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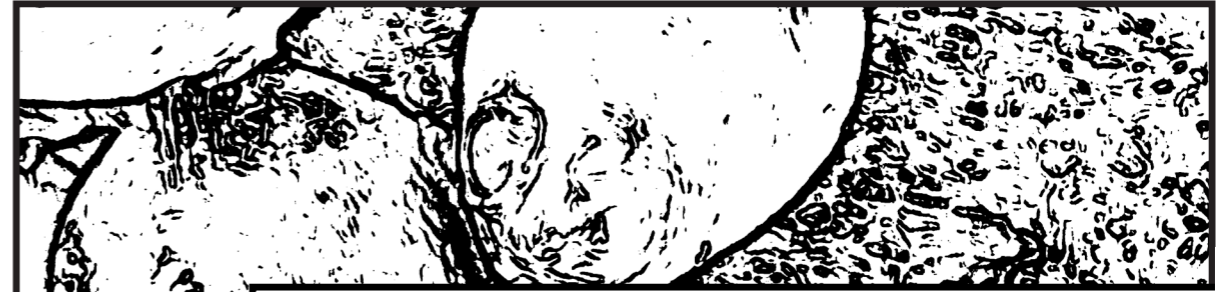
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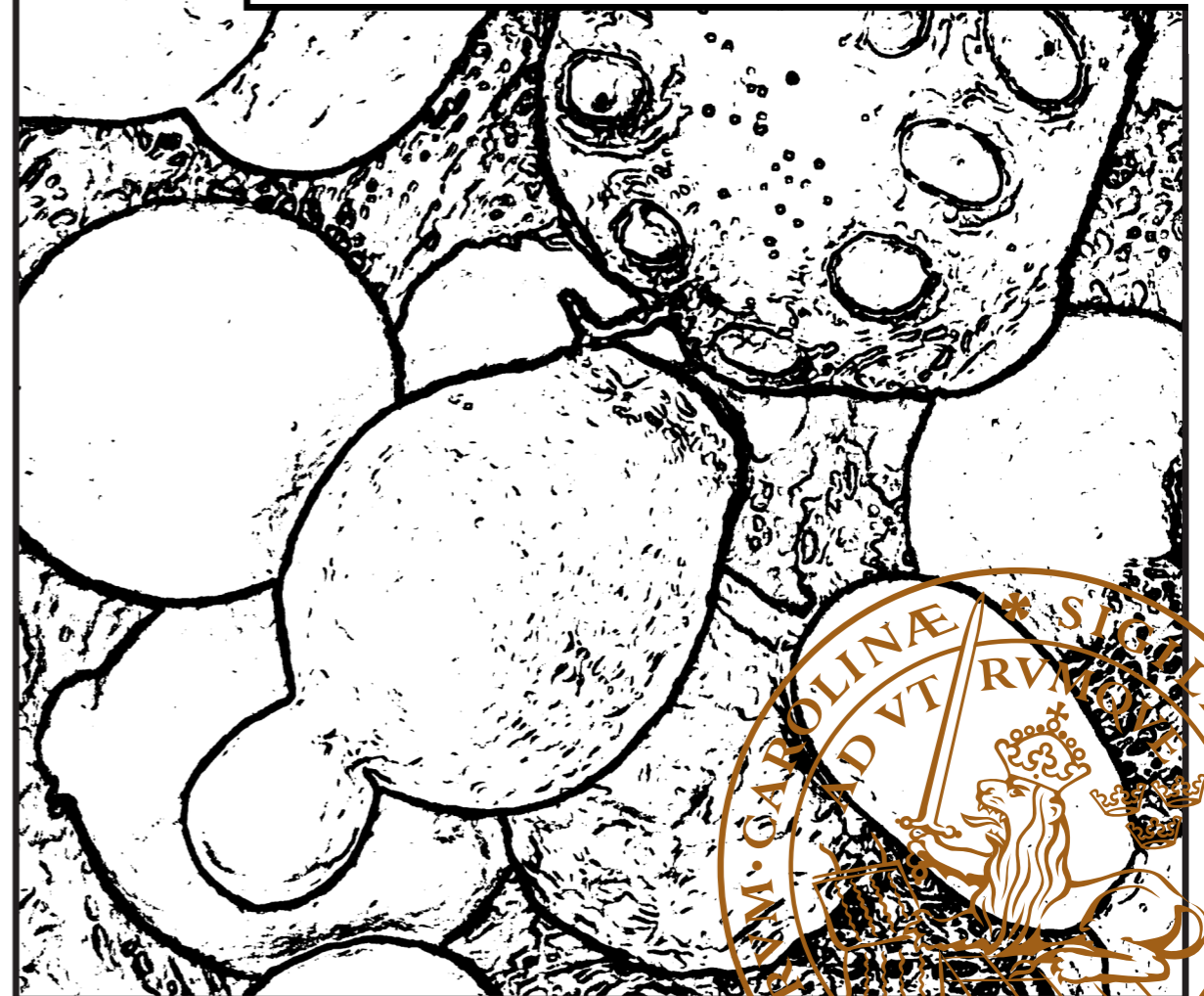
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Application of Synthetic Biology for Biopolymer Production using *Saccharomyces cerevisiae*

APPLIED MICROBIOLOGY | FACULTY OF ENGINEERING | LUND UNIVERSITY
ALEJANDRO MUÑOZ DE LAS HERAS



ALEJANDRO MUÑOZ DE LAS HERAS Application of Synthetic Biology for Biopolymer Production using *Saccharomyces cerevisiae*

2017

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I have been curious about how Nature works since I was a child. After I obtained my degree in biology I wanted to apply that knowledge for the benefit of society. I moved to Sweden for that purpose and during that period, I realized that microbes are fascinating living systems that can be modified to synthesize many chemicals in a more sustainable manner. Yeasts have been used for centuries to make beer, wine and bread, and nowadays, with the application of synthetic biology, they can be modified to produce other products such as biofuels, pharmaceuticals and even bioplastics. The work presented in this thesis is my contribution to ways in which baker's yeast can be engineered to produce bio-based and biodegradable polymers from some of the sugars present in waste products from agriculture and forestry. Welcome to the complex, beautiful and powerful world of yeast.

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Department of Chemistry
Faculty of Engineering
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Cover photo: “The art of bioengineering” by Alejandro Muñoz de las Heras.
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Application of Synthetic Biology for Biopolymer Production using *Saccharomyces cerevisiae*

Alejandro Muñoz de las Heras



DOCTORAL DISSERTATION

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on Friday 12th of May 2017 at 10:00.

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Associate Professor Verena Siewers, Department of Biology and Biological
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Title and subtitle: Application of Synthetic Biology for Biopolymer Production using <i>Saccharomyces cerevisiae</i>		
<p>Abstract:</p> <p>Plastics are versatile, cheap and durable materials that are omnipresent in modern society. Since most of them are derived from crude oil and are not biodegradable, their production leads to the depletion of fossil fuels and the accumulation of enormous amounts of plastic waste that pollutes ecosystems worldwide. For this reason, the European Union and other organisations are investing in research aimed at developing eco-friendly alternatives such as bioplastics.</p> <p>Poly-3-D-hydroxybutyrate (PHB) is a type of biodegradable bioplastic that is naturally synthesized and used by specific microorganisms as an energy source and for carbon storage under stressful environmental conditions. However, these microorganisms are not well suited for growth in biomass hydrolysates. Baker's yeast, <i>Saccharomyces cerevisiae</i>, might represent an interesting host for PHB production since it is a well-known industrial platform for the effective conversion of various carbon sources into key precursor metabolites, and has high tolerance to low pH and fermentation inhibitors that are present in biomass hydrolysates. The aim of the present work was to use synthetic biology tools to "rewire" <i>S. cerevisiae</i> metabolism, and to investigate whether it can be used for the efficient production of PHB from xylose feedstock.</p> <p>Recombinant <i>S. cerevisiae</i> strains carrying the oxido-reductive xylose pathway from <i>Scheffersomyces stipitis</i> were engineered for heterologous gene expression of the biosynthetic PHB pathway from <i>Cupriavidus necator</i>. This enabled PHB production from xylose as the sole carbon source, although the production was low and oxygen-dependent. Further improvements were achieved by modification of the cofactor balance through the introduction of alternative enzymes with different cofactor requirements. The introduction of a xylose reductase variant with increased affinity for NADH cofactor enabled better redox homeostasis, allowing PHB production from xylose under anaerobic conditions. PHB biosynthesis was also improved by the substitution of the NADPH-dependent acetoacetyl-CoA reductase by an NADH-dependent counterpart.</p> <p>As the availability of cytosolic acetyl-CoA, which is the precursor of PHB, remained a major challenge, several strategies were tested to redirect the carbon flux towards acetyl-CoA, such as the use of alternative pathways and the downregulation of the ethanol route. However, the production of PHB could only be improved under aerobic conditions due to tight cell regulation at the cytosolic NADH and acetyl-CoA levels.</p> <p>The xylose-fermentation capabilities of different <i>Spathaspora</i> species were explored in parallel, with the aim of finding better enzymes for further improvement of anaerobic xylose fermentation by <i>S. cerevisiae</i>. The xylose reductase gene <i>XYL1.2</i> from <i>Sp. passalidarum</i> was found to encode an enzyme with a high affinity for NADH. When transferred to <i>S. cerevisiae</i>, xylose fermentation and ethanol production were improved under anaerobic conditions.</p> <p>This work demonstrates how the <i>S. cerevisiae</i> genome can be reprogrammed for PHB production from sugars derived from lignocellulosic biomass. However, further modification of the metabolism will be necessary before industrial implementation is possible.</p>		
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Abstract

Plastics are versatile, cheap and durable materials that are omnipresent in modern society. Since most of them are derived from crude oil and are not biodegradable, their production leads to the depletion of fossil fuels and the accumulation of enormous amounts of plastic waste that pollutes ecosystems worldwide. For this reason, the European Union and other organisations are investing in research aimed at developing eco-friendly alternatives such as bioplastics.

Poly-3-D-hydroxybutyrate (PHB) is a type of biodegradable bioplastic that is naturally synthesized and used by specific microorganisms as an energy source and for carbon storage under stressful environmental conditions. However, these microorganisms are not well suited for growth in biomass hydrolysates. Baker's yeast, *Saccharomyces cerevisiae*, might represent an interesting host for PHB production since it is a well-known industrial platform for the effective conversion of various carbon sources into key precursor metabolites, and has high tolerance to low pH and fermentation inhibitors that are present in biomass hydrolysates. The aim of the present work was to use synthetic biology tools to “rewire” *S. cerevisiae* metabolism, and to investigate whether it can be used for the efficient production of PHB from xylose feedstock.

Recombinant *S. cerevisiae* strains carrying the oxido-reductive xylose pathway from *Scheffersomyces stipitis* were engineered for heterologous gene expression of the biosynthetic PHB pathway from *Cupriavidus necator*. This enabled PHB production from xylose as the sole carbon source, although the production was low and oxygen-dependent. Further improvements were achieved by modification of the cofactor balance through the introduction of alternative enzymes with different cofactor requirements. The introduction of a xylose reductase variant with increased affinity for NADH cofactor enabled better redox homeostasis, allowing PHB production from xylose under anaerobic conditions. PHB biosynthesis was also improved by the substitution of the NADPH-dependent acetoacetyl-CoA reductase by an NADH-dependent counterpart.

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aerobic conditions due to tight cell regulation at the cytosolic NADH and acetyl-CoA levels.

The xylose-fermentation capabilities of different *Spathaspora* species were explored in parallel, with the aim of finding better enzymes for further improvement of anaerobic xylose fermentation by *S. cerevisiae*. The xylose reductase gene *XYL1.2* from *Sp. passalidarum* was found to encode an enzyme with a high affinity for NADH. When transferred to *S. cerevisiae*, xylose fermentation and ethanol production were improved under anaerobic conditions.

This work demonstrates how the *S. cerevisiae* genome can be reprogrammed for PHB production from sugars derived from lignocellulosic biomass. However, further modification of the metabolism will be necessary before industrial implementation is possible.

Popular scientific summary

Plastics have become an indispensable component of everyday life, they can be found in objects ranging from bags to aircraft, and from food wrap to mobile phones. This polymeric compound, which is mostly derived from crude oil, is considered the most versatile manmade material. Due to their non-natural structure, plastics cannot be biodegraded, and can persist in the environment for hundreds of years, causing pollution of land and seas, and severely affecting the biodiversity of ecosystems. Fortunately, social awareness concerning the negative impact of the production and disposal of plastics has led to the search for eco-friendly alternatives, so-called bioplastics. Progressive substitution of conventional plastics with bio-based and biodegradable plastics such as poly-3-D-hydroxybutyrate (PHB) would contribute to reducing our dependency on oil, while reducing the environmental impact of plastics on our planet.

Some types of bacteria can produce PHB naturally as a nutrient storage molecule that use to survive during periods of nutrient deficiency. The instructions for the molecular machinery (enzymes) that produces PHB are contained in the organisms' DNA. This information can be extracted and transferred to other microorganisms that are more suitable for industrial applications. The aim of the present work was to apply this principle, called synthetic biology, to construct genetically modified yeasts that are able to produce PHB from renewable carbon sources. Baker's yeast, *Saccharomyces cerevisiae*, was chosen as the host for PHB production. This microorganism has been used throughout human history to make bread, beer and wine; nowadays, the application of synthetic biology allows the modification of *S. cerevisiae* for the production of chemicals such as fuels, pharmaceuticals and biopolymers.

In this work, the genes that allow microbes to produce PHB and the genes for utilization of xylose were introduced into *S. cerevisiae*. This is because xylose, a five-carbon molecule, and glucose, a six-carbon molecule, are the most abundant sugars present in lignocellulosic biomass, a cheap raw material obtained from agricultural and forest residues. As result is expected that the production cost of PHB from lignocellulosic biomass may be reduced since both glucose and xylose fractions of the substrate can be used.

Although the generated yeast strains were able to consume xylose and produce PHB, the production remained low. To tackle this problem, other enzymes from different

PHB-producing bacteria were tested, resulting in improved PHB production, even when oxygen was not available (called anaerobic conditions). In order to further optimize this “cell factory”, other biological reactions that are involved in the central metabolism were also modified; for example, of the metabolism of ethanol, which is a major by-product of xylose fermentation, was attenuated. Although these modifications resulted in growth retardation or the formation of other by-products when the strains were tested under anaerobic conditions, the PHB production was increased when oxygen was available.

Aiming at reducing the cost of xylose-based processes other improvements were investigated. Modified *S. cerevisiae* strains consume xylose when oxygen is available; however, their performance is not optimal anaerobic conditions. As pumping air into bioreactors is expensive, one of our goals was to improve anaerobic xylose consumption by yeast. For this purpose, several other yeasts isolated from rotten wood in the Amazon rainforest were investigated. The genetic information carried by these yeasts, which are able to consume xylose anaerobically, was transferred to *S. cerevisiae*. The resulting new strains showed improved anaerobic xylose consumption capacity due to better performance of the new enzymes, thereby offering a promising option for the industrial implementation of PHB-producing strains or other products derived from xylose.

Resumen de divulgación científica

Los plásticos son materiales indispensables de nuestro día a día que podemos encontrar desde en bolsas y envoltorios hasta en componentes de aviones y teléfonos móviles. Este compuesto polimérico derivado del petróleo, es considerado el material más versátil creado por el hombre. Debido a su estructura artificial, los plásticos no pueden ser biodegradados y pueden persistir en el medio ambiente por cientos de años. Su acumulación causa la contaminación de tierras y mares que resulta en la reducción severa de la biodiversidad en los ecosistemas. Por suerte, la sociedad comienza a ser consciente del impacto que conlleva la producción y la acumulación de desechos plásticos, impulsando la búsqueda de nuevas alternativas ecológicas como, por ejemplo, los bioplásticos. La sustitución progresiva de plásticos convencionales por plásticos biodegradables y de origen biológico, como el polihidroxibutirato (PHB), pueden contribuir a disminuir la dependencia del petróleo, a la par que reducir el impacto medioambiental por la acumulación de desechos en nuestro planeta.

Algunos tipos de bacterias son capaces de producir PHB de manera natural como molécula de almacenamiento de energía, que podrá ser utilizada posteriormente durante períodos de escasez de nutrientes. Las instrucciones de las máquinas moleculares (enzimas) que sintetizan PHB se encuentran en el ADN de las bacterias, y esta información se puede extraer y transferir a otros microorganismos mejor adaptados a procesos industriales. El objetivo del trabajo presentado ha sido aplicar este principio, llamado biología sintética, para construir levaduras genéticamente modificadas capaces de producir PHB a partir de fuentes de carbono renovables. El microorganismo escogido ha sido la levadura del pan, *Saccharomyces cerevisiae*. Este microorganismo ha sido usado a lo largo de la historia para producir pan, cerveza y vino; hoy en día, la aplicación de la biología sintética en *S. cerevisiae*, nos permite la utilización de este microorganismo para la producción de compuestos químicos como por ejemplo combustibles, fármacos y biopolímeros.

En este trabajo, los genes que codifican las enzimas de los microorganismos que producen PHB y los genes que se utilizan para el consumo de xilosa han sido introducidos en *S. cerevisiae*. La xilosa, junto a la glucosa, constituyen los azúcares más abundantes de la biomasa lignocelulósica, un material que puede obtenerse fácilmente de los residuos agrícolas y forestales. Como resultado se espera que los

costes de producción de PHB a partir de la biomasa lignocelulósica puedan ser reducidos ya que ambas fracciones de glucosa y xilosa pueden ser utilizadas.

Aunque las cepas de las levaduras diseñadas fueron capaces de consumir xilosa y producir PHB, la producción fue baja. Para abordar este problema, se implementaron enzimas de otras bacterias productoras de PHB, resultando en la mejora de la producción incluso en condiciones de ausencia de oxígeno (anaerobiosis). Para continuar mejorando esta “factoría celular”, se modificaron otras reacciones biológicas del metabolismo central: por ejemplo, se atenuó metabolismo de la producción de etanol, que es un subproducto de la fermentación de la xilosa. Aunque estas modificaciones cambiaron los mecanismos de regulación celular, causando una desaceleración en el crecimiento y la formación de otros subproductos en condiciones anaerobias, la producción de PHB aumentó en presencia de oxígeno.

Con el objetivo de reducir el coste de productos derivados de la xilosa, se investigaron otras mejoras genéticas. Pese a que las cepas modificadas de *S. cerevisiae* son capaces de consumir xilosa en presencia de oxígeno, el funcionamiento no es óptimo en condiciones de anaerobiosis. Puesto que el bombeo de oxígeno en los biorreactores es un proceso caro, uno de nuestros objetivos fue mejorar el consumo de xilosa en levaduras en condiciones de anaerobiosis. Para ello, se investigó con otras levaduras aisladas en la selva del Amazonas. La información genética que portaban estas levaduras capaces de consumir xilosa fue transferida a *S. cerevisiae*, cuando se evaluó el rendimiento de las nuevas cepas en condiciones de anaerobiosis, el consumo de xilosa mejoró. Este resultado fue debido al mejor funcionamiento de las nuevas enzimas aisladas, ofreciendo una prometedora opción para la implementación industrial en cepas productoras de PHB u otros productos derivados de la xilosa.

List of publications

This thesis is based on the following published scientific articles and manuscript, which will be referred to by their Roman numerals in the text. The articles can be found at the end of the thesis.

- I. Engineering of *Saccharomyces cerevisiae* for the production of poly-3-D-hydroxybutyrate from xylose**
Sandström, A. G., de las Heras, A. M., Portugal-Nunes, D. J., & Gorwa-Grauslund, M. F. (2015). *AMB Express*, 5(1), 1.
- II. Anaerobic poly-3-D-hydroxybutyrate production from xylose in recombinant *Saccharomyces cerevisiae* using a NADH-dependent acetoacetyl-CoA reductase**
de las Heras, A. M., Portugal-Nunes, D. J., Rizza, N., Sandström, A. G., & Gorwa-Grauslund, M. F. (2016). *Microbial Cell Factories*, 15(1), 197.
- III. Rewiring the central carbon metabolism for poly-3-D-hydroxybutyrate production in *Saccharomyces cerevisiae***
de las Heras, A. M., Bjurman, N., Landberg, J., & Gorwa-Grauslund, M. F. Manuscript.
- IV. Exploring xylose metabolism in *Spathaspora species: XYLI. 2* from *Spathaspora passalidarum* as the key for efficient anaerobic xylose fermentation in metabolic engineered *Saccharomyces cerevisiae***
Cadete, R. M., de las Heras, A. M., Sandström, A.G., Ferreira, C., Gírio, F., Gorwa-Grauslund, M. F., Rosa C. A., Fonseca, C., (2016). *Biotechnology for Biofuels*, 9(1), 167.

My contributions to the studies

- I. I performed the molecular genetic engineering and strain constructions, I analysed the corresponding data and contributed to the writing of the manuscript.
- II. I participated to the design of the study, I carried out the molecular genetic engineering, enzymatic assay, growth assays and HPLC metabolite analysis. I wrote the manuscript.
- III. I designed the study, and performed the molecular genetic engineering and strain constructions, the yeast physiological and biochemical characterization, and data analysis. I wrote the manuscript.
- IV. I participated to the strain engineering work, the strain characterization and commented on the manuscript.

Abbreviations

A-ALD	NAD ⁺ -dependent acetylating acetaldehyde dehydrogenase
AAR	Acetoacetyl-CoA reductase
ACL	ATP-citrate lyase
ACS	Acetyl-CoA synthase
ACT	Acetyl-CoA acetyltransferase
ADH	Alcohol dehydrogenase
ALD	Acetaldehyde dehydrogenase
ATP	Adenosine triphosphate
CDW	Cell dry weight
CoA	Coenzyme A
CRISPR	Clustered regularly interspaced short palindromic repeats
gRNA	Guide RNA
NAD ⁺ /H	Nicotinamide adenine dinucleotide
NADP ⁺ /H	Nicotinamide adenine dinucleotide phosphate
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
PHB	Poly-3-D-hydroxybutyrate
PHS	Poly-3-D-hydroxyalkanoate synthase
PPP	Pentose phosphate pathway
TCA	Tricarboxylic acid cycle
XDH	Xylitol dehydrogenase
XR _{mut}	Mutated xylose reductase
XR _{wt}	Wild type xylose reductase

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1. Introduction

Plastics are indispensable in modern society, and are mainly synthesized from fossil fuels. The word plastic is derived from the Greek *plastikos* (πλαστικός), meaning “able to be mould”. This feature makes plastic an extremely versatile material. Plastics are found in a wide range of products, from water bottles to spacecraft. Since the industrial production of plastics started more than half century ago, they have gradually replaced natural products such as wood, paper, glass, metal, etc. The plastics industry plays an important role in the global economy. The production of plastics has grown from 1.5 million tons in 1950, to 322 million tons in 2015 (Figure 1). This means that the production of plastic during the past 10 years is equal to the production during the whole of the 20th century, and the trend is increasing, with production expected to triple between 2015 and 2050 [1, 2].

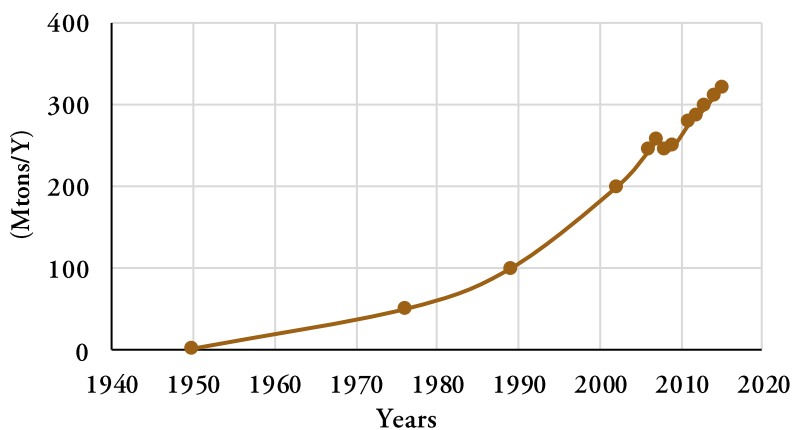


Figure 1. Global plastic production from 1950 to 2015 (adapted from PlasticsEurope 2016 [1]).

Advances made over the years have enabled the production of lightweight, durable and cheap plastics; however, the characteristics that make plastics so attractive are also the reason why they pose such a serious hazard to the environment [3]. As a result of their low cost and slow degradation, plastics have become widely spread, and our modern “disposable” society is responsible for millions of tons of non-degradable plastic waste that will continue to pollute the terrestrial and marine

environments for centuries to come [4]. Fortunately, we have become more aware of the results of our actions on the environment, including the utilization of fossil fuels, and disposable materials. This had led to a social demand for eco-friendly alternatives, the so-called bioplastics.

1.1 Bioplastics

1.1.1 Bio-based, biodegradable, or both?

Bioplastics are a family of plastic materials with different properties and applications. They may be *bio-based* or *biodegradable*, or may have both features.

- **Bio-based plastics** are plastics synthesized from renewable resources, such as sugar, vegetable oil or wood pulp, in contrast to traditional plastics made from fossil resources.
- **Biodegradable plastics**, are plastics that, after use, can be utilized as a substrate by microorganisms that convert the material into basic components such as water, carbon dioxide and organic matter.
- **Bio-based and biodegradable plastics** are plastics made from renewable resources that can be biologically degraded by microorganisms at the end of their life.

Plastic made from fossil fuels is thus not bio-based but can be biodegradable, for example, polycaprolactone. In contrast, a bio-based plastic, such as polyethylene (bio-PE), made from bioethanol may not be biodegradable. The most environmentally friendly alternatives are bio-based, biodegradable plastics such as polyhydroxyalkanoate (PHA) (Figure 2) (Table 1) [5].

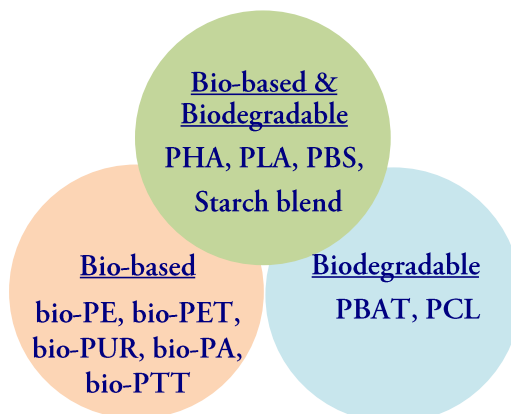


Figure 2. Classification of bioplastics.

Table 1. Examples of the most common types of bioplastics and their properties

Name	Abbreviation	Bio-based	Biodegradable
Polyhydroxyalkanoate	PHA	Yes	Yes
Poly lactate	PLA	Yes	Yes
Polybutylene succinate	PBS	Yes	Yes
Starch (blends)	-	Yes	Yes
Bio-Polyethylene	Bio-PE	Yes	No
Bio-Poly (ethylene terephthalate)	Bio-PET	Yes	No
Bio-Polyurethane	Bio-PUR	Yes	No
Bio-Polyamide	Bio-PA	Yes	No
Bio-Polytrimethylene terephthalate	Bio-PTT	Yes	No
Polybutyrate adipate terephthalate	PBAT	No	Yes
Polycaprolactone	PCL	No	Yes

1.1.2 Waste management

Various end-of-life options can be used to ensure that bioplastics contribute positively to the circular economy, by re-use and recycling of products to avoid pollution, as illustrated in Figure 3 and discussed below.

- **Mechanical recycling** is the best end-of-life option for most of plastics. Based on the type of material, plastics and their bioplastic alternatives can be recycled together, for example, bio-PE in PE streams and bio-PET in PET streams.
- **Organic recycling or composting** is used when mechanical recycling is not an option. Bioplastics and organic matter are mixed and subjected to biodegradation by bacteria, fungi or other organisms [5]. Home composting is not usually an option for most biodegradable plastics, as specific environmental conditions (e.g., temperature, pH and humidity) are required for the adequate biodegradation of bioplastics in a reasonable time [6].

- **Energy recovery** is an alternative when there is no infrastructure for waste management. This can be achieved by the production of heat, electricity or fuel by combustion, gasification, pyrolyzation, anaerobic digestion, or landfill gas.

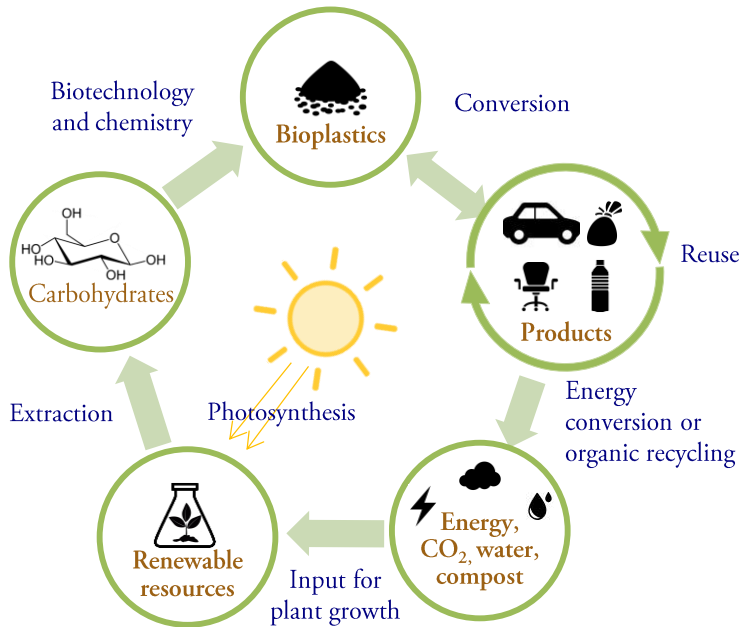


Figure 3. Closing the bioplastics loop (adapted from PlasticsEurope 2016 [1]).

1.1.3 Market and applications

The bioplastics market is growing rapidly, and currently represents about 1% of the total plastics market, of which about 20% is bio-based and biodegradable plastics. The increase in the proportion of bioplastics is due to government policies, cheap and easily accessible renewable feedstocks, and the use of more eco-friendly plastics by consumers (Figure 4) [7].

Today, there is usually a bioplastic alternative to every type of traditional plastic. As a result, bioplastics have the potential to cover a wide range of products and industries, including packaging, consumer goods, automotive and transport, building and construction, textile, agriculture and electronics (Figure 4) [8]. Some important brands in the food packaging industry are already packaging products in bioplastics. Biodegradability is one of the most desirable features in reducing the accumulation of waste, but other properties, such as rigidity, gloss, barrier effect, antistatic behavior and printability are also important [8]. In agriculture,

biodegradable mulching of film made of PLA can be ploughed in after use, saving time for the farmer in collecting, cleaning and recycling [9]. Biodegradable bioplastic containers are also available for bulbs which dissolve as the plant begins to grow, allowing the customer to plant them in the soil. Within the consumer electronics market, light, tough bioplastics for circuit boards, casings and data storage, are starting to replace conventional polymers. The use of bioplastics is also foreseen in the automotive sector, and for the construction of cockpits as well as passenger and cabin panels in the aeronautic, terrestrial and maritime transport sectors [8, 10].

The market is clearly divided between biodegradable and bio-based plastics. [8, 11]. Among all the PHA the most well-studied is poly-3-D-hydroxybutyrate (PHB). This polymer can be synthesized by microorganisms, and research is currently focused on finding a suitable host for its production from cheap and renewable carbon sources such as lignocellulosic biomass.

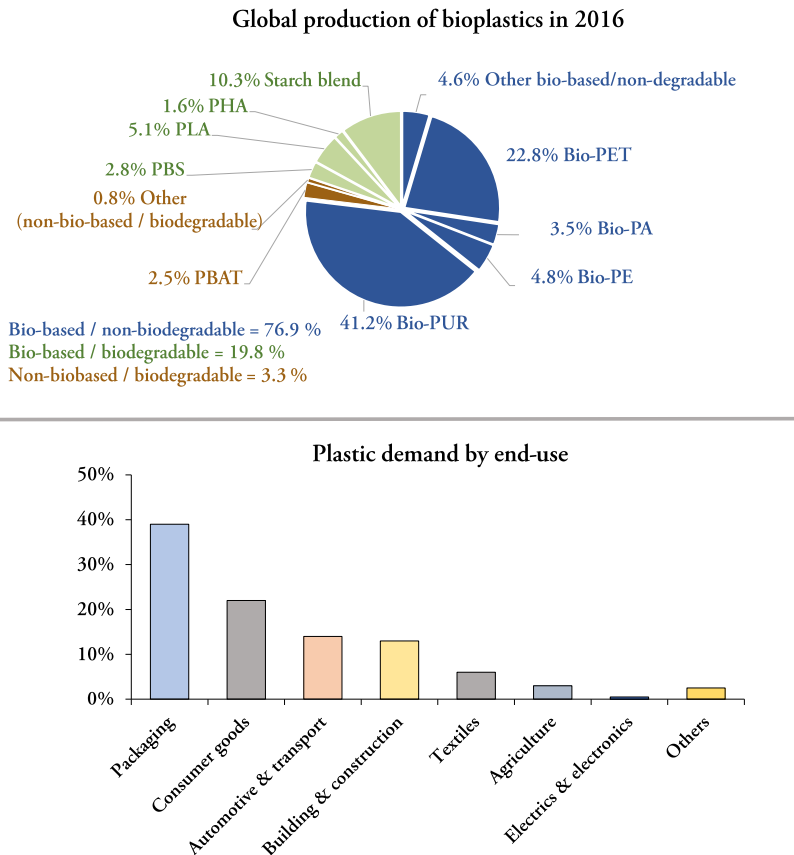


Figure 4. Global bioplastic production in 2016 and plastic demand according to end-use (adapted from PlasticsEurope 2016 [1]).

1.2 Aims of this work and outline of the thesis

The aim of the work presented in this thesis was to investigate the potential use of *S. cerevisiae* for the production of poly-3-D-hydroxybutyrate from sugars derived from lignocellulosic biomass.

Paper I describes strategies for the production of PHB from xylose, while Paper II describes how PHB production from xylose was increased under anaerobic conditions through enzyme substitutions with different cofactor preferences. Strategies for engineering the central carbon metabolism to enhance the level of cytosolic acetyl-CoA, a major PHB precursor, are reported in Paper III. Parallel efforts to improve anaerobic xylose fermentation in recombinant *S. cerevisiae* are discussed in Paper IV.

The production of bioplastics as alternatives to conventional plastics is presented in Chapter 1. While the properties, challenges and biotechnological production of PHB are discussed in Chapter 2. The advantages and the engineering possibilities associated with the use of *Saccharomyces cerevisiae* as an industrial platform for bulk chemical production, and PHB in particular, are discussed in Chapter 3. As the production of PHB, and other chemicals, by *S. cerevisiae* is associated with changes in the redox metabolism, Chapter 4 discusses the redox metabolism in *S. cerevisiae* and the impact of metabolic engineering strategies on its homeostasis. As PHB is derived from cytosolic acetyl-CoA, Chapter 5 focuses on the metabolism of acetyl-CoA and engineering strategies for its enhancement.

2. Poly-3-D-hydroxybutyrate – Properties, synthesis and applications

Polyhydroxyalkanoate (PHA) is a class of natural bioplastics synthesized and degraded by many different gram-positive and gram-negative bacteria. Among them, poly-3-D-hydroxybutyrate (PHB) is an amorphous polymer made up of units of hydroxybutyric acid ($C_4H_6O_2$) (Figure 5) [12]. The present section focuses on what makes PHB interesting, how and why it is produced by microorganisms, and the challenges in its industrial production.

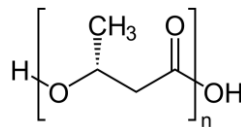


Figure 5. Chemical structure of PHB. ('n' denotes the number of monomer units in each polymer chain.)

2.1 Properties of PHB

PHB has attracted commercial interest because of its physical properties, which resemble those of polypropylene. Both polymers have compact, right-handed helical configurations, similar melting temperatures (close to 180°C), and similar degrees of crystallinity, making PHB a potential candidate for applications in plastics and fibres [13]. These similarities also make PHB suitable for injection moulding, extrusion blow moulding and fibre-spray-gun moulding; hence the same equipment can be used as for polyolefins and other plastics [14]. However, PHB has some chemical and physical properties that differ from those of polypropylene; for example, its stiffness and brittleness are higher, and it is less resistant to solvents. However, these properties can be improved by using it as a copolymer together with monomers of 3-hydroxyvalerate, resulting in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV) [15].

The most important feature of PHB by far is its biodegradability. A wide range of microorganisms are capable of degrading PHB and other PHAs by secreting hydrolytic enzymes and depolymerases. This allows degradation of the polymer in periods sometimes shorter than 6 months, depending on the physical form, dimensions, and, most importantly, the environmental conditions (Figure 6) [16]. Another important property of PHB is its biocompatibility, making it very attractive for medical use. It is an excellent candidate for long-term tissue engineering or drug delivery, and can be implanted in the body without causing inflammation [17].

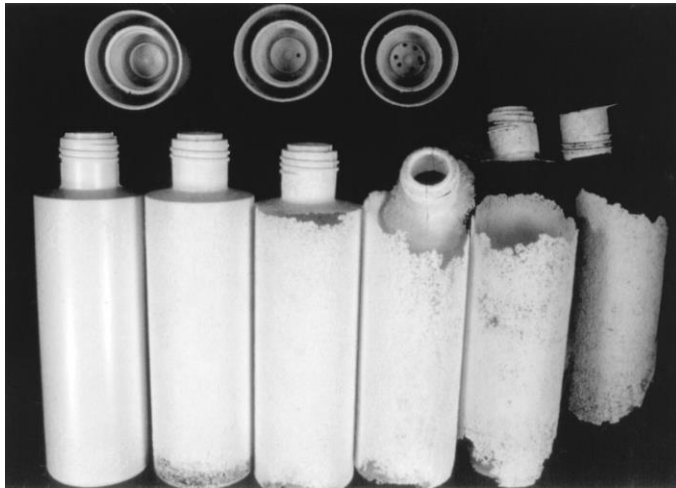


Figure 6. Degradation of PHBHV bottles by aerobic sewage sludge at an average temperature of 20°C. From left to right: after 0, 2, 4, 6, 8 and 10 weeks (adapted from Madison and Huisman, 1999 [27]).

2.2 Occurrence and physiological function of PHB

In 1926, the French microbiologist Maurice Lemoigne discovered intracellular granules in the gram-positive bacterium *Bacillus megaterium* [18], which he later identified as PHB [19]. These PHB granules were found to be formed during sporulation, and were degraded before the encystment process in *B. megaterium* and in *Azotobacter chroococcum* [20, 21]. In 1958, Macrae and Wilkinson observed that PHB levels increased four-fold in glucose-limited cultures when the nitrogen source was limited (NH_4Cl), and also that PHB yields increased significantly when the medium was supplemented with acetate, pyruvate or 3-hydroxybutyrate [22].

Since these first studies, hundreds of microorganisms, including gram-negative and gram-positive, aerobic and photosynthetic, lithotrophic and organotrophic bacteria, and some extremely halophilic archaea, have been identified as PHB-producing organisms. The accumulation of PHB is the result of a biological strategy to store excess carbon as insoluble molecules without compromising the viability of the host under non-optimal conditions. When normal conditions are restored the depolymerization of PHB provides a source of carbon for the microorganism [12, 23]. The formation of low-molecular-mass PHA complexed to proteins or polyphosphates has been observed in eukaryotes, and some other prokaryotes, but their metabolic function is still unknown [24, 25].

The number and size of PHB granules can differ between species. For example, *Cupriavidus necator* (formerly known as *Alcaligenes eutropha* or *Ralstonia eutropha*) accumulates between 8 and 13 granules with sizes between 0.2 and 0.5 μm (Figure 7) [23]. In this organism, accumulation of up to 80% of the cell dry weight (CDW) has been observed; above that, PHB formation ceases, apparently because of physical cellular limitations, rather than enzyme attenuation [14, 26].

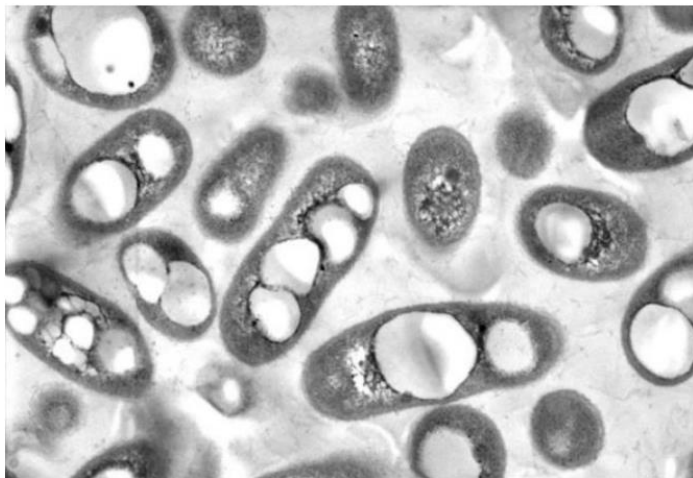


Figure 7. Scanning transmission electron microscopy image of *Cupriavidus necator* showing cells harbouring granules of PHB, seen in white (magnification 1/65000) (adapted from Koller, Salerno et al., 2013 [25]).

2.3 PHB biosynthetic pathways

Many microorganisms synthesize PHB granules, but most biochemical research has been performed on *Zoogloea ramigera* and *C. necator* [27]. In most organisms, PHB formation requires a sequence of three enzymes, and the precursor for its synthesis is the central metabolite acetyl coenzyme A (acetyl-CoA) (Figure 8) [26, 28].

- **Acetyl-CoA acetyltransferase (ACT)** (also known as β -ketoacyl thiolase) (EC: 2.3.1.9), encoded by the *phaA* gene in *C. necator*, catalyses the first step in PHB formation. These enzymes are divided in two classes, and can be found in prokaryotes and eukaryotes. Class I is involved in the degradation of fatty acids, while Class II is involved in PHA, ketone body, steroid and isoprenoid biosynthesis [29].
- **NAD(P)H-dependent acetoacetyl-CoA reductase (AAR)** (EC 1.1.1.36) is encoded by a *phaB* gene in *C. necator*, and catalyses the second reaction in PHB formation. In most organisms, this oxidoreductase is NADPH-dependent. However, in few of them, this reaction depends not only on NADPH, but also on NADH, such as *Allochromatium vinosum* (formerly known as *Chromatium vinosum*) [30], *Syntrophomonas wolfei* [31] and *Halomonas boliviensis* [32]. An alternative route for PHB synthesis has been identified in *Rhodospirillum rubrum*. Its AAR yields 3-L-hydroxybutyryl-CoA, which is thereafter used for the synthesis of 3-D-hydroxybutyryl-CoA by employing two stereospecific enoyl-CoA hydratases (ECH) (Figure 8) [33].
- **Poly-3-D-hydroxyalkanoate synthase (PHS)** (EC 2.3.1.B2) is encoded by the *phaC* gene in *C. necator* and catalyses the polymerization reaction. This enzyme exists in both soluble and granule-bound forms; as the granule grows, more enzyme is added to the surface [34].

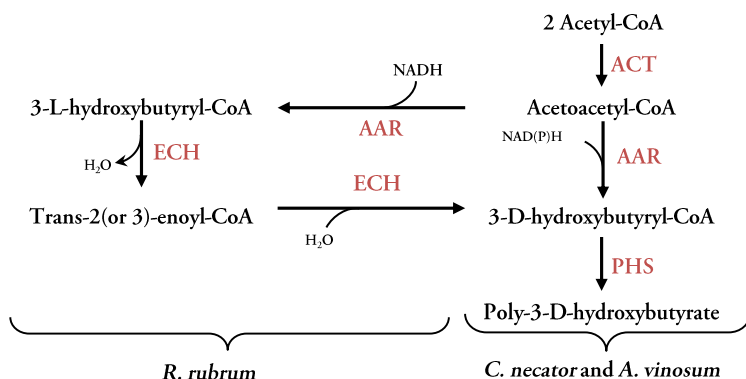


Figure 8. Biosynthetic pathway for PHB in different bacteria.

2.4 Hosts for biotechnological PHB production

The choice of organism and substrate has a major influence on the product yield and the type of by-products generated, and thus the production cost [35]. PHB production can be performed with natural/wild type organisms or with recombinant organisms (e.g. plants and yeasts). In the next section, a review of the most prominent organisms and their performance is presented.

2.4.1 Production by natural microorganisms

Improvement of PHB production by *C. necator* has been the aim of many lab-scale studies, the most relevant of which are reported below. As in many other organisms, PHB formation is triggered in response to nutrient limitation under non-growing conditions. Fed-batch fermentation is usually the preferred strategy, where biomass is first accumulated (usually ~100 g/L), followed by the establishment of low nitrogen-carbon ratio conditions.

One means of inducing PHB accumulation during cell growth, used by Kim et al., was to combine glucose feeding (without nitrogen addition) with pH regulation in bioreactors. Increasing the pH upon carbon depletion triggered the addition of glucose, and led to the accumulation of 10 g/L PHB, representing 17% of the biomass, with a volumetric productivity of 0.25 g/L·h [36]. Since pH changes are slow, another strategy is to use oxygen-dissolved values instead of pH variations as a trigger for glucose pulses. When nitrogen was limited at biomass concentrations of 70 g/L, PHB was produced with titres of 121 g/L, representing 75% of the biomass, and yields of 0.3 g PHB/g glucose and a volumetric productivity of 2.42 g/L·h were obtained [36]. To maintain a stable pH at high cell density, Rye et al. replaced NaOH (which is toxic) with NH₄OH, and phosphate limitation was induced instead of nitrogen limitation. Under these conditions, PHB production reached titres of 232 g/L, representing 80% of the biomass, and yields of 0.38 g PHB/g glucose with a volumetric productivity of 3.14 g/L·h were reported [37]. Continuous culture studies have shown that at low dilution rates (0.05 h⁻¹) under ammonia limitation, yields of 0.45 g PHB/g glucose could be achieved, a value close to the maximum theoretical yield (0.48 g/g) [38, 39]. Research has also been focused on replacing glucose with cheaper substrates, such as tapioca hydrolysates, which contained up to 90% glucose, in an attempt to reduce the production cost. However, the presence of inhibitory compounds limited the productivity by *C. necator* to 1 g/L·h, reaching 61 g/L and PHB equivalent to 58% of the CDW [40].

Scientists at ImCI (Imperial Chemical Industries) investigated *C. necator* as a candidate for PHB production due to its capacity to use a variety of substrates, such

ethanol, carbohydrates, sucrose or molasses, and mixtures of H₂ and CO₂. Although the theoretical yields from autotrophic growth in mixtures of H₂ and CO₂ mixture were high (1.0 g PHB/g substrate), fermentation with flammable hydrogen/air mixtures and the capital cost of the plant made the process uneconomic [35]. Using ethanol as the carbon source also gave high yields of PHB, 0.5 g/g, which is comparable to the theoretical yield of 0.62 g PHB/g substrate (when NADPH regeneration is taken in consideration) [39]. However, ethanol is more expensive than carbohydrates as a carbon source. Also, the use of complex carbohydrates requires pre-processing, which increases the production cost. For instance, sucrose must be converted into glucose and fructose, and molasses must be purified to reduce the nitrogen and phosphorus contents. Glucose was therefore chosen as the substrate as it does not require any processing. ImCI produced PHB in fed-batch fermentation at 30°C in 200,000 L air-lifted. using glucose medium and phosphate as the growth-limiting factor. Biomass was formed after the first 60 hours of growth in the glucose and phosphate medium. The medium was then supplemented with glucose only, reaching a maximum PHB of 75% of the total dry biomass in 48 h [35]. However, ImCI (later part of AstraZeneca) ceased production of PHB in 1998. The technology was then transferred to Monsanto where PHB was produced with bacteria until 2004, after which PHB was produced in engineered plants [25, 41].

Methylobacterium sp. has also been an industrially attractive organism for PHB production as it can utilize methanol, which is a cheap carbon source. It has been reported that *M. extorquens* was able to produce 149 g/L of PHB, in 170 h, while the yield was 0.18 g/g of methanol, which can be compared to the maximum theoretical yield of 0.54 g/g (taking NADPH regeneration into consideration) [39]. However, the composition of the medium, and operational parameters such as temperature and pH, affected the molecular weight of the PHB granules [42]. At temperatures and pHs that differed from those for optimal growth, the molecular weight of the PHB produced increased significantly, while the yield and productivity were reduced [37]. It was also observed that the molecular mass decreased at high methanol concentrations, whereas it increased at low concentrations giving from 30 to 60% PHB of the biomass [37]. Using slow methanol feeding, Bourque et al. obtained PHB corresponding to 45% of the biomass, corresponding to a volumetric productivity of 0.56 g/L·h and a yield of 0.20 g/g methanol [43]. *M. extorquens* was also tested in the pilot plant at ImCI, but it was rejected because it produced low-molecular-weight polymers that were difficult to extract [35].

In contrast to the bacteria described above, *Azotobacter vinelandii* UWD is able to produce PHB during growth under oxygen-limited conditions, as a result of a defective NADH oxidase [44]. Growth has been reported in complex carbon sources such as corn syrup, cane molasses, beet molasses and malt extract, fatty acids and

swine waste liquor [35, 45-47]. The wide range of carbon sources and the possibility of using a one-step fermentation process initially attracted the interest of ImCl. However, the microorganism was rejected due to poor genetic stability, which led to a decrease in PHB yield and increases in the yields of other carbohydrates [35].

During the late 1980s, Chemie Linz GmbH in Austria reported the production of 1000 kg PHB per week from glucose using *Alcaligenes latus* [25, 48]. As for *A. vinelandii* UWD, *A. latus* produces PHB during the growth phase, but in this case production was triggered by a higher affinity of acetyl-CoA acyltransferase for acetyl-CoA. But, as in the case of *M. extorquens*, low-molecular-weight PHB granules made the process of polymer extraction too expensive [49].

2.4.2 Production by recombinant prokaryotes

The commercialization of PHB production will require further improvement of the final PHB content per unit biomass, increased process yield and higher productivity. Although natural organisms have the metabolic machinery required for PHB biosynthesis, and its accumulation does not affect their viability, they also have disadvantages, such as the presence of cellular machinery for intracellular polymer degradation, slow growth, slow PHB accumulation, poor tolerance to inhibitors and sometimes a narrow range of substrates that can be metabolized [27]. These problems led to the engineering of prokaryotic organisms.

C. necator strains have been engineered for the overexpression of the poly-3-D-hydroxyalkanoate synthase gene (*phaC*), resulting in an increase in productivity by 1.2 times and in titre by 1.9 times [50]. However, *C. necator* has major limitations: it has poor inhibitor tolerance, and is poorly genetically characterized, which complicates strain engineering in attempts to improve industrial performance [27].

The gram-negative bacterium *Escherichia coli* has been engineered for PHB production due to its widespread use as a cell factory. A strain expressing the *phaA*, *phaB* and *phaC* genes in a high-copy-number vector has been found to be able to accumulate 80% PHB the biomass in fed-batch fermentation with 2% glucose in complex Luria-Bertani broth [51]. However, the performance was significantly poorer when cells were grown in minimum medium, and cell filamentation was observed. The maximum yield was only 0.07 g PHB/g glucose and only 22.5% PHB of biomass was accumulated [51]. It has been reported that PHB accumulation increased to 38% of the biomass with a yield of 0.13 g PHB/g glucose in engineered strains expressing *ftsZ*, which suppressed filamentation [52]. However, a major challenge that must be overcome when utilizing *E. coli* as the host for PHB production is the limited availability of NADPH cofactor [53], as well as a reduction

in PHB yields from heterogeneous populations originating from the utilization of high-copy-number plasmids [54]. As the production cost was high, other carbon sources, such as sugar cane bagasse, have been investigated using recombinant *E. coli*. However, PHB production and growth were found to be significantly reduced when 6% sugar cane molasses was used in minimal medium, due to the presence of inhibitory compounds [55].

2.4.3 Production by recombinant eukaryotes

Although eukaryotes do not produce PHB as carbon storage molecules, eukaryotic plants and yeasts can be engineered to produce PHB. Plants have several advantages, such as the low cost of substrates (water, soil nutrients, atmospheric CO₂ and light), and the fact that they can be grown under non-sterile conditions [27]. PHB production using the model plant *Arabidopsis thaliana* has been investigated. As eukaryotic systems are compartmentalized, the protein expression of the PHB pathway was tested in different compartments: the cytosol, peroxisomes and plastids. The expression of *C. necator* genes in *A. thaliana* resulted in the accumulation of PHB granules of sufficient molecular weight in the cytosol for industrial applications [41]. However, the accumulation of PHB led to retardation of plant growth due to the depletion of acetyl-CoA and acetoacetyl-CoA, which are essential molecules in the synthesis of sterols [56].

Plant peroxisomes are known to produce high levels of acetyl-CoA since they are involved in the β -oxidation of fatty acids; but when PHB genes were expressed in this organelle, PHB accumulation was low [57]. Plastids also contain high levels of acetyl-CoA. Plants with PHB genes targeting this compartment accumulated approximately 14% PHB of the dry weight in pre-senesce leaves, with no significant impact on plant growth. However, chlorosis was affected, and the gene number varied after a few generations due to low plasmid stability [58]. Another approach that has been investigated is the expression of the pathway in one plasmid in plastids. This resulted in a PHB accumulation of 40% of the dry mass; however, fertility, growth and sugar metabolism were seriously affected [59].

Other eukaryotic systems apart from plants have been investigated. For example, the poly-3-D-hydroxyalkanoate synthase gene (*phaC*) has been produced in the caterpillar *Trichoplusia ni* (cabbage looper) due to their ease of purification [56]. Yeast can be used for PHB production from cheap carbon sources such as lignocellulosic biomass; in addition, genetic engineering of yeast is less time consuming and more effective than engineering of plants. Several yeast species have been engineered for PHB production, such as *Komagataella pastoris* (formerly known as *Pichia pastoris*). PHB has been synthesized from rich media, at values between 19% and 27% CDW under different oxygenation conditions [60]. PHB

production has also been demonstrated with *Yarrowia lipolytica*, which was capable of accumulating 10% of CDW using acetate as the sole carbon source [61, 62]. Many trials have also been performed using the well-known baker's yeast *Saccharomyces cerevisiae*, which will be discussed in the following chapters. In conclusion, some progress has been made in the improvement of PHB yields and titres using various organisms and substrates, but the results obtained so far are not sufficient for sustainable industrial production. A summary of companies that produce PHB and other PHAs are presented in Table 2.

TABLE 2. Semi-industrial PHB production, add it as landscape format in the next page

2.5 Economic challenges in industrial PHB production

In 2011, the prices of conventional polyolefins such as polyethylene terephthalate and polystyrene were in the range of 1.38 to 1.63 €/kg, while the price of PHA was 3.7 to 4.5 €/kg [63]. The major barrier preventing bioplastics from being economically competitive remains the capital cost of the plant, which is determined by the complexity of the fermentation and recovery steps [35]. Therefore, every step of the biotechnological production of PHA is economically critical (Figure 9). Some of the key steps are summarized below.

The recovery of PHAs after cell harvesting is part of the downstream processing. The use of hazardous solvents and high amounts of energy not only increases the production cost, but also counteracts the character of bioplastics as being ecological materials. Therefore, new efficient solvents as well as new biological methods for autolysis are being investigated [25]

The process design for PHA production is usually based on fed-batch techniques. But if efficient continuous cultivation can be established using fast-growing organisms, then high productivities, uniform product quality and a reduction in costs could be achieved [25].

One of the major costs is related to the type of substrate used for PHA production. In most laboratory trials and many industrial applications PHAs are still synthesized from glucose. In order for the plastics industry to be regarded as eco-friendly, while remaining competitive, the cost of the substrate must be reduced. Moreover, the substrate should not interfere with human nutrition and animal feed supply chains. The list of potential substrates investigated for PHA production includes waste effluents such as olive mill and palm oil mill effluents, glycerol and cheese whey, waste plastics, activated sludge, swine waste and agricultural residues such as sugar cane bagasse, molasses, corn steep liquor, sawdust, wheat straw and forest biomass [63, 64]. The major problem when utilizing these types of substrate is the presence of substances that inhibit natural PHB producers and engineered prokaryotes. For this reason, more robust microorganisms, such as *S. cerevisiae*, can be considered as a better option.

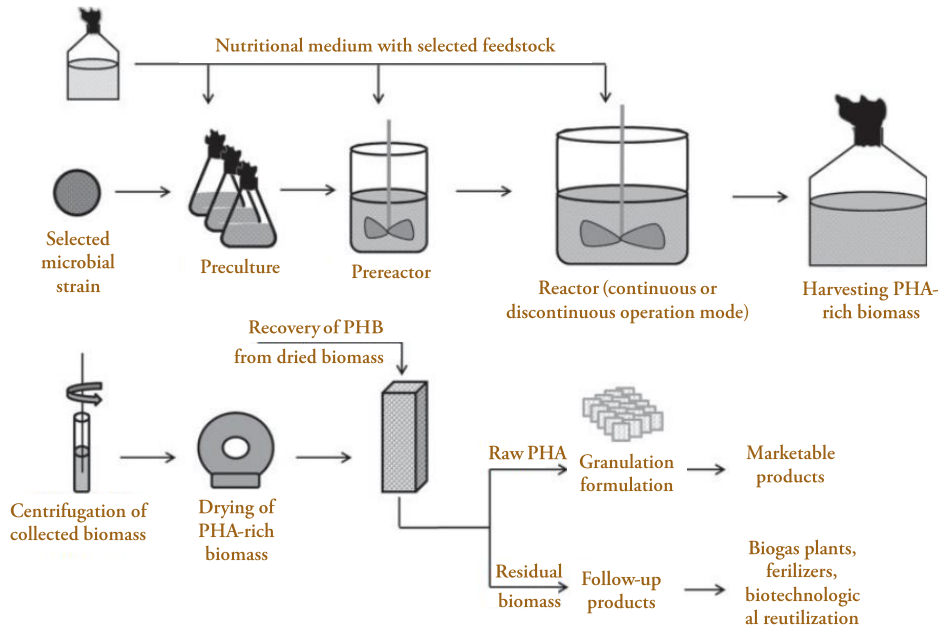


Figure 9. Schematic illustration of the entire PHA production process. (Adapted from Koller, et al., 2013 [25]).

3. *Saccharomyces cerevisiae* as a cell factory

3.1 From an old workhorse to a versatile cell factory

The use of yeast by humans was reported by civilisations as far back as the Babylonians (ca. 6000 BC) and the ancient Egyptians (ca. 5000 BC), for the production of wine, beer and bread. Yeasts are unicellular eukaryotes belonging to the largest branch of the tree of life, namely the kingdom of fungi [65]. The first microscope observations of yeast were made by van Leeuwenhoek in 1680, but it was not until 1838 that brewer's and baker's yeast was given the name *Saccharomyces* (meaning "sucrose eating") *cerevisiae* (beer) [66].

Our dependency on ever-decreasing oil resources has increased interest in using yeast for the production of chemicals from renewable resources using biotechnological approaches. Although yeasts are often associated with food products, their utilization as a microbial platform for chemical production offers a number of advantages. *S. cerevisiae* has been defined as GRAS microorganism (generally recognized as safe) by the American Food and Drug Administration (FDA) [67], and in 2007, it got Qualified Presumption of Safety by the European Food Safety Authority (EFSA).[68]. Since it is the simplest unicellular eukaryote, a vast amount of knowledge has been acquired about its physiology and genetic regulation, increasing the availability of tools for its modification [69]. *S. cerevisiae* can also grow on cheap substrates, and is characterized by its robustness under the stressful conditions encountered in industrial processes [70], which include low pH, high osmotic pressure, and inhibitory compounds [71]. Bioethanol made with *S. cerevisiae* is still the most important product made in the biotechnology industry, although proof of concept has been reported for hundreds of chemicals using *S. cerevisiae*, thus expanding the potential of this key cell factory (Figure 10) [71, 72].

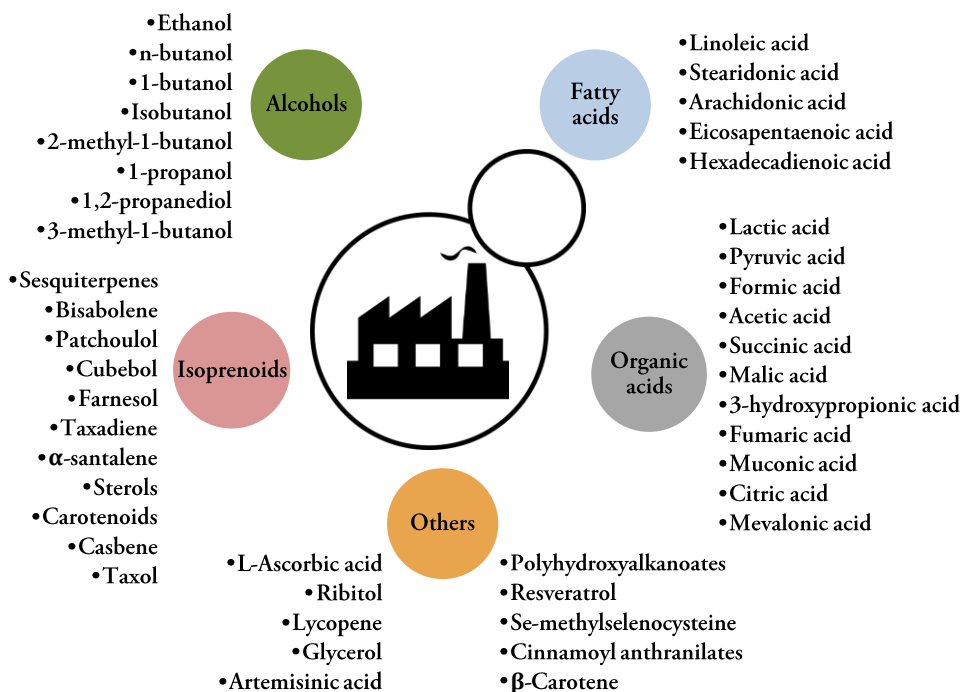


Figure 10. Examples of chemicals whose synthesis has been reported using *S. cerevisiae*.

3.2 Synthetic biology of *S. cerevisiae*

The rational and systematic engineering of biological systems to exhibit new biological functions is known as synthetic biology [73]. The application of synthetic biology has the potential to develop robust and versatile cell factories, leading to a successful bio-based economy which can replace chemical oil-based industry [74]. Two approaches can be adopted in the biotechnological production of chemicals.

- **Manipulation of a natural host** able to synthesize the product of interest in order to obtain high yields and productivities. The metabolism of the host can be modified by genetic engineering. If this is not possible, random mutagenesis or evolutionary engineering can be employed.
- **Metabolic engineering of a well-described microorganism** for the synthesis of the product of interest. *S. cerevisiae* is an interesting candidate since a wide range of synthetic biology tools are available. This approach is the subject of this chapter.

The definition of a suitable cell factory is one that produces the desired chemical at high titre, rate and yield, in a large-scale fermentation plant using a cheap substrate as carbon source. In the next section, some of the key steps the introduction of new genetic traits in a cell-factory are described.

3.2.1 Identification of enzymes

Cell factory engineering starts with the identification of the metabolic reaction(s) and pathway(s) that are involved in the synthesis of the desired compound. Bioinformatics tools have been developed for this purpose. Databases such as BRENDA, KEGG, the *Saccharomyces cerevisiae* Genome Database (SGD), Biocyc, NCBI are excellent sources of information on the genes and enzymes associated with different organisms. It is also possible to predict possible metabolic pathways from heterologous organisms based on the information available in these databases with the help of an algorithm called “From Metabolite to Meta-bolite” (FMM) [75]. Another algorithm, eQuilibrator, can also be used to obtain information about the thermodynamic profile of biochemical reactions and to estimate how much energy will be required and in which direction the reaction will proceed under particular cellular conditions (intracellular concentration, pH and ionic strength) [76]. Further information on the bioinformatics tools used for metabolic engineering design can be found in a review by Li and Borodina [71].

3.2.2 Pathway design

The appropriate genetic design of a metabolic pathway can significantly enhance titres, yields and productivities, improving the overall performance of a cell factory. This section summarises some of the factors that should be taken into consideration in genetic design.

DNA synthesis and assembly

Once the relevant pathways have been identified, their activities must be assembled in the host. Conventional methods of DNA fragment construction have previously relied on polymerase chain reaction (PCR) fragments plugged in standard vectors. However, it will soon be possible to make whole *de novo* DNA constructs synthetically in so-called *BioBricks*. This method is still expensive, but offers advantages such as DNA codon optimization for the host organism. Meanwhile, fragment assembly techniques, including classical restriction-ligation-based cloning and other new methods, can be used. These new methods improve the speed, precision and flexibility of cloning and they rely mostly on fragment overlapping for *in vitro* and *in vivo* homologous recombination. Some of the most well-know

are: the Gibson assembly [77], the uracil-specific excision reaction (USER) [78], In-fusion [79] and *in vivo* recombination in *S. cerevisiae* [80].

Selection markers

Until now, successive genetic modifications of *S. cerevisiae* required the use of selection markers in order to identify clones after engineering. Prototrophic gene markers complementing certain amino acid (e.g., *LEU2*, *TRP1*, *HIS3*, *LYS2*), or nucleotide auxotrophies (e.g., *URA3*, *ADE2*) are very popular, but they require the use of an auxotrophic yeast strain. Antibiotic resistance markers for geneticin (*KanMX*), hygromycin (*HYG*, *hphNT1*) and nourseothricin (*NAT*) are also commonly used for the modification of industrial strains that usually lack auxotrophies. However, the addition of antibiotics to maintain the selection pressure increases the production cost significantly [81].

To enable multiple genetic modifications in the same strain, it is sometimes necessary to reuse the same marker. The Cre/*loxP* system adds *loxP* sequences adjacent to the marker, which are detected by Cre recombinase excising the marker, allowing its utilization in the next engineering step [82]. Another tool is the *A. nidulans amdSYM* gene. This marker encodes a protein that confers the ability to utilize acetamide as the sole carbon source on an organism. This protein also confers sensitivity to fluoroacetamide, which is used as counter selection so that only cells that are able to remove the marker remain [83].

Gene expression regulators: promoters and terminators

Promoters are elements in gene expression that play a key role in metabolic pathway optimization. Constitutive promoters maintain relatively constant levels of gene expression over time. These promoters are usually obtained from genes encoding glycolytic enzymes, such as phosphoglycerate kinase (*PGK1p*), triose-phosphate isomerase (*TPI1p*) or glyceraldehyde-3-phosphate dehydrogenase (*TDH3p*) but also from the fermentative route, such as alcohol dehydrogenase I (*ADH1p*), from the translation elongation factors (*TEF1p*), or from hexose transport genes (*HXT7p*) [84]. Other types of promoters can be regulated, and are useful for the control of gene expression over time, for instance, to avoid the accumulation of toxic intermediates that can inhibit enzymatic reactions. Several inducible promoters have been reported, including *GAL1p*, *GAL7p* and *GAL10p*, which are induced by galactose and repressed by glucose; *CUP1p*, which is induced by copper; and *ADH2p*, which is induced when glucose is depleted and ethanol consumption starts [67].

Although terminators have not been studied in as much detail as promoters, they play an important role in the mRNA half-life, thus influencing the translation event. Some of these high-capacity terminators originate from genes encoding cytochrome

c (*CYC1t*), pheromone-regulated protein (*PRM9t*) and the vacuolar carboxypeptidase (*CPS1t*) [85].

Compartmentation, scaffolding and fusion proteins

The production of chemicals can be enhanced by modifying the mechanisms of protein assembly on the gene level.

During the genetic design of metabolic pathways, the genes introduced can contain nucleotide sequences that enable the localization of the encoded protein(s) in specific organelles. One example is the localization of acetyl-CoA-requiring pathways in the yeast mitochondria, for example, isobutanol. When the isobutanol pathway was targeted in the mitochondria the production was improved 2.6-fold in comparison to the cytosolic production [86]. The reason for this is that high amounts of acetyl-CoA are present in the mitochondria during aerobic respiration.

Proteins can be scaffolded into other proteins or RNA molecules to increase the protein stability and also to localize products and substrates of enzymatic reactions close to each other. For instance, the resveratrol pathway was constructed in ligand-fusion protein in *S. cerevisiae*, resulting in a 5-fold increase in resveratrol titres compared with the control strain [87]. Another example is the 3-fold increase in PHB production when the pathway was scaffolded in *E. coli* [88].

Fusion proteins can be used in microbial cell factories for product enhancement by increasing the proximity between the substrate and the enzyme, thus facilitating the biochemical reaction. Fusion proteins consist of two or more genes joined to form a single protein. This strategy has been used for the production of amorphadiene, a key precursor molecule of artemisinin (an antimalarial drug). A chimeric fusion protein was constructed using farnesyl diphosphate synthase from yeast and amorphadiene synthase from *Artemisia annua*. The introduction of this fusion protein led to a 4-fold increase in artemisinin production [89].

3.2.3 Pathway introduction: expression vectors and CRISPR/Cas9

The regulation and integration of pathways in *S. cerevisiae* can be achieved in two different ways: using expression vectors, or by directly integrating the constructed DNA fragment into the chromosome using techniques such as the CRISPR/Cas9 system.

Expression vectors

There are two types of expression vectors: autonomously replicating vectors and integrative vectors (Table 3). Replicating vectors are useful for gene overexpression, but they have some drawbacks, such as limited control of copy number and

segregational stability [90]. The homologous recombination machinery of *S. cerevisiae* allows the integration of vector-based and PCR-based DNA fragments into a specific target region in the chromosome, offering high genetic stability [67].

Table 3. List of the most common expression vectors for *S. cerevisiae*.

Name	Abbrev.	Features
Yeast Replicating plasmids	YRp	Contain an autonomously replicating sequence (ARS) derived from the yeast chromosome. Can replicate independently, but are rather unstable.
Yeast Centromeric plasmids	YCp	Carry an ARS together with part of a centromere sequence (CEN). Very stable as their replication is similar to a small chromosome, but low copy number.
Yeast Episomal plasmids	YEpl	Contain the 2-micron circle (2 μ , a natural yeast plasmid), and are considered “high copy” (~50 copies). Most similar to bacterial plasmids.
Yeast Artificial Chromosome	YAC	Carry an origin of replication (ORI), a CEN and telomeres. Used to introduce a large number of genes simultaneously, e.g., a metabolic pathway. Can be stable for more than 50 generations.
Yeast Integrating plasmids	YIp	Lack an ORI and must be integrated directly into the chromosome via homologous recombination. Offers very high genetic stability and can be used for gene deletion. In multiple integrations, these can be targeted to repeated genomic regions, such as rDNA, transposon elements or δ -sites (long terminal repeats) of retrotransposons (Ty1/2); however, multiple integration events can vary, and the exact positions are not known due to duplications.

CRISPR/Cas9

During recent years, conventional homologous recombination methods have been replaced by more efficient techniques that rely on site-specific endonucleases. Some of these techniques are: yeast oligo-mediated genome engineering (YOGE) [91], zinc finger endonucleases [92], transcription-activator-like (TAL) effector nucleases [93] and the CRISPR-Cas9 system [91]. The CRISPR-Cas9 system appears to be the most promising, and is described in detail below.

In 2007, the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems were discovered to be prokaryotic mechanisms for viral defence [94]. This system has since been modified for use in the genetic modification of various organisms. This application allows simultaneous gene deletions/integrations and the introduction of specific mutations more rapidly than classical cloning [95]. In 2013, this genetic tool was adapted for use in *S. cerevisiae* [91]. The method relies on 3 pillars.

- *Cas9* is an RNA-guided endonuclease from *Streptococcus pyogenes* which is part of the bacterium's immune system [91]. The endonuclease forms a ribonucleoprotein complex with two small RNA molecules. This complex performs a sequence-specific double-strand break (DSB) in genomic DNA [96].
- *CRISPR RNA* (crRNA) contains a target recognition sequence composed of 20 base pairs, followed downstream by a genomic sequence of NGG, i.e., the protospacer adjacent motif (PAM). *Cas9* recognises the trans-activating (tracrRNA) molecule that is bound to crRNA. Both RNAs constitute the guide RNA (gRNA) [97, 98].
- The *Cas9* gRNA complex induces a DSB. To repair it, the homologous recombination machinery of *S. cerevisiae* uses a donor DNA cassette. This donor DNA contains the metabolic pathway of interest flanked by 40 bp of overlap homology to the cleaved site (Figure 11) [98].

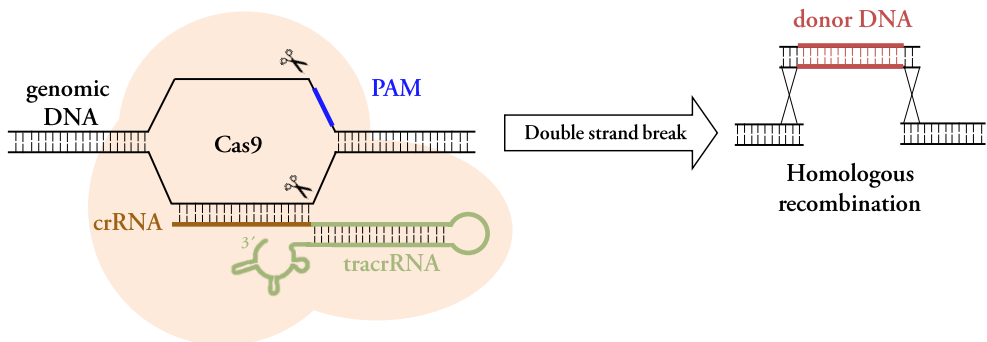


Figure 11. Schematic representation of the Cas9 complex attached to target genomic DNA. The host is transformed with: a vector encoding Cas9; a vector containing the gRNA and a donor DNA containing the DNA of interest. First, Cas9 forms a ribonucleoprotein complex with gRNA and performs a sequence-specific cleavage in the genomic DNA. After the DSB, the homologous recombination system of the host incorporates the donor DNA, repairing the DSB. PAM: protospacer adjacent motif; crRNA: CRISPR RNA, tracrRNA: trans-activating RNA.

The main advantages of the CRISPR-Cas9 system is its efficiency, accuracy and rapidity, and it promises to be a useful technique for genome editing. Moreover, the creation of gRNA libraries to target loci with high transcriptional activity, and the design of gRNA plasmids with multiple target sequences will accelerate the strain construction process when multiple engineering is needed [74].

3.3 *S. cerevisiae* and lignocellulosic biomass

The typical carbon sources used by *S. cerevisiae* are glucose, fructose and sucrose. These are usually derived from corn starch or sugar cane, but in order for *S. cerevisiae* to be successfully implemented as a microbial platform for chemical production, the range of substrates must be widened. Cheap carbon sources from non-edible biomass are being explored, and one of the most investigated is lignocellulosic biomass [74].

3.3.1 Composition and processing of lignocellulosic biomass

Lignocellulosic biomass is the most abundant renewable organic resource on Earth [99], and it can be obtained from forest products (e.g., hardwood and softwood), agricultural residues (e.g., wheat straw, sugar cane bagasse and corn stover), as well as dedicated crops (e.g., switchgrass and *Salix*) [100]. About 90% of the plant dry weight consists of cellulose, hemicellulose and lignin. The remaining 10% is made up of minor components such as pectins, fats and waxes [101].

Cellulose is a uniform linear polymer of β -1,4-linked D-glucopyranose units which constitute about 33-51% of the lignocellulosic biomass. It forms tissue connecting the cells, controlling the shape of the plant and allowing the plant to withstand the turgor pressure of the fluids contained in it [99]. Hemicellulose is a heteropolysaccharide consisting of hexose sugars (glucose, galactose and mannose), pentose sugars (xylose and arabinose) and various carboxylic acids (e.g. 4-*O*-methyl-glucuronic acid). It constitutes about 19-34% of the lignocellulosic biomass, and reinforces the rigidity and shape of the plant [102]. Finally, lignin consists of a complex of irregular cross-linked, water-insoluble, hydrophobic and highly branched polymers. It represents about 20-30% of the lignocellulosic biomass, and serves as a protective barrier preventing degradation of the plant by bacteria and fungi, and also increases the rigidity of the plant (Figure 12) [103].

Biomass must be treated to release the sugars contained in lignocellulose. Pretreatment increases the digestibility of the raw material. Various methods have been tested, including physical (milling and grinding), physico-chemical (steam pretreatment, freeze explosion), chemical (acid treatment [sulphuric acid, hydrochloric acid, sulphur dioxide or phosphoric acid], alkaline treatment [ammonia or sodium hydroxide] and organic solvents [ethanol, ethylene glycol]), biological (with white rot fungi), electrical, or a combination of these [100]. Each method has a different effect on the cellulose, hemicellulose and lignin fractions, so the method

chosen depends on the configurations of the following hydrolysis and fermentation processes.

During hydrolysis, the cellulose and hemicellulose are converted into fermentable sugars using either chemical or enzymatic methods. Dilute-acid hydrolysis is carried out at high temperatures (160-230 °C) and pressures (~10⁶ atm). This method is usually combined with weak-acid prehydrolysis. Hydrolysates can also be obtained using highly concentrated acids, giving higher sugar yields, but more inhibitory compounds. This method is carried out at lower temperatures (<50 °C) and at atmospheric pressure [100, 104]. In enzymatic hydrolysis, enzyme cocktails from fungal species are used to break down the cellulose fibrils into sugars [105, 106]. This method requires prehydrolysis steps to expose the plant fibres to the enzymes, and longer retention times are required. [100].

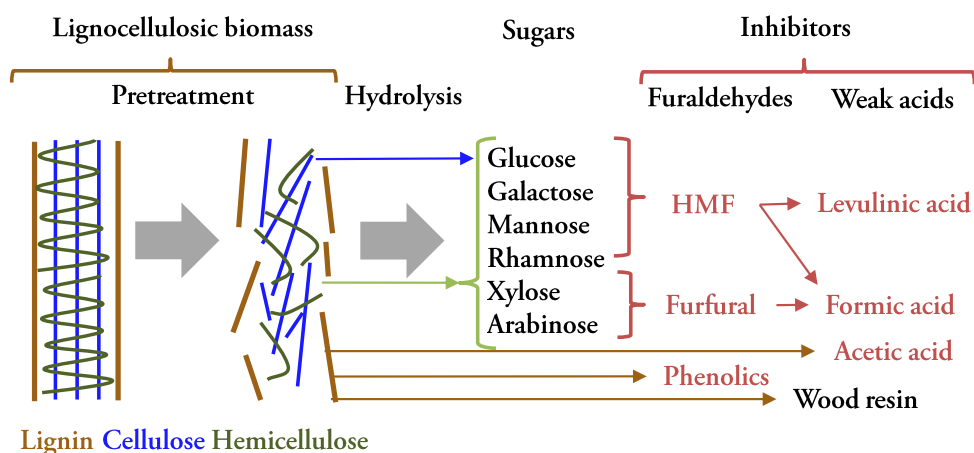


Figure 12. Reactions occurring during the treatment of lignocellulosic biomass. The raw biomass is first pretreated to expose the plant fibres for the hydrolysis step. During hydrolysis, the cellulose, hemicellulose and lignin are digested by chemical or enzymatic methods, releasing a mixture of sugars and inhibitors. HMF: hydroxymethylfurfural.

3.3.2 Yeast and lignocellulosic inhibitors

The most common conditions for pretreatment and hydrolysis are high temperatures and acidic conditions. This leads to the formation of microbial inhibitory compounds such as furaldehydes (furfural, hydroxymethylfurfural [HMF]), weak acids (acetic acid, formic acid, levulinic acid) and phenolic compounds [107] (Figure 12). Furaldehydes have been shown to decrease the specific growth rate of the yeast, so it is necessary to reduce them into less inhibitory alcohols in order to restore growth [108]. Furfural causes a decrease in cell viability during the lag phase, and leads to

the formation of reactive oxygen species that affect yeast metabolism [109, 110]. HMF causes a decrease in protein content and lipid accumulation [111]. As for weak acids, these liposoluble molecules can diffuse across the cell membrane, causing a reduction in growth rate and biomass [112]. Phenolic compounds are a very large, heterogenic group of lignocellulosic inhibitors. They also reduce the fermentation rate, biomass production and ethanol yield of *S. cerevisiae* [113].

Studies on inhibitors have helped to understand the mechanisms behind yeast inhibition, as well as the cells' response to them. For example, reduction of furaldehydes decreases the level of NAD(P)H, thus causing a reduction in amino acid synthesis [114]. When weak acids are located in the cytosol, they dissociate generating free protons and decreasing intracellular pH. To maintain internal pH, cells pump out the excess protons by hydrolysing ATP through the plasma membrane H⁺-ATPase [115], which affects cell growth. The mechanisms of action of phenolic compounds inside the cell are still not clear, due to their broad heterogeneity [116]. Moreover, in complex media such as hydrolysates, these inhibitory compounds have synergistic effects on the performance of the yeast, making the situation more complex [117].

3.3.3 Yeast and pentose sugars

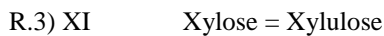
Among the cell factories studied to date, *S. cerevisiae* is one of the preferred due to its high inhibitor tolerance and high ethanol yield from the hexose sugars present in lignocellulosic biomass [118]. However, to increase the ethanol yield and to make the process economically feasible, *S. cerevisiae* must be able to utilize not only the hexose sugars, but also the pentose sugars, such as xylose, which constitutes the second most abundant sugar in lignocellulosic material [119]. Xylose-fermenting organisms and the implementation of relevant pathways in *S. cerevisiae* have been investigated over the past 30 years [104]. Two pathways have been found to confer xylose utilization on *S. cerevisiae*: the oxido-reductive xylose pathway and the xylose isomerization pathway [120].

The oxido-reductive xylose pathway is present in yeast species that can utilize xylose aerobically or under oxygen-limited conditions, and these are usually isolated from wood-related environments [104, 121]. The most popular heterologous oxido-reductive xylose pathway for engineering *S. cerevisiae* is obtained from *Scheffersomyces stipitis*. The oxido-reductive xylose pathway is a two-step reaction in which xylose is first reduced to xylitol by a NAD(P)H-dependent xylose reductase (XR) (R.1; EC 1.1.1.10). XR has a dual co-enzyme specificity, although NADPH utilization is predominant. Xylitol is then converted into xylulose by a NAD⁺-dependent xylitol dehydrogenase (XDH) (R.2; EC 1.1.1.9) [121]. The

difference in cofactor specificity between XR and XDH results in a cofactor imbalance, causing the accumulation of xylitol due to the depletion of NADPH and NAD⁺ [118].



The **xylose isomerase (XI) pathway**, in contrast to the oxido-reductive-pathway, does not require the use of cofactors (R.3; EC 5.3.1.5). This route directly converts xylose into xylulose, and is found in bacteria and some fungi [104, 122]. After various unsuccessful trials, efficient XI expression in *S. cerevisiae* was eventually reported in the early 2000s [123]. There are now many XI alternatives from different organisms, however, most of them are susceptible to inhibition by xylitol. To avoid natural xylitol production, the aldose reductase encoded by *GRE3* can be deleted [124]. Among the enzymes most recently expressed in recombinant *S. cerevisiae* strains is *Burkholderia cenocepacia xylA*. Strains expressing this enzyme do not accumulate xylitol and can co-consume glucose and xylose under anaerobic conditions [125]. However, after implementation, the strain required evolutionary engineering based on sequential batch cultivations in order to improve the xylose consumption rate [126].



A natural pathway that allows xylose oxidation generating α -ketoglutarate through a multistep pathway, has also been found, namely the **Weimberg pathway** [127]. However, this pathway has not yet been successfully engineered in *S. cerevisiae*.

3.4 Exploring *S. cerevisiae* for PHB production

In order for the production of PHB to be commercially viable, the production cost must be competitive with that for conventional petroleum-derived plastics. The major limitation on PHB production by bacteria is the low yield on complex substrates and the low efficiency of product formulation in downstream processing [11]. Natural PHB producers possess the metabolic machinery for PHB depolymerization, but often have long generation times. These are some of the main reasons why researchers are exploring eukaryotic systems. *S. cerevisiae* is considered interesting due to its ability to utilize non-detoxified lignocellulosic hydrolysates, and the fact that it can be engineered for xylose utilization [118].

The first attempt to produce PHB using *S. cerevisiae* was made by expressing the poly-3-D-hydroxybutyrate synthase (PHS) gene from *C. necator* in a multi-copy plasmid. The strain generated was able to accumulate up to 0.5% PHB per CDW, simultaneously with the production of ethanol [128]. Although the PHB accumulation was low, the study demonstrated that *S. cerevisiae* strains possessed the machinery for the synthesis of PHB precursor molecules (3-D-hydroxybutyryl-CoA and acetoacetyl-CoA). Leaf et al. hypothesized that the low PHB yields could be the result of insufficient endogenous acetyl-CoA acetyltransferase and acetoacetyl-CoA reductase activities [128]. A study involving combinatorial enzyme analysis demonstrated that PHB formation was enhanced by co-expression of the three enzymes of the biosynthetic PHB pathway in *S. cerevisiae*. Expression of PHB synthase allowed PHB accumulation up to 0.2% of the CDW, while co-expression of PHB synthase and acetoacetyl-CoA reductase led to a PHB accumulation of 3.5% of the CDW, and co-expression of all three enzymes (ACT, AAR and PHS) led to the accumulation of up to 9% of the CDW after 4 days of cultivation [129].

During anaerobic cell growth in excess of glucose, the carbon flux has been found to be directed mainly to ethanol production due to the overflow/Crabtree mechanism, and therefore, very little PHB was detected [129]. Thus, under these conditions most of PHB production occurred during growth on ethanol, and not during growth on glucose (Table 4). During the ethanol phase, NADPH is supplied by the conversion of acetaldehyde to acetate via the NADP⁺-dependent acetaldehyde dehydrogenase encoded by *ALD6* [130]. Acetate is then used for the synthesis of cytosolic acetyl-CoA, which is the substrate for PHB and biomass synthesis [131] (Figure 13). When glucose was used as substrate under anaerobic conditions, PHB and ethanol were co-produced [129].

Engineered strains growing on xylose synthesized PHB simultaneously with biomass production (Table 4) (Figure 14) (Paper I). Less ethanol is formed

aerobically from xylose than from glucose. This observation suggests that although there is no glucose-mediated repression, the respiratory capacity is limited (Paper III) [132]. Although acetyl-CoA is synthesized from acetate, its synthesis is limited (See Chapter 5), and acetate accumulation lowers the pH, inhibiting xylose fermentation (Papers I, II and III).

Table 4. Overall stoichiometry for the formation of PHB from one mole of substrate glucose, ethanol and xylose by recombinant *S. cerevisiae*. Calculations were performed assuming PHB formation via NADP⁺-dependent acetaldehyde dehydrogenase (ALD6) and NADPH-dependent AAR, and xylose utilization via NADPH-dependent XR.

Stoichiometry
1Glucose + 2ATP + 2NAD ⁺ + 1NADP ⁺ = 1PHB + 2ADP + 2(NADH+ H ⁺) + 1(NADPH+ H ⁺) + 2CO ₂
1Ethanol + 2ATP + 1NAD ⁺ + 0.5NADP ⁺ = 0.5PHB + 2ADP + 1(NADH+ H ⁺) + 0.5(NADPH+ H ⁺)
1Xylose + 1ATP + 2.2NAD ⁺ + 0.4NADPH = 0.6PHB + 1ADP + 2.2(NADH+ H ⁺) + 0.4(NADP ⁺ + H ⁺) + 1.2CO ₂

The effect of supplementing the growth medium with different precursors of the PHB pathway has also been investigated in *S. cerevisiae*. When acetate was added to the medium (0.5 g/L, pH adjusted to 4.5) PHB synthesis was improved, and similar results were obtained when adding pantothenate, a coenzyme A precursor [129]. In addition to the effects of carbon source and precursor supply, it was also found that the use of episomal plasmids led to population heterogeneity. This effect was detected when cells were stained with Nile red and were observed in fluorescence microscopy, only half of the cells contained PHB inclusions [129].

The cell morphology of *S. cerevisiae* strains that synthesize PHB has also been investigated. Cell staining with fluorescent dyes (BODIPY or Nile red) and subsequent analysis with flow cytometry revealed that cells with higher PHB content tended to be smaller. It was suggested that this could be due to the fact that smaller cells have a higher surface area for nutrient transport into the cell per unit cell volume than larger cells [133].

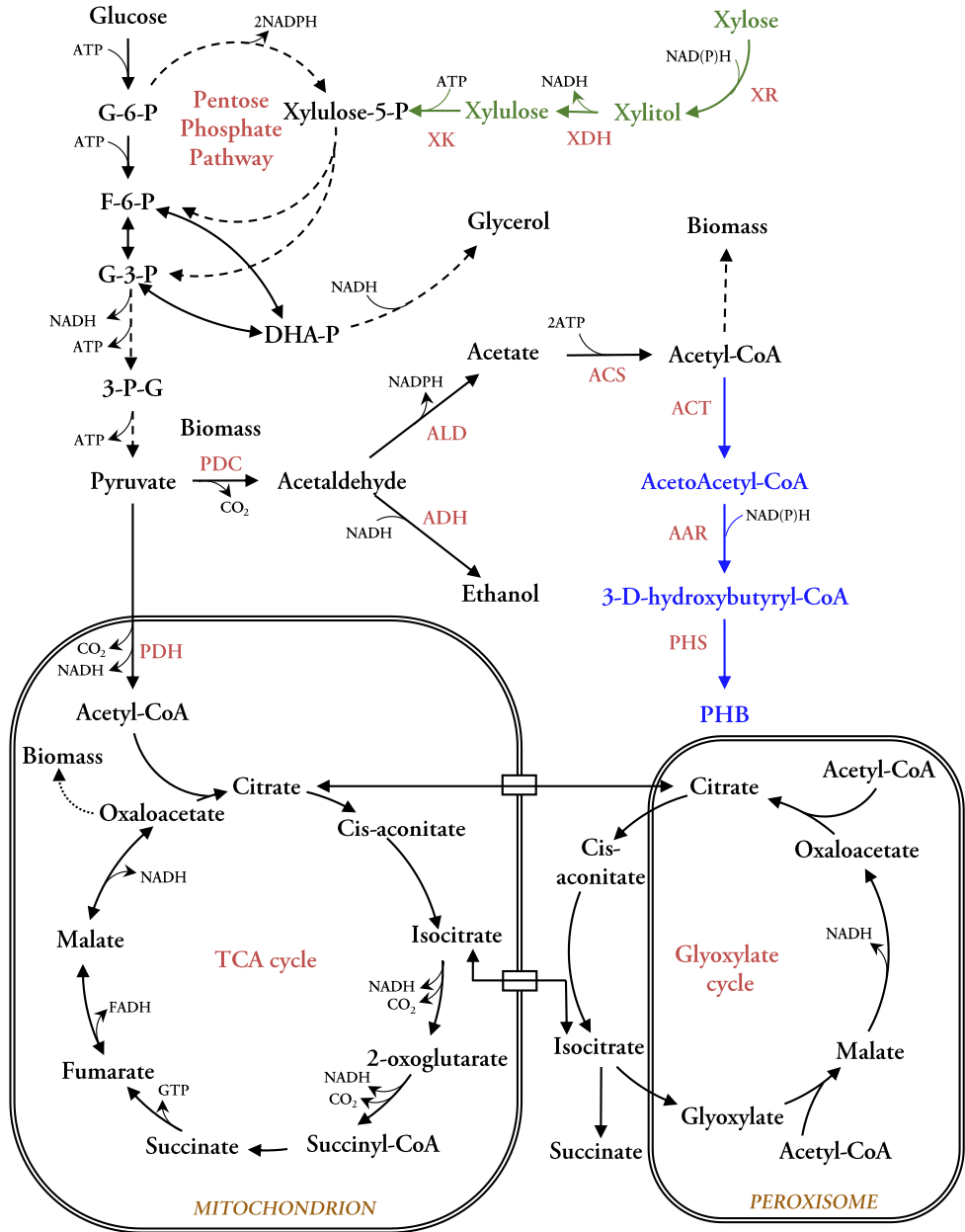


Figure 13. Overview of the metabolism of engineered *S. cerevisiae*. Continuous lines represent sequential biochemical reactions. Dashed lines represent pools of biochemical reactions. Red denotes genes or names of metabolic pathways. Xylose metabolism is represented by green lines and PHB biosynthesis by blue lines. Xylose is taken up in engineered strains via xylose reductase (XR) converted into xylitol (XDH) and then into xylulose via xylulose kinase (XK). Xylulose is further incorporated into the pentose phosphate pathway and from there it is incorporated into the glycolytic pathway. Glucose is naturally taken up and incorporated into glycolysis, which leads to pyruvate formation. Pyruvate can enter the mitochondria during aerobic respiration, or remain in the cytosol in anaerobic fermentation or during respiration-fermentative metabolism. In the mitochondria, pyruvate is converted into acetyl-CoA via pyruvate dehydrogenase (PDH), and is then incorporated into the tricarboxylic acid (TCA) cycle. Citrate can be transported from the mitochondria to the cytosol, where it becomes part of the glyoxylate cycle required for the assimilation of C₂ carbon sources (ethanol and acetate). If pyruvate remains in the cytosol it is decarboxylated by pyruvate decarboxylase (PDC) and converted into acetaldehyde, where it can generate ethanol via alcohol dehydrogenase (ADH) or acetate via acetaldehyde dehydrogenase (ALD). Acetate can be secreted, remain in the cytosol or be transported to the peroxisome or nucleus, where it is further converted into acetyl-CoA via acetyl-CoA synthase (ACS). Cytosolic acetyl-CoA can be taken up as a substrate for PHB formation in engineered strains through a three-step reaction, or used for the synthesis of fatty acids and sterols. Two molecules of cytosolic acetyl-CoA are first converted into acetoacetyl-CoA by acetyl-CoA acyltransferase (ACT). This is then converted into 3-D-hydroxybutyryl-CoA by an NAD(P)H-dependent AAR, yielding NAD⁺ or NADP⁺ depending on whether the enzyme is taken from *A. vinosum* or *C. necator*. The last step is carried out by poly-3-D-hydroxyalkanoate synthase (PHS), in which one monomeric unit of PHB is generated.

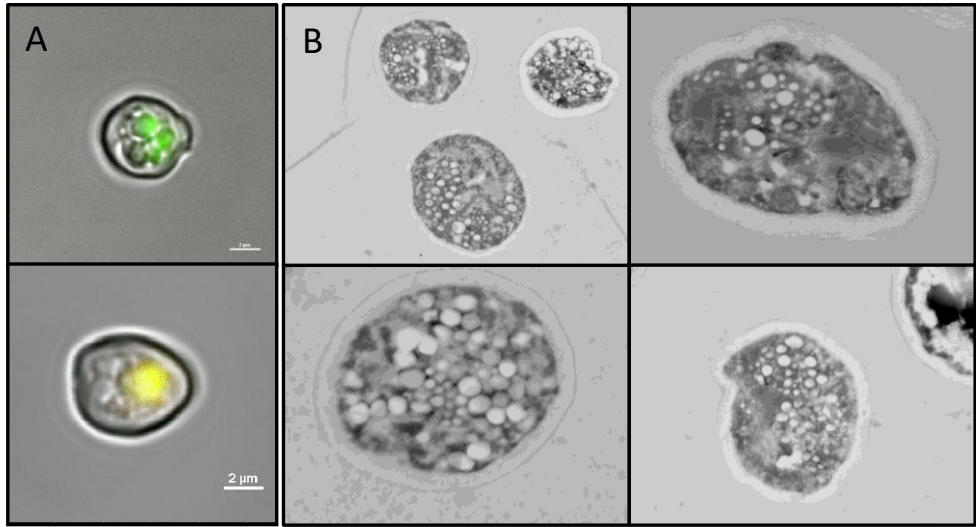


Figure 14. Confocal laser scanning fluorescence microscopy (A) and STEM images (B) of recombinant *S. cerevisiae* TMB4425 cells expressing the biosynthetic PHB pathway from *C. necator* in combination with the *A. vinosum* AAR, when grown on xylose. The fluorescence microscopy images show the fluorescence obtained from BODIPY emission at 525 ± 25 nm and Nile red emission at 595 ± 25 nm. The white inclusions in the STEM images are granules of PHB. (Photographs by Sebastian Wasserstrom and Alejandro Muñoz.)

4. Redox balance and its impact on metabolic engineering *S. cerevisiae*

The implementation of biochemical pathways *S. cerevisiae* often requires the utilization of the redox cofactors NADH and/or NADPH, leading to disturbance of the natural equilibrium in the cellular metabolism [134]. These cofactors play a major role in the connections between central and peripheral reactions in the metabolism of carbon and nitrogen, i.e., the redox metabolism [135]. The redox metabolism in *S. cerevisiae*, as well as its impact on metabolic engineering strategies, is discussed in this chapter, with special focus on PHB production from xylose.

4.1 Redox cofactors and their involvement in metabolism

More than 200 metabolic reactions are dependent on the pyridine-nucleotide cofactors $\text{NADP}^+/\text{NADPH}$ and NAD^+/NADH in *S. cerevisiae*. NADPH is preferentially utilized in assimilatory pathways or anabolism, such as the biosynthesis of fatty acids, whereas NADH is mostly involved in dissimilatory pathways or catabolism, such as the glycolytic pathway [136]. This implies that $\text{NAD(P)}^+/\text{NAD(P)H}$ cofactors play a central role in the homeostasis of metabolism, energy generation and growth in *S. cerevisiae* [137].

$\text{NAD(P)H}/\text{NAD(P)}^+$ molecules consist of an adenine nucleotide and a nicotinamide nucleotide bound by a phosphate group. As they cannot cross biological membranes, they are oxidized and reduced in the cell compartment in which they are generated, e.g. the cytosol of the mitochondria [138]. The *de novo* synthesis of cytosolic or mitochondrial pyrimidine nucleotide $\text{NADP}^+/\text{NADPH}$ is catalysed by the $\text{ATP-NAD}^+/\text{ATP-NADH}$ kinases *UTR1*, *YEF1* and *POS5* (R.4; EC 2.7.1.23) (R.5; EC 2.7.1.86), and the cells become inviable if *UTR1* and *POS5* are deleted [139, 140]. These reactions provide a pool of NADPH that can subsequently be utilized in assimilatory pathways such as synthesis of amino acids, lipids and nucleotides as

well as this cofactor is also involved in oxidative stress responses (Figure 15) [141]. Thereafter, cytosolic NADPH is mainly regenerated from NADP^+ through the pentose phosphate pathway by glucose-6-phosphate dehydrogenase (G6PDH) (R.6; EC 1.1.1.49) [142, 143], and the decarboxylating enzymes glucose 6-phosphogluconate dehydrogenases (GND) (R.7; EC 1.1.1.44) [144]. NADPH acetaldehyde dehydrogenase (ALD) (R.8; EC 1.2.1.5) and isocitrate dehydrogenases (IDP) (R.9; EC 1.1.1.42) also contribute to the regeneration of cytosolic and mitochondrial NADPH, respectively [145, 146].

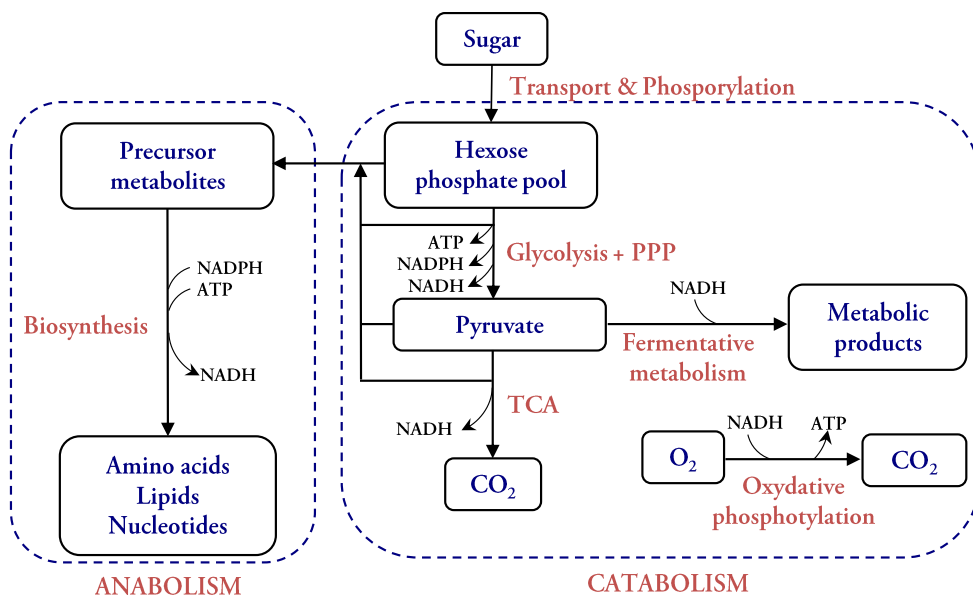
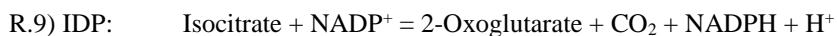
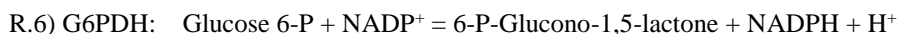


Figure 15. Schematic illustration of the involvement of nicotinamide nucleotides (NADH and NADPH) in the dissimilatory metabolism (catabolism) and assimilatory metabolism (anabolism) of biomass. (Adapted from Stephanopoulos et al., 1998 [148]).

During catabolism (or dissimilatory reactions) NADH from NAD^+ is generated via the metabolic routes that release energy, in particular, glycolysis in the cytosol and

the TCA cycle in the mitochondria [135]. NADH is also generated in anabolism during mitochondrial and cytosolic amino acid synthesis [147] (Figure 15). In yeast, *de novo* synthesis of NAD⁺ originates from the tryptophan pathway, and from the salvage pathway from nicotinamide riboside [140, 148].

4.2 Redox balance mechanisms

Limited amounts of NAD(P)H/NAD(P)⁺ are present in the cell, and the reduction of NAD(P)⁺ must therefore be matched to the oxidation of NAD(P)H to maintain cellular metabolism. *S. cerevisiae* possesses several mechanisms of cofactor regeneration to ensure this balance, and they depend on the carbon source and oxygen availability [121], as discussed below.

4.2.1 Aerobic respiration

Under aerobic conditions, the respiratory chain is the major pathway for the reoxidation of NADH to NAD⁺, where oxygen acts on the final electron acceptor. In *S. cerevisiae*, NADH dehydrogenases (or NADH:ubiquinone oxidoreductase) are located in the external mitochondrial membrane (encoded by *NDE1* and *NDE2*) and in the internal membrane (encoded by *NDII*). These enzymes mediate oxidation by transferring the electrons from NADH to the ubiquinone pool, which are subsequently transferred to the bc1 complex and to cytochrome c. This last enzyme catalyses the final step in the conversion of oxygen and protons into water (Figure 16) [149].

The external NADH dehydrogenase is considered to be the main system for oxidizing cytosolic NADH; however, *S. cerevisiae* also employs the glycerol-3-phosphate shuttle as an alternative to decrease the elevated levels of NADH [135]. This shuttle consists of a cytosolic NAD⁺-dependent glycerol-3-phosphate dehydrogenase encoded by *GPD1* and *GPD2*, and a mitochondrial-bound glycerol-3-phosphate:ubiquinone oxidoreductase encoded by *GUT2* [135]. In contrast to the complex I-type of many eukaryotic cells, including yeast, these enzymes do not contribute to the proton motive force [149]. Hence, *S. cerevisiae* has a low ATP stoichiometry of oxidative phosphorylation compared to other organisms. As a result, complete dissimilation of a glucose molecule yields approximately 16 ATP molecules, 12 of which are from oxidative phosphorylation and 4 from substrate-level phosphorylation (2 in glycolysis and 2 formed in the TCA cycle) [150].

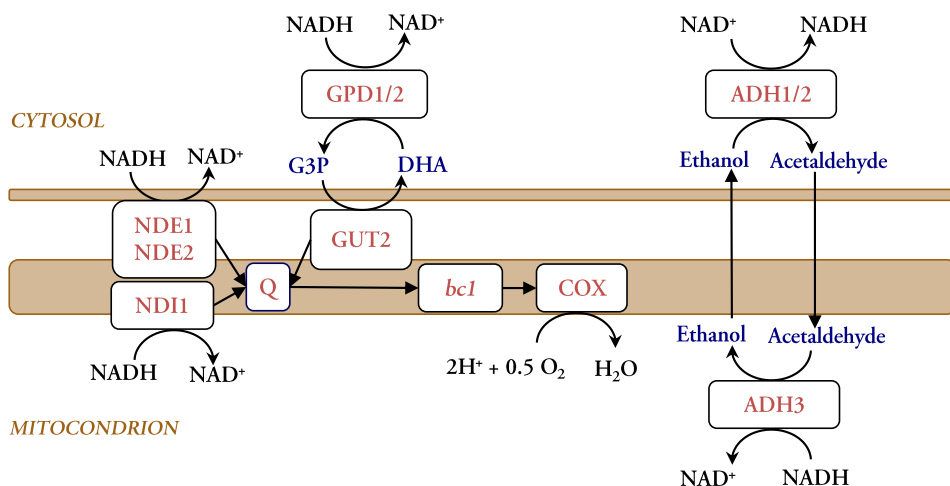
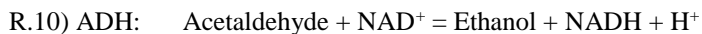


Figure 16. The respiratory chain of *S. cerevisiae*. ADH1/2/3: alcohol dehydrogenase; bc1: bc1 complex; COX: cytochrome c oxidase; GPD1/2: glycerol-3-P dehydrogenase; GUT2: glycerol-3-P dehydrogenase membrane-bound; NDE1/2: external NADH dehydrogenase; NDI1: internal NADH dehydrogenase; Q: ubiquinone (adapted from Bakker et al., 2001 [135]).

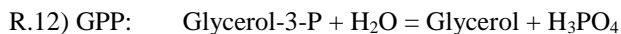
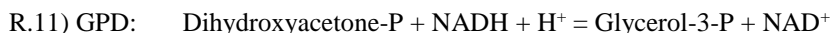
4.2.2 Anaerobic fermentation

One molecule of glucose is converted into two pyruvate molecules, two ATP molecules and two NADH molecules by glycolysis. In the absence of oxygen, alcoholic fermentation enables the regeneration of cytosolic NAD⁺ in 2 steps: decarboxylation of pyruvate to acetaldehyde, and reduction of acetaldehyde to ethanol via alcohol dehydrogenases (ADHs) (R.10; E.C.1.1.1.1) [151]. This process yields eight-fold less ATP than aerobic respiration. The anaerobic dissimilation of one molecule of glucose yields only 2 ATP molecules, from substrate-level phosphorylation, which is reflected in an approximately five-fold lower biomass yield in anaerobic culture than in aerobic sugar-limited cultures [150].



The additional NADH that is generated via biomass synthesis (mainly amino acids) is reoxidized to NAD⁺ through the formation of glycerol in a two-step reaction: the glycolytic intermediate dihydroxyacetone phosphate (DHAP) is reduced to glycerol-3-phosphate (G-3-P) using an NAD⁺-dependent glycerol-3-phosphate dehydrogenase (encoded by *GPD1/GPD2* genes) (R.11; EC 1.1.1.8), after which G-3-P is converted to glycerol with help of glycerol-3-phosphatase (encoded by *GPP1/GPP2*) (R.12; EC 3.1.3.21) [135]. Strains harbouring knock-outs of genes

gpd1 and *gpd2* are unable to grow anaerobically, unless an external electron acceptor (NADH-oxidizing agent) is added to the medium, for example, acetoin or acetaldehyde [152], which indicates that glycerol formation is the sole redox valve under anaerobic conditions.



The overall synthesis of glycerol requires the hydrolysis of ATP, and in many cases its production in *S. cerevisiae* is used as a redox sink for the reoxidation of NADH. Glycerol also plays an important role in osmoregulation. When cells are exposed to decreased extracellular water activity, glycerol is accumulated intracellularly to counteract the outflow of water molecules since it is a compatible solute [121, 153]. In addition, glycerol is a precursor for triacylglycerol, which is an important precursor in lipid synthesis [154].

The mechanism of transferring electrons from mitochondrial NADH to a cytosolic electron carrier is known as a redox shuttle. Under anaerobic conditions oxidation of NADH inside the mitochondria is not feasible because oxygen cannot be used as the final electron acceptor. Several shuttles can be found in *S. cerevisiae*: malate–oxaloacetate, malate–aspartate and ethanol–acetaldehyde. For example, in the ethanol–acetaldehyde shuttle, different alcohol dehydrogenases located in the mitochondria and in the cytosol oxidize ethanol and acetaldehyde in different compartments since these molecules can cross the membrane without the need of a transporter (Figure 16) [138].

4.2.3 Respiro-fermentative metabolism

Alcoholic fermentation by yeasts occurs under conditions of oxygen limitation or depletion. However, in some yeast species, such as *S. cerevisiae*, alcoholic fermentation can also occur under aerobic conditions, a phenomenon known as the Crabtree effect or overflow metabolism [121]. This represents a major industrial challenge in the production of metabolites other than ethanol when high concentrations of glucose are used [74].

High glucose levels (> 5 g/L) repress the genes involved in the utilization of alternative carbon sources (ethanol and acetate), gluconeogenesis, peroxisomal functions and limits the cell respiration capacity and triggers ethanol formation [151, 155]. Under such conditions, the predominant mode of NADH oxidation is alcoholic fermentation [156]. In the first stage, glucose is quickly consumed until depletion. The cell then undergoes the diauxic shift, where the metabolism

assimilating ethanol as a carbon source is activated, and the transcript levels of the NADH oxidases increase. During this stage, ethanol is consumed aerobically, generating acetic acid and NADPH that is oxidized in the respiratory chain generating ATP [121].

4.3 Yeast engineering and redox metabolism

S. cerevisiae is a suitable cell factory for the production of many chemicals, however, most of the engineering approaches require cofactors which often alter the redox status of the cell. Table 5 gives some examples of the chemicals that can be produced by *S. cerevisiae*, and the limitations on their synthesis. In this section, various metabolic strategies for the enhancement of NADH reoxidation, xylose assimilation and PHB synthesis will be discussed in more detail.

Table 5. Examples of products that can be made using engineered *S. cerevisiae* and the associated cofactors

Product (associated cofactor) Precursor	Approach	Limitation	Reference
Glycerol (NADH) DHA	Oxidation of NADH through glycerol by deletion of pyruvate decarboxylases (<i>pdc1/5/6</i> , <i>nde1/2</i> and <i>gut2</i>) and overexpression of formate dehydrogenase (<i>FDH1</i>)	Requires co-feeding with formate	[157]
	Deletion of external NADH oxidases (<i>nde1/2</i> and <i>gut2</i>) and accumulation of DHAP by deletion of triose phosphate isomerase (<i>tpi1</i>)	Accumulation of DHA derives to methylglyoxal, which is toxic, resulting in slow growth rate	[158]
	Cofactor shift to NADPH by heterologous NADPH-dependent DHAP reductase (<i>gpsA</i>)	Low specific activity of GpsA	[159]
Isobutanol (NADPH) Pyruvate	Compartmentalization of isobutanol pathway in the mitochondria by overexpression of the L-valine pathway (<i>LLV2/3/5</i> , <i>BAT2</i> , <i>α-KDC</i> and <i>ADH</i>)	Limited carbon flux. Toxic at high concentrations	[86]
Propanediol (NAD[P]H) DHA	Overexpression of methylglyoxal synthase (<i>mgs</i>) and glycerol dehydrogenase (<i>gldA</i>) from <i>E. coli</i>	High concentrations of GldA enzyme are inhibitory.	[160]
Lactic acid (NADH) Pyruvate	Overexpression of L-lactic acid dehydrogenase and deletion of pyruvate decarboxylases (<i>pdc1/5/6</i>) to avoid ethanol production	Unable to grow anaerobically possibly due to energy-dependent lactate transport	[161]
Succinic acid (NAD[P]H) TCA cycle intermediates	Deletion of succinic dehydrogenases (<i>sdh1/2</i>) and isocitrate dehydrogenase (<i>idh1</i> , <i>idp1</i>)	Repression of oxidative metabolism. Acetate accumulation	[162]

4.3.1 Enhancing NADH reoxidation

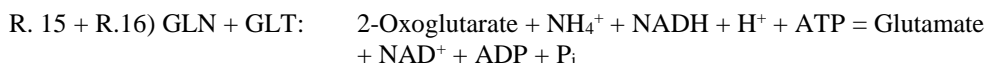
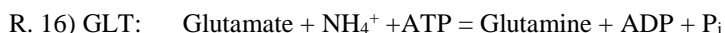
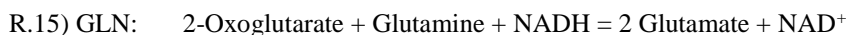
The production of chemicals often implies the formation of by-products causing carbon loss. These by-products (e.g. xylitol and glycerol) are usually triggered by excess NADH, which is generated under anaerobic conditions, or by overflow metabolism due to excess glucose. In this section, the various metabolic engineering strategies aimed at reducing excess NADH are discussed.

Transhydrogenase (TH) is found in many prokaryotes that use it to convert NADH to NADPH [163]. As yeast lacks TH activity, several attempts have been made to overexpress functional heterologous TH. Under anaerobic conditions, TH was

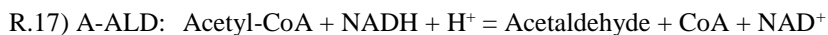
expected to cope with excess cytosolic NADH, thus reducing the carbon lost by glycerol formation and the synthesis of NADPH for biomass formation. Unfortunately, despite efforts to express THs from different origins (*E. coli* and *A. vinelandii*) (R.13; EC 1.6.1.1), their expression resulted in the accumulation of glycerol, 2-oxoglutarate and acetic acid due to increased NADH and decreased NADPH levels [134, 164, 165].



Ammonium assimilation occurs in yeast in a NADPH-dependent process. The NADPH-dependent glutamate dehydrogenase gene *GDH1* (R.14; EC 1.4.1.4) was removed and substituted with two genes encoding a two-step reaction for ammonium assimilation. This consisted of overexpression of a glutamine synthase encoded by *GLN1* (R.15; EC 6.3.1.2) and glutamate synthase encoded by *GLT1* (R.16; EC. 1.4.1.14). This successfully reduced the glycerol yield by 38% providing an alternative redox sink for NADH [166].



When strains lacking the glycerol pathway were engineered with the **NAD⁺-dependent acetylating acetaldehyde dehydrogenase** (A-ALD) (R.17; EC 1.2.1.10) from *E. coli*, anaerobic growth was restored in the presence of glucose when acetate was added to the medium. A-ALD catalyses the NADH-consuming reaction that converts acetyl-CoA obtained from acetate into acetaldehyde, thus allowing reoxidation of NADH [167].

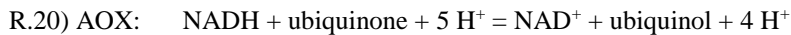
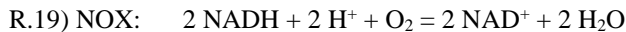


NADH kinases catalyse the last step in the *de novo* formation of NADPH from NADH and ATP (R.4; EC 2.7.1.86). It has been demonstrated that overexpression contributes to a decrease in NADH levels in *S. cerevisiae*. Under conditions of excess glucose the overexpression of the native mitochondrial *POS5* lowered the NADH/NAD⁺ ratio, which contributed to de-repression of the mitochondrial enzymes of the TCA cycle, increasing the carbon flux, and subsequently increasing biomass formation [134]. On the other hand, if the enzyme is truncated such that it is only expressed in the cytosol, the formation of glycerol decreases, but so does the biomass, due to a drain of NADH and ATP [134].

The **NADP⁺-dependent glyceraldehyde-3-phosphate dehydrogenase** (GapN) (EC 1.2.1.9) has been introduced as a substitute for the native NAD⁺-dependent glycerate-3-phosphate dehydrogenase (TDH) (R.18; EC. 1.2.1.12). *In silico* results showed a 40% lower glycerol yield on glucose with no effect on the maximum specific growth rate [168]. Results have also been obtained *in vivo* by the overexpression of GapN combined with the deletion of the glycerol exporter Fps1p and the overexpression of the cytosolic NADH kinase UTR1. Fps1p was disrupted to accumulate intracellular glycerol, in an attempt to induce other regulatory systems, in order to reduce glycerol formation [169]. This resulted in 38% lower glycerol yields, however, the strains showed limited anaerobic growth due to the drain of ATP [170].



The expression of **water-forming NADH oxidase** (NOX) (R.19; EC 1.6.3.4) by *Streptococcus pneumoniae* in the cytosol, and an **alternative NADH oxidase** (AOX) (R.20; EC 1.10.3.11) in cultures grown at high glucose levels under aerobic conditions, resulted in strains with lower ethanol levels caused by a decrease in NADH[171].



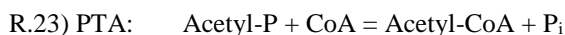
4.3.2 Enhancing xylose conversion

Xylose fermentation through the oxido-reductive pathway suffers redox limitation due to cofactor imbalance. The difference in cofactor specificity between XR and XDH results in the accumulation of xylitol due to the depletion of NADPH and NAD⁺ [118]. NADPH can be regenerated from the oxidative PPP, whereas NAD⁺ is regenerated through the respiratory chain under aerobic conditions, and anaerobically from metabolic pathways that require NADH oxidation (Figure 17). Since glycerol synthesis prevents ATP formation from glycolysis, anaerobic xylose utilization becomes limited [121].

The challenge of cofactor regeneration has been addressed through various metabolic engineering strategies targeting the xylose metabolic pathway either directly or indirectly. One series of approaches was aimed at rebalancing the cofactor level by introducing additional redox reactions (see section above). Intracellular cofactor concentrations have also been altered by shutting down the PPP through the **deletion of NADP⁺-dependent glucose-6-phosphate dehydrogenase (G6PDH)** (EC 1.1.1.49), while overexpressing an **NADP⁺-dependent glyceraldehyde-3-phosphate dehydrogenase (GDP)** from *Kluyveromyces lactis* (R.21; EC 1.2.1.9). This resulted in lower xylitol accumulation and higher ethanol production, however, the xylose consumption rate was significantly decreased [172].



Ethanol production has been enhanced by modifying the xylose metabolization route through the introduction of a **phosphoketolase (PK)** (R.22; EC 4.1.2.9) and a **phosphotransacetylase (PTA)** (R.23; EC 2.3.1.8) (Figure 17). The ethanol yield on xylose was improved by about 25% without affecting the xylose fermentation rate. However, acetate formation inhibited xylose fermentation. This problem was solved by the deletion of acetaldehyde dehydrogenase, resulting in an improvement in the xylose fermentation rate [173].



Another improvement was achieved by exploring **alternatives XR and XDH with more balanced cofactor affinity**. For instance, a library of mutated XR variants was generated and expressed in *S. cerevisiae* using error-prone PCR on the cofactor binding site. After sequential anaerobic batch cultivations, the selected strain harboured a XR mutated gene with an increased NADH/NADPH utilization ratio [174]. Another example is the construction of a strictly NADP⁺-dependent XDH generated by multiple site-directed mutagenesis, resulting in enhanced ethanol production and less xylitol accumulation [175]. A search has also been made for

naturally occurring alternative XR genes were also searched in Nature. When expressed in *S. cerevisiae*, the XR isolated from *Spathaspora passalidarum*, *XYL1.2*, resulted in an enzyme with higher affinity for NADH than for NADPH, enhancing xylose fermentation under anaerobic conditions (Figure 17) (Paper IV).

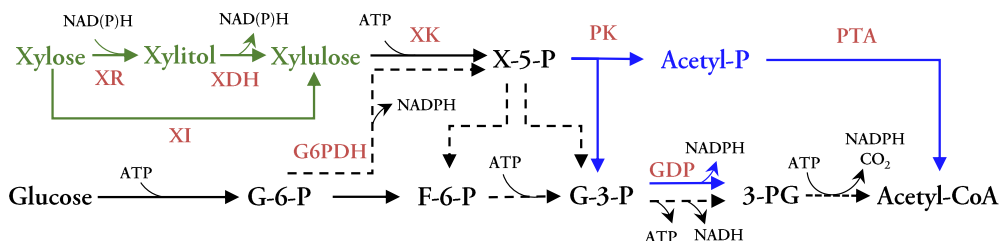
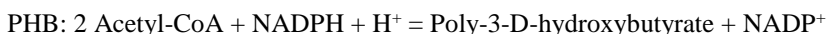


Figure 17. Schematic overview of metabolic engineering approaches used for cofactor-balanced xylose utilization. Continuous lines represent sequential biochemical reactions. Dashed lines represent pools of biochemical reactions. In red encoded enzymes. Heterologous xylose metabolism is shown in green. XR: Xylose reductase; XDH: xylitol dehydrogenase; XI: xylose isomerase. Endogenous reactions are indicated by black arrows; XK: xylulokinase; G6PDH: glucose-6-phosphate dehydrogenase. Heterologous reactions are indicated by blue arrows; GDP: NADP⁺-dependent glyceraldehyde-3-phosphate dehydrogenase; PK: phosphoketolase; PTA: phosphotransacetylase.

4.3.3 Enhancing PHB production

When the biosynthetic pathway from *C. necator* is implemented in *S. cerevisiae* PHB synthesis requires the reduction of half a mole of NADPH per mole of acetyl-CoA. Although the functionality of the pathway has been demonstrated in *S. cerevisiae*, PHB yields, titres and productivities are still much too low for industrial application.



The growth rate of *S. cerevisiae* strain expressing engineered xylose reductase with increased affinity for NADH (XR_{mut}), was reduced under aerobic conditions compared with strains expressing wild type XR, but this favoured the accumulation of PHB per cell. Furthermore, the increased affinity for NADH allowed anaerobic growth on xylose (Paper II).

As NADPH is involved in biosynthetic pathways, the availability of NADPH is one of the bottlenecks in PHB production using *S. cerevisiae* (Paper I). Therefore, metabolic engineering strategies were implemented to increase the availability of NADPH or modify the cofactor usage in the PHB pathway.

Cofactor supply

One alternative strategy to increase NADPH availability has been focused on introducing an alternative source of NADPH. In practice, the NADP⁺-dependent glyceraldehyde-3-phosphate dehydrogenase GapN from *Streptococcus mutans* (R.18; EC 1.2.1.9) was expressed in PHB-producing strains [176]. This resulted in a decreased flux towards glycerol, due to a reduction in NADH formation, and an increase in the production of PHB (Figure 18).

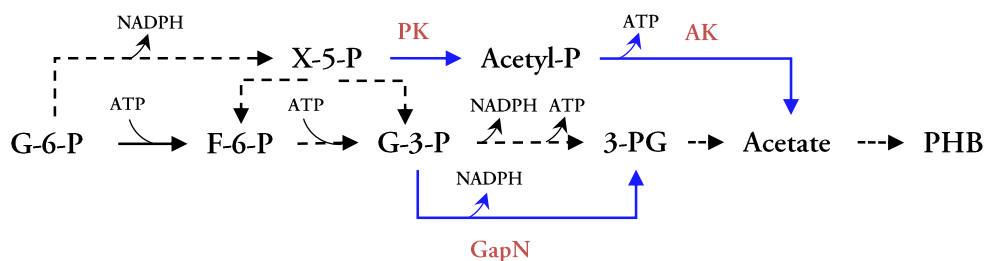


Figure 18. Schematic illustration of metabolic engineering strategies for PHB production. Continuous lines represent sequential biochemical reactions. Dashed lines represent pools of biochemical reactions. In red encoded enzymes. Endogenous reactions are indicated by black arrows. Heterologous reactions are indicated by blue arrows: GapN: NADP⁺-dependent glyceraldehyde-3-phosphate dehydrogenase. PK: phosphoketolase. AK: acetate kinase (Adapted from Kocharin, et al., 2013 [176]).

To avoid competition for NADPH between biomass formation and PHB production, an NADH-dependent acetoacetyl-CoA reductase was evaluated (Paper II). The NADH-dependent AAR from *A. vinosum* (R.24; EC 1.1.1.36) was expressed in *S. cerevisiae* instead of the NADPH-dependent AAR from *C. necator*. When the new constructed strains were evaluated, the PHB titres, yields and content per cell were significantly increased. Moreover, in strains that expressed XR_{mut} with high affinity for NADH, PHB could also be produced from xylose anaerobically (Paper II). Although NADH-PHB synthesis represents an alternative redox sink for excess NADH, the capacity for reoxidation of NADH via PHB formation is lower than that for glycerol synthesis (Paper II).



These strategies showed that modification of the redox metabolism can increase PHB formation, the next step to improve PHB production is enhancement of the precursor supply. This aspect will be discussed in the next chapter.

5. Acetyl coenzyme A, a central building block in *S. cerevisiae*

Acetyl coenzyme A (acetyl-CoA) is an important precursor molecule for the biosynthesis of many industrially relevant products such as isoprenoids, polyketides, lipids and PHAs [177]. These compounds can be synthesized in *S. cerevisiae* by implementation of the relevant heterologous pathways. However, in order to maximize their synthesis and minimize the production of by-products, it is necessary to engineer the central carbon metabolism to redirect carbon towards acetyl-CoA [178]. In this chapter the synthesis and metabolic engineering approaches used for the enhancement of the acetyl-CoA pool in *S. cerevisiae* will be discussed.

5.1 Role and synthesis of acetyl-CoA

Acetyl-CoA consists of a coenzyme A (CoASH or CoA) linked to acetic acid by a high-energy thioester bond. This ubiquitous building block derives many different molecules, some of which are of commercial interest (Table 6). But above all, acetyl-CoA is an essential metabolite for many biochemical reactions within protein, lipid and carbohydrate metabolism [179].

The function of acetyl-CoA is highly dependent on the cell compartment (Figure 19). In the nucleus, acetyl-CoA is used for protein acetylation as a mechanism for cellular signalling and gene transcription [180], whereas mitochondrial acetyl-CoA acts as a substrate in the TCA cycle for the generation of ATP, and as an intermediate for amino acid and porphyrin synthesis [181]. Cytosolic acetyl-CoA is used in the biosynthesis of lipids and fatty acids [181]. Finally, the β -oxidation of fatty acids leads to the production of peroxisomal acetyl-CoA, which is further oxidized in the glyoxylate cycle for the provision of precursors for the biosynthesis and utilization of C_2 carbon compounds such as ethanol and acetate (Figure 19) [182].

Despite its requirement in all cell compartments, eukaryotic membranes are impermeable to acetyl-CoA, which prevents its movement from one compartment

to another. The mechanisms of transport are explained at the end of this section. [183].

Fermentable carbon sources are converted into pyruvate through glycolysis, and this can remain in the cytosol or enter the mitochondria, which determines the compartment of acetyl-CoA [184].

Table 6. Examples of derived acetyl-CoA products of industrial interest and their applications

Type	Example	Application
Polyhydroxyalkanoate	Polyhydroxybutyrate	Biopolymers
Alcohol	1-butanol	Biofuels and chemical building blocks
Sterol	Ergosterol	Dietary supplement
Polyphenol	Resveratrol	Antioxidant and Nutraceutical ingredient
Isoprenoids	Farnesene	Biofuel
	Artemisinic acid	Antimalarial drug
	Santalene	Fragrance
	β -carotene	Nutraceutical ingredient
	Lycopene	Nutraceutical ingredient
Alkanes/alkenes	Terminal alkenes	Advanced biofuels
Fatty alcohols	C ₈ -C ₁₈ fatty alcohols	Biofuels and chemical building blocks
Waxes	Fatty acid ethyl esters	Detergents, lubricants and cosmetics

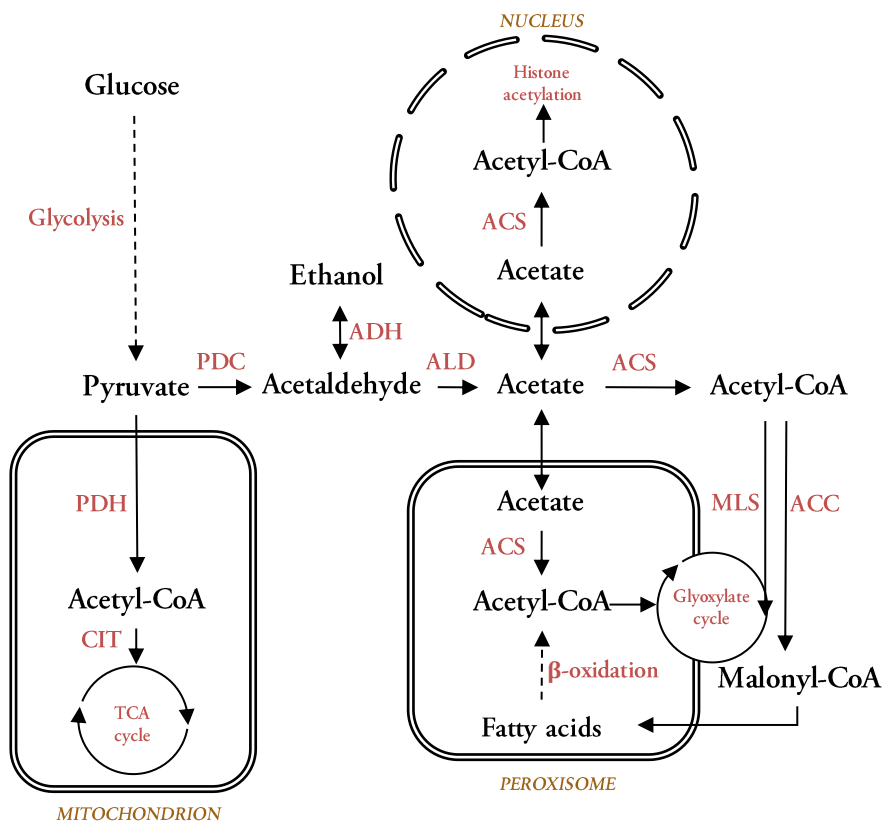


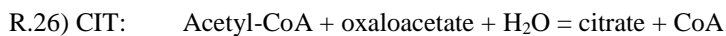
Figure 19. Compartmentation and metabolism of acetyl-CoA in *S. cerevisiae*. Continuous lines represent sequential biochemical reactions. Dashed lines represent pools of biochemical reactions. PDH: pyruvate dehydrogenase; CIT: citrate synthase; PDC: pyruvate decarboxylase; ADH: alcohol dehydrogenase; ALD: acetaldehyde dehydrogenase; ACS: acetyl-CoA synthase; MLS: malate synthase; ACC: acetyl-CoA carboxylase (adapted from Krivoruchko, et al., 2015 [181]).

5.1.1 Mitochondrial acetyl-CoA

During aerobic respiration (e.g. under glucose-limiting conditions), acetyl-CoA is generated from the pyruvate dehydrogenase complex (PDH), and is used as a substrate in the first reaction of the TCA cycle by citrate synthase (CIT) [178]. The PDH protein complex of *S. cerevisiae* is formed by three catalytic subunits: pyruvate dehydrogenase (E1) (EC 1.2.4.1), dihydrolipoyl transacetylase (E2) (EC 2.3.1.12), and dihydrolipoyl dehydrogenase (E3) (EC 1.8.1.4), and the size of the complex can exceed that of a ribosome (~10 MDa) [185]. This complex catalyses the irreversible

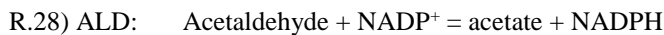
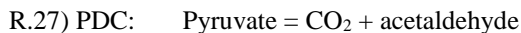
conversion of pyruvate to acetyl-CoA, NADH and CO₂ in a reaction that requires four different cofactors: thiamine pyrophosphate, lipoic acid, flavin adenine dinucleotide, CoA, plus NAD⁺ (R.25) [186]. In *S. cerevisiae* E3 is inactivated at high NADH/NAD⁺ ratios, which occurs under anaerobic fermentation or during overflow metabolism as a consequence of blockage of the TCA cycle and the need for reoxidation of the NADH generated [186, 187].

The mitochondrial citrate synthase, CIT1 (R.26; EC 2.3.3.1), uses acetyl-CoA as a substrate together with oxaloacetate in the condensation of two C₂ molecules to yield citrate. But, as for the PDH complex, elevated NADH/NAD⁺ ratios cause its inhibition, blocking the TCA cycle [188].



5.1.2 Cytosolic Acetyl-CoA

Cytosolic acetyl-CoA is generated from pyruvate through multistep conversion, known as the PDH bypass, which consists of 3 reactions catalysed by pyruvate decarboxylase, PDC (R.27; EC 4.1.1.1), acetaldehyde dehydrogenase, ALD (R.28; EC 1.2.1.4) and acetyl-CoA synthase (ACS) (R.29; EC 6.2.1.1) [184], as shown below.



Yeasts have three PDC isoenzymes, encoded by *PDC1*, *PDC5* and *PDC6* [184]. When these are inactivated, the microorganism is no longer able to produce ethanol. But under conditions of excess glucose, pyruvate cannot be channelled to the TCA cycle due to the repression of *PDH* and *CIT2* genes, so *S. cerevisiae* cannot grow [189].

In *S. cerevisiae*, acetaldehyde can be converted to ethanol or acetate. However, ethanol formation is favoured to maintain redox homeostasis anaerobically or in excess glucose (see Chapter 4). Moreover, ADH1 (alcohol dehydrogenase) has a higher affinity for acetaldehyde than ALD6 (aldehyde dehydrogenase) [145, 190]. However, *S. cerevisiae* still relies on acetic acid formation as a source of NADPH and as a precursor for cytosolic acetyl-CoA for lipid and fatty acid biosynthesis [181].

The conversion of acetate into acetyl-CoA is catalysed by ACS, which in *S. cerevisiae* is encoded by *ACS1* and *ACS2*. This reaction is highly energetically demanding since it requires the net hydrolysis of 1 ATP to 1 AMP, releasing 2 pyrophosphates (P_i), similar to the conversion of 2 ATP molecules to 2 ADP molecules and 2 P_i molecules [181]. *ACS1* is repressed by high glucose concentrations, and it is activated by the presence of C_2 sources [191, 192]. *ACS2*, on the other hand, is constitutively expressed under anaerobic and aerobic conditions, but the *ACS2* V_{max} is three times lower compared to *ACS1*; and *ACS2* K_m is thirty times lower for acetate than *ACS1* [192]. Despite gene transcription, the enzymes can become inactive by acetylation, which occurs during growth on fermentable carbon sources under aerobic conditions [193, 194]. Disruption of *ACS1* results in unviable growth on C_2 sources [195], whereas disruption of *ACS2* leads to an inability to grow in high levels of glucose since *ACS1* is repressed [192]. Consequently, the double deletion is unviable since cytosolic acetyl-CoA cannot be synthesized [192].

Cytosolic acetyl-CoA is needed for the synthesis of fatty acids and sterols. For fatty acid synthesis acetyl-CoA is converted into malonyl-CoA by acetyl-CoA carboxylase (ACC) (R.30; EC 6.4.1.2) [196], and for sterol synthesis two acetyl-CoA molecules are condensed into one acetoacetyl-CoA molecule via the acetoacetyl-CoA thiolase, (ACT) encoded by *ERG10* (R.31; EC 2.3.1.9) [197]. Both components are constituents of cellular membranes and fatty acids and are also used for energy storage and for post-translational protein modifications [198].



5.1.3 Peroxisomal acetyl-CoA

S. cerevisiae has the ability to utilize fatty acids as a carbon source when other nutrients are unavailable [199]. The degradation of fatty acids occurs in the peroxisome, through a metabolic pathway known as fatty acid β -oxidation. Acetyl-CoA and acyl-CoA result from the cleavage of 3-ketoacyl-CoA by the 3-ketoacyl-CoA thiolase, POT1 (EC 2.3.1.16) [200]. Peroxisomal acetyl-CoA can also be produced from acetate by the ACS. Acetyl-CoA is then incorporated into the glyoxylate cycle, which takes place between the peroxisome and the cytoplasm. This cycle is used to convert C_2 units into C_4 units (succinate) to function as precursors for the synthesis of amino acids or carbohydrates, as well as to replenish the TCA cycle (Figure 20). [201]. Succinate originates from isocitrate via the isocitrate lyase, ICI (EC 4.1.3.1) which re-enters the TCA cycle where it is converted into malate, which can re-enter the cytoplasm. The peroxisomal enzymes

isocitrate synthase, CIT2 (EC 2.3.3.1) and malate synthase, MLS1 (EC 2.3.3.9), catalyse the condensation reaction of acetyl-CoA with oxaloacetate and glyoxylate, respectively [188]. When *CIT2* is deleted the utilization of acetate is reduced [195], whereas if *MLS1* is deleted acetate can still be consumed since the homologue malate synthase *DAL7* can take over [202].

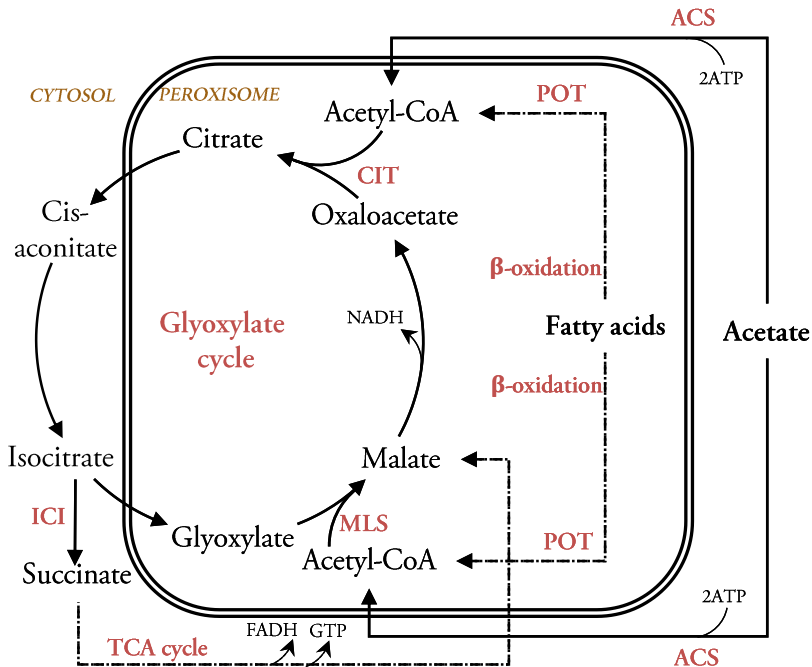


Figure 20. Overview of glyoxylate metabolism in *S. cerevisiae*. Continuous lines represent sequential biochemical reactions. Dashed lines represent pools of biochemical reactions. Genes and the names of metabolic pathways are given in red. 3-ketoacyl-CoA thiolase (POT) catalyses the last step of the β -oxidation of fatty acids providing a peroxisomal source of acetyl-CoA. This can also be produced through the conversion of acetate into acetyl-CoA through acetyl-CoA synthase (ACS). Malate synthase (MSL) and citrate synthase (CIT) are the enzymes that take up peroxisomal acetyl-CoA for its incorporation into the glyoxylate cycle. Isocitrate lyase (ICI) generates succinate which crosses the mitochondrial membrane forming part of the TCA cycle. Mitochondrial malate can cross the mitochondrial membrane and be incorporated into the glyoxylate cycle.

5.1.4 Transportation of acetyl-CoA

There are three indirect mechanisms of transportation of acetyl-CoA: the citrate–oxaloacetate shuttle, the carnitine shuttle, and acetate conversion; only the last two are present in *S. cerevisiae*.

The carnitine shuttle is a mechanism for transporting the acetyl groups of acetyl-CoA from the mitochondria to the cytosol. Carnitine acetyltransferases (CAT) (R.32; EC 2.3.1.7) shuttle activated acetyl groups across the membranes to carnitine to form acetylcarnitine [203]. Despite the presence of the carnitine shuttle, *S. cerevisiae* cannot synthesize carnitine *de novo*. Although it can be taken up if present in the medium, but supplementation would increase the production cost of the desired chemical to an unacceptable level [183].

The other indirect transport system of acetyl-CoA in *S. cerevisiae* is the acetyl-CoA hydrolase (ACH) (R.33; EC 3.1.2.1). This enzyme is used to shuttle acetate from the mitochondria to the cytosol, but it can also transfer CoA between acetate and succinate (R.34; EC 2.8.3.18). In Δpdh strains ACH can play the role of the PDH complex to provide a source of intramitochondrial acetyl-CoA. Strains lacking *ach* showed poorer growth in acetate [204], and strains containing Δpdh and Δach showed respiratory deficiencies when grown in glucose [205].

R.32) CAT: Acetyl-CoA + carnitine = CoA + O-acetylcarnitine

R.33) ACH: Acetyl-CoA + H₂O = CoA + acetate

R.34) ACH: Acetyl-CoA + succinate = succinyl-CoA + acetate

5.2 Enhancing the cytosolic acetyl-CoA supply

When *S. cerevisiae* is used as a cell factory, heterologous pathways with acetyl-CoA as a precursor are usually expressed in the cytosol to minimize problems associated with product secretion [205]. There are, however, some exceptions in which the heterologous pathways are expressed in the mitochondria, for instance, in the synthesis of isobutanol and the isoprenoid farnesyl diphosphate, since these products can be transported across the mitochondrial membrane [86, 206].

Since acetyl-CoA is compartmentalized, it is often necessary to manipulate the central carbon metabolism to artificially increase its production. Some of the most relevant strategies will be discussed in this section (Figure 21), with particular focus on the ways in which these have been applied to increase the production of PHB.

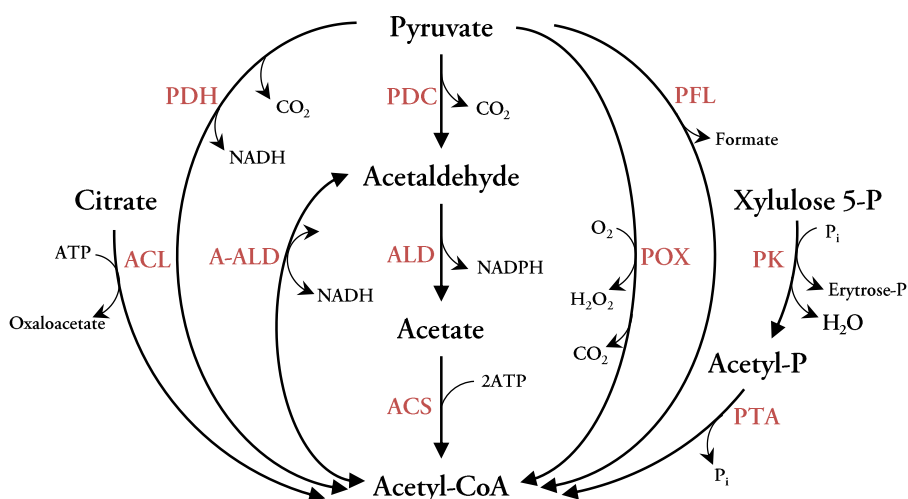


Figure 21. Overview of the strategies used for cytosolic acetyl-CoA enhancement. In red: PDH: pyruvate dehydrogenase; ACL: ATP-citrate lyase; A-ALD: acetylating acetaldehyde dehydrogenase; PDC: pyruvate decarboxylase; ALD: acetaldehyde dehydrogenase; ACS: acetyl-CoA synthase; POX: pyruvate oxydase; PFL: pyruvate formate lyase; PK: phosphoketolase; PTA: phosphotransacetylase.

5.2.1 Strategies based on existing *S. cerevisiae* routes

Downregulation of the ethanol pathway

Cytosolic acetyl-CoA is produced via acetaldehyde, which can also be diverted to ethanol (Figures 13 and 19). Theoretically, the deletion of ADH-encoding genes

would thus direct acetaldehyde towards acetate, which is the precursor of acetyl-CoA. However, *S. cerevisiae* contains many ADHs, and ethanol production is necessary for redox balancing under aerobic conditions with excess glucose (Crabtree effect) and under anaerobic conditions. As the effect of ADH inactivation during aerobic xylose utilization is unknown, the deletion of *ADH1* encoding the most active ADH was tested in PHB-producing strains (Paper III). Deletion of *ADH1* led to an increase in PHB yield, titre and accumulation when xylose was used as the carbon source in the presence of oxygen. As expected, the same modification caused redox imbalance under anaerobic conditions, and this was compensated for by the production of the reduced compounds glycerol and xylitol, rather than by PHB production. Redox imbalance also retarded cell growth, allowing the cells to increase their production of PHB.

Upregulation of the acetyl-CoA node

Another method of increasing the production of PHB is a 2-stage process in which ethanol is used to generate acetyl-CoA via an upregulated ethanol assimilation pathway. For this purpose, the alcohol dehydrogenase responsible for ethanol assimilation, ADH2 was overexpressed. As the accumulation of acetaldehyde would reduce the growth rate and the biomass due to toxicity, the acetaldehyde dehydrogenase-encoding gene *ALD6* is also overexpressed to increase acetate production from acetaldehyde [207]. Finally, the conversion of acetate to acetyl-CoA can be mediated by an engineered ACS from *Salmonella enterica* in which the target residue for acetylation is substituted, generating an active enzyme during growth [194, 208]. The combination of ADH2, ALD6 and ACS^{L641P} improved the production of PHB (Figure 22) [131], 3-hydroxypropionic acid [209], fatty acyl ethyl esters [210] and 1-butanol [211].

However, none of these strategies could overcome the problem of ethanol of being the major product. Regardless of the strategy, the stoichiometric ATP requirement for cytosolic acetyl-CoA formation limits the production of acetyl-CoA-derived products [205], especially under anaerobic conditions, where ATP relies only on substrate level phosphorylation [150].

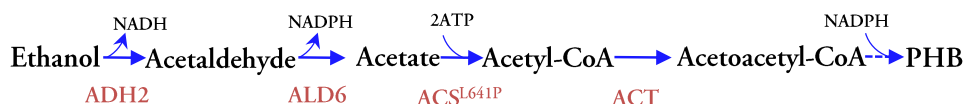


Figure 22. Schematic pathway illustrating metabolic engineering strategies to increase PHB production. Blue arrows indicate over expression of enzymes. Dashed lines represent various pathway steps. ADH2: alcohol dehydrogenase; ALD6: acetaldehyde dehydrogenase; ACS^{L641P} acetyl-CoA synthase. ACT: acetyl-CoA acyl transferase. (Adapted from Kocharin, et al., 2012 [131])

Downregulation of the glyoxylate cycle

An alternative strategy is to reduce the drain of cytosolic acetyl-CoA. The peroxisomal citrate synthase *CIT2* and malate synthase *MLS1* have been deleted with the aim of increasing PHB production. However, since glucose was used as the substrate, PHB was produced by ethanol consumption, and deletion of the genes that were integrated into the glyoxylate cycle resulted in strains with reduced biomass yields and lower accumulation of PHB due to poor assimilation of C₂ carbon sources (Figure 20) [131].

Cytosolic PDH complex

Relocation of the yeast PDH complex in the cytosol could lead to the conversion of pyruvate into cytosolic acetyl-CoA, carbon dioxide and NADH in a single step, without the need of ATP. However, previous attempts have failed due to the complexity of the PDH subunits [178]. In addition, the functionality of the PDH complex (R.25) is limited to aerobic cultures under glucose-limited conditions since high levels of NADH/NAD⁺ are known to inactivate this complex [185].

Kozak et al. were successful in expressing the heterologous PDH complex from *Enterococcus faecalis* in *S. cerevisiae* strains lacking ACS, and demonstrated functionality *in vivo* under both aerobic and anaerobic conditions [212]. In contrast to other PDH complexes, this one was not inhibited by the high NADH/NAD⁺ ratio. Furthermore, since it originated from a prokaryote, it did not contain a subcellular localization signal [185]. However, this specific PDH complex requires lipoic acid, which is not present in the cytosol of *S. cerevisiae*. Therefore, the strategy is dependent on the addition of lipoic acid to the medium [212].

5.2.2 Strategies based on heterologous routes

Alternative pathways are used for the regulation of acetyl-CoA in other prokaryotes and eukaryotes, which may be implementable in *S. cerevisiae*. The most relevant ones are discussed below.

Acetylating acetaldehyde dehydrogenase

The acetylating acetaldehyde dehydrogenase (A-ALD) (R.35; EC 1.2.1.1.10) catalyses the reversible reaction:



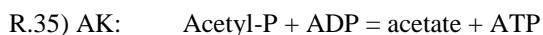
In contrast to the native PDH bypass, A-ALD does not require ATP and yields NADH. This enzyme has been successfully expressed in *S. cerevisiae* [167], and it has been demonstrated that the reaction can provide cytosolic acetyl-CoA in mutant

strains lacking both ACS and ALD, despite ATP is not required, low biomass yields were observed due to acetaldehyde accumulation [207].

Implementation of A-ALD for PHB synthesis was attempted in the present work as a strategy to supplement cytosolic acetyl-CoA in parallel with the PDH bypass. However, due to the reversibility of this enzyme, the expression of A-ALD resulted in the formation of acetaldehyde from acetyl-CoA thus, preventing PHB accumulation (Paper III). Under anaerobic conditions, the utilization of A-ALD served as an alternative redox sink for NADH oxidation. An attempt was made to change the directionality of A-ALD towards acetyl-CoA by elevating intracellular acetaldehyde levels through the deletion of *ADHI*, but the cells upregulated other ADHs as a response (Paper III).

Phosphoketolase pathway

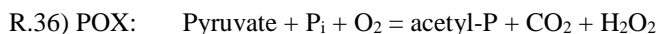
Heterofermentative lactic acid bacteria and some fungi possess an alternative route for acetyl-CoA that utilizes sugar-phosphate intermediates from the PPP and P_i to provide acetyl-phosphate (acetyl-P), which can be further converted into acetate or cytosolic acetyl-CoA in a two-step reaction mediated by PK (R.22; EC 4.1.2.9) and phosphate acetyltransferase PTA (R.23; EC 2.3.1.8) or an acetate kinase, AK (R.35; EC 2.7.2.1) [213, 214]:



Genes from the corresponding enzymes in *Aspergillus nidulans* and *B. subtilis* have been successfully expressed in *S. cerevisiae*, and used in an attempt to increase cytosolic acetyl-CoA in strains that produce PHB and fatty-acid ethyl esters. However, no significant improvement in PHB or fatty-acid ethyl ester production was observed [176, 210]. It was later found that *S. cerevisiae* had the ability to break down acetyl-P into acetate through the phosphatases encoded by *GPP1* and *GPP2* [215]. When these are removed, a more efficient pathway flux is foreseen, as has already been demonstrated in the production of farnesene [216].

Pyruvate oxidase

The AK/PTA precursor acetyl-P can also be generated from pyruvate and oxygen via pyruvate oxidase (POX) (R.36; EC 1.2.3.3). This reaction occurs in many prokaryotes where oxygen donates electrons to form hydrogen peroxide [217].

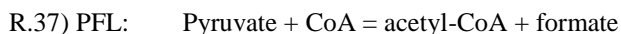


Although the hydrogen peroxide generated is toxic, it can be removed by the expression of a catalase to form water and oxygen. These reactions, in combination with PTA, can provide cytosolic acetyl-CoA from glucose independently of ATP consumption. The incorporation of POX from *Aerococcus viridans* in *S. cerevisiae*

has been reported in combination with PTA and the inactivation of PDC for the synthesis of cytosolic acetyl-CoA, resulting in a strain that did not produce ethanol and had a higher growth rate [178].

Pyruvate formate lyase

In many anaerobic bacteria and some eukaryotes, pyruvate can be converted into acetyl-CoA and formate by pyruvate formate lyase (PFL) (R.37; EC 2.3.1.54) [218]:



Since formate is a weak acid and its production can deplete the ATP pool to maintain a stable internal pH through the proton pumps, it is possible to express a formate dehydrogenase (FDH) (R.38; EC 1.2.1.2) gene, whose product can oxidize formate to CO₂ [218]:



It has been reported that cytosolic acetyl-CoA levels were restored when the PFL gene was expressed together with a PFL-activating enzyme and FDH in a *S. cerevisiae* strain lacking the PDH bypass [207]. One main advantage of this strategy is that it results in an ATP-independent reaction for the synthesis of cytosolic acetyl-CoA. However, PFL is sensitive to oxygen, and formate accumulation causes a reduction in biomass yields [207].

Protein engineering has been already reported of a mutant FDH with cofactor preference shifted from NAD⁺ to NADP⁺ so that the enzyme can contribute to the production of chemicals that require NADPH for their synthesis, e.g. mevalonate [219].

ATP-citrate lyase

Oleaginous yeasts (but not *S. cerevisiae*) contain genes encoding ATP-citrate lyase (ACL) (R.39; EC 2.3.3.8), which catalyses the following reaction:



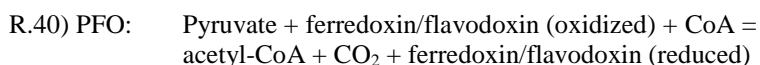
ACL is used to shuttle acetyl-CoA equivalents from the mitochondria to the cytosol, where it is required by oleaginous yeasts for the accumulation of triacylglycerides under nitrogen-limited conditions [220].

ACL has been expressed in *S. cerevisiae* for the synthesis of mevalonate. Cells lacking mitochondrial isocitrate dehydrogenase (IDH1) were able to accumulate high levels of citrate that could be exported outside the mitochondria. Under these conditions the expression of ACL induces the formation of cytosolic acetyl-CoA, thus enhancing the precursor for the formation of mevalonate [221].

An *in vitro* metabolic model of *S. cerevisiae* has been developed for PHB production [222]. This model was used to evaluate the impact of implementing ATP-citrate lyase, and predicted a 26% increase in the yield. However, no experimental data confirming this have been reported. Moreover, this strategy is limited anaerobically since it requires ATP consumption.

Pyruvate-ferredoxin and pyruvate-NADP⁺ oxidoreductase

Pyruvate-ferredoxin oxidoreductase (PFO) (R.40; EC 1.2.7.1) has been shown to play the same role as PDH in the formation of acetyl-CoA. This enzyme is typical of prokaryotes, but some eukaryotes also express it. This reaction releases two electrons that reduce ferredoxin/ flavodoxin only under anaerobic conditions, since it contains several iron–sulfur clusters that are sensitive to oxygen [223].



As an alternative to PFO, some protists express pyruvate-NADP⁺ oxidoreductase (PNO) (R.41; EC 1.2.1.51), which is also sensitive to oxygen, and only functions under anaerobic conditions [224].



To the best of the author's knowledge, none of these enzymes has been successfully expressed in *S. cerevisiae*.

Summary of heterologous pathways for acetyl-CoA enhancement

A summary of the strategies described above intended to enhance cytosolic acetyl-CoA, together with their advantages and their disadvantages, is presented in the table below.

Table 7. Summary of heterologous pathways for cytosolic acetyl-CoA formation and their stoichiometry. The stoichiometry is calculated from one mole of glucose or xylose for cytosolic acetyl-CoA synthesis from the described pathways (adapted from van Rossum et al., 2016 [205]).

Strategy	Abbrev.	Advantage	Disadvantage	Stoichiometry
Acetylating acetaldehyde dehydrogenase	A-ALD	ATP-independent	Reversible	Eq. 1 & Eq. 2
Phosphoketolase pathway	PK + AK/PTA	Higher carbon yield	Low flux	Eq. 3
Pyruvate oxidase	POX	ATP-independent	Oxygen dependent	Eq. 4 & Eq. 5
Pyruvate formate lyase	PFL	ATP - independent	Oxygen-sensitive	Eq. 1 & Eq. 2
Pyruvate ferredoxin/flavodox in oxidoreductase	PFOR and PNO	ATP - independent	Oxygen-sensitive	Eq.1
ATP-citrate lyase	ACL	Redox neutral	ATP-dependent	Eq. 6 & Eq. 7
Cytosolic pyruvate dehydrogenase complex	PDH _{cyt}	ATP-independent	Lipoic acid must be supplemented	Eq. 1 & Eq. 2
Equations	Stoichiometry			
Eq. 1	$1\text{Glucose} + 4\text{NAD}^+ + 2(\text{ADP} + 2\text{P}_i) + 2\text{CoA} = 2\text{acetyl-CoA} + 4(\text{NADH} + \text{H}^+) + 2\text{CO}_2 + 2\text{ATP} + 2\text{H}_2\text{O}$			
Eq. 2	$1\text{Xylose} + 4.6\text{NAD}^+ + 1(\text{NADPH} + \text{H}^+) + 1.4(\text{ADP} + \text{P}_i) + 1.2\text{CoA} = 1.2\text{acetyl-CoA} + 4.6(\text{NADH} + \text{H}^+) + 1\text{NADP}^+ + 1.2\text{CO}_2 + 1.4\text{ATP}$			
Eq. 3	$1\text{Glucose} + 1\text{ATP} + 3\text{CoA} = 3\text{acetyl-CoA} + 1(\text{ADP} + \text{P}_i) + 2\text{H}_2\text{O}$			
Eq. 4	$1\text{Glucose} + 2\text{NAD}^+ + 2(\text{ADP} + \text{P}_i) + 2\text{CoA} + \text{O}_2 = 2\text{acetyl-CoA} + 2(\text{NADH} + \text{H}^+) + 2\text{CO}_2 + 2\text{ATP} + 2\text{H}_2\text{O}$			
Eq. 5	$1\text{Xylose} + 1(\text{NADPH} + \text{H}^+) + 1.4\text{NAD}^+ + 1.4(\text{ADP} + \text{P}_i) + 1.2\text{CoA} + 0.6\text{O}_2 = 1.2\text{acetyl-CoA} + 1\text{NADP}^+ + 1.4(\text{NADH} + \text{H}^+) + 1.4\text{ATP} + 1.2\text{H}_2\text{O}$			
Eq. 6	$1\text{Glucose} + 4\text{NAD}^+ + 2\text{CoA} = 2\text{acetyl-CoA} + 4(\text{NADH} + \text{H}^+) + 2\text{CO}_2 + 2\text{H}_2\text{O}$			
Eq. 7	$1\text{Xylose} + 4.6\text{NAD}^+ + 1(\text{NADPH} + \text{H}^+) + 1.2\text{CoA} = 1.2\text{acetyl-CoA} + 4.6(\text{NADH} + \text{H}^+) + 1\text{NADP}^+ + 1.2\text{CO}_2$			

6. Conclusions and outlook

During the past years, significant improvements have been achieved in the development of *S. cerevisiae* strains for the production of chemicals. Progress has been accelerated by the recent establishment of new genetic tools, such as CRISPR/Cas9, which allow faster strain engineering and more efficient investigation of new pathways.

The work presented in this thesis has contributed to a better understanding of the metabolic challenges associated with the synthesis of PHB by *S. cerevisiae*, which it is hoped will facilitate industrial production. Several conclusions could be drawn from the results obtained, and these are presented below, together with some suggestions for future studies.

The first study demonstrated that xylose could be used as a substrate for PHB synthesis using *S. cerevisiae* (Paper I), which paves the way for PHB production from lignocellulosic feedstock. However, metabolic engineering must be applied to improve the production efficiency. At the xylose level, it is known that the oxidoreductive xylose pathway affords high productivity, but low yield, compared to the xylose isomerization pathway [120]. Implementation of the xylose reductase *XYL1.2* isolated from *Sp. passalidarum* (Paper IV) in PHB-producing strains could improve the anaerobic co-production of ethanol and PHB. The high affinity of this enzyme for NADH would contribute to maintaining better redox homeostasis in the cell under anaerobic conditions, hence, decreasing by-product accumulation (xylitol and glycerol). The most recently reported xylose isomerases could also be considered for implementation in *S. cerevisiae*. Since no cofactor is involved, the utilization of XI could decrease by-product formation and increase ethanol and PHB production. However, if strain adaptation is needed, PHB production could be affected as it is still not known how evolutionary engineering would affect PHB-producing *S. cerevisiae*.

Modification of the biosynthetic PHB pathway itself can lead to improvements in PHB yields, titres and accumulation (Paper II). This was demonstrated by the replacement of *C. necator* NADPH-dependent acetoacetyl-CoA reductase by the NADH-dependent counterpart from *A. vinosum*. This enzyme exhibits improved enzyme kinetics, allowing for faster conversion of acetoacetyl-CoA in PHB synthesis. More importantly, the cofactor shift towards NADH, eliminated the dependency of NADPH in the pathway, which is a limiting factor during growth.

Based on this observation, more efficient alternatives for the other enzymes in the PHB pathway should be explored. Since it has been shown that an increase in the gene copy number of PHS improved the formation of PHB in *C. necator*, [50] multiple chromosomal integration of PHS in the genome of *S. cerevisiae* or any other recombinant host may improve PHB synthesis. However, the search for ACT in different organisms appears to be a key strategy for the improvement of PHB production by *S. cerevisiae*, since ACT competes with more efficient routes for cytosolic acetyl-CoA utilization, as demonstrated in the work presented in Paper III. Most PHB-producing organisms synthesize PHB as a result of high concentrations of acetyl-CoA arising from nutrient limitation[225], which implies that the affinity for ACT does not have to be high. *A. latus* is a bacterium that possesses high ACT activity, which allows PHB accumulation without any nutrient limitation [226]. The utilization of this enzyme, or engineering of ACT for high affinity for acetyl-CoA, could thus improve PHB production in engineered *S. cerevisiae*.

In addition to the optimization of the PHB pathway, it appears to be necessary to apply a combination of metabolic engineering strategies to increase the production of cytosolic acetyl-CoA, from which not only PHB, but many other chemicals, are derived. It was shown in this work that increasing the carbon flux towards acetyl-CoA contributed to an increase in PHB yields under aerobic conditions (Paper III) [131]. However, increasing the flux towards acetyl-CoA also increased the formation of acetate, which causes inhibition of xylose fermentation due to a decrease in pH (Papers I, II, III). It would therefore be preferable to perform strain characterization in pH-controlled bioreactors to obtain more accurate information on metabolite regulation. It was also shown in the present work that downregulating ethanol formation contributed positively to the formation of PHB under aerobic conditions (Paper III), since acetaldehyde was shuttled towards acetate, and the excess NADH generated was taken care of by respiration. However, under anaerobic conditions, the imbalance of redox cofactors had a detrimental effect, as the NADH-dependent PHB production was not sufficient to balance the NADH consumption through the ethanol production.

Based on the discussion of the different approaches for the enhancement of cytosolic acetyl-CoA production given in the previous chapter, it is suggested that the utilization of *S. cerevisiae* Δpdc strains expressing the cytosolic PDH complex from *E. faecalis* could offer an interesting platform for PHB production. ATP-independent acetyl-CoA synthesis would be advantageous to increase PHB yields and titres, especially under anaerobic conditions. However, limitations in redox metabolism might be encountered in NADH oxidation. Moreover, the xylose pathway could be implemented in this type of strain to investigate the metabolism of xylose since the mechanism for glucose repression might not be active under these conditions.

Techniques such as fluorescence-activated cell sorting (FACS) and whole genome sequencing can also be used to improve PHB production by *S. cerevisiae* strains. Strains with high contents of PHB can be easily identified by staining with fluorescent dyes such as BODIPY and Nile red and separated by FACS. Whole genome sequencing of these strains can then provide information on the mechanism behind the high accumulation of PHB, and this information can be used to improve native strains.

To conclude, large-scale biopolymer synthesis in the robust cell factory offered by *S. cerevisiae* cannot yet compete with bacterial production. However, the information obtained from the present studies contributes to our understanding of the regulatory mechanisms of cells in response to the addition of a NAD(P)H-dependent heterologous pathway requiring a supply of cytosolic acetyl-CoA. Moreover these findings provide useful information on ways to enhance the production of acetyl-CoA-derived bulk chemicals from xylose using *S. cerevisiae* and other microbial cell factories.

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