of 1.0 GPa to 0.79-0.9 for a Young's modulus of 3.0 GPa.

$$MI = \frac{E^{1/3}}{\rho} \quad (2\text{-}1)$$

In addition, the product's production process and disposal can give different results. The PHB composite production process involved an extrusion and an injection moulding step. Plastic Europe reported a high amount of natural gas use for injection moulding, and the NREU for injection molded polypropylene was at 113.2 GJ/t compared to 73.0 GJ/ for polypropylene production. For the monitor housing, the contribution of injection moulding per housing was higher for the PHB composites (60-70 MJ/housing) compared to the conventional polymer (54 MJ/housing). Furthermore, when the composite material was nanoscale organophilic montmorillonite, composite production was up to 19% of the positive NREU value, while sugarcane bagasse as filler did not contribute to NREU. Lastly, the materials gained energy credits because the assumed disposal was postconsumer waste incineration, which gave a gain of 26-30 MJ/housing.

For specific applications, the product design can therefore improve the environmental properties with improvement of the material index, for example by designing PHAs with higher Young's modulus and lower density for internal car panel use.

2.5.6 Biomass utilization efficiency

Recently, Iffland et al. [78] introduced the Biomass Utilization Efficiency to measure what fraction of the feedstock atoms end up in the product. The approach is comparable to the atom economy, which measures the percent of all reactant atoms in the end product and to the carbon efficiency, which measures the amount of biomass carbon in the product. The BUE is the ratio of the product molecular mass to the biomass feedstock molecular mass multiplied by the conversion yield. It therefore reflects the transfer of chemical complexity to the end product by including oxygen and hydrogen content. The theoretical conversion yield based on the stoichiometric equation gives the theoretical efficiency (stoichiometric BUE: BUE_S,

Equation 2-2), the highest and lowest published conversion yield gives the realistic efficiency (BUE-high: BUE_H, BUE-low: BUE_L, Equation 2-3).

The BUE for PHB production from glucose with a recovery yield of 84% were 47.8 for BUE_S and 40.1 for BUE_H as shown in the following equations:

$$BUE_S(PHB) = \frac{\text{Molar mass product } [\frac{g}{mol}]}{\text{Molar mass feedstock } [\frac{g}{mol}]} \times 100 = \frac{86\,[\frac{g}{mol}]}{180\,[\frac{g}{mol}]} \times 100 = 47.8 \quad (2\text{-}2)$$

$$BUE_{H/L}(PHB) = \frac{BUE_S}{100} \times \text{Total Product Recovery Yield}_{H/L}[\%] = \frac{47.8}{100} \times 84 = 40.1 \quad (2\text{-}3)$$

In comparison, PLA had a BUE_S of 80 and BUE_H of 76.6. The low efficiency for PHB from glucose was due to the loss of two CO_2 molecules in the acetyl-CoA production step according to the following reaction:

$C_6H_{12}O_6 + 2\ CoA + 1.5\ O_2 \rightarrow 2\ C_2H_3O\text{-}CoA + 2\ CO_2 + 3\ H_2O$
$2\ C_2H_3O\text{-}CoA \rightarrow -(C_4H_6O_2)- + 2\ CoA$
$C_6H_{12}O_6 + 1.5\ O_2 \rightarrow -(C_4H_6O_2)- + 2\ CO_2 + 3\ H_2O$

Consequently, the conversion to PHB meant a loss of up to 59.9% of the glucose feedstock. A higher BUE would mean lower area of feedstock cultivation and therefore increased land use efficiency. Due to the consideration of the stoichiometric reaction of a feedstock, the BUE of PHAs does not change with alternative feedstocks that contain mainly glucose and other C6 sugars such as certain lignocellulose hydrolysates. However, it should be noted that feedstocks containing non C6 sugars such as C5 sugars, acetic acid or fatty acids may alter the BUE. The higher process impact on land use can be compensated for with a better functionality such as the biodegradability of PHB or with a high value of the product.

2.6 Integrating PHA production in second-generation biorefineries

Many lignocellulose conversion processes to high value chemicals and materials already exist. However, one major obstacle is that many of the technologies are not yet considered technologically mature. Indeed, among the integrated biorefineries that are currently commercialized, only few use lignocellulose feedstock with other techniques than gasification or pyrolysis [88]. In contrast, processes that retain the intrinsic chemical diversity of the feedstock, usually in the form of biochemicals and biomaterials, can be more efficient [89]. For example,

lignocellulose contains sugars, that are chiral, and phenols, that have aromatic bonds. These functionalities are lost when syngas is the conversion product. Furthermore, lignocellulose has a higher oxygen to carbon ratio than crude oil. Since carbon-carbon bonds have a higher energy than carbon-oxygen bonds, replacing transportation fuels would require twice as much biomass than crude oil. In contrast, these carbon-oxygen bonds and the chemical diversity can be desired properties for commodity chemicals and polymers [90], such as in the production of acrylic acid [91].

2.6.1 Conversion processes: pretreatment and fractionation

The emerging interest in both biofuels and biochemicals lead to the development of diverse lignocellulose conversion processes [92]. Principally, technology development follows either a top down or bottom up approach. The bottom up approach employs new processes that are still at the research stage and have not yet been validated in pilot plants, while the top down approach modifies existing industrial infrastructure. Overall, the processes differ in their recovery of the hemicellulose and lignin fractions. Initially, lignocellulose pretreatments were developed to increase enzymatic conversion of cellulose to glucose. The pretreatments increase cellulose accessibility, decrease crystallinity and/or remove inhibitors [92]. However, in integrated processes, both hemicellulose and lignin are feedstocks for biobased products. Such pretreatment processes that separate the lignocellulose components are typically called fractionation processes [4].

The quality criteria for fractionation processes are the material recovery of all three lignocellulose components [93]. One example is a three-stage process using concentrated phosphoric acid as cellulose solvent (cellulose solvent-based lignocellulose fractionation) [94-95]. In the other stages, lignin is dissolved with an organic solvent, such as acetone, and finally hemicelluloses are completely recovered by addition of water. As a result, lignocellulose is separated into three fractions. Another approach is the use of ionic liquids which can dissolve cellulose as well as lignin. This allows for an easy recovery of the fractionated streams. The ionic liquid that is most commonly used to dissolve cellulose is 1-butyl-3-methylimidazolium chloride, while lignin is typically dissolved in 1-ethyl-3-methylimidazolium acetate.

In contrast for a top down approach, existing pulp and paper infrastructure could be modified for biofuel and biochemical production. Zhang, et al. [96] suggested three processes

that are easily adaptable. One opportunity is a pre-extraction step prior to sulfite (Kraft) pulping. In this approach, a combination of acids, pulping liquor and water can be used to extract the hemicelluloses from the wood chips [97]. Mechanical pulping is a process that combines heat and mechanical forces to deconstruct fiber cell walls. A modified mechanical pulping process to conduct continuous steam explosion improves enzymatic digestibility as compared to other pretreatment processes such as dilute acid pretreatment and ammonia fiber explosion [98]. Finally, existing pulp bleaching equipment such as a peg or kettle mixer can improve lignocellulose conversion. In brief, this type of mixer has rotating pegs that create shear forces which expose fiber surfaces thereby increasing enzymatic accessibility to cellulose [96]. Using existing industrial lignocellulose conversion processes for PHA production can accelerate the market adoption of lignocellulose-based PHA since it reduces technical uncertainties and equipment.

2.7 Metabolic pathways of lignocellulosic sugar consumption

The metabolic pathways for PHA production from sugars are intrinsically linked to the essential cata- and anabolism of microorganisms. In general, PHA production starts with sugar transport into the cell, followed by catabolic conversion to pyruvate and finally to acetyl-Coenzyme A (acetyl-CoA) before the final three-step conversion to PHAs [11, 81, 99-110]. The sugars serve as sources of energy, carbon and electrons thereby generating adenosine triphosphate (ATP), and the reduced forms of nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) with the concomitant formation of pyruvate [111]. Error! Reference source not found.illustrates the three main pathways for lignocellulose sugar catabolism to PHB, Embden-Meyerhof-Parnas (EMP), Entner-Doudoroff (ED) and the Pentose-Phosphate Shunt (PPS). The C6 sugars D-mannose and D-galactose can enter the same pathways as D-glucose after conversion, while the C5 sugars D-xylose and L-arabinose are converted via the PPS [21] .

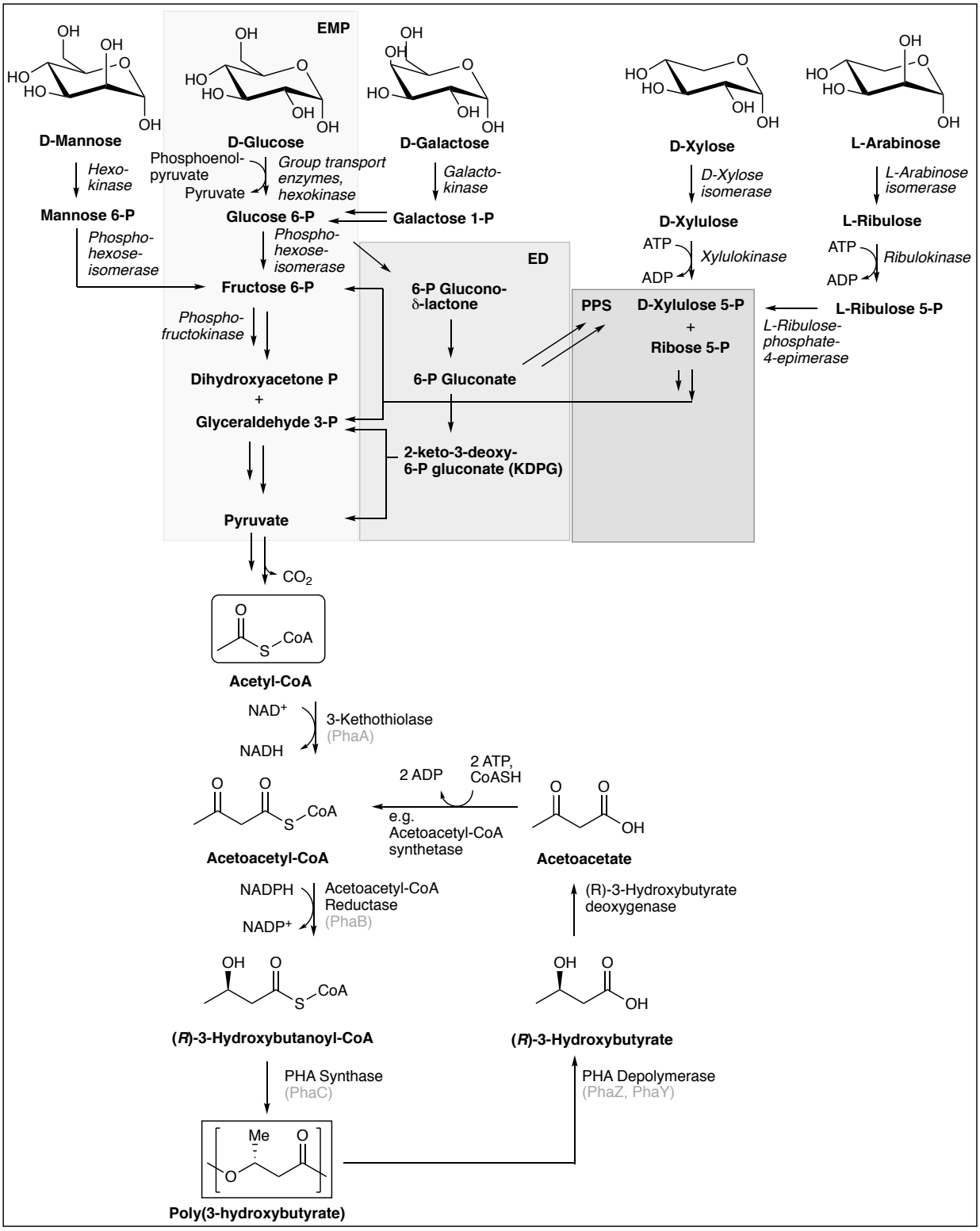


Figure 2-9: Overview of general catabolic pathways for lignocellulose sugars to PHB. EMP-Embden-Meyerhof-Parnas, ED-Entner-Doudoroff, PPS-Pentose Phosphate Shunt (adapted from [31, 103, 112-114]).

The most common glucose conversion pathway in bacteria, yeast and fungi is the EMP [115]. However, the PHB model organism *C. necator* H16 and *P. sacchari* IPT101 are lacking the gene for the key EMP enzyme, phosphofructokinase and use the ED pathway instead [113, 116].

The main sugars of lignocellulose hydrolysates (glucose, xylose, arabinose, galactose and mannose) are all catabolized by either EMP, ED or PPS (**Error! Reference source not found.**[115]. Mannose is phosphorylated to mannose 6-phosphate and directly isomerized to fructose 6-phosphate to enter the catabolic pathways. In contrast, galactose is converted to the intermediate uridine diphosphate (UDP) galactose and enters the catabolic pathway via glucose 6-phosphate [31]. In contrast to hexoses, the pentoses xylose and arabinose can only enter catabolism through the pentose-phosphate pathway [21, 115, 117].

Overall, pentose conversion leads to lower pyruvate and therefore PHA yields. In the case of *P. sacchari*, a recent study showed that overexpression of the sequence *xylA* and *xylB* increases xylose catabolism, increasing the maximum specific growth rate on xylose by 31% [118]. Introducing multiple copies of the sequences increases expression of endogenous xylose isomerase and xylulokinase genes. The increased catabolic rate also increased PHB production [119].

2.8 PHA production from lignocellulose feedstock

2.8.1 PHA from hemicellulose hydrolysates

The low price of hemicellulose feedstock, few concurrent product streams, and milder hydrolysis conditions to monomer sugars make hemicellulose an attractive candidate for PHA feedstock. However, only few microbial strains are able to metabolize C5 sugars and accumulate PHAs, and if they do, conversion efficiencies have been low [21]. Table 2-3 summarizes the various pretreatments and their respective PHA yields. The most common method employed is dilute acid treatment with sulfuric acid. However, the main disadvantage is that even under mild temperatures and pressures, this treatment leads to inhibitory amounts of furfural and (5-hydroxymethylfurfural) 5-HMF.

Table 2-3: Overview of PHA yields from hemicellulose hydrolysate from different pretreatment methods. PHA concentration (PHA), Cell dry mass (CDM), PHA mass fraction in biomass (mass fraction), PHA productivity (Pro), Substrate yield ($Y_{P/S}$). n.a.-not applicable, n.s.-not specified.

Pretreatment conditions	Carbon source	Strain	Type	PHA [g L^{-1}]	CDM [g L^{-1}]	Mass fraction [(g/g)]	Pro [g L^{-1} h^{-1}]	$Y_{P/S}$ (g/g)	Scale	Ref.
Steam explosion										
Steam explosion followed by enzymatic hydrolysis	Trembling Aspen	*P. cepacia*	PHB	1.56	2.60	0.60		0.11	shake flask, bioreactor	[120]
Dilute Acid										
2 wt% H_2SO_4, 1h @ 30 °C, 1h @ 120 °C	*Pinus radiata* sawdust	*B. vesicularis,* *S. macrogoltabida*	PHBVP	0,162 0,231	0,253 0,320	0.64 0.72	n.s.	n.s.	shake flask	[121]
loading 150 g·L^{-1}, 1% H_2SO_4, 120 min, 115 °C	Sugarcane bagasse	*C. necator*	PHB	6.27	11.10	0.57	n.s.	n.s.	shake flask	[122]
160 °C, 120 min; then 2% H_2SO_4, 95 °C, 20 min Two-stage filtration	Sugar maple wood chips	*B. cepacia*	PHB	8.72		0.51	0.09	0.19	(fed-)batch in 1L bioreactor	[123-124]
loading 15% (g/g), 2% (g/g) H_2SO_4, 60 min, 121 °C, 15 lb pressure	Rice Straw	*B. firmus*	PHB	1.70	1.90	0.89			shake flask	[125]
loading 100 g, 600 mL of 1% H_2SO_4, 60 °C overnight 3-fold concentration at 85 °C	Sugarcane bagasse	*Burkholderia* sp. F24	PHB	12.25	25.00	0.49	0.28	n.s.	shake flask 5 L in fed-batch bioreactor	[126]
N.s.	Sugarcane trash	*Bacillus* spp.	PHBV	0.23	2.89	0.08	0.00	n.s.	shake flask	[127]
Hot Water										
loading 10 g·L^{-1}, 70 ˚C, 100 rpm, 2h	Orange peel	recombinant *B. subtilis*	PHB	1.24	3.10	0.40	0.02	n.s.	shake flask, 0,5 L in batch bioreactor	[128]
Consolidated Bioprocessing										
Fermentation by recombinant E. coli	Beechwood Xylan/ xylose *co*-feed	recombinant *E. coli*	PLAHB	3.70	8.90	0.42	n.s.	n.s.	shake flask	[129]

Bertrand, et al. [130] reported the first microorganism, *Pseudomonas pseudoflava*, to grow PHA from hemicellulosic sugars. The strain grew on a medium with pure glucose, xylose and arabinose. The PHA mass fraction in biomass was low (0.17-0.22 (g/g)), which possibly resulted from the high maintenance energy of the strain with xylose. When the pretreatment minimized inhibitor formation, *Pseudomonas cepacia* successfully grew on hemicellulose hydrolysate [120]. The combination of steam explosion and enzymatic hydrolysis with xylanase on trembling aspen produced a xylose-rich feedstock. Subsequent fermentation yielded a PHB concentration of 1.56 g/L at a PHB mass fraction in biomass of 0.60 (g/g). The product yield was 0.11 g PHB per g xylose. However, it was determined that a minimum yield of 0.33 g/g was required for economic competitiveness.

Using dilute sulfuric acid pretreatment, Silva, et al. [121] tested *Pinus radiata* sawdust hydrolysate with a total sugar concentration of 112.5 g/L. In shake flasks with *Brevundimonas vesicularis* and *Sphingopyxis macrogoltabida*, they produced the *co*-polymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxypropionate) (PHBVP) at a PHA mass fraction in biomass of 0.64-0.72 (g/g), but due to low cell growth, PHA concentrations were only 0.162-0.231 g/L. Yu and Stahl [122] used similar hydrolysis conditions and characterized the hydrolysate composition. The dilute acid pretreatment of sugarcane bagasse yielded 27.6 g/L total sugars with 70% xylose, 6% glucose and 6% arabinose in the hydrolysate. Formic acid, acetic acid, furfural, 5-HMF and acid soluble lignin were present as inhibitors. By starting with a high inoculum concentration and the selection of a resistant strain of *Cupriavidus necator*, they obtained a PHB concentration of 6.27 g/L at a PHB mass fraction in biomass of 0.57 (g/g) in shake flasks.

Pan, et al. [123] investigated the inhibitory effects of several components of hemicellulose hydrolysate (for example acetic acid, furfural, vanillin, vanillic acid and syringic acid) on growth and PHA production of *Burkholderia cepacia*. Experimental design using response surface methodology revealed acetic acid and syringic acid as the most inhibitory compounds. However, *B. cepacia* metabolized the phenolic inhibitors during the first 24 h in the order of vanillin, vanillic acid and syringic acid. In a subsequent study [124], they compared several detoxification methods such as overliming, activated charcoal, anion and cation exchange to improve the conversion of hemicellulose hydrolysates to PHB with *B. cepacia.* The dilute acid pretreatment of sugar maple wood chips yielded a total sugar concentration of 81 g/L with 85% xylose

content. The lignin degradation products in the hydrolysate were vanillin, cinnamic acid, syringaldehyde, vanillic acid, syringic acid and *p*-coumaric acid in addition to furfural, 2-furanyl methanol and acetic acid. Overliming followed by low temperature sterilization had the highest sugar recovery and reduced the concentration of acetic acid and phenolics to 0.05 M and 0.6 g/L respectively, and achieved the near quantitative removal of furfural and 5-HMF while maximizing sugar recovery. Fermentation in a bioreactor yielded a PHB concentration of 8.72 g/L at a PHB mass fraction in biomass of 0.51 (g/g).

Sindhu, et al. [125] used experimental design to optimize PHB cell content of *Bacillus firmus* with rice straw hydrolysate in shake flasks. The hydrolysate had a total sugar concentration of 24.05 g/L with 94% xylose content and formic acid, acetic acid, furfural and 5-HMF as identified inhibitors. The shake flask experiments reached a PHB mass fraction in biomass of 0.89 (g/g). However, because of low cell growth, the resulting PHA concentration was only 1.7 g/L.

[126] obtained a PHB concentration of 12.3 g/L and a PHB mass fraction in biomass of 0.49 (g/g) with a detoxified hemicellulose hydrolysate of sugarcane bagasse by isolating a resistant *Burkholderia* strain from soil and using a high cell density inoculum concentration in a fed-batch bioreactor. The hydrolysate had a sugar concentration of 21 g/L and contained 78% xylose, 12% glucose and 10% arabinose. Formic acid, acetic acid, furfural and small amounts of 5-HMF were removed by overliming.

Moorkoth and Nampoothiri [127] reported a new halophilic, Gram-positive *Bacillus* species capable of converting sugarcane trash to PHBV. The hydrolysate had a total sugar content of ca. 20 g/L with 65% xylose, 25% glucose and 10% arabinose content. In shake flask experiments, the *Bacillus* species yielded 0.231 g/L PHBV at a PHA mass fraction in biomass of 0.08 (g/g).

Sukan, et al. [128] hydrolyzed orange peel with a hot water pretreatment. The water with the immersed orange peel had a temperature of 70 ˚C for 2 h. Without detoxification, a recombinant strain of *Bacillus subtilis* yielded 1.24 g/L PHB at a PHB mass fraction in biomass of 0.40 (g/g) in a batch bioreactor.

Consolidated bioprocessing combines carbohydrate hydrolysis with PHA production, thus eliminating the need for a pretreatment or detoxification process [131]. Salamanca-Cardona, et al. [129] introduced an endoxylanase and β-xylosidase gene into a PHA-producing *Escherichia coli*. The resulting recombinant strain produced the *co*-polymer poly(lactate-*co*-

3-hydroxybutyrate) (PLAHB) at a concentration of 3.7 g/L and a PHA mass fraction in biomass of 0.42 (g/g) from beechwood xylan in the presence of xylose.

The studies on hemicellulose hydrolysate fermentation to PHA show significant improvement in pure xylose and hemicellulose hydrolysate conversion, but validation on bigger scale is still missing. Xylose conversion improved by strain development through genetic modification and strain isolation [118]. Additionally, carbon catabolite repression has been overcome for some strains, depending on the feeding strategies [132-133]. Furthermore, the processes for hemicellulose hydrolysate are economically feasible and well-established, and several detoxification methods are capable of removing the inhibitory compounds allowing hemicellulose hydrolysate fermentation [120, 122-124, 126]. However, the remaining challenge is to translate the fundamental knowledge into applied results. More studies are needed that ferment hemicellulose hydrolysate in bioreactors, thus allowing high-cell density cultivations. Currently, the PHA concentrations and productivities are too low. Lignocellulose hydrolysate has already achieved PHA concentrations of 105 g/L in 61h [132]. In contrast, the highest PHA concentration with hemicellulose hydrolysates reported reached 12.3 g/L with *Burkholderia* sp. f24 [126], which is only 6-12% of the industrial concentrations with glucose feedstock. Nevertheless, *S. macrogoltabida* [121] and *B. firmus* [125] already achieved the necessary PHA cell content for industrial feasibility of a PHA mass fraction in biomass of over 0.70 (g/g).

To increase PHA production from hemicellulose, one approach is to use hemicellulose hydrolysates with a higher hexose sugar content. This is the case for softwood hemicellulose hydrolysates, which consist of glucose, mannose, galactose, xylose and arabinose. In a model study, the main softwood hemicellulose sugar mannose and galactose afforded respectively 97%, and 60% of the maximum specific growth rate of glucose [134]. However, the inhibitor content in softwood hemicellulose hydrolysates currently prevents their use as a carbon source for PHA production [134]. For example, a softwood holocellulose hydrolysate stopped cell growth and PHA production when used as the sole carbon source, both in a low and a high concentration. Nevertheless, feeding the hydrolysate dropwise after an initial 15h growth phase with glucose afforded a high max. PHA concentration of 60.5 g/L [135]. Using the halophile *Halomonas halophila* could also improve PHA production, as it converts hemicellulose sugars (notably mannose, galactose and xylose) without carbon catabolite repression and can grow on pine

sawdust hydrolysate in inexpensive media [136]. Furthermore, a recent study found that bacterial growth and PHA productivity increases when softwood holocellulose hydrolysate is detoxified with either over-liming, activated charcoal, or lignite [137]. In general, several detoxification methods successfully removed inhibitors in other lignocellulose hydrolysates [138]. These methods are also likely to be suitable for the corresponding hemicellulose hydrolysates.

An alternative approach to reduce PHA production costs is the use of microbiomes that allow non-aseptic fermentations [81]. A microbiome is a mixed microbial community, where the members have efficient metabolic interactions due to natural selection processes [139]. For PHA production, mixed microbial communities, such as activated sludge, are subjected to transient conditions such as an alternating feast-famine regime or variations in electron donors and acceptors to increase PHA producing organisms [140]. Microbiomes can reach 0.77 (g/g) PHA in 5h [141]. Hemicellulose-rich carbon substrates, such as municipal solid waste, are suitable carbon sources for these fermentations. However, an excess of sugars in mixed microbial cultures can lead to the enrichment of glycogen, exopolysaccharide and lipid producers, and thus a pre-fermentation to volatile fatty acids (e.g. acetic acid, propionic acid, butyric acid) can improve yields [142]. In future, the use of hemicellulose hydrolysate as substrate for mixed microbial communities is a potential improvement to decrease PHA production costs. However, the material properties (e.g. molar mass distribution, crystallinity) would need to be controlled in the presence of diverse PHA-producing microorganisms [81].

2.8.2 PHA from fractionated lignocellulose streams

Keenan et al., [143-144] proposed an integrated process to produce PHBV using hemicellulose hydrolysate from woody biomass as the main substrate *co*-fed with levulinic acid from the cellulose stream under simultaneous recovery of lignin. The study was based on findings that levulinic acid is a precursor to produce high-quality PHBV *co*-polymers [145-147]. Keenan et al. compared the National Renewable Energy Laboratory's clean fractionation process [148] to hydrothermal autohydrolysis with subsequent membrane-diafiltration to produce a fermentable hemicellulose stream from Aspen and maple wood, respectively. The cellulose fraction is intended as feedstock for levulinic acid from the Biofine process [149]. The hemicellulose hydrolysate was detoxified to remove phenolics, furfuraldehydes and sulfates to yield the feedstock for shake-flask cultures with *B. cepacia*. Since the fermentations were only

conducted on the shake flask scale, the yields were low at 1.6-2.0 g/L PHA concentration, 0.39-0.40 (g/g) PHA mass fraction in biomass and 4-67 mol% 3HV content in the *co*-polymer.

Using wheat straw, Gasser, et al. [150] followed a similar fractionation approach based on the conversion of hemicellulose hydrolysate. Fractionation by thermal pressure hydrolysis of wheat straw afforded hydrolyzed hemicellulose and a solid cellulose-lignin pulp. The straw was treated in dilute nitric acid to yield a hydrolysate with xylose (11.87 g/L), glucose (3.59 g/L) and arabinose (1.79 g/L). Enzymatic hydrolysis of the pulp afforded glucose (13 g/L) and xylose (1.7 g/L). Both hemicellulose and cellulose hydrolysates were tested for PHB production. Detoxification of the hemicellulose hydrolysate removed furfural and 5-HMF through concentration, overliming, deep bed filtration to separate solids, and filtration over activated charcoal. Using *Bacillus licheniformis* and *Bacillus megaterium,* the hemicellulose hydrolysate in shake flask cultures yielded PHB concentrations of 1.91-2.48 g/L at a PHA mass fraction in biomass of 0.38-0.46 (g/g). Similarly, the cellulose hydrolysates resulted in 1.11-1.23 g/L PHB at a PHB mass fraction in biomass of 0.53-0.55 (g/g).

Lopes, et al. [126] followed the same fractionation approach with sugarcane bagasse. Pretreatment of sugarcane bagasse with mild acid hydrolysis using 1% sulfuric acid at 60 ˚C overnight produced a hemicellulose stream with low furfural content and a cellulose-lignin solid residue. Overliming followed by neutralization, filtration and concentration produced the feedstock for PHA fermentation. They isolated bacteria from soil samples and screened them for PHA production from xylose and tolerance to inhibitors. Among the isolates, a *Burkholderia* sp. was the most efficient and reached 12.3 g/L PHB concentration at 0.49 (g/g) PHB mass fraction in biomass in a fed-batch bioreactor cultivation. The strain was also capable of consuming levulinic acid as *co*-feed to produce 9-43 mol% 3HV content. Based on these results, the authors suggested a sugar-ethanol-PHA biorefinery. In the biorefinery concept, PHA is produced from bagasse hemicellulose hydrolysate, levulinic acid and ethanol from enzymatically hydrolyzed cellulose, and sucrose from sugar juice [151].

Naranjo, et al. [152] modeled the economic performance of a multiproduct biorefinery based on rejected bananas. The model assumed a combination of the xylose and glucose for PHB production to a PHB concentration of 35 g/L at a PHB mass fraction in biomass of 0.58 (g/g) and a substrate yield of 0.22 g/g. Additionally, part of the glucose was used as a feedstock for bioethanol production. In the integrated biorefinery, the economic margin of PHB almost

doubled compared to single product PHB production from banana pulp alone. Recycling of the process water resulted in key savings. Finally, the integration reduced the production cost of the *co*-products glucose (-22%) and ethanol (-53%).

Integrated lignocellulose biorefinery concepts can also be the source of composite blends of lignocellulosic materials and PHAs. Blending lignin with PHAs reduces the product price and valorizes lignin. Camargo, et al. [153] created lignin-PHBV composites ranging from 0-80 wt% lignin content. Up to 50 wt% lignin could be used to extrude the composites. However, lignin decreased the mechanical properties (elongation at break and Young's modulus) due to no physical contact between the lignin filler and the polymeric matrix. Nevertheless, for a lower lignin content of maximum 10 wt%, Kovalcik, et al. [154] demonstrated a smooth lignin particle distribution in blends with PHBV. It was also shown that lower lignin contents (1-5 wt%) changed crystallization and melting behavior of the polymer and did not affect the mechanical properties compared to pure PHBV. A strong advantage for applications in food packaging was that the gas permeability of oxygen and carbon dioxide decreased up to 77% and 91%, respectively. Additionally, to make PHA-based composites with improved mechanical properties, lignocellulose fibers can improve strength properties. For example, Hodzic, et al. [155] used sugarcane bagasse fibers, which resulted in a composite with improved flexural strength than polypropylene (65 MPa compared to 23.8-50 MPa). For overall PHA market success, the mechanical properties and the processability in injection moulding and blow extrusion must be improved, while keeping the compostability of the biopolymer. Pérez Amaro, et al. [156] reported several components added to PHBV to create an improved composite, that contained 5 wt% of lignocellulosic filler, a plasticizer, other biodegradable polymers, an antioxidant and a nucleating agent. Thus, lignocellulose can be both a starting material for PHA production and a material for post-synthesis improvement.

2.9 Conclusion

The bioeconomy is a broad political concept that combines targets of green economic growth with innovation in biotechnology. Bio-based materials are rarely included in bioeconomy policies, even though they can impart a high degree of environmental savings by reducing greenhouse gas emissions and waste. The higher value of biobased materials as compared to

fuels makes them interesting *co*-products that can drive the development of integrated biorefineries by improving their economic competitiveness.

PHAs are already produced at commercial scale with a full scale production capacity of 32,000 tonnes per year and many more facilities are planned. While PHAs have the important benefits of being biobased, compostable and non-toxic, the specific environmental savings depend on various factors. The production can be CO_2 neutral when part of the biomass feedstock generates energy but can cause GHG emissions with conventional energy use and low agricultural efficiencies. Among various feedstocks, sugarcane with *co*-production of energy through burning of bagasse have shown the highest greenhouse gas emission savings, while lignocellulose such as corn stover can increase the environmental benefits of PHAs as compared to sugar beet or corn starch. Furthermore, the GHG emission reduction from PHA use depends on the material properties for a given product application.

PHAs can be an attractive material for a sustainable bioeconomy if the process and product design incorporates sustainability criteria. A high potential for sustainable PHA production is in the use of lignocellulosic biomass feedstock, as it occurs as waste material and can therefore reduce land use, feedstock GHG emissions and other feedstock related impacts such as fertilizer and water use. However, since biomass feedstock prices are likely to increase with increased interest in biobased products, the support of an adequate policy framework that penalizes the high impact on pollution and climate change of petrochemicals is necessary to drive the market success of sustainable PHAs.

Connecting Statement to Chapter 3

In Chapter 2, PHAs were identified as performing products for cleaner plastic consumption in the bioeconomy. Lignocellulose biorefinery schemes were discussed as efficient conversion units for the production of PHAs at decreased costs. To allow for a quick scale up and market uptake, the lignocellulose feedstock must ideally have an existing harvest and collection infrastructure, which is the case for Canadian forest resources.

In the next chapter, a forest biorefinery scheme based on the hemicellulose extraction of softwood chips was tested for PHB production. The chips were subjected to a prehydrolysis treatment, which hydrolyzes hemicelluloses to sugar-rich hydrolysates. Based on the composition analysis of the hemicellulose hydrolysates, the hydrolysate sugar and by-product composition was mimicked in simulated hemicellulose hydrolysates. These simulated hemicellulose hydrolysates were tested as feedstock for PHB production using *P. sacchari* while the solid residue, composed of cellulose and lignin, was recovered as feedstock for conventional pulping. The chapter is based on the following article: Dietrich, K.; Dumont, M.-J.; Schwinghamer, T.; Orsat, V.; Del Rio, L. F., Model Study To Assess Softwood Hemicellulose Hydrolysates as the Carbon Source for PHB Production in Paraburkholderia sacchari IPT 101. *Biomacromolecules* **2018,** *19* (1), 188-200.

Chapter 3: Model Study To Assess Softwood Hemicellulose Hydrolysates as the Carbon Source for PHB Production in Paraburkholderia sacchari IPT 101

3.1 Abstract

Softwood hemicellulose hydrolysates are a cheap source of sugars that can be used as a feedstock to produce polyhydroxybutyrates (PHB), which are biobased and compostable bacterial polyesters. To assess the potential of the hemicellulosic sugars as a carbon source for PHB production, synthetic media containing softwood hemicellulose sugars (glucose, mannose, galactose, xylose, arabinose) and the potentially inhibitory lignocellulose degradation products (acetic acid, 5-hydroxymethylfurfural (HMF), furfural and vanillin) were fermented with the model strain *Paraburkholderia sacchari* IPT 101.

Relative to pure glucose, individual fermentation for 24h with 20 g/L mannose or galactose exhibited maximum specific growth rates of 97% and 60% respectively. With sugar mixtures of glucose, mannose, galactose, xylose and arabinose, the strain converted all sugars simultaneously to reach a maximum PHB concentration of 5.72 g/L and 80.5% PHB after 51h. The addition of the inhibitor mixture at the following concentration: sodium acetate (2.11 g/L), HMF (0.67 g/L), furfural (0.66 g/L), and vanillin (0.93 g/L), to the sugar mixture stopped the growth entirely within 24h. Individually, the inhibitors either had no effect or only reduced growth (from optical density, OD of 28.7 to 19.3, compared to 1.5 for inhibitor mixtures). Moreover, it was found that a bacterial inoculum with high initial cell density (OD $\geq$ 5.6) could overcome the growth inhibition to yield an OD of 13 within 24h. Therefore, softwood hemicellulose sugars are viable carbon sources for PHB production. Nevertheless, real softwood hemicellulose hydrolysates need detoxification or a high inoculum to overcome inhibitory effects and allow bacterial growth.

3.2 Introduction

Compostable biopolymers can be environmentally friendly alternatives to meet the growing demand for polymers as they reduce land and water pollution, greenhouse gas emissions, and resource depletion [158]. Among them, polyhydroxyalkanoates (PHAs) are microbiologically-

produced polyesters with similar material properties as polyethylene and polypropylene [159]. Presently, corn is the main carbon source for the industrial production of PHAs [160]. However, in the long term, this carbon source is undesirable as it is expensive, and competes with food production [13]. Hemicelluloses are promising alternative carbon sources for PHA production as they are cheaper than glucose, non-edible, and currently considered as a waste product [161]. They are by-products of the existing pulp and paper infrastructure and are industrially readily available [89]. However, the heterogeneous sugar composition and the high amounts of the pentose sugar xylose are challenges to the fermentation of hemicellulose feedstock [162].

Softwoods are the main type of tree used for pulp production in the northern hemisphere [163] and their hemicelluloses contain a higher proportion of the hexose sugars mannose and galactose (in the form of galactoglucomannans) compared to the hemicelluloses from agricultural residues or hardwoods [162, 164]. This difference in hemicellulose composition is significant as both mannose and galactose can enter the same glycolytic pathway as glucose [115, 117]. Therefore, softwood hemicellulose hydrolysates have been extensively studied for bioethanol conversion [165-168] and lactic acid production [169].

While softwood hemicellulose hydrolysates have a favorable sugar profile, they can contain compounds that inhibit the growth of microorganisms [170-171]. The compounds are released in side reactions during the pretreatment and depend on the pretreatment type and conditions [138], which can be severe in the case of softwood. The main inhibitor types are furans (e.g. HMF and furfural), organic acids (e.g. acetic, formic, and levulinic acid), and phenolics (e.g. vanillin, and syringic acid). Among the softwood phenolic compounds, vanillin was found to be the most abundant in a dilute acid hydrolysate of spruce [172].

The use of hemicellulose hydrolysates for PHB production at an industrial scale necessitates the use of high productivity strains [22]. The ability to consume xylose and arabinose simultaneously with glucose, and the production of different PHAs, makes *P. sacchari* an ideal model organism for the production of PHAs from mixed sugar substrates such as lignocellulose hydrolysates [116]. Raposo, et al. [133] described the optimal glucose-xylose mixtures in synthetic medium with pure sugars. Kucera, et al. [173] used *P. sacchari* and *Burkholderia cepacia* to ferment spruce sawdust hydrolysates (holocellulose). The hydrolysate inhibited cell growth and was detoxified with activated carbon, overliming, and lignite. Despite detoxification, cell growth reached only 4 g/L with 2 g/L PHB after 72h for both strains. To

improve softwood fermentability to PHB, a better mechanistic understanding of the influence (including potential inhibition) of mannose and galactose on the model strain *P. sacchari* must be understood.

The goal of this study was to identify the conditions that make softwood hemicellulose hydrolysate a suitable carbon source for PHB production. The first objective was to compare the fermentation of softwood hemicellulose sugars (mixture of glucose, mannose, galactose, arabinose and xylose) with pure glucose. The second objective was to quantify the effects of inhibitors on bacterial growth using statistical analysis. For individual inhibitors, the effects were compared using a generalized linear mixed linear model. For inhibitor mixtures, the effects were analyzed using a mixture design.

3.3 Materials & methods

3.3.1 Chemical composition of softwood chips

Black spruce (*Picea mariana*) wood logs were debarked and chipped to a maximum size of 10 mm. The wet chips were stored in the fridge and had a solid content of 70.09±0.93%. A sample of the chips was Wiley-milled through a 40-mesh screen [174]. The ground wood (ca. 2 g) was soxhlet-extracted with acetone (200 mL) for 8 h to determine the extractives content [175]. The residual wood was dried at 105 °C and cooled to room temperature in a desiccator prior to weighing. The weight loss after extraction consisted of the extractives content. The carbohydrate and lignin composition of the extractives-free biomass was analyzed following the National Renewable Energy Laboratory procedure [175]. The oven-dried ground wood (ca. 200 mg) was hydrolyzed with 72% H_2SO_4 (3 mL) at room temperature for 2 h. Subsequently, 97 mL of water was added to obtain 4% H_2SO_4 and autoclaved at 121 °C for 1 h. The mixture was weighed before and after and the lost water was compensated. The solids were separated by filtration, washed, and dried at 105 °C, to yield the acid-insoluble lignin. The liquor was collected for monosaccharide analysis using ion chromatography (IC) (Dionex DX 600). Acid-soluble lignin was measured using a UV-VIS spectrophotometer at a wavelength of 205 nm. The aromatic portion of acid soluble lignin has an absorption maximum at 280 nm, but due to possible interference with HMF (λ_{max} 284 nm) and furfural (λ_{max} 266 nm), the measurement was

conducted at a lower wavelength. The chemical composition of the wood chips is presented in Section 3.1 (Softwood hemicellulose composition).

3.3.2 Dilute acid hydrolysis of softwood chips

The wood chips were pretreated with a dilute sulphuric acid hydrolysis. The wood chips (200 g oven-dry basis) were pre-steamed to remove air and to saturate the chips with water. In a 56 L digester, the chips were subjected to three cycles of steam at 138 kPa for 3 min followed by water soaking. The digester was left to cool for 5 min, and then filled with water, and soaked for 15 min. The water was drained and the soaking step was repeated a second time.

The pre-steamed chips were hydrolyzed in a 1:3 wood-to-liquor ratio based on the oven-dried wood mass. They were placed in 2 L bomb digesters with 600 mL water and sulphuric acid (4 g corresponding to 5.56 mL of 72% H_2SO_4) with a pH of 1.17. The hydrolysis temperature was 160 °C. Upon reaching 160 °C, the reaction time was 15 min. The combined severity (CS) of the hydrolysis was calculated according to Equation 3-1 [165]. The parameters are the reaction time (min), the temperature (°C) and the pH. The combined severity of the reaction was 1.77.

$$\mathrm{CS} = \log_{10}\left[\mathrm{t} \cdot \exp\left(\frac{\mathrm{T}-100}{14.75}\right)\right] - \mathrm{pH} \tag{3-1}$$

After the reaction time, the bomb digesters were cooled in cold water. The hydrolysate was drained and collected for further use. The chips were washed three times in cold water, soaked overnight, drained and oven-dried at 105 °C. The final mass was recorded to determine the solid yield.

3.3.3 Fermentation

3.3.3.1 Microorganism and media

The PHB producing strain was *Paraburkholderia sacchari* IPT 101 (DSM 17165) and was purchased from DSMZ (Germany). The strain was resuscitated and grown on agar plates using R2A medium as well as in the seeding medium. They were stored in glycerol stocks.

The growth medium composition was adapted from Cesário, et al. [132]. In brief, the nitrogen-limited medium was composed of medium stock solutions (buffer, $MgSO_4$, and trace element) and the carbon source. The buffer stock was concentrated two-fold. In the final

medium, it had a concentration of $(NH_4)_2SO_4$ 1 g/L, Na_2HPO_4 x$7H_2O$ 6.78 g/L, KH_2PO_4 1.5 g/L, and 1 g/L of yeast extract. The buffer stock was adjusted to pH 6.8. The magnesium sulfate stock was concentrated 100-fold to yield a final concentration of 0.2 g/L. The trace element stock had the composition reported by [176] to yield a final concentration of 1 mL/L. The buffer stock and the magnesium stock were sterilized by autoclaving, and the trace element stock was sterilized by filtration (0.22 µm). The carbon stock solution was prepared from pure sugars (glucose, mannose, galactose, xylose, arabinose) purchased from Sigma Aldrich and sterilized by filtration (0.22 µm). The inhibitor stock solutions were prepared from pure compounds (vanillin, furfural, HMF, sodium acetate) purchased from Sigma Aldrich and sterilized by autoclaving.

The medium was prepared by adding in % total volume: 50% buffer stock, 1% $MgSO_4$ stock, 0.1% trace element stock, and varying amounts of carbon source, inhibitors and autoclaved water.

3.3.3.2 Sugar fermentations

The fermentation procedure was adapted from Cesário, et al. [132]. The seeding medium was inoculated with a pure colony from the agar plates in 500 mL shake flasks with 50 mL seeding medium. The seeding medium was incubated at 30 °C at 170 rpm for 12-15 h (to approx. 1 g/L cell dry mass (CDM)).

The fermentation of the sugars was carried out in 500 mL shake flasks. The inoculum varied between 5-10% (v/v) to obtain an OD of 0.5-1. The culture was grown for 24-51 h, harvested and analyzed for sugar consumption, CDM and PHB content.

3.3.3.3 Effect of individual inhibitors on fermentation

The inhibitors found in softwood hemicellulose hydrolysates were added to a synthetic sugar mixture composed of 18.02 g/L total sugars. The concentration of individual sugars in the mixture was 7.43 g/L mannose (41%), 3.61 g/L xylose (20%), 3.41 g/L glucose (19%), 2.49 g/L galactose (14%) and 1.08 g/L arabinose (6%). The individual inhibitor concentrations were set at 0.1 g/L, 0.5 g/L and 1 g/L for vanillin, furfural and HMF; and 0.5 g/L, 1 g/L and 2 g/L for sodium acetate. The inhibitor mixture concentration was set at 0.93 g/L vanillin, 0.66 g/L furfural, 0.67 g/L HMF, and 2.11 g/L acetate.

The fermentation experiments were conducted using 12 mL of fermentation medium in 50 mL shake flasks operating at 170 rpm. The experiments were conducted in triplicate in separate randomized batches. The inoculum was grown overnight in the synthetic sugar mixture.

1 mL of seeding medium was transferred to obtain an initial OD_{600} of 0.8±0.2. The culture was grown for 24 h at 30 °C. The OD_{600} was measured in triplicates at the beginning (t=0 h) and at the end (t=24 h) of the experiments.

3.3.3.4 Effect of multiple inhibitors on fermentation

Sixteen fermentation experiments were conducted where the first 15 runs contained inhibitors at a concentration of 2 g/L and the 16th run did not contain inhibitors (blank). All runs were repeated on three separate days in a randomized order. They all contained fresh inoculum.

The initial sugar concentration was 12.01 g/L total sugars composed of 4.95 g/L mannose (41%), 2.41 g/L xylose (20%), 2.27 g/L glucose (19%), 1.66 g/L galactose (14%) and 0.72 g/L arabinose (6%). The total fermentation volume was 15 mL. The OD_{600} was measured in duplicates at the beginning (t=0 h) and at the end (t=24 h) of the experiments.

3.3.4 Analytical methods

3.3.4.1 Bacterial growth

Cellular growth was optically measured by OD_{600} and gravimetrically measured by CDM. The OD_{600} was measured in duplicates or triplicates at 600 nm in a UV-Vis spectrophotometer. CDM was determined by centrifuging triplicates of 1.5 mL of culture broth in a centrifuge (1300 rpm for 5 min) using previously dried and weighted microtubes. The supernatant (1.3 mL) was removed and kept for carbohydrate analysis. The pellet was washed twice with distilled water (1 mL) and freeze-dried until constant mass (ca. 24 h.)

3.3.4.2 Polyhydroxybutyrate determination

For PHB determination, the pellet from the CDM measurement was subjected to acidic methanolysis [177]. Samples of the organic phase were analyzed in a gas chromatograph (Agilent Technologies 5890 series II) equipped with a MS detector and a 7683B injector. The capillary column was a HP-5 from Agilent J&W Scientific, 30 m in length and 0.32 mm of internal diameter. The oven, injector, and detector temperatures were kept constant at 60 °C, 120 °C and 150 °C, respectively. Data acquisition and integration were performed by a Shimadzu CBM-102 communication Bus Module and Shimadzu GC Solution software (Version 2.3), respectively. The PHB signal was identified using a standard 3-methyl hydroxybutyrate (Sigma Aldrich). Calibration curves were obtained using samples of commercial PHB purchased from Sigma Aldrich, which were subjected to the same methylation process as the cells.

3.3.4.3 Sugars

The monosaccharides concentration was determined with a DX-600 Ion Chromatography system (Dionex, Sunnyvale, CA), equipped with an anion exchange column (Dionex CarboPac PA1) and an ED40 electrochemical detector, with 2-deoxy-D-glucose (0.25 mg mL^{-1}) as the internal standard. The column was eluted with deionized water at a flow rate of 1 mL min^{-1}. Aliquots (20 µl) were injected after being passed through a 0.45 µm nylon syringe filter (Chromatographic Specialties Inc., Brockville, ON, Canada). Baseline stability and detector sensitivity were optimized by post column addition of 0.2 M NaOH at a flow rate of 0.5 mL·min-^{1} using a Dionex AXP pump. The column was reconditioned using 1 M NaOH after each analysis. Monosaccharides (arabinose, galactose, glucose, xylose and mannose) in the substrates were quantified with reference to standards.

3.3.5 Statistical analysis

3.3.5.1 Comparison of individual inhibitors

Statistical analysis (test for normality and generalized mixed linear models) was carried out in SAS® Studio.

3.3.5.2 Mixture design

The mixture design of four components (sodium acetate, furfural, HMF, vanillin) was analyzed in Statsgraphic. The experimental runs were determined by a simplex centroid design and the response was fitted with a special cubic model. The maximum inhibitor concentration was set at 2 g/L and all combinations summed up to the maximum concentration. Furthermore, a blank sample was measured under the same conditions. Table 3-5 summarizes the experimental setup. A total of 15 combinations with a 16th run corresponding to a blank sample were run in triplicates. The resulting model has four single terms, six binary interaction terms and four tertiary interaction terms (Equation 3-2).

$$E(Y)=\beta_1x_1+\beta_2x_2+\beta_3x_3+\beta_4x_4+\beta_{12}x_1x_2+\beta_{13}x_1x_3+\beta_{14}x_1x_4+\beta_{23}x_2x_3+\beta_{24}x_2x_4+\beta_{34}x_3x_4$$
$$+\beta_{123}x_1x_2x_3+\beta_{234}x_2x_3x_4+\beta_{134}x_1x_3x_4+\beta_{124}x_1x_2x_4 \quad (3\text{-}2)$$

3.4 Results & discussion

3.4.1 Softwood hemicellulose composition

Softwood hemicelluloses have a high amount of galactoglucomannans (20-25% w/w) [178]. Their hydrolysis releases the hexose sugars mannose, galactose and glucose, making them potentially attractive carbon sources for biotechnological processes [162]. For example, 100 g of softwood chips contained 9.09-12.9 g of mannan and 1.8-2.6 g of galactan in previous studies [179-181]. Typically, the ratio of galactose:glucose:mannose is between 1:1:3 and 0.1:1:4 [178]. This allows an estimation of the hemicellulose-derived glucan content to be 4.3 g/100 g (one third of 12.9 g mannan). In this study, the composition of black spruce was determined and was similar to other softwoods. In brief, the composition of the black spruce was: (g/100 g) 44.25±0.38 glucan, 12.39±0.10 mannan, 5.80±0.04 xylan, 2.66±0.03 galactan, 0.81±0.02 arabinan, 3.09±0.68 extractives, 28.77±0.20 acid insoluble lignin, and 0.49±0.01 acid soluble lignin. The mass balance was of 99.02±0.37.

The aim of softwood hemicellulose separation processes is to maximize sugar recovery in the form of monosaccharides while minimizing the generation of degradation products [163]. Among the separation processes, dilute acid hydrolysis is simple, cheap and feasible within the current pulp and paper mill structure [89]. The reaction mechanism is comprised of a series of complex reactions breaking multiple types of bonds. The most important cleavages are proton-catalyzed hydrolysis of the glycosidic bonds, the bonds between hemicellulose-lignin, and the non-covalent interactions between hemicellulose and cellulose fibrils [163]. The cleavages release the monomeric hemicellulose sugars in the liquor. Additionally, acetate, HMF, furfural and phenols arising from the sugar and lignin degradation are present.

In this study, a low severity dilute acid hydrolysis was used. The conditions were 160 °C, 2% (w/w) H_2SO_4, 15 min reaction time. The composition of the softwood hemicellulose hydrolysate is displayed in Table 3-1. The mild conditions prevented cellulose hydrolysis, so that the sugars are primarily derived from the hemicelluloses. The most abundant sugar is mannose, followed by xylose, glucose, galactose and arabinose. Furthermore, hemicellulose oligomers are present (data not shown), that make up between 8% (for arabinose) to 25% (for glucose, mannose and xylose) of the total respective sugar content. The composition table only shows the monomeric sugars, since it is not clear if *P. sacchari* can utilize oligomers for growth. A

posthydrolysis was not performed due to the potential increase in inhibitory compounds, while the presence of oligomers is less likely to inhibit bacterial growth.

In the literature, the total sugar concentrations are often higher due to higher severity. For example, Boucher, et al. [165] used 160 °C, 1% (w/w) H_2SO_4, and 120 min reaction time. The total monomeric sugar concentration was around 40 g/L (the exact value was not shown). However, the degradation product concentration was also higher with 4.6 g/L acetic acid, 3.0 g/L HMF and 2.70 g/L furfural. In this study, the concentrations of degradation products were lower (2.12 g/L acetic acid, 0.67 g/L HMF, 0.66 g/L furfural and 0.93 g/L phenols). Among the degradation products, phenols are important components of the hydrolysates due to their high inhibitory effect. The phenols are complex aromatic mixtures (e.g. vanillin, dihydroconiferylalcohol, vanillic acid, hydroquinone, catechol, acetoguaiacone, homovanillic acid, 4-hydroxy-benzoic acid) [172]. In softwood hydrolysates of both cellulose and hemicellulose sugars, vanillin was the most abundant phenol in the mixtures [172].

Table 3-2: Softwood (black spruce) hemicellulose hydrolysate composition (g/L) from pretreatment with dilute acid (160 °C, 2% (w/w) H_2SO_4, 15 min).

Glucose	Mannose	Xylose	Galactose	Arabinose	Total sugars	HMF	Furfural	Acetic acid	Phenols
3.41±0.39	7.43±0.84	3.61±0.36	2.49±0.26	1.08±0.12	18.0±1.78	0.67±0.01	0.66±0.00	2.12±0.02	0.93±0.14

3.4.2 Fermentability of the softwood sugars mannose and galactose

The heterogeneous composition of hemicellulose hydrolysates consisting of both hexoses and pentoses typically makes hemicellulose hydrolysates less efficient feedstocks for PHB production than glucose solutions. One challenge is the uptake of C5 sugars, while another one is carbon catabolite repression, which leads to sequential rather than simultaneous sugar uptake. The model strain *P. sacchari* can both convert pentoses and hexoses simultaneously and overcome carbon catabolite repression, showing high performance in wheat straw hydrolysates [132]. Previous studies showed that *P. sacchari* could grow on glucose, xylose and arabinose [133, 173]. However, its growth on mannose and galactose has not been reported yet. To determine its ability to utilize these sugars, mannose and galactose were used as the sole carbon sources with an initial sugar concentration of 20 g/L. Additionally, the growth of *P. sacchari* on

mannose and galactose was compared to its growth on glucose. In the exponential phase, the strain exhibited its best growth on glucose and mannose and had similar maximum specific growth rates (μ_{max}) of 0.40 and 0.39 h^{-1} respectively (Table 3-4). For galactose, μ_{max} was significantly lower (0.24 h^{-1}). Cesário, et al. [132] reported a maximum specific growth rate for the same strain and medium ranging between 0.27-0.28 h^{-1} for 10-60 g/L glucose concentrations. For 10-30 g/L xylose, the reported maximum specific growth rate was 0.18-0.21 h^{-1}. Furthermore, Raposo, et al. [133] found a μ_{max} of 0.30 h^{-1} in glucose-xylose mixtures with 10 g/L glucose and up to 30 g/L xylose. The growth difference can result from different factors such as the physiological state of the inoculum, and the shake flasks design (with or without baffles), which can change the oxygen availability in the medium.

To compare the effect of the individual sugars within the same experimental conditions, the growth rates can be expressed as the percent of the glucose growth rate ($\%\mu_{max,glu}$). An initial mannose concentration of 20 g/L yielded 97% $\mu_{max,glu}$, while galactose yielded 60% $\mu_{max,glu}$. In comparison, Cesário, et al. [132] reported 67-78%$\mu_{max,glu}$ for 20 g/L xylose. This indicates that *P. sacchari* grows fastest on glucose, followed by mannose, xylose, and galactose.

Table 3-3: *P. sacchari* IPT101 growth parameters on glucose, galactose and mannose.

Sugar	S_{0h} [g/L]	S_{24h} [g/L]	ΔS [g/L]	μ_{max} [h^{-1}]	$\%\mu_{max,glu}$	CDM_{24h} [g/L]	PHB_{24h} [g/L]	$Y_{PHB/CDM}$ [%]	$Y_{PHB/S}$	$Y_{CDM/S}$
Glucose	19.8±0.4	2.52±0.29	17.3±0.5	0.40	100	6.42±0.04	4.56±0.05	71.0±0.9	0.26±0.01	0.37±0.01
Mannose	19.3±0.4	1.99±0.11	17.3±0.4	0.39	97	6.91±0.20	4.24±0.02	61.4±1.8	0.21±0.01	0.40±0.02
Galactose	19.4±0.2	6.22±0.23	13.2±0.3	0.24	60	4.93±0.07	2.20±0.03	44.6±0.9	0.11±0.00	0.37±0.01

S_{0h} = Initial sugar concentration (after 0h), S_{24h} = Final sugar concentration (after 24h), ΔS = sugar consumption (difference between initial sugar concentration and final sugar concentration), μ_{max} = maximum specific growth rate, $\%\mu_{max,glu}$ = maximum specific growth rate per maximum specific growth rate of glucose, CDM_{24h} = cell dry mass after 24h, PHB_{24h} = PHB concentration after 24h, $Y_{PHB/CDM}$ = PHB yield per cell dry mass, $Y_{PHB/S}$ = PHB yield per sugar consumption, $Y_{CDM/S}$ = cell dry mass yield per sugar consumption.

After 24 h, the flasks containing mannose reached the same OD (OD = 33) as glucose, while those containing galactose exhibited slower growth (OD = 21) (data shown as natural logarithm, Figure *3-2*). The strain produced PHB from both mannose and galactose. Similar to the growth patterns, the PHB concentration after 24 h was higher for mannose (4.24 g/L) than for galactose (2.20 g/L). The PHB yields ($Y_{PHB/CDM}$) were 71.0% for glucose, 61.4% for mannose and 44.6% for galactose. At the same initial concentrations, Cesário, et al. [132] found PHB

yields of 60.3%, 44.4% and 62.0% for glucose, xylose and arabinose respectively. Both mannose and galactose were therefore used for PHB production, but galactose conversion resulted in a lower PHB yield.

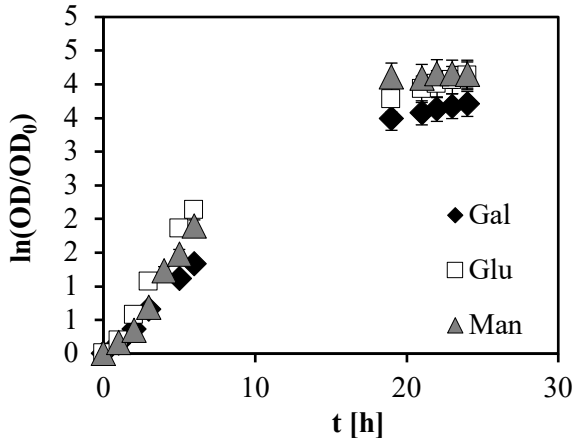


Figure 3-10: Growth profile of *P. sacchari* IPT 101 on glucose (Glu), mannose (Man) and galactose (Gal).

The difference in the fermentation efficiencies of the sugars can be explained by the difference in their catabolism. While mannose and galactose can enter the same pathways as glucose after initial conversion, the galactose conversion is more complex [162]. More specifically, after transport into the cells, mannose is phosphorylated to mannose 6-phosphate and directly isomerized to fructose 6-phosphate through mannose 6-P isomerase-PMI, where it follows catabolic breakdown to pyruvate [162]. In contrast, galactose needs two conversions to glucose 6-phosphate via the intermediate uridine diphosphate (UDP) galactose in the Leloir pathway [31]. Galactose conversion needs the expression of the enzymes uridylyltransferase and UDP-Glc:Gal1-P uridyltransferase, which might consume part of the energy provided by the sugar.

Since softwood hemicellulose hydrolysates contain a higher concentration in mannose and xylose than galactose, their composition can be favorable for PHB production. However, in sugar mixtures, the fermentability can differ depending on the initial concentrations of the individual sugars [133]. To the best of our knowledge, the effect of mannose and galactose on sugar mixtures with glucose, xylose and arabinose has not yet been described.

3.4.3 Fermentability of synthetic hemicellulose sugar mixtures

To determine whether softwood hemicellulose hydrolysates can be a suitable feedstock for PHB production, a typical hydrolysate was simulated with pure sugars at varying initial concentrations. The ratios of the individual sugars simulated those observed in a softwood hemicellulose hydrolysate produced under low severity conditions and contained a relatively high amount of arabinose (Table 3-1). Briefly, the simulated hydrolysate was composed of 31% mannose, 26% arabinose, 19% galactose, 16% xylose, and 8% glucose. For pure glucose, an initial concentration of 20 g/L had previously showed the best results [132]. For the effect of concentration, the most abundant sugar (mannose) was fixed at four concentrations (7.65, 21.8, 41.6 and 61.5 g/L), with the other sugars added in corresponding amounts. Fermentation was performed for 24 h. At initial mannose concentrations of 7.65 g/L and 21.8 g/L, cell growth and PHB production were similar and reached 6.34 g/L and 6.44 g/L, respectively. The PHB contents were 59% and 62%, respectively. These values are comparable to the PHB production on glucose under the same conditions. In contrast, at higher initial concentrations, the cell growth was inhibited and the PHB concentration decreased (Figure 3-2a).

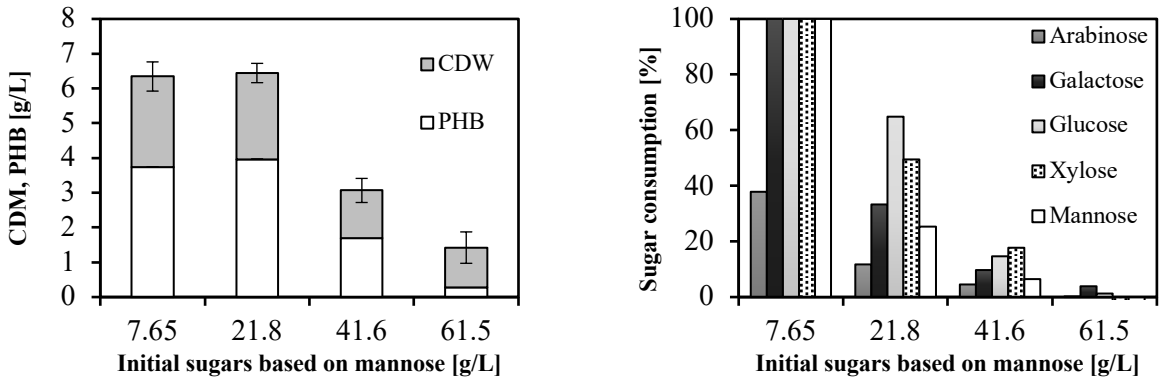


Figure 3-11: a) Effect of initial sugar concentration on PHB production using mixtures of pure sugars in similar ratios as softwood hemicellulose hydrolysates. The initial sugar concentration was based on mannose as the most abundant sugar. b) Sugar consumption of the five sugars expressed as percent of initial sugars.

The effect of the initial sugar concentration shows that sugar mixtures with 8-20 g/L initial mannose concentration give comparable growth to pure glucose. To determine the growth behavior at a fixed sugar concentration, a growth curve with a simulated sugar mixture was recorded over 51 h. The synthetic sugar mixture had an initial concentration of 26.5 g/L

mannose, 19.4 g/L arabinose, 12.6 g/L galactose, 12.1 g/L xylose and 5.02 g/L glucose. The biomass reached a maximum after 48 h of 7.22 g/L with 77.28% PHB, while the PHB concentration continued to increase up to 5.72 g/L and 80.46% PHB after 51 h. Bacterial growth started to decline after 24 h, where the biomass reached 5.58 g/L (Figure 3-3a). The sugars were all consumed simultaneously. The final sugar consumption was 18.16 g/L with 6.07 g/L xylose, 4.68 g/L mannose, 3.42 g/L galactose, 2.53 g/L glucose and 1.46 g/L arabinose (Figure 3-3b).

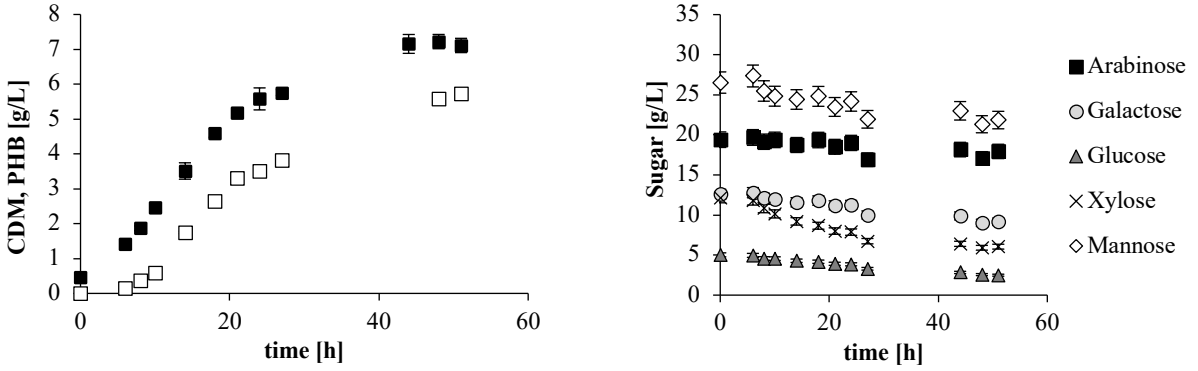


Figure 3-12: a) Growth curve of *P. sacchari* IPT101 on simulated hydrolysate with an initial mannose concentration of 26.5 g/L. PHB in white squares, CDM in black squares. In some cases, the standard deviations are smaller than the squares and therefore not visible. b) Sugar concentration in the fermentation medium.

The simultaneous uptake of all sugars might be possible due to the low initial glucose concentration (5 g/L). For example, a previous study by Raposo, et al. [133] found that xylose consumption was enhanced by reduced glucose concentrations. Specifically, initial concentrations of 5 g/L glucose and 25 g/L xylose yielded specific xylose uptake rates equal to those of glucose. At higher initial glucose concentrations, Cesário, et al. [132] observed preferential consumption of glucose in sugar mixtures of 10 g/L glucose and 10 g/L xylose, indicating carbon catabolite repression. However, xylose consumption started before complete exhaustion of glucose. Consequently, Raposo, et al. [133] hypothesized that a glucose threshold for the repression was present. In this study, the initial glucose concentration was also 5 g/L, which supports the hypothesis of a glucose threshold somewhere between 5-10 g/L. Carbon catabolite repression was due to a difference in the initial sugar uptake with the transport and phosphorylation steps regulated by the phosphotransferase system (PTS) in *E. coli* [133]. In *P. sacchari*, similar genes were found, which explains the preferential uptake of glucose over

pentoses. Nevertheless, the advantage of *P. sacchari* over *E. coli* is that even at higher initial glucose concentrations, the uptake of other sugars is only reduced, but not stopped entirely. Consequently, the combination of *P. sacchari* and low glucose, but high hexose sugar mixtures are promising for PHB production. The sugar composition of softwood hemicellulose hydrolysates makes them a suitable carbon source to produce PHB with a xylose-converting strain.

3.4.4 Effect of inhibitory compounds in softwood hemicellulose hydrolysates

Since the sugar consumption and PHB production with *P. sacchari* is high for simulated softwood hemicellulose hydrolysates, the presence of other compounds in the hydrolysates are likely to be responsible for their poor fermentability. To identify these inhibitory compounds, *P. sacchari* was grown in the simulated hydrolysates in the presence of different amounts of potentially inhibitory compounds in the range found in the softwood hemicellulose hydrolysates (see Table 3-1). The purpose of this initial screen was to select compounds for further investigation of their inhibitory effects since the inhibition thresholds for *P. sacchari* are not known. Four potential inhibitors (vanillin, furfural, HMF and acetate) were selected and individually added to the synthetic medium at three concentrations (The concentrations are given in Table S3-1 in the supporting information). Their growth after 24 h was compared to a blank run and a synthetic mixture of inhibitors. Overall, fourteen synthetic media were tested in 3-6 replicates and 3 replicate measurements of each OD_{600} (24 h), resulting in 159 observations (see supporting information). The runs were performed in random order on different days with different inocula to randomize the error from medium preparation.

Comparing the bacterial growth after 24 h showed that while individual inhibitors reduced the OD_{600} (24 h), only a mixture of all four components stopped their growth entirely (see supporting information). The difference in the growth between the blank (OD_{600} (24 h) 28.31-30.32) and the mixture (OD_{600} (24 h) 0.817-1.905) clearly showed substantial growth inhibition. However, the addition of inhibitors also increased the variability of the observations. For example, with the addition of vanillin at 1 g/L, the OD_{600} ranged from 1.884 to over 15.21 to 29.43. Therefore, some runs with higher inhibitor concentrations were repeated more than three times.

To assess the inhibitor effects as well as other sources of variation, the data were analyzed statistically using SAS. First, the correlation between the OD_{600} (24 h) and the increasing levels of individual inhibitors was measured by the Spearman correlation coefficient. Compared to a linear relationship measured by the Pearson's correlation coefficient, Spearman's rho is a measure of (nonlinear) monotonic (increasing or decreasing) relationships between variables. The calculation of Spearsman's rho is done based on the ranked values of the response variable OD_{600} (24 h). Based on Spearman's correlation coefficients, all inhibitors were significantly negatively correlated to the OD_{600} (24 h) (Table S3-2). To illustrate the correlation, the scatter plots are provided in the supporting information.

The first assumption was to use a general linear model for analysis of variance to verify if the data were normally distributed. The statistical analysis of variance showed that the residuals are not normally distributed based on graphical and numerical tests (the Shapiro Wilk test [182], the Kolmogorov-Smirnov test [183], Cramer-von Mises and the Anderson-Darling test [184] , quantile-quantile plot and residual scatter plot [185], data not shown). The residual scatter plot shows the residuals versus the predicted value from the model [185]. The residuals of predicted low OD's are very small as compared to the residuals of higher OD's. This can be explained by the different dilutions for the OD measurement. OD's above 10 were diluted 100-fold, while OD's of 1 were diluted by only 10-fold, resulting in an additional source of variation. Another possible source of variation is the physiological state of the bacteria in the inocula. The runs were repeated on different days with different inocula and the variability between days was higher than within days (data not shown). If the number of viable cells is lower between inocula, the ratio of inhibitor molecules per living cells is higher, which can lead to longer lag phases. This variation did not occur in the inhibitor-free runs.

To analyze the differences between each inhibitor concentration, the model needs to account as close as possible to the distribution of the error. Generalized linear models are models that allow for non-normal error distributions and fit an appropriate distribution [186]. In this experiment, the variance of the OD increased with the mean, and therefore the inverse Gaussian response distribution was the best model fit as shown by the lowest Bayesian information criterion (BIC). To account for the different sources of variation and isolate the inhibitor effect as effectively as possible, the date and flask number were included in the model as random effects.

The model accounted for the heteroscedasticity of the variance that may arise from the different inhibitor levels. The covariance parameters of different sources of variation on the OD_{600} (24 h) are in the supporting information (Table S3-3). The table shows that the fixed effects were at least one to two orders of magnitude higher than the random effects. The treatments had a significant effect on the bacterial growth $F(13,105) = 8.45$, $p<.0001$ (Type III Tests of Fixed Effects, Table S3-4). Figure *3-5* displays the least square means for each treatment combination on the (inverse linked) scale of observation. The whiskers indicate 95% confidence limits. The treatment effect was compared to the blank run by testing the difference of the treatment least square means after adjustment for multiple comparisons by Dunnett-Hsu. Among the treatments, 1 g/L HMF (treatment 7), 0.5 g/L vanillin (treatment 12), 1 g/L vanillin (treatment 13) and the mixture (treatment 14) were significantly different to the blank run (Table S3-5).

In summary, the statistical analysis showed a significant negative correlation between vanillin, HMF, furfural, acetate and bacterial growth. The OD_{600} (24h) was significantly reduced by high levels of HMF (1 g/L) and vanillin (0.5 and 1 g/L), but the highest reduction arose in presence of the inhibitor mixture.

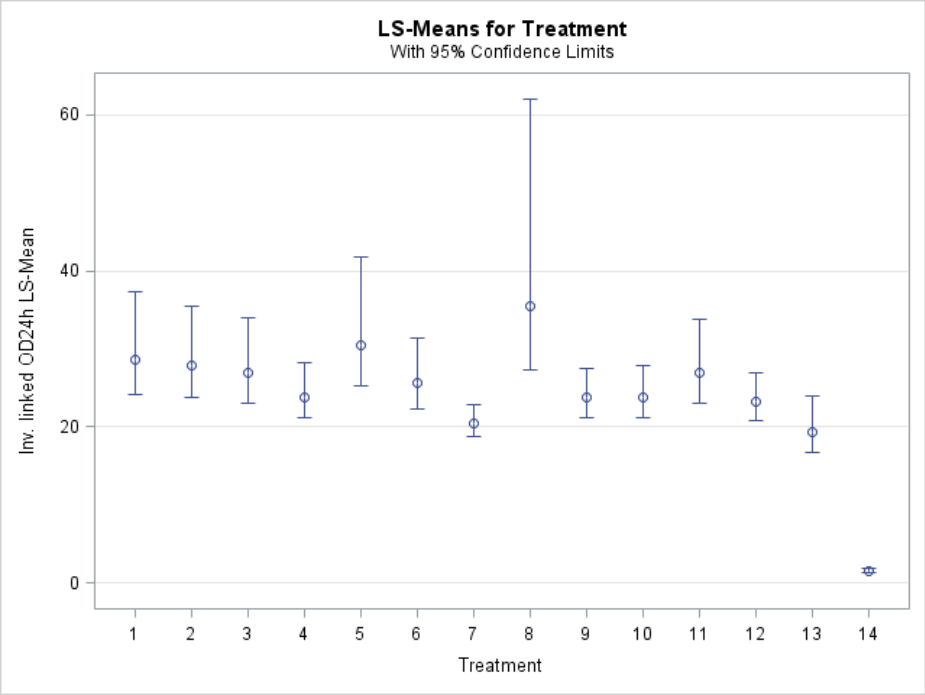


Figure 3-13: Difference of LS-means for inhibitor mixture in inverse-linked observation**.** Effect on *P. sacchari* IPT 101 growth of different hemicellulose hydrolysate components after 24 h in synthetic medium at three individual levels compared to blank and inhibitor mixture.

Organic acids, furans and phenols can act as growth inhibitors as well as substrates. They are metabolized by numerous bacteria, often after a longer lag phase [187]. These compounds can be substrates under the following conditions: if an existing metabolic pathway exists, if the gene expression is not hindered, and if the expressed enzymes are not inhibited. Furthermore, the inhibitory effect can come from a terminal metabolite of the substrate that accumulates in the cells, rather than the substrate itself.

Regarding the mechanism of inhibition, Kucera, et al. [173] discussed the mechanisms for the case of *P. sacchari*. The specific mechanism of phenols on *P. sacchari* is not known but is believed to interfere with the cells' membranes. The inhibitory effect can be dependent on the type of functional groups present in the phenol. In general, there are 12 main pathways for aromatic degradation in proteobacteria, such as the ß-ketoadipate pathway [188]. Pérez-Pantoja, et al. [188] provided a detailed description of the pathways and the corresponding genes. *Burkholderia cepacia* ATCC 17759 grows with low levels of vanillin, but displays early inhibition at 1 g/L vanillin and full inhibition at 2 g/L vanillin [124].

In contrast, the mechanism by which acetate (and other organic acids) inhibits microbial growth is well understood. Undissociated weak organic acids diffuse into the cells, where their

dissociation decreases the intracellular pH and causes cell death. Nevertheless, Yu and Si [189] showed in *Ralstonia eutropha* that low amounts of acetate as sole carbon source was converted by acyl-CoA synthetases to acetyl-CoA, which can lead to cell growth by channeling into the glyoxylate shunt for anabolic reactions and act as a PHB precursor [189]. Moreover, in *Burkholderia* sp. f24, acetic acid (below 2.5 g/L) and formic acid (below 1.25 g/L) stimulated growth as compared to glucose only [126]. Finally, for *P. sacchari*, Mendonça, et al. [116] showed that *P. sacchari* IFM 101 can grow on 1 g/L acetic acid in presence of 5 g/L glucose.

In the case of furans, the inhibitory effects differ among previous studies. In general, furfural is considered more toxic than HMF [190]. However, Wierckx, et al. [191] found that bacterial isolates capable of growing on furfural grew on HMF as well, indicating a shared metabolic pathway. Indeed, the authors identified the common furan converting pathways in *Cupriavidus basilensis* HMF14, located in the same gene cluster (hmfABCDE) [192]. Both aldehydes are first rapidly reduced to the respective alcohol, followed by reoxidation by non-specific dehydrogenases. The pathways then converge at the intermediate 2-furoic acid.

Regarding the inhibition thresholds, both HMF and furfural increased the doubling time of *Cupriavidus basilensis* HMF14. In *Burkholderia* sp. f24, concentrations of 0.5 g/L of either furfural or HMF inhibited growth, but also were both metabolized [126]. The effect was relieved by increasing buffering strength, which could indicate that furoic acid accumulation caused the toxicity. Lopes, et al. [126] found a higher inhibitory effect of furfural. Similarly, Pan, et al. [124] found strong inhibition of *Burkholderia cepacia* ATCC 17759 at 1 g/L furfural and early inhibition at 0.5 g/L furfural. They observed no inhibition by HMF, but the HMF concentration was only a maximum of 0.3 g/L, which was lower than in this study. In comparison, *Ralstonia eutropha* exhibited good growth on furfural [122]. Furthermore in yeast cells, furfural did not result in cell death but rather a longer lag phase that was due to substrate oxidation [193]. The differences between the inhibition thresholds could be due to a different catabolic activity for *P. sacchari.* As a general conclusion, no inhibitor alone causes complete growth inhibition, indicating that the individual toxicity can be overcome. However, the inhibitor mixture stop growth entirely. This agrees with the fermentation of the real softwood hemicellulose hydrolysate, which did not show any growth even after four days of fermentation (data not shown).

A main reason for growth inhibition can be the intracellular accumulation of the substrates since the required enzymes were not expressed fast enough [122]. The authors found that the initial phenols could be extracted from the harvested cells of *Ralstonia eutropha* when no growth occurred. The strain that had been exposed to inhibitors before inoculation grew on higher initial inhibitor thresholds. Inducing higher activity of inhibitor metabolizing enzymes could therefore be a strategy to alleviate the growth inhibition.

3.4.5 Mixture optimization of growth inhibitors

The inhibitory effects indicate the presence of synergistic effects in inhibitor mixtures. For efficient detoxification of the softwood hemicellulose hydrolysates, it is necessary to quantify the synergistic effects and determine the maximum inhibitor concentrations that still result in efficient growth. Among experimental designs, mixture designs predict a response surface depending on the proportions of mixture components. To estimate the interaction of two, three and four inhibitors, the four inhibitors were tested in a simplex centroid mixture design (Table *3-5*-3). The response was fitted with a special cubic model, which is one type of the standard mixture models.

Table 3-4: Mixture Design (Simplex Centroid) with 4 components run in triplicates and with a blank. Vanillin (V), Furfural (F), HMF (H), Acetate (A).

	Code				Level (g/L)				Results (OD_{600} (24h))		
Run	V	F	H	A	V	F	H	A	Rep 1	Rep 2	Rep 3
1	1	0	0	0	2.00	0.00	0.00	0.00	0.5797	0.4680	0.4420
2	0	1	0	0	0.00	2.00	0.00	0.00	8.126	11.17	11.45
3	0	0	1	0	0.00	0.00	2.00	0.00	7.833	8.675	3.205
4	0	0	0	1	0.00	0.00	0.00	2.00	18.24	18.26	18.64
5	0.5	0.5	0	0	1.00	1.00	0.00	0.00	0.4930	0.3760	0.4075
6	0	0.5	0.5	0	0.00	1.00	1.00	0.00	3.896	3.676	5.021
7	0	0	0.5	0.5	0.00	0.00	1.00	1.00	14.65	14.10	16.15
8	0.5	0	0	0.5	1.00	0.00	0.00	1.00	5.550	8.775	8.825
9	0	0.5	0	0.5	0.00	1.00	0.00	1.00	17.58	19.05	20.24
10	0.5	0	0.5	0	1.00	0.00	1.00	0.00	0.9163	0.7430	0.6155
11	0.33	0.33	0.33	0.00	0.67	0.67	0.67	0.00	1.044	0.5110	0.6620
12	0.00	0.33	0.33	0.33	0.00	0.67	0.67	0.67	11.50	11.99	10.72
13	0.33	0.33	0.00	0.33	0.67	0.67	0.00	0.67	0.8263	0.6005	0.6980
14	0.33	0.00	0.33	0.33	0.67	0.00	0.67	0.67	3.788	4.330	3.805
15	0.25	0.25	0.25	0.25	0.50	0.50	0.50	0.50	0.9323	0.6615	0.8180
Blank											
16	0	0	0	0	0.00	0.00	0.00	0.00	20.67	27.07	22.52

To predict the OD_{600} after 24 h, the best model fit was the special cubic model. Comparing the special cubic model with simpler models such as the quadratic or linear models, the additional terms in the special cubic model were significant (supplementary information). Likewise, the special cubic model had a lower standard error and a higher adjusted R-square, which makes it the most suitable among the three models.

The analysis of variance assesses the quality of the model with respect to the experimental error (Table 3-4). Since each mixture point was carried out in triplicates, the degree of freedom for the pure error is high and allows for a strong estimation of the error. The F-ratio is the ratio of the mean square of the model by the mean squared error. Since the probability is below 5%, there is a statistically significant relationship between the OD_{600} (24 h) and the inhibitory components. The adjusted R-square measures how well the model fits the observed OD_{600} (24 h)

adjusted for the degrees of freedom. It indicates that the fitted model explains 96.96 of the OD_{600} (24 h) variability.

With an adequate model, the next step is to use diagnostic plots to check the residuals. Based on the set of diagnostic plots (observed versus predicted, residual versus predicted, residuals versus factor and normality plot), the residuals appear to be random and normally distributed.

The resulting estimates for each parameter of the special cubic model are shown in the supporting information (Table S3-6). The smaller the term, the higher the inhibitory effect. Among the individual terms, vanillin has the highest impact on growth inhibition (0.4881), followed by HMF (6.563), furfural (10.24) and finally acetate (18.37). It should be noted, however, that the acetate concentration was below its inhibitory concentration. Among the binary terms, all combinations with vanillin are negative and therefore inhibitory to growth. The combinations with acetate and furfural or HMF are positive, thus showing the least inhibitory effect. Regarding the tertiary components, the combination of vanillin, furfural and HMF was not significant. All other tertiary combinations of three components were inhibitory, even without vanillin. The highest reduction came from the combinations with vanillin, furfural and acetate. The resulting model to predict the OD_{600} (24 h) in each combination of four inhibitors is in Equation 3-3 (for coded component values between 0-1).

$$OD_{600}\,(24h) = 0.4881*V + 10.24*F + 6.563*H + 18.37*A - 19.48*V*F - 10.80*V*H - 6.580*V*A - 16.54*F*H + 18.86*F*A + 10.26*H*A - 1.320*V*F*H - 227.3*V*F*A - 106.3*V*H*A - 52.63*F*H*A \quad (3\text{-}3)$$

Table 3-5: ANOVA of special cubic model for OD_{600} (24 h).

ANOVA for OD_{600} (24h)

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-value*
Special cubic model	1869.16	13	143.78	109.49	>0.00
Lack-of-fit	1.57	1	1.57	1.20	0.28
Pure error	39.40	30	1.31		
Total (corr.)	1910.13	44			

R-Square = 97.86 percent
R-Square (adjusted for df) = 96.96 percent
Standard error of est. = 1.15
Mean absolute error = 0.64

Figure 3-14 shows the resulting contour plots for the predicted OD_{600} (24 h) over the range of the mixture. The OD is overall highest when vanillin is absent from the mixture. Even raising vanillin concentrations to 0.5 g/L decreases the overall response to an OD below 2 arbitrary units (a.u.). for most of the contour plot. Figure *3-7* illustrates the influence of each parameter on the final OD. The trace plot displays the change of the OD when following one fixed mixture setting, like a path on a topographic map. Starting from a reference mixture, the change in response when one component changes is displayed, with the other components kept at the same ratio, but the total value adjusts according to the change. Each of the trace plot of the reference mixture of 0.5 g/L illustrates how an increase in vanillin in the quaternary mixture sharply decreases the OD to no growth at a share of 0.4 (corresponding to 0.8 g/L). In contrast, increasing all other components increased growth, the steepest growth in the case of acetate. This decline is even more visible when starting from the optimum mixture settings (calculated below), where the OD dropped from ca. 20 to ca. 8 upon addition of vanillin. In conclusion, the response suggests strong mixture effects among the inhibitors. The strong inhibition effect of softwood hemicellulose hydrolysates is therefore likely to come from the inhibitor combination.

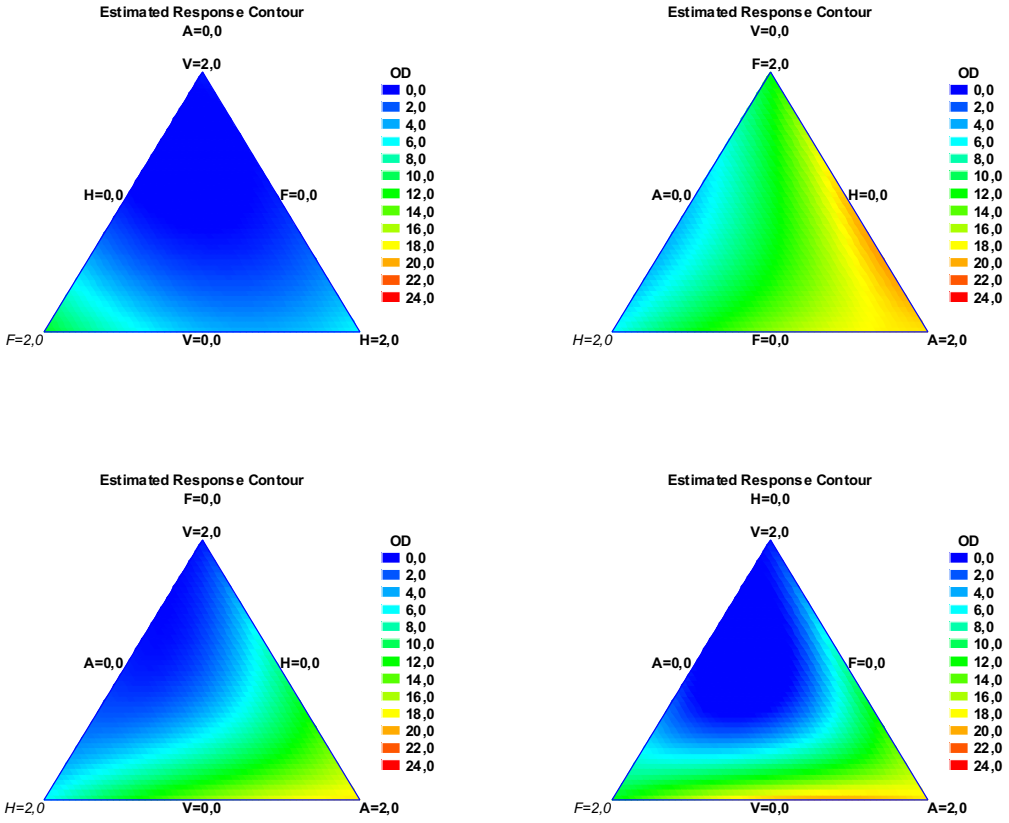


Figure 3-14: Contour plots of OD_{600} (24 h) as a function of mixture components. Vanillin (V), Furfural (F), HMF (H), Acetate (A).

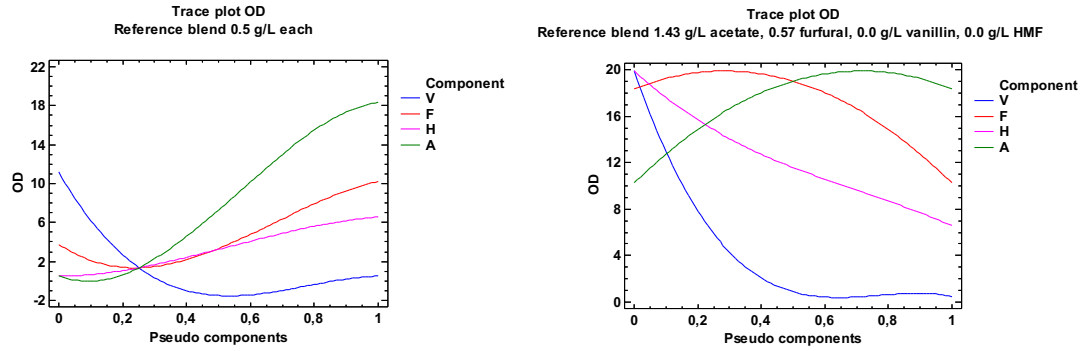


Figure 3-15: Trace plots to visualize inhibitor effects from center point blend (0.5 g/L each) and optimal blend (1.43 g/L acetate, 0.57 g/L furfural, 0.0 g/L HMF and Vanillin).

Within the range of 2 g/L of total inhibitor concentration, the optimal combination of inhibitors results from the optimization of the response surface (Table 3-5). A mixture with

1.43 g/L acetate, 0.57 g/L furfural, no vanillin, and no HMF yields the highest OD_{600} value (24 h) of 19.90. Consequently, the detoxification of softwood hydrolysate should target phenols and HMF removal.

Table 3-6: Response optimization.

Goal: maximize OD_{600} (24 h)

Optimal value = 19.90

Factor	*Low*	*High*	*Optimum(g/L)*
V	0.0	2.0	0.00
F	0.0	2.0	0.57
H	0.0	2.0	0.00
A	0.0	2.0	1.43

Pienkos and Zhang [194] reviewed the inhibitory effects of lignocellulose hydrolysate components on ethanol production and found that the toxicity assessment moves towards inhibitor mixtures due to many examples of synergistic effects. However, for softwood hemicellulose hydrolysates for PHB producing bacteria, these effects have not been quantified. The inhibitor thresholds depend on the microorganism and inhibitor concentrations. Furthermore, albeit the authors have discussed systems biology as a tool to understand the effects of inhibitors on bacterial metabolism, the reasons for the specific synergistic interactions between the inhibitors remain unknown.

3.4.5.1 Inhibition of *P. sacchari*

Kucera, et al. [173] reported *P. sacchari* cultivation in softwood hydrolysate containing 1.205 g/L polyphenols, 0.052 g/L furfural, 0.53 g/L acetic acid and 0.0099 g/L levulinic acid, which is in the concentration range of the inhibitor mixture design. Similar to the results of this study, the strain grew only to 0.87 g/L. Removal of 90% polyphenols to 0.12 g/L did not significantly increase biomass growth (1.01-1.57 g/L), which means that the remaining inhibitor thresholds were still too high. In this study, vanillin levels as low as 0.2 g/L highly reduced the predicted biomass growth. Furthermore, Cesário, et al. [132] used wheat straw hydrolysates containing only furfural (0.01-0.27 g/L) with *P. sacchari*. They observed no growth inhibition,

which agrees with the results of this study. Silva, et al. [195] observed that activated charcoal treatment was obligatory for efficient *P. sacchari* growth from sugarcane bagasse hydrolysate. The detoxification reduces the inhibitor concentration to 12.6 mg/L furfural, 59.0 g/L HMF and 205 mg/L acetic acid. A hydrolysate with 867 mg/L furfural, 116 mg/L HMF and 286 mg/L acetic acid gave poor CDM for the strain. The toxicity thresholds are therefore in the range of the previously described hydrolysates for *P. sacchari* growth.

3.4.5.2 Inhibition of PHB producers from lignocellulose

Although several studies reported PHB production from other lignocellulose hydrolysates and other bacteria in the presence of multiple inhibitors [122, 126, 196], the maximum mixture thresholds have not been assessed. One assessment of the mixture thresholds exists for hardwood hemicellulose hydrolysate [123]. That study showed that the inhibitor mixture of mixed phenolic compounds (0.2-0.8 g/L), acetate (2.88-8.65 g/L), levulinic acid (1-3 g/L) and furfural (0.1-0.3 g/L) showed strong synergistic effects between acetate and phenolic compounds as well as levulinic acid and furfural on *Burkholderia cepacia* ATCC 17759 [123]. However, the design used in that study did not allow for an estimation of tertiary interactions. In comparison, softwood hemicellulose hydrolysate has a lower acetate and levulinic acid content, and higher amounts of furfural and HMF. Accordingly, in hardwood hemicellulose hydrolysates, acetate concentrations ranging from 2.88 to 8.65 g/L showed the greatest inhibition individually. In comparison to this study, vanillin was the most inhibitory in the softwood hemicellulose inhibitor mixture since the maximum acetate concentration was 2 g/L.

3.4.5.3 Inhibitor mixture effects

Some studies investigated the mechanisms behind the synergistic effect of inhibitor mixtures. For example, in acetate-phenol mixtures, the presence of acetate blocks the synthesis of phenol-converting enzymes in *R. eutropha* [187]. In bioethanol production, mixtures of furfural with acetic acid, HMF, 4-hydroxybenzaldehyde, syringaldehyde, vanillin, furfuryl alcohol, and guaiacol were especially toxic. Palmqvist and Hahn-Hägerdal [197] reported an antagonistic effect in mixtures of 10 g/L acetic acid and 3 g/L furfural on the bacterial growth rate, while ethanol yield was not affected to a significant extent. Ezeji, et al. [198] found that a mixture of 1 g/L of each furfural, HMF and glucuronic acid reduced *C. beijerinckii* BA101 growth by 10% for acetone–butanol–ethanol (ABE) production. However, in that case the inhibition was an additive effect rather than synergistic interaction, which differed from previous

studies with *E. coli*. More recent studies with different strains and a range of fermentation products have also found interaction effects. For example Cao, et al. [199] studied the effect of lignocellulose-derived inhibitors on growth and hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* W16. Synthetic mixtures of furfural, HMF, vanillin, syringaldehyde and sodium acetate reduced the cell concentrations as compared to individual inhibitors. In corn stover hydrolysates, the inhibitor threshold for complete growth inhibition was found with 7.31 g/L acetate, 2.32 g/L furfural, 0.92 g/L HMF, 0.13 g/L vanillin, 0.35 g/L syringaldehyde. More dilute hydrolysates merely reduced growth and created longer lag phases. The mixture effect was attributed to furfural-phenol interactions due to the high concentrations of furfural. Bellido, et al. [200] investigated the inhibitory effect of the main inhibitors (acetic acid, furfural and HMF) for ethanol production with *Pichia stipitis* in synthetic mixtures and wheat straw hydrolysates from steam explosion. Complete cell growth inhibition occurred in presence of 1.5 g/L acetic acid, 0.15 g/L furfural and 0.05 g/L HMF together in hydrolysates, while hydrolysates with only acetic acid at 0.5 g/L were fermentable. Synthetic tertiary mixtures had an effect on sugar consumption and inhibited xylose uptake entirely while reducing glucose consumption. Chen and Wan [201] assessed the mixture effect of different phenols and sodium acetate with glucose on *Rhodococcus jostii* RHA1 for lipid synthesis. A mixture of four phenols each at 0.5 g/L (total 2 g/L) with acetate at 8 g/L and 40 g/L glucose inhibited growth, while up to 0.32 g/L phenols, 5 g/L acetate, and 20 g/L glucose had no effect. The reduced growth was due to longer lag phases of up to 72 h, however, eventually growth started and reached the same final OD. The tolerated acetate concentration in inhibitor mixtures thus varies and has been investigated for up to 10 g/L. The inhibitory mixture thresholds therefore depend on the strain, while the overall range of inhibitory concentrations is similar between 0.1-2 g/L for furans and phenols and 1-10 g/L for acetic acid.

Like individual inhibitors, low amounts of substrate mixtures can also stimulate growth. Sodium acetate stimulated growth in initial concentrations up to 5 g/L [199] and 8.9 g/L [198], and furfural and HMF at up to 2 g/L [198]. Mixtures below 1.2 g/L phenols, 5 g/L acetate and low glucose concentrations of 0.1 g/L allowed metabolic conversion of the compounds as substrates [201]. A potential improvement for PHA production from lignocellulose hydrolysates can come from the identification of strains that both metabolize lignocellulose sugar mixtures and other components.

3.4.6 Effect of initial OD

In previous studies, a high cell density inoculum was an effective method to overcome hydrolysate toxicity [138]. To assess the potential of growing *P. sacchari* in the presence of inhibitors, the inoculation was tried with five initial OD from 0.6 to 5.6 a.u.. An inoculum of 5.6 a.u. led to significant bacterial growth, effectively overcoming the inhibitory effect of the mixture (Figure *3-8*). While the growth was still lower than without inhibitors, it was the simplest method to use hydrolysates with inhibitors as a carbon source for *P. sacchari* growth.

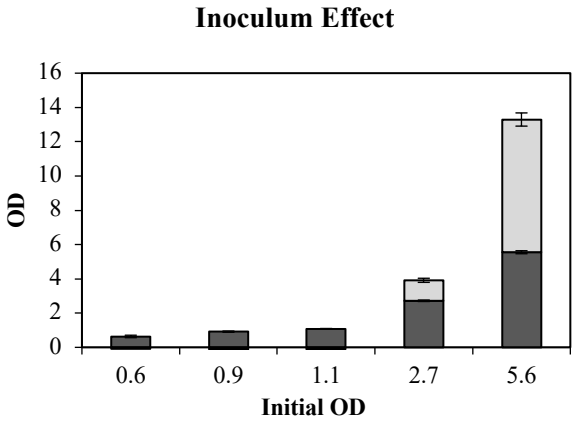


Figure 3-16: Bacterial growth in presence of inhibitor mixture at increasing inoculum sizes (light grey: OD_{600} (24 h), dark grey: OD_{600} (0h))**.** Inhibitor concentrations are 0.93 g/L vanillin, 0.66 g/L furfural, 0.67 g/L HMF and 2.11 g/L acetate.

The inoculum effect is in agreement with observations for other PHB producing bacteria. For example, with *Ralstonia eutropha*, an initial cell density of 3-6 g/L relieved the inhibition effects of the lignocellulose hydrolysate and allowed inhibitor consumption to levels below 0.1 g/L [122]. In *Cupriavidus basilensis* HMF14, higher inhibitor concentrations required a 4-fold increase of the inoculum and a longer fermentation time to reach the same growth as with lower inhibitor concentrations [191]. In *Bacillus firmus* NII 0830, both inoculum concentration and inoculum age increased bacterial growth as compared to the control [125]. Finally, in *Burkholderia* sp. f24, for initial cell densities between 0.5-6.5 g/L, the strain grew only on the hydrolysate with an inoculum above 1.5 g/L to 10.5 g/L with 35.7% PHA [126].

The reason for the higher inhibitor tolerance observed when the inoculum is increased has been investigated in the context of bioethanol production. Using yeast, Cassells, et al. [202] found that a minimum inoculum density of 4 g/L was necessary to overcome the inhibitor

toxicity of wheat straw hydrolysates with acetic acid (2.5 g/L), HMF (0.3 g/L) and furfural (3.5 g/L) as the quantified inhibitors. They studied the effect of inoculum densities between 1-8 g/L. Low inocula of 1 g/L lead to irreversible cell damage, while increasing inoculum size resulted in increased growth. They observed that furfural first needed to be metabolized before the cells started glucose conversion and a higher inoculum converted furfural faster (furfural consumption rate was 0.25 $g/g \cdot h^{-1}$), thus reducing exposure to the inhibitor. Besides the inoculum density, the cell conditions can influence the ability to grow in inhibitor mixtures. To understand the effect of different physiological states of the inoculum, Narayanan, et al. [203] investigated the inoculum in different growth phases as well as different cell types and their intracellular pH as well as reactive oxygen species. They found that inhibitor tolerance increased with cells in early stationary phase (18-24 h) as compared to cells in logarithmic growth phase (8-12 h) and late stationary phase (48 h). Furthermore, the inoculum of pre-adapted cells exposed to low inhibitor concentrations were more inhibitor tolerant. The growth correlated with lower intracellular pH and reactive oxygen species, while the viability of the cells from both inocula was similar. Furthermore, only a subpopulation of the cells in early stationary phase tolerated the inhibitor mixture, while the preadapted cells reacted uniformly. Consequently, optimizing the use of softwood hemicellulose hydrolysates as substrates for PHA production can target inhibitor reduction by detoxification, process adaptation or the control of fermentation inoculum.

3.5 Conclusion

This study investigated softwood hemicellulose hydrolysates as potential carbon sources for PHB production by assessing the effect of individual components and their mixtures on the growth of a model strain *P. sacchari*. The softwood hemicellulose sugars mannose and galactose are effective carbon sources for *P. sacchari*, with mannose being more efficient than galactose. *P. sacchari* grows equally well in sugar mixtures, consuming all sugars simultaneously without carbon catabolite repression.

The presence of furans (HMF and furfural), organic acids (acetic acid) and phenols (e.g. vanillin) can inhibit *P. sacchari* growth. The individual components reduced growth rates, with vanillin being to most inhibitory. The inhibition was the strongest in inhibitor mixtures.

For optimal predicted performance of *P. sacchari* with softwood hemicellulose hydrolysates, the hydrolysates should be free of vanillin and HMF, and have maximum

concentrations of 1.4 g/L acetate and 0.6 g/L furfural. In a mixture of 0.93 g/L vanillin, 0.66 g/L furfural, 0.67 g/L HMF and 2.11 g/L acetate, a high initial OD of 5.6 a.u. also overcame the toxicity and is a simple method to achieve bacterial growth from hemicellulose hydrolysates.

3.6 Supporting information

The supporting information contains summary tables with the setup and results of the statistical analysis (Table S3-1-S3-6), and the original SAS output of the Spearman correlation coefficient and the generalized linear mixed model used to assess the effect of inhibitory compounds on bacterial growth after 24 h (section 3.4)

Table S3-7: Inhibitor concentration in synthetic medium.

No.	Treatment
1	Blank
2	Sodium acetate 0.5 g/L
3	Sodium acetate 1 g/L
4	Sodium acetate 2 g/L
5	HMF 0.1 g/L
6	HMF 0.5 g/L
7	HMF 1 g/L
8	Furfural 0.1 g/L
9	Furfural 0.5 g/L
10	Furfural 1 g/L
11	Vanillin 0.1 g/L
12	Vanillin 0.5 g/L
13	Vanillin 1 g/L
14	Sodium acetate 2.11 g/L & HMF 0.67 g/L & Furfural 0.66 g/L & Vanillin 0.93 g/L

Table S3-8: Measure of correlation between bacterial growth (OD_{600} (24 h)) and increasing levels of individual inhibitors.

Spearman Correlation Coefficients, Prob > |r| under H0: Rho=0	
	OD_{600} (24 h)
Vanillin, N = 54	-0.6381
	<.0001
Furfural, N = 48	-0.5754
	<.0001
HMF, N = 39	-0.8424
	<.0001
Acetate, N=35	-0.8330
	<.0001

Table S3-9: Covariance parameters of different sources of variation (date, flask as random factors, treatments as fixed factors) with OD_{600} (24 h). A dot indicates that the error was too small to compute.

Covariance Parameter Estimates			
Cov Parm	Group	Estimate	Standard Error
Date		2.32E-07	.
Flask		5.95E-08	.
Residual (VC)	Treatment 1	5.72E-06	3.32E-06
Residual (VC)	Treatment 2	0.00001	5.89E-06
Residual (VC)	Treatment 3	0.000018	0.000012
Residual (VC)	Treatment 4	0.000016	9.10E-06
Residual (VC)	Treatment 5	5.02E-06	.
Residual (VC)	Treatment 6	0.000015	8.42E-06
Residual (VC)	Treatment 7	0.000025	0.000013
Residual (VC)	Treatment 8	0.000014	7.90E-06
Residual (VC)	Treatment 9	0.000069	0.000028
Residual (VC)	Treatment 10	0.000012	5.81E-06
Residual (VC)	Treatment 11	0.00013	0.000073
Residual (VC)	Treatment 12	0.000031	0.000014
Residual (VC)	Treatment 13	0.008143	0.00271
Residual (VC)	Treatment 14	0.0646	0.0323

Table S3-10: Fixed effects from generalized linear mixed model on OD_{600} (24 h).

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr >F
Treatment	13	105	8.45	<.0001

Table S3-11: Comparison of Least Squares Means for different inhibitor concentrations and 95% confidence intervals.

Treatment Least Squares Means					Differences of Treatment Least Squares Means Adjustment for Multiple Comparisons: Dunnett-Hsu			
Treatment	Mean	Standard error mean	Lower mean	Upper mean	DF	t Value	Pr > |t|	Adj P
1	28.672	2.9647	24.1457	37.3295	/	/	/	/
2	27.8715	2.6712	23.7253	35.3987	105	0.31	0.755	1
3	26.9526	2.507	23.0367	33.9266	105	0.71	0.4812	0.9979
4	23.8755	1.6908	21.0963	28.1538	105	2.43	0.0167	0.1424
5	30.5225	3.5957	25.1988	41.8143	105	-0.63	0.5274	0.9993
6	25.6906	2.1409	22.2726	31.3972	105	1.29	0.2011	0.8432
7	20.4289	1.0058	18.6859	22.7727	105	5.76	<.0001	<.0001
8	35.4516	6.0211	27.4044	62.045	105	-1.83	0.0703	0.4529
9	23.7052	1.5168	21.1708	27.4411	105	2.83	0.0055	0.0529
10	23.828	1.5872	21.1927	27.7776	105	2.68	0.0085	0.0785
11	26.889	2.4847	23.0026	33.7819	105	0.79	0.4341	0.9946
12	23.2832	1.4609	20.835	26.8641	105	3.08	0.0026	0.0267
13	19.3583	1.6812	16.6957	23.9082	105	3.21	0.0017	0.0181
14	1.5031	0.1562	1.2649	1.9603	105	4.8	<.0001	<.0001

Table S3-12: Estimated model terms to predict OD_{600} (24 h). Vanillin (A:V), Furfural (B:F), HMF (C:H), Acetate (D:A).

Special Cubic Model Results for OD_{600} (24 h)

Parameter	*Estimate*	*Standard Error*	*T Statistic*	*P-Value*
A:V	0.488137	0.663689		
B:F	10.2385	0.663689		
C:H	6.56269	0.663689		
D:A	18.3699	0.663689		
AB	-19.482	3.23973	-6.01345	0.0000
AC	-10.7991	3.23973	-3.33335	0.0022
AD	-6.58003	3.23973	-2.03104	0.0509
BC	-16.5426	3.23973	-5.10617	0.0000
BD	18.8614	3.23973	5.82189	0.0000
CD	10.2642	3.23973	3.16822	0.0034
ABC	-1.32037	21.4638	-0.0615163	0.9513
ABD	-227.281	21.4638	-10.589	0.0000
ACD	-106.272	21.4638	-4.95122	0.0000
BCD	-52.6266	21.4638	-2.45187	0.0200

Connecting Statement to Chapter 4

The results found in the third chapter showed that the sugars present in softwood hemicellulose hydrolysates (glucose, mannose, galactose, xylose and arabinose) were suitable carbon sources for PHB production. Mannose and galactose exhibited maximum specific growth rates of 97% and 60% relative to pure glucose, respectively. Sugar mixtures could be converted to PHB and reached a maximum PHB concentration of 5.72 g/L and 80.5% PHB after 51 h. However, the presence of other components such as sodium acetate, HMF, furfural, and vanillin completely inhibited bacterial growth. The inhibitory effects of these components were assessed with a mixture design. Finally, in simulated hydrolysates, the inhibitory effects were overcome by increasing the initial cell density of the inoculum from OD 0.5-1.0 to $\geq$ 5.6.

In the fourth chapter, a real softwood hemicellulose hydrolysate was tested as feedstock for PHB production. To study the effects of increasing sugar diversity and inhibitor concentration, the softwood hydrolysate was mixed in varying proportions with a hardwood holocellulose hydrolysate obtained from the TMP-Bio process (a thermomechanical pulping-based process producing glucose and xylose). This allowed tracking of the consumption of all softwood hemicellulose hydrolysate components. The chapter is based on the following article: Dietrich, K.; Dumont, M.-J.; Orsat, V.; Del Rio, L. F., Consumption of sugars and inhibitors of softwood hemicellulose hydrolysates as carbon sources for polyhydroxybutyrate (PHB) production with Paraburkholderia sacchari IPT 101. *Cellulose* **2019**.

Chapter 4: Consumption of sugars and inhibitors of softwood hemicellulose hydrolysates as carbon sources for polyhydroxybutyrate (PHB) production with *Paraburkholderia sacchari* IPT 101

4.1 Abstract

The future industrial success of the compostable bio-polyesters known as polyhydroxyalkanoates (PHAs), which includes polyhydroxybutyrate (PHB), depends mainly on their production using cheaper carbon sources than food-derived glucose. The existing pulp and paper infrastructure enables an alternative sugar supply in the form of wood hydrolysates. Softwood hemicellulose hydrolysates have a favourable sugar profile for fermentations and can be produced in such emerging integrated forest biorefineries. The processes can lead to varying amounts of inhibitors which can lead to the reduction or prevention of bacterial growth.

A dilute acid pretreatment was used to produce a softwood hemicellulose hydrolysate containing the sugars mannose, xylose, glucose, galactose, and arabinose. To study the effects of increasing sugar diversity and inhibitor concentration, the softwood hydrolysate was mixed in varying proportions with a hardwood holocellulose hydrolysate obtained from the TMP-Bio process (a thermomechanical pulping-based process producing glucose and xylose). In fermentations with *P. sacchari* IPT 101, the sugars were depleted at different rates in the following order: glucose, mannose, xylose, galactose, and arabinose. All potential inhibitors except phenols were metabolized. The maximum cell dry mass reached 6.7±0.1 g/L with 71±5% PHB with hardwood holocellulose hydrolysate after 48h.

The analyses of the sugar and inhibitor consumption provided valuable information to validate approaches for the detoxification of softwood hemicellulose hydrolysates. Overall, the detoxification would allow PHB production in an integrated softwood biorefinery scheme.

4.2 Introduction

As of 2015, the increasing production of synthetic polymers resulted in approximately 6300 million metric tons of plastic wastes, of which 79% were disposed in landfills or in the environment [204]. Polyhydroxyalkanoates (PHAs) are a family of microbiologically-produced polyesters that can be sustainable alternatives to polyethylene and polypropylene [81].

Industrially produced PHAs are poly[(*R*)-3-hydroxybutyrate] (PHB), poly[(*R*)-3-hydroxybutyrate-*co*-(R)-3-hydroxyvalerate] (PHBV), poly[(*R*)-3-hydroxybuty-rate-*co*-4-hydroxybutyrate] (PHB4HB), and poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] (PHBHHx) [59]. However, despite their favorable environmental profile, the current global PHA production capacity is merely 1% of all bioplastics. This low production has been partly attributed to the high cost of the typical carbon sources (glucose from corn starch and vegetable oils [205-206]). To reduce the cost, softwood and hardwood [21] are being evaluated as cheaper carbon sources. Softwoods are abundantly available through the infrastructure of the pulp and paper industry [18]. The established softwood supply is a promising carbon source for industrial-scale PHA production.

The chemical composition of softwood biomass can be both beneficial and challenging for its use as a carbon source as compared to hardwood. Both are composed of cellulose (37-43%), hemicelluloses (20-35%), and lignin (20-33%), and minor amounts of extractives and ash [18, 207]. Specifically, softwood hemicelluloses are composed of hexose sugars (D-glucose, D-mannose, D-galactose) and pentose sugars (D-xylose, L-arabinose). In contrast, hardwood hemicelluloses consist mainly of xylose and arabinose, with only minor amounts of glucose and glucuronic acid. Consequently, the sugar composition of softwood hemicellulose has higher hexose sugar content (mannose and galactose) than hardwood hemicelluloses [18, 207]. Furthermore, softwoods have a higher lignin content of 25-33% as compared to 20-25% in hardwoods [18], which can result in higher amounts of inhibitory phenols in softwood hydrolysates, such as vanillin and guaiacol [138].

The effect of the sugar and inhibitor diversity of softwood hydrolysates on PHA production is still little understood. Particularly as this diversity depends on the wood species, process and conditions used to generate the hydrolysate. There are two approaches to process wood into fermentable carbon sources. One approach is to produce holocellulose hydrolysates (hydrolysis of cellulose and hemicelluloses to monosaccharide mixtures). The second approach is to generate hemicellulose hydrolysates (extraction and hydrolysis of hemicelluloses to monosaccharide mixtures). In softwood holocellulose hydrolysate, glucose is the most abundant sugar due to the depolymerization of cellulose. The holocellulose hydrolysate composition is therefore less diverse in sugars and inhibitors. The fermentation of softwood holocellulose hydrolysates to PHA has been successful. For example, the use of spruce sawdust as carbon source in a

bioreactor yielded 61.8 g/L PHBV [135]. However, the wood hydrolysate was added only after 15h growth phase in pure glucose. The resulting high cell density overcame the inhibitory effect of the hydrolysate. In contrast, direct use of the hydrolysate stopped bacterial growth completely [135]. Moreover, hydrolysate characterization was not included in the study. The authors concluded that due to the complex and "crude" composition of spruce hydrolysates, further studies are needed to elucidate the toxic compounds and their removal. Indeed, a preceding study on softwood hydrolysate derived from *Pinus radiata* wood chips only yielded 0.39 g/L PHB [208].

In a similar vein, softwood hemicellulose hydrolysate fermentation produced low PHA concentrations so far. The hydrolysate of *Pinus radiata* sawdust yielded 0.23 g/L poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxypropionate) (PHBVP) [121]. Nevertheless, a recent study found lignite as an effective detoxification treatment for a spruce hemicellulose hydrolysate [173]. The lignite detoxification increased the cell dry mass (CDM) to around 4 g/L as compared to around 1 g/L with the non-detoxified hydrolysate. However, the mechanism of detoxification remained unclear. The lignite treatment did not have a high reduction of any inhibitors reported in the hydrolysate, which were initially 1.2 g/L polyphenols, 0.05 g/L furfural, 0.5 g/L acetic acid and 0.01 g/L levulinic acid. In contrast, using activated charcoal instead of lignite reduced polyphenols and furfural below 0.03 g/L, but increased the CDM to only 1.5 g/L.

To understand the effective fermentation of softwood hydrolysates, this study reports the consumption of the wood sugars (arabinose, galactose, glucose, mannose, xylose) and potential inhibitors (phenols, 5-hydroxymethylfurfural (HMF), furfural, volatile fatty acids) of non-detoxified hydrolysates. The softwood hemicellulose hydrolysate (SHH) was chosen due to its potential for integrated forest biorefineries. Specifically, the biorefinery concept of hemicellulose hydrolysate is to recover cellulose separately as it has multiple high value applications such as pulp, cellulose acetate, rayon, and glucose hydrolysates for subsequent fermentation to products such as ethanol, butanol, and lactic acid [209]. The hemicellulose extraction was performed by dilute acid treatment [134], which can be integrated in softwood pulp mills as a pre-hydrolysis step [210]. The dilute acid treatment (160 °C, 2% (w/w on wood) H_2SO_4, 15 min reaction time) hydrolyzed hemicelluloses to monomeric sugars while leaving most of the cellulose and lignin in the solid residue. However, the process promoted the formation of acetic acid, HMF, furfural and

phenols arising from the degradation of sugar and lignin. While each degradation product on its own only reduced the growth of *P. sacchari*, a mixture of all degradation products resulted in complete inhibition of growth [134]. Since the inhibitors are derived from degradation reactions, further reducing the reaction time to 10 min was hypothesized to decrease the inhibitor concentration without significantly affecting the sugar concentration.

Due to the toxicity of SHH, the hydrolysates were mixed with an inhibitor-free hardwood holocellulose hydrolysate (HHH). The HHH comes from the enzymatic hydrolysis of pretreated biomass generated by the thermomechanical pulping-based process (TMP-Bio) developed by FPInnovations [211]. The only potentially inhibitory compound was acetate, which was used as a buffer in the enzymatic hydrolysis. However, hardwood hemicelluloses consist mainly of the pentose sugar xylose, resulting in binary sugar mixtures of glucose and xylose (at a ratio of ~3:1) that can lead to carbon catabolite repression and reduce the PHA concentration [132]. For glucose-xylose mixtures, the sugarcane field isolate *Paraburkholderia sacchari* IPT101 (*P. sacchari*) had the best performance in overcoming carbon catabolite repression [132-133]. With the same strain, the specific growth rate on mannose and galactose were 97% and 60% of glucose [134].

Neither of the hydrolysates had been tested as a carbon source for PHA production. Monitoring the sugar and inhibitor consumption profile in the two sugar streams during fermentation by *P. sacchari* IPT101 with regards to bacterial growth and PHB production can inform ongoing and future efforts to detoxify softwood hydrolysates.

4.3 Materials & methods

4.3.1 Wood hydrolysates

4.3.1.1 Hardwood holocellulose hydrolysate from the TMP-Bio process

The hardwood holocellulose hydrolysate (HHH) was generated by FPInnovations using their proprietary biomass pretreatment process (TMP-Bio) [211]. After pretreatment, the biomass was subjected to enzymatic hydrolysis (20 wt% solids, 50 °C, 50 mM sodium acetate buffer (pH 4.8)) using a cocktail of commercially available cellulases and xylanases at an enzyme dosage of 10 filter paper units (FPU)/g glucan. After 72 hours, the resulting slurry was separated by centrifugation into a liquid sugar stream and a lignin-rich solid. The resulting sugar concentration was 120-140 g/L with an average of approximately 100 g/L glucose and 30 g/L xylose. The

hydrolysate was heated to 90 °C to denature the enzymes and prevent microbial contamination and was stored at –20 °C.

4.3.1.2 Softwood hemicellulose hydrolysate from dilute acid treatment

Two softwood hemicellulose hydrolysates (SHH) were produced from wood chips by dilute acid treatment as previously described [134]. In short, the wood chips (200 g oven-dry basis) were pre-steamed and hydrolyzed at a 1:3 (w:v) wood-to-liquor ratio based on the oven-dried wood mass. The samples were placed in 2 L bomb digesters with 600 mL water and sulphuric acid (4 g corresponding to 5.56 mL of 72% H_2SO_4). The hydrolysis temperature was 160 °C and the pH was 0.87. Upon reaching 160 °C, two reaction times were compared to maximize total sugar concentration and minimize degradation product formation. The reaction times were 10 and 15 min, respectively.

4.3.2 Fermentation

4.3.2.1 Microorganism and media

The PHB producing strain was *Paraburkholderia sacchari* IPT 101 (DSM 17165) and was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). The strain was resuscitated in R2A medium as recommended by DSMZ and stored at –70 °C.

The growth medium composition was adapted from a previous study [132]. In brief, the nitrogen-limited medium was composed of medium stock solutions (buffer, $MgSO_4$, and trace elements) and the carbon source. The buffer stock was concentrated two-fold. In the final medium, it had a concentration of $(NH_4)_2SO_4$ 1 g/L, Na_2HPO_4 x7H_2O 6.78 g/L, KH_2PO_4 1.5 g/L, and 1 g/L of yeast extract. The buffer stock was adjusted to pH 6.8. The magnesium sulfate concentration in the final medium was 0.2 g/L, and the stock solution had a concentration of 20 g/L (100-fold concentration). The trace element stock had a previously reported composition yielding a final concentration of 1 mL/L [176]. The buffer stock and the magnesium stock were sterilized by autoclaving, and the trace element stock was sterilized by filtration (0.22 μm). The medium was prepared by adding in % total volume: 50% buffer stock, 1% $MgSO_4$ stock, 0.1% trace element stock, and varying amounts of carbon source and autoclaved water.

The carbon source was either from pure sugar stock solution, SHH or mixtures of both. The sugar stock solution was prepared from pure sugars, glucose and xylose, purchased from Sigma Aldrich and sterilized by filtration (0.22 μm). The HHH feed was prepared by diluting the

sterilized hydrolysate in the buffer to a total sugar concentration of 10 g/L and resulted in a pH of 7. The two SHH feeds were prepared by adjusting the pH to 7 by addition of 10 M KOH. The precipitate was then separated by centrifugation and filtration. The buffer salts were added to the hydrolysates followed by filter sterilization. The wood hydrolysate mixtures were prepared by mixing the corresponding volumes of the feed solutions. The HHH initial medium was prepared by adding the sterilized hydrolysate to the medium stock solutions to reach a final total sugar concentration of 20 g/L.

4.3.2.2 Wood hydrolysate fermentation with sugar medium

The fermentation procedure was adapted from a previous study [132]. The strain from the frozen stock was streaked on agar plates with R2A medium. A pure colony was taken to inoculate seeding medium (100 mL) in 250 mL shake flasks. The seeding medium had the same composition as the culture medium, except for a lower total sugar concentration of 15 g/L (glucose:xylose 2:1). Following inoculation, the seeding medium was incubated at 30 °C at 170 rpm for 12-15 h (to approx. 1 g/L CDM). An aliquot of the seeding medium (1 mL) was transferred to the sugar medium (24 mL) and grown for 24h. The sugar medium had a total sugar concentration of approximately 20 g/L (glucose:xylose 2:1). The wood hydrolysates were added as feed (10 mL) at 24, 48 and 72h (see Figure 4-1).

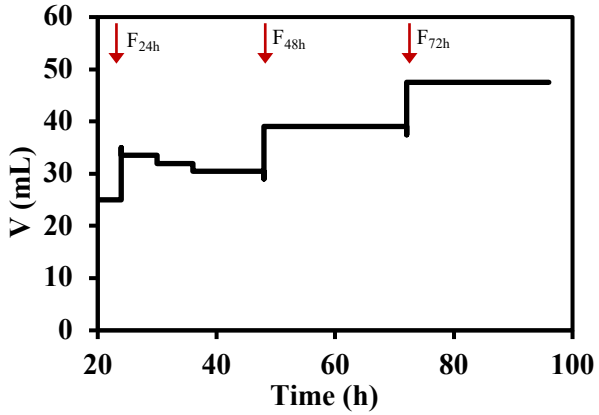


Figure 4-1: The total volume (mL) during the fermentations used to calculate the mass (g). The red arrows indicate the feeding points at 24, 48 and 72h.

Samples (1.5 mL) were taken at 24, 30, 36, 48, 72 and 96h to monitor the initial component consumption duration in the first 12h and the metabolic activity over increasing

inhibitor amount. The total volume (V_{total}) was calculated with Equation 4-1 based on the values given in Table 4-1.

$$V_{\text{total}} = V_{\text{initial}} - n \cdot V_{\text{sample}} + m \cdot V_{\text{feed}} \quad (4\text{-}1)$$

The experiment was performed in triplicate in three separate blocks with addition of the 6 feed solutions in randomized order.

Table 4-7: Values to calculate the total volume (mL) of the fermentation medium.

$V_{initial}$ = 25 mL		
V_{sample} = 1.5 mL		
V_{feed} = 10 mL		
Time (h)	n	m
24	0	1[a]
30	1	1
36	2	1
48	3	1[b]
72	4	2[b]
96	5	3[b]

[a]At 24h, the feed was added before the samples were taken. [b]At 48 and 72h, the samples were taken before the feed was added.

4.3.2.3 Wood hydrolysate fermentation with HHH medium

The fermentation was repeated with HHH medium followed by either HHH or SHH feed solution for 48h. The experiment was carried out in triplicates in a single block. The other conditions were kept constant.

4.3.3 Analytical methods

4.3.3.1 Bacterial growth

Cellular growth was measured gravimetrically (eq. 2) by CDM. In brief, CDM (g/L) was determined by centrifuging 1.5 mL of culture broth in a centrifuge (13000 rpm for 5 min) using previously dried and weighed microtubes. The supernatant (1.3 mL) was removed and kept for

carbohydrate analysis. The pellet was washed twice with distilled water (1 mL) and freeze-dried until constant mass (ca. 24 h.

$$\mathrm{CDM} = \frac{m(\text{full vial}) - m(\text{empty vial})}{V_{\text{sample}}} \quad (4\text{-}2)$$

The bacterial mass in g was calculated by multiplying the CDM with the total volume. All experiments were performed in triplicate.

4.3.3.2 PHB determination

For PHB determination, the pellet from the CDM measurement was subjected to acidic methanolysis with hexanoic acid as internal standard [177] . Samples of the organic phase were analyzed in a gas chromatograph (Agilent Technologies 5890 series II) equipped with a MS detector and a 7683B injector. The capillary column was a HP-5 from Agilent J&W Scientific, 30 m in length and 0.32 mm of internal diameter. The injector and the detector temperatures were kept constant at 120 °C and 150 °C, respectively. Data acquisition and integration were performed by a Shimadzu CBM-102 communication Bus Module and Shimadzu GC Solution software (Version 2.3), respectively. Calibration curves were obtained using samples of commercial PHB (Sigma Aldrich), which were subjected to the same methylation process as the cells. The PHB concentration was calculated using the slope and the y-intercept of a simple linear regression.

4.3.3.3 Sugars

The monosaccharide concentration was determined with a DX-600 Ion Chromatography system (Dionex, Sunnyvale, CA), equipped with an anion exchange column (Dionex CarboPac PA1) and an ED40 electrochemical detector, with 2-deoxy-D-glucose (0.25 mg mL^{-1}) as the internal standard. The column was eluted with deionized water at a flow rate of 1 mL min^{-1}. Aliquots (20 µl) were injected after being passed through a 0.45 µm nylon syringe filter (Chromatographic Specialties Inc., Brockville, ON, Canada). Baseline stability and detector sensitivity were optimized by post column addition of 0.2 M NaOH at a flow rate of 0.5 $mL \cdot min^{-1}$ using a Dionex AXP pump. The column was reconditioned using 1 M NaOH after each analysis. Monosaccharides (arabinose, galactose, glucose, xylose and mannose) in the substrates were quantified with reference to standards.

4.3.3.4 Furans

The furan concentration (furfural and HMF) was determined in an HPLC (Waters 2695 separation module), equipped with a C18 column (Nova-Pak C-18, 4µm, 3.9mm x 150mm) and photodiode array detector (Waters 2996). The column was eluted in a gradient solvent mixture (solvent A: methanol (5%), acetic acid (2%), water (93%); solvent B: methanol (90%), acetic acid (2%), water (8%)) at a flow rate of 1.0 mL min^{-1}. Solvent B was increased from 0-50% within 31 min. Aliquots (10 µl) were injected after being passed through a 0.45 µm PVDF syringe filter (Steriltech). Furfural (absorption 278 nm) and HMF (284 nm) in the substrates were quantified with reference to their standards.

4.3.3.5 Volatile fatty acids

Volatile fatty acids (VFA: formate, acetate, propionate, butyrate, glycolate) concentration was measured with an Ion Chromatography system (Dionex, Sunnyvale, CA), equipped with an ion exclusion column (Dionex ICE-AS1, 9x250mm), GP40 gradient pump, eluent suppressor (AMMS-ICE 300), and ED40 electrochemical detector. The column was eluted with 0.4 mM heptafluorobutyric acid at a flow rate of 0.8 mL min^{-1}. The suppressor regenerant was 5 mM tetrabutylammonium hydroxide. Aliquots (20 µl) were injected after being passed through a 0.45 µm PVDF syringe filter (Steriltech). VFAs in the substrates were quantified with reference to standards.

4.3.3.6 Phenols

The content of phenolic compounds was estimated with an adapted Folin-Ciocalteu phenol assay using vanillin as the standard. The results were expressed as vanillin equivalents [212]. The standards and samples were measured in duplicates. The blank, vanillin calibration standard (0.05-1 mg/mL) or sample (each 100 µL) were transferred into glass test tubes. Distilled water (3 mL) and Folin-Ciocalteu reagent (250 µL) were added and vortexed. After exactly 5 minutes, 750 µL of 20% Na_2CO_3 and 900 µL of distilled water were added to bring the volume to 5 mL. The tubes were incubated for 1.5 hours at room temperature with occasional vortexing. The phenol concentration was measured by recording the absorbance at 760 nm in a spectrophotometer.

4.3.4 Statistical analysis

Statistical analysis (ANOVA, Tukey's comparison of means) was carried out in SAS® Studio. In the fermentation of the hydrolysate mixtures, the statistical analysis was performed for

the CDM at 24, 30, 36, 48, 72 and 96h, respectively (Supplementary information, p. S.1-6). In the fermentation of the wood hydrolysates, the statistical analysis was carried out for CDM, PHB and total sugars at 24, 30, 36 and 48h (Supplementary information, p. S.7-18). The analysis was based on triplicate measurements and the confidence interval was $p<0.05$.

4.4 Results & discussion

4.4.1 Wood hydrolysate composition / Fermentation strategy

Two SHH were produced at 10 and 15 min reaction times (SHH (10min) and SHH (15min), respectively). The glucose concentration increased with longer reaction time, but the inhibitor concentration also increased. As previously mentioned, the HHH was obtained from the FPInnovations' pilot plant and was diluted to a similar total sugar concentration as the SHH. With the dilution, the acetate concentration was below 0.5 g/L, while the inhibitory threshold of acetate is above 2 g/L for *P. sacchari* [134]. Table 4-2 shows the composition of the hydrolysates.

Table 4-2: Composition (g/L) of the wood hydrolysate feed solutions after pH adjustment, filtration, sterilization and buffer addition. Softwood (black spruce) hemicellulose hydrolysate (SHH) from pretreatment with dilute acid (160 °C, 2% (w/w on wood) H_2SO_4, 15 min and 10 min), hardwood holocellulose hydrolysate (HHH) from the TMP-Bio process, diluted to a similar total sugar concentration as the softwood hemicellulose hydrolysate.

	Sugar profile					
Hydrolysate	Arabinose	Galactose	Glucose	Xylose	Mannose	Total sugars
SHH (15min)	1.18±0.08	3.16±0.18	3.55±0.2	4.71±0.27	8.69±0.49	21.28±1.22
SHH (10min)	1.52±0.04	3.15±0.07	2.87±0.07	4.80±0.25	8.81±0.21	21.15±0.64
HHH	n.d.	n.d.	14.77±0.90	6.28±0.40	n.d.	21.05±0.80
	Inhibitor profile					
	HMF	Furfural	Acetic acid	Phenols		
SHH (15min)	0.47±0.01	0.43±0.00	2.20±0.02	1.67±0.14		
SHH (10min)	0.20±0.00	0.22±0.00	1.95±0.01	0.87±0.09		
HHH	n.d.	n.d.	0.47±0.00	n.d.		

The fermentation strategy of the wood hydrolysates was designed to overcome the inhibitory effect of SHH. Using synthetic mixtures simulating SHH, an initial inoculum of high cell density (optical density, OD >5) overcame the inhibitor toxicity and resulted in bacterial growth. However, the 24h growth yield was reduced from an OD_{600} of 23 with only sugars to 14 [134]. In this study, the fermentation strategy was to use SHH as a feed after the initial growth phase for 24h. The hypothesis was that similar to a previous study [135] , the higher cell density and physiological maturity would enable the bacterial population to consume the sugars in the presence of inhibitory compounds. The initial growth was first tested with pure sugar mixtures as carbon source, which was then substituted with HHH. Both conditions were run with HHH and SHH as feed solutions. The fermentations using HHH for initial growth followed by HHH and SHH feed were successful. Consequently, it is feasible to produce PHB with solely wood-based carbon sources from integrated forest biorefineries.

4.4.2 Consumption of hydrolysates after initial growth in a synthetic sugar seed medium

Six wood hydrolysates were evaluated as feed solutions for PHA production after 24h. They were added to the fermentation broth three times at 24h, 48h, and 72h (Figure 4-1). Table 4-3 shows the name and composition of the feed solutions (H100-S0, H75-S25, H50-S50, H25-S75, H0-S100, and S10min), which are either mixtures of HHH and SHH (15min), or pure SHH (10min). The mixtures gradually increased the sugar diversity and inhibitor content. This allowed observing the effects of the sugar profile and inhibitor content on the bacterial growth and PHB production. Furthermore, the two SHH solutions could be compared. The six hydrolysates were fermented in triplicate in three blocks on different days. The fermentation profile of the replicates for each hydrolysate differed only in the case of H0-S100. This was also observed in synthetic sugar mixtures that simulated SHH, where higher inhibitor concentrations increased the variance in cell growth [134]. Among the hydrolysates, H0-S100 had the highest inhibitor concentration, and the highest cell growth variance, which reflected the inhibitory effect of the hydrolysate component on bacterial cells (Table 4-2, Figure 4-2, CDM).

Table 4-3: List of six wood hydrolysates as the feed solutions for fermentation. The hydrolysates are hardwood holocellulose hydrolysate (HHH), softwood hemicellulose hydrolysate, extracted for 15 min (SHH (15min)), softwood hemicellulose hydrolysate, extracted for 10 min (SHH (10min)), or mixtures.

		HHH	**SHH (15min)**	**SHH (10min)**
Label		%	%	%
H100-S0	=	100	0	0
H75-S25	=	75	25	0
H50-S50	=	50	50	0
H25-S75	=	25	75	0
H0-S100	=	0	100	0
S10min	=	0	0	100

4.4.2.1 CDM and PHB

The bacteria were grown in the sugar seed medium for 24h to 4.4 g/L. Upon addition of fresh carbon source in the form of the six wood hydrolysates, the CDM increased to 4.8-5.9 g/L (Figure 4-2, CDM), the lowest for H0-S100 and highest for H100-S0. Since H100-S0 does not contain inhibitors and has the highest glucose content, it was anticipated that its use as a carbon source would result in the highest CDM and mass among the hydrolysates. Yet, within the first 48h, the difference in CDM and mass amongst the hydrolysates was not statistically significant ($P<0.05$) (Figure 4-2, CDM indicated by *). Upon the second and third addition of hydrolysates, the fermentation broth was diluted (see Figure 4-1), which was accompanied by a concomitant decrease in CDM. The mass stayed constant for H100-S0 and decreased for the other hydrolysates (Figure 4-2, Mass). As fermentation time increased, the differences in CDM became more significant. For example, at 72h, H100-S0 had the highest mean CDM and was significantly different to the lowest mean CDM corresponding to H0-S100. Moreover, when the fermentation time was extended to 96h, H100-S0 became significantly different to the two lowest means corresponding to the two softwood hydrolysates (H0-S100 and S10min). Finally, throughout the entire fermentation period, the hydrolysate mixtures from H75-S25 to H25-S75 were not significantly different from either the highest or the lowest means. This could indicate a comparable sugar consumption pattern among the hydrolysates provided that the inhibitor concentration remains comparatively low. Upon further feed addition at 48h, the non-metabolized inhibitors accumulated (see section on Degradation products), which might have

caused the decreased CDM for the hydrolysates with higher inhibitor content (H0-S100 and S10min).

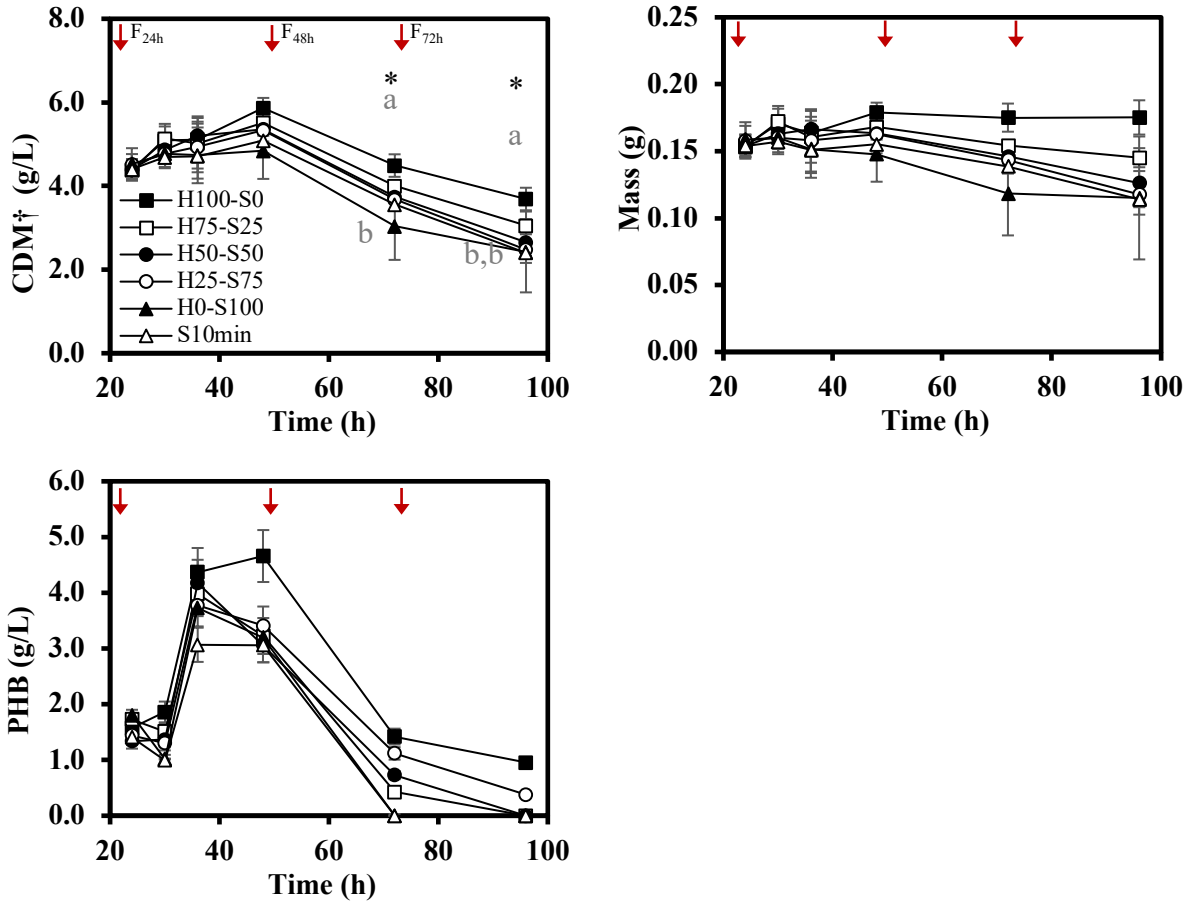


Figure 4-2: Cell dry mass (CDM) (g/L), mass (g) and PHB (%, g/g) with wood hydrolysate feeds over time for mixtures of hardwood and softwood hydrolysates. Wood hydrolysates (10 mL) were added to pure sugar medium (25 mL) after 24h, 48h and 72h. The mass is a measure of CDM that accounts for the change in volume with feed addition. [1]The CDM was analyzed for statistical difference for each time point. *Significant difference of means between hydrolysates ($P<0.05$) is indicated with different letters (a, b) next to symbols. When letters are present, symbols in between highest and lowest values are both ab, therefore not significantly different to any other mean. No star (*) indicates no significant difference. The red arrows indicate the feeding points at 24, 48 and 72h.

During the fermentation, the changing environment in the different media can have an effect on the PHB metabolism of the cells. The PHB content at 24h was low (average 33%), which can be explained by the low sugar concentration in the seed medium (Figure 4-2, PHB). This sugar concentration was chosen to allow for bacterial growth, while fully depleting glucose within 24h thereby promoting xylose consumption. Within 48h, the PHB concentration increased to 3.1-4.7 g/L, being highest for H100-S0. Upon further addition of the hydrolysates, the PHB was consumed which was possibly due to carbon depletion. The total sugar concentration

dropped to 3 g/L at 72h and 96h, among which glucose and mannose concentrations dropped to 0 g/L. Between 24-48h, the sugars consumption was used for PHB production and after 48h, the cells were saturated with PHB. Further sugar feed is likely consumed for cell maintenance. Since PHB is produced in an excess of carbon source, the depletion of glucose and mannose might lead to PHB mobilization to maintain the cells.

The maximum PHB content reached was 79%. A previous study, which also used the same *P. sacchari strain,* reported 60.3% and 72% PHB in shake flask cultures with glucose (20 g/L, 29h) and simulated hydrolysate (32.4 g/L glucose, 12.9 g/L xylose and 4.5 g/L arabinose, 70h) as respective carbon source, reaching an overall CDM of 6.3 g/L and 6.0 g/L respectively [132]. Together these results indicate that the strain is able to metabolize the wood carbon sources to PHA, reaching similar yields as with pure sugar mixtures.

4.4.2.2 Sugars

The total sugar consumption showed a similar tendency as the CDM. Specifically, after 48h, 3.9-6.7 g/L sugars were consumed (Figure 4-3, Total Sugars). The lowest sugar consumption was exhibited by cultures fed with H0-S100, while the highest by those fed with H75-S25 and H100-S0. This can be attributed either to the higher glucose content of the hardwood hydrolysate or to the lower inhibitor concentration. After 48h, the total sugars remained constant. Among the sugars, glucose was fully depleted at 36h for all hydrolysates (Figure 4-3, Glucose). The S10min hydrolysate had the lowest glucose content and as expected, showed the fastest depletion at 30h. As was also expected due to their differences in metabolism [114], mannose consumption exhibited a lower rate than that of glucose, yet it was fully consumed within 48h (Figure 4-3, Mannose). Surprisingly, galactose content increased with the second and third hydrolysate addition despite having a low initial concentration (<1 g/L) (Figure 4-3, Galactose). Only in the case of the H75-S25 hydrolysate, the initial galactose (0.242 g/L) was consumed within 48h and was metabolized upon further hydrolysate addition. In contrast, higher initial galactose content, as in the case for the hydrolysates composed of 50% SHH and higher, galactose was not fully metabolized within 48h.

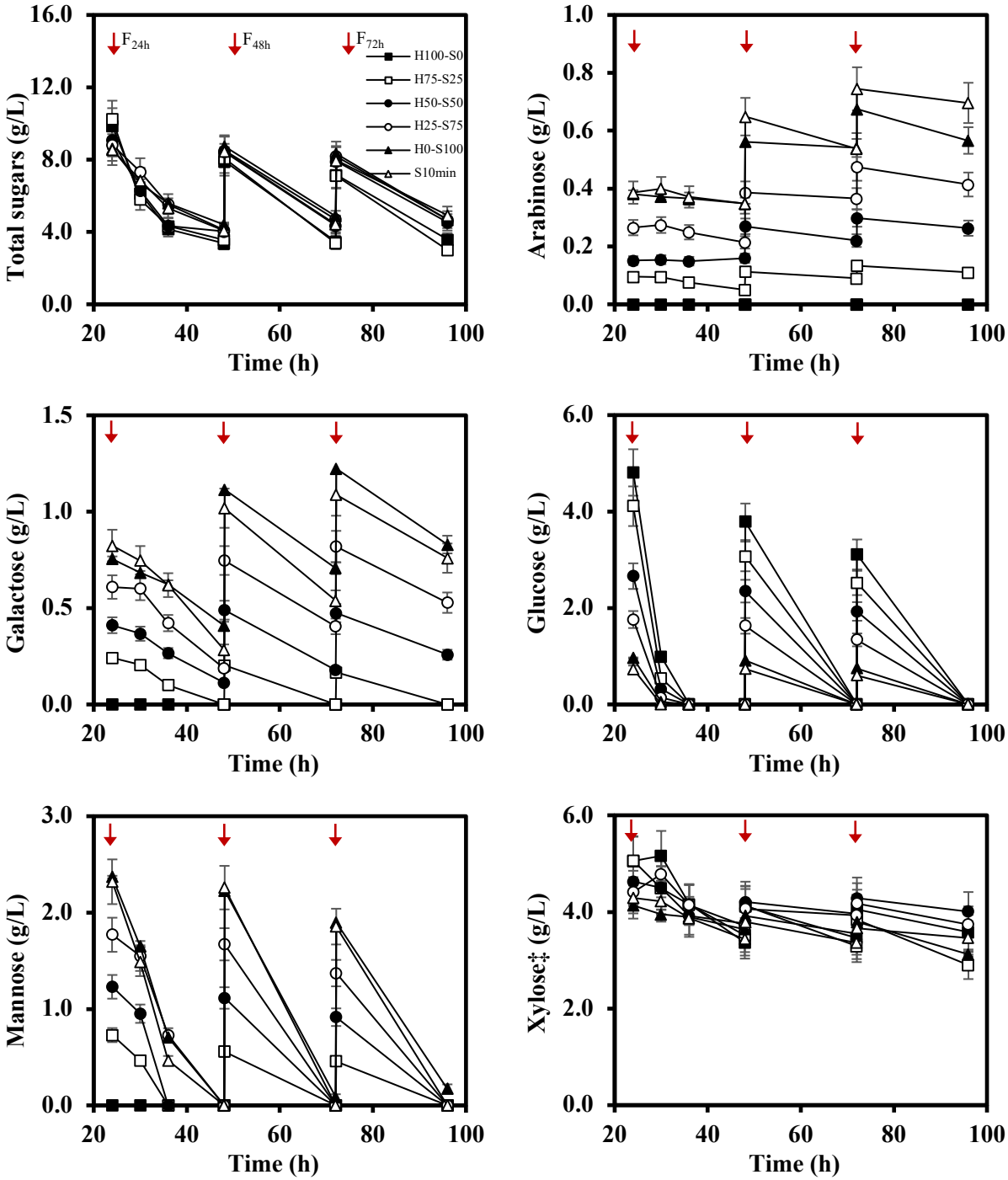


Figure 4-3: Total sugars, arabinose, galactose, glucose, mannose, xylose (in g/L) with wood hydrolysate feeds over time for mixtures of hardwood and softwood hydrolysates. Each wood hydrolysate (10 mL) was added after growth in pure sugar medium (glucose and xylose, 25 mL) at 24h, 48h and 72h. The red arrows indicate the feeding points at 24, 48 and 72h. [a]An average of 3.06 g/L xylose was carried over to each flask from the initial sugar growth medium, resulting in a higher initial xylose concentration than solely from the feed addition.

Due to a similar xylose concentration in the initial softwood and hardwood hydrolysates, and the leftover xylose from the initial sugar medium, the initial xylose concentration ranged between 4.29-5.06 g/L for all hydrolysates (Figure 4-3, Xylose). Interestingly, *P. sacchari* exhibited a similar xylose consumption pattern for all hydrolysates. Specifically, the initial xylose concentration decreased by 0.550-1.680 g/L within 48h and remained constant afterwards. On the other hand, arabinose was not consumed even at concentrations as low as 0.095 g/L. While previous studies have used softwood hydrolysates as a carbon source for PHA production, the consumption patterns of the different sugars were not discussed. This was likely due to the fact that glucose was the major component in the holocellulose hydrolysates. For example, two softwood holocellulose hydrolysates contained 18.3 g/L and 20.6 g/L glucose, with each of the remaining sugars (xylose, mannose, galactose, arabinose) having a concentration below 2.5 g/L, and thus being negligible in comparison to glucose [208]. Similarly, a different softwood hydrolysate had a total sugar concentration of 14.9 g/L with 10.4 g/L xylose and 4.5 g/L glucose [173]. The high xylose content indicated that mostly hemicellulose was hydrolyzed during the treatment. Due to the softwood hemicellulose composition, it is likely that the hexose sugars mannose and galactose were also present in similar concentrations as xylose. In comparison, many studies on sugar consumption with glucose-xylose mixtures exist [134]. For example, one study showed the optimal glucose-xylose mixtures in synthetic medium with pure sugars [133].

4.4.2.3 Degradation products

While softwood hemicellulose sugars were known to be metabolized by *P. sacchari* [134], the consumption of sugar and lignin degradation products was unknown. The potentially inhibitory compounds had a low initial concentration as a result of the three-fold dilution (see Table 4-2). In the case of the furans, both HMF and furfural were depleted within 48h and continued to be metabolized for the duration of the experiment (Figure 4-4, HMF, furfural). Additionally, VFAs were also depleted (Figure 4-4, acetate, glycolate, formate) which indicates their metabolic consumption. Glycolate and formate increased again after 48h, which indicates that they are also formed during metabolic activity.

In contrast to HMF and furfural, phenolic compounds were not consumed over the course of the fermentation (Figure 4-4, phenols). This is problematic for fed-batch bioreactor fermentations, since the feed is continuously added upon carbon source consumption. Thus, the phenols would accumulate with increasing feed addition and inhibit bacterial growth in the

course of the fermentation. In a previous model study [134], phenols were indeed identified as the most inhibitory compound for *P. sacchari* growth, especially when present in mixtures with acetate, HMF and furfural. As an example, a softwood hydrolysate had to be detoxified to be a suitable carbon source for PHA production with *P. sacchari* and *Burkholderia cepacia* [173]. Despite detoxification, cell growth and PHB content for both species reached only 4 g/L and 2 g/L PHB after 72h respectively. Therefore, fermentation inhibitors present in softwood hydrolysates are a challenge that needs to be addressed to obtain high PHA yields. However, since *P. sacchari* is able to metabolize all inhibitors except phenols, for further bioreactor use it might be necessary to remove phenolic compounds with the detoxification treatment.

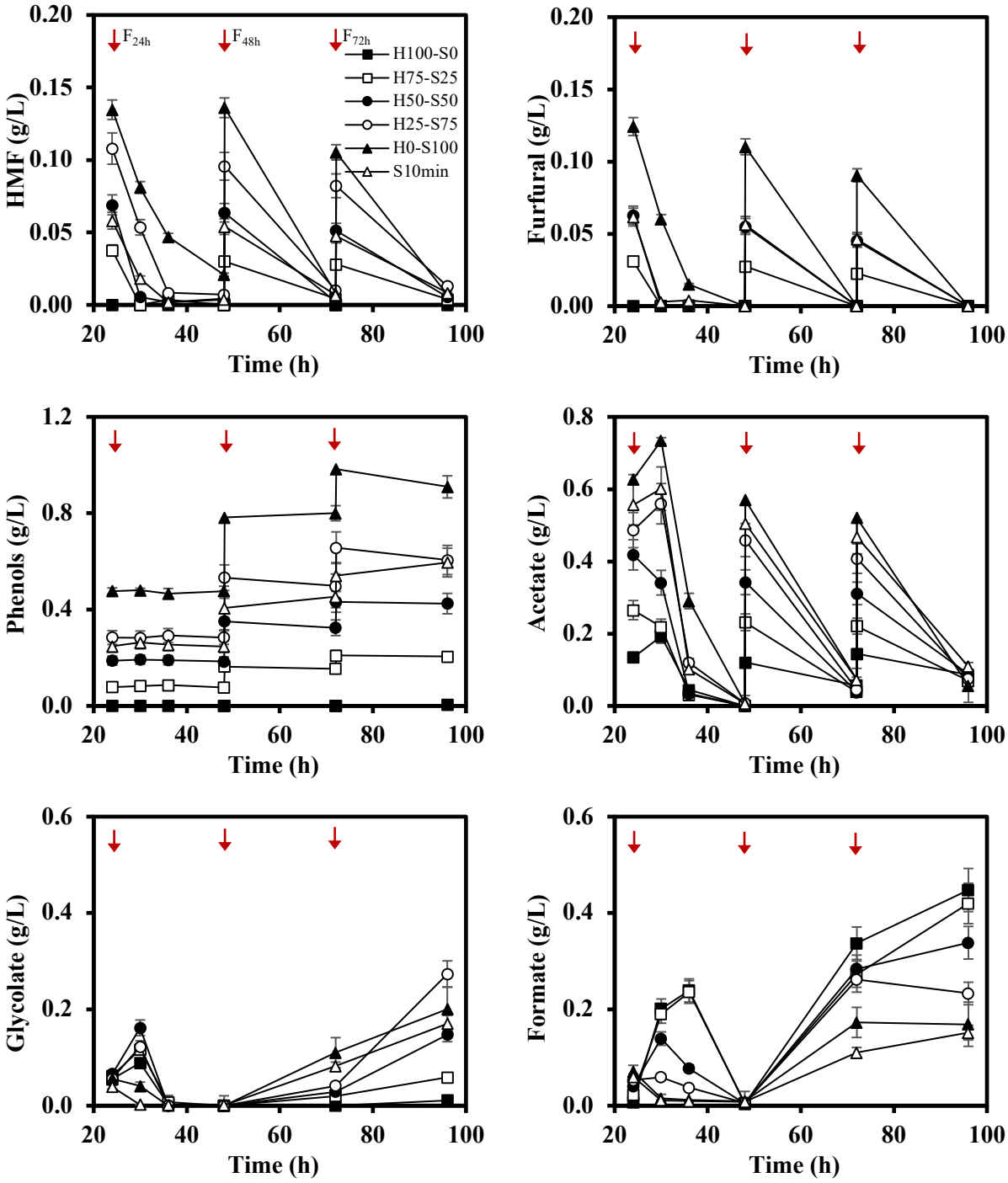


Figure 4-4: HMF, furfural, phenols, acetate, formate and glycolate (in g/L) with wood hydrolysate feeds over time for mixtures of hardwood and softwood hydrolysates. Each wood hydrolysate (10 mL) was added after growth in pure sugar medium (glucose and xylose, 25 mL) at 24h, 48h and 72h. The red arrows indicate the feeding points at 24, 48 and 72h.

4.4.3 Consumption of wood hydrolysate components with HHH seed medium

After the successful uptake of wood carbon sources following the initial growth phase, the next step was to use wood hydrolysates as sole carbon source. To this end, the inhibitor-free HHH medium was used directly in the seed medium, followed by the addition of either H100-S0 or H0-S100. All analyses were performed in triplicates. The statistical difference was evaluated for the means of CDM, PHB content and total sugars.

After 24h, the average CDM reached 5.9 g/L (Table 4-4). This value is 1.5 g/L higher than that obtained with the pure sugar seed medium. In previous fermentations to lactic acid, the HHH from the TMP-Bio process showed that the glucose consumption rate increased by a factor of two as compared to the same amount of synthetic sugars [213]. The author attributed the faster fermentation to higher nutrient levels in the HHH (for example calcium, phosphorous, and sulfur), which are released into the medium during enzymatic hydrolysis. As a consequence of the higher initial CDM, the component consumption changed. The maximum CDM reached 6.2 g/L for H0-S100 and 6.7 g/L for H100-S0 at 48h. This made little difference to the initial CDM, since the starting CDM was already very high for *P. sacchari* fermentation in similar conditions [134]. Nevertheless, the CDM was significantly different for H100-S0 (HHH) and H0-S100 (SHH) at 30h and 48h (see Table 4-4, indicated by a, b). However, the actual difference between the averages are rather small (0.3 g/L at 30h, 0.2 g/L at 36h and 0.5 g/L at 48h), so that the statistical difference reflects the low variation within the experimental replicates (Supplementary information, p. S.7-10).

Table 4-4: Wood hydrolysate components over time after 24h growth phase in hardwood holocellulose hydrolysate medium. Average (μ) and standard deviation (σ) of triplicates. The feed solutions were 100% softwood hemicellulose hydrolysate, extracted for 15 min (H0-S100) and 100% hardwood holocellulose hydrolysate (H100-S0).

		0h		24h		30h		36h		48h	
g/L	Feed	μ	σ	μ	σ	μ	σ	μ	σ	μ	σ
CDM	H0-S100	0.3	0.0	5.9	0.1	5.8	0.0	5.8	0.0	6.2	0.0
	H100-S0	0.3	0.0	5.9	0.0	6.1	0.1	6.0	0.1	6.7	0.1
PHB	H0-S100	0.0	0.0	4.5	0.5	4.1	0.1	4.4	0.1	4.6	0.3
	H100-S0	0.0	0.0	4.0	0.3	4.3	0.1	4.4	0.5	4.7	0.4
PHB (%)	H0-S100	0.0	0.0	76	7	70	2	75	2	74	5
	H100-S0	0.0	0.0	68	5	71	2	73	7	71	5
Arabinose	H0-S100	0.0	0.0	0.38	0.03	0.37	0.01	0.34	0.02	0.29	0.00
	H100-S0	0.0	0.0	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Galactose	H0-S100	0.0	0.0	0.73	0.06	0.68	0.01	0.57	0.03	0.25	0.03
	H100-S0	0.0	0.0	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00
Glucose	H0-S100	4.81	0.34	0.95	0.07	0.03	0.01	0.01	0.00	0.00	0.00
	H100-S0	4.81	0.34	4.41	0.12	0.06	0.05	0.00	0.00	0.00	0.00
Xylose	H0-S100	5.05	0.35	3.25	0.10	3.15	0.11	2.91	0.13	2.41	0.18
	H100-S0	5.05	0.35	3.67	0.16	3.33	0.10	2.72	0.13	1.54	0.12
Mannose	H0-S100	0.00	0.00	2.34	0.18	1.48	0.04	0.04	0.00	0.04	0.00
	H100-S0	0.00	0.00	0.06	0.00	0.01	0.01	0.00	0.00	0.00	0.00
Total Sugars	H0-S100	9.86	0.69	7.64	0.43	5.72	0.15	3.87	0.17	2.99	0.21
	H100-S0	9.86	0.69	8.16	0.28	3.41	0.13	2.73	0.13	1.55	0.12
HMF*	H0-S100	n.d.	n.d.	0.13	0.00	0.01	0.00	0.01	0.00	0.01	0.00
	H100-S0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Furfural*	H0-S100	n.d.	n.d.	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	H100-S0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acetate	H0-S100	0.13	0.01	0.62	0.01	0.35	0.02	0.05	0.01	0.02	0.01
	H100-S0	0.13	0.01	0.06	0.01	0.11	0.02	0.03	0.00	0.04	0.01
Glycolate	H0-S100	0.06	0.00	0.13	0.06	0.00	0.00	0.00	0.00	0.00	0.00
	H100-S0	0.06	0.00	0.06	0.02	0.27	0.05	0.02	0.01	0.00	0.00
Formate	H0-S100	0.01	0.00	0.15	0.03	0.20	0.03	0.21	0.04	0.03	0.02
	H100-S0	0.01	0.00	0.01	0.00	0.36	0.05	0.40	0.02	0.03	0.02
Phenols*	H0-S100	n.d.	n.d.	0.38	0.01	0.39	0.00	0.39	0.00	0.37	0.01
	H100-S0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

*The CDM, PHB and total sugars were analyzed for statistical difference at each time point. Significant difference of means between hydrolysates ($P<0.05$) is indicated with different letters (a, b) next to the value. No letter indicates that there is no significant difference. [c]H100-S0 does not contain HMF, furfural and phenols. n.d.: not determined.

Similar to the constant CDM, the PHB content stays constant at approximately 70% for all time points (Table 4-4). Indeed, the PHB content is not statistically different for the two hydrolysates (Supplementary information, p. S.11-14). Since the high CDM at 24h indicates that the cells are in stationary phase, then the constant PHB content shows that the cells use the carbon sources of the medium for maintenance, instead of consuming the accumulated PHB.

With the high initial CDM, the consumption of the components was fast (Table 4-4). Glucose was completely consumed after 36h. Furthermore, xylose consumption after 48h reached 0.84 g/L (H0-S100) and 2.13 g/L (H100-S0). The total sugar consumption is 4.65 g/L and 6.61 g/L for H0-S100 and H100-S0, respectively. The total sugars concentrations are significantly different at 30h, 36h, and 48h (indicated by a, b). HMF and furfural both depleted at 30h. The VFAs are depleted after 48h while phenols, as expected, remained constant as they are not metabolized by *P. sacchari*. Therefore, the lack of inhibitors and high glucose content make HHH an effective carbon source for PHB production with *P. sacchari*, while SHH requires further detoxification to be used for PHB production.

4.5 Conclusion

The sugar and inhibitor profile of softwood hemicellulose hydrolysates influenced the CDM throughout the course of the fermentation. The sugars were consumed in decreasing order as follows: glucose, mannose, xylose, galactose, and arabinose. Additionally, potential inhibitors (i.e. volatile fatty acids and furans) were metabolized while phenols were not metabolized and accumulated when a fed batch approach was employed. Softwood hemicellulose hydrolysate was fermented to PHB when added after an initial growth phase to overcome its inhibitory effects. Yet, the softwood hydrolysates reduced the CDM compared to the inhibitor-free hardwood hydrolysate. Since phenols are not consumed, further detoxification strategies should focus on their removal.

The inhibitor free hardwood holocellulose hydrolysate from the TMP-Bio process is a promising carbon source with the bacterium *P. sacchari* IPT 101. The strain grew to 5.9 g/L CDM within 24h, as compared to 4.4 g/L using pure sugars. The hardwood and softwood hydrolysates represent promising carbon sources from emerging forest biorefinery concepts. To further investigate the potential of softwood hemicellulose hydrolysates, targeted detoxification is a challenge that needs to be addressed.

Connecting Statement to Chapter 5

In the fourth chapter, it was hypothesized that growing the bacteria to a early stationary phase would increase their resistance to the inhibitors in the hydrolysate. An initial growth of 24 h was sufficient to overcome the inhibitory effect of softwood hemicellulose hydrolysate and allowed to track the consumption of all hydrolysate components. The sugars were depleted at different rates in the following order: glucose, mannose, xylose, galactose, and arabinose. While acetate, HMF, and furfural were all metabolized by *P. sacchari,* phenols were not consumed. Even though softwood hemicellulose hydrolysate was consumed by the strain, it was the hardwood holocellulose hydrolysate that resulted in a maximum cell dry mass.

In the fifth chapter, the higher growth of *P. sacchari* with the inhibitor-free hardwood holocellulose hydrolysate was further explored in fed-batch bioreactors. In this biorefinery scheme, hardwood chips were subjected to thermomechanical pretreatment followed by enzymatic hydrolysis, which hydrolyzed cellulose and hemicelluloses with the recovery of a pure lignin stream. The lignin was the main biorefinery product, and the holocellulose hydrolysate was evaluated as a feedstock for PHB production. The chapter is based on a manuscript to be submitted for publication in the journal Industrial Crops and Products under the title “Increasing PHB production using wood: The fermentation of an industrially scalable hardwood hydrolysate to PHB” with the co-authors Edmar R. Oliveira-Filho, Prof. Luiziana F. da Silva, José G. C. Gomez, Marilda K. Taciro, Dr Marie-Josée Dumont, Prof. Valérie Orsat, and Dr. Luis F. Del Rio.

Chapter 5: Increasing PHB production using wood: The fermentation of an industrially scalable hardwood hydrolysate to PHB

5.1 Abstract

Biobased polymers, notably polyhydroxybutyrate (PHB), have a tremendous and yet underexplored potential to address several global environmental problems, such as ocean pollution and greenhouse gas emissions. The key challenge to produce PHB at minimum cost and maximum societal benefits is to convert non-edible and abundant carbon sources. Lignocellulosic feedstocks, such as wood, is a promising sustainable carbon source, but so far, the pretreatments investigated for PHB production have not yet reached industrial maturity. In this study, we report an industrially feasible conversion of wood hydrolysate from a pilot-scale forest biorefinery. The wood hydrolysate was compared to a synthetic hydrolysate with similar carbon composition (i.e. glucose, xylose, and acetate) to assess the effect of additional components present in wood hydrolysate. To maximize the PHB concentration, cultivation in high-cell density bioreactors was conducted for 52 h using the bacterium *Paraburkholderia sacchari* IPT 101.

The fermentation of wood hydrolysate yielded a PHB concentration of 34.5 g/L, which is among the highest reported for lignocellulose hydrolysates. In comparison, the use of a synthetic hydrolysate resulted in substantially lower PHB concentration (22.0 g/L). This could be attributed to a higher maximum specific growth rate of 0.36 vs. 0.33 per h for cells grown in wood hydrolysate vs. synthetic hydrolysate. The higher growth rate led to a higher residual cell mass and therefore more cells to accumulate PHB. Furthermore, the bacterial growth phase lasted longer, since after 12.2 h, the PHB content with wood hydrolysate was 26.3% as compared to 35.3% g/g. For the wood hydrolysate, the final PHB content per cell mass reached 58% g/g and a PHB productivity of 0.72 g/(L·h), while the synthetic hydrolysate reached 55% g/g and 0.46 g/(L·h), respectively.

Given the increase in bacterial growth using wood hydrolysate, the chosen lignocellulose conversion process is beneficial for subsequent fermentative conversion to key bioproducts. The industrially mature process prevents inhibitor formation and preserves nutrients. As such, PHB production from wood is technically feasible on a large scale.

5.2 Introduction

Polyhydroxyalkanoates (PHAs) are polyesters that are produced during secondary metabolism of bacteria and archaea [214]. Among over 150 possible monomer combinations [215], polyhydroxybutyrate (PHB) was been the first PHA discovered and has since been studied as a reference polymer [99]. Compared to other bioplastics such as PLA or PBS, PHB biodegrades faster in various environments (e.g. home compost, anaerobic digestion, and marine environment) [8]. One of the major challenges for industrial production is the reliable and scalable supply of carbon sources, as it makes up a major fraction of the PHA production costs [216]. Currently, the industry employs mostly edible feedstocks such as glucose from corn starch and vegetable oils as carbon sources for the fermentation process [160].

Wood is a natural composite of cellulose, hemicelluloses and lignin and makes up the fibrous material that constitutes the plant cell wall. As such, it belongs to the category of lignocellulosic biomass, which has the highest potential to ensure a sustainable supply as it is abundant and underutilized [217]. Notably, the forest industry provides a solid infrastructure for fractionated lignocellulose streams that can be explored for PHA production [218] However, woody biomass requires pretreatment to release fermentable sugars, which are often still industrially immature [218]. One of the few examples of a pilot scale pretreatment is the thermomechanical pulping-based process, developed by FPInnovations (TMP-Bio). Enzymatic hydrolysis of pretreated hardwood chips produces an inhibitor-free hardwood holocellulose hydrolysate (WH) [211]. The WH consists of binary sugar mixtures of glucose and xylose (at a ratio of ~2.5:1), and minor amounts of acetate derived from the buffer used in enzymatic hydrolysis. As a main advantage compared to other lignocellulose pretreatments, lignin is recovered as a product in the form of hydrolysis lignin (H-lignin), which allows to recover a maximum mass balance of products per feedstock.

Several lignocellulose hydrolysates have been fermented to PHB, but pretreatments used have limited potential for large-scale hydrolysate production. For example, wheat straw hydrolysate in fed-batch bioreactor cultivations of *Paraburkholderia sacchari* IPT101 (*P. sacchari*) with an automated feeding strategy afforded a PHB concentration of 105 g/L, 72% cell content (% g/g), and productivity of 1.6 g/(L·h) [132]. The main drawback was the use of ammonium fiber expansion as a pre-treatment which can be hazardous due to the ammonium present and has not yet been proven at the pilot or demonstration scale. Softwood holocellulose

hydrolysate was also fermented to a high PHBV concentration of 61.8 g/L, 77% cell content (% g/g), and 2.84 g/(L·h) [135] . However, in that study the wood was liquefied in a microwave process, which is difficult for large scale operation. Furthermore, due to the presence of inhibitory compounds, the softwood hydrolysate could only be added after a 15h growth phase in pure glucose. The next highest PHB concentration (15.6 g/L) was achieved with perennial ryegrass [219]. Fermentable monomers were obtained by ensilation, which is an acidic fermentation process. While simple and scalable, this pretreatment is mostly adequate for herbaceous feedstock, and it takes seven weeks to obtain a pretreated biomass having a heterogenous composition due to variations in the occurring metabolism.

Building on the high cell density cultivations described above, this study investigates the potential to include PHB production in the hardwood biorefinery pilot plant. The sugarcane field isolate *P. sacchari* was chosen as it is a promising strain for glucose-xylose mixtures due to its high growth rate on xylose [21, 119, 133].

5.3 Materials & methods

5.3.1 Wood hydrolysate from the TMP-Bio process

Hardwood chips were pretreated in a proprietary process (TMP-Bio) in a pilot plant to generate hardwood holocellulose hydrolysate (WH) [211]. The final products were WH and a lignin-rich solid. To convert the chips, a thermomechanical pulping step was applied to increase enzyme accessibility to cellulose and hemicellulose. Next, the polysaccharides were hydrolysed (20 wt% solids, 50 °C, 50 mM sodium acetate buffer (pH 4.8)) by commercially available cellulases and xylanases. The resulting WH had a sugar concentration of 120-140 g/L with an average of 100 g/L glucose and 30 g/L xylose. After heating to 90 °C to denature the enzymes and prevent microbial contamination, WH was stored at –20 °C [220]. To prepare the bioreactor feed solution, the WH was concentrated to a final concentration of 414 g/L glucose, 168 g/L xylose and 13 g/L acetate (Table 5-1).

5.3.2 Fermentation

5.3.2.1 Microorganism

The PHB producing strain, *Paraburkholderia sacchari* IPT 101, was a soil isolate [221]. The strain was stored at –80 ºC.

5.3.2.2 Culture media

The cells were first grown in shake flasks in nutrient broth (Table 5-1) for 8 h at 30 °C with shaking at 250 rpm. The suspended cells (10% v/v) were transferred to the seed medium, which was a minimal medium (MM) containing limited amounts of nutrients to sustain growth (Table 5-1) and incubated for 15 h at 30 °C. The growth medium composition was adapted from a previous study [222]. The initial batch medium (Table 5-1) was inoculated with 16% (v/v) to an initial volume of 1.85 L. The pH was maintained at 7.0 with 2 N H_2SO_4 and 28% NH_3 to keep the nitrogen above the limiting concentration. Prior to the two runs, two bioreactor runs with SH were conducted in different growth media (MM and yeast-rich medium) to test the bacterial growth and reproducibility (data not shown).

5.3.2.3 Carbon sources

The WH (concentrated TMP-Bio) was used as is in the bioreactor feed (Table 5-1). For the SH, to reach similar concentration as in WH, glucose and xylose were gradually dissolved in distilled water by heating the solution in a microwave for 20 s until the solubility increased. Sodium acetate was added separately following pasteurization at 70 °C for 3 h to avoid sugar degradation.

Table 5-1: Growth media composition.

	Nutrient broth [g/L]	Seed culture (MM) [g/L]	Initial batch (MM) [g/L]	Feed solution [g/L]
SH				
Glucose	-	10.0	15.9	407.4
Xylose	-	5.0	6.8	174.6
Acetate	-	-	0.5	11.6
WH				
Glucose	-	-	17.3	414.1
Xylose	-	-	7.0	168.2
Acetate	-	-	0.6	13.2
KH_2PO_4	-	1.5	0.64	-
Na_2HPO_4	-	4.45	-	-
$(NH_4)_2SO_4$	-	1	1.86	-
$MgSO_4 \cdot 7\ H_2O$	-	0.2	0.53	-
$CaCl_2 \cdot 2H_2O$	-	0.01	0.03	-
Ammoniac citric ferric	-	0.06	0.05	-
NaCl	-	-	0.81	-
TES (mL/L)[a]	-	0.2	9.73	-
Meat extract	3.0	-	-	-
Peptone	5.0	-	-	-

[a] The trace element solution (TES) composition was per L: H_2BO_4 (0.3 g), $CoCl_2 \cdot 6\ H_2O$ (0.2 g), $ZnSO_4 \cdot 7\ H_2O$ (0.1 g), $NaMoO_4 \cdot 2\ H_2O$ (0.03 g), $NiCl_2 \cdot 6\ H_2O$ (0.02 g), $CuSO_4 \cdot 5\ H_2O$ (0.01 g), $MnCl_2 \cdot 6\ H_2O$ (0.03 g).

5.3.2.4 Bioreactors

High-cell density fermentations were conducted in two 5-L bench scale bioreactors (Applikon Biotechnology, Delft, The Netherlands). The jacketed reactors included a gas sparger, two impellers (45 mm), a sampling port, a pH probe (12 mm classic pH sensor), a dissolved oxygen (DO) probe (12 mm classic polarographic DO sensor), a temperature sensor (Pt-100 sensor in thermowell in the top plate), a foam sensor, an exhaust gas condenser and multiple inlet ports for the acid, base and feed solutions. Oxygen was supplied by a constant airflow of 1 L/min and varying stirrer speed (operated between 200 – 1200 rpm, the bioreactor standard ranges from 50 – 1250 rpm). The feed solutions were added in separate pulses by peristaltic pumps upon an increase of DO. The WH and SH runs were conducted for 52 h and were operated in parallel.

5.3.3 Analytical methods

The analytical methods were previously described [195]. Briefly, the total cell concentration (CDM) (g/L) was determined gravimetrically after centrifugation for 10 min at 4 ºC. The supernatant was separated, and the solids washed with distilled water. The solids were freeze-dried to constant mass. The PHB concentration was measured after methanolysis to methyl esters by gas chromatography (GC) in a Varian 3700 gas chromatograph (Sunnyvale, Calif., USA) [221]. Separation occurred in a Varian DB-5 capillary column (30 m by 0.25 mm, Varian, Sunnyvale, Calif., USA) and the components were detected with a flame-ionization detector [221]. Glucose and xylose were analyzed with high performance liquid chromatography (HPLC) using the supernatant after filtration (0.22 pore membrane). Samples (10 µL) were injected into a Waters 510 HPLC (Waters-Millipore, Milford, Mass., USA) equipped with a sugar-separation column (Shodex SC-1011, Waters-Millipore, Milford, Mass., USA) and a differential Waters 410 refractometer [221]. The nitrogen concentration was determined with a NH_4^+ electrode. Inorganic phosphorus (Pi) was determined with the ascorbic acid colorimetric method using a standard curve of NaH_2PO_4 solution [222]. The total bioreactor volume (L) was calculated following Equation 5-1. The masses (g) of the total cell concentration, PHB and substrate were calculated by multiplying the measured concentrations (total cells, PHB and substrate, respectively) with the total bioreactor volume of the broth at the corresponding time point. The PHB content (% g/g) was calculated by PHB (g/L) per CDM (g/L). Residual biomass (Xr) (g/L) was calculated as CDM (g/L) minus PHB (g/L). Total sugar concentration was calculated as the sum of glucose (g/L) and xylose (g/L). The substrate consumption (glucose, xylose, and acetate) was calculated as the difference between substrates added and substrate measured, adjusted for the change in volume. The acetate concentration was not measured, as it was assumed to be metabolized quickly based on previously reported consumption patterns for acetate [220].

$$V_{\text{total}} = V_{\text{initial}} - V_{\text{sample}} - V_{\text{condenser}} + V_{\text{base}} + V_{\text{acid}} + V_{\text{feed}} \quad (5\text{-}1)$$

5.3.4 Kinetic parameters

The maximum specific growth rate ($\mu_{\max}$) was determined by linear regression of ln (Xr/X_0) versus time (h). The yields ($Y_{P/S}$ and $Y_{Xr/S}$) were calculated by Equation 5-2.

$$Y_{\mathrm{P/S}} = \frac{\Delta PHB}{\Delta S},\ Y_{\mathrm{Xr/S}} = \frac{\Delta Xr}{\Delta S} \tag{5-2}$$

The productivities (P_{PHB} and P_{Xr}) were calculated by Equation 5-3.

$$P_{PHB} = \frac{\Delta PHB}{\Delta t}, P_{Xr} = \frac{\Delta Xr}{\Delta t} \tag{5-3}$$

5.4 Results & discussion

5.4.1 Bacterial growth

Sustainable carbon sources for efficient (high-cell density and high volumetric productivity) production of PHB are necessary to make this biopolymer affordable and environmentally benign. The WH had previously given promising results as an inhibitor-free substrate [220]. To further evaluate its potential use for PHB production, WH was compared to SH, a synthetic sugar and acetate solution. The process design aimed to enable two growth phases and an accumulation phase, as reported previously [222]. During the initial growth phases, the substrate should be used for cell growth to maximize Xr. Upon depletion of phosphorus (the limiting nutrient), the PHB accumulation phase starts and substrate consumption is mainly directed towards PHB synthesis. One limitation of the available bioreactors was that an automated feeding strategy was not available, which can optimize the supply of carbon source in the medium compared with a manual feed addition.

Both fermentations (WH and SH) displayed growth and accumulation phases, starting with a low PHB content of around 20% (g PHB/g CDM). Growth was visualized as ln Xr/X_0 against time (Figure 5-1a), and the kinetic parameters were calculated for the exponential growth phase (Figure 5-1b). In the initial growth phase, μ_{max} was higher for WH (0.36 h^{-1}) than for SH (0.33 h^{-1}) (Table 5-2). Indeed, after 12.2 h, 21.7 g/L of total WH substrates were consumed, while the SH substrates consumption was 8.8 g/L. Consequently, a higher Xr of 6.28 g/L (WH) compared to 4.23 g/L (SH) was attained. One major factor affecting the cell growth could be the early onset of PHB production. With SH, the PHB content (% g PHB/g CDM) was 35.3 as compared to 26.3 (WH) and 20 (sucrose [222]) (Table 5-2). This could indicate that the MM itself contained fewer growth-stimulating nutrients. Therefore, the additional nutrients contained

in the WS allowed the flow of acetyl-CoA to the citric acid cycle for a longer period thus allowing for higher cell growth.

The initial exponential growth phase stopped, for both WH and SH, between 12-21h, while cell growth continued at a lower rate (Figure 5-1c**,** d, e, f). This is comparable with the onset of the accumulation phase observed in previous studies [132, 135, 222]. With SH, the cell concentration reached a maximum of 16.1 g/L after 51 h. On the other hand, with WH, the maximum cell concentration reached 23 g/L after already 31 h and stayed approximately constant. However, the cell mass, which accounts for the dilution due to feed addition, continued to increase and reached its maximum of 68.3 g after 48 h. In comparison, a maximum of 24 g/L Xr was obtained after 72h in a previous study [195]. The authors had adjusted the salt concentrations in the medium to provide nutrients for approximately 24 g/L cells as that corresponds to the oxygen transfer limit of the bioreactors used in this study. As the same bioreactors were used in this experiment, the same medium composition was used. Indeed, in the literature, the highest PHB concentrations from lignocellulose were obtained with 18.5 g/L residual biomass (both wheat straw and black spruce, Table 5-3). This shows that the cell density obtained is among the highest reported from lignocellulose hydrolysates.

Table 5-8: *P. sacchari* IPT 101 growth parameters on different carbon sources.

Carbon source	**Time** [h]	**S_{0h}** [g/L]	**ΔS** [g/L]	**μ_{max}** [h^{-1}]	**CDM** [g/L]	**Xr** [g/L]	**PHB** [g/L]	**$Y_{PHB/CDM}$** [%]	**$Y_{PHB/S}$** [g/g]	**$Y_{Xr/S}$** [g/g]	**Ref.**
SH	12.2	23.2±1.2	8.8±0.5	0.33	6.53±0.10	4.23±0.19	2.30±0.04	35.3	0.26	0.48	This study
WH	12.2	24.9±1.3	21.7±1.1	0.36	8.52±0.03	6.28±0.28	2.20±0.05	26.3	0.10	0.29	This study
Glucose, xylose	18	17.6 15.7	n.a	0.25	~10	~7.5	~2.5	25	n.a	0.4	[195]
Sucrose	12	30	n.a.	0.40	13	10.4	2.6	20	0.22	0.4	[222]
Glucose	24	19.8±0.4	17.3±0.5	0.40	6.42±0.04		4.56±0.05	71.0	0.26	0.37	[134]

S_{0h} = Initial sugar concentration (at 0h), **ΔS** = sugar consumption (difference between initial sugar concentration and sugar concentration at given time), **μ_{max}** = maximum specific growth rate, **CDM** = cell dry mass, **PHB** = PHB concentration, **$Y_{PHB/CDM}$** = PHB yield per cell dry mass, **$Y_{PHB/S}$** = PHB yield per sugar consumption.

For the purpose of comparing WH and SH in this study, the cell growth limited by the medium composition was sufficiently adequate to compare the effect of the carbon sources on

the growth parameters (maximum specific growth rate and Xr at the end of the exponential growth phase). The three cultivation phases observed in the bioreactor runs (exponential growth, second growth phase, and accumulation phases) were also reported in a study using the same conditions [195]. The μ_{max} obtained in this study is higher than a previously reported growth rate from a glucose-xylose mixture (0.25 h^{-1}), which can be explained by the higher ratio of glucose:xylose (~2.5:1 as compared to ~1:1) in the carbon source of this study. With other carbon sources (i.e. sucrose or glucose), μ_{max} can reach 0.4 h^{-1} (Table 5-2). To further optimize cell growth, the growth medium and the bioreactor type could be optimized for WH. In the literature, the exponential growth phase in an airlift bioreactor continued until a CDM of 60 g/L, during which the PHB content in the cells was low (~20% g/g). As a consequence, the final Xr was 87 g/L [222]. While the airlift bioreactor used sucrose as carbon source and is therefore not comparable to WH, it was the increased oxygen transfer capacity in the airlift reactor that allowed for a higher Xr, which could also be operated with WH. Changing the cultivation conditions (bioreactor type, corresponding medium composition) could therefore further improve cell growth using WH.

5.5 PHB production

In a typical PHB fermentation, PHB production starts after the exponential growth phase, in which the cells have a low PHB content (up to ~20% g PHB per g cell mass). At the onset of the PHB accumulation phase, the PHB content increases (Figure 5-1c, d, e, f). For WH, the PHB content started rising after 12 h, and for SH, the PHB accumulation started after 10 h. The same effect was reported in the literature, [195] where the intracellular PHB content increased after 10 h and stabilized after 25 h. While this could be attributed to fewer nutrients available in the medium to sustain the exponential growth, the PHB accumulation proceeded faster for WH. Indeed, 50% PHB content was reached after 23 h as compared to 30 h with SH. Nevertheless, at the end of the fermentation, there was a negligible difference in the PHB content between SH (55%) and WH (58%). The main difference between the feedstocks was therefore the higher cell growth (Xr), as discussed in the previous section. As a result, the PHB productivity was higher for WH (0.72 g/(L·h)) as compared to SH (0.46 g/(L·h)).

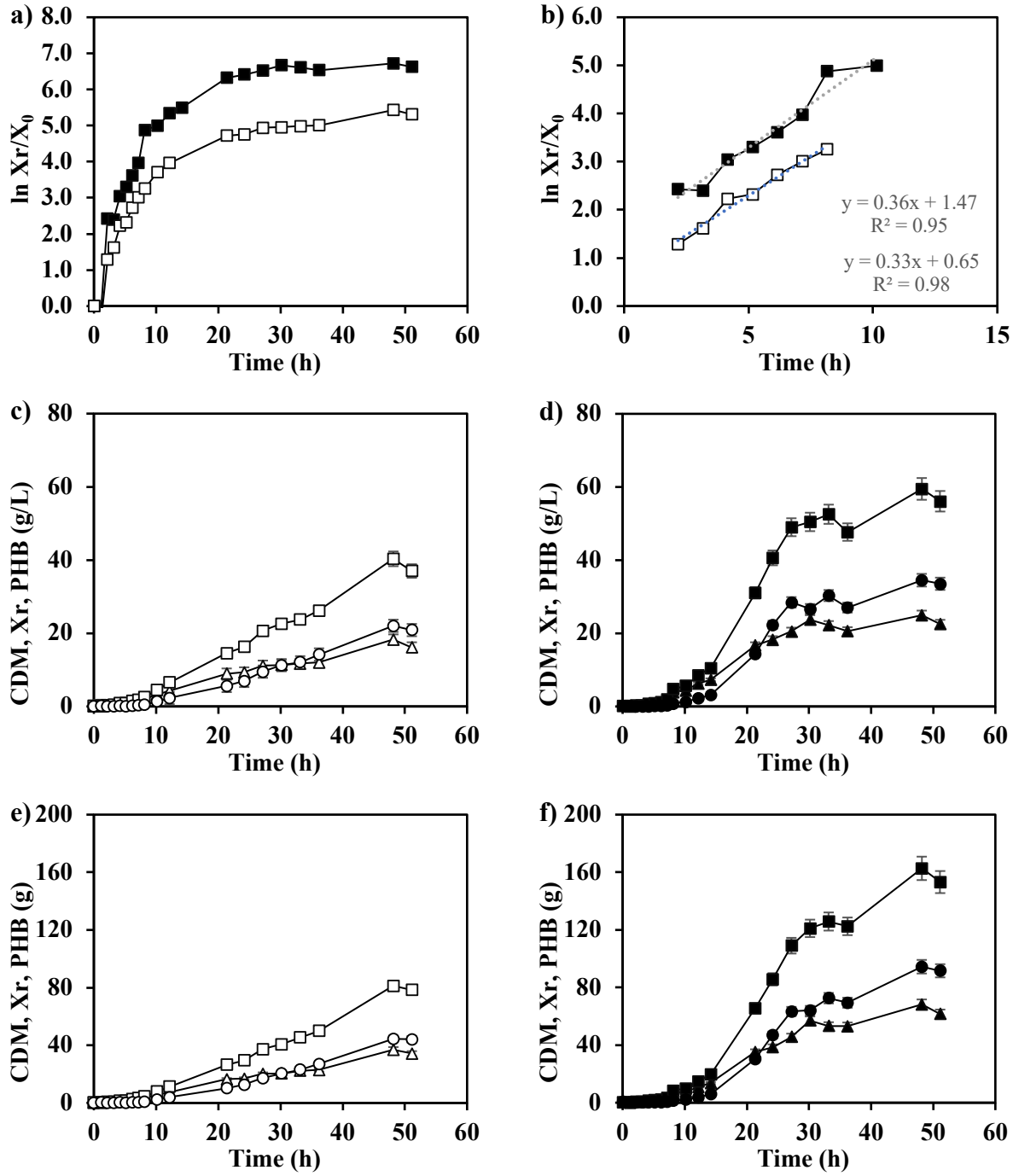


Figure 5-1: High-cell density PHB production with *P. sacchari* using SH (white symbols, c, e) and WH (black symbols, d, f) as carbon source. a) ln Xr/X_0 (square) against time for the bioreactor run. b) ln Xr/X_0 against time in the first hours. c,d) Cell dry mass (CDM) (square), residual bacterial mass (triangle), PHB (circle) in [g/L]. e,f) CDM (square), residual bacterial mass (triangle), PHB (circle) in [g].

The PHB cell content was comparable to literature reports using the same strain and medium (58%) [195], and was in the range of the maximum values reported for *P. sacchari* (60-80%) [116, 132, 134]. For economic competitiveness, maximizing the PHB cell content is important to

reduce the PHB extraction costs [59]. Here we found that WH components are effectively converted to PHB.

5.6 Substrate consumption

The initial substrate concentration varied between 20-30 g/L total sugars (Figure 5-2a, b), with a ratio of glucose:xylose of 2.5:1. Fermentation experiments conducted with either WH or SH showed clear differences in sugar consumption patterns, corresponding to the differences in the respective cell growth. Glucose depletion was monitored by DO, which increased when glucose was consumed and only xylose remained in the medium [132]. Furthermore, the sugar concentration was measured by HPLC, albeit with a delay of approximately 30 min. For WH, the first feed pulse was added at 12.2 h, when glucose was depleted and 2.67 g/L xylose was left. Seven feed pulses were added in total, corresponding to 1 L WH feed (Figure 5-2b). In contrast, SH was first fed after 15.3 h and only four pulses were added (Figure 5-2a). Between 10-35 h, the sugar consumption (g) was constant and as expected, glucose was consumed at a higher rate than xylose (Figure 5-2c,d). For WH, sugar consumption decreased at 35 h and remained constant until the harvest. This could arise from reaching a maximum Xr and nearly maximum PHB content after 30 h. Consequently, the substrate was mostly used for maintenance. For SH, the total sugar consumption increased after 35 h, which could be attributed to ongoing cell growth and increase in PHB content. Overall, 119.98 g/L total sugars were consumed for SH as compared to 237.04 g/L WH. In the course of the fermentation, feeding started after partial xylose depletion, so that it would not accumulate in the medium, as described by Cesário, et al. [132]. Therefore, the ratio of glucose:xylose gradually decreased with the consumption of all sugars (Figure 5-2e). The Xr yield per total substrate (% g/g) was the highest after the growth phase (0.45 for WH after 8.2 h and 0.48 for SH after 12.2 h) and gradually decreased to 0.09 (WH) and 0.16 (SH) at the end of the fermentation. Instead, the PHB yield per total substrate (% g/g) increased to a maximum of 0.2 after 27.2 h (WH) and 0.26 after 12.2 h (SH). This corresponds to the early onset of PHB production with SH. At the end of the fermentation, the PHB yield per total substrate dropped to 0.15 (WH) and 0.19 (SH).

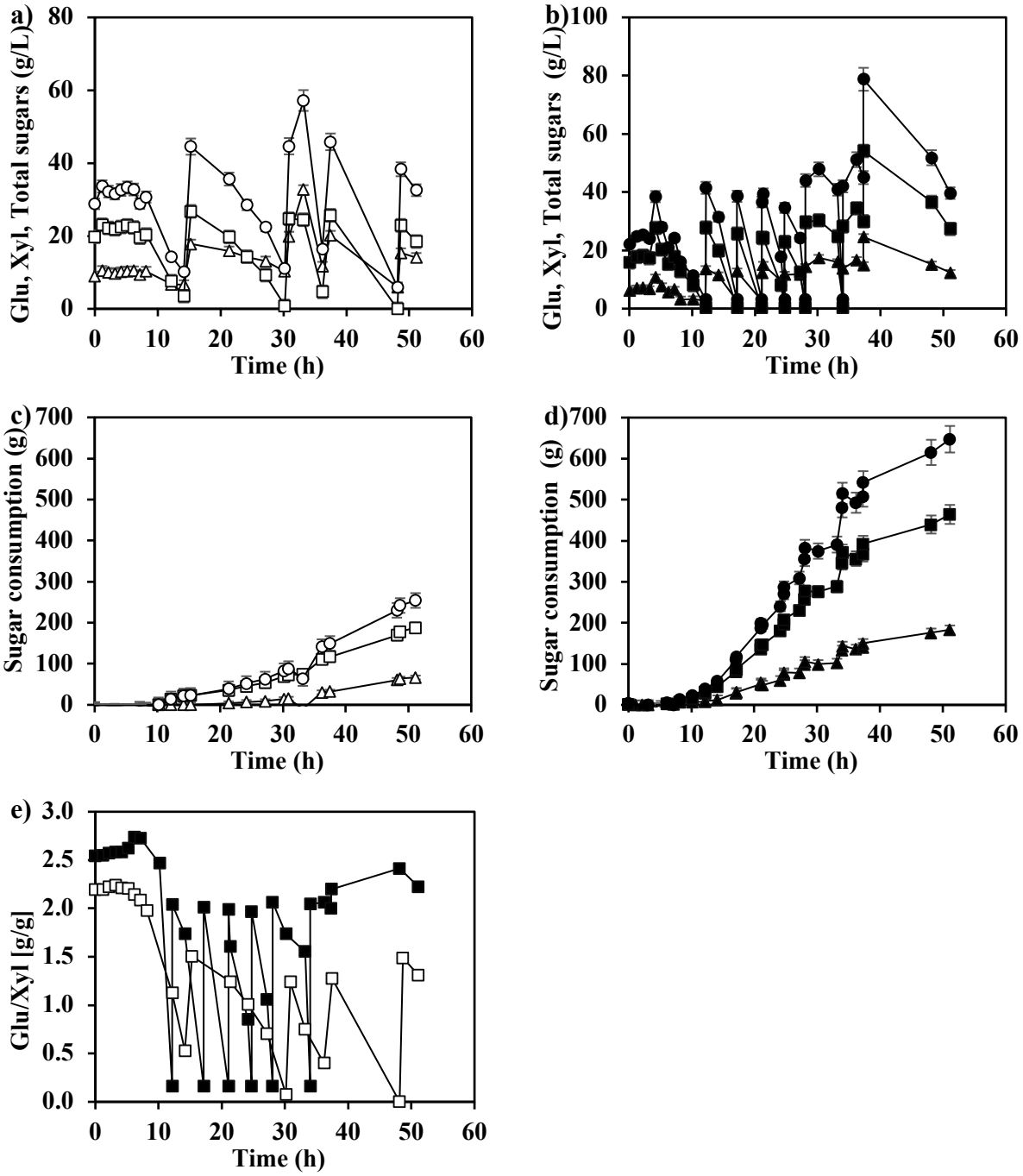


Figure 5-2: Carbon source consumption in high-cell density PHB production with *P. sacchari* using SH (white symbols, a, c, e) and WH (black symbols, b, d, e) as carbon source. a,b) Glucose (Glu) (square), xylose (Xyl) (triangle), total sugars (circle) in [g/L]. c,d) Consumption of glucose (Glu) (square), xylose (Xyl) (triangle), total sugars (circle) in [g]. e) Ratio of glucose to xylose (Glu/Xyl) using SH (white square) and WH (black square) against time.

The consumption of glucose-xylose mixtures has been the subject of several studies [132, 135, 195]. In brief, *P. sacchari* is capable of simultaneous consumption of glucose and xylose without carbon catabolite repression, which makes it a promising strain to convert lignocellulose

hydrolysates to PHB [218]. Compared to the literature, the PHB yield per total substrate in this study was in the range of most lignocellulose hydrolysates (typically 0.22, Table 5-3). While glucose-xylose mixtures from various biomass sources (wheat straw, and spruce sawdust) have shown promising results, it is noteworthy that forest biomass such as hardwood chips have an established collection infrastructure and are therefore sustainably available year round. Since global plastic demand has risen to over 300 million metric tonnes, alternatives will need to be produced in large quantities and therefore will require large amounts of feedstock [204]. Converting WH to PHB in a pilot plant would provide a large quantity of PHB on the market and allow the development of a global bioplastic strategy [204].

5.7 Reactor kinetics

In the current study, phosphorus was chosen as the limiting nutrient and was depleted after 21 h (WH) and 36 h (SH) (Figure 5-3a, b). These results are comparable to those obtained in a previous study with similar medium, where phosphorus consumption started after 10 h and was depleted after 30 h[195]. As the cells grew faster on WH, they consumed phosphorus faster to build phosphorus-containing cell constituents. The ammonium concentration in the bioreactor medium was intended to be above a limiting concentration of >0.19 g/L [222] by having a nitrogen-containing base (NH_4OH) which was automatically added by the pH-regulation of the bioreactor. The regulation was more successful for WH, where the minimum concentration was 0.3 g/L throughout the fermentation (Figure 5-3a and b). For SH, the concentration dropped to 0.07 g/L at 48h. It should be noted that between 30-48 h, the base addition was low, and increased suddenly after 48 h. Although the reason for the lag in regulation is unclear, possible explanation is a delay in communication between the pH electrode and the base pump. Nevertheless, during the exponential growth phase, the concentration of ammonium in both SH and WH was above limiting.

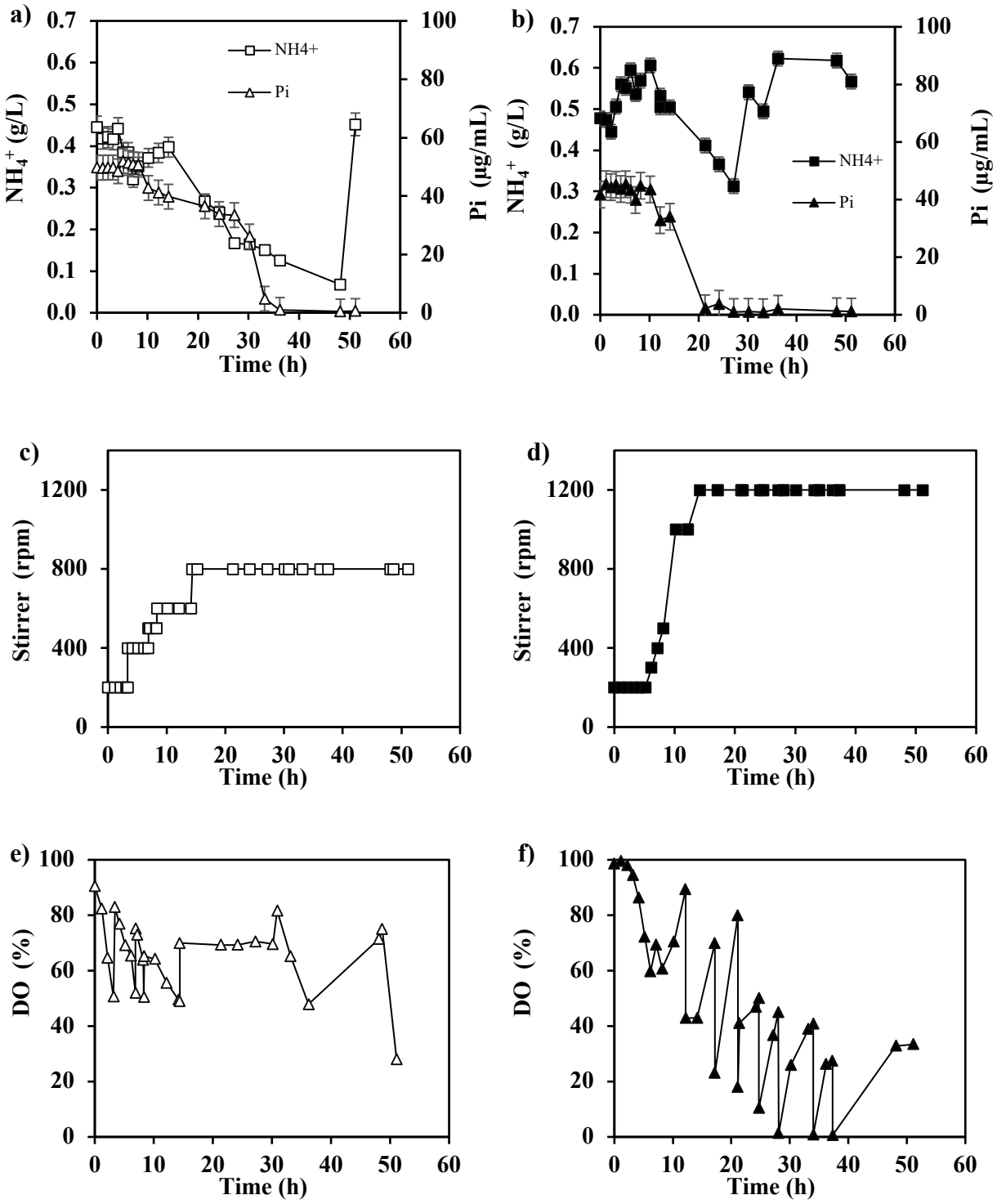


Figure 5-3: PHB production in high-cell density cultivation with SH (white symbols, a, c, e) and WH (black symbols, b, d, f). a, b) Ammonium (NH_4^+) (square), phosphorus (Pi) (triangle) in [g/L]. c,d) Manual setting of stirrer speed (square) in [rpm]. e, f) Dissolved oxygen (DO) (triangle) in [%].

The oxygen supply was regulated with a constant air flow of 1 L/min and a stirrer speed. The stirrer speed needed to be manually adjusted upon a decrease in DO (Figure 5-3c, d). After 17 h, the stirrer speed of WH was set to the maximum of 1200 rpm. Since the stirrer speed is related to the oxygen depletion in the medium, which is correlated to the cell concentration, the highest setpoint of the stirrer speed of SH was lower than that of WH (800 rpm). For WH, once the maximum stirrer speed was set, the oxygen flow into the medium could not be increased, and therefore the cells were cultivated under oxygen limitation. Indeed, the DO dropped to 0% several times after 30 h (Figure 5-3e, f). Increases in DO, which indicate a decrease in metabolic activity due to carbon source exhaustion triggered a feed pulse to the medium.

Table 5-3: Comparison of PHA production from lignocellulose hydrolysates. PHA concentration (PHA), Cell dry mass (CDM), PHA content ($Y_{PHA/CDM}$), PHA productivity (P_{PHA}), Substrate yield ($Y_{PHA/S}$).

Carbon source	Bacterial Strain	Type	PHA [g/L]	CDM [g/L]	$Y_{PHA/CDM}$ [% g/g]	P_{PHA} [g/(L h)]	$Y_{P/S}$ (g/g)	Scale	Ref.
SH	*Paraburkholderia sacchari IPT 101*	PHB	22.0	40.3	54.6	0.46	0.19	fed-batch bioreactor	This study
WH	*Paraburkholderia sacchari IPT 101*	PHB	34.5	59.5	58.0	0.72	0.15	fed-batch bioreactor	This study
Glucose-xylose	*Paraburkholderia sacchari IPT 101*	PHB	35	60	58	0.5	0.22	fed-batch bioreactor	[195]
Sucrose	*Paraburkholderia sacchari IPT 189*	PHB	63	150	42	1.7	0.22	Airlift bioreactor	[222]
Paulownia Wood extract	*Burkholderia cepacia*	3-HB	16.8	n.a.	n.a.	0.08	n.a.	batch bioreactor	[223]
Pinus radiata woodchips	*Sphingobium scionense*	PHB	0.394	1.23	32	0.005	0.22	shake flask	[208]
Wheat Straw (+ GBL)	*Paraburkholderia sacchari*	PHB PHB4HB	105 24	123,5 88	72 27	1,6 0,5	0.22	fed-batch bioreactor	[132] [224]
Spruce sawdust	*Cupriavidus necator*	PHBV	61.8	80.3	77	2.84	n.a.	shake flask, fed-batch bioreactor	[135]
Perennial ryegrass	*Paraburkholderia sacchari*	PHB	15.6	44.5	35	0.38	0.47	fed-batch bioreactor	[219]
Waste office paper	*Cupriavidus necator* NCIMB 11599	PHB	4.45	7.74	58	0.061	0.21	Shake flask	[225]

Oxygen limitation was also reported in high cell density cultivation using wheat straw hydrolysate [132], and was shown to occur concurrently with the phosphorus limitation that was intended to initiate PHB accumulation. To supply sufficient oxygen to the cells, a different type of bioreactor would need to be used. For example, an airlift bioreactor allowed to maintain the DO above 10% throughout the fermentation [222]. However, while the resulting CDM was

150 g/L, the PHB yield was lower (42%) than typical values for the strain (around 70%). This was attributed to a possible connection between oxygen limitation and the trigger of PHB biosynthesis. Therefore, it is unclear if an unlimited oxygen supply would in fact increase the final PHB concentration in the broth.

5.8 Conclusion

A hardwood hydrolysate increased bacterial growth as compared to a synthetic solution with similar amounts of carbon source. The high cell-density fermentation afforded PHB concentrations among the highest reported for lignocellulose hydrolysates (CDM = 59.5 g/L, PHB = 34.5 g/L, $Y_{PHA/CDM}$ = 58% g/g, P_{PHB} = 0.72 g/(L·h), $Y_{P/S}$ = 0.15 g/g). The improved performance is probably due to the higher nutrient content of the hydrolysate as compared to the synthetic solution. Therefore, it could be advantageous to use enzymatically hydrolyzed wood sugars for biotechnological conversion processes as compared to chemical processes, where the nutrient would be unnecessary. Further research should enable the optimization of PHB concentration and productivity by adapting the fermentation medium to the carbon source, and by using a bioreactor with adjusted oxygen transfer capabilities and an automated feeding system.

Connecting Statement to Chapter 6

In Chapter 5, the fermentation of wood hydrolysate yielded a PHB concentration of 34.5 g/L, which is among the highest reported for lignocellulose hydrolysates. In comparison, the use of a synthetic hydrolysate resulted in a lower PHB concentration of 22.0 g/L. This could be attributed to a higher maximum specific growth rate of 0.36 vs. 0.33 per h for cells grown in wood hydrolysate vs. synthetic hydrolysate. It was thus feasible to produce PHB from wood in this forest biorefinery scheme.

In Chapter 6, the harvested cells from wood hydrolysate and simulated hydrolysate fermentation were extracted to recover the PHB. The material properties of the isolated PHB samples were characterized and benchmarked against a commercial PHB obtained from Sigma Aldrich. All samples were also compression moulded into films to test their viscoelastic properties. The chapter is based on a manuscript to be submitted for publication in the journal Polymers under the title “In-Depth Material Characterization of Polyhydroxybutyrate (PHB) from a Forest Biorefinery” with the co-authors Prof. Marie-Josée Dumont, Prof. Valérie Orsat, and Dr. Luis F. Del Rio.

Chapter 6: In-Depth Material Characterization of Polyhydroxybutyrate (PHB) from a Forest Biorefinery

6.1 Abstract

The large scale replacement of petroplastics with compostable plastics, such as polyhydroxybutyrates (PHB), will contribute to the elimination of plastic pollution, decrease greenhouse gas emissions and valorize local biomass resources. Lignocellulose hydrolysates have emerged as potentially sustainable carbon sources for PHB production. For industrial processing, such as extrusion, injection moulding, or compression moulding, it is necessary to know the polymer properties. Yet, most studies on PHB samples from lignocellulose report few material properties.

Here we characterized PHB samples produced from a hardwood holocellulose hydrolysate from a pilot scale conversion process and compared it to PHB from a sugar hydrolysate and a commercial PHB powder. The samples were analyzed as extracted (in form of granules) and as solvent-cast and compression moulded films. Fourier transform infrared spectroscopy and nuclear magnetic resonance confirmed that the samples were homopolymers comprised of hydroxybutyrate units. Differential scanning calorimetry and thermal gravimetric analysis showed that the samples had the same thermal behavior. The melting temperature was around 176 °C and the decomposition temperature was around 293 °C. From the melting enthalpy, a crystallinity of 63% was determined for all samples. Dynamic mechanical analysis showed a glass transition temperature at around 5 °C and a crystallization temperature of 57 °C. The only difference in the samples was found in the number average molecular mass, which ranged from 246.4 kDa for wood hydrolysate to 670.3 kDa for sugar hydrolysate.

The material properties of PHB from wood hydrolysates are comparable to commercial PHB, which is made from edible sugars derived from corn starch or vegetable oils. PHB produced in a wood biorefinery would therefore be suitable for commercial applications, while bringing about an increase in social benefits (e.g. more land availability to grow food crops).

6.2 Introduction

Plastic production has grown from 2 million metric tons in 1950 to 380 million metric tons in 2015, and the demand is still rising. Of the total global generated plastic waste, 79% has ended up in landfills and the natural environment. As a consequence, the current end-of-life management of plastics leads to unprecedented amounts of plastics in terrestrial and aquatic ecosystems [204]. Notably in the marine environment, trillions of floating plastic pieces can have a negative impact on wildlife by ingestion and entanglement [226]. With its excellent biodegradation properties, the intracellular bacterial polyester polyhydroxybutyrate (PHB) prevents plastic waste accumulation [8]. Indeed, PHB biodegrades in industrial composts, home composts, anaerobic digesters under biogas production, and the marine environment. This is an important advantage over the current market leader of biodegradable plastics, polylactic acid (PLA), which neither degrades in home composts nor in marine environments [8]. Since industrial PHB is usually produced from edible biomass such as corn-derived sugars and vegetable oils, lignocellulosic feedstocks (e.g. wheat straw, sugarcane bagasse, hardwood and softwood) is currently explored as a potentially cheaper and more sustainable raw material [218].

The physicochemical properties determine the polymers' processability into consumer products. For example, the thermal stability and crystallization kinetics influence the recovery and cycle time in the melt [227]. For PHB, the main disadvantage is the high melting temperature of about 180 °C, while its decomposition starts at temperatures above 170 °C and shows complete weight loss between 225 and 300 °C [228]. Therefore, assessing the thermal and mechanical properties of PHB is important for its industrial application. Most studies on the production of PHB from lignocellulosic biomass report some material properties, but the choice of characterisation is often not consistent between different studies. In general, the most commonly reported properties are the molecular number and molecular mass average as well as the melting and glass transition temperatures. In contrast, mechanical properties such as the tensile strength and the young's modulus are not reported.

Standard PHB (from other carbon sources) is a well-characterized polymer, and the material properties mostly change when different monomers are used (such as 3-hydroxyvalerate or aromatic side chains from lignin precursors). Short chain length PHAs have a high degree of crystallinity of 60-80% [110]. As a semi-crystalline polymer, PHB has a glass transition temperature (T_g) and a crystalline melting temperature (T_m) [228]. For PHB from lignocellulose,

the T_g is between 2.4 °C [219] and 7.3 °C [124], which is close to known values of PHB of 5-9 °C. The reported PHB polymers melt between 170 °C [229] and 176.3 °C [208] , compared to a literature value of 177 °C [228]. Depending on various factors such as the type of organism and culture conditions, the molecular mass of PHAs ranges from 200-300 kDa [110]. The molecular mass average for PHB from lignocellulose ranges from 360 to 1700 kDa. Bowers, et al. [208] reported a high molecular mass of 1233 kDa with a very high polydispersity index (PDI) of 8.4 from high-temperature mechanical pre-treatment softwood hydrolysate. Exceptionally high molecular mass of 1400–1700 kDa were found by Yu and Stahl [122]. Their *co*-polymer was produced in *R. eutropha* grown on bagasse hydrolysates containing various byproducts such as formic acid, acetic acid, furfural and acid soluble lignin. The PDI was rather uniform and ranged between 1-3. For mechanical properties, while the Young's modulus (3.5-4 GPa) and tensile strength (40 MPa) of standard PHB are similar to isotactic polypropylene, the elongation at break of PHB is very poor (3-8%) as compared to the petropolymer (400%) [228]. For PHB from lignocellulose, these values are not reported.

Since the carbon source has the highest impact on the PHB price in industrial scale PHB production, here the material properties of a PHB made from a wood hydrolysate (WH) to PHB made from a sugar solution that simulates wood hydrolysate (SH) were compared. The WH was obtained by enzymatic hydrolysis of pretreated hardwood chips in the thermomechanical pulping-based process (TMP-Bio), developed by FPInnovations [211]. The forest industry provides a solid infrastructure for sustainable and accessible fractionated lignocellulose streams that can be explored for PHB production [218]. If the PHB samples have material properties that are comparable to those of standard PHB, the wood hydrolysate can be combined with other precursors (such as levulinic acid, gamma-butyrolactone and lignin units) to produce forest-based *co*-polymers with tailored material properties. Furthermore, PHB can also be processed in the melt if the conditions are controlled to prevent degradation [228].

6.3 Materials & methods

6.3.1 PHB standard

Commercial PHB from natural origin was purchased from Sigma-Aldrich (MilliporeSigma Canada Co., Oakville, Canada). The product number was 363502 and the PHB was in form of white powder with a T_m of 172 °C.

6.3.2 PHB extraction

The PHB extraction followed a previously described protocol [224]. After lyophilization, the harvested biomass was ground in a mortar. The PHB was extracted in a capped glass flask (1 L) by stirring the dried cell mass (50 g) in chloroform (500 mL) at 4 °C for 40 h. The suspension was heated in a water bath to 50 °C to increase the viscosity prior filtration. The remaining cell debris in the warm suspension was separated by vacuum filtration using a Whatman #4 filter paper (cellulose, 20-25 µm) and glass microfiber filters GF5 (0.7 µm pore). The liquor was condensed by removing the chloroform under vacuum to a final volume of 150 mL. PHB was precipitated from the solution by slowly pouring the liquid phase into 2 L of previously ice cooled ethanol in a 2 L glass flask under gentle agitation. The precipitate was allowed to settle overnight at 4 °C. The solid was recovered by vacuum filtration using Whatman #4 filter paper. Prior to material characterization, PHB (1 g) was redissolved in chloroform (10 mL) at 50 °C and precipitated by slowly pouring the liquid phase into ice cooled ethanol (100 mL) under gentle agitation. The purified PHB was recovered by vacuum filtration using glass microfiber filters GF5 (0.7 µm pore). Lastly, the remaining solvent was allowed to evaporate by air drying in Petri dishes for 24h at room temperature. The extraction yield (Y_e) was calculated by Equation 6-1.

$$Y_e[\%] = \frac{\text{PHB [g]}}{\text{CDM [g]}} \cdot 100 \tag{6-1}$$

6.3.3 Fourier transform infrared spectroscopy (FTIR)

The polymers' functional groups were determined by FTIR [230]. The spectra were recorded at room temperature on a Nicolet iS5 FTIR spectrometer (Thermo Scientific). The wavenumber range was between 500-4000 cm^{-1}. The spectra were recorded with a resolution of 4 cm^{-1} and 32 scans per sample.

6.3.4 Nuclear magnetic resonance (NMR)

One milligram of PHB was dissolved into 0.7 ml of deuterated chloroform for NMR analysis (Bruker Ascend 500, Bruker Co., Billerica, MA). ^{1}H and ^{13}C NMR spectra were

obtained at 500 MHz and the data were collected and analyzed using the software TopSpin 4.0.7 (Bruker Co., Billerica, MA).

6.3.5 X-ray diffraction (XRD)

For the XRD measurement, thin films with a smooth surface area were prepared by solvent-casting. PHB granules (1 g) were dissolved in chloroform (10 mL) at 50 °C and poured into a 100 mL glass beaker. The samples were kept in the fumehood for 2 h to allow for the solvent to evaporate. The films were separated from the beaker and air dried overnight.

Diffractograms of powder and film samples were obtained using a X-Ray diffractometer (Bruker D8 Discovery Diffractometer with scintillation counter detector and Cu-anode for X-Rays at 1.54 Å wavelength). The scans were conducted within the 2θ region of 4 to 104 degrees at a step size of 0.005° and 5° per min scan speed. The X-ray tube was energized at 40 kV and 40 mA mode for the data collection. The analysis was performed against the Powder Diffraction File (PDF) database (PDF4+) of the International Centre for Diffraction Data (ICDD) for the identification of the crystalline phases possibly present in the samples.

6.3.6 Gel permeation chromatography (GPC)

Samples were determined using an Agilent 1200 gel permeation chromatography (GPC) system, with Shodex 804L-805L-806L columns. The samples were dissolved in HPLC-grade chloroform at a concentration of 1 mg/mL and filtered through 0.22 µm PTFE membrane. Chloroform was employed as the mobile phase at a rate of 0.7 mL/min at 50 °C. Polystyrene with low polydispersity was used to construct a conventional calibration with a range from 605 to 2,996,000 Da (Agilent).

6.3.7 Thermogravimetric analysis (TGA)

The thermal decomposition was measured with a TGA Q50 (TA Instruments, Texas, USA). Samples of 10±2 mg were heated at a heating rate of 20 °C/min over a temperature range of 25-400 °C. A plot of mass percentage against temperature was obtained for each polymer. Samples were vacuum dried at 30 °C for 24 h prior to testing. The summarized results are averages of three samples tested.

6.3.8 Differential scanning calorimetry (DSC)

The heat capacity and phase transitions were measured with a Q2000 (TA instruments, Texas, USA). The samples (2-3 mg) were measured in the heat/cool/heat mode between -80 °C

and 190 °C. The cycle started with cooling to -80 °C by 20 °C/min ramping. Afterwards, the first heat cycle started with ramping at 20 °C/min to 190 °C, followed by cooling and heating cycles. The recorded signal provided the T_m and crystallization temperature (T_c). The measurements were conducted in duplicates. The percent crystallinity (X_c) was calculated by Equation 6-2. The melting enthalpy per gram PHB sample (ΔH^*) was obtained experimentally from the integration of the endothermic melting peak, and the melting enthalpy for 100% crystalline PHB (ΔH^0) was previously reported as 146 J/g [231].

$$X_c(\text{PHB}) = \frac{\Delta H^*}{\Delta H^0} \cdot 100 = \frac{\Delta H^*}{146\ \text{J/g}} \cdot 100 \quad (6\text{-}2)$$

6.3.9 Dynamic mechanical analysis (DMA)

For the DMA measurements, films were prepared by compression moulding following a procedure by Abdelwahab, et al. [232]. The PHB granules from WH and SH were ground in a metal grinder (Arthur H. Thomas Co. / Thomas Scientific, Philadelphia, U.S.A.) and collected through a mesh of size 10. Next, the polymers were dried at 85 ºC for 24 h in a vacuum oven. SG powder, ground SH and WH (each 750 mg) were pressed on a Teflon-sheet in a laboratory press (Dake Corporation, Grand Haven, Michigan) at 180 °C for 3 min at 3851 psi. The hot Teflon-sheet was immediately put into the freezer for 5 min and afterwards air dried at room temperature. The films had a thickness ranging from 0.10 to 0.40 mm.

The dynamic mechanical properties were measured with a DMA Q800 from TA instruments. The tension clamp was used in multifrequency-strain mode to measure rectangular film specimen of approximately 10 x 8 x 0.5 mm. An amplitude of 10.0 µm, a frequency of 1 Hz, a preload force of 0.01 N, and a force track of 0.125% was used. The specimens were heated at a rate of 3 °C/min from -50 °C to 120 °C. The start and end temperatures were kept constant for 1 min. Nitrogen was used as the purge gas.

6.4 Results & discussion

6.4.1 Extraction yield

The extraction of PHB from bacterial cells yielded 14.84 g of PHB granules for SH and 16.31 g for WH. This corresponds to an extraction yield of 29.68% for SH and 32.61% of WH,

which is about 50% of the PHB present in the cells when the bioreactor runs were stopped. The extraction technique applied was a cold extraction at 4 °C, which is used to preserve the crystallinity of the PHB as synthesized in the bacterial cells [224]. Other extraction techniques, such as Soxhlet-extraction, are optimized to recover the maximum amount of solute, but in this study, the interest was focused on the materials' properties. Therefore, the low temperature treatment was chosen. In general, extractions with less toxic solvents, such as 1,2-propylene carbonate, have been developed by other groups, reporting extraction yields of up to 95% [233].

PHB is the most common biological polyester. Independent of the microorganism, the product of the synthesis is an isotactic polymer of (*R*)-3-hydroxybutyric acids. Figure 6-1 shows the FTIR spectra of the three PHB samples. In the fingerprint region, the peaks at 1165 cm^{-1} and 1280 cm^{-1} correspond to the typical C-O-C vibration of PHAs [234]. In the group frequency region, the characteristic peak at around 1721 cm^{-1} is the C-O stretching vibration of the carbonyl group. The transmission bands at 2900 cm^{-1} correspond to the C-H stretching [234]. The infrared transmission pattern is therefore typical for PHB samples and shows no difference between the samples.

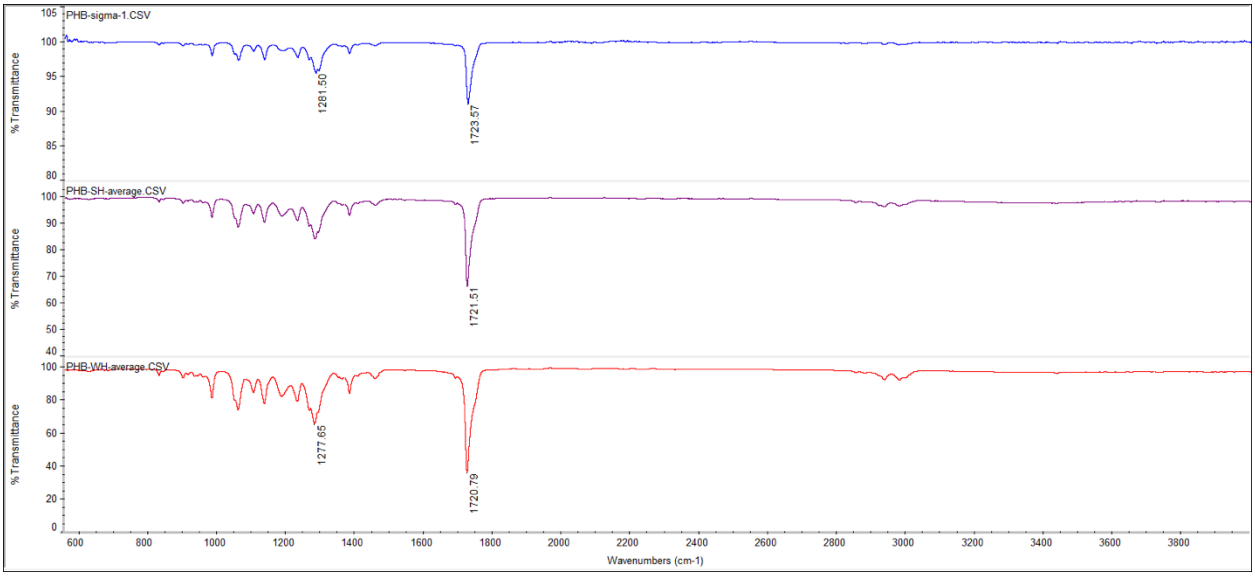
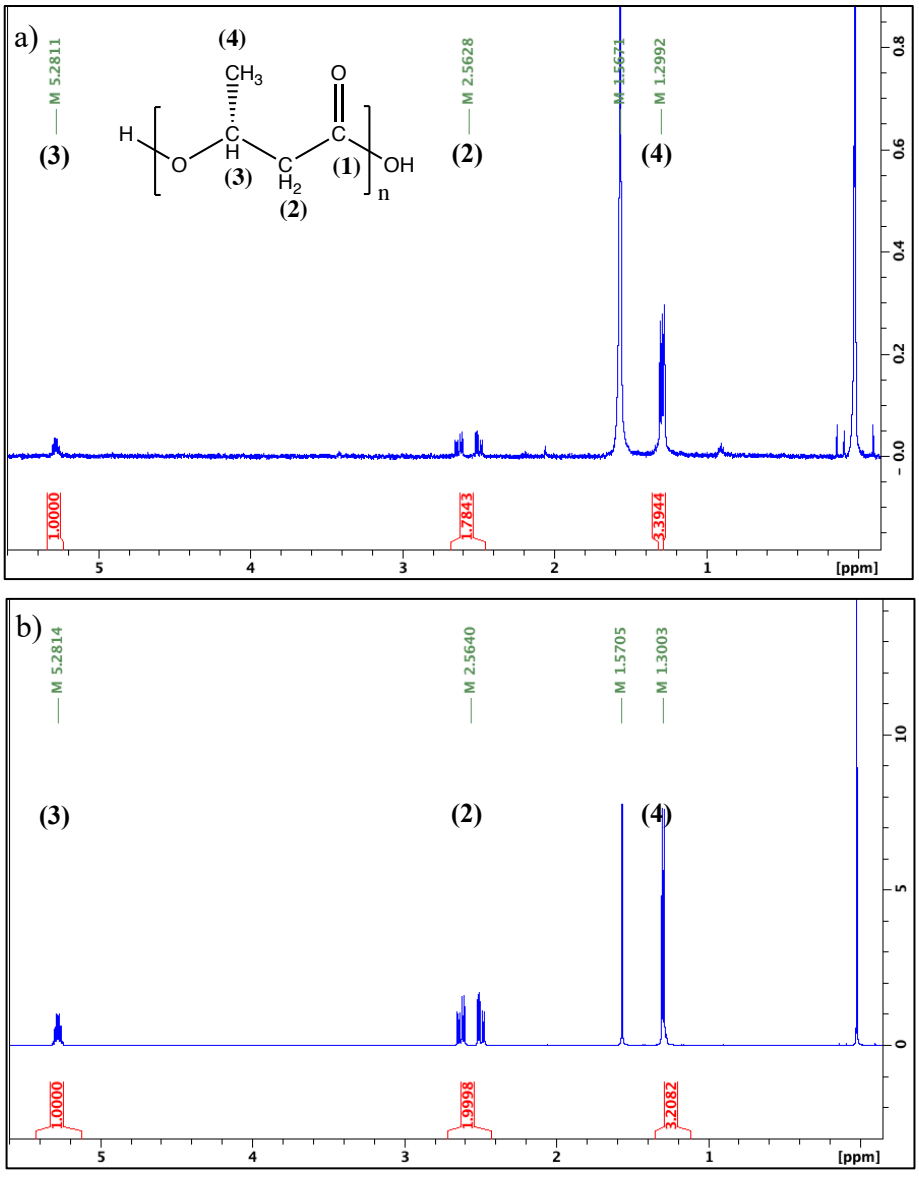
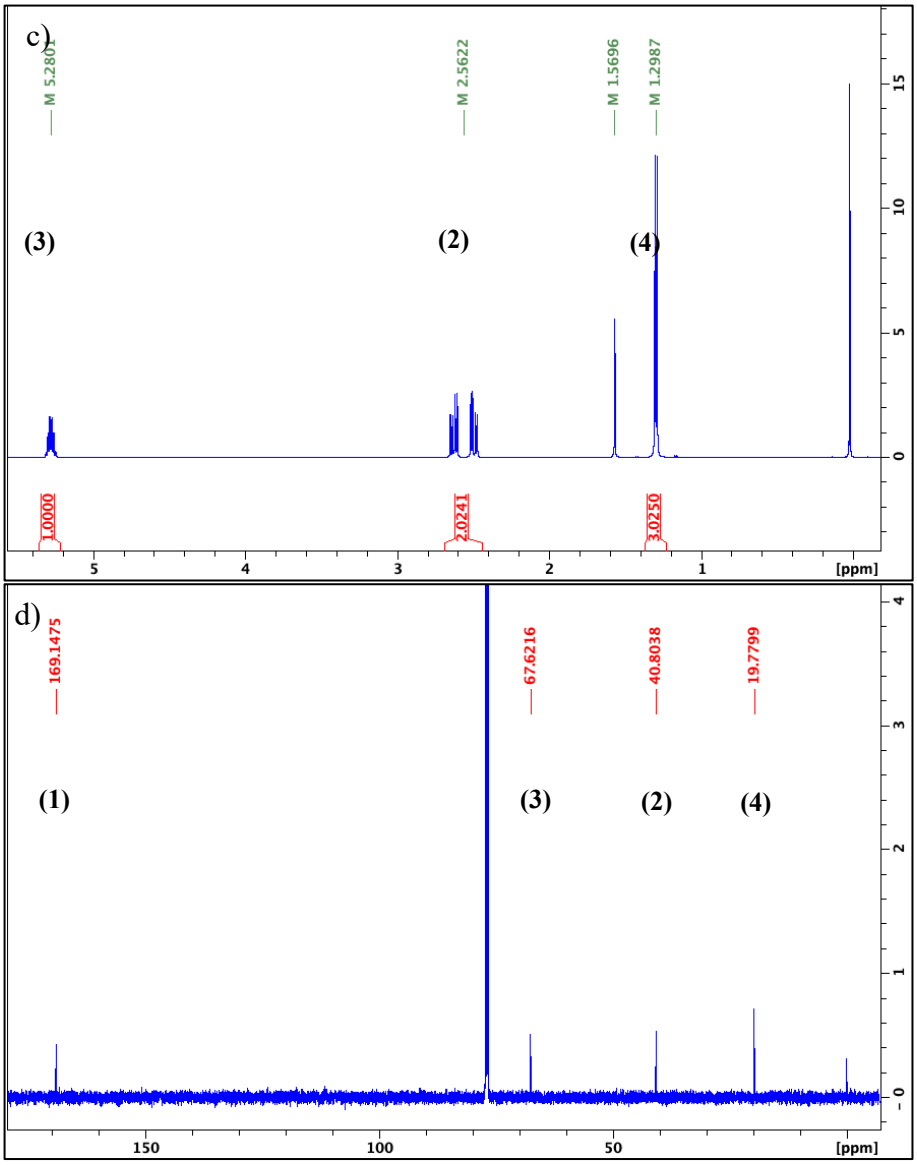


Figure 6-1: FTIR transmission spectra of PHB standard (top spectrum, PHB-sigma-1), PHB from sugar hydrolysate (middle spectrum, PHB-SH-average) and PHB from wood hydrolysate (bottom spectrum) in the 500-4000 cm^{-1} region. The spectra are an average of triplicate measurements.

The ^{1}H and ^{13}C NMR (Figure 6-2) spectrum confirmed the chemical composition of the PHB (Figure 6-2, the corresponding proton and carbon signals labelled as 1-4). In the ^{1}H NMR

spectra, the typical chemical shifts of PHB are a doublet at 1.30 ppm from -CH_3 (4), a multiplet at 2.56 ppm from -CH_2 (2), and a multiplet at 5.28 ppm for -CH (3). The peak at 1.5 ppm is from water. The ^{13}C NMR chemical shifts are 20 ppm (4), 41 ppm (2), 68 ppm (3) and 169 ppm (1). Similar spectra, including residual water, have been obtained of PHB produced from softwood hydrolysate [208] .

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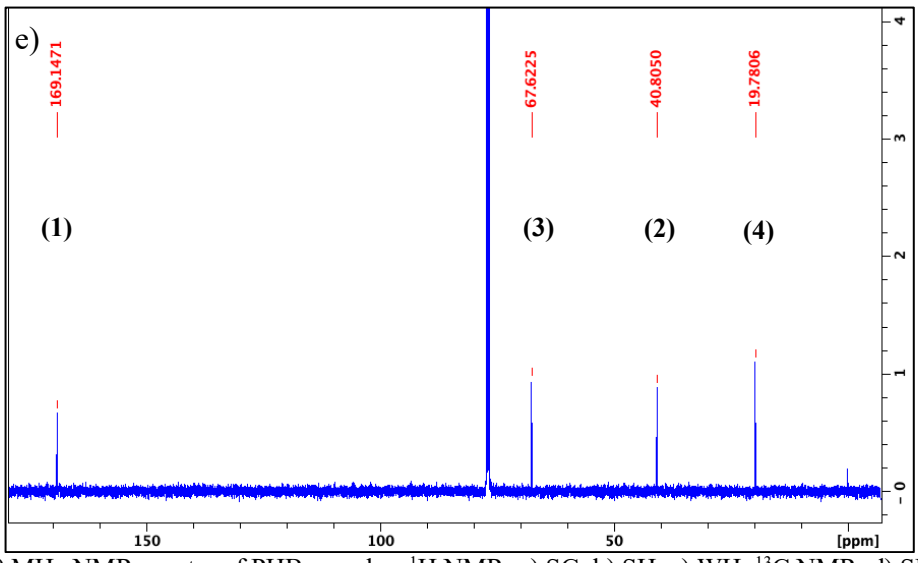

Figure 6-2: 500 MHz NMR spectra of PHB samples. ^{1}H NMR: a) SG, b) SH, c) WH. ^{13}C NMR: d) SH, e) WH. The ^{13}C NMR of SG was not measured because of poor solubility of the powder in chloroform. The resonance peaks (1–4) correspond to the hydrogen and carbon atoms present in PHB and correspond to literature values [208] .

The crystalline regions of PHB are in an orthorhombic unit cell (a=0.576 nm, b=1.320 nm, c=0.596 nm) in the space group P212121 [235]. The crystallinity of PHB samples depends largely on the sample preparation method. For example, PHB can be in the form of a single crystal, which are monolamellar systems, or in form of films and plates, which are multilamellar to varying degrees. PHB chains form spherulites at a maximum rate at 90 °C, while the overall crystal growth is at a maximum between 50-60 °C. Here, SH and WH solvent-cast films were measured by XRD. Since the films were cast at room temperature, there were fewer crystalline regions. XRD analysis (Figure 6-3) showed that there is no noticeable difference between SH and WH. The red bars show the predicted PHB peaks for crystalline PHB. The film samples differ from the predicted values and indicated a mostly amorphous structure.

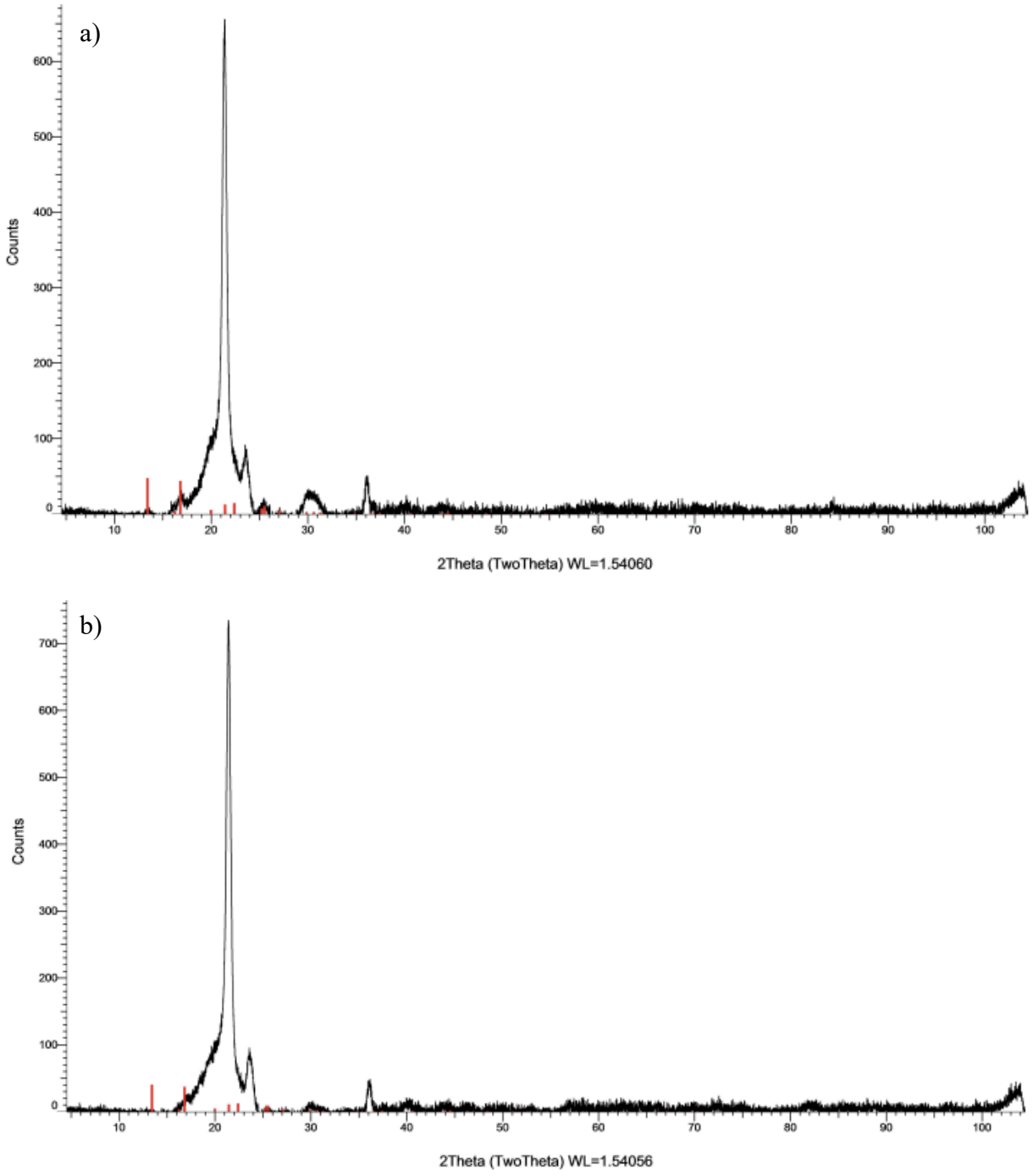

Figure 6-3: XRD patterns of PHB. a) SH b) WH.

Different carbon sources, microorganism and cultivation conditions influence the molecular mass of PHB [235]. One reason that was suggested was the influence on the activity of PHB synthase. A higher activity was shown to result in lower M_n. Ultra-high M_w is desirable since it improves the mechanical properties of PHB (increased stretching for films). After PHB

extraction, sample preparation can reduce the molecular mass through random chain scission. The rate increases at higher temperatures. Here, the cold-extracted PHB samples were measured by GPC. M_n, M_w and pdi are shown in Table 6-1. SH had both a higher M_n and M_w than WH. This could mean that cells grown on WH have a higher activity of PHB synthase, but this would need to be validated. For WH, the M_n and M_w was within the literature values for PHB from lignocellulose hydrolysates. The PDI of WH is higher than for SH and also within literature values. For example, PHB from softwood hydrolysates had a high PDI of 8.4, which indicates that lignocellulose hydrolysates can result in a higher difference in polymer chain length. The molecular mass of PHB from WH is therefore not ultra-high, but within literature values.

Table 6-1: Material properties of PHB from lignocellulose hydrolysates. T_g - Glass transition temperature, T_m - melting temperature, T_c - crystallization temperature, T_d - Thermal degradation onset temperature, X_c – ratio of crystallinity, ΔH_m - melting enthalpy, M_n - molecular number, M_w - molecular mass, pdi - polydispersity index (M_w/M_n).

Carbon source for PHB	T_g,[1] °C	T_m,[2] °C	T_c,[1] °C	T_d,[3] °C	X_c,[2] %	ΔH_m,[2] J/g	M_n,[4] kDa	M_w,[4] kDa	PDI[4]	Ref
SG[5]	5.2±0.3	174±0	57±3	287±0	62.5	91.19	/	/	/	This study
SH	4.4±0.5	177±1	61±3	291±4	64.7	94.42	670.3	951.1	1.42	This study
WH	6.1±0.3	177±1	54±2	300±1	60.4	88.25	246.4	809.5	3.29	This study
Softwood hydrolysate (WP01-HTMP)	2.5	176.3	/	/	55.8	81.5	146	1233	8.4	[208]
Ensiled grass press juice	2.44	172.6	/	288	/	-16	238	548	2.3	[219]
Wheat straw hydrolysate & gamma-butyrolactone	/	171.7	68.8	279	/	94.6	580	790	1.4	[224]
Maple Hemicellulose hydrolysate	7.3	174.4	/	267	/	/	451	1015	2.3	[124]

[1] Determined by DMA.
[2] Determined by DSC.
[3] Determined by TGA.
[4] Determined by GPC.
[4] The GPC could not be measured due to an insufficient solubility in chloroform.

The decomposition temperature was determined with a TGA (Figure 6-4). The decomposition occurred in a single step starting at an extrapolated onset temperature above 280 °C under N_2 (Table 6-1). Other PHB samples from lignocellulose showed similar decomposition temperatures ranging from 267-288 °C [124, 219, 224]. The thermal degradation is a result of chain scission and hydrolysis, which leads to lower molecular mass polymers and crotonic acid [236]. The onset temperature can vary by 15 °C, depending on the sample's

morphology, which changes the surface area. WH particles were denser than SH and SG, which can explain the delayed onset of the decomposition.

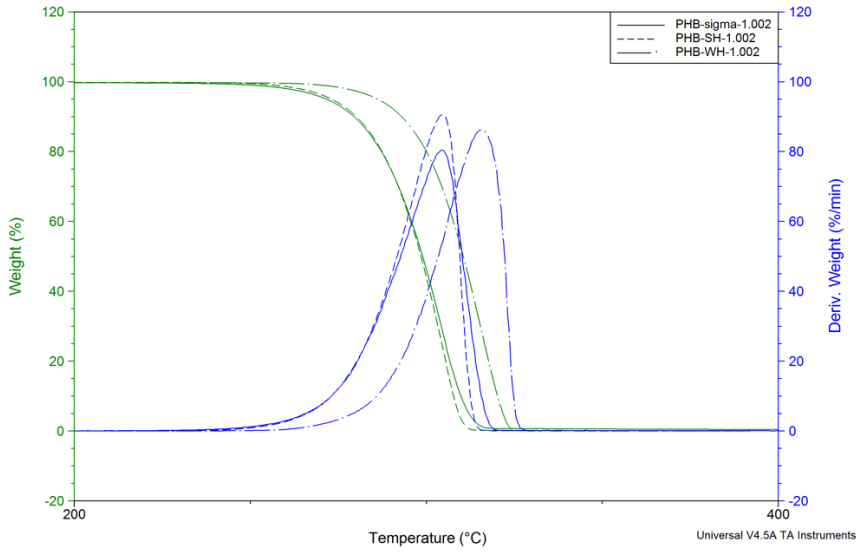

Figure 6-4: Thermograms (green = weight loss and blue = derivate of the weight loss curves) of three PHB materials (SG, SH, WH) obtained from TGA.

The thermal transitions of the extracted PHB samples were recorded in thermograms (Figure 6-5). The thermograms were the same for all PHB samples. Upon heating, the crystalline samples started to melt at an onset temperature of 160 °C, and the peak of the endothermic transition occurred between 174-177 °C (Table 6-1). This is in good agreement with the literature values (171-176 °C) [124, 208, 219, 224]. Upon cooling, the exothermic crystallization started at an onset temperature of 108-117 °C and the peak had a maximum temperature between 96-109 °C. Typically, T_c occurs between 55-69 °C when it is determined in the second heating scan after quenching of the melt to a fully amorphous state at -100 °C. In this study, the T_c was obtained from the controlled cooling of the melt, thus the difference can be attributed to the experimental setup rather than the polymer samples. The melting enthalpy and crystalline enthalpy were similar across the samples and were between 88-91 J/g and 72-77 J/g, respectively. The crystallinities of the PHB samples were thus uniform between 60-65%, which are typical values for PHB after several days of storage (58-68%) [237].

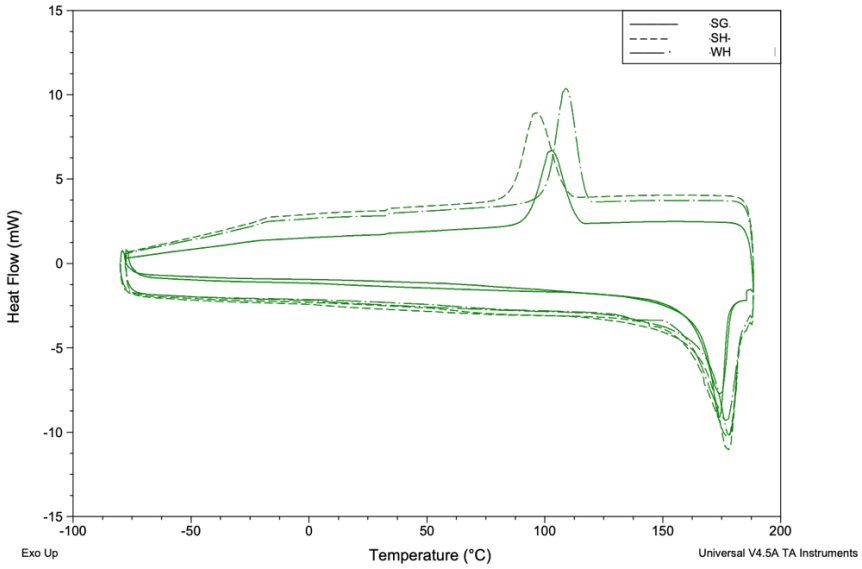

Figure 6-5: Calorimetric curves of PHB samples showing T_m and T_c with heat/cool/heat cycle.

The viscoelastic properties (storage modulus (E'), loss modulus (E'') and $\tan \partial$) of compression-moulded PHB films were determined with a DMA (Figure 6-6). The dissipation of energy manifests itself as internal friction in a polymeric material. The internal friction can be quantified by $\tan \partial$, which is the ratio of the energy dissipated per cycle to the energy stored during the cycle (i.e., E''/E'). All PHB samples showed the same transitions in the measurement. Temperature dependent changes in E' showed the onset of T_g between 4.40-6.09 °C (Table 6-1), which is in good agreement with literature values. Furthermore, a smaller transition occurred between 54-61 °C, which can be attributed to the onset of T_c. As the temperature allows a higher rate of crystallization, the molecular movement of the chains is more restricted and thus the drop of E' is slower. Dynamic mechanical properties are further influenced by the presence of cracks and voids in the semicrystalline films [238]. Between the samples, the results differed little, which indicated that the spherulite growth, after the compression moulding, was similar for all samples.

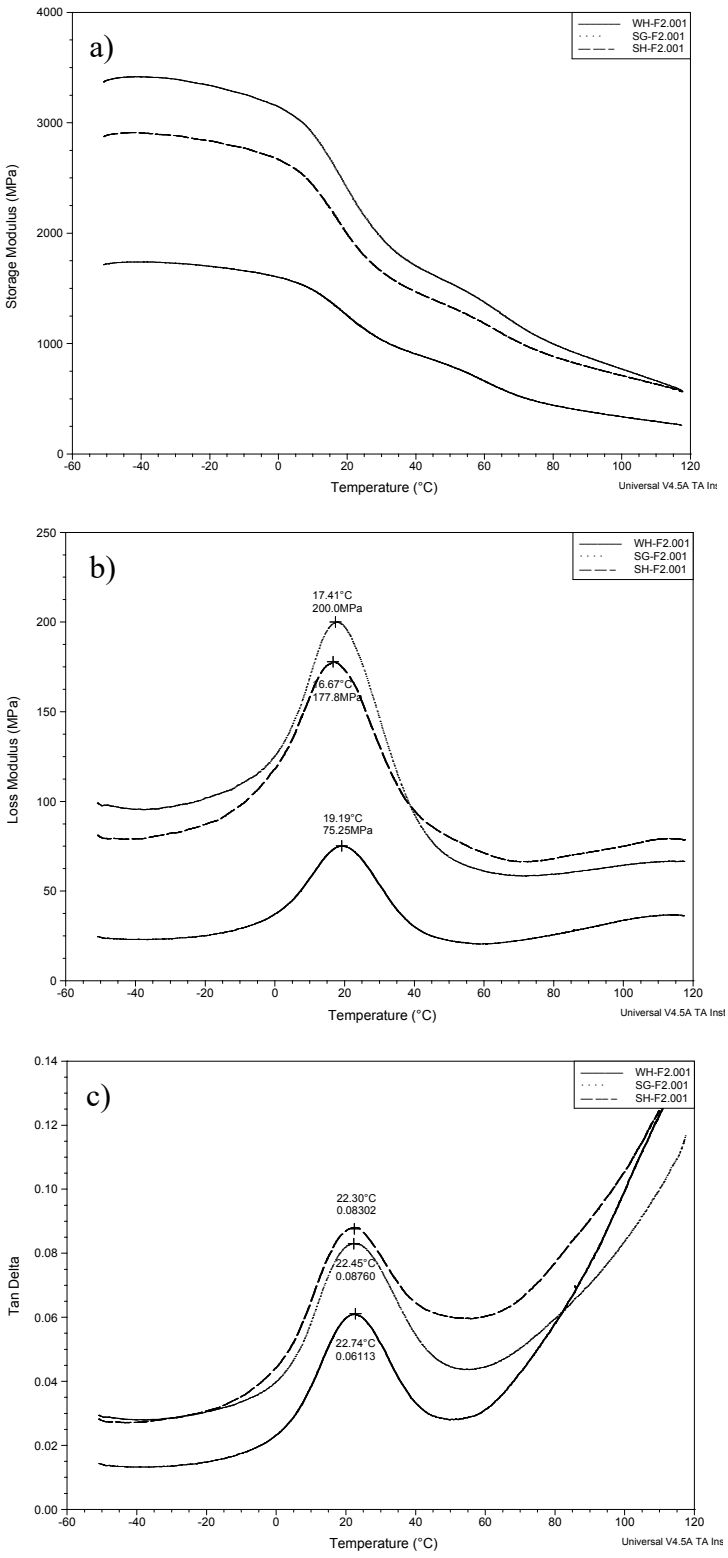

Figure 6-6: a) Storage modulus (*E′*), b) Loss modulus (*E″*) and c) Tan δ for SG (solid line), SH (dotted line) and WH (dashed line). Compression-moulded samples were measured during temperature sweeps at 3 °C /min with a DMA.

6.5 Conclusion

PHB from wood hydrolysate exhibits the same material properties as from synthetic hydrolysate and as commercial PHB. The main difference between the polymers were the average molecular masses, which were higher for SH than for WH (M_n of 670.3 kDa and PDI of 1.42 compared with M_n of 246.4 kDa and PDI of 3.29, respectively). The polymer composition was confirmed by Fourier transform infrared spectroscopy and nuclear magnetic resonance. All samples showed similar thermal behavior with a melting temperature around 176 °C and a decomposition temperature around 293 °C. The solid PHB samples, obtained as granules after extraction, had a crystallinity of 63%. The glass transition occurred around 5 °C and the crystallization at a temperature of 57 °C, as is typical for PHB samples. These results confirm the feasibility of replacing PHB made from edible plants (e.g. corn starch) with PHB produced in wood biorefineries.

Chapter 7: General Conclusions and Recommendations

7.1 General conclusion & summary

The integration of PHB production in forest biorefineries has been explored for two different feedstocks, which can be applied to two biorefinery schemes. In the first scheme, softwood chips were subjected to a prehydrolysis treatment, which hydrolyzes hemicelluloses. The hemicellulose hydrolysate was evaluated as a feedstock for PHB production, while the solid residue, composed of cellulose and lignin, was a feedstock for conventional pulping. In the second scheme, hardwood chips were subjected to thermomechanical pretreatment followed by enzymatic hydrolysis, which hydrolyzed cellulose and hemicelluloses with the recovery of a pure lignin stream. The lignin was the main biorefinery product, and the holocellulose hydrolysate was evaluated as a feedstock for PHB production.

In Chapter 3, softwood hemicellulose hydrolysate components were studied for their fermentability with *P. sacchari*. Softwood hemicellulose sugars (glucose, mannose, galactose, xylose and arabinose) are more diverse than most hemicellulose sugars, which are typically glucose, xylose and arabinose. The study showed that these sugars were suitable carbon sources for PHB production. Mannose and galactose exhibited maximum specific growth rates of 97% and 60% relative to pure glucose, respectively. Sugar mixtures could be converted to PHB and reached a maximum PHB concentration of 5.72 g/L and 80.5% PHB after 51 h. However, the presence of side components (sodium acetate, HMF, furfural, and vanillin) completely inhibited bacterial growth. A mixture design allowed to predict the inhibitory effect based on antagonistic effects of the compounds when present in mixtures, which could be overcome in simulated hydrolysate solutions by increasing the initial optical density of the inoculum to $OD \geq 5.6$. Nevertheless, the strong inhibitory effect showed that real softwood hemicellulose hydrolysates need detoxification to allow bacterial growth.

In Chapter 4, the results from the simulated softwood hydrolysate were applied to real softwood hemicellulose hydrolysate fermentation. In contrast to the simulated softwood hydrolysate, increasing optical density did not work for the real hydrolysates. To further explore this effect, it was hypothesized that growing the bacteria to a later growth stage (early stationary phase) would increase their resistance to the inhibitors in the hydrolysate since the cells are

larger, more numerous and physiologically fitter. Furthermore, to study the effects of increasing sugar diversity and inhibitor concentration, the softwood hydrolysate was mixed in varying proportions with a hardwood holocellulose hydrolysate obtained from FPInnovations' TMP-Bio process. This allowed tracking the consumption of all softwood hemicellulose hydrolysate components. The sugars were depleted at different rates in the following order: glucose, mannose, xylose, galactose, and arabinose. While acetate, HMF, and furfural were all metabolized by *P. sacchari,* phenols were not consumed. Softwood hemicellulose hydrolysate was thus consumed by the strain, but it was the hardwood holocellulose hydrolysate that resulted in a maximum cell dry mass of 6.7±0.1 g/L with 71±5% PHB after 48 h.

In Chapter 5, the promising results found for hardwood hydrolysate fermentation in shake flasks were further evaluated in high-cell density fermentations in fed-batch bioreactors. The wood hydrolysate was compared to a simulated hydrolysate consisting of glucose, xylose and acetate. The fermentation of wood hydrolysate yielded a PHB concentration of 34.5 g/L, among the highest reported for lignocellulose hydrolysates. In comparison, the use of a synthetic hydrolysate resulted in a lower PHB concentration of 22.0 g/L. This could be attributed to a higher maximum specific growth rate of 0.36 vs. 0.33 per h for cells grown in wood hydrolysate vs. synthetic hydrolysate. The industrially mature production process of the wood hydrolysate prevented inhibitor formation and preserved nutrients, with the latter being the likely cause of the higher growth rate. These results showed that PHB production from wood is feasible using this forest biorefinery scheme.

In the last chapter, the PHB from wood hydrolysate and from simulated hydrolysate was extracted from the cell harvest and characterized for its material properties. The PHB samples were compared to commercial PHB from Sigma Aldrich. All samples were also compression moulded into films to test their viscoelastic properties. The samples consisted of pure homopolymers, as identified by Fourier transform infrared spectroscopy and nuclear magnetic resonance. Differential scanning calorimetry and thermal gravimetric analysis showed that the samples had the same thermal behavior. The melting temperature was around 176 °C and the decomposition temperature around 293 °C. From the melting enthalpy, a crystallinity of 63% was determined for all samples. Dynamic mechanical analysis showed a glass transition around 5 °C and the crystallization temperature of 57 °C. The main difference was found in the average molecular mass, which was higher for SH (M_n of 670.3 kDa and PDI of 1.42) than for WH (M_n of

246.4 kDa and PDI of 3.29). This could arise from a higher PHB synthase activity in cells grown on WH but would need to be validated. The material properties of PHB from wood hydrolysates were comparable to commercial PHB, which confirmed the potential of producing PHB from wood biorefineries for commercial applications.

7.2 Contribution to knowledge

i. An assessment was conducted on the suitability of galactose and mannose as a carbon source for PHA production in *P. sacchari*.
ii. Maximum thresholds and mixture combinations were determined for softwood-derived inhibitors in synthetic hemicellulose sugar mixtures for optimal *P. sacchari* growth.
iii. Consumption patterns of all softwood hemicellulose hydrolysate components were elucidated in fermentations with *P. sacchari.*
iv. The benefits of inhibitor-free hardwood hydrolysate compared to simulated hydrolysate were assessed for high-density PHB production.
v. Polymer applications were evaluated for PHBs from hardwood hydrolysate based on the characterization of their material properties.

7.3 Impact & relevance

This work explored the utilization of affordable, abundant, and sustainably managed Canadian hardwood and softwood hydrolysates to produce the compostable biopolymer PHB. The research provided protocols for the PHB production from industrially available wood streams in shake flasks, high-cell density bioreactors, and its material characterization.

Global polymer production has continuously risen over the past fifty years to 299 million tonnes in 2013 [24]. This is equivalent to the weight of 45 pyramids of Giza per year that need to be disposed at the end of their use. Between 22-43% of polymers end up in landfills, thus wasting the carbon feedstock and potentially leading to groundwater pollution by the leaching of toxic additives. Furthermore, polymer wastes accumulate in the natural environment where they can remain for up to 2000 years [239]. The pollution caused by polymers is especially harmful in

the marine environments, where an estimated 100 million tonnes of polymers cause an ecosystem service damage of approximately US$ 13 billion per year [26].

In contrast, PHAs are both compostable and biodegradable in marine environments by ASTM standards. This is an important difference with other bio-based polymers such as polylactic acid (PLA), which are compostable in industrial composts, but may remain in marine environments for up to a thousand years [239]. Replacing fossil-based polymers with PHAs can potentially reduce greenhouse gas emission by 200% and fossil energy use by 95% [239].

Integrating PHA production into biorefinery plants can improve its economic and environmental performance [240]. To avoid major investments, sugar streams of already operating industrial plants, as in the case of modified pulp and paper mills, are efficient feedstocks [240]. Furthermore, since the support for industrial development of new biorefineries is high, PHA production from new refining processes at the research scale (as in the case for softwood hemicellulose hydrolysate) or pilot scale (as in the case for the TMP-Bio process) have a high potential for scale up.

7.4 Recommendations for future research

The main challenges for PHA production in the future are to ensure that the products meet price, functional and sustainability criteria, so that they can compete on the market and contribute to healthier lives for the next generations. In the case of softwood biorefineries, there are still many opportunities to valorize the feedstock into PHA. Future research should try different hemicellulose extraction methods and evaluate if they can reduce the inhibitory effect of the hydrolysates. Furthermore, it is still questionable if acetate, HMF, furfural and phenols are the only inhibitory compounds in softwood hydrolysates. Consequently, solving the inhibitory effect of softwood hemicellulose hydrolysate would unlock the potential of the abundant feedstock.

In the case of hardwood biorefineries, the next challenge is to find applications for wood-based PHB that can replace petrochemical alternatives. Currently, PHAs are often used in the biomedical field, but this low production volume will not unlock the environmental benefits that come from replacing large volumes of petroplastics. Instead, food packaging would be an ideal

application. Here, an area of innovation would be the interfaces of cellulose-based packaging with PHA-based packaging.

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