Polyhydroxyalkanoate production in yeast Saccharomyces cerevisiae

Anna Ylinen
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Anna Yinen

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Abstract

This thesis was inspired by the global need to find replacements for plastic materials originating from fossil sources. Microbiologically produced polyhydroxyalkanoates (PHAs) are diverse group of fully biobased and biodegradable polyesters with interesting properties for many applications. However, wide use of PHAs is still hindered by their limited mechanical properties and relatively high costs of sugar feedstocks. This thesis explored possibilities to use lignocellulose derived cellulose as a carbon source for PHA production in yeast Saccharomyces cerevisiae. In addition, it focused on polymerization of a 2-hydroxacid (D-lactic acid) and controlling PHA copolymer structure in yeast S. cerevisiae with synthetic biology. These approaches contribute to the wider themes of using lignocellulose-based carbon sources for PHA production and improving PHA polymer properties for use in even wider range of different applications in future.

Polymerization of 2-hydroxyacids in S. cerevisiae was shown for the first time in Publication 1. As an example, D-lactic was produced and polymerized in vivo into poly(D-lactic acid) (PDLA) and copolymer poly(D-lactate-co-3-hydroxybutyrate) [P(LA-3HB)]. In Publication 2, the D-lactic acid production was controlled with a doxycycline-based Tet-On system. Increase in D-lactic acid production improved PLDA and P(LA-3HB) accumulation in the cells to 5.2% and 19% of CDW, respectively. Adjustable idhA expression allowed also control of D-lactic acid content in the copolymer from 6 to 92 mol%.

In Publication 3, S. cerevisiae was engineered to utilize cellulose for poly(3-hydroxybutyrate) (PHB) production. Two pathways containing either cellulose phosphorylase gene cbp or β-glucosidase gene GH1-1 were compared. Both pathways allowed accumulation of high molecular weight PHB (Mw 450-500 kDa) in S. cerevisiae. The strains expressing GH1-1 consumed cellulose faster than strains expressing cbp, which lead also to faster growth and PHB accumulation in GH1-1 strains. However, both strains accumulated more PHB on cellulose (as % of CDW and per consumed sugar) in comparison to control strain grown on glucose. The highest PHB accumulation levels of 13.4±0.9% and 18.5±3.9% PHB of CDW for cbp and GH1-1 strains, respectively, were obtained in pH controlled (pH 6) bioreactor experiments.

Keywords
Polyhydroxyalkanoate, PHA, Saccharomyces cerevisiae, Yeast, Poly(hydroxybutyrate), PHB, copolymer, Tet-On
Tekijä
Anna Yinen

Väittöskirjan nimi
Polyhydroksialkanoaatteen tuotto Saccharomyces cerevisiae -hiivalla

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Kemian tekniikan korkeakoulu

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Kemian tekniikan korkeakoulu

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Kieli
Englanti

Monografia
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Artikkeliväittöskirja
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Tiivistelmä


Julkaisussa 3 S. cerevisiae -hiivaa lisättiin reitti sellbioosin hyödyntämistä varten. Samalla tuotettiin poly(3-hydroxybutyraattia) (PHB:ta). Tutkimuksessa vertailtiin kahta eri reittiä, joissa oli joko sellbioosifosforylasian geeni cbp tai β-glukosidaasin geeni GHI-1. Tuotetut PHB-polymeerit molemmilla reiteillä olivat pitkiä, noin 450–500 kDa (Mw). GHI-1 -kannat hyödynsivät sellbioosia nopeammin kuin cbp-kannat, minkä vuoksi GHI-1 -kannat myös kasvoivat ja tuottivat PHB:ta nopeammin. Molemmat kannat tuottivat sellbioosilla enemmän PHB:ta kuin vertailukanta gluokosin avulla (suhteutettuna sekä solun kuivapainoon, että kulutettuun sokerimäärään). Isoommat PHB:n tuottomäärät solun kuivapainosta, 13.4±0.9 % (cbp-kannalla) ja 18.5±3.9 % (GHI-1 -kannalla) mitattiin bioreaktorikasvatuksessa, jossa pH oli säädetty (pH 6).

Avainsanat
Polyhydroksialkanoatti, PHA, Saccharomyces cerevisiae, hiiva, polyhydroksibutyratta, PHB, Tet-On

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Preface

This Thesis was written at VTT Technical Research Centre of Finland during the years 2017-2022. Work was carried in the Production host engineering team under supervision of Principal Scientist Mervi Toivari, Senior Scientist Laura Salusjärvi, and Professor Merja Penttilä. I am grateful for all their support, advice, and feedback during the past five years. I have been lucky to have such talented and committed team to work with, both in ups and downs.

The funding from Maj and Tor Nessling foundation (grant numbers 201700259 and 201800005) enabled the very first three years of this work. These personal grants for doctoral studies allowed me to focus on this challenging topic and build my knowledge for all the future biopolymer projects to come. Thesis work was continued with funding from Jenny and Antti Wihuri foundation to The Centre of Young Synbio Scientist (CYSS) at Aalto University and with a grant for doctoral studies from the Kalle and Dagmar Foundation, which belongs to the Finnish Cultural Foundation (grant number 00201193). In addition, my work was supported by VTT in the form of laboratory and office space and resources, supervision, and dedicated time for writing this Thesis.

I am deeply grateful for my Team Leader Jussi Jäntti and Vice President Arto Forsberg for supporting my thesis through all the different phases and for providing continuity for my research carrier. I would like to thank also Kirsi-Marja Oksman-Caldentey giving me opportunity to study this topic at VTT and start my carrier as a researcher.

During this Thesis, I had privilege to work with many experienced scientists in the field of synthetic biology and polymer chemistry. These colleagues and co-authors have shared their knowledge about different aspects of biotechnology with me and helped me to push forward the challenging, multidisciplinary experiments. I want to thank Research Scientist Jorg de Ruijter for continuous support in the cellobiose work, Research Scientist Anssi Rantasalo for guidance in the gene expression control in yeast, Senior Scientist Dorothee Barth for help in the processing of the bioreactor data, Senior Scientist Hannu Maaheimo for teaching me the essential basics of the amazing NMR science, Senior Scientists Adina Angelescu-Hakala and Jukka Niskanen for all their insights in the polymer chemistry world, and Research Team Leader Paula Jouhten for her guidance in understanding the different metabolic states of yeasts.

Special thanks belong also to Professor Gunnar Lidén and Docent Miia Mäkelä for pre-examination of this Thesis and their highly useful feedback to the text. In addition, I would like to present my gratitude also to Annemari Linnatsalo for helping me with transcriptional analysis and microbial cultures, Anna-Liisa Ruskeepää, Matti Hölttä and Airi Hyrkäs for carrying out GC-MS analysis, Pia Willberg-Keyriläinen for DSC analysis, Atte Mikkelsen for SEC analysis, Juha Tähtiharju for guidance with bioreactor cultivations, and Nina Pulkkis for shooting, editing, and distributing a great video clip out of my research.

In addition, I would like to say that working at VTT has been a really nice experience this far. The general atmosphere is safe, supporting, and inclusive. It is very easy to ask help from anyone and brainstorm all kinds of ‘Beyond the Obvious’ ideas for future projects. I have had
so many nice lunch and coffee breaks with my colleagues from our ‘Yeast and Mold lab’ and rest of the department of Industrial biotechnology and food solutions, but also with my new friends I have met through VTT young professionals -network.

Last, but not least, I would like to thank my parents and siblings for support and understanding through the stressful times, and my friend Julia Ribeck who encouraged me to follow my heart in 2015-2016 when I was still only dreaming about a research carrier and applying for PhD grants. My free time during this Thesis has been unexpectedly rich and fun, mostly due to the lively Lindy hop dancing community in Helsinki, my wonderful scuba diving friends at Kupla (diving club of Aalto University students), and my spontaneous climbing friends from VTT and abroad.

Helsinki, 21st June 2022
Anna Maria Ylilinen
List of Publications

This doctoral dissertation consists of a summary and of the following Publications, which are referred to in the text by their numerals:


2. Ylinen, Anna; Salusjärvi, Laura; Toivari, Mervi; Penttilä, Merja. 2022. Control of D-lactic acid content in P(LA-3HB) copolymer in the yeast *Saccharomyces cerevisiae* using a synthetic gene expression system. *Metabolic Engineering Communications*. ISSN of journal 2214-0301. Publication date: 30 April 2022, https://doi.org/10.1016/j.mec.2022.e00199

Author’s Contribution

Publication 1: Production of D-lactic acid containing polyhydroxyalkanoate polymers in yeast *Saccharomyces cerevisiae*

AY was responsible for experimental design and work including strain engineering, sample preparation, metabolite analysis, growth assays, and polymer extraction and analysis. In addition, AY analysed the results, visualized the data, and drafted the manuscript under supervision of the other authors. HM planned and carried out the NMR analysis and wrote the NMR Chapters of the manuscript. AAH contributed to the experimental design of polymer analysis. LS, MT, and MP participated in conceptualization, and editing and reviewing of the manuscript.

Publication 2: Control of D-lactic acid content in P(LA-3HB) copolymer in the yeast *Saccharomyces cerevisiae* using a synthetic gene expression system.

AY was responsible for experimental design and work including strain engineering, sample preparation, metabolite analysis, growth assays, and polymer extraction and analysis. In addition, AY analysed the results, visualized the data, and drafted the manuscript. LS, MT, and MP participated in conceptualization, and editing and reviewing of the manuscript.

Publication 3: PHB production from cellobiose with *Saccharomyces cerevisiae*

AY was responsible for experimental design of the polymer extraction, polymer analysis, and introduction of the PHB pathway to the strains. AY and JR divided the experimental work regarding cultivations and analysis equally. AY and JR drafted the manuscript under the supervision of MP and PJ.
## List of Abbreviations and Symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>3HB</td>
<td>3-hydroxybutyric acid</td>
</tr>
<tr>
<td>3HB-CoA</td>
<td>3-hydroxybutyryl-CoA</td>
</tr>
<tr>
<td>3HHx</td>
<td>3-hydroxyhexanoic acid</td>
</tr>
<tr>
<td>3HV</td>
<td>3-hydroxyvaleric acid</td>
</tr>
<tr>
<td>4HB</td>
<td>4-hydroxybutyrate</td>
</tr>
<tr>
<td>Cbp</td>
<td>Cellobiose phosphorylase</td>
</tr>
<tr>
<td>Cdt-1</td>
<td>Cellodextrin transporter</td>
</tr>
<tr>
<td>CDW</td>
<td>Cell dry weight</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>D</td>
<td>Dispersity</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>Gh1-1</td>
<td>β-glucosidase</td>
</tr>
<tr>
<td>LA</td>
<td>Lactic acid</td>
</tr>
<tr>
<td>LdhA</td>
<td>Stereospecific D-lactate dehydrogenase</td>
</tr>
<tr>
<td>Mn</td>
<td>Number average molecular weight</td>
</tr>
<tr>
<td>Mw</td>
<td>Weight average molecular weight</td>
</tr>
<tr>
<td>Pct</td>
<td>Propionyl-CoA transferase</td>
</tr>
<tr>
<td>PDLA</td>
<td>Poly(D-lactic acid)</td>
</tr>
<tr>
<td>PHA</td>
<td>Poly(hydroxyalkanoate)</td>
</tr>
<tr>
<td>PhaA</td>
<td>Acetyltransferase</td>
</tr>
<tr>
<td>PHB</td>
<td>Poly(hydroxybutyrate)</td>
</tr>
<tr>
<td>PhaB1</td>
<td>Acetoacetyl-CoA reductase</td>
</tr>
<tr>
<td>PhaC</td>
<td>PHA synthase</td>
</tr>
<tr>
<td>P(LA-3HB)</td>
<td>Copolymer of D-lactic and 3-hydroxybutyric acids</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>$T_g$</td>
<td>Glass transition temperature</td>
</tr>
</tbody>
</table>
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Introducing the Basis of Biobased and Biodegradable Polymer Production

1. Introduction

Different plastic materials have become an essential part of our lives on many levels. They have ideal properties for many applications being at the same time cheap, versatile, durable, and easy to process. It has been estimated that from 1950’s to 2015, approximately 8300 million metric tons of virgin plastic were produced globally and that the amount of produced petrochemical plastics still increases in the future. But what happens to plastic materials after their use? By 2015, around 75% of all the plastics ever produced was already disposed (Geyer et al., 2017). Almost 80% of the plastic waste was disposed to landfills and environment, while 12% was incinerated and only 9% was recycled. Either way, the plastic disposal creates many environmental challenges. When petroleum-based plastics are disposed by incineration, they increase the carbon dioxide load in our atmosphere, intensifying thus the global warming. Incineration can also release other harmful by-products to the environment, including persistent organic compounds, acidic gases, and heavy metals (Zhang et al., 2021). When plastics are disposed to environment, they can accumulate in water areas either as visible items or as microplastics. It is estimated that approximately eight million tons of plastics would enter oceans annually and that, in addition to obvious sources, microplastics could originate for example from wearing of car tires and fishing nets, cosmetics, and from washing of synthetic clothing (Napper and Thompson, 2020). Even the plastic storage at landfills can result in toxic leaches to the environment (Zhang et al., 2021). To conclude, efficient plastic waste collection, advanced recycling technologies, and biodegradable and biobased alternatives are needed to reduce the negative environmental effects of the plastic materials. Fully biodegradable plastic materials become important especially when plastic discharge to environment is likely or cannot be avoided.

This thesis focuses on production of biobased and biodegradable polyhydroxyalkanoate (PHA) polymers, which are microbially made polyesters consisting of different natural hydroxyacids. Many bacterial strains accumulate PHAs in vivo in nutrient limited conditions either for carbon storage or for protection from challenging environmental conditions, such as high UV radiation or high salt concentrations. These bacterial cells are able to degrade both intra- and extracellular PHAs back to different organic compounds (Jendrossek and Handrick, 2002; Knoll et al., 2009). As these microbes exist widely in different soil, sea, and freshwater environments, PHAs are considered as fully biodegradable polymers. The biodegradation speed in different environments depends however on many parameters including the microbial activity, material thickness and crystallinity, and PHA monomer composition.

The PHAs can be produced by feeding the bacterial hosts with sugars from edible crops, such as corn or sugar cane (Levett et al., 2016). The large-scale use of edible sugars is however expensive and contributes negatively to global food security. Thus, the PHA industry and academic PHA research have shifted their focus towards the use of alternative organic raw materials.
material and wastes (Choi et al., 2020; Zheng et al., 2019). These include for example n-alkanes, n-alcohols, methane, agricultural waste, frying oil waste, plant oil mill effluents, food waste, and lignocellulosic biomass (Koller et al., 2010; Sandström et al., 2015). These raw materials can be however challenging for many bacterial hosts which are sensitive to low pH and toxic compounds.

In this thesis, the PHA production was studied in yeast *S. cerevisiae* due to its robustness and tolerance for acidic conditions. These resilient features could simplify e.g., the use of lignocellulosic hydrolysates and organic acids as raw materials for PHA production.

Publication 1 describes the polymerization of 2-hydroxyacids in *S. cerevisiae*. As an example, D-lactic acid is polymerized into poly(D-lactic acid) (PDLA) and copolymer poly(D-lactate-co-3-hydroxybutyrate) [P(LA-3HB)]. The 2-hydroxyacid polymerization is further developed in Publication 2. It demonstrates how the PDLA production can be enhanced and how the P(LA-3HB) copolymer structure can be modified *in vivo* in *S. cerevisiae*. Tools for polymer modification are important as native PHAs have rather limited properties in comparison to traditional petrochemical polymers. Novel PHAs are required for applications, which require properties beyond the existing PHAs. In Publication 3, the *S. cerevisiae* is supplied with two distinct pathways to utilize cellobiose as a sole carbon source for poly(3-hydroxybutyrate) (PHB) production. It explores possible energetic benefits of cellobiose phosphorolysis on the PHB production in comparison to cellobiose hydrolysis by β-glucosidases and paves the way for efficient utilization of lignocellulose-based raw materials for PHA production.

### 1.1 Polyhydroxyalkanoate (PHA) production pathways *in vivo*

PHA production has been studied in many different bacterial hosts including native producers such as *Cupriavidus necator* (Peoples and Sinskey, 1989) and *Bacillus megaterium* (Tsuge et al., 2015) and various engineered hosts such as *Escherichia coli* (Li et al., 2016; Yang et al., 2018; Zhang et al., 2018) and *Halomonas bluephagenesis* (Yu et al., 2020). Recent review articles describe the current state of PHA production in bacterial hosts (Choi et al., 2020; Yañez et al., 2020; Zheng et al., 2019). In addition, few studies describe introduction of PHA producing pathway in eukaryotic hosts such as plants (Poirier et al., 1995) and yeasts (described in Chapter 1.5).

PHA synthesis pathways vary a lot depending on the host cell and target PHA. However, each pathway contains a PHA synthase and enzymes for production of Coenzyme-A (CoA) activated hydroxyacid monomers. The polymerization starts when PHA synthase combines the very first two hydroxyacids to each other. Then the rest of the monomers are added to growing chain one by one, in a linear manner. PHA synthases stay covalently attached to the polymer chain during the entire polymerization process (Gerngross et al., 1993). The hydrophobic nature of the PHA chains leads to formation of PHA granules in the hydrophilic cytosol (Figure 1). Formation is estimated to occur by a self-assembly method, similar to micelles (Rehm, 2003). Hydrophilic PHA synthase and other enzymes stay on the surface of the granule towards the cytosol.
1.1.1 Production of PHA monomers, the building blocks for PHA polymers

Over 160 different PHA monomers are known to date (Choi et al., 2020). These natural and synthetic monomers are produced in vivo through different pathways. For example, some microbial hosts are able convert sugars, such as glucose, first into acetyl-CoA and then further into different PHA monomers. In addition, some cells import fatty acids from their environment and convert them to R-3-hydroxyacyl-CoA monomers. However, all PHA monomers have also some common features. They all derive from R-enantiomeric carboxylic acids carrying a hydroxyl group (-OH) in the 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup>, 5<sup>th</sup>, or 6<sup>th</sup> carbon from the carbonyl group (-COOH). The remaining part after the hydroxyl group forms the side chain of the monomer (Figure 2). Side chains can contain for example long or short hydrocarbon chains with unsaturated bonds or aromatic groups. This huge variety in possible side chains, in addition to the different lengths of the main chain, allows production of many different PHAs. However, the hydroxyacids cannot be polymerized as such. In all the PHA monomers, the hydroxyl group of the carbonyl group is replaced by Coenzyme A (CoA) -group. This CoA group is later released when monomer is added to the polymer chain.

Monomers can be classified based on the total amount of the carbons in the monomer, including both side chain and main chain. Short chain length (scl) -monomers contain only 3 to 5 carbons in total and medium chain length (mcl) -monomers 6 to 16 carbons. This classification is often used to describe which kind of monomers certain PHA synthase enzyme is able to polymerize (Chapter 1.1.2).

One of the most studied PHAs is PHB homopolymer. It consists of scl R-3-hydroxybutyryl (3HB) units, which are produced from acetyl-CoA by two consecutive steps (Figure 2). First, two acetyl-CoA molecules are combined into one acetoacetyl-CoA by an acetyl-CoA acyltransferase, such as β-ketothiolase (phaA) from C. necator (Peoples and Sinskey, 1989). Acetoacetyl-CoA is then converted into 3-hydroxybutyryl-CoA by an acetoacetyl-CoA reductase phaB1. Depending on the source, the phaB1 enzymes can use either NADPH or both NADPH and NADH as cofactors, respectively (de Las Heras et al., 2016).
Figure 2. Examples of short chain length (scl) and medium chain length (mcl) monomers and their polymerization into PHA polymers \textit{in vivo}. The 2-hydroxyacids, such as D-lactic acid, have a hydroxyl group (-OH) attached to the 2\textsuperscript{nd} carbon from carbonyl group (-COOH). In R-3-hydroxyacids, the hydroxyl groups is attached to the 3\textsuperscript{rd} carbon from the carbonyl group. The remaining part of the acid (after the hydroxyl group) forms the side chain (grey circles).

Many R-hydroxyacids can be processed into R-hydroxyacid-CoA monomers in a one-step manner. Some 2-hydroxyacids, e.g., D-lactic acid, glycolic acid, and 2-hydroxybutyric acid are converted to CoA-monomers by propionyl-CoA transferases. For example, a native propionyl-CoA transferase PctMe from \textit{Megasphaera elsdenii} has been successfully used for production of D-lactyl-CoA (Taguchi et al., 2008). In addition, engineered enzyme Pct540Cp from \textit{Clostridium propionicum} with one active mutation V193A and three silent mutations T78C, T669C, and A1125G, has been used for production of D-lactyl-CoA, glycolyl-CoA, 2-hydroxybutyryl-CoA, and 2-hydroxyisovaleryl-CoA (Chae et al., 2016; Choi et al., 2016; Park et al., 2012; Yang et al., 2016). Some aromatic hydroxyacids, including phenyllactate, D-mandelate, and D-3-hydroxy-3-phenylpropionate, can be prepared for polymerization with iso-caprenoyl-CoA:2-hydroxyisocaproate CoA-transferase (HadA) from \textit{Clostridium difficile} (Yang et al., 2018).

Mcl-monomers are formed \textit{in vivo} usually through \textit{β}-oxidation pathway, which converts fatty acids to energy. The R-3-hydroxyacyl-CoA intermediates can be used as PHA monomers as such. However, each \textit{β}-oxidation cycle decreases the chain length of the monomer by two carbons resulting in formation of many different mcl-monomers in the same strain. Modifications in \textit{β}-oxidation can be used for terminating the cycle after R-3-hydroxyacyl-CoA is formed. This enables production of homopolymeric mcl-PHAs (Haddouche et al., 2011; Rigouin et al., 2019). The mcl-monomers are rather large group of different monomers. The content of mcl-monomers with even or odd number of carbons, or unsaturated bonds, can be adjusted in PHA polymer by feeding the cells with corresponding fatty acids (Poirier et al., 2001; Zhang et al., 2006).

1.1.2 Monomer polymerization by different PHA synthases

The actual polymerization of hydroxyalkanoates is carried out by different native and engineered PHA synthase enzymes. They can be categorized into four classes depending on their
substrate specificity and subunit structure (Rehm, 2003). Active PHA synthases always require presence of two subunits, which can be either identical or different from each other.

Class I synthases accept usually only scl-monomers and contain two identical subunits. This enables expression of active PHA synthase dimer in recombinant hosts only from one gene. As an example, Class I PHA synthase from the bacterium C. necator (formerly known as Hydrogenomonas eutrophus, Alcaligenes eutropha, or Ralstonia eutropha) is one of the most studied PHA synthases and used for PHB production. Class II synthases originate often from Pseudomonas species and polymerize mainly mcl-monomers. They also contain two identical subunits. Class III synthases originate for example from Allochromatium vinosum and contain one subunit that is similar to subunits from class I and II, and one different subunit PhaE. These class III synthases prefer scl-monomers like Class I enzymes. Class IV synthases originate for example from Bacillus species. They are similar to Class III synthases but contain subunit PhaR instead of PhaE.

Some native PHA synthases have showed wider substrate specificity than others. For example, class II PHA synthase PhaC1 from Pseudomonas sp. 61-3 can polymerize both scl- and mcl-3-hydroxyacids (Matsusaki et al., 1998). Also, PhaC1 from C. necator has shown some activity towards mcl-monomers in addition to their preferred scl-monomers (Antonio et al., 2000; Dennis et al., 1998).

Wider substrate specificities can be obtained by modifying amino acids in the active sites of the PHA synthase enzymes. In 2003 and 2004, mutagenesis studies of PHA synthase PhaC1Ps from Pseudomonas. sp 61-3 revealed two amino acids changes (S325 and Q481) which increased the PHB production up to 400-fold in comparison to wild type enzyme (Takase et al., 2004, 2003). In 2008 mutation of these sites, S325T and Q481K, was tested in all four PHA synthase classes and it was found that only mutated class II PhaC1Ps could incorporate also lactic acid (LA) into P(LA-3HB) copolymer (Taguchi et al., 2008). It was discussed that the wild type PhaC1Ps would have exceptionally broad substrate specificity in comparison to other wild type PHA synthases. This would allow the mutated version to accept wide range of different monomers with unusual side- and main chains. The development of lactic acid polymerizing PHA synthases did not end there. In 2010 and 2011 it was shown that when these mutations were combined with two additional amino acid changes (E130D, and S477G), PHA synthases from Pseudomonas sp. MBEL 6–19 (PhaC1437Ps6-19), Pseudomonas resinovorans (PhaC1Pre), Pseudomonas chlororaphis, Pseudomonas putida KT2440 (PhaC1Ptt), and Pseudomonas aeruginosa PAO1 were able to include LA into copolymer P(LA-3HB) (Yang et al., 2011, 2010). Expression of these mutated PHA synthases in E. coli resulted in accumulation of more than 40% copolymer of cell dry weight (CDW). However, LA content in the formed P(LA-3HB) copolymers varied. The PhaC1Ptt incorporated only 30% LA while PhaC1437Ps6-19 and PhaC1Pre reached LA percentages over 50% and 60% respectively. Later studies also report incorporation of other 2-hydroxyacids monomers with PHA synthase PhaC1437Ps6-19. These include glycolic acid, 2-hydroxybutyrate, and D-phenyllactate (Chae et al., 2016; Choi et al., 2020, 2016; Yang et al., 2018).

1.2 Properties of poly(hydroxybutyrate) (PHB) and poly(lactic acid) (PLA) homopolymers and PHA random copolymers

PHAs are a diverse group of biobased and biodegradable polyesters. They all consist of organic R-hydroxyacid monomers which contribute to their physical and chemical properties.
PHAs with scl-monomers, such as 3HB or 3-hydroxyvalerate (3HV), are often highly crystalline, whereas polymers with mcl-monomers are mostly amorphous. Crystallinity is an essential feature in PHA materials as it affects many features including thermal, mechanical, and barrier properties (Follain et al., 2014). A high degree of crystallinity can offer good barrier properties, e.g., low water permeability, but result in stiff and brittle materials.

The different behaviour of the scl- and mcl-polymers is supported also by a recent in silico modelling study which explains how the atomistic monomer structure affects PHA homopolymer properties (Bejagam et al., 2022). Mechanical strength of the polymer, in terms of Young’s modulus and yield stress, decreased systematically when the number of carbon atoms either in the polymer backbone or side chain increased.

PHAs are not limited to scl- or mcl-homopolymers, but include also large number of different random, alternating, and block copolymers with different molecular weights, monomer contents, and degree of crystallinity, with different polymer properties. The large variety in these structures allows tailoring of the discussed mechanical and thermal properties of the materials.

### 1.2.1 PHB

The 3HB homopolymer (PHB) is a common PHA produced in vivo in various microorganism (Chapter 1.1.1). PHB chains can contain up to 60,000 3HB-units covalently attached to each other, which means that weight average molecular weight (Mw) of the PHB can be up to 6000 kDa (Castillo et al., 2017). The high molecular weights of PHB, combined with regular structure consisting of only one type of scl-monomer, results in a high degree of crystallinity (50-90%). The presence of rigid (crystalline) and mobile (amorphous) phases affect the physical properties of PHB such as impact strength and flexibility (elongation % at break) (Turco et al., 2021).

PHB has many features similar to polypropylene (PP). For example, a melting temperature of 175-180°C, stiffness in terms of Young’s modulus (1-3.5 GPa), and tensile strength (15-40 MPa). However, the extension to break value of PHB is only 5%, while for PP it is around 40% (Możejko-Ciesielska and Kiewisz, 2016), indicating that PHB is less flexible than PP. Water vapor transmission rate of PHB (1-5 g mm / m² / day) is slightly higher than of the PP (0.2-0.4 g mm / m² / day) (Turco et al., 2021). However, oxygen transmission rate of PHB (2 cc mm / m² / day) is approximately 30-times lower than of PP, indicating that PHB is a better oxygen barrier (Turco et al., 2021). Good barrier properties, combined with thermoformability and biodegradability are promising features especially for future packing applications.

### 1.2.2 PLA

Poly(lactic acid) (PLA) is a biodegradable and biobased hydroxyacid polyester. It is traditionally produced by fermenting different carbon sources with various microbial hosts into L- and D-lactic acids (Baek et al., 2016; Gao et al., 2011; Ilmén et al., 2007; Koivuranta et al., 2014; Zhong et al., 2019), which can be polymerized chemically into PDLA, poly-(L-lactic acid) (PLLA), random copolymer of L- and D-lactic acids (PDLLA), or PDLLA block copolymers with distinct D-lactic acid (D-LA) and L-lactic acid (L-LA) block sequences. Recent advancements in PHA synthase enzyme engineering have enabled polymerization of D-lactic acid into PDLA with an in vivo hydroxyacid polymerization pathway (Taguchi et al., 2008; Yang et al., 2010) (Chapters 1.1.1 and 1.1.2). This pathway offers an alternative one-step production
method of PDLA for the bioplastic industry, which does not require high temperatures, metal catalysts, or extensive purification of the monomers prior to their polymerization.

The isotactic PLAs (PLLA and PDLA) have different degrees of crystallinities, in comparison to the atactic random PDLLA. The differences in the degree of crystallinity affect mechanical and thermal properties and biodegradation rates of the different PLAs. For example, incorporation of only 8% D-LA monomers into PLLA in a random manner can decrease the degree of crystallinity of the PLA polymer from 57% to 32% and lower the melting point from 180°C to 138°C (Reeve et al., 1994). PDLLA block copolymers and blends of PLLA and PDLA expand the PLA family even further by formation of PLA stereocomplexes, which have higher melting temperatures (220°C-230°C) and better barrier and mechanical properties in comparison to PLLA or PDLA (Tsuji, 2016). Controlled monomer content in PDLLA random copolymer can be used for adjusting the biodegradation rate of the PDLLA and thus for tailoring material properties for example for medical applications, such as drug delivery or tissue engineering (Ginjupalli et al., 2017). In fact, PLA has gained a GRAS status and is considered as biocompatible material as neither the PLA material nor its degradation products cause toxic effects or affect healing of human tissues (Rasal et al., 2010). In addition to medical applications, PLA is widely used in biodegradable consumer goods such as disposable cutleries and 3D-printing filaments. PLA also shows potential for higher value applications such as speciality textiles and fibres due to its ease of processing, soft feel, and resistance for different stains and UV-radiation (Farah et al., 2016).

1.2.3 PHA random copolymers

Scl-homopolymers such as PHB are often stiff and brittle. Random PHA copolymers offer interesting alternatives for applications where more flexible polymers are required. Their flexibility and degree of crystallinity can be adjusted by changing ratio of different monomers in the polymer. This has been studied for example by combining 3HV, 3-hydroxyhexanoic acid (3HHx), 4-hydroxybutyrate (4HB), or D-LA units with 3HB monomers (Doi et al., 1995; Li et al., 2010; Yamada et al., 2011). The flexibility and lower processing temperatures of the resulting copolymers have gained commercial interest, as copolymers poly(3HB-co-3HV), poly(3HB-co-3HHx), and poly(3HB-co-4HB) belong to the few industrially produced PHAs.

In addition to experimental studies, the effect of PHA copolymer structure on the polymer properties has been recently investigated by in silico methods. A model based on machine learning and deep neural networks was used for detecting the most relevant parameters defining the PHA copolymer glass transition temperature (Tg) (Jiang et al., 2020). The study revealed that while the choice of monomer had the highest impact on final Tg, the relative amount of the monomers followed right after. For comparison, the molecular weight and dispersity of the polymers had smaller effect on the Tg. Another study explored Tg values of different copolymers by simulating their molecular dynamics on an atomistic level (Bejagam et al., 2021). The simulation results support findings from the previous studies: the Tg of a copolymer depends on the chosen monomers and their ratio in the copolymer. For example, the Tg of P(3HB-4HB) could be estimated based on known Tg values for PHB and P(4HB) homopolymers and the ratio of the two monomers in the copolymer. However, the study reports also that when two monomers have different atomic weights, the correlation might not be completely linear and possible interchain reactions can alter the thermal behaviour especially in block copolymer structures (Bejagam et al., 2021).
1.3 Control of the PHA copolymer structure in vivo

Traditional approaches for adjusting monomer composition of PHA include control of cultivation parameters, such as substrate feeding and aeration, and use of PHA synthases with different substrate specificities (Figure 3). These methods have been used for example for controlling lactic acid content in P(LA-3HB) copolymer from 20 to 50 mol% (Yang et al., 2010), from 55 to 86 mol% (Jung et al., 2010), from 3 to 30 mol% (Kadoya et al., 2015), and from 8 to 73 mol% (Nduko et al., 2014). Similar approaches, together with modified β-oxidation, have been also used to control the scel- and mcl-monomer composition in yeasts Y. lipolytica and S. cerevisiae (De Oliveira et al., 2004; Gao et al., 2015; Haddouche et al., 2011, 2010; Poirier et al., 2001; Rigouin et al., 2019; Zhang et al., 2006).

The control of the monomer ratio in PHA with synthetic biology methods, at the gene expression level, has not been exploited as widely as the previously described methods. Synthetic biology could however enable precise control of the polymer structure without externally supplied monomers or extensive changes in the cultivation conditions. This could be useful especially when required monomers that are too expensive, toxic, or unavailable for feeding in industrial scale, or when the changes in cultivation conditions would decrease cell growth notably.

Synthetic biology techniques have been applied mainly in bacterial hosts either directly to the monomer pathway or to repress their competing pathways. As an example of direct approach, the expression of 3HB-CoA pathway was adjusted with arabinose in E. coli (Wang et al., 2013b). Higher arabinose concentrations resulted in increased expression of the 3HB-CoA pathway and higher 3HB content in copolymer poly(3-hydroxypropionate-co-3-hydroxybutyrate) P(3HP-3HB). The 3HB content was adjusted from 5.4 mol% up to 88.5 mol% by inducing the pathway with different arabinose concentrations. This study demonstrates also a common method used in PHA copolymer engineering: availability of certain monomer is adjusted while quantity of another monomer is kept constant.

It is also possible to adjust gene expression level by using different promoters and transcription activation domains with varying strengths (Da Silva and Srikrishnan, 2012; Lee et al., 2015; Ottoz et al., 2014). These methods are efficient but require often building of large
strain libraries. They have been used for example for increasing the 3-hydroxyhexanoic acid (3HHx) content in poly(3HB-co-3HHx), from 2.8 to 10.7 mol% in C. necator and from 0 to 45 mol% in Halomonas bluephagenesis (Arikawa et al., 2016; Yu et al., 2020).

The universal clustered regularly interspaced short palindromic repeat (CRISPR) interference (CRISPRi) can be used to induce repression of competing pathways (Qi et al., 2013). CRISPRi is based on CRISPR system in which cleavage of foreign deoxyribonucleic acid (DNA) sequences by Cas9 protein is guided by small ribonucleic acid (RNA) sequences. In CRISPRi, the Cas9 protein is catalytically inactive but interferes transcription elongation of the recognized sequence. This leads to repression of target sequence. The CRISPRi and other CRISPR based systems might require testing of multiple different guide ribonucleic acids (gRNAs), as their prediction in silico is not yet fully accurate (Jensen et al., 2017), but they enable control of many different genes at the same time. CRISPRi has been successfully used for controlling the 4-hydroxybutyric acid (4HB) ratio in poly(3HB-co-4HB) in E. coli (Lv et al., 2015) and the 3-hydroxyvaleric acid (3HV) ratio in poly(3HB-co-3HV) in Halomonas sp. TD01 (Tao et al., 2017). In these studies, the expression of succinate semi-aldehyde dehydrogenases and 2-methylcitrate synthase, respectively, were gradually decreased. The 4HB and 3HV content in the copolymers remained however low, approximately at 1-19 mol%.

Similar synthetic biology methods have not yet been applied in fungal host for controlling PHA copolymer structures but could be employed in future. In addition, gene expression in eukaryotic hosts could be linked to the presence of synthetic transcription factors (sTFs), which function either as repressors or activators upstream from the core promoter of the gene of interest. Different sTFs are used for example in doxycycline-based Tet-Off and Tet-On systems (Bellí et al., 1998; Das et al., 2016) and in recently developed universal synthetic expression system (SES) (Rantasalo et al., 2018a, 2018b, 2016). In these methods, the sTFs are co-expressed with transcription activation domains under constitutive promoters. In the Tet-On and Tet-Off approaches, the binding of sTFs to their binding sites is either improved (Tet-On) or repressed (Tet-Off) by the presence of tetracycline compounds, such as doxycycline. These differences in sTF binding reflect also to the expression level of the gene of interest, as higher binding increases the expression. The use of externally added inducer enables smooth control of the different expression level in one strain. The Tet-On and Tet-Off approaches are thus highly useful tools for screening proper expression levels in the metabolic engineering studies. In this thesis, the D-lactic acid content in P(LA-3HB) was adjusted from 6 to 94 mol% with doxycycline-based Tet-On method (Publication 2). However, the need for external inducers is not convenient when strains are grown in industrial applications. In SES, the sTFs bind continuously to their binding sites, without need of any external compounds. Optimal gene expression levels can be implemented in the SES systems by choosing optimal number of sTF binding sites prior to the gene of interest.

1.4 Effect of glass transition temperature (Tg) of a polymer for polymerization in vivo

Recent in vitro study proposes that the elongation of the PDLA polymer in vivo halts when the PDLA polymer reaches molecular weight of approximately 3 kDa (Matsumoto et al., 2018b). The suggested reason for this inhibition is the high Tg of PDLA (60°C), which is higher than the cultivation temperature of common host organisms. Thus, at 30°C or 37°C, which
are common cultivation temperatures for most microbial hosts, the mobility of the formed PDLA chain would be very low. This low mobility is discussed to stall the polymerization.

Even though the highest reported molecular weights of PDLA homopolymers in vivo are over ten-fold higher, 30 or 56 kDa (Mw) (Jung et al., 2010; Lajus et al., 2020; Yang et al., 2011), than presented in the in vitro study (Matsumoto et al., 2018b), none of the in vivo PDLAs reach the high molecular weights above 1000 kDa (Mw), which are frequently reported for the PHB polymer (Fei et al., 2016; Meixner et al., 2018) (Figure 4). The difference in molecular weights in the in vitro and in vivo studies could result from differences in the used PHA synthases and environmental conditions. PHA synthase PhaC1PsSTQK in the in vitro study originated from Pseudomonas sp. 61-3 and carried only two mutations in the active site, S325T and Q481K, while PHA synthase in the in vivo study (which reports PDLA with 56 kDa (Mw)) originated from P. resinovorans and contained four mutations: E130D, S325T, S477G, and Q481K (Yang et al., 2011). Slightly different, engineered active sites might have allowed the PHA synthase in the in vitro study to continue PHA elongation to higher molecular weights, in comparison to PHA synthase in the in vivo study. However, also the molecular weights of 30-55 kDa (Mw) reported in the in vivo studies are still rather low.

The theory of high $T_g$ by Matsumoto et al. (2018) is supported by experimental data from studies focusing on P(LA-3HB) production in vivo (Yamada et al., 2011, 2009). While the $T_g$ of the PHB homopolymer is low, around -7°C to -1°C, the addition of D-lactic acid monomers into the polymer chain has shown to increase the $T_g$ of P(LA-3HB) and to result in formation of polymers which are shorter than PHB homopolymer. The high $T_g$ theory has been also studied by exploring 2-hydroxybutyric acid (2HB) polymerization at different temperatures below and above $T_g$ of P(2HB), which is 30°C (Matsumoto and Kageyama, 2019). Studied strains produced over five-fold more P(2HB) when cultivation temperature increased from 32°C to 34°C and Mw increased from 10 kDa to over 40 kDa when temperature increased from 30°C to 32°C. To exclude possible other effects on cell metabolism, authors compared also PHB production at these temperatures, but did not observe significant differences. These observations indicate that it is important to consider also the $T_g$ of a novel PHA polymer when the aim is to produce PHAs with high molecular weights.

### Figure 4

The glass transition temperatures ($T_g$) of polymers containing different D-lactic acid (LA) and 3-hydroxybutyric acid (3HB) contents. Figure is adapted from in vitro study (Matsumoto et al., 2018b) and complemented with data from experimental studies in vivo. a: (Yang et al., 2011), b: (Castillo et al., 2017), c: (Yamada et al., 2011).

### 1.5 PHA production in engineered yeasts

The production of PHAs has interested scientists ever since PHB was discovered in B. megaterium (Lemoigne, 1926). Engineered bacterial species are able to accumulate high amounts
of PHAs in relation to their cell dry weight, up to 94-96% of CDW (Ling et al., 2018; Nduko et al., 2014). However, the nutritional needs and sensitivity for acidic conditions of the bacterial strains have drawn interest also towards yeasts as alternative production hosts (Table 1).

Yeast are industrially important unicellular eukaryotic hosts, which can use a wide range of different carbon sources and grow to high cell densities. Their cultivation is simple since they tolerate high pressure and low pH and are not prone to infectious viruses and bacteriophages. In addition, yeasts have different subcellular compartments which offer e.g., distinct physiochemical environments with different cofactor and metabolite availabilities which can be used for varying industrial purposes (Hammer and Avalos, 2017; Lajus et al., 2020; Wagner and Alper, 2016). For example, mcl-PHAs are often produced in peroxisomes in which β-oxidation is used to supply required R-hydroxyacyl-CoA monomers.

Native yeast strains are not usually reported to polymerize PHAs, the only exception being a wild type Rhodotorula minuta, which was demonstrated to produce small amount PHA copolymer, 2% of CDW, when grown with glucose, oleic acid, and Tween (Abd-El-Haleem, 2009). The expression of bacterial PHA synthases (Chapter 1.1.2) can be however introduced also into yeast strains and combined with the endogenous pathways for 3-hydroxyacid-CoA production or with synthetic monomer pathways (Chapter 1.1.1).

Table 1. PHA production studies in yeasts.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>PHA % of cell dry weight</th>
<th>PHA synthase origin (Targeting C: Cytosol, P: Peroxisomes)</th>
<th>Focus and other genetic modifications</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arxula adeninivorans</td>
<td>2.2% PHV 0.019 % PHB</td>
<td>Cupriavidus necator (C)</td>
<td>PHB production with or without β-ketothiolase gene (phaA) and acetoacetyl-CoA reductase gene (phaB1) from C. necator</td>
<td>(Terentiev et al., 2004)</td>
</tr>
<tr>
<td>A. adeninivorans</td>
<td>52.1% PHBV</td>
<td>C. necator (C)</td>
<td>Thl thiolase from Clostridium acetobutylicum, phaA, phaB1, and phasin genes from C. necator</td>
<td>(Biernacki et al., 2017)</td>
</tr>
<tr>
<td>Kloeckera sp and Saccharomyces cerevisiae</td>
<td>7.06% PHV (K.s.) 2.68% PHB (S.c.)</td>
<td>C. necator (C)</td>
<td>phaA and phaB1 genes from C. necator</td>
<td>(Abd-El-Haleem et al., 2007)</td>
</tr>
<tr>
<td>Pichia pastoris</td>
<td>1% mcl-PHA</td>
<td>Pseudomonas aeruginosa (P)</td>
<td>Different concentrations of oleic acid in media</td>
<td>(Poirier et al., 2002)</td>
</tr>
<tr>
<td>P. pastoris</td>
<td>30% PHB</td>
<td>C. necator (C)</td>
<td>Nitrogen limitation, inducible Gfp reporter protein for isolating strains with two copies of the expression cassette. Comparison of ethanol and glucose as carbon sources and aerobic and oxygen limiting conditions. phaA and phaB1 genes from C. necator</td>
<td>(Vijayasankaran et al., 2005)</td>
</tr>
<tr>
<td>Rhodotorula minuta</td>
<td>2% P(3HB-co-3HV-co-SHV)</td>
<td>-</td>
<td>Screening of wild type yeasts, comparison of glucose, oleic acid, and tween as carbon sources</td>
<td>(Abd-El-Haleem, 2009)</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>0.6% PHB</td>
<td>Cupriavidus necator (C)</td>
<td>PHB production in yeast</td>
<td>(Leaf et al., 1996)</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>0.45% mcl-PHA</td>
<td>P. aeruginosa (P)</td>
<td>Polymerization of even and odd chain fatty acids</td>
<td>(Poirier et al., 2001)</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>6.2% PHB</td>
<td>Methylbacterium extorquens (C)</td>
<td>Increased PHB accumulation with phaA and phaB1 genes from C. necator</td>
<td>(Breuer et al., 2002)</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>-</td>
<td>C. necator (C)</td>
<td>Elementary mode analysis: Effect of ATP citrate lyase, transhydrogenase, anaerobic conditions, and phaA and phaB1 genes from C. necator</td>
<td>(Carlson et al., 2002)</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>0.5% scl-</td>
<td>Aeromonas cavi-</td>
<td>Increase in polymer accumulation by using a</td>
<td>(De Oliveira et al., 2001)</td>
</tr>
</tbody>
</table>
food, and bioethanol. The recent biotechnological developments have increased the list of its potential applications for example with artemisinic acid, which is an antimalarial drug pre-

1.5.1 The PHB production in *S. cerevisiae*

The baker’s yeast *S. cerevisiae* has been used traditionally for fermentation of beverages, food, and bioethanol. The recent biotechnological developments have increased the list of its potential applications for example with artemisinic acid, which is an antimalarial drug pre-
cursor (Ro et al., 2006), and stereopure D- and L-lactic acids, which are precursors of PLA biopolymers (Borodina and Nielsen, 2014). The *S. cerevisiae* genome is well known, and its efficient homologous recombination DNA repair mechanisms have inspired researchers to develop molecular biology tools for it. In addition, *S. cerevisiae* has the Generally Regarded As Safe (GRAS) status from the US Food and Drug Administration (FDA) (Da Silva and Srikrishnan, 2012; Lian et al., 2018).

In 1996 Leaf et al. demonstrated PHB production in yeast *S. cerevisiae* for the first time (Leaf et al., 1996). Accumulation of 0.5% of CDW was obtained by expressing PHA synthase from *C. necator*, indicating that *S. cerevisiae* was able to produce at least a small amount of precursors with its endogenous pathways. PHB production was however later increased over 40-fold to 9% of CDW, when the entire PHB pathway from *C. necator*, including β-ketothiolase (*phaA*) and acetoacetyl-CoA reductase (*phaB1*) genes, was expressed in the same strain (Carlson and Srienc, 2006). Addition of acetate and panthothenate into culture media increased PHB accumulation even further. These compounds serve as precursors for acetyl-CoA and CoA, respectively.

The importance of efficient precursor pathway is supported also by other studies. In 2012 and 2013, Kocharin et al. demonstrated that PHB production can be enhanced by increasing acetyl-CoA and NADPH availability (Kocharin et al., 2013, 2012). The NADPH supply was increased in *S. cerevisiae* by expressing a glyceraldehyde-3-phosphate dehydrogenase encoding gene from *Streptococcus mutans*. Acetyl-CoA supply was improved by enhancing the ethanol degradation pathway with alcohol dehydrogenase (Adh2) and by directing more acetaldehyde towards acetyl-CoA with acetaldehyde dehydrogenase (Ald6) and acetyl-CoA synthetase variant from *Salmonella enterica*.

In 2015-2017, PHB production in *S. cerevisiae* was studied using xylose as carbon source (de Las Heras et al., 2016; Portugal-Nunes et al., 2017; Sandström et al., 2015). Addition of xylose utilization pathway from *Scheffersomyces stipitis* enabled PHB production in both aerobic and anaerobic conditions from xylose. In addition, the authors demonstrated that expression of an alternative acetoacetyl-CoA reductase from *A. vinosum*, which can use both NADPH and NADH as cofactors, is beneficial for PHB production especially in anaerobic conditions when xylose is used as sole carbon source.

### 1.5.2 Medium chain length (mcl) PHA production in *S. cerevisiae*

In addition to PHB, also different mcl-PHAs have been produced in *S. cerevisiae*. This yeast processes fatty acids through a β-oxidation, which results in formation of different R-3-hydroxyacyl–CoA intermediates, which can be used as monomers for PHA production.

Poirier et al. demonstrated production of different mcl-PHAs in *S. cerevisiae* by targeting PHA synthase from *P. aeruginosa* to peroxisomes (Poirier et al., 2001). Even though accumulated PHA percentages of CDW were low, only up to 0.45%, the authors controlled the monomer structure by feeding cells with glucose and with either oleic acid (C18:1 Δ9cis) or heptadecenoic acid (C17:1 Δ10cis). Oleic acid resulted in production of even-chain monomers with 6-14 carbons and heptadecenoic acid in production of odd-chain monomers with 5-15 carbons. The cis-unsaturated monomers of C15:1, C13:1, and C14:1 were observed. Free fatty acids were found to function as a better substrate for mcl-PHA production in comparison to esterified fatty acids found for example in Tween.

Zhang et al. expanded mcl-PHA production in *S. cerevisiae* also to cytosol by retaining the key peroxisomal enzymes in cytosol (Zhang et al., 2006). These included an Acyl coenzyme A
oxidase (Fox1p) and hydratase (Fox2p). The authors obtained trace amounts of mcl-PHA, 0.0022-0.069% of CDW, when PHA synthase from \textit{P. oleovorans} was targeted to cytosol and cells were fed with C10—C18 fatty acids and other substrates such as formate and acetate. When the PHA synthase from \textit{C. necator} was targeted to peroxisomes, the cells produced up to 7% PHA of CDW, containing shorter C4-C8 monomers. It was observed that neither intermediates of β-oxidation nor untargeted PHA synthases could pass the peroxisome membranes.

1.5.3 Engineering of mcl-PHA, PHB, and poly(D-lactic acid) (PDLA) production in other yeasts

Even though \textit{S. cerevisiae} is without doubt, the most studied yeast for production of PHAs and many other bioproducts, other yeasts with certain unique features are considered as potential alternatives. Especially the oleaginous yeast \textit{Y. lipolytica} has been studied for PHA production as it contains natively high amounts of acetyl-CoA derived lipids.

In 2010-2011 Haddouche et al. demonstrated production of different mcl-PHAs in \textit{Y. lipolytica} strains by targeting \textit{P. aeruginosa} PHA synthase to peroxisomes and by altering the β-oxidation cycle with acyl-CoA oxidase deletions and modifications on multifunctional enzyme MFE (Haddouche et al., 2011, 2010). Deletion of lipid biosynthesis genes \textit{LRO1}, \textit{DGA1}, \textit{DGA2}, and \textit{ARE1} increased PHA accumulation to 7.3% of CDW. Cells were grown with odd chain fatty acids: heptadecenoic acid (C17), undecanoic acid (C11), tridecanoic acid (C13), nonanoic acid (C9), and heptanoic acid (C7). In 2015, Gao et al. confirmed the PHA production in \textit{Y. lipolytica}. Targeting of PHA synthase from \textit{P. aeruginosa} PAO1 to peroxisomes and cultivating cells with oleic acid or triolein resulted in production of mcl-PHAs (Gao et al., 2015). Gao et al. also enhanced PHA accumulation from 0.044% up to 1.46% of CDW by increasing expression of PHA gene with codon optimization and addition of a Kozak sequence, and by integrating multiple copies of the PHA synthase gene. In addition, an increase in oleic acid concentration from 0.1% to 1% increased the PHA accumulation from 1.13% to 2.84%. However, the highest PHA accumulation of 5% of CDW was obtained growing the cells with 2% triolein. In 2019, Rigouin et al. continued PHA studies in \textit{Y. lipolytica} (Rigouin et al., 2019). They produced homopolymer P(3-hydroxydodecanoate) (PHDD) (C12) by expressing only hydratase domain of the MFE enzyme. In comparison, copolymer consisting of even chain fatty acids; 3-hydroxyoctanoic acid (C8), decanoic acid (C10), dodecanoic acid (C12), and tetradecanoic (C14) was obtained by expressing the entire MFE enzyme. For both polymers, mix of glucose and either methyl myristate (C14) or methyl laurate (mC12) was used as a carbon source. The study showed that mutations E130, S325, S477, and Q481 in PHA synthase from \textit{P. aeruginosa} increased the mcl-PHA accumulation from 7% to 27% of CDW. The use of these four mutations together had been studied earlier in bacterial species (Yang et al., 2011, 2010). The highest PHA accumulation levels of 27% and 28% of CDW, for copolymer and PHHD, respectively, are the highest reported values this far for \textit{Y. lipolytica}. Rigouin et al. also extracted and analysed these polymers thoroughly. NMR and GC-MS analysis confirmed the polymer structures. The weight average molecular weight (Mw) of PHDD was 316 kDa and Mw of copolymer was 128 kDa. The copolymer had smaller Young’s modulus, but higher elongation % at brake, in comparison to PHDD, indicating that copolymer was more flexible.

In 2016, production of PHB in \textit{Y. lipolytica} was studied for the first time (Li et al., 2017). Li et al. expressed \textit{C. necator} PHB pathway including genes \textit{phaA}, \textit{phaB1}, and \textit{phaC1} in \textit{Y. lipo-
lytica. In addition, engineered PHA synthase from *Pseudomonas* sp. 61-3 with S325T and Q481K mutation was tested in the study, but it showed lower transcriptional level and lower PHB accumulation and additional experiments were carried only with *C. necator* pathway. When cells were grown with glucose in shake flasks, they accumulated 1.5% PHB of CDW. Higher accumulation levels of 3.84% and 10.2% of CDW were obtained growing cells with acetate in shake flasks and bioreactors, respectively.

In 2020, Lajus et al. continued to widen the range of different PHA polymers produced by *Y. lipolytica* (Lajus et al., 2020). PDLA accumulation level of 2.6% of CDW was achieved by targeting propionyl-CoA transferase (Pct) from *Clostridium propionicum* into the cytosol and engineered PHA synthase into peroxisomes. Authors compared Pct enzymes from different bacterial species and discovered that *C. propionicum* variant with V193A mutation was the only efficient enzyme for activation of D-lactic acid precursors. *In silico* and *in vivo* experiments showed that the endogenous Ldh enzyme, coded by *YILDL1* gene, was responsible for D-lactic acid conversion to pyruvate. Authors deleted the *YILDL1* gene to ensure the D-lactic acid availability for PDLA production. In addition, *POX1-6, DGA1, IRO1*, and *DGA2* genes were deleted to avoid production of mcl-precursor and triacylglycerols, which could inhibit the production of PDLA homopolymers and prevent efficient analysis of PHA accumulation with fluorescent BODIPY dye.

PHA production has been also demonstrated in few other yeast species besides *S. cerevisiae* and *Y. lipolytica*. In 2002, Poirier et al. showed production of mcl-PHAs in *Pichia pastoris* (Poirier et al., 2002). A modest 1% mcl-PHA accumulation was obtained when PHA synthase from *P. aeruginosa* was targeted to peroxisomes and cells were fed with oleic acid. *P. pastoris* was considered for the study as it grows well using fatty acids or methanol as a sole carbon source. Three years later Vijayasankaran et al. produced PHB in the cytosol of *P. pastoris* cells (Vijayasankaran et al., 2005). Integration of two copies of PHB synthase pathway from *C. necator* lead to production of 27% PHB of CDW in bioreactors under severe oxygen stress. However, reduced oxygen availability also decreased cell growth, in comparison to aerobic bioreactor cultivations. Fluorescence microscopy showed large clumps of PHB granules in *P. pastoris*, indicating that lack of phasins was possibly limiting proper granule formation.

Terentiev et al. studied in 2004 production of PHB and poly(hydroxyvalerate) (PHV) in non-conventional yeast *Arxula adeninivorans* (Terentiev et al., 2004). These cells were able to produce up to 2.2% and 0.019% of PHV and PHB, respectively, when cells expressed the *C. necator* PHB pathway and were grown with ethanol. PHA accumulation was later increased to record high level of 52.1% poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) copolymer of CDW by overexpression of PHA pathway genes together with gene encoding phasins (Biernacki et al., 2017). However, the molecular weights of the formed PHBV were relatively small, only 17.3 kDa (Mw). Non-conventional yeasts were also studied by Abd-El-haleem et al. in 2007 and 2009 (Abd-El-haleem et al., 2007; Abd-El-Haleem, 2009). Engineered *Kloeckera* sp. with *C. necator* PHB pathway accumulated 7% copolymer PHBV of CDW when cells were grown with glucose and oleic acid. In addition, wild type *R. minuta* produced a copolymer P(3-hydroxybutyrate-co-3-hydroxyvalerate-co-5-hydroxyvalerate) with accumulation level 2% of polymer of CDW. *R. minuta* was the only PHA producing yeast strain out of forty yeast isolates found from soil environment and from different molasses, foods, and concentrated juices. Both copolymer structures were verified by extracting the polymers and analysing them with NMR.
The listed PHA production studies with different yeast species increase the understanding of yeasts as production hosts in general. The highest PHA accumulation levels to date have been obtained by targeting monomer production and PHA synthase into suitable cellular spaces, deleting lipid synthesis pathways, controlling the cultivation condition such as oxygen availability, considering activity and expression efficiency of the PHA pathway, and by co-expression of phasins genes together with the PHA pathway.

1.6 Alternative carbon sources to glucose for production of PHAs in *S. cerevisiae*

Native *S. cerevisiae* strains are inherently able to catabolize simple hexose sugars like glucose. In industrial applications, these sugars are often derived from edible crops like sugar-cane or corn (Walker and Walker, 2018). However, the high price and unsustainability of these carbon sources have shifted the research efforts towards utilization of non-edible crops like cellulosic feedstocks (Wyman, 2007).

The main components of the complex lignocellulosic feedstocks include lignin, cellulose, and hemicellulose. The cellulose fraction can be hydrolysed into glucose by using different cellulase enzymes. These include for example β-glucosidase, which carries out the last step in the hydrolysis by hydrolysing one cellobiose molecule (β-glucose disaccharide) into two glucose units. High amount of β-glucosidase is needed to hydrolyse cellobiose and to prevent inhibition of other cellulases by the cellobiose molecules (Gruno et al., 2004). The cost of different cellulase enzymes reduces the cost-effectiveness of the process (Aditiya et al., 2016) and has thus inspired researchers to find alternative ways to use different plant waste feedstocks.

The native *S. cerevisiae* strains are not able to utilize cellobiose as their carbon source. They lack both celloextrin transporters carrying the cellobiose through the cell membrane and enzymes needed for the cellobiose catabolism. However, it has been demonstrated recently, that the expression of a celloextrin transporter gene (*CDT-1*) from a filamentous ascomycete fungus *Neurospora crassa*, together with an intracellular β-glucosidase gene (*GH1-1*) from *N. crassa* or a cellobiose phosphorylase gene (*cbp*) from bacterium *Ruminococcus flavefaciens*, allows *S. cerevisiae* to directly ferment celloextrins, including cellobiose (Galazka et al., 2010; Ha et al., 2013). The β-glucosidase enzyme converts one cellobiose disaccharide into two glucose molecules, while the cellobiose phosphorylase converts it into one glucose molecule and one glucose-1-phosphate molecule. Use of cellobiose phosphorylase could thus benefit especially systems with high ATP requirements as the conversion of glucose-1-phosphate to glucose-6-phosphate does not consume ATP. However, the different Michaelis constants of these enzymes indicate that the cellobiose phosphorylase could require higher cellobiose concentration than the β-glucosidase to process cellobiose with same rates. The importance of high cellobiose concentration was observed when native and engineered versions of celloextrin transporter Cdt-1 from *N. crassa* were compared (Ha et al., 2013). The amino acid change F213L in Cdt-1 increased cellobiose consumption in strain expressing cellobiose phosphorylase gene from *Saccharophagus degradans* while the difference in cellobiose consumption was smaller with strain expressing a β-glucosidase gene *GH1-1*. The studies focusing on improvement of cellobiose fermentation are not limited to the Cdt-1 engineering but cover also screening on novel transporters and glucosidases (Bae et al., 2014; de Ruijter et al., 2020) and methods like adaptive evolution and random mutagenesis (Oh et al., 2017, 2016).
Cellobiose utilization has been studied especially for ethanol fermentation, the productivity being over 1 g l^-1 h^-1 (Oh et al., 2016) and the highest titers over 30 g l^-1 (Ha et al., 2013; Kim et al., 2018; Oh et al., 2016; Yuan and Zhao, 2013). In addition, cellobiose has been successfully converted to lactic acid (Turner et al., 2016) and 2,3-butanediol (Nan et al., 2014).

Lactic acid is a common organic acid, which can be produced for example from organic kitchen waste by lactic acid bacteria (Wakamatsu et al., 2013) or from various sugars by different engineered yeast species (Baek et al., 2016; Ilmén et al., 2007; Koivuranta et al., 2014; Park et al., 2018). D- and L-lactic acids are also present in kraft black liquor of paper and pulp industry (Niemi et al., 2011). Both enantiomers can be transported into S. cerevisiae cells either actively through Jen1 and Ady2 transporters, or as ionic lactate through the cell membrane (Casal et al., 1999; Pacheco et al., 2012). The imported D-lactic acid and L-lactic acid are converted in vivo to pyruvate with D-lactate dehydrogenase (Dld1) and flavocytochrome b2 (Cyb2), respectively.

Xylose is a pentose sugar found in the hemicellulose fraction of the lignocellulosic feedstocks. It is present for example in spent sulphite liquor (SSL) originating from hard wood biomass (Pereira et al., 2013). Native S. cerevisiae is not able to use xylose as a carbon source. However, metabolic engineering of the S. cerevisiae allows xylose conversion to xylose which can be further metabolized by pentose phosphate pathway (Demeke et al., 2013). Two pathways for xylose conversion exists. Xylose can be either converted to xylitol by a xylose reductase and then into xylulose by a xylitol dehydrogenase. Alternatively, xylose can be converted directly to xylose by xylose isomerase (Walfridsson et al., 1996). Xylose utilisation has been enhanced for example by optimizing xylose reductase coenzyme preferences, overexpression of xylulokinase, and by combining both xylose conversion pathways into same strain (Bengtsson et al., 2009; Cunha et al., 2019; Jeppsson et al., 2006; Toivari et al., 2001). Xylose utilization in S. cerevisiae has been studied specially to improve ethanol production from lignocellulosic hydrolysates. However, recent studies indicate that xylose is an interesting carbon source also for PHB production (de Las Heras et al., 2016; Portugal-Nunes et al., 2017; Sandström et al., 2015).

1.7 The aims of the study

This thesis was inspired by the urgent need to find new environmentally friendly alternatives for non-biodegradable fossil-based plastics. The aim of this thesis was to study the possibilities to produce 2-hydroxyacid containing PHAs in S. cerevisiae, to control monomer composition of a PHA copolymer, and to use cellobiose as the sole carbon source for PHB production. These goals contribute to the wider themes of diversifying PHA properties so that they could be used in new applications and increasing sustainability of PHA production.

In the Publication 1, aim was to study if two wide substrate specificity PHA synthases are active in yeast S. cerevisiae and if their expression, combined with D-lactyl-CoA monomer production, would result in PDLA homopolymer formation. In addition, aim was to test P(LA-3HB) copolymer production in similar strains which carry also additional 3HB-CoA pathway genes. This goal was chosen as P(LA-3HB) copolymer has shown in literature improved mechanical properties over the rather stiff and brittle PHB and PLA homopolymers. The activity of chosen PHA synthase and copolymerization of 2-hydroxyxoids and 3-hydroxyacids had not been studied in yeast hosts before. The 2-hydroxyxid polymerization especially in robust yeast hosts is interesting as different 2-hydroxyxoids could be potential-
ly obtained for PHA production from black liquor, which is an abundant paper and pulp industry side stream. The purified hemicellulose fraction from black liquor is reported to contain lactic acid, glycolic acid, and 2-hydroxybutyric acid which are suitable precursors for PHA production with studied PHA synthase enzymes.

In Publication 2, the aim was to adjust the LA fraction in P(LA-3HB) precisely by controlling D-lactic acid production with Tet-On based synthetic biology method. This goal was chosen as P(LA-3HB) polymer properties are known to vary depending on the ratio of the two monomers. In vivo control method was selected for establishing a tool that could be used in future also for control of many other PHA copolymers. An additional target in Publication 2 was to study how D-lactic availability affects the total polymer accumulation in the selected strains and if PHA production efficiency could be increased by boosting PHA synthase expression. The understanding of limitations in PHA accumulation was chosen as target since previous literature has reported rather low PHA accumulation in yeast in comparison to bacterial hosts.

In Publication 3, the aim was to study if S. cerevisiae is able to produce PHB using cellobiose as a sole carbon source. Use of lignocellulose derived sugars instead of cultivated sugars could improve overall sustainability of the PHA production. Direct use of cellobiose by the cells process could improve the cellulose hydrolysis in simultaneous saccharification and fermentation by lowering the concentration of free cellobiose, which is an intermediate of hydrolysis process acting as a competitive inhibitor for many essential cellulases. In addition, direct use of cellobiose could also reduce the need for extracellular β-glucosidases in the hydrolytic enzyme mixture. Additional goal in the Publication 3 was to compare cellobiose metabolism in vivo either through cellobiose hydrolysis or cellobiose phosphorolysis to understand how their different kinetic and energetic mechanisms affect PHA production.
2. Materials and methods

2.1 Strain engineering

2.1.1 Genes and parental strains

Experiments in Publications 1-3 were carried with different prototrophic and auxotrophic haploid *S. cerevisiae* CEN.PK strains since these yeast strains are easy to modify with synthetic biology tools. Parental strains were kindly provided by Dr. P. Kötter (Institut für Mikrobiologie, J.W. Goethe Universität Frankfurt, Germany). These parental strains included prototrophic strain CEN.PK113-7D and auxotrophic strains CEN.PK102-5B (ura3-52, his3-Δ1, leu2-3-12), CEN.PK113-5D (ura3-52), and CEN.PK111-9A (his3-Δ1). The used genes are listed in the Table 2. All genes were codon optimized for expression in yeast *S. cerevisiae* and ordered from Integrated DNA Technologies.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>phaA</em></td>
<td>Acetyl-CoA acetyltransferase from <em>Cupriavidus necator</em>, GenBank KP681582</td>
<td>(Sandström et al., 2015)</td>
</tr>
<tr>
<td><em>phaB1</em></td>
<td>Acetoacetyl-CoA reductase from <em>C. necator</em>, GenBank KP681583</td>
<td>(Sandström et al., 2015)</td>
</tr>
<tr>
<td><em>phaC1</em></td>
<td>PHA synthase from <em>C. necator</em>, GenBank KP681584</td>
<td>(Sandström et al., 2015)</td>
</tr>
<tr>
<td><em>ldhA</em></td>
<td>Stereospecific D-lactate dehydrogenase from <em>Leuconostoc mesenteroides</em></td>
<td>(Baek et al., 2016)</td>
</tr>
<tr>
<td><em>phaC1437Ps6-19</em></td>
<td>PHA synthase from <em>Pseudomonas</em> sp. MBEL 6-19, with amino acid substitutions E130D, S325T, S477G, and Q481K</td>
<td>(Yang et al., 2010)</td>
</tr>
<tr>
<td><em>phaC1Pre</em></td>
<td>PHA synthase from <em>Pseudomonas resinovorans</em>, with amino acid substitutions E130D, S325T, S477G, and Q481K</td>
<td>(Yang et al., 2011)</td>
</tr>
<tr>
<td><em>pct540Cp</em></td>
<td>Propionyl-CoA transferase <em>pct540</em> from <em>Clostridium propionicum</em>, with V193A and four silent nucleotide mutations T78C, T669C, A1125G, and T1158C</td>
<td>(Yang et al., 2010)</td>
</tr>
<tr>
<td><em>pctMe</em></td>
<td>Propionyl-CoA transferase from <em>Megasphaera elsenii</em> BAU59368.1</td>
<td>(Prabhu et al., 2012)</td>
</tr>
<tr>
<td><em>cbp</em></td>
<td>FD-1 cellobiose phosphorylase from <em>Ruminococcus flavefaciens</em>, GenBank NZ_ACOK01000116.1</td>
<td>(Aeling et al., 2012)</td>
</tr>
<tr>
<td><em>GH1-1</em></td>
<td>β-glucosidase from <em>Neurospora crassa</em></td>
<td>(Galazka et al., 2010)</td>
</tr>
<tr>
<td><em>CDT-1</em></td>
<td>The cellodextrin transporter from <em>N. crassa</em></td>
<td>(Galazka et al., 2010)</td>
</tr>
</tbody>
</table>
2.1.2 Cloning and transformations

In Publication 1 genes were cloned into 2μ multicopy plasmids of the modular cloning kit (Lee et al., 2015). In Publications 2 and 3 into EasyClone integration vectors (Jessop-Fabre et al., 2016). Cloning was carried with modular cloning methods or with Gibson Assembly (E2611S, New England BioLabs). All plasmid constructions were carried in E. coli TOP10 cells. E. coli cells were grown in Luria–Bertani medium with either kanamycin (50 μg ml⁻¹) or ampicillin (100 μg ml⁻¹) at 37°C.

Lithium acetate methods was used for all yeast transformations (Gietz and Schiestl, 2007). The EasyClone expression cassettes were linearized with NotI enzyme (FD0596, Thermo Scientific) and the CRISPR/Cas9 protocol of the EasyClone kit was used for selection.

2.2 Cell cultures

2.2.1 Multiwell plate cultivation in Bioscreen C equipment

In Publication 2, the growth of the integrated PDLA and P(LA-3HB) strains, and growth of their corresponding control strains, was followed in one experiment with Bioscreen C equipment (Oy Growth Curves Ab Ltd). Bioscreen C was chosen for this purpose as it can measures the growth of up to 200 samples simultaneously. The expression ldhA gene was controlled with doxycycline concentrations from 0 to 10 mg l⁻¹. The cells were grown in honeycomb multiwell plates in 200 μl volume using continuous shaking at 30°C. The cell growth was followed with OD₆₀₀ measurements every 10 minutes and samples were analysed in three biological and technical replicates. Media evaporation from the wells was decreased by filling outer wells with water.

2.2.2 Shake flask cultivations

Synthetic complete (SC) media were used in all shake flask experiments as they allow yeast transformation with auxotrophic strains and better control of cultivation conditions in comparison more complicated media. SC media contained 6.7 g L⁻¹ yeast nitrogen base (Becton Dickinson) and 790 mg L⁻¹ complete supplement mixture (Formedium). In Publication 1, the complete supplement mixture lacked either leucine or uracil (SC-URA, SC-LEU). In Publication 3, the complete supplement mixture lacked uracil. SC media was also supplemented with proper carbon source depending of the cultivation. All shake flask experiments were carried in 250 ml Erlenmeyer flasks in 50 ml volume, at 30°C temperature with 220 rpm shaking in two or three biological replicates.

In Publication 3, the cell growth was followed in shake flasks also by using 20 g l⁻¹ of the EnPump 200 substrate (Enpresso GmbH) in growth media instead of glucose. EnPump 200 was dissolved into media and sterilized with filtration. Glucose was slowly released from the media (Figure 5) by the addition of Reagent A (Enpresso GmbH) to a final concentration of 0.5 U l⁻¹. This slow glucose release mimicked the cellobiose uptake of the cellobiose consuming strains in the same study.
2.2.3 Batch bioreactor

The cellobiose consuming strains were compared in Publication 3 in batch bioreactor which allowed better control of pH and aeration than shake flasks. Inocula were grown over night in shake flasks in SC medium containing 20 g l$^{-1}$ cellobiose. The cells were washed with sterile water prior suspension in 50 ml of sterile water for inoculation. The bioreactor experiments were carried in 1-liter Biostat-Q benchtop bioreactors (Sartorius) in 750 ml working volume. Strains were grown in SC-URA medium with 35 g l$^{-1}$ cellobiose. Temperature was kept at 30°C. The pH was maintained at pH 6 with 15% phosphoric acid and 1 M NaOH. The aeration was maintained with an impeller rotation set to 600 rpm and filtered (0.2 μm) continuous ambient air flow at rate of 1.5 l min$^{-1}$.

2.3 Analysis methods

2.3.1 Analysis of metabolites with chromatography methods

In Publications 1-3 the following media components and extracellular metabolites were measured with high-performance liquid chromatography (HPLC): glucose, cellobiose, ethanol, acetate, glycerol, and D-lactic acid. The samples were centrifuged for 10-20 minutes at 13500 rpm prior to dissolving the supernatants with 50 mM sulfuric acid in ratio of 1:1. Dissolved supernatants were run with 5 mM sulfuric acid eluent at 55°C using 0.5 ml min$^{-1}$ flow rate. Components were separated with Waters module 2690 and with two columns from Bio-Rad Laboratories: an organic acid analysis column (300 × 7.8 mm, Aminex HPX-87H) and Fast Acid Analysis Column (100 × 7.8 mm). Separated metabolites were detected with a Waters a differential refractometer 2414 and data processing was carried using Waters Empower 3 software.

In Publication 3 the cellotetraose and cellotriose concentrations were measured with high pressure ion chromatography (HPIC) using a Dionex ICS-6000 system (Thermo Scientific). Bioreactor samples were first centrifuged at 13 500 rpm. Supernatants were diluted in water 100 to 500-fold and separated in CarboPac Sa10-4μm column (Thermo Scientific) using 12 mM KOH eluent at 0.380 ml min$^{-1}$ flow rate at 40°C.
2.3.2 Analysis of cell growth

In Publications 1-3, cell growth was monitored either by measuring the CDW of the filtrated and washed samples or by measuring optical densities (OD) of the cultures at 600 nm with VitroSpec 2100 Pro (Amersham Biosciences). For CDW analysis, samples of 2-10 ml were filtered through pre-weighted Whatman glass microfiber filters (55 mm, GF/B), washed three times with distilled water, dried overnight at 100°C, and weighted. When necessary, linear correlation between CDW and OD600 values was estimated by calculating R² values for their linear trend lines. The linear trend line functions were used for conversion of OD600 values to CDW values if the R² value was higher than 0.96.

2.3.3 Measurement of pH

The pH of the cultivations was measured with WTW Sentix Mic electrode Innolab pH 720 instrument.

2.3.4 Analysis of PHA content with gas chromatography mass spectrometry (GC-MS)

In Publications 1-3, monomer compositions of the polymers and amount of polymer produced by each strain were measured with gas chromatography mass spectrometry (GC-MS) (Braunegg et al., 1978). The GC-MS method allows identification of different monomers reliably and is sensitive enough for detection of small monomer quantities of around 0.1 % of CDW or higher. Prior to analysis, cells were collected by centrifugation at 4000 rpm, washed two or three times with distilled water, and lyophilized overnight. Samples were subjected to methanolysis at 100°C for 140 minutes. Methanolysis was carried out in 1 ml chloroform, 830 μl methanol, 150 μl sulfuric acid, and 20 μl 3-hydroxybutyric acid as internal standard. Distilled water (0.5 ml) was used for removing the water-soluble particles prior to analysis of chloroform phase with HP-FFAP column (19091F-102, Agilent) and gas chromatography system (7890, Agilent). In Publication 1, the control strain without PHA synthase (H5531) contained less than 0.03% of 3HB-units, indicating that the possible free 3HB or 3HB-CoA units in the strains did not distort the GC-MS result.

2.3.5 Transcriptional analysis with quantitative polymerase chain reaction (qPCR)

In Publication 2, transcriptional analysis of *ldhA* gene was carried out to observe how different doxycycline concentrations affect the expression efficiency of Tet-On system. Transcriptional analysis was carried out with quantitative polymerase chain reaction (qPCR) using primer pairs (Table 3) which produce approximately 200 bp products of gene of interests (Rantasalo et al., 2018a). Cells were first collected by centrifugation, washed with water, and frozen quickly with liquid nitrogen. Frozen samples were stored at -80°C prior to analysis. RNeasy Mini kit (Qiagen) was used for total RNA extraction and DNase treatment (DNase I RNAse-free, Thermo Scientific) for removing the residual genomic DNA. The cDNA synthesis was carried out with cDNA synthesis kit (Roche). The prepared cDNA was combined with primers and LightCycler 480 SYBR Green I Master (Roche). The measured mRNA levels were compared to ubiquitin-protein ligase gene *UBC6*, which has been reported to maintain high transcriptional stability (Teste et al., 2009).

Table 3. Primers used for the transcriptional analysis.
<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBC6_qPCR_F</td>
<td>ACTTCCCGTCTGATTATCCA</td>
<td>(Rantasalo et al., 2018a)</td>
</tr>
<tr>
<td>UBC6_qPCR_R</td>
<td>TAATTGATCCTGCGGTGGCT</td>
<td>(Rantasalo et al., 2018a)</td>
</tr>
<tr>
<td>ldhA_1 qPCR_F</td>
<td>ATGCCATAAAGTCGTAATGCC</td>
<td>Publication 2</td>
</tr>
<tr>
<td>ldhA_1 qPCR_R</td>
<td>AAATTCCTTTTCCACTCCAGTC</td>
<td>Publication 2</td>
</tr>
<tr>
<td>ldhA_2 qPCR_F</td>
<td>AGAGTGGCGGATTAACATCT</td>
<td>Publication 2</td>
</tr>
<tr>
<td>ldhA_2 qPCR_R</td>
<td>GCCGTCTATATCCATCAGATTACC</td>
<td>Publication 2</td>
</tr>
</tbody>
</table>

### 2.3.6 Polymer extraction

Polymers were extracted for analysis of polymer properties in Publications 1-3. Cells were centrifuged for 6 minutes at 4000 rpm, washed with water, and lyophilized overnight. Lyophilized cell pellets were powdered and boiled in chloroform in glass tubes for three hours in 95°C water bath. Ten to twenty-fold times higher chloroform volume was used in comparison to cell pellets. After heating, solutions were stirred overnight at room temperature, filtered with PTFE filters (0.45 μm), and concentrated to approximately 300 μl. Ten-fold higher volume of ice-cold methanol was added to the concentrate for removal of possible lipid residues. Samples were centrifuged 3000 rpm for 20 minutes and supernatants were removed. If required, samples were washed also with additional non-solvent diethyl ether.

### 2.3.7 Size exclusion chromatography (SEC)

The lengths of the polymers were estimated based on molecular weight analysis with size exclusion chromatography (SEC) in Publications 1-3. Samples were dissolved either in 1,1,3,3,3-hexafluoro-2-propanol (HFIP) with 5 mM sodium trifluoroacetate or in chloroform by mixing the components in glass vials for one to seven days at room temperature. Samples were filtered with 0.45 μm syringe filters prior to analysis.

Samples dissolved in HFIP were run in 40°C HFIP eluent supplied with 5 mM sodium trifluoroacetate at flow rate of 0.5 ml min⁻¹. The HFIP system was equipped with Waters columns HR-4E and HR 5 and results were analysed against nine Agilent poly(methyl methacrylate) standards in the range of 2710 - 1 667 000 g mol⁻¹. Samples dissolved in chloroform were run in 30°C chloroform eluent at a flow rate of 0.6 ml min⁻¹, separated with HR 4 and 3 columns, and analysed against ten polystyrene standards of 1260- 3 040 000 g mol⁻¹ in chloroform. Both systems contained a Waters refractive index detector (2414) and a pre-column. Results were analysed with Waters Empower 3 software using third order fit (R2= 0.998-0.999).

### 2.3.8 Nuclear magnetic resonance (NMR)

The monomer compositions of and polymer structures were analysed also with nuclear magnetic resonance (NMR) in Publication 1. NMR analysis is important especially for verification of copolymer structure as it reveals which kind of monomers are covalently attached to each other. It can thus detect whether a sample contains e.g., random copolymers, diblock copolymers, or blend of two different homopolymers. The polymers were dissolved in 5 mm NMR tubes to final concentration of 830 mg l⁻¹ in 600 μl chloroform containing 0.03% (v/v) TMS standard. The NMR spectra were analysed at 22°C without solvent suppression with a Bruker Avance III NMR spectrometer (600 MHz) equipped with an inverse detection QCI H-P/C/N-
D cryoprobe. The repetition rate for the 1D 1H NMR analysis was 4.6 s. The zero quantum filtered total correlation spectroscopy (TOCSY) spectra were recorded using the pulse program dpsi2gpphzs with 120 ms DIPSI2 spinlock. The multiplicity edited, phase sensitive heteronuclear single quantum correlation (HSQC) spectra were recorded with pulse program hsqcedetgpsisp2.2. It uses shaped pulses (CHIRP) and Echo/Antiecho-TPPI selection for 180° pulses. The 13C decoupling during acquisition was achieved using the adiabatic CHIRP decoupling. The one-bond 13C – 1H coupling constant was set to 150 Hz (1/4J = 1.6667 ms) with relaxation delay of 1 s. For TOCSY and HSQC experiments, a matrix with 2048 x 256 data points was collected and zero-filled once in F1. The π/2 shifted squared sine-bell weighting function was used in both dimensions prior to the Fourier transformation. The absolute value heteronuclear multiple bond correlation (HMBC) spectra were recorded without decoupling by using the pulse program hmbcgpdqf. The one-bond 13C – 1H coupling constant was set to 150 Hz while the long-range 13C – 1H coupling constant was set to 8 Hz (1/2J = 3.3333 ms). A matrix of 4096 x 256 points was collected and zero-filled with linear prediction to 4096 x 1024 points. The unshifted sine-bell weighting function was applied in both dimensions prior to the Fourier transformation. All spectra were analysed with Bruker Topspin 3.5 pl 5 software.

2.3.9 Differential scanning calorimetry (DSC)

Polymer melting temperatures were analysed with differential scanning calorimetry (DSC) in Publication 1. The Mettler Toledo instrument DSC2 was equipped with Huber intra-cooler TC100MT and run under nitrogen atmosphere. Samples were weighed into 40 μl aluminium crucibles and lids were pricked prior to closing the crucibles with cold-pressing. Samples were heated twice with heating rate of 10°C min⁻¹. The samples were first cooled to -60°C and heated to 75°C. Then samples were cooled down to -60°C and heated to 250°C. Results were analysed with Mettler Toledo STARe software (version 13.0).
3. Results and discussion

The work is divided into three parts, which cover the production three different PHAs in *S. cerevisiae*. In the first part, the production homopolymeric PDLA is described. The second part focuses on P(LA-3HB) copolymer production and optimization of the monomer composition with synthetic biology. The last part describes the use of cellubiose as a carbon source for PHB production.

3.1 PDLA production in *S. cerevisiae*

In this thesis, the D-lactic acid production was introduced into the *S. cerevisiae* strains by integration of stereospecific D-lactate dehydrogenase gene from *L. mesenteroides* (*ldhA*) with simultaneous deletion of endogenous *DLD1* gene (Figure 6, Table 4). The *DLD1* gene was deleted to prevent D-lactic acid conversion back to pyruvate.

Figure 6. The PDLA pathway introduced into *S. cerevisiae*. The *ldhA* gene was expressed either with constitutive *TDH3* promoter (in Publication 1) or with doxycycline-controlled Tet-On synthetic expression system (in Publication 2). Genetic modifications are written in boxes.

In Publication 1, the *ldhA* gene was expressed from constitutive *TDH3* promoter, but later, in Publication 2, the expression was controlled with doxycycline-based Tet-On system. All studied PDLA pathways included propionyl-CoA transferase and PHA synthase. Two different propionyl-CoA transferases were compared: PctMe from *M. elsdenii* and Pct540Cp from *C. propionicum* with five amino acid changes (V193A, T78C, T669C, A1125G, and T1158C).
Also, two different PHA synthases were compared: PhaC1437_Ps6-19 from *Pseudomonas sp.* MBEL 6-19 and PhaC1Pre from *P. putida*. Both PHA synthases carried same amino acid changes (E130D, S325T, S477G, and Q481K) enabling the polymerization of D-lactic acid.

### Table 4. The PDLA producing *S. cerevisiae* strains and their corresponding control strains studied in Publications 1 and 2.

<table>
<thead>
<tr>
<th>Name</th>
<th>Number</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEN.PK102-5B</td>
<td>H3900</td>
<td><em>S. cerevisiae</em> (MATa his3-Δ1 ura3-52 leu2-3,112 TRP1 MAL2-8c SUC2)</td>
<td>-</td>
</tr>
<tr>
<td>pTDH3-lldHA</td>
<td>H5513</td>
<td>H3900 with DLD1 deletion and integration of pTDH3-lldHA (MATa his3-Δ1 ura3-52 leu2-3,112 TRP1 MAL2-8c SUC2, did1Δ::ldhA)</td>
<td>Publication 1</td>
</tr>
<tr>
<td>pTDH3-lldHA-2u_pTDH3_</td>
<td>H5520</td>
<td>H5513 with plasmid B9664 (pTEF1-pctMe-tENO1-pTDH3-phaC1Pre-tSSA1-LEU2)</td>
<td>Publication 1</td>
</tr>
<tr>
<td>PhaC1Pre</td>
<td>CEN.PK111-9A</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>pTEF1-pctMe</td>
<td>H5720</td>
<td>H3892 with pTEF1-pctMe (EasyClone integration site X-3)</td>
<td>Publication 2</td>
</tr>
<tr>
<td>pTDH3-tetR-VP16</td>
<td>H5721</td>
<td>H3892 with LEU2:pTDH3-tetR-VP16-LEU2</td>
<td>Publication 2</td>
</tr>
<tr>
<td>pTEF1-pctMe-TetOn-lldHA</td>
<td>H5723</td>
<td>H3892 with leu2-3,112::pTDH3-tetR-VP16-LEU2, pTEF1-pctMe (EasyClone integration site X-3), did1Δ::tetSES-ldhA</td>
<td>Publication 2</td>
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<tr>
<td>PhaC1Pre_1x</td>
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<td>H5723 with pTDH3-phaC1Pre (EasyClone integration site X-4)</td>
<td>Publication 2</td>
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<tr>
<td>PhaC1437_1x</td>
<td>H5725</td>
<td>H5723 with pTDH3-phaC1437Ps6-19 (EasyClone integration site X-4)</td>
<td>Publication 2</td>
</tr>
<tr>
<td>PhaC1Pre_3x</td>
<td>H5726</td>
<td>H5723 with 3x pTDH3-phaC1Pre (EasyClone integration sites X-4, XII-5, XI-3)</td>
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</tr>
<tr>
<td>PhaC1437_3x</td>
<td>H5728</td>
<td>H5723 with 3x pTDH3-phaC1437Ps6-19 (EasyClone integration sites X-4, XII-5, XI-3)</td>
<td>Publication 2</td>
</tr>
<tr>
<td>PhaC1Pre_6x</td>
<td>H5727</td>
<td>H5723 with 6x pTDH3-phaC1Pre (EasyClone integration sites X-4, XII-5, XI-3, X-2, XI-4, XI-5)</td>
<td>Publication 2</td>
</tr>
<tr>
<td>PhaC1437_6x</td>
<td>H5729</td>
<td>H5723 with 6x pTDH3-phaC1437Ps6-19 (EasyClone integration sites X-4, XII-5, XI-3, X-2, XI-4, XI-5)</td>
<td>Publication 2</td>
</tr>
</tbody>
</table>

#### 3.1.1 The polymer structure of yeast PDLA was confirmed with NMR and SEC analysis

In Publication 1, the production of PDLA homopolymer in yeast *S. cerevisiae* was demonstrated for the first time. Strains with different PHA synthases (PhaC1437_Ps6-19 or PhaC1Pre) and propionyl-CoA transferases (PctMe or Pct540Cp) were grown in shake flasks on glucose for 72 hours. All combinations of these two enzymes resulted in accumulation of PDLA. The highest accumulation level, 0.88% PDLA of CDW, was observed in strain expressing phaC1Pre and pctMe genes (strain H5520). This strain was chosen for PDLA extraction for polymer analysis. In Publication 2, the strains phaC1Pre_1x and phaC1437_1x accumulated 3.6% and 4.6% PDLA of CDW, respectively, when ldhA gene expression was adjusted with 6 mg l⁻¹ doxycycline. The molecular weights of extracted PDLA in these two studies are presented in Table 5. The measured Mw and Mn results are relatively small, but similar to each other, around 5.3 to 8.5 kDa, dispersity (D) being 1.18-1.3. They are both similar or smaller than molecular weights reported in literature for PDLA: 5-25 kDa (Mn) and 20-55 kDa (Mw) (Jung et al., 2010; Lajus et al., 2020; Yang et al., 2011). However, all of the PDLA molecular weights are rather short in comparison to molecular weights of PHB homopolymer, which can reach 1000 or 6000 kDa (Castillo et al., 2017; Fei et al., 2016; Meixner et al., 2018). These small molecular weights of PDLA could result from high $T_g$ of PDLA polymer (60°C) which exceeds the cultivation temperature of 30°C, as described in Chapter 1.4 (Figure 4) (Matsumoto et al., 2018b).

### Table 5. The molecular weights of extracted PDLA polymer in Publication 1 and 2. Mn: number average molecular weight, Mw: weight average molecular weight, D: dispersity

<table>
<thead>
<tr>
<th>Sample (Sampling time)</th>
<th>Strain</th>
<th>Mn (Da)</th>
<th>Mw (Da)</th>
<th>D</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The similarity of the $^1$H NMR peaks of the yeast PDLA (Publication 1) and the commercial PLA (95468-1G-F, Sigma Aldrich, Mw of 259 kDa and Mn of 103 kDa) confirms the polymer structure of PDLA (Figure 7). In addition, the $^1$H NMR spectra of the yeast PDLA does not show any scattering in 1.6 ppm and 5.2 ppm signals, which was seen in oligomeric PLA standard due the proximity of polymer ends. The oligomeric PLA sample was produced by acidic hydrolysis of commercial PLA (Ingeo 3251D, NatureWorks) and it contained PLA dimer, trimer, tetramer, and pentamer units (Patent PC19083FI).

Figure 7. The $^1$H -NMR spectra PDLA extracted from S. cerevisiae strain H5520, PLLA standard (Sigma Aldrich, 95468-1G-F, Mn 103 kDa and Mw 259 kDa), and oligomeric PLA standard obtained by acidic hydrolysis of commercial PLA (NatureWorks, Ingeo 3251D) containing dimer, trimer, tetramer, and pentamer oligomers. Figure modified from Publication 1.

3.1.2 Tet-On system enabled a control of expression of ldhA gene in S. cerevisiae

In Publication 2, the expression of ldhA gene was adjusted with doxycycline controllable Tet-On system presented in Figure 8. Different doxycycline concentrations were used for adjusting the attachment of TetR-VP16 (Tet-On) synthetic transcription factors (sTFs) to eight binding sites upstream from ENO1 core promoter (ENO1cp) and ldhA gene. Higher doxycycline concentrations increased sTF attachment and ldhA expression accelerating thus the D-lactic acid production.
Figure 8. The expression of D-lactate dehydrogenase gene (ldhA) was adjusted with Tet-On method (Bellí et al., 1998; Das et al., 2016; Gossen et al., 1995; Rantasalo et al., 2018a). The synthetic transcription factor TetR-VP16 contained an engineered TetR (Tet-On) domain and VP16 activation domain (AD) (Kakko et al. in preparation). The binding of the TetR-VP16 to the binding sites (BS) upstream from the ldhA gene was adjusted with different doxycycline concentrations. This in turn, affected ldhA expression and production of D-lactic acid.

The transcriptional analysis of ldhA gene was carried out in shake flask cultivations on glucose with two control strains lacking the PHA synthase, and in addition, with strain H5520 from Publication 1. Results show that highest ldhA expression with described Tet-On system, obtained with 10 mg l⁻¹ doxycycline, exceeded the ldhA expression with constitutive TDH3 promoter by 30% (Figure 9). In addition, measured mRNA levels correlated positively with doxycycline concentrations of 3 to 10 mg l⁻¹. This correlation fitted a polynomial function (y = 0.5103 x² - 1.4577 x + 0.7897) with high R² value of 0.9983, indicating that the used Tet-On construct is precisely adjustable for different expression levels.

Figure 9. The transcriptional level of ldhA gene relative to the ubiquitin-protein ligase gene UBC6. The ldhA expression of strain pTEF1-pctMe-TetOn-ldhA was adjusted with doxycycline and analysed at 6 hours. The ldhA gene expression of strains H5520 and pTDH3-ldhA was carried with constitutive TDH3 promoter and analysed at 16 hours. All transcription levels were measured in three biological replicates with two oligo pairs (Table 3). A: Data points represent averages of each oligo pair. B: Data points represent averages of two oligo pairs of strain pTEF1-pctMe-TetOn-ldhA.

3.1.3 High expression of stereospecific D-lactate dehydrogenase gene from Leuconostoc mesenteroides (ldhA) decreased the growth of S. cerevisiae

The effect of different doxycycline concentrations and expression of sTFs (tetR-VP16), PctMe, and ldhA on the growth of S. cerevisiae CEN.PK111-9A was studied in Publication 2. Strains (Table 4) were grown in Bioscreen C instrument on 20 g l⁻¹ glucose. The highest doxycycline level of 10 mg l⁻¹ had only minor effect on the final OD₆₀₀ and the specific growth rates of the control strains CEN.PK111-9A and pTEF1-pctMe (Table 6). In addition, the expression of pctMe or tetR-VP16 genes under strong constitutive promoters
did not affect the cell growth. However, the high expression of the \textit{ldhA} from the Tet-On construct with 10 mg l\(^{-1}\) doxycycline or from the strong constitutive \textit{TDH3} promoter resulted in approximately 50\% slower growth in comparison the parent strain CEN.PK111-9A. The slower growth rate, that correlated with the increase in doxycycline concentration, occurred only in \textit{ldhA} expressing strains indicating that growth was reduced due to the \textit{ldhA} expression or consequent D-lactic acid production.

**Table 6.** The growth of the \textit{S. cerevisiae} strains in the Bioscreen C equipment with doxycycline concentrations of 0-10 mg l\(^{-1}\) during the 15-hour experiment.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doxycycline (mg l(^{-1}))</th>
<th>Specific growth rate (h(^{-1}))</th>
<th>Max OD(_{600})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEN.PK111-9A</td>
<td>0</td>
<td>0.21</td>
<td>1.7</td>
</tr>
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<td>CEN.PK111-9A</td>
<td>10</td>
<td>0.2</td>
<td>1.68</td>
</tr>
<tr>
<td>pTEF1-pctMe</td>
<td>0</td>
<td>0.21</td>
<td>1.7</td>
</tr>
<tr>
<td>pTEF1-pctMe</td>
<td>10</td>
<td>0.19</td>
<td>1.71</td>
</tr>
<tr>
<td>pTDH3-tetR-VP16</td>
<td>0</td>
<td>0.21</td>
<td>1.7</td>
</tr>
<tr>
<td>pTDH3-tetR-VP16</td>
<td>10</td>
<td>0.19</td>
<td>1.57</td>
</tr>
<tr>
<td>pTDH3-ldhA</td>
<td>0</td>
<td>0.11</td>
<td>1.22</td>
</tr>
<tr>
<td>pTDH3-ldhA</td>
<td>10</td>
<td>0.08</td>
<td>0.96</td>
</tr>
<tr>
<td>pTEF1-pctMe-TetOn-ldhA</td>
<td>0</td>
<td>0.19</td>
<td>1.64</td>
</tr>
<tr>
<td>pTEF1-pctMe-TetOn-ldhA</td>
<td>1</td>
<td>0.16</td>
<td>1.58</td>
</tr>
<tr>
<td>pTEF1-pctMe-TetOn-ldhA</td>
<td>5</td>
<td>0.15</td>
<td>1.49</td>
</tr>
<tr>
<td>pTEF1-pctMe-TetOn-ldhA</td>
<td>10</td>
<td>0.12</td>
<td>1.23</td>
</tr>
</tbody>
</table>

3.1.4 The PDLA accumulation in \textit{S. cerevisiae} was enhanced by increasing expression of \textit{ldhA} gene and D-lactic acid production

In Publication 2, the effect of \textit{ldhA} expression on D-lactic acid production and PDLA accumulation was studied with strains phaC1Pre_1x and pTEF1-pctMe-TetOn-ldhA grown on 20 g l\(^{-1}\) glucose with doxycycline concentrations of 0 to 10 mg l\(^{-1}\). The results indicate that it is possible to increase D-lactic acid production linearly from 1 to 6.5 g l\(^{-1}\) by increasing the doxycycline concentration from 0 to 7.5 mg l\(^{-1}\) (Figure 10B). This increase in D-lactic acid production improved also PDLA accumulation in the cells (Figure 10E). The increase in doxycycline concentration above 7.5 mg l\(^{-1}\) did not increase D-lactic acid production notably, indicating that other factors such as the availability of pyruvate or the \textit{ldhA} mRNA translation efficiency were possibly limiting the D-lactic acid formation at doxycycline concentration of 10 mg l\(^{-1}\). Both strains produced around 1 g l\(^{-1}\) D-lactic acid also when doxycycline was not added (Figure 10A,C). However, no PDLA was observed without doxycycline (Figure 10D). The leakage of Tet-On system has been described previously (Bellí et al., 1998) and could be potentially reduced by lowering the sTF expression (Roney et al., 2016).
Figure 10. The effect of doxycycline concentrations of 0 to 10 mg l⁻¹ on D-lactic acid production and PDLA accumulation in *S. cerevisiae* expressing *ldhA* gene with Tet-On expression system. Strains pTEF1-pctMe-TetOn-ldhA (A, B) and phaC1Pre_1x (C-E) were grown in shake flasks for 72 hours on 20 g l⁻¹ glucose. The results represent averages of three biological replicates, A, C, D: individual data points are presented with circles.

3.1.5 *S. cerevisiae* accumulated most of the PDLA (as % of cell dry weight [CDW] and as mg l⁻¹) already during the first 24h of the cultivation

The time profile of PDLA accumulation in Publication 2 demonstrates that *S. cerevisiae* is able to accumulate up to 3.7-4.6% PDLA of CDW already in 24 hours (Figure 11) when glucose is available. The PDLA production increased only by less than 15% (as % of CDW and mg l⁻¹) during the following 48 hours when cells were consuming formed ethanol from the media.

Figure 11. The PDLA accumulation in strain phaC1Pre_1x with different doxycycline concentrations at 24 hours, 48 hours and 72 hours. The results represent averages of three biological replicates and individual data points are presented with circles. A: PDLA accumulation of CDW. B: PDLA titer (mg l⁻¹).

The production of homopolymeric PDLA in *vivo* has been studied previously only in few studies. PDLA accumulation was shown recently in yeast *Y. lipolytica* by feeding the strains D-lactic acid instead of producing it *in vivo* (Lajus et al., 2020). Strains accumulated PDLA from 48 hours onward reaching the highest accumulation level of 2.8% of CDW by the end of the cultivation at 120 hours. In an earlier bacterial study, up to 11% PDLA of CDW was pro-
duced in *E. coli* in 72 hours experiment (Jung et al., 2010), but accumulation was not followed during the cultivation.

### 3.1.6 Record high PDLA accumulation in *S. cerevisiae* was obtained with PHA synthase PhaC1437Ps6-19 and high D-lactic acid supply

The highest PDLA accumulation level in *S. cerevisiae* in this thesis, 5.6% of CDW, was measured when strain phaC1437_1x was grown on 20 g l\(^{-1}\) glucose for 48 hours and expression of *ldhA* gene was adjusted with 7.5 mg l\(^{-1}\) doxycycline (Figure 12H). Slightly lower accumulation levels of 4.2-4.3 % PDLA of CDW were measured when *ldhA* gene expression was adjusted with lower doxycycline concentrations of 5 or 6 mg l\(^{-1}\) or strains were expressing PHA synthase gene *phaC1Pre*. Positive correlation between D-lactic acid production and PDLA accumulation (Figure 13) was observed in all strains expressing PHA synthase gene *phaC1437Ps6-19* and in strain phaC1Pre_1x. The differences in PDLA accumulation in the studied strains could result from the differences D-lactic acid production. However, also the copolymer P(LA-3HB) production results (Chapter 3.2.3) indicate that strains expressing PHA synthase gene *phaC1437Ps6-19* would be able to accumulate more biomass and D-lactic acid containing PHAs, than strains expressing PHA synthase gene *phaC1Pre*.

The highest obtained PDLA accumulation level of 5.6% is over two-fold higher than that reported previously for *Y. lipolytica* (2.6% of CDW) in which the PHA synthase was targeted to peroxisomes (Lajus et al., 2020). Both of the yeast results are lower than the highest reported PDLA accumulation in bacterial strains, 11% of CDW in *E. coli* (Jung et al., 2010). However, all of these PDLA accumulation levels are relatively low in comparison to the highest obtained PHB or mcl-PHA accumulation levels in engineered yeast and bacteria. This could result from few different phenomena, e.g., from the high T\(_g\) of PDLA, which might restrict the PDLA elongation (described in Chapter 1.4). In addition, results from this thesis (Figures 11 and 12) indicate that the PDLA strains polymerized less than 3% of the produced D-lactic acid. This raised a question whether PDLA pathway was enough active to convert available D-lactic acid into PDLA polymer *in vivo*. In literature, 16% and over 400% increase in PDLA accumulation has been reported in yeast *Y. lipolytica* (Lajus et al., 2020) and bacterium *C. glutamicum* (Matsumoto et al., 2014), respectively, when the expression of PHA synthase has been increased. To study this in *S. cerevisiae*, the PHA synthase expression was increased in Publication 2 by adding two or five additional copies of each PHA synthase gene to the PDLA producing strains. However, no significant increase in polymer accumulation was observed when the copy number of the PHA synthases was increased (Figure 12G,H). Instead, the increase in PHA synthase copy number delayed the growth and production of D-lactic acid (Figure 12A-C). Only the strain expressing PHA synthase gene *phaC1Pre* accumulated slightly more PDLA when copy number increased from one to three. With all other strains, the PDLA accumulation stayed same or decreased with higher gene copy number. These results indicate that PDLA accumulation in studied strains was limited by the availability of the monomers rather than PHA synthase enzymes. This observation supports findings from other studies in which PHB production has been improved in *S. cerevisiae* e.g., by addition of acetate and panthothenate or by improving in acetyl-CoA and NADPH supply (Carlson and Srienc, 2006; Kocharin et al., 2012; Kocharin and Nielsen, 2013). As PHAs accumulate inside the cells, PHA titers are dependent on both amount of produced biomass and PHA accumulation level as % of biomass. The studied CEN.PK laboratory strains do not usually grow into high cell densities which limits possibilities to reach high titers. In Publication 1 obtained
PDLA titer was only 9.4 mg L⁻¹ and in Publication 2 around 75 mg L⁻¹. These could be however increased in future by using other *S. cerevisiae* strains which grow into higher cell densities and by improving PDLA accumulation in the cells. In both Publications, cells produced significant amounts of extracellular ethanol, D-lactic acid, and acetate and approximately 10- to 20-fold more biomass than PDLA, resulting in rather low PDLA yields per consumed sugar. In Publication 1 the PDLA yield was only 0.47 mg g⁻¹ glucose and in Publication 2 approximately 3.8 mg g⁻¹ glucose. PDLA yield could be improved in the future by reducing production of secreted metabolites and by improving PDLA accumulation.

In general, PHA accumulation levels in yeast are still rather low, max around 30% of CDW (Table 1), in comparison highest PHA accumulation levels obtained in bacterial hosts which reach 94-96% of CDW (Ling et al., 2018; Nduko et al., 2014). However, research in lipid production has shown that traditionally non-oleaginous *S. cerevisiae* can be modified to accumulate around 60% acetyl-CoA derived lipids of their cell dry weight (Arhar et al., 2021). This high lipid accumulation indicates in two ways that *S. cerevisiae* could be possibly engineered to accumulate also high PHA concentrations. Firstly, *S. cerevisiae* seems to have enough intracellular space to fit high quantity of storage compounds. Secondly, *S. cerevisiae* metabolism seems to be adjustable for production of high amount of reducing agents (NADPH) and acetyl-CoA, which are important precursor also for PHA production. In practice, following engineering steps could be adapted also for PHA production. Parent strains could be chosen amongst naturally lipid accumulating variants (Arhar et al., 2021; He et al., 2018) and competing pathways, such as glycogen and steryl ester synthesis, could be reduced (Arhar et al., 2021). However, in contrast to lipid production, the optimization of PHA production might also require reduction in TAG biosynthesis (Haddouche et al., 2011) and adjustments in PHA pathway downstream from acetyl-CoA (de Las Heras et al., 2016; Kocharin et al., 2012; Liu et al., 2017). Albeit the increase in PHA synthase gene copy number did not improve PHA accumulation in this thesis, improved PHA synthase expression has enhanced PHA production in few other studies (Gao et al., 2015; Lajus et al., 2020; Matsumoto et al., 2014) and could possibly raise PHA levels when precursor formation is improved. Depending on the target PHA, the PHA synthase activity could be also increased by using other efficient PHA synthases (Bhubalan et al., 2011) or by improving PHA synthase efficiencies (Amara et al., 2002; Bhubalan et al., 2011; Rigouin et al., 2019; Takase et al., 2003).
Figure 12. Comparison of the strains expressing PHA synthase genes *phaC1Pre* and *phaC1437P46-19* from one, three, or six copies of each gene. D-lactic acid production was adjusted with 5, 6, and 7.5 mg l$^{-1}$ doxycycline. A-C: D-lactic acid production, D-F: Cell growth, G-H: PDLA accumulation. Results represent averages of the three biological replicates and individual data points are presented with squares, circles, and triangles.

Figure 13. The correlation of produced D-lactic acid (g l$^{-1}$) to the PDLA accumulation in the cells (% of CDW). The studied strains were expressing one, three, or six copies of PHA synthase gene *phaC1437P46-19* or *phaC1Pre*. A, D: 5 mg l$^{-1}$ doxycycline, B, E: 6 mg l$^{-1}$ doxycycline, C, F: 7.5 mg l$^{-1}$ doxycycline
3.2 P(LA-3HB) production

The mechanical properties of chemically produced PLA and some common PHAs, e.g., PHB, are rather limited due their brittle and rigid structures. These mechanical properties can be improved by combining different monomers to copolymers. In the Publication 1, the combination of D-lactic and 3-hydroxybutyric acids into copolymer P(LA-3HB), was shown for the first time in a fungal host. In Publication 2, the P(LA-3HB) monomer contents were controlled with a gene expression-based method. This was achieved by expressing the stereospecific D-lactate dehydrogenase gene (ldhA) from *L. mesenteroides* with an adjustable Tet-On system (Table 7, Figure 14). The propionyl-CoA transferase gene *pct540Cp* and PHA synthase genes *phaC1Pre* and *phaC1437* were expressed either from multi copy plasmids (Publication 1) or were integrated into yeast genome (Publication 2). The acetyl-CoA acetyltransferase (phaA) and acetoacetyl-CoA reductase (phaB1) genes from *C. necator* were integrated to yeast genome in both studies.

Table 7. The P(LA-3HB) producing *S. cerevisiae* strains and their corresponding control strains studied in Publications 1 and 2.

<table>
<thead>
<tr>
<th>Name</th>
<th>Number</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEN.PK102-5B</td>
<td>H3900</td>
<td><em>S. cerevisiae</em> (MATa his3-Δ1 ura3-52 leu2-3,112 TRP1)</td>
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</tr>
<tr>
<td>-</td>
<td>H5514</td>
<td>H3900  did1Δ::ldhA, HIS3::phaA,phaB1,HIS3</td>
<td>Publication 1</td>
</tr>
<tr>
<td>-</td>
<td>H5523</td>
<td>H5514 with plasmid B9665 (pct540Cp and phaC1437)</td>
<td>Publication 1</td>
</tr>
<tr>
<td>-</td>
<td>H5524</td>
<td>H5514 with plasmid B9666 (pct540Cp and phaC1Pre)</td>
<td>Publication 1</td>
</tr>
<tr>
<td>CEN.PK111-9A</td>
<td>H3892</td>
<td><em>S. cerevisiae</em> (MATa his3-Δ1 URA3 leu2-3,112 TRP1)</td>
<td>-</td>
</tr>
<tr>
<td>phaC1Pre_3HB</td>
<td>H5730</td>
<td>H3892 with leu2-3,112::pTDH3-tetR-VP16-LEU2, pTEF1-pctMe (EasyClone integration site X-3); did1Δ::tetSES-ldhA; pTEF1-phaA, pTDH3-phaB1 (EasyClone integration site XI-I); 3x pTDH3-phaC1Pre (EasyClone integration sites X-4, XII-5, XI-3)</td>
<td>Publication 2</td>
</tr>
<tr>
<td>phaC1437_3HB</td>
<td>H5731</td>
<td>H3892 with leu2-3,112::pTDH3-tetR-VP16-LEU2, pTEF1-pctMe (EasyClone integration site X-3); did1Δ::tetSES-ldhA; pTEF1-phaA, pTDH3-phaB1 (EasyClone integration site XI-I); 3x pTDH3-phaC1437 (EasyClone integration sites X-4, XII-5, XI-3)</td>
<td>Publication 2</td>
</tr>
</tbody>
</table>
3.2.1 NMR and SEC results confirmed the copolymer structure and presence of D-lactic acid repeating sequences in the P(LA-3HB) copolymer

In Publication 1, both the strains H5523 and H5524 (Table 7) showed production of P(LA-3HB) copolymer when strains were grown in shake flasks on glucose. The H5523 strain expressing PHA synthase gene phaC1437Ps6-19 showed the highest accumulation of copolymer, 3.0% of CDW, in GC-MS analysis, and was selected for analysis of polymer properties. Later cultivations showed accumulation levels of 2.54% and 3.65% of CDW, with D-lactic acid content of approximately 65% and 40%, respectively. These obtained D-lactic acid contents are similar to previously reported D-lactic acid contents of approximately 50% in *E. coli* expressing PHA synthase gene *phaC1437Ps6-19* (Yang et al., 2011).

The P(LA-3HB) copolymer structure was confirmed in Publication 1 with 1H and 2D NMR (Figure 15). The triad LA-LA-LA signals and 3HB-3HB-3HB indicate presence of LA and 3HB repeating sequences within the polymer chain (Figure 15B). However, the slightly sifted signals also confirm presence of both monomers next to each other in the same chain. The TOCSY spectrum shows that shifted signals were part of lactate type spin systems and corresponded 3HB-LA-3HB and 3HB-LA-LA monomer structures (Figure 15A). The integration of these signals gave following estimation for the copolymer structure: 79% for LA-LA-LA triad, 5% for 3HB-LA-3HB triad, and 16% for 3HB-LA-LA triad. The small shift in signals was observed also with 3HB CH signals. The presence of high amount of LA-LA-LA triad signals indicates that copolymer P(LA-3HB) contained D-lactic acid repeating sequences. These have been found also in some bacterial studies focusing on P(LA-3HB) production (Ochi et al., 2013; Tajima et al., 2012; Yamada et al., 2009). The repeating monomer sequences might influence the thermal properties of material by for example enabling the crystallization of otherwise amorphous polymer (Peacock and Calhoun, 2006).

One explanation for LA or 3HB repeating sequence could be that LA-CoA and 3HB-CoA were available at different concentrations in different phases of the polymerization. In Publication 1, both LA-CoA and 3HB-CoA pathways were active at the same time which should result in simultaneous availability of the both monomers and thus production of random copolymer. It is however known from literature, that block structures can be formed when different monomers are sequentially added (Hu et al., 2011; Pederson et al., 2006; Wang et al., 2013a) or when PHA synthase exhibits very different affinities to the monomers (Matsumoto et al., 2018a). The activity of studied PHA syntheses towards D-LA-CoA or 3HB-CoA was not measured in this study, but results from recent *in vitro* paper reports that activity of another engineered PHA synthase (PhaC1PsSTQK) towards D-lactyl-CoA (20.4 U g⁻¹ PhaC1PsSTQK) is in similar range, but approximately half of activity towards 3HB-CoA (46.8 U g⁻¹ PhaC1PsSTQK) (Matsumoto et al., 2018b). However, the PHA syntheses used in Publication 1 and 2 might have different activities towards these substrates as PhaC1PsSTQK originates from different strain, *Pseudomonas* sp. 61–3, and carries only two mutations in its active site, S325T and Q481K.
Figure 15. The $^1$H and 2D NMR results of copolymer P(LA-3HB). A-F: Expansions of heteronuclear single quantum correlation (HSQC) spectra, heteronuclear multiple bond correlation (HMBC) spectra, and 2D total correlation spectroscopy (TOCSY) of the P(LA-3HB) copolymer. Figures are from Publication 1. A) Part of the CH2 and methyl region of TOCSY demonstrating the lactate type methyl correlations to the small CH signals present in HSQC (Figure B). B) HSQC CH signals of the polymers. Green and violet areas are overlaid spectra of the homopolymers poly(hydroxybutyrate) (PHB) (b) and poly(D-lactic acid) (PDLA) (a), respectively. Three slightly shifted PDLA type signals (c - e) have TOCSY correlations to methyl regions (panel A). Some of the 3HB signals are possibly overlapped with CH signal from the glycerol backbone of triglycerides (tg). C) HMBC correlations of PHB and PDLA CO carbons to CH protons. D) CH2 signals of 3HB blocks in HSQC show a slightly shifted doublet. E) HMBC correlations of the CH carbon of PHB to the CH2 protons of 3HB blocks. F) HMBC correlations of CO carbon to PHB CH2 protons. G: The $^1$H -NMR spectra P(LA-3HB) extracted from *S. cerevisiae* strain H5523. Figure is modified from Publication 1. tg: CH signal from the triglyceride glycerol backbone.

In Publication 2, the strains phaC1Pre_3HB and phaC1437_3HB produced 8.7 and 10.5 % copolymer of CDW, respectively, already in 24 hours. The molecular weights of the formed copolymers were however two to three-fold smaller than in Publication 1, only 7.5-12.2 kDa (Mw) (Table 8). This difference in molecular weight could result from different D-lactic acid contents in P(LA-3HB) copolymers, which were approximately 50 mol% in Publication 1 and 90 mol% in Publication 2. Increase in D-lactic acid content in P(LA-3HB) from 47 mol% to 100 mol% has been shown to increase the $T_g$ from 15°C to 63°C (Yamada et al., 2009). As mentioned in Chapter 1.4, the recent *in vitro* study proposes that high $T_g$ above cultivation temperatures and consequent polymer stiffness could halt the polymer elongation already when P(LA-3HB) and PDLA polymers reach molecular weight of 3 kDa (Matsumoto et al., 2018b). This could apply especially to copolymers obtained in Publication 2 with high 90 mol% LA content, but also to copolymers in Publication 1 with high amount of LA-repeating sequences. In addition, differences between these two studies could result from other param-
eters, e.g., from different cultivation conditions, possibly different expression levels of PHA synthase genes, and robustness of the SEC analysis.

In both Publications 1 and 2, the measured P(LA-3HB) molecular weights (Table 8) were lower than previously reported for *E. coli* expressing PHA synthase gene *phaC1437Ps6-19* and producing P(LA-3HB) with similar lactic acid content of around 50 mol% (Mw 39 kDa, Mn 22kDa) (Yang et al., 2010). However, the SEC analysis results in this Thesis confirm the successful polymer production in *S. cerevisiae*. The narrow Đ and unimodal distribution of molecular masses indicate that polymers were free of contaminants with smaller molecular weights. The polymers with low molecular weight might suit for example for packaging materials or aqueous dispersion coatings.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Strain (PHA synthase)</th>
<th>Mn (kDa)</th>
<th>Mw (kDa)</th>
<th>Đ</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(LA-3HB)</td>
<td>H5523 (phaC1437Ps6-19)</td>
<td>19.2</td>
<td>24.6</td>
<td>1.29</td>
<td>Publication 1</td>
</tr>
<tr>
<td>P(LA-3HB)</td>
<td>phaC1Pre_3HB</td>
<td>6.1±0.13</td>
<td>7.5±0.12</td>
<td>1.3±0.01</td>
<td>Publication 2</td>
</tr>
<tr>
<td>P(LA-3HB)</td>
<td>phaC1437_3HB</td>
<td>8.5±0.33</td>
<td>12.2±0.70</td>
<td>1.3±0.03</td>
<td>Publication 2</td>
</tr>
</tbody>
</table>

### 3.2.2 The D-lactic acid content in P(LA-3HB) was controlled in vivo in *S. cerevisiae* by adjusting the expression of *ldhA* gene

In the Publication 2, the lactic acid content of P(LA-3HB) copolymer was adjusted by expressing *ldhA* gene with doxycycline adjustable Tet-On expression system. Strains *phaC1437_3HB* and *phaC1Pre_3HB* were grown in shake flasks on 20 g l⁻¹ glucose with 0-7.5 mg l⁻¹ doxycycline for 48 hours. The concentration of formed D-lactic acid and D-lactic acid content in the P(LA-3HB) correlated positively in a linear manner in both strains (Figure 16). Wide range of different D-lactic acid contents, from 6 to 93 mol% were obtained (Table 9). This result indicates that the Tet-On control of the *ldhA* gene expression is a powerful alternative for tuning the P(LA-3HB) composition in vivo, when compared to the monomer feeding and the control of the oxygen availability used in literature.

Increase in D-lactic acid production resulted in lower acetate, ethanol, and biomass formation (Table 9). The strains without doxycycline produced trace concentrations of D-lactic acid, leading to incorporation of 6 mol% and 17 mol% D-lactic acid in the P(LA-3HB). This basal D-lactic acid level could be potentially lowered by decreasing the expression of the *tetR-VP16* genes as high sTF production has been reported to cause leakages in the Tet-On systems (Roney et al., 2016). The lower background level of D-lactic acid could result in even wider range of different lactic acid contents in the future, starting from less than 6 mol% of D-lactic acid.
Figure 16. The correlation of produced D-lactic acid (g l⁻¹) to the P(LA-3HB) copolymer accumulation cells as % of CDW and to the D-lactic acid content in the copolymer in the strains expressing PHA synthase gene phaC1Pre (A) or phaC1437Ps6-19 (B). The results represent averages of three biological replicates.

Table 9. Comparison of the P(LA-3HB) copolymer strains phaC1437_3HB and phaC1Pre_3HB. The expression of ldhA gene was adjusted with the Tet-On system using 0 to 7.5 mg l⁻¹ doxycycline. Results are averages of two or three replicates. CDW: Cell dry weight.

<table>
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<tr>
<th>Strain</th>
<th>Doxycycline (mg l⁻¹)</th>
<th>Polymer % of CDW</th>
<th>Polymer titer (mg l⁻¹)</th>
<th>Polymer yield (mg g⁻¹ glucose)</th>
<th>D-lactic acid mol % in polymer</th>
<th>Ethanol (g l⁻¹)</th>
<th>D-lactic acid (g l⁻¹)</th>
<th>Acetate (g l⁻¹)</th>
<th>CDW (g l⁻¹)</th>
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<td>PhaC1437_3HB</td>
<td>0</td>
<td>5</td>
<td>135</td>
<td>6.4</td>
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<td>8.7</td>
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<td>50%</td>
<td>8.5</td>
<td>1.8</td>
<td>1.4</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>17.8</td>
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<td>88%</td>
<td>7.9</td>
<td>4</td>
<td>0.8</td>
<td>1.4</td>
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<tr>
<td></td>
<td>6</td>
<td>19</td>
<td>232</td>
<td>11</td>
<td>88%</td>
<td>7.9</td>
<td>4.3</td>
<td>0.6</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>9.1</td>
<td>168</td>
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<td>7.1</td>
<td>3.2</td>
<td>1</td>
<td>1.8</td>
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<tr>
<td>PhaC1Pre_3HB</td>
<td>0</td>
<td>3</td>
<td>56</td>
<td>2.7</td>
<td>17%</td>
<td>9.4</td>
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<td>63</td>
<td>3</td>
<td>29%</td>
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<td>5</td>
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<td>6.2</td>
<td>88%</td>
<td>8.6</td>
<td>3.3</td>
<td>0.7</td>
<td>1.3</td>
</tr>
</tbody>
</table>

3.2.3 The strain expressing phaC1437Ps6-19 gene produced more P(LA-3HB) copolymer per consumed sugar, volume, and cell dry weight, in comparison to the strain expressing phaC1Pre gene

Comparison of the two PHA synthases in Publication 2 showed that strains with PHA synthase PhaC1437Ps6-19 produced 30-140% more P(LA-3HB) as mg g⁻¹ glucose with all used doxycycline concentrations, in comparison to strain with PHA synthase PhaC1Pre. For example, when strain PhaC1437Ps6-19 was grown with 6 mg l⁻¹ doxycycline it reached the highest copolymer accumulation level of 19% of CDW (with 88 mol% D-lactic acid content), the highest titer of 232 mg polymer l⁻¹, and the highest yield of 11 mg polymer g⁻¹ glucose of the Publication 2 (Table 9). For comparison, when the strain expressing PHA synthase gene phaC1Pre was grown in similar conditions, it produced less P(LA-3HB), 14.2% of CDW, with higher D-lactic acid content of 93 mol%. An earlier study in *E. coli* supports this small, but recurring difference in D-lactic acid content in P(LA-3HB) produced by these engineered PHA synthases (Yang et al., 2011). Observed D-lactic acid content in P(LA-3HB) were 50 mol% for strains expressing phaC1437Ps6-19 and 65 mol% for strains expressing phaC1Pre. (Yang et al., 2011).

The highest P(LA-3HB) accumulation level of 19% of CDW (Table 9) of strain PhaC1437_3HB, is approximately four-fold higher than PDLA accumulation in similar strain which didn’t contain 3HB-CoA pathway (PhaC1437_3x) (Figure 12H). Cultivation conditions are not expected to affect these results as both strains were grown in similar conditions (50 ml SC media in 250 ml shake flasks, 20 g l⁻¹ glucose, rotation 220 rpm, temperature 30°C, doxycycline concentration 6 mg l⁻¹) for 48 hours and extracellular pH decreased with both strains to approximately pH 3 in 24 hours. However, there are few reasons why more P(LA-
3HB) than PDLA was formed. The inclusion of 3HB monomers into the P(LA-3HB) polymer could have resulted in polymers with lower Tg and allow thus more efficient polymerization, as described in Chapter 1.4. This is supported by the finding from the Publication 2 in which in P(LA-3HB) strains polymerized approximately 5% of the produced D-lactic acid but PDLA strains only around 2%. However, the polymerization of such small percentages of produced D-lactic acid in both strains indicates also that cells were inefficient in converting D-lactic acid into D-lactyl-CoA. Perhaps D-lactic acid was quickly exported from the cells to avoid pH decrease in the cells or the PHA pathway was not able to add CoA-group to all the D-lactic acid precursors. Simultaneous production and polymerization of another monomer, 3HB-CoA, could have thus improved total polymer accumulation in copolymer strains. The 3HB-CoA precursors, acetyl-CoA and acetoacetyl-CoA, contain CoA-groups and are not exported from the cells as easily as D-lactic acid.

The effect of formation of biomass and different metabolites on PHA titers and yields are described in general level in Chapter 3.1.6. In addition, same chapter describes possible methods to increase PHA accumulation in yeast hosts.

3.3 PHB production in *S. cerevisiae* on glucose and on cellobiose

In literature, production of PHB has been demonstrated in *S. cerevisiae* only on glucose, xylose, acetate, and ethanol, but not on cellobiose. In addition, none of previous studies with *S. cerevisiae* report analysis of PHB polymer properties. In this thesis, PHB was produced and analysed on glucose in Publication 1 and on cellobiose in Publication 3. PHB molecular weights were analysed in both publications with SEC.

Lignocellulose derived sugars offer alternatives for sugars originating from edible crops in biotechnical applications. For example, cellulose can be hydrolysed into glucose using different cellulase enzymes. One of the hydrolysis products is cellobiose, which is further processed into two glucose units by β-glucosidases. This conversion is important as cellobiose accumulation decreases efficiency of the cellulase enzymes (Gruno et al., 2004) and wild type *S. cerevisiae* strains are not able to use cellobiose as their carbon source. However, cellobiose hydrolyzation requires high quantities of extracellular β-glucosidases. The direct use of cellobiose by *S. cerevisiae* could thus simplify cellulose hydrolysis in simultaneous saccharification and fermentation process by decreasing both negative effects of cellobiose accumulation on the different cellulase enzymes and need for extracellular β-glucosidases.
The PHB pathway contains three steps from acetyl-CoA to PHB. An acetyltransferase (PhaA) combines two acetyl-CoA molecules into acetoacetyl-CoA, which is then further converted acetoacetyl-CoA by an acetoacetyl-CoA reductase (PhaB1) and finally added to the polymer chain by a PHA synthase (PhaC1) (Figures 2 and 17). This PHB pathway from C. necator was expressed either from multicopy plasmids (strain H5529) in Publication 1 or from same pathway integrated into the yeast genome in Publication 3 (Table 10). The cellobiose metabolism was enabled in Publication 3 by integrating either a cellobiose phosphorylase gene from R. flavefaciens (cbp), or β-glucosidase gene from N. crassa (GH1-1) into strain carrying a cellodextrin transporter gene (CDT-1) from N. crassa in CEN/ARS low copy plasmid. Expression of CDT-1 gene from a plasmid was chosen as one integrated copy had shown in other studies to limit cellobiose consumption (Oh et al., 2016; Wei et al., 2015). Cellobiose phosphorylase converts cellobiose into glucose and glucose-1-phosphate units. Formed glucose-1-phosphate can be further converted into glucose-6-phosphate without ATP, unlike glucose. This ATP saving by cellobiose phosphorylase could benefit especially bioprocesses with limited ATP supply or with high ATP requirements.

Shake flask experiments were carried with 20 g l⁻¹ glucose, 20 g l⁻¹ cellobiose, or with EnPump 200 system releasing glucose slowly to the media by addition of Reagent A. Glucose cultivations allowed us to compare PHB production and growth on cellobiose and on glucose. The glucose release speed from EnPump 200 was adjusted to match the cellobiose consumption speed of the cellobiose strains (Figure 5). In the EnPump 200 flask cultivations no glucose was measured, indicating that all the released glucose was consumed by the cells. In
bioreactor experiments the strains PHB_cbp and PHB_GH1-1, were grown in two replicates on 35 g l⁻¹ cellobiose.

### Table 10. The PHB producing *S. cerevisiae* strains and their corresponding control strains studied in Publications 1 and 3.

<table>
<thead>
<tr>
<th>Name</th>
<th>Number</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEN.PK113-5D</td>
<td>H3895</td>
<td><em>S. cerevisiae</em> (MATa HIS3 ura3-52 LEU2, TRP1 MAL2-8c SUC2)</td>
<td>-</td>
</tr>
<tr>
<td>CEN.PK111-9A</td>
<td>H3892</td>
<td><em>S. cerevisiae</em> (MATa his3-Δ1 URA3 leu2-3,112 TRP1 MAL2-8c SUC2)</td>
<td>-</td>
</tr>
<tr>
<td>CEN.PK113-7D</td>
<td>H3887</td>
<td><em>S. cerevisiae</em> (MATa HIS3 URA3 LEU2 TRP1 MAL2-8c SUC2)</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>H5529</td>
<td>H3895 with plasmid B9660 (pTEF1-phaA1-ENO1-pTDH3-phaB-SSA1-pPGK1-phaC1-ADH1-URA3)</td>
<td>Publication 1</td>
</tr>
<tr>
<td>PHB_glu</td>
<td>H5696</td>
<td>H3887 with pTEF1-phaA1-ENO1-pTDH3-phaB1-SSA1-pPGK1-phaC1-ICYC (EasyClone integration site X-3)</td>
<td>Publication 3</td>
</tr>
<tr>
<td>PHB_cbp</td>
<td>H5716</td>
<td>H3892 with pTEF1-phaA1-ENO1-pTDH3-phaB1-SSA1-pPGK1-phaC1-ICYC (EasyClone integration site X-3); ura3Δ::pPGK1-cbp-1-ENO1,LEU2; and plasmid B8444 (pPGK1-CPT-1-ENO1, URA3, CEN/ARS, kanR)</td>
<td>Publication 3</td>
</tr>
<tr>
<td>PHB_GH1-1</td>
<td>H5717</td>
<td>H3892 with pTEF1-phaA1-ENO1-pTDH3-phaB1-SSA1-pPGK1-phaC1-ICYC (EasyClone integration site X-3); ura3Δ::pADH1-GH1-1-ENO1, LEU2; and plasmid B8444 (pPGK1-CPT-1-ENO1, URA3, CEN/ARS, kanR)</td>
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<td>cbp_control</td>
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<td>H3892 with ura3Δ::pADH1-GH1-1-ENO1, LEU2; and plasmid B8444 (pPGK1-CPT-1-ENO1, URA3, CEN/ARS, kanR)</td>
<td>Publication 3</td>
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</table>

### 3.3.1 Strains grown on cellobiose accumulated record high PHB % per CDW in *S. cerevisiae*

The highest obtained PHB accumulation level of CDW in this study was 21.2% PHB of CDW (Figure 18C). It was measured in PHB_GH1-1 strain grown on cellobiose in bioreactors for 144 hours. The average result of two PHB_GH1-1 replicates in the experiment was 18.5% PHB of CDW. Both of these PHB results are higher than previously reported in literature for *S. cerevisiae* on glucose (9% of CDW) (Carlson and Srienc, 2006) or on xylose (15-16% of CDW) (de Las Heras et al., 2016; Portugal-Nunes et al., 2017). It is also very similar to the highest reported P(LA-3HB) result, 19% of CDW, in Publication 2. These results indicate that laboratory strain CEN.PK is able to accumulate up to 18-20% PHA of its CDW when one copy of each PHA pathway gene is expressed constitutively. These PHA levels are however slightly lower than the highest levels reported in other yeasts, and over four-fold lower than reported in engineered bacterial hosts. Possible methods for increasing PHA accumulation in general in yeasts are described in Chapter 3.1.6.
in Publication 3. Strains were grown either on 20 g l\(^{-1}\) glucose, 20 g l\(^{-1}\) cellobiose, or EnPump 200 media, which releases glucose slowly to the media. C: Bioreactor results from Publication 3. Strains were grown on 35 g l\(^{-1}\) cellobiose. The results represent averages of three biological replicates, individual data points are marked with circles.

3.3.2 The SEC, \(^1\)H –NMR, and DSC analysis confirm the polymer structure of purified PHB

In the Publication 1, the PHB polymer was extracted and purified from the PHB strain H5529 grown on glucose in flasks for 72 hours. The structure of purified PHB polymer was confirmed with DSC and NMR. The melting temperature (T\(_m\)) of the yeast PHB was on average 176.7°C, which is similar to the measured T\(_m\) of commercial PHB of 174.5°C (Figure 19A). The similarity of the \(^1\)H NMR peaks of the yeast PHB and the commercial PHB (363502, Sigma Aldrich) confirms the PHB polymer structure (Figure 19C).

Figure 19. Analysis results of yeast PHB, extracted from strain H5529, and commercial PHB (363502, Sigma). Figure is modified from Publication 1. A: SEC chromatograms. B: Differential scanning calorimetry (DSC) thermograms. Each line represents one replicate. C: \(^1\)H -NMR spectra.

The extracted PHB polymer was analysed also with SEC in Publications 1 and 3. In the Publication 3, the PHB was produced in strains PHB_cbp and PHB_GH1-1 grown on cellobiose in bioreactors for 144 or 168 hours respectively. Measured chromatograms are presented in Figure 19B and calculated molecular weights in Table 11. The 1,1,3,3,3-hexafluoro-2-propanol (HFIP) based method used in Publication 1 gave higher M\(_w\) and D\(_\rho\), but lower M\(_n\), for the same commercial PHB standard, in comparison to analysis with chloroform-based method. The molecular weight results from these two publications are thus not directly comparable to each other. However, both studies indicate production of high molecular weight polymers and demonstrate that yeast are able to produce PHB with similar polymer properties to PHB extracted previously from bacterial hosts, such as *C. necator* (Fei et al., 2016; Meixner et al., 2018). Measured PHB molecular weights in *S. cerevisiae* on glucose and cellobiose are higher than reported from yeast *Y. lipolytica* expressing the same PHA synthase, but using acetate as carbon source, 130 kDa (M\(_n\)) and 200 kDa (M\(_w\)) (Li et al., 2017).
Table 11. The molecular weights of extracted PHB polymer and commercial PHB (363502, Sigma). Abbreviations: HFIP: 1,1,3,3,3-hexafluoro-2-propanol, Mn: number average molecular weight, Mw: weight average molecular weight, Đ: dispersity

<table>
<thead>
<tr>
<th>Strain (Sampling time)</th>
<th>Carbon source</th>
<th>Solvent used in analysis</th>
<th>Mn (kDa)</th>
<th>Mw (kDa)</th>
<th>Đ</th>
<th>Reference</th>
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<tr>
<td>H5529 (72 h)</td>
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<td>1090</td>
<td>3.2</td>
<td>Publication 1</td>
</tr>
<tr>
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<td>Glucose</td>
<td>HFIP</td>
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<td>1193</td>
<td>3.5</td>
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</tr>
<tr>
<td>H5529 (72 h)</td>
<td>Glucose</td>
<td>HFIP</td>
<td>335</td>
<td>1108</td>
<td>3.3</td>
<td>Publication 1</td>
</tr>
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<td>PHB standard</td>
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<td>HFIP</td>
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<td>1529</td>
<td>12.4</td>
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<td>PHB_cbp (168 h)</td>
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<td>526</td>
<td>2.2</td>
<td>Publication 3</td>
</tr>
<tr>
<td>PHB_cbp (168 h)</td>
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<td>Chloroform</td>
<td>216</td>
<td>482</td>
<td>2.2</td>
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</tr>
<tr>
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<td>444</td>
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</tr>
<tr>
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<tr>
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<td>258</td>
<td>563</td>
<td>2.18</td>
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3.3.3 The strains expressing β-glucosidase gene (GH1-1) consumed cellobiose faster than the strains expressing cellobiose phosphorylase gene (cbp)

In Publication 3, the biomass formation was highly dependent on the sugar utilization rate. The strains expressing gene GH1-1 consumed cellobiose faster than the strains expressing gene cbp, leading to faster PHB and biomass formation in GH1-1 expressing strains. For example, in the shake flask experiment, the strain PHB_GH1-1 consumed 2.8 g l\(^{-1}\) cellobiose between 32 and 80 hours, which is approximately twice as much as the strain PHB_cbp consumed during the same time (Figure 20A). Faster cellobiose consumption resulted also in higher biomass formation by 56 hours (OD\(_{600}\) of 5.16). However, the difference in biomass accumulation decreased towards the end of the cultivation as the strain PHB_cbp reached OD\(_{600}\) of 4.84 by 96 hours.

![Figure 20](image-url)

**Figure 20.** Comparison of cellobiose consumption (g l\(^{-1}\)), cell growth (OD\(_{600}\)), and PHB titer (mg l\(^{-1}\)) of cellobiose using strains PHB_cbp (blue) and PHB_GH1-1 (black). A-C: Results from the shake flask experiment. Lines represent averages of two or three biological replicates. Individual data points are presented with circles. D-F: Results from the batch bioreactor experiment. Continued lines (strains PHB_cbp and PHB_GH1-1) represent averages of two biological replicates. Dashed lines (cbp_control and GH1-1_control) represent results of one biological replicate. Individual data points are presented with circles.

The faster growth of the strains expressing GH1-1 gene, in comparison to strains expressing cbp gene, has been demonstrated also in few other studies and suggested to result from the
two-fold lower Michaelis constant $k_m$ of Gh1-1, in comparison to Cbp (Ha et al., 2013; Kim et al., 2018), which might allow the strains expressing GH1-1 gene to drive cellobiose metabolism more efficiently at lower cellobiose concentrations than strains expressing cbp gene. However, cellobiose phosphorolysis has shown in literature to result in higher biomass and ethanol production in comparison to cellobiose hydrolysis (Ha et al., 2013). In addition, expression of cellobiose phosphorylase does not lead to formation of cellotriose and cellotetraose as it lacks transglycosylation activity typical for β-glucosidases (Ha et al., 2013). Cellobiose uptake and thus cellobiose metabolism with cellobiose phosphorylase could be accelerated in future by increasing intracellular concentration of cellobiose for example by choosing novel transporters (Bae et al., 2014), by increasing the gene copy numbers of the transporters (Oh et al., 2016), or by using higher extracellular cellobiose concentration in media (Oh et al., 2017). The improved cellobiose utilization through phosphorylation could be useful in many applications as it consumes less ATP in comparison to cellobiose hydrolysis. Smaller ATP need could be beneficial especially when ATP production is limited e.g., due to anaerobic conditions, or when high concentrations of ATP are needed elsewhere in metabolism, e.g., in PHA production.

In bioreactor experiment in Publication 3, the strain PHB_GH1-1 produced approximately 1 and 6 g l$^{-1}$ extracellular cellotetraose and cellotriose, respectively (Figure 21 B, C). These values were reduced from the measured cellobiose consumption (Figure 21A) to evaluate how much cellobiose was consumed by the strains e.g., for PHB production and growth (Figure 21D). The biomass and PHB yields per consumed cellobiose were calculated with these cellular cellobiose consumption values (Figure 21D).

![Figure 21. Cellobiose consumption, formation of cellotriose and cellotetraose, and cellular cellobiose consumption in the bioreactor experiment. Lines represent averages of two biological replicates and individual data points are marked with circles.](image)

In the Publication 3, both cellobiose utilizing strains, PHB_cbp and PHB_GH1-1, consumed more cellobiose and grew better in bioreactors than in shake flasks (Figure 20). This is probably related to improved culture conditions in bioreactors, including controlled pH (at pH 6). The efficiency of the studied cellobiose transporter, Cdt-1, is reported in literature to decrease when pH drops below pH 4 (Turner et al., 2016). However, not all cellobiose transporters are as sensitive to low pH as Cdt-1. For example, a mutated variant of Cdt-2 from N. crassa has shown rather similar specific cellobiose consumption rate both at pH 3 and pH 6 (Oh et al., 2017). Transporter functionality at pH 3 is highly useful as the pH of buffered yeast cultivations often decreases below pH 4. Albeit being a challenge for some celdextrin transporters, acidic conditions reduce the need for buffering agents, lower the risks of contamination by other microorganisms, and allow passive transport of organic acids if necessary.
3.3.4 The strains grown on cellobiose reached higher PHB yield per consumed sugar in comparison to strains grown on glucose

The results from shake flask experiment in Publication 3 demonstrate that strains grown on cellobiose had a higher PHB yield per consumed sugar in comparison to strains grown on glucose (Figure 22). For the first 72 hours the PHB yield per consumed sugar was very low on both glucose media, around 10 mg PHB g\(^{-1}\) glucose (Figure 22). For comparison, by 72 hours, the strains PHB\_GH1-1 and PHB\_cbp grown on cellobiose had reached 3.7- and 7.0-fold higher PHB yields per consumed c mol of sugar (p<0.0005), 37 mg and 70 PHB mg g\(^{-1}\) cellobiose, respectively. After 72 hours, the strain grown on EnPump 200 media showed increase in PHB yield per consumed sugar but results between replicates varied a lot. Exact reason for this variance is not known but it might derive at least from small variation in enzymatic sugar release between different replicates (Figure 5).

![Figure 22. PHB yield per consumed sugar in shake flask experiment in Publication 3. Strains PHB\_GH1-1 and PHB\_cbp were grown with 20 g l\(^{-1}\) cellobiose. Strain PHB\_glu was grown with EnPump 200 slow glucose release media (PHB\_glu slow) or with 20 g l\(^{-1}\) glucose (PHB\_glu). Individual data points are marked with circles. Lines show averages of two or three biological replicates.](image)

In the same flask experiment in Publication 3, approximately 60% of the available carbon was converted to 9.4 g l\(^{-1}\) ethanol during the first 19 hours when strain PHB\_glu was grown with free glucose (Figure 23A). Cellobiose strains did not show any ethanol production and strain grown on EnPump 200 media started to produce ethanol only after 72 hours (less than 1 g l\(^{-1}\)). The strain PHB\_glu grown on free glucose produced 2.2 g l\(^{-1}\) acetate by 72 hours, which corresponds to approximately 5% of the available carbon (Figure 23B). Strain PHB\_GH1-1 started to produce acetate after the first day and reached 1.4 g l\(^{-1}\) by the fourth day. Strain PHB\_cbp produced less acetate, on average only 0.24 g l\(^{-1}\) after three days. The lack of fermentative side products, especially ethanol, indicates that cellobiose strains PHB\_cbp and PHB-GH1-1 were mainly in respirative metabolic state during the flask cultivation. The respirative metabolism allows *S. cerevisiae* to direct more sugars into pentose phosphate pathway and to produce more NADPH than the fermentative metabolism (Jouhten et al., 2008; Maaheimo et al., 2001). This might improve production of NADPH derived products such as PHB. However, it is possible that the overall metabolism and PHB production on cellobiose will be similar to glucose cultivations (fermentative) if cellobiose uptake and conversion to glucose-1-phosphate are improved in the future (Huberts et al., 2012).
The flask experiment in Publication 3 indicates also that absence of glucose in growth media could be beneficial for PHB production. Cultivation of *S. cerevisiae* on cellobiose and EnPump 200 substrate resulted in similar growth patterns and in lack of ethanol as side product, indicating that use of both of these carbon sources led to respiratory metabolism. However, the PHB yield per consumed sugar (during the first 72 hours) was significantly higher with cellobiose than with EnPump 200 glucose media.

### 3.3.5 Maximum PHB productivity coincided with maximum growth rate when cells were grown on cellobiose

The daily analysis of PHB accumulation in flasks in Publication 3 showed, that the cellobiose strains PHB_GH1-1 and PHB_cbp accumulated PHB steadily during the 96 hour -cultivation, reaching final levels of 8.4% and 10.0% PHB of CDW by the end of the cultivation, respectively (Figure 20B). The PHB accumulation coincided with growth. This was observed also in the bioreactor experiment in Publication 3, where the maximum PHB productivity, the highest specific growth rate, and the highest growth rate of strain PHB_GH1-1 all occurred around 60 hours (Table 12 and Figure 20 E,F).

The growth of strains PHB_cbp and PHB_GH1-1 occurred at two distinct phases in bioreactors. The faster growth phase started around 24 hours and continued for 48 hours (strain PHB_GH1-1) or for 72 hours (strain PHB_cbp), until strains reached biomass concentrations of around 4.8 g l⁻¹. During this fast growth phase, both strains consumed same amount of cellobiose, 9.4 g l⁻¹, but the strain PHB_cbp gained on average 8% more biomass and 19 % less PHB per consumed cellobiose in comparison to the strain PHB_GH1-1 (Table 2). This higher biomass accumulation by cellobiose phosphorolysis was observed also in shake flasks and in a previous study where it was discussed to result from improved ATP availability in comparison to cellobiose hydrolysis (Ha et al., 2013).

In addition, due to the faster cellobiose consumption, strain PHB_GH1-1 had on average 40% and 80% higher maximum PHB productivity and maximum specific PHB productivity, respectively, in comparison to strain PHB_cbp. However, these differences and differences in PHB accumulation per consumed cellobiose are not significant due to high variation in PHB accumulation in the two PHB_GH1-1 replicates (Figure 20F).

<p>| Table 12. The maximum growth rates, maximum biomass and PHB yields per consumed sugar, and maximum PHB accumulation % of CDW during the fast growth phase of strains PHB_GH1-1 and PHB_cbp. Strains were grown in bioreactors and results describe the fast growth phase of each replicate separately. Table adapted from Publication 3. |</p>
<table>
<thead>
<tr>
<th>Strain</th>
<th>Fast growth phase timing</th>
<th>Maximum growth rate (mg CDW l⁻¹ h⁻¹)</th>
<th>Maximum specific growth rate (h⁻¹)</th>
<th>Biomass yield per cellobiose (mg g⁻¹)</th>
<th>Max PHB productivity (mg PHB l⁻¹ h⁻¹)</th>
<th>Max specific PHB productivity (mg PHB g CDW⁻¹ h⁻¹)</th>
<th>PHB yield on cellobiose (mg g⁻¹)</th>
<th>Max PHB accumulation per biomass (% of CDW)</th>
</tr>
</thead>
<tbody>
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<td>0.092 (at 58.5 h)</td>
<td>0.032 (at 58.1 h)</td>
<td>440</td>
<td>13.4 (at 62.6 h)</td>
<td>3.84 (at 54.9 h)</td>
<td>43.1</td>
<td>8.1 (at 72 h)</td>
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</tr>
<tr>
<td>PHB_GH1</td>
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<td>0.047 (at 56.9 h)</td>
<td>428</td>
<td>17 (at 62.5 h)</td>
<td>5.6 (at 53.9 h)</td>
<td>49.4</td>
<td>9.4 (at 72 h)</td>
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<tr>
<td>PHB_cbp</td>
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<td>0.061 (at 30.1 h)</td>
<td>470</td>
<td>11.5 (at 90.3 h)</td>
<td>2.81 (at 82.5 h)</td>
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<tr>
<td>PHB_cbp</td>
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<td>0.07 (at 84 h)</td>
<td>0.062 (at 28.1 h)</td>
<td>466</td>
<td>10.5 (at 96 h)</td>
<td>2.39 (at 87.1 h)</td>
<td>37.4</td>
<td>7.2 (at 96 h)</td>
</tr>
</tbody>
</table>
4. Conclusions and future perspectives

This thesis was inspired by the global need to find replacements for non-biodegradable plastic materials originating from fossil sources. Microbially produced PHAs are a diverse group of fully biobased and biodegradable polyesters. Especially some thermoplastic PHAs have shown high potential for medical and consumer applications which require good barrier properties and biodegradability. However, wider use of PHAs is still hindered by their rather limited mechanical properties and relatively high costs of sugar feedstocks. This thesis focused on these bottlenecks by exploring possibilities to modify PHA structure in the yeast \textit{S. cerevisiae} and by using cellobiose as a sole carbon source. PHA modifications were enabled by combining a wide substrate specificity PHA synthase with a precise synthetic biology tool for controlling monomer availability.

In Publication 1, two engineered bacterial PHA synthases, PhaC1437Ps6-19 and PhaC1Pre, were studied for polymerization of D-lactic acid in \textit{S. cerevisiae}. Both enzymes were able to produce both PDLA homopolymer and P(LA-3HB) copolymer. Their demonstrated activity in \textit{S. cerevisiae} paves the way for production of many new PHAs in yeasts in future as both of them are known to polymerize also other small 2- and 3-hydroxyacids and larger mcl-monomers. Especially PhaC1437Ps6-19 has shown potential for monomers with diverse properties, including aromatic side chains (Yang et al., 2018).

In Publication 2, a precise doxycycline-based Tet-On regulatory approach was developed for adjusting copolymer structure of P(LA-3HB) random copolymer. This method enabled control of \textit{ldhA} expression resulting in linear range of different D-lactic acid contents from 6 mol% to up to 93 mol% in the copolymer P(LA-3HB) depending on the used doxycycline concentration. Precise tools for controlling monomer content \textit{in vivo} could become essential in the future if \textit{in silico} modelling tools are able to predict PHA structures with novel properties and those structures need to be produced \textit{in vivo}. Previous methods relying on controlled addition of monomers from cultivation media are not functional if target monomers are too toxic, expensive, or unavailable for feeding in high concentrations. However, if monomers are available for feeding in high concentrations, feeding could be combined with the described Tet-On method. For example, adjusted D-LA-CoA production \textit{in vivo} could be combined with controlled feeding of glycolic or propionic acids, or the Tet-On approach could be applied for controlling Pet availability and thus CoA activation of monomers.

In this thesis, \textit{S. cerevisiae} accumulated at maximum 5.4% PDLA, 19% P(LA-3HB), and 18-21.3% PHB of CDW. These results are in similar range to PHA accumulation than in other yeasts, but two- to five-fold lower than those obtained in bacterial hosts. Therefore, the low polymer accumulation level remains as the main challenge in PHA production in yeast, especially in \textit{S. cerevisiae}. In the Publication 2, the highest PDLA and copolymer accumulation levels were obtained when cells produced highest amounts of D-lactic acid. Increasing PHA synthase expression did not lead to a significant increase in PDLA accumulation. These two
findings indicate that the precursor supply was most likely limiting the polymerization, rather than the final polymerization step by the PHA synthase enzymes. The PDLA and copolymer producing strains exported over 90% of the produced D-lactic acid into the media, which also suggests that the D-lactic acid conversion to D-lactyl-CoA precursor was limiting the polymerization process. Further engineering of the PDLA and P(LA-3HB) production should thus focus especially on the acetyl-CoA availability or on the efficiency and substrate specificity of the Pct enzymes carrying this step.

Industrially relevant yeast *S. cerevisiae* strains are inherently unable to use cellobiose as a carbon source. In this study, *S. cerevisiae* strains carrying the PHB production pathway were engineered to use cellobiose as their sole carbon source by enabling cellobiose transport with a cellodextrin transporter and by metabolising cellobiose intracellularly either with the β-glucosidase Gh1-1 or cellobiose phosphorylase Cbp. Both pathways lead to PHB accumulation levels on cellobiose (per cell dry weight and per consumed sugar) that exceeded the PHB production on glucose. The produced PHB polymers had similar molecular weights to bacterial PHBs (Mw of around 450-500 kDa). Even though strains consumed cellobiose slower than glucose, leading to slower growth, they showed potential for PHA production on cellobiose. This is interesting as direct use of cellobiose in an SSF process (simultaneous saccharification and fermentation) could potentially benefit cellulose hydrolysis by decreasing the inhibition of cellulolytic enzymes by free cellobiose. It could also reduce the need for extracellular β-glucosidases in the hydrolytic enzyme mixture, which converts cellobiose to glucose.

In this thesis, intracellular cellobiose hydrolysis by β-glucosidase resulted in faster cellobiose utilization, but slightly lower cellobiose conversion to biomass, than with cellobiose phosphorylation. Cellobiose phosphorylase consumes less ATP than cellobiose hydrolysis and could thus be useful in future in different bioprocesses requiring high ATP supply or suffering from low ATP production. Improvement in cellobiose uptake could increase cellobiose consumption rate through phosphorylation in future.

The role of biotechnological approaches in material production will increase in the future. Microbially produced PHAs offer plastic industry interesting materials with fully biodegradable, biobased, biocompatible, safe, thermoformable, and watertight properties. Improvements in PHA synthase activities and adjustment of monomer availability *in vivo* allow tailoring of PHA structures for novel applications in future. The ability of the yeast *S. cerevisiae* to uptake and utilize glucose, cellobiose, lactic acid, and xylose, combined with recent improvements in PHA production could enable utilization of many different organic side streams as sustainable raw materials for PHA production in coming years.
References


Meixner, K., Kovalcik, A., Sykacek, E., Gruber-Brunhumer, M., Zeilinger, W., Markl, K., Haas, C., Fritz,


This thesis was inspired by the global need to find replacements for non-biodegradable plastic materials originating from fossil sources. Microbially produced polyhydroxyalkanoates (PHAs) are diverse group of fully biobased and biodegradable polyesters with interesting properties for many applications. However, wide use of PHAs is still hindered by their limited mechanical properties and relatively high costs of sugar feedstocks.

This thesis explored possibilities to use lignocellulose derived celllobiose as a carbon source for PHA production in yeast *Saccharomyces cerevisiae*. In addition, it focused on polymerization of a 2-hydroxacid (D-lactic acid) and controlling PHA copolymer structure in yeast *S. cerevisiae* with synthetic biology. These approaches contribute to the wider themes of using lignocellulose-based carbon sources for PHA production and improving PHA polymer properties for use in even wider range of different applications in future.