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Exploring Yeast as a Cell Factory for the Production of Carboxylic Acids and Derivatives

APPLIED MICROBIOLOGY | FACULTY OF ENGINEERING | LUND UNIVERSITY
DIOGO PORTUGAL-NUNES



Exploring Yeast as a Cell Factory for the Production of Carboxylic Acids and Derivatives

Diogo Portugal-Nunes



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DOCTORAL DISSERTATION

which, by due permission of the Faculty of Engineering, Lund University, Sweden,
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Kemicentrum, Naturvetarvägen 12-18, Lund, for the degree of Doctor of Philosophy.

Faculty opponent

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Title: Exploring Yeast as a Cell Factory for the Production of Carboxylic Acids and Derivatives		
<p>Abstract</p> <p>Baker's yeast, <i>Saccharomyces cerevisiae</i>, is a promising cell factory for the sustainable utilization of renewable resources for the formation of products with commercial value. Among these, poly-3-D-hydroxybutyrate (PHB) is an extensively studied biopolymer naturally accumulated in some bacteria and archaea species through the formation of carbon granules. Its bio-based origin, biodegradability and applications in several industries makes it one of the most interesting biopolymers. In the present study, aerobic production of PHB from xylose was achieved in <i>S. cerevisiae</i> through the engineering of an optimized xylose oxido-reducing pathway and the expression of the genes involved in the PHB-producing pathway from the bacterium <i>Cupriavidus necator</i>. As anaerobicity is generally preferred in industrial applications, leading to an excess of NADH in the yeast metabolism, <i>S. cerevisiae</i> was further engineered by the introduction of a NADH-dependent acetoacetyl-CoA reductase from the bacterium <i>Allochromatium vinosum</i>. PHB formation clearly benefited from this modification and its formation from pure carbon sources under both anaerobic and oxygen-limited conditions was observed. The influence of nitrogen availability on PHB accumulation was also investigated. In contrast to the natural producers, PHB formation in <i>S. cerevisiae</i> was favored by high levels of nitrogen. These engineering strategies together resulted in one of the highest PHB contents reported in <i>S. cerevisiae</i> to date.</p> <p>The production of carboxylic acids, i.e. organic compounds that can be used as building blocks for a wide range of products, was also investigated in yeast due to its robustness and ability to grow at low pH. Cytosolic production of alpha-ketoglutarate (AKG) from xylose was attempted by rewiring the carbon flux towards the glyoxylate cycle in <i>S. cerevisiae</i>. Although AKG production was low, the study contributed to a deeper understanding of the mitochondrial and cytosolic formation of carboxylic acids in <i>S. cerevisiae</i>, revealing novel routes for their bio-production and for further optimization studies.</p> <p>In the last part of this work, AKG production was attempted by using a heterologous oxidative pathway that bypasses glycolysis and links xylose directly to the tricarboxylic acid cycle – the so-called Weimberg pathway. The Weimberg pathway was found to be partially active and highlighted the fact that the assembly and activity of the proteins converting xylonate into AKG require further development.</p>		
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Diogo Portugal-Nunes



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Para os meus pais

Abstract

Baker's yeast, *Saccharomyces cerevisiae*, is a promising cell factory for the sustainable utilization of renewable resources for the formation of products with commercial value. Among these, poly-3-D-hydroxybutyrate (PHB) is an extensively studied biopolymer naturally accumulated in some bacteria and archaea species through the formation of carbon granules. Its bio-based origin, biodegradability and applications in several industries makes it one of the most interesting biopolymers. In the present study, aerobic production of PHB from xylose was achieved in *S. cerevisiae* through the engineering of an optimized xylose oxido-reducing pathway and the expression of the genes involved in the PHB-producing pathway from the bacterium *Cupriavidus necator*. As anaerobicity is generally preferred in industrial applications, leading to an excess of NADH in the yeast metabolism, *S. cerevisiae* was further engineered by the introduction of an NADH-dependent acetoacetyl-CoA reductase from the bacterium *Allochromatium vinosum*. PHB formation clearly benefited from this modification and its formation from pure carbon sources under both anaerobic and oxygen-limited conditions was observed. The influence of nitrogen availability on PHB accumulation was also investigated. In contrast to the natural producers, PHB formation in *S. cerevisiae* was favored by high levels of nitrogen. These engineering strategies together resulted in one of the highest PHB contents reported in *S. cerevisiae* to date.

The production of carboxylic acids, i.e. organic compounds that can be used as building blocks for a wide range of products, was also investigated in yeast due to its robustness and ability to grow at low pH. Cytosolic production of alpha-ketoglutarate (AKG) from xylose was attempted by rewiring the carbon flux towards the glyoxylate cycle in *S. cerevisiae*. Although AKG production was low, the study contributed to a deeper understanding of the mitochondrial and cytosolic formation of carboxylic acids in *S. cerevisiae*, revealing novel routes for their bio-production and for further optimization studies.

In the last part of this work, AKG production was attempted by using a heterologous oxidative pathway that bypasses glycolysis and links xylose directly to the tricarboxylic acid cycle – the so-called Weimberg pathway. The Weimberg pathway was found to be partially active and highlighted the fact that the assembly and activity of the proteins converting xylonate into AKG require further development.

Popular scientific summary

Microorganisms, or microbes, are microscopic living organisms with great diversity. It has recently been estimated that 300 trillion different microbial species are present on Earth. Although microorganisms are commonly associated with health-related issues, many bacterial and fungal species are essential in our daily lives. For instance, the production of bread, beer, wine, cheese, yoghurt and insulin, among many other products, depends on fermentative processes carried out by microorganisms. In this regard, the fungus *Saccharomyces cerevisiae*, also called baker's or brewer's yeast, is of particular interest since it has been widely used in the food and beverage industries.

The interesting features of microorganisms, together with the need for more sustainable processes to replace those based on fossil fuels and refineries, triggered the development of the biorefinery concept. Biorefineries aim at the efficient utilization of natural substrates (biomass), for the formation of various products with commercial value. Microorganisms such as yeast or bacteria are the heart of biorefineries, since they can convert sugars present in raw materials into different forms of energy (power and heat), transportation fuels and/or chemical compounds. The global biorefinery market is currently valued at more than 400 billion euros, and is growing at approximately 14% per year.

Initially, only the natural abilities of microbes were utilized. This is illustrated by the production of the fuel bioethanol by brewer's yeast, based on its natural ability to produce ethanol during beer or wine production. However, the development of genetic engineering, has significantly expanded the range of compounds that can be obtained by fermentation.

In the present work, the yeast *S. cerevisiae* was genetically modified to evaluate its potential as a producer of biopolymers and carboxylic acids, compounds that have a higher market value than, for example, ethanol. Importantly, the studies focused on the utilization of xylose, which is the second most abundant sugar in nature, after glucose. Since *S. cerevisiae* cannot utilize this sugar naturally, genetic modifications described in the literature were used to increase the assimilation of xylose.

Poly-3-D-hydroxybutyrate (PHB) is a biodegradable polymer that can be used to produce plastics with unique properties. There are bacteria in nature capable of producing PHB under very specific conditions. Therefore, the genes responsible for its formation were introduced into yeast to elucidate whether this microorganism could also form PHB. In this work, PHB was obtained from xylose using yeast for the first time. A number of genetic modifications were then implemented resulting in increased PHB formation, especially in the absence of oxygen, a condition that is desirable in industry since it reduces the need for aeration, and therefore the overall process cost.

The production of the carboxylic acid alpha-ketoglutarate (AKG) by yeast was also investigated. This compound has interesting properties, and is used, for example, as a supplement for wound healing and in fine chemistry. AKG has an important biological role, taking part in several processes essential for cell viability, which prevents its accumulation in microbes. Although only low levels of AKG could be obtained from xylose using yeast, this study revealed a possible route for further optimization strategies. A novel route for xylose utilization, called the Weimberg pathway, was also introduced and evaluated in brewer's yeast, as an alternative to the commonly engineered pathways, for more direct conversion of xylose into AKG.

In conclusion, *Saccharomyces cerevisiae* is being transformed from a brewer's or bakery's yeast into a microbial cell factory, for the production of fuels, polymers, pharmaceutical compounds, and vaccines, among many others. The knowledge acquired on yeast engineering and physiology through the present work is expected to contribute to increasing the economic competitiveness of yeast-based bioprocesses, in comparison with fossil-based chemical synthesis, facilitating the transition to a biorefinery-based market.

Resumo de divulgação científica

Microorganismos, ou micróbios, são organismos vivos de tamanho muito reduzido e que apresentam uma grande diversidade. Recentemente, estimou-se a existência de 300 triliões de espécies diferentes de microorganismos na Terra. Embora este tipo de organismos esteja normalmente associado a problemas de saúde, muitas bactérias e fungos são essenciais para o nosso dia-a-dia. Por exemplo, produtos como o pão, cerveja, vinho, queijo, iogurte ou até insulina, dependem de processos fermentativos que ocorrem devido à actividade de microorganismos. A levedura *Saccharomyces cerevisiae*, também denominada levedura da cerveja ou fermento de padeiro, é de grande importância nesta área tendo influenciado significativamente a produção de diversos bens alimentares.

As características únicas dos microorganismos e a necessidade de processos mais sustentáveis do que os actuais baseados em combustíveis fósseis e refinarias químicas, impulsionaram o desenvolvimento do conceito de biorrefinaria. O principal objectivo da biorrefinaria é a utilização de substratos naturais (biomassa) para a produção de compostos com valor comercial. Microorganismos, como as leveduras ou bactérias, são o cerne das biorrefinarias, pois convertem os açúcares presentes em diversas matérias-primas em diferentes formas de energia (eléctrica e térmica), combustíveis e/ou compostos químicos. O mercado global associado às biorrefinarias encontra-se em franca expansão, estando neste momento avaliado em mais de 400 biliões de euros e a crescer, aproximadamente, 14% ao ano.

Inicialmente, apenas as características intrínsecas dos microorganismos foram exploradas. A produção do combustível bioetanol pela levedura da cerveja é um exemplo paradigmático, baseando-se na capacidade deste microorganismo para produzir álcool durante a produção de cerveja ou vinho. O desenvolvimento da área da engenharia genética resultou no alargamento significativo do tipo de compostos que podem ser produzidos através de processos fermentativos.

Neste projecto, a levedura *S. cerevisiae* foi modificada geneticamente com o objectivo de determinar o seu potencial para a produção de biopolímeros e ácidos carboxílicos - compostos com valor comercial superior ao do etanol. Os estudos efectuados focaram-se na utilização de xilose, o segundo açúcar mais abundante na natureza a seguir à glucose. Visto que *S. cerevisiae* não possui a capacidade de assimilar este açúcar, foram necessárias várias modificações genéticas descritas na literatura para obter uma utilização eficaz da xilose.

O polímero biodegradável poli-3-D-hidroxitirato (PHB) pode ser utilizado para a produção de plásticos com propriedades únicas. Existem bactérias na natureza com a capacidade intrínseca de produzir PHB sob condições específicas. Assim, os genes

associados à síntese de PHB foram inseridos na levedura para avaliar se a produção deste polímero também seria possível neste microorganismo. Neste projecto, a produção de PHB a partir de xilose foi descrita pela primeira vez. As modificações genéticas introduzidas de seguida resultaram numa melhoria da produção de PHB, destacando-se os resultados obtidos na ausência de oxigénio, condição normalmente requisitada pela indústria devido à redução de custos associados ao arejamento.

A produção do ácido carboxílico alfa-cetoglutarato (AKG) através da actividade da levedura da cerveja foi também investigada. Este composto tem características interessantes, podendo ser usado como suplemento para a cicatrização de feridas ou como precursor para a química fina, normalmente associada a compostos de valor comercial mais elevado. AKG desempenha um papel essencial a nível biológico, participando em diversos processos relacionados com a viabilidade celular, o que, naturalmente, dificulta a sua acumulação em microorganismos. Embora apenas níveis reduzidos de AKG tenham sido obtidos, este estudo abre diversas possibilidades para a optimização da produção de ácidos carboxílicos em projectos futuros. Paralelamente, uma via metabólica para a utilização de xilose com características únicas, denominada Weimberg, foi introduzida e avaliada na levedura da cerveja como alternativa às vias metabólicas normalmente utilizadas. Esta via permite que a conversão de xilose para AKG ocorra de forma mais directa.

Em suma, a levedura *Saccharomyces cerevisiae* deixou de ser apenas a levedura da cerveja ou do fermento de padeiro para se transformar numa fábrica celular, produzindo combustíveis, polímeros, medicamentos, entre muitos outros compostos. Este estudo permitiu aprofundar o conhecimento nas áreas da engenharia genética e fisiologia dos microorganismos, contribuindo para o crescendo de competitividade dos bioprocessos em relação aos processos baseados na síntese química, o que facilitará a transição para um mercado cada vez mais ligado ao conceito de biorrefinaria.

List of publications

This thesis is based on the following scientific papers, which will be referred to in the text by their Roman numerals.

Paper I **Engineering of *Saccharomyces cerevisiae* for the production of poly-3-D-hydroxybutyrate from xylose**

A.G. Sandström, A. Muñoz de las Heras, D. Portugal-Nunes and M.F. Gorwa-Grauslund. (2015) *AMB Express* 5(1):14.

Paper II **Anaerobic poly-3-D-hydroxybutyrate production from xylose in recombinant *Saccharomyces cerevisiae* using a NADH-dependent acetoacetyl-CoA reductase**

A. Muñoz de las Heras, D. Portugal-Nunes, N. Rizza, A.G. Sandström and M.F. Gorwa-Grauslund. (2016) *Microbial Cell Factories* 15(1):197.

Paper III **Effect of nitrogen availability on the poly-3-D-hydroxybutyrate accumulation by engineered *Saccharomyces cerevisiae***

D. Portugal-Nunes, S.S. Pawar, G. Lidén and M.F. Gorwa-Grauslund (2017) *AMB Express* 7(1):35.

Paper IV **Redirecting the carbon flux towards the glyoxylate cycle in *Saccharomyces cerevisiae*: alpha-ketoglutarate as a case-study**

D. Portugal-Nunes, L. Wasserstrom, A. Guibert, B. Bergdahl, G. Lidén and M.F. Gorwa-Grauslund (2017) Submitted.

Paper V **Establishing the Weimberg pathway in *Saccharomyces cerevisiae***

L. Wasserstrom, D. Portugal-Nunes, H. Almqvist, S.S. Pawar, A.G. Sandström, G. Lidén and M.F. Gorwa-Grauslund (2017) Manuscript.

During my studies, I have also contributed to the following scientific articles which are not included in this thesis:

Effect of cell immobilization and pH on *Scheffersomyces stipitis* growth and fermentation capacity in rich and inhibitory media.

D. Portugal-Nunes, V. Sánchez i Nogué, S.R. Pereira, S.C. Craveiro, A.J. Calado and A.M.R.B. Xavier. (2015) *Bioresources and Bioprocessing* 2(1):13.

***Saccharomyces cerevisiae*: a potential host for carboxylic acid production from lignocellulosic feedstock?**

A.G. Sandström*, H. Almqvist*, D. Portugal-Nunes*, D. Neves*, G. Lidén* and M.F. Gorwa-Grauslund*. (2014) *Applied Microbiology and Biotechnology* 98(17): 7299-7318.

* Equal contributions

My contributions to the studies

- Paper I I performed the physiological characterization of the recombinant strains and contributed to the optimization of the HPLC method.
- Paper II I participated in the design of the study, performed the physiological characterization in the bioreactor and helped draft the manuscript.
- Paper III I designed the study, performed the experimental work together with Sudhanshu S. Pawar, and wrote the manuscript.
- Paper IV I participated in the design of the study, performed the physiological analysis of the recombinant strains and wrote the manuscript.
- Paper V I participated in the design of the study, performed the physiological characterization of the recombinant strains in shake-flasks, and helped draft the manuscript.

List of abbreviations

AAR	acetoacetyl-CoA reductase
ACO1	aconitase
AKG	alpha-ketoglutarate
ATP	adenosine triphosphate
FADH ₂	flavin adenine dinucleotide
G6PD	glucose-6-phosphate dehydrogenase
GHG	greenhouse gas(es)
IDH	isocitrate dehydrogenase
IDP2	cytosolic NADP-specific isocitrate dehydrogenase
KDY	2-keto-3-desoxyxylonate dehydratase
KGD2	dihydrolipoyl transsuccinylase
KGDH	alpha-ketoglutarate dehydrogenase complex
KSH	α-ketoglutarate semialdehyde dehydrogenase
NADH/NAD ⁺	nicotinamide adenine dinucleotide
NADPH/NADP ⁺	nicotinamide adenine dinucleotide phosphate
PDC	pyruvate decarboxylase
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
PGI	phosphoglucose isomerase
PHA	polyhydroxyalkanoate
PHB	poly-3-D-hydroxybutyrate
PLA	polylactic acid
PPP	pentose phosphate pathway
TCA	tricarboxylic acid
XDH	xylitol dehydrogenase
XDY	xylonate dehydratase

XI	xylose isomerase
XK	xylulokinase
XLS	xylonolactonase
XR	xylose reductase
XylB	xylose dehydrogenase
3-HP	3-hydroxypropionic acid

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1. Introduction

1.1. Bioeconomy and biorefineries

Today's economy is largely based on fossil fuels which will become increasingly scarce as these resources are being consumed at a faster rate than their natural formation. In addition, the price of oil is volatile, and the general public is becoming aware of the environmental issues associated with its utilization. Therefore, renewable resources must be explored in order to reduce our dependency on fossil fuels, while increasing the sustainable utilization of the natural sources available (Van Dien 2013). The sustainability and appropriate management of natural resources have become major concerns, leading to the introduction of political directives promoting the bioeconomy over recent years. For instance, the European Union has declared that at least 20% of total energy needs in the EU countries must be obtained from renewable sources by 2020. This requirement will be further increased to 27% by 2030 (EU 2009; EU 2015). Biomass is a completely renewable resource with a complex composition whose breakdown can result in a wide variety of products. Biomass has been defined by the European Union as *the biodegradable fraction of products, waste and residues from biological origin, including agricultural, forestry and municipal wastes* (EU 2009). Therefore, biomass is recognized as having the potential to replace many of the oil-based processes in commercial use today (Cherubini 2010).

In analogy with traditional refineries, the biorefinery concept was developed based on the sustainable and efficient utilization of biomass for the formation of various commercial products, such as fuels and chemicals (Jong et al. 2009; Kamm et al. 2006; Mussatto and Dragone 2016). It is important to mention that a facility, a process, a plant, or even a cluster of facilities using biomass, are all included in the biorefinery concept. In 2006, Kamm and coworkers identified four types of biorefinery system based on the type of biomass used: (1) lignocellulosic feedstock (e.g. agricultural and forestry waste); (2) whole crop (e.g. cereals and maize); (3) green biomass (e.g. grass and alfalfa), and (4) the two-platform concept (sugar and syngas) (Kamm et al. 2006). Several companies around the world are already applying the biorefinery concept on large and commercial scale, most of them utilizing lignocellulosic biomass as the substrate for ethanol production (Table 1).

Table 1 – Examples of biorefinery-based companies/plants. The main compounds produced by these biorefineries are given, together with the major carbon sources utilized. The type of microorganism is also specified.

Company	Main product(s)	Substrate(s)	Producing organism	Webpage	Country
Abengoa Bioenergy	- Ethanol	Lignocellulosic biomass	Yeast ¹	www.abengoa.bioenergy.com	Spain, USA
Alpena Biorefinery	- Ethanol - Wood molasses	Lignocellulosic biomass	Yeast ¹	alpenabiorefinery.com	USA
Amyris	- Farnesene	Sugar and starch crops	ND	amyris.com	Brazil, USA
Beta Renewables	- Ethanol	Lignocellulosic biomass	Yeast ¹	www.betarenewables.com	Italy
BioAmber	- Succinic acid - Butanediol - Disodium succinate	Renewable sugars	Yeast ¹	www.bio-amber.com	Canada, USA
BioGasol	- Ethanol	Lignocellulosic biomass	“Pentocrobe”	www.biogasol.com	Denmark
Borregaard	- Lignin derivatives - Specialty cellulose - Ethanol	Lignocellulosic biomass	<i>S. cerevisiae</i>	www.borregaard.com	Norway
Domsjö Fabriker	- Specialty cellulose - Lignin derivatives - Ethanol	Lignocellulosic biomass	<i>S. cerevisiae</i>	www.domsjo.adityabirla.com	Sweden
Dupont	- Ethanol	Corn crop residues	<i>Z. mobilis</i>	www.dupont.com	USA
Fiberight	- Ethanol	Residential/organic waste	ND	fiberight.com	USA
Gevo	- Isobutanol - Jet fuel	Renewable carbon sources	<i>S. cerevisiae</i>	www.gevo.com	USA

GlycosBio	- Lactic acid	Crude glycerol				USA, Malaysia
	- Isoprene	Fatty acids	ND		www.glycosbio.com	
	- Nutraceuticals	Starch/lignocellulosic biomass				
		Fish oil				
Godavari Biorefineries	- Ethanol					
	- Acetaldehyde - Ethyl acetate	Sugarcane	ND		www.somaiya.com	India
Green Biologics	- n-butanol - acetone	Lignocellulosic biomass	<i>Clostridium</i> sp.		www.greenbiologics.com	UK, USA
	- Ethanol - Hydrogen	Carbon-rich industrial gases	<i>C. autoethanogenum</i> and other acetogens		www.lanzatech.com	USA, China, India
Petrobras	- Ethanol	Sugarcane bagasse	<i>S. cerevisiae</i> <i>S. stipitis</i>		www.petrobras.com	Brazil
	- Ethanol	Corn crop residues	<i>S. cerevisiae</i>		poet-dsm.com	USA
Reverdia	- Succinic acid	Renewable sugars	Yeast ¹		www.reverdia.com	Italy, France
	- Ethanol - Biogas - Lignin	Starch and lignocellulosic biomass	<i>S. cerevisiae</i>		www.sekab.com	Sweden
Tembec	- Ethanol - Specialty cellulose - Lignin derivatives	Lignocellulosic biomass	<i>S. cerevisiae</i>		www.tembec.com	Canada
	- Adipic acid	Low-cost plant-sourced oil	Yeast ¹		verdezyne.com	USA
Zeachem	- Ethanol	Lignocellulosic biomass C2/C3 compounds	Bacteria		www.zeachem.com	USA

¹ The specific species was not disclosed ND: not disclosed

Lignocellulosic biomass is one of the most abundant materials in nature and it does not impact the food-chain to the same extent as a whole-crop biorefinery, for example. Forestry and industrial wastes are two important kinds of lignocellulosic biomass being investigated as sources of carbohydrates, also contributing to a reduction in the amount of waste accumulated. Apart from the important impact on waste management, processes based on lignocellulose can lead to a greater reduction in the emission of greenhouse gases (GHG) than other types of biomass, depending on the product and geographical area (Hoefnagels et al. 2010). For instance, simulations have indicated that changing the substrate for ethanol production from corn to switchgrass could lead to approximately a 45% greater reduction in GHG emissions (Wang et al. 2012).

Lignocellulosic biomass is composed of cellulose (40-45%), hemicellulose (20-30%), and lignin (20-35%) (see reviews by e.g. Pereira et al. 2013 and Sun and Cheng 2002). The first two are carbohydrate polymers from which monomeric sugars can be obtained after being subjected to chemical or enzymatic hydrolysis, whereas lignin is commonly described as a recalcitrant polymer of aromatics.

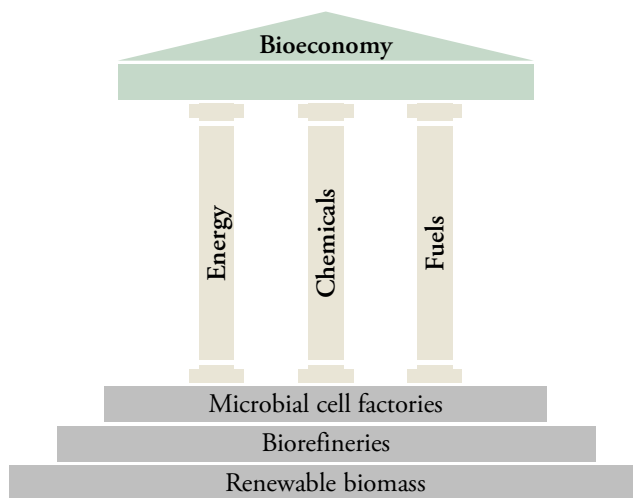


Figure 1 – The three pillars of a bio-based economy (energy, chemicals and fuels), having renewable biomass, biorefineries and microbial cell factories as strong supporting bases.

The global biorefinery market was valued at 407 billion euros in 2014, with a predicted annual growth rate of 14% between 2015 and 2020 (P&S_MarketResearch 2015), demonstrating the enormous potential of this field. The successful implementation of biorefineries is naturally dependent on the efficient conversion of the carbohydrates present in biomass into products with commercial value (Nielsen et al. 2013). Several bacteria and yeasts have demonstrated a significant ability to utilize biomass as a carbon source, leading to their classification as microbial cell factories. Microbial cell factories can be considered the heart of biorefineries, transforming renewable biomass into

different forms of energy (power and heat), transportation fuels and/or commodity/fine chemicals. These form the basis and the main pillars for the creation of a completely sustainable bioeconomy (Figure 1), which is expected to have beneficial effects on both society and the environment.

1.2. Microbial cell factories

1.2.1. Main characteristics of reported cell factories

Microbial cell factories are the core of the biorefinery process, determining the commercial output. The choice of microbial host is therefore crucial, and the substrate, process technology and desired product must be taken into consideration. Fletcher and coworkers recently summarized the key characteristics of an ideal microbial host (Fletcher et al. 2015):

- a) Easy to engineer;
- b) Robust and resistant to industrial stresses;
- c) Capable of expressing complex heterologous metabolic pathways;
- d) Efficient producer (high metrics on titer, yield and productivity).

A diversity of prokaryotic and eukaryotic organisms, such as bacteria, yeasts, filamentous fungi and mammalian cell lines (insect, hybridoma, hamster and human) have been explored as candidates for cell factories (Ferrer-Miralles et al. 2009). The bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae* have emerged as the most promising ones (Krivoruchko and Nielsen 2015). *E. coli* is considered the preferred organism for the production of recombinant proteins, especially for pharmaceutical applications (Ferrer-Miralles et al. 2009), whereas research on *S. cerevisiae* is more focused on the production of fuels, bulk chemicals and high-value metabolites.

The impact of producing fuels with the aid of microbes can be tremendous, since it constitutes a long-term sustainable alternative to fossil-based fuel production. The development of efficient microbial cell factories for the production of bulk and fine chemicals is also of interest. Currently, a variety of high-value compounds are directly extracted from natural biological sources, which is an inefficient process due to their low abundance. Chemical synthesis has also been extensively applied in the production of such natural compounds and other high-value metabolites, for example, in the pharmaceutical industry. However, the chemical synthesis of bulk and fine chemicals usually involves complex multi-step processes with low yields. These problems can be

circumvented by the utilization of efficient microbial cell factories, such as *S. cerevisiae* (Dai et al. 2015; Otto et al. 2011). The following section focuses on the properties of *S. cerevisiae* as a cell factory, describing the main advantages and drawbacks associated with industrial implementation.

1.2.2. *Saccharomyces cerevisiae* as a cell factory

The application of the eukaryotic unicellular model *S. cerevisiae* in industry started decades ago with its implementation in baking and brewing processes. It was one of the first microorganisms to be classified “as generally regarded as safe” (GRAS) and has been approved by the European Food Safety Authority, with qualified presumption of safety (QPS). As mentioned above, this yeast has been well characterized in many studies, in the fields of both fundamental and applied microbiology.

The sequencing of the complete genome of *S. cerevisiae* together with the enormous pool of genotypic and physiological data available were essential to the development of novel molecular tools, which is still ongoing (Goffeau 2000; Goffeau et al. 1996). This continuous development has expanded the range of obtainable metabolites and optimized the production parameters, leading to an efficient and sustainable cell factory. The most significant advances in synthetic biology, systems biology and metabolic engineering over recent years include the following:

- (a) rapid assembly of entire biosynthetic pathways in a single step (Gibson et al. 2008; Naesby et al. 2009; Shao et al. 2009);
- (b) modulation of the expression of heterologous genes (Dai et al. 2012; Zhou et al. 2012);
- (c) acquisition of stable and high-level gene expression (Mikkelsen et al. 2012);
- (d) implementation of the CRISPR/Cas9 technique in *S. cerevisiae* (this technique is classified as one of the most powerful tools for genome engineering, allowing simultaneous genetic modifications in a single-step without the need for antibiotics and/or markers (Jakočiūnas et al. 2015; Mans et al. 2015);
- (e) localization of enzymes to a specific cell compartment or scaffold (Avalos et al. 2013; Farhi et al. 2011);
- (f) development of biosensors that facilitate high-throughput screening of the best producing strains (riboswitches, protein-based biosensors) (Knudsen et al. 2014; Zhang et al. 2015); and
- (g) rewiring of the central carbon metabolism by expression of synthetic pathways (Dai et al. 2015; Meadows et al. 2016).

The robustness, especially in terms of tolerance to different pH levels and to the presence of inhibitory compounds such as weak acids, together with its non-

susceptibility to phage contamination, are the main advantages of *S. cerevisiae* over bacterial cell factories (Krivoruchko and Nielsen 2015). Furthermore, *S. cerevisiae* can functionally express enzymes that are involved in the synthesis of many complex plant-derived molecules, for example, cytochrome P450 (Pompon et al. 1996), which is not possible in bacteria. The unique characteristics of *S. cerevisiae* and the genetic engineering toolbox available have made this yeast a platform cell factory, with the demonstrated ability to produce a wide range of fuels and chemicals, varying in market value and complexity (Figure 2). The production of metabolites such as carboxylic acids, flavonoids, isoprenoids, and polyphenols, among others, has been accomplished by redirecting the carbon flux from the central carbon metabolism to the metabolic route(s) of interest.

The potential of *S. cerevisiae* as a microbial cell factory was initially explored for the production of **ethanol as a biofuel**, relying on the efficient anaerobic conversion of monomeric sugars, such as glucose, into ethanol. Ethanol was first produced from food feedstock such as sugarcane, as it contains a considerable amount of easily accessible fermentable sugars, forming the so-called “first generation bioethanol”. However, the production of high volumes of ethanol with this feedstock was not beneficial in terms of GHG emission, as initially hypothesized, and it created a link between the price of food and that of fuel, generating a wave of controversy and criticism in society (Peralta-Yahya et al. 2012). Furthermore, economic analysis showed that the production of first generation biofuels was not cost-efficient (Hamelinck and Faaij 2006).

Attention was then directed to the production of second generation biofuels, using non-edible renewable carbon substrates containing cellulose, hemicellulose, lignin or pectin (e.g. forest and agricultural residues). The existing research on ethanol production using yeast was applied to production from these alternatives and more complex substrates. However, the formation of microbial inhibitory compounds became one of the major bottlenecks preventing an economically viable production (Almeida et al. 2007). Although significant advances have been made, the International Energy Agency stated in 2010 that the biotechnological production of ethanol and other biofuels would not be sufficient to enable the complete replacement of diesel and jet fuel (IEA 2010). Thus, exploration started on a new generation of fuels – so-called **advanced fuels** – with improved characteristics, using *S. cerevisiae* and other microbial hosts. Advanced biofuels can be used as drop-in fuels and have been defined by the European Industrial Bioenergy Initiative as *biofuels produced from lignocellulosic feedstocks, non-food crops or industrial waste and residue streams that have a low CO₂ emission or high GHG reduction and a zero or low impact on land usage* (EBTP 2016) (Buijs et al. 2013). Examples of advanced fuels with promising properties are isobutanol, 1-butanol, farnesene and long-chain alkanes. The higher energy density and better blending properties of isobutanol compared with bioethanol makes its commercial application as a transportation fuel very appealing (Weber et al. 2010). Furthermore, isobutanol can be converted into the jet fuel kerosene (Borodina and Nielsen 2014), and its industrial

Isoprenoids/Terpenoids

- Artemisinin acid
- Cholesterol
- Ferruginol
- Miltiradiene
- Taxadiene
- Beta-carotene

Vitamins

- Vitamin C

Fuels

- Amorphaadiene
- Biodiesel
- Bioethanol
- 1-butanol
- Farnesene
- Isobutanol
- Long-chain alkanes

Polyphenols

- Resveratrol

Carboxylic acids

- Acrylic
- Fumaric
- Glycolic
- Lactic
- Malic
- Muconic
- Poly-3-D-hydroxybutyric
- Pyruvic
- Succinic
- 3-hydroxypropionic

Flavonoids

- Genistein
- Kaempferol
- Naringenin
- Quercetin

Vaccines

- Coccidioidomycosis
- Hepatitis B
- Tumor antigen

Polyketides

- Rubrofusarin

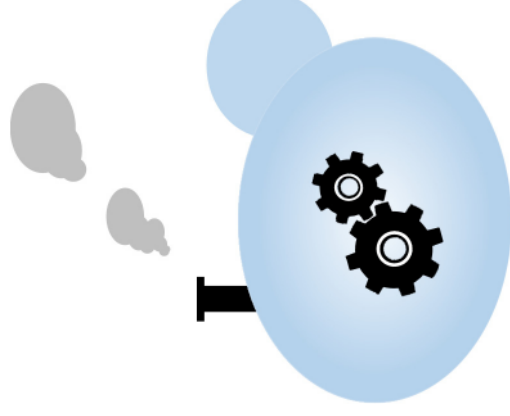


Figure 2 – *Saccharomyces cerevisiae* as a cell factory. A diversity of metabolites whose production has been reported in yeast through metabolic engineering, and synthetic and systems biology are given (corresponding references are given on the next page).

References for Figure 2:

Carboxylic acids: acrylic (Straathof et al. 2005), fumaric (Xu et al. 2012b), glycolic (Koivistoinen et al. 2013), lactic (Ishida et al. 2005), malic (Zelle et al. 2008), muconic (Curran et al. 2013; Weber et al. 2012), poly-3-D-hydroxybutyric (PHB) (Leaf et al. 1996), pyruvic (van Maris et al. 2004), succinic (Raab et al. 2010), 3-hydroxypropionic (Chen et al. 2014). **Flavonoids:** genistein (Trantas et al. 2009), kaempferol (Trantas et al. 2009), naringenin (Koopman et al. 2012), quercetin (Trantas et al. 2009). **Fuels:** amorphadiene (Westfall et al. 2012), biodiesel (Shi et al. 2012), bioethanol (Van Zyl et al. 2007), 1-butanol (Steen et al. 2008), farnesene (Renninger et al. 2010), isobutanol (Brat et al. 2012; Chen et al. 2011), long-chain alkanes (Buijs et al. 2015). **Hormones:** insulin (Kjeldsen 2000). **Isoprenoids/Terpenoids:** artemisinic acid (Paddon et al. 2013), cholesterol (Souza et al. 2011), ferruginol (Guo et al. 2013), multiradiene (Dai et al. 2012; Zhou et al. 2012), taxadiene (Ding et al. 2014; Engels et al. 2008), beta-carotene (Verwaal et al. 2007). **Polyketides:** rubrofusarin (Rugbjerg et al. 2013). **Polyphenols:** resveratrol (Becker et al. 2003). **Vaccines:** coccidioidomycosis (Capilla et al. 2009), hepatitis B (McAleer et al. 1984), tumor antigen (Wansley et al. 2008). **Vitamins:** Vitamin C (Branduardi et al. 2007).

production from renewable carbon sources has already been initiated by Gevo (Table 1). The production of farnesene by *S. cerevisiae*, which can be utilized as diesel and jet fuel, is also being commercially explored by the company Amyris (in collaboration with Total and Cosan) (Renninger et al. 2010). However, most advanced biofuels are still in the research and development stage, and their industrial implementation will require some more years. In addition to the technical developments required, a variety of metabolic engineering strategies are under investigation to further optimize the production yields by *S. cerevisiae* (see reviews by Buijs et al. 2013 and Peralta-Yahya et al. 2012).

In addition to fuels, *S. cerevisiae* is being explored as a cell factory for the production of **carboxylic acids**. Some of these acids with commercial applications are intermediates of the central carbon metabolism in yeast, mainly in the mitochondrial tricarboxylic acid (TCA) and in the glyoxylate cycles. Thus, their microbial production constitutes a logical alternative to the present production via petroleum-based processes and precursors (Abbott et al. 2009; Sandström et al. 2014). Due to the acidic environment caused by the formation of acids, the robustness of yeast can be a significant advantage over other microbial hosts. The production of succinic acid by yeast has been extensively investigated and optimized, leading to its large-scale implementation by Reverdia and BioAmber. Carboxylic acids such as adipic, acrylic and 3-hydroxypropionic (3-HP) are other important precursors for the polymer industry. The efficiency of *S. cerevisiae* in producing most of these carboxylic acids has so far been significantly lower than those required by industry, although yeast engineering for 3-HP production appears to be proceeding along a promising route (Borodina et al. 2015; Chen et al. 2014). The accumulation of the polymer poly-3-D-hydroxybutyrate (PHB), which was initially found in bacteria, was also attempted in recombinant *S. cerevisiae*, although with limited success (Leaf et al. 1996). However, recent studies,

including the present work, focused on increasing the formation of PHB in yeast (Chapter 3, Papers I-III).

Although *S. cerevisiae* is not the organism of choice for the production of **recombinant proteins**, it can provide a suitable alternative in cases where the target protein cannot be produced in a soluble form by bacteria and/or when its activation requires a specific post-translational modification that can only occur in eukaryotic organisms. Most recombinant proteins produced by yeast have medical/pharmaceutical applications, within the fields of infectious diseases or endocrine, nutritional and metabolic disorders (Ferrer-Miralles et al. 2009). The large-scale production of insulin by Novo Nordisk is a good example of the successful application of *S. cerevisiae* as a cell factory, providing life-changing benefits for patients with diabetes (Kjeldsen 2000).

The engineering of *S. cerevisiae* for the production of **resveratrol** and **artemisinic acid** are two other success stories. The first is a powerful antioxidant associated with important health benefits, and its production in yeast was implemented by Fluxome Sciences and further explored by Evolve (Becker et al. 2003). The biosynthesis of artemisinic acid, which is used as an antimalarial drug, represents a significant advance in the production of high-value metabolites by yeast. This constituted a proof of concept since the challenges associated with the expression of complex heterologous pathways (plant-based in this case) could be overcome, and a functional metabolic route was achieved (Paddon et al. 2013).

Although the formation of specific **high-value metabolites** has been achieved using *S. cerevisiae*, the complexity of the heterologous metabolic routes required can be a drastic bottleneck. For example, the identification of the complete biosynthetic pathway, or the toxicity of specific intermediate metabolites, or even of the compound of interest, to the host cell can constitute serious challenges (Dai et al. 2015). The identification of novel genes can be vital for the optimization of specific reaction steps and also to identify other links between the heterologous pathway of interest and the endogenous metabolism. It is important to mention here that *S. cerevisiae* may not always be the preferred microbial host, since each microorganism has particular intrinsic characteristics that may be essential for the efficient production of the metabolite of interest. For instance, some bioprocesses require high temperatures to achieve efficient production, and *S. cerevisiae* is generally not best suited for these, although some studies have demonstrated that adaptive laboratory evolution or targeted engineering can increase its thermotolerance (An et al. 2011; Wallace-Salinas and Gorwa-Grauslund 2013).

1.3. Industrial implementation of biorefineries

The diversity of the compounds produced by natural and recombinant microbial cell factories is immense. Nevertheless, there is a tremendous gap between the studies performed on laboratory and small scale, and implementation on commercial scale. One of the main reasons for this gap is the production metrics: levels of approximately 5 g/L are typically obtained on laboratory scale, which are far below those required in commercial applications (over 50 g/L) (Van Dien 2013). In order to reduce this gap, several problems associated with microbial cell factories, biomass composition and bioprocess technology are being addressed.

1.3.1. Challenges associated with microbial cell factories

1.3.1.1. *The central carbon metabolism: energy and cofactors*

The production of heterologous compounds by *S. cerevisiae* competes, in the majority of cases, with the native processes involved in cell maintenance and growth (**anabolism**). Therefore, the production of adenosine triphosphate (ATP), redox cofactors and other precursor molecules may be limited when pathways designed to produce other products with high demands on these compounds are expressed. Considering the conversion of glucose (**catabolism**), the formation of redox and energy cofactors at the end point of glycolysis is equivalent under aerobic and anaerobic conditions. However, when oxygen is present, pyruvate is mostly directed to respiration through the TCA cycle, where the formation of cofactors in the reduced form, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂), is favored (Figure 3). Those cofactors are molecules capable of conserving energy and are oxidized for ATP formation in the mitochondrial electron transport chain. The resulting NAD⁺ can be utilized for anabolic processes and to supply the glycolytic reactions. Under anaerobic conditions, on the other hand, the carbon is deflected into fermentative metabolism, where oxidized cofactors are formed from pyruvate via ethanol formation, without ATP formation (Figure 3). Considering the above factors, aerobic conditions should be beneficial for the expression of energy-demanding heterologous pathways as the amount of ATP generated is estimated to be 8 times higher than under anaerobic conditions (Vos et al. 2015). Coupling the formation of the metabolite of interest with cell growth may be an alternative way of increasing the amount of product obtained through specific heterologous pathways (Vos et al. 2015), as explored in the present work (Paper IV). This means that the formation of biomass should be dependent on and/or occur simultaneously with one of the compounds of interest.

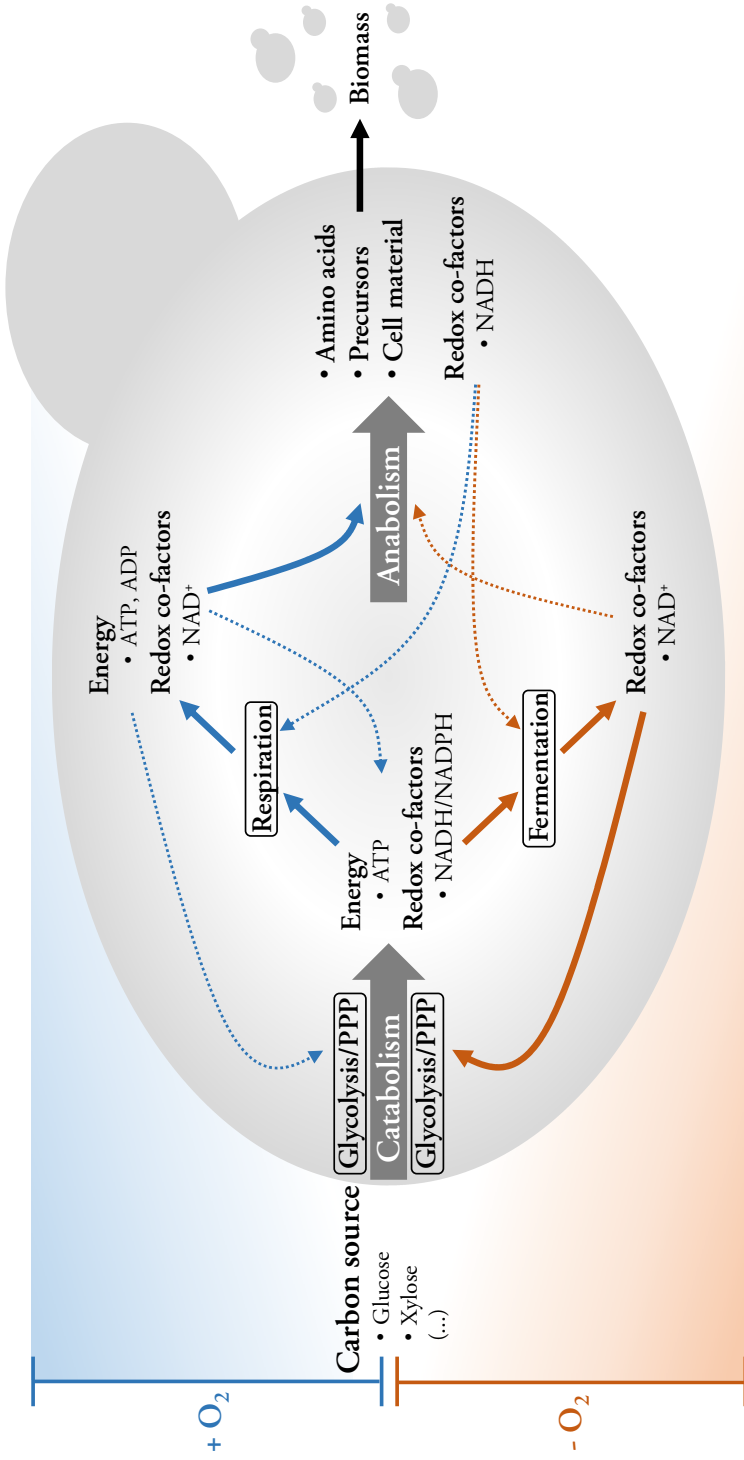


Figure 3 – Simplified scheme of the key processes involved in the catabolism and anabolism of *S. cerevisiae* under aerobic (blue arrows) and anaerobic (orange arrows) conditions. Heavy lines represent the processes that are favored under aerobic or anaerobic conditions. Other metabolic processes that take place to a lesser degree are indicated by dashed arrows.

In the initial design of the engineering strategy, it must be borne in mind that the cell metabolism should be redox-balanced at all times, while redirecting the carbon towards the metabolite of interest (Dugar and Stephanopoulos 2011). For instance, the re-oxidation of excess NADH requires the activity of futile cycles and/or the formation of side products, reducing the yield of the compound of interest (Dugar and Stephanopoulos 2011).

1.3.1.2. *Transport and secretion*

Some of the compounds produced by *S. cerevisiae* are intracellular (Figure 2), meaning that the downstream processing should include extra-steps, for example, to disrupt the cells. Therefore, engineering or expression of functional heterologous **transporters** can facilitate the **secretion** of the metabolites of interest, reducing the time and cost of downstream processing.

1.3.1.3. *Synthetic and systems biology*

The continuous development of **synthetic biology** and molecular tools is essential for the design and construction of novel and more efficient biological routes from scratch (see reviews by e.g. Borodina and Nielsen 2014, Nielsen and Keasling 2016, Siddiqui et al. 2012 and Stephanopoulos 2012). The application of **genome-scale metabolic models** and **systems biology** for the rationalization of strain engineering strategies can be used to further optimize cell factories (Jouhten et al. 2016). An important point here is that some of these novel high-throughput methods lead to the creation of massive amounts of data. Thus, advances in “machinery” technology must be accompanied by the development of powerful computational tools that can handle large amounts of data and present the output to the user in a comprehensible way (Fletcher et al. 2015).

1.3.2. Challenges associated with lignocellulosic biomass

Lignocellulosic biomass is characterized by having a low energy density and high moisture content, which increases the cost of transportation due to the large volumes and weight (Miao et al. 2012). Transportation actually represents a significant cost in the overall process, and it has been demonstrated that transporting biomass can be more expensive than energy (e.g. ethanol) (Searcy et al. 2007). Therefore, a revised biorefinery concept, the **territorial biorefinery**, is currently being investigated. In this case, the biorefinery utilizes the biomass formed in the surrounding region, reducing the transportation cost and contributing to a sustainable surrounding environment.

The utilization of lignocellulosic biomass also requires a chemical and/or enzymatic hydrolysis step to release the fermentable sugars. This may involve harsh conditions such as high temperature and the addition of acids/bases. This process generally leads to the formation of compounds such as weak acids or furaldehydes, which have negative

effects on the performance of the microorganism, thus reducing the bioprocess yield and productivity (Almeida et al. 2007). Considering the natural ability of microorganisms to produce enzymes, the concept of a **consolidated bioprocess** was developed as an alternative to physico-chemical pretreatment. This means that the cell factory solubilizes the biomass, cleaves the carbohydrate polymers and further converts the resulting fermentable sugars. In order to be capable of hydrolyzing the biomass, *S. cerevisiae* has been engineered for the functional expression of different cellulases and hemicellulases, showing promising results (see reviews by e.g. Lambertz et al. 2014 and Lynd et al. 2005). More generally, complete and simultaneous conversion of the available carbon sources into the product of interest could significantly increase the production metrics and lower the process cost. A major fraction of the fermentable sugars in lignocellulosic material is usually composed of the hexose sugar glucose and the pentose sugar xylose. Whereas glucose can be rapidly and efficiently converted, non-recombinant *S. cerevisiae* cannot utilize xylose, which has led to extensive strain engineering (see Chapter 2). Although important advances have been made, an efficient anaerobic consolidated bioprocess using yeast as a cell factory has yet to be described (Olson et al. 2012).

1.3.3. Challenges associated with bioprocess technology

The scale-up of a bioprocess represents a critical stage in industrial implementation. It is a very complex process, in which not only the physiological characteristics of the cell factory in question must be considered, but also the product of interest and, more importantly, the design of the bioreactor (Lidén 2002; Schmidt 2005). This must involve careful planning of the processes used for sterilization, stirring, heating, further extraction of the compound of interest, etc. The principles of biochemical engineering and the development of methods based on physiology and energy/mass balances are crucial for the correct design of the bioreactor and bioprocess. From a cost point of view, the application of the biorefinery concept to existing refineries is very appealing. However, it is essential that the facilities are adapted and/or re-designed in order to optimize the associated processes and technologies (Kamm et al. 2006). For instance, the raw material used in a biorefinery is often heterogeneous and in polymeric form, requiring pretreatment prior to fermentation. This contrasts with the usually homogeneous feedstock utilized in refineries (de Jong and Jungmeier 2015). The chemical compounds used during the chemical or metabolic reactions are quite different, which can also require changes in the materials used in the construction of the reactors. Another example of technical adaptation is the downstream purification of the compound of interest. In contrast to chemical synthesis, where the solvents and solution are well defined, the growth of yeast and fermentation can result in the secretion of a complex mixture of metabolites into the broth, whose interactions with the compound of interest are unknown. This can complicate purification and lead to

more process steps. Despite all these challenges, the transformation of oil refineries into biorefineries has already begun, and more are planned by companies such as Eni (Italy) and Total (France) (Kotrba 2015).

1.4. Scope and outline of this work

The work described in the present thesis focused on exploring the yeast *S. cerevisiae* as a cell factory for the production of biopolymers and carboxylic acids from xylose-rich substrates, envisaging the future utilization of lignocellulosic biomass as a carbon source. Metabolic engineering strategies based on both rational and systems biology models were applied, resulting in the functional expression of novel metabolic routes towards the products of interest. This was followed by extensive physiological characterization and bioprocess optimization to identify the best-producing strains. Papers I-III report on the production of the biopolymer PHB by recombinant yeast, including strategies to improve the PHB-producing pathway through metabolic engineering (Papers I-II), and the evaluation of the impact of nitrogen availability (Paper III). The redirection of the carbon flux from central carbon metabolism towards carboxylic acids was also investigated, first by increasing the flux in the cytosolic glyoxylate cycle and using alpha-ketoglutarate (AKG) accumulation as a case-study (Paper IV); and also by working on the establishment of a novel xylose-utilizing route independent of glycolysis (Paper V).

The following chapters constitute an overview of microbial processes that will guide the reader to a better understanding of the studies discussed in Papers I-V. In Chapter 2, cell mechanisms involved in the sugar metabolism in *S. cerevisiae* are reviewed, particularly those associated with the assimilation of glucose and xylose. The recent advances on the microbial production of PHB and carboxylic acids, with special focus on *S. cerevisiae*, are reviewed in Chapters 3 and 4.



2. Sugar utilization in *Saccharomyces cerevisiae*

2.1. Metabolic routes for glucose utilization

S. cerevisiae can grow on a wide variety of carbon sources such as glucose, mannose, galactose, pyruvate and ethanol. Glucose is the preferred carbon source, and yeast metabolism is optimized for the rapid conversion of this sugar. Glucose metabolism has been extensively studied in yeast, and key points will be discussed in this section.

Baker's yeast is a facultative anaerobe, meaning that it can switch from respiration to fermentative metabolism in response to oxygen level. The type and availability of the carbon source also determines which metabolic routes are favored at a certain time. Complete oxidation of glucose can only be achieved through mitochondrial respiration via the TCA cycle, which maximizes the formation of energy and cofactor molecules which are essential to the anabolic processes. However, when glucose is the sole carbon source, *S. cerevisiae* has a **respiro-fermentative metabolism** and, at concentrations higher than 0.15 g glucose/L, fermentation and consequently ethanol formation are observed (Verduyn et al. 1984). Higher levels of glucose result in overflow metabolism in glycolysis and the enzyme pyruvate dehydrogenase, which is the entry point of the TCA cycle, appears not to be able to cope with that carbon flux (see review by Pronk et al. 1996). Therefore, pyruvate is also converted through fermentative metabolism in order to rapidly restore the levels of NAD⁺ needed for the glycolytic reactions. This behavior is characterized by alcoholic fermentation under aerobic conditions, and is commonly called the **Crabtree effect** (De Deken 1966). This leads to a decrease in biomass yield, since part of the carbon is redirected towards the formation of mostly ethanol, but also glycerol and weak acids (Marc et al. 2013). In practice, the cultivation of yeast in glucose usually involves two growth phases: (1) the conversion of glucose into biomass and ethanol; and (2) as ethanol becomes the only carbon source available when the glucose is depleted (diauxic shift) it is converted to acetate and further into acetyl-CoA, which is an important precursor in the cells. The formation of biomass from ethanol and acetate takes place via the activity of the cytosolic glyoxylate cycle, by the conversion of two-carbon into four-carbon compounds (with a supply of ATP), which can be further metabolized into intermediates of glycolysis. This shunt has an

anaplerotic role, being essential for **gluconeogenesis** and, therefore, for the supply of cofactors and intermediates needed for biomass formation (Daran-Lapujade et al. 2004).

The catabolism of glucose and similar carbon sources by the cell initiates anabolic processes such as the biosynthesis of nucleic acids and amino acids which, ultimately, results in biomass formation (Figure 3). The activity of the pentose phosphate pathway (PPP) determines the supply of nicotinamide adenine dinucleotide phosphate (NADPH) required by anabolic reactions, being the main production pathway of this cofactor in the cell (Stanton 2012). The PPP is also involved in the protection of cells from oxidative stress (Slekar et al. 1996).

Glucose enters the cell in a process mediated by hexose transporters (see review by Özcan and Johnston 1999). In the presence of glucose the rate of synthesis of the enzymes needed for the utilization of alternative carbon sources becomes very low, resulting in partial or complete repression of the associated genes, also called **catabolite repression** (Gancedo 1998; Trumbly 1992). This non-simultaneous uptake of other sugars together with glucose can be challenging when complex media are used as carbon source, since the productivities can be severely compromised due to the increased time needed to deplete all the sugars.

A complete understanding of glucose catabolism and its associated regulatory mechanisms is of great importance for the application of *S. cerevisiae* as a cell factory, since this would allow the production metrics of the compound of interest to be increased. The recognition of extracellular glucose by the cells occurs through **sugar sensing** mechanisms, initiating a cascade of **signaling** and **regulatory pathways** that maximize the uptake of this sugar (see reviews by e.g. Peeters and Thevelein 2014, and Santangelo 2006). Although remarkable advances have been made, the complex cellular mechanisms governing glucose metabolism are still the subject of ongoing research.

Figure 4 shows a simplified representation of the central carbon metabolism of *S. cerevisiae* and the metabolic reactions that take place in glycolysis, the PPP, fermentative metabolism, the TCA cycle and the glyoxylate shunt. Heterologous pathways for xylose consumption and the production of PHB are also shown in the metabolic map, and their importance in the present work will be described in greater detail in the following chapters. The key enzymes investigated and/or targeted by metabolic engineering in the present work, as well as the required cofactors, are also shown in the scheme.

Sugar utilization in *S. cerevisiae*

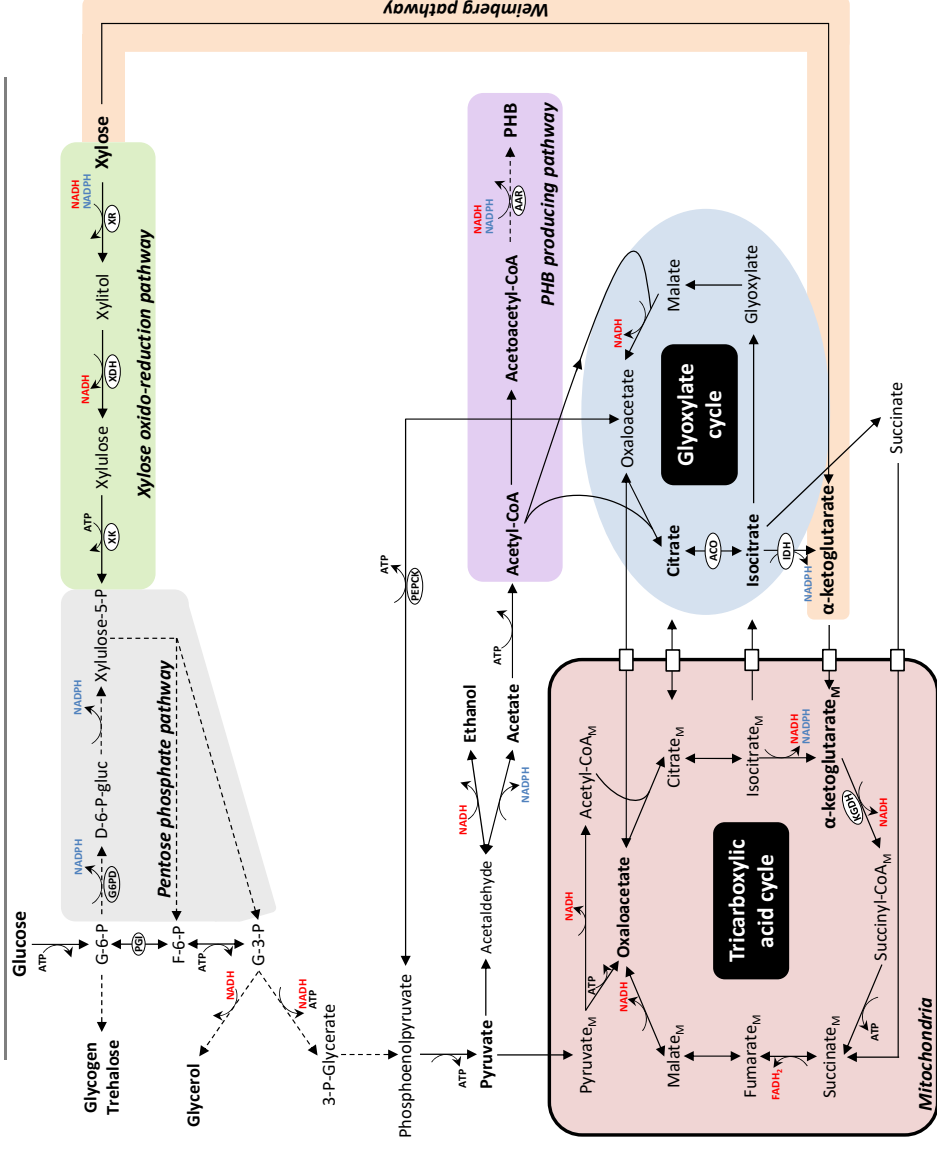


Figure 4 – Illustration of the metabolic pathways in *Saccharomyces cerevisiae*. Key enzymes targeted in the present study are indicated in white-shaded ellipses in the reaction steps, and the required cofactors are shown in red (NADH or FADH₂) and blue (NADPH).

Abbreviations for Figure 4:

AAR acetoacetyl-CoA reductase, ACO1 aconitase, ATP adenosine triphosphate, FADH₂ flavin adenine dinucleotide, G6PD glucose-6-phosphate dehydrogenase, IDH isocitrate dehydrogenase, IDP2 cytosolic NADP-specific isocitrate dehydrogenase, KGD2 dihydrolipoyl transsuccinylase, KGDH alpha-ketoglutarate dehydrogenase, NADH nicotinamide adenine dinucleotide, NADPH nicotinamide adenine dinucleotide phosphate, PEPCK phosphoenolpyruvate carboxykinase, PGI phosphoglucose isomerase, PHB poly-3-D-hydroxybutyrate, XR xylose reductase, XDH xylitol dehydrogenase, XK xylulose kinase.

2.2. Metabolic routes for xylose utilization

Xylose is the second most abundant sugar in nature, after glucose, and it is commonly found in lignocellulosic biomass. Due to the inability of native *S. cerevisiae* to convert xylose, metabolic engineering strategies have been widely explored since the late 1980s in attempts to achieve efficient xylose utilization. Three main metabolic pathways in *S. cerevisiae* have been investigated for the conversion of xylose: 1) xylose oxido-reduction, 2) xylose isomerization and, more recently, 3) xylose oxidation. The first two metabolic engineering strategies were based on the xylose-consuming pathways of natural xylose-fermenting organisms, both prokaryotic and eukaryotic. Interestingly, most of the prokaryotes isomerize xylose, whereas eukaryotes perform the two-step reduction-oxidation reaction (Jeffries 1983).

2.2.1. Xylose oxido-reduction

In yeast species that cannot ferment xylose, such as *S. cerevisiae*, it has been found that the keto-isomer xylulose could be naturally phosphorylated to xylulose-5-P by the endogenous enzyme xylulokinase (XK), and then channeled into the PPP and central carbon metabolism (Wang and Schneider 1980) (Figure 4). This finding indicated that the conversion of xylose into xylulose could be the main bottleneck in obtaining an efficient xylose-consuming strain of *S. cerevisiae*, especially under anaerobic conditions.

Xylose conversion to xylulose in *S. cerevisiae* has been attempted by introducing the enzymes xylose reductase (XR) and xylitol dehydrogenase (XDH) (Kötter and Ciriacy 1993; Tantirungkij et al. 1993), whose associated genes *XYL1* and *XYL2* originated from the efficient xylose-utilizing yeast *Scheffersomyces stipitis* (Du Preez and Prior 1985). In the XR-XDH pathway, XR reduces xylose to xylitol, which is then oxidized by XDH, forming xylulose (Figure 5). However, *S. stipitis* XR has a higher affinity for NADPH than for NADH (Verduyn et al. 1985), which creates a cofactor imbalance since XDH only uses NAD⁺ as cofactor. Thus, xylose fermentation resulted in xylitol accumulation due to a lack of intracellular NAD⁺. Other studies showed that XRs with a more balanced cofactor affinity or with NADH preference would be needed to

achieve efficient xylose fermentation (Bruinenberg et al. 1984). Interestingly, it was later found that the genome of wild-type *S. cerevisiae* contained the genes for a complete xylose utilization pathway: XR, XDH and XK (Aguilera and Prieto 2001; Kuhn et al. 1995; Richard et al. 1999; Rodriguez-Peña et al. 1998). However, only residual expression was observed, and the XR was found to be strictly NADPH-dependent (Toivari et al. 2004).

The cofactor imbalance observed when the XR-XDH pathway was expressed in *S. cerevisiae* led to a wide range of investigations on the cofactor preference of the two enzymes. Redox cofactor engineering was initially attempted by the introduction of bacterial transhydrogenases that catalyze the interconversion between NADH and NADPH, to compensate for the imbalance caused by the XR-XDH route. Unfortunately, the reaction occurred in the reverse direction, resulting in NADPH consumption and NADH formation (Nissen et al. 2001). Site-directed and random mutagenesis proved to be a useful approach to obtain XR with increased NADH preference or XDH with NADP⁺ affinity, resulting in decreased xylitol formation and improved xylose fermentation (Bengtsson et al. 2009; Jeppsson et al. 2006; Kostrzynska et al. 1998; Watanabe et al. 2007a; Watanabe et al. 2007b). However, although the NADH affinity was increased, the XR-engineered enzymes still had a preference for NADPH. A breakthrough occurred in 2010, when a mutated *S. stipitis* XR was reported to exhibit higher affinity for NADH than NADPH (Runquist et al. 2010). This was achieved by mutating the cofactor-binding region by error-prone PCR and further library selection through sequential anaerobic batch fermentation. *S. cerevisiae* expressing this XR showed a significant increase in xylose fermentation efficiency and in ethanol productivity. Since the cofactor preference of the enzymes XR and XDH is species- and strain-dependent, screening studies that aimed at finding enzymes with different cofactor affinities have also been performed. A native XR in the yeast *Spathaspora passalidarum* with significantly higher NADH preference was identified and successfully introduced into *S. cerevisiae*, resulting in a considerable increase in xylose fermentation efficiency (Cadete et al. 2016). It should also be emphasized that efficient fermentation of xylose could only take place when additional genetic modifications were implemented, in particular the overexpression of genes encoding XK and enzymes from the non-oxidative branch of the PPP (as described below).

2.2.2. Xylose isomerization

Xylose isomerase (XI), an enzyme catalyzing the reversible isomerization of xylose to xylulose and of glucose to fructose, is found widely in bacteria but in only a few eukaryotes (Jeffries 1983). The isomerization of xylose through XI rapidly became a target of interest since it was expected to allow anaerobic redox-neutral conversion of xylose in *S. cerevisiae* (Bruinenberg et al. 1983) (Figure 5).

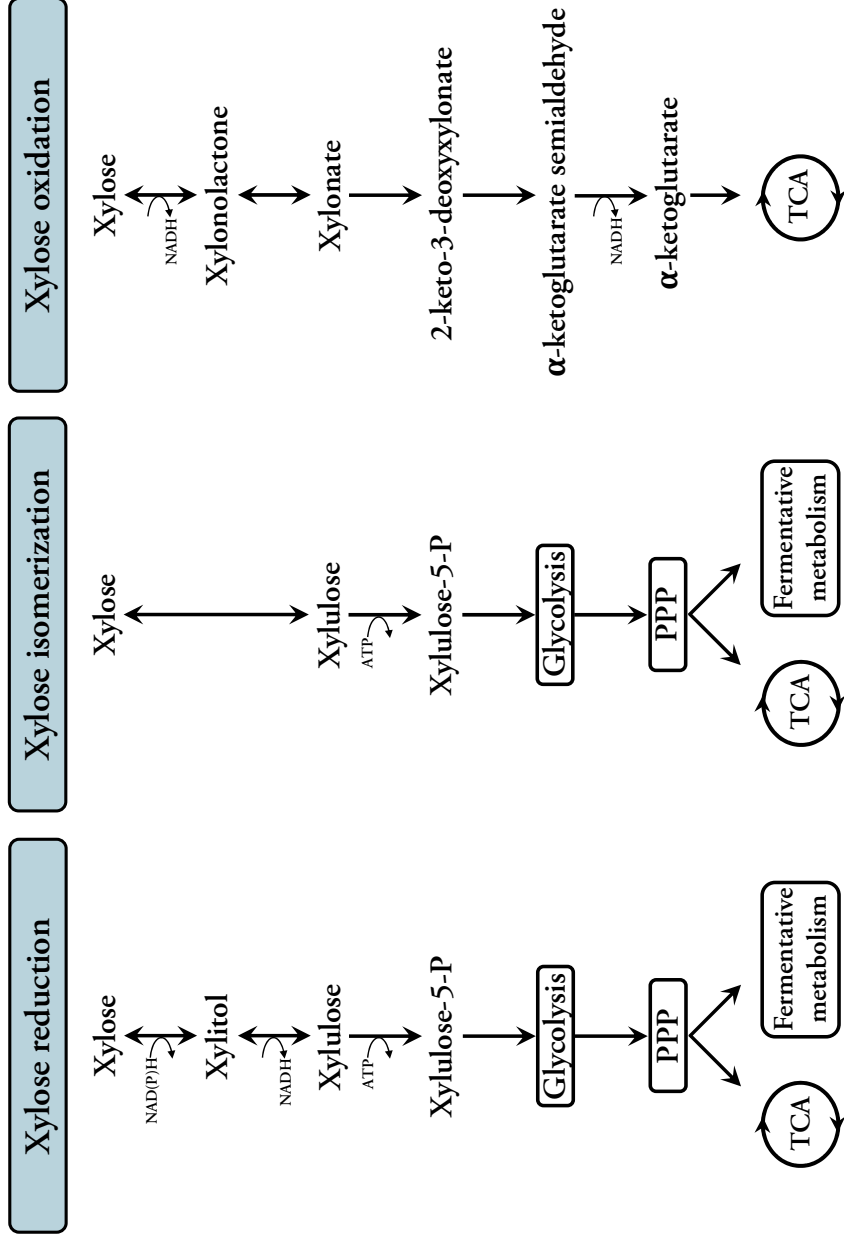


Figure 5 – Main metabolic steps that link xylose to the central carbon metabolism of three different xylose-consuming pathways that have been expressed in *S. cerevisiae*.

Heterologous expression of XI genes in *S. cerevisiae* was reported already in 1984, but no enzyme activity was detected (Briggs et al. 1984). In fact, a number of unsuccessful attempts to express XI genes from different microorganisms in yeast showed that this task was very difficult (Amore et al. 1989; Gárdonyi and Hahn-Hagerdal 2003; Moes et al. 1996; Sarthy et al. 1987). The XI gene from the bacterium *Thermus thermophilus* was the first to be functionally expressed in *S. cerevisiae*, but the XI activity was very low, and further metabolic engineering was required to obtain an efficient xylose utilization (Walfridsson et al. 1996).

Similarly to the XR-XDH pathway, it was thought that XI with different intrinsic characteristics and/or from different sources would allow efficient xylose fermentation. Initially, random mutagenesis was tried in an attempt to improve the XI from *T. thermophilus* and increase its activity in yeast, but this strategy was not successful (Lönn et al. 2002). Several research groups focused instead on finding and testing alternative sources of XI (see review by Van Maris et al. 2007 for more detailed information). The expression of the native enzyme from the fungus *Piromyces* sp. E2 in *S. cerevisiae* eventually resulted in considerable XI activity (Kuyper et al. 2005). However, growth on xylose was still low, which suggested that XI alone, without further metabolic engineering was not sufficient for efficient xylose fermentation. Later, a prokaryotic XI with high activity was successfully introduced into *S. cerevisiae*, and this enzyme was found to be less susceptible to xylitol inhibition than the XI from *Piromyces* sp. E2 (Brat et al. 2009). It is important to mention that the codon-optimization for *S. cerevisiae* of this XI gene, native in *Clostridium phytofermentans*, was important to obtain the desired trait (Wiedemann and Boles 2008). More recently, directed evolution of the XI gene has resulted in further improvement of xylose utilization under both aerobic and anaerobic conditions, exhibiting high consumption rates (Lee et al. 2012).

In addition to the optimization of XI expression or cofactor utilization in the case of XR-XDH, other genetic modifications have proven to be necessary to improve xylose utilization in both pathways. Overexpression of the endogenous XK gene in *S. cerevisiae* increased the flux of xylulose into the central carbon metabolism (Johansson et al. 2001). Reduced activity or inactivation of the native aldose reductase encoded by the *GRE3* gene resulted in lower xylitol formation and increased ethanol formation for both xylose-consuming pathways (Träff-Bjerre et al. 2004; Träff et al. 2001). This deletion was particularly important to the isomerization pathway since many XIs were found to be inhibited by xylitol (Yamanaka 1969). It is, however, important to note that biomass formation was negatively affected by $\Delta gre3$, meaning that the pros and cons of this modification should be judged depending on the final goal of the study. When high activities of XR-XDH or XI were obtained in *S. cerevisiae*, it was shown that the overexpression of *XKS1*, encoding XK, together with the genes encoding the four enzymes involved in the non-oxidative branch of the PPP (transaldolase, transketolase, ribose 5-phosphate ketol-isomerase and ribulose 5-phosphate epimerase) was crucial to

direct enough of the carbon flux towards glycolysis to achieve efficient xylose consumption (Karhumaa et al. 2007a; Karhumaa et al. 2005; Kuyper et al. 2005). For more detailed information about these genetic modifications and other metabolic engineering strategies, the reader is referred to the specialized review articles by Hahn-Hägerdal et al. 2007 and Kim et al. 2013.

Another common trait of the XI and XR-XHD pathways is the transport of xylose into the cell, which occurs by facilitated diffusion, and is mediated by hexose transporters (Hamacher et al. 2002). These transporters are not optimal for xylose, and it has been shown that with the significantly improved xylose uptake using the strategies mentioned above, the transport rate of this pentose may become a limiting step, especially when the concentration of xylose is low (Gárdonyi et al. 2003; Tanino et al. 2012). In addition, hexose transporters have higher affinity for glucose, meaning that if both sugars are present, xylose uptake will start only after glucose depletion. Several research groups are working on finding or generating a xylose transporter that is not inhibited by glucose, allowing co-consumption of the two sugars. Recently, a mutated version of the Gal2 transporter (*GAL^{2N376F}*), which originally transports glucose, galactose and xylose, was demonstrated to have a high affinity for xylose and to have lost the ability to transport hexoses (Farwick et al. 2014).

A comparative study of isogenic strains of *S. cerevisiae* carrying either the XR-XDH-XK or the XI-XK integrated pathways, demonstrated that xylose consumption was more efficient with the xylose reduction approach, resulting in higher ethanol productivity. On the other hand, xylose isomerization resulted in higher ethanol yields (Karhumaa et al. 2007b).

2.2.3. Xylose oxidation

Xylose oxidation was first described in *Pseudomonas fragi*, where xylose was converted into xylonate and further to the TCA cycle intermediate AKG (Weimberg 1961). This pathway, called the Weimberg pathway, was later found in the bacterium *Caulobacter crescentus* (Stephens et al. 2007), in the archaeon *Haloferax volcanii* (Johnsen et al. 2009), and also in the solvent-tolerant bacterium *Pseudomonas taiwanensis* VLB120 (Köhler et al. 2015). In the Weimberg pathway (Figure 5), xylose is initially oxidized to xylonolactone by a xylose dehydrogenase (XylB, encoded by *xylB*), which is further converted to the intermediate xylonate by a xylonolactonase (XLS, encoded by *xylC*). The xylonate-forming reaction can also occur spontaneously at neutral pH, with the lactone ring opening at a low rate (Buchert and Viikari 1988; Toivari et al. 2012a). Xylonate is then subjected to two dehydration reactions by a xylonate dehydratase (XDY, encoded by *xylD*) and a 2-keto-3-deoxyxylonate dehydratase (KDY, encoded by *xylX*), yielding 2-keto-3-deoxyxylonate and then alpha-ketoglutarate semialdehyde. In the final step, the semialdehyde is

oxidized by alpha-ketoglutarate semialdehyde dehydrogenase (KSH, encoded by *xylA*) into AKG. The first and last metabolic reactions of the Weimberg pathway require the cofactor NAD⁺.

Functional implementation of the complete Weimberg pathway originating from *C. crescentus* has been accomplished in *Pseudomonas putida* S12 (Meijnen et al. 2009), and more recently in *Corynebacterium glutamicum* (Radek et al. 2014). In both studies, biomass formation from xylose was observed, proving that the complete pathway was expressed and active, with the end product AKG being further metabolized in the central carbon metabolism. However, it highlighted difficulties associated with the implementation of the Weimberg pathway. In *Pseudomonas putida* S12, it was found that a native periplasmic glucose dehydrogenase could also oxidize xylose and that it was the major source of xylonolactone and xylonate in the cell, despite the fact that the complete Weimberg pathway was expressed. It was hypothesized that the XylB activity was lower than that of the endogenous enzymes to avoid the cytosolic accumulation of toxic intermediates of the Weimberg pathway (Meijnen et al. 2009). In the case of *C. glutamicum*, a long lag phase was observed and xylonate was the main product when xylose was the sole carbon source, suggesting that dehydration by XDY may constitute a limiting step (Radek et al. 2014).

The functional expression of the whole Weimberg pathway in *S. cerevisiae* may be particularly interesting in the biotechnological production of TCA intermediates such as carboxylic acids (Mihasan et al. 2013). The production of xylonate, which is an intermediate of the Weimberg pathway, was investigated in *S. cerevisiae* through the functional expression of the genes encoding the enzymes XylB and XLS found in *C. crescentus* (Toivari et al. 2012a). XylB showed a high specificity for xylose, and the expression of the *xylC* gene encoding XLS resulted in increased carbon flux through the oxidative route, meaning that the xylonate productivity was increased. However, the rapid accumulation of xylonate led to a decrease in intracellular pH, which had a negative effect on cell viability. As the conversion of the lactone into the acid form can occur naturally at neutral pH, the absence of XLS, leading to a lower conversion rate, proved to be beneficial in *S. cerevisiae* since the xylonate accumulation was more gradual (Toivari et al. 2012a). In addition, deletion of the native aldose reductase encoded by *GRE3* increased the flux in the upper part of the Weimberg pathway since the oxidation route became the only xylose utilization pathway available in the cell; and it also reduced xylitol accumulation to negligible amounts (Toivari et al. 2010). Finally, the process requires aerobic conditions since the resulting excess NADH must be oxidized. The ATP generation associated with the aerobic process was also predicted to be beneficial for cell maintenance and pH homeostasis (Toivari et al. 2012b).

Native genes from *C. crescentus* encoding the whole Weimberg pathway were introduced into *S. cerevisiae* in the present work (Paper V), although the functional expression of all of them proved to be challenging. Xylonate accumulated and no

further conversion into AKG and/or biomass could be observed. Thus, xylonate dehydration appeared to be a major limitation, in line with observations made on *C. glutamicum* (Radek et al. 2014). XDY from *C. crescentus* was recently purified and its crystallization allowed a preliminary prediction of its structure, which offers the possibility of optimization through protein engineering (Rahman et al. 2016). Additionally, the role of [FeS] clusters was found to be crucial for the catalytic activity of this specific XDY (Andberg et al. 2016). More generally, the expression of non-eukaryotic [FeS] cluster-harboring enzymes, such as XDY, in *S. cerevisiae* has not yet been accomplished, despite remarkable efforts made in a recent study (Benisch and Boles 2014).

Due to the limited success of the xylose oxidation pathway so far, no comparative studies have been performed between the XR-XDH-XK, the XI-XK and the Weimberg pathways. The choice of xylose utilization pathway in engineered yeast is expected to depend on the metabolite of interest, the bioprocess conditions and the substrate characteristics, among others. The advent of synthetic biology, together with increasing knowledge on the signaling and regulation of metabolic pathways, is expected to provide the possibility of alternative routes for xylose utilization. For example, a novel synthetic route with altered xylose utilization stoichiometry that involves the formation of xylulose-1-P and glycolaldehyde was recently investigated in *E. coli* (Cam et al. 2015). An alternative assimilation pathway involving the phosphorylation of xylulose-5-P into xylulose-1-P, which can be further converted into ethylene glycol, was recently demonstrated to partially bypass the PPP in *S. cerevisiae* (Chomvong et al. 2016). In addition to the investigation of novel pathways, previously described pathways can also be further developed and optimized.

Table 2 summarizes the main advantages and drawbacks associated with the implementation of the three xylose-utilization pathways described above. The main optimization strategies used to overcome those challenges are also included.

Table 2 – Comparative analysis of the advantages and drawbacks of the three xylose-consuming pathways expressed in *S. cerevisiae*.

	Advantages	Drawbacks	Main optimization strategies
Xylose oxidoreduction via XR-XDH-XK	<ul style="list-style-type: none"> - Requires fewer gene copies - Extensively studied and optimized 	<ul style="list-style-type: none"> - Causes redox imbalance - Dependent on the flux through the PPP (carbon loss) and glycolysis - Xylitol formation 	<ul style="list-style-type: none"> - Use of XR and/or XDH with improved activity - Change in the cofactor preferences of XR and XDH - $\Delta gre3$ - Overexpression of genes in the non-oxidative branch of the PPP and XKSI - Evolutionary engineering
Xylose isomerization via XI-XK	<ul style="list-style-type: none"> - Redox neutral - Extensively studied and optimized 	<ul style="list-style-type: none"> - Requires more XI gene copies - Dependent on the flux through the PPP (carbon loss) and glycolysis - Unfavorable equilibrium towards xylulose formation - May require addition of metals 	<ul style="list-style-type: none"> - Use of XI with improved activity - $\Delta gre3$ - Overexpression of genes in the non-oxidative branch of the PPP and XKSI - Evolutionary engineering
Xylose oxidation via Weimberg pathway	<ul style="list-style-type: none"> - Independent of the central carbon metabolism - No carbon loss 	<ul style="list-style-type: none"> - Less studied than xylose reduction and isomerization pathways - Expression of dehydratases can be problematic 	<ul style="list-style-type: none"> - Optimization of xylose conversion into the intermediate xylonate - Novel sources of dehydratases - Investigation of the mechanisms related with [FeS] cluster harboring enzymes



3. Production of PHB in yeast

3.1. Why bioplastics?

In modern society, it is almost impossible to imagine what life would be like without plastics. In 2015, more than 250 million tons of plastic were produced worldwide, approximately 65% in the USA, Europe and China, and this is predicted to increase over the next few years (PlasticsEurope 2016). Plastics are used in almost every aspect of society, being especially important in the packaging, construction, automotive and electronics industries. In Europe alone, the plastics industry generated more than 340 billion euros in 2015 (PlasticsEurope 2016), demonstrating the huge impact of this industry on the world market.

The manufacturing of conventional plastics is entirely dependent on oil-based processes. The different chemical structures of a large variety of polymers can be easily manipulated through these processes, to achieve the desired properties in terms of shape, elasticity, resistance, etc. (Reddy et al. 2003; Yang et al. 2004). The advent of modern plastics was welcomed as many products had high chemical resistance and durability. However, these properties soon became a threat to the environment, due to the accumulation of large quantities of non-degradable materials, for example, in landfills and the marine environment (Reddy et al. 2003). The recalcitrance, complex three-dimensional structure and hydrophobicity of conventional plastics difficult their natural degradation by environmental factors and microbes (Gu 2003; Kale et al. 2015). The consequences can be harmful not only to the environment, but also to wildlife (Sigler 2014; Thompson et al. 2009). For instance, the continuous and significant accumulation of plastics in the oceans will continue to threaten the marine ecosystem unless radical changes are made in waste management (Jambeck et al. 2015).

The production of polymers from sustainable resources is of great importance, not only because it would drastically reduce our dependency on oil-based processes, but also as some of these materials are biodegradable, both of which would reduce the negative effects on the environment. In a recent study it was estimated that replacing conventional plastics that are classified as hazardous with safer and reusable materials could reduce the predicted plastic waste in 2015 from 33 to 4 billion tons (Rochman et al. 2013). Therefore, it is essential to investigate the large-scale production and

application of other types of plastics and plastic precursors, in particular so-called bioplastics.

3.2. Bioplastic types and market

3.2.1. Definition and classification

According to European Bioplastics, **bioplastics** are plastic materials that are bio-based and/or biodegradable (EuropeanBioplastics). Thus, it is essential to clarify the difference between these two concepts:

- **bio-based plastics** are materials or products derived from renewable biomass (e.g. lignocellulose and sugarcane);
- **biodegradable plastics** are polymers that can be converted into natural substances such as water, carbon dioxide or compost by microorganisms under specific conditions (e.g. temperature and oxygen availability).

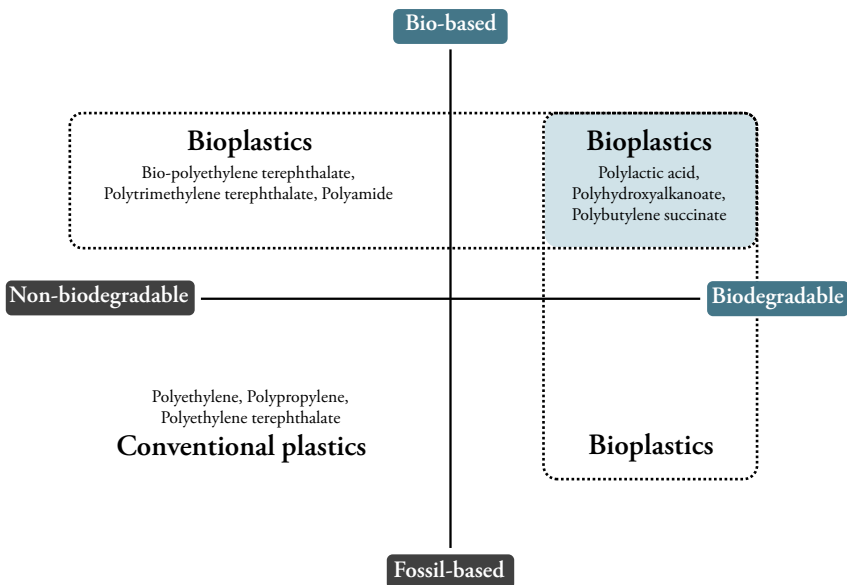


Figure 6 – Distinct characteristics of conventional and bioplastics in terms of origin (fossil- or bio-based) and biodegradability. The bioplastics in the blue-shaded box are those with the most interesting environmentally related characteristics, being both bio-based and biodegradable.

(Adapted from <http://www.european-bioplastics.org/bioplastics/>).

Bioplastics can be divided into three distinct groups based on their origin and biodegradability (Figure 6):

- 1) Fossil-based and biodegradable: highly flexible polymers with applications in the packaging and 3D-printing industries;
- 2) Bio-based and non-biodegradable: very durable polymers used as textile fibers and foams;
- 3) Bio-based and biodegradable: mostly used in food packaging due to their poor durability.

A bio-based plastic is not necessarily biodegradable, and a biodegradable plastic may not originate from renewable biomass. Among the bioplastics that are bio-based and biodegradable, which are the complete antithesis to conventional plastics, **polyhydroxyalkanoate (PHA)**, polylactic acid (PLA) and polybutylene succinate (PBS) are some of the most interesting polymers for commercial exploration.

3.2.2. Institutional directives and government policies

The cost of producing most bioplastics is higher than that associated with conventional plastics (Petersen et al. 1999; Verlinden et al. 2007). Therefore, political directives and strategies will be required to promote the production and use of bioplastics. Both the USA and the European Union have developed and implemented programs intended to support bioplastics research and production, such as Europe 2020, FP7 and Horizon 2020, the Bioeconomy Strategy and BioPreferred. In addition, rising oil prices will have a significant impact on fossil-based processes, increasing the cost of conventional plastics.

3.2.3. Market and applications

Bioplastics currently represent less than 1% of the total plastics market, but are one of the fastest growing sectors in the industry, with an annual growth of approximately 25% (Müllhaupt 2013; PlasticsIndustryAssociation 2017). The development of novel bioplastics by, for example, blending different kinds of biopolymers, results in the formation of materials with unique properties (e.g. elasticity, bio-compatibility), which may create applications that were not possible with conventional plastics. A number of companies, including BASF, Brasken, DSM, DuPont, Metabolix, Succinity, and NatureWorks, amongst many others, are therefore focusing on the bioplastics market. For instance, BASF is producing PLA for organic waste bags, carrier bags and agricultural films, while Succinity is instead turning its attention to PBS due to its application in food packaging and the automotive industry. Plastic filaments for use in

3D printing are one of the most recent applications of bioplastics, and this market is being explored, by, for example, NatureWorks. The Coca-Cola Company has developed a drinking bottle partly composed of bio-PET, and Solaplast is integrating algae waste in plastic materials, to form a bio-based material. Another interesting approach is being developed by Metabolix in collaboration with Honeywell, for the production of microbeads, commonly used in shampoo and facial scrubs, from the versatile polymer PHA.

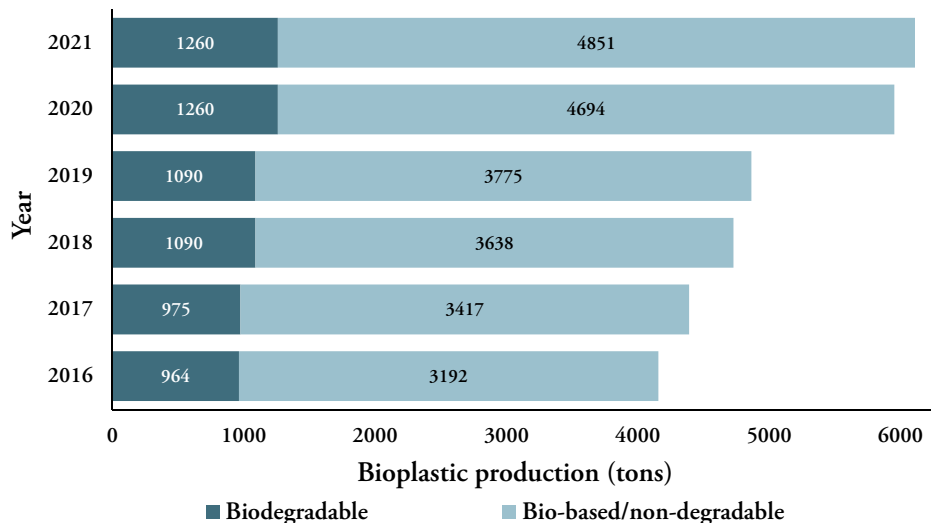


Figure 7 – Global production of bioplastics in 2016 and market forecast for the following five years, showing biodegradable and bio-based but non-degradable fractions.

(Adapted from <http://www.european-bioplastics.org/market/>).

Of the total of 4.16 million tons of bioplastic produced in 2016, only 23% was biodegradable (Figure 7) (EuropeanBioplastics). The fraction of bio-based and biodegradable plastics is still very low, and there is considerable room for improvement. As mentioned above, PHAs have both properties and are very interesting for commercial exploration, exhibiting thermo-plastic and elastomeric properties (Hazer and Steinbüchel 2007; Philip et al. 2007). In addition, PHAs are biocompatible, as the degradation products are non-toxic, which means they can be used in medical applications. The low acidity of these polymers also gives them a competitive advantage over other bioplastics with similar properties (e.g. PLA) (Ali and Jamil 2016). Approximately 150 different types of PHAs have been reported in the literature, and **PHB**, the first PHA described, is commonly considered to be the one most extensively studied (Steinbüchel and Lütke-Eversloh 2003).

3.3. Poly-3-D-hydroxybutyrate

The accumulation of PHB was first described in *Bacillus* species in the 1920s, but its properties as a plastic material were not explored until the 1960s (Lemoigne 1926; Noel 1962; Philip et al. 2007). This short-chain, linear polyester is composed of C₃-C₅ hydroxyacid monomers and its carbon content is entirely bio-based (Aeschelmann and Carus 2016; Horng et al. 2010). Among other interesting characteristics, PHBs are: (1) water-insoluble (Madison and Huisman 1999), (2) relatively resistant to hydrolytic degradation (Pilla 2011), (3) resistant to ultra-violet light (Kaplan 1998), and (4) similar to the conventional plastic polypropylene, for example, in terms of tensile strength (Madison and Huisman 1999). However, PHB is a brittle material (Savenkova et al. 2000) and its chemical modification is difficult (Ikejima and Inoue 2000). In addition, the melting point of PHB is very close to the temperature at which it decomposes, which limits its application (Madison and Huisman 1999). Therefore, several research groups are working on improving the mechanical and physical properties of PHB (see reviews by e.g. Anbukarasu et al. 2015, Bhatt and Jaffe 2015 and Chen et al. 2012).

PHB production was initially investigated in bacteria that naturally accumulate the polymer. With the development of recombinant engineering techniques, strategies to produce PHB in other microbial hosts with distinct features were implemented. The following section summarizes the main advances made in both natural and recombinant PHB producers.

3.3.1. Microbial production of PHB

3.3.1.1. Natural producers

The accumulation of PHB occurs naturally in some bacteria and halophilic archaea species by the formation of carbon granules that serve as energy storage material (Hezayen et al. 2000; Suriyamongkol et al. 2007; Verlinden et al. 2007). Besides this important role, it has been hypothesized that PHB also acts as a redox regulator (Senior and Dawes 1971). Generally, PHB production is optimal under excess carbon and limiting levels of nitrogen, phosphorus and/or oxygen (Shang et al. 2003; Verlinden et al. 2007).

A number of bacterial hosts reported to naturally accumulate considerable amounts of PHB are listed in Table 3. Among them, the gram-negative soil bacterium *Cupriavidus necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*) is considered a model organism for PHB biosynthesis, and has been extensively studied due to its efficient polymer accumulation, achieving PHB content levels exceeding 70% (Eggers and Steinbüchel 2014). The biosynthesis of PHB in *C. necator* takes place from the

central carbon metabolism intermediate acetyl-CoA in a three-step process: (1) an acetyl-CoA acetyltransferase, encoded by *PhaA*, which catalyzes the combination of two acetyl-CoA molecules into acetoacetyl-CoA (Peoples and Sinskey 1989b); (2) an acetoacetyl-CoA reductase (AAR) encoded by *PhaB*, which reduces acetoacetyl-CoA to 3-D-hydroxybutyryl-CoA (Peoples and Sinskey 1989b); and (3) a PHB synthase, encoded by *PhaC*, which catalyzes the final polymerization step forming PHB (Peoples and Sinskey 1989a) (Figure 8). The last reaction is considered the key step in PHB formation, since the synthase is responsible for the stereo-selective polymerization process with the release of a CoA molecule (Rehm and Steinbüchel 1999). Approximately 60 different synthases have been described and classified into four different classes according to substrate preference, chemical structure and amino acid sequence (see review by Rehm 2003). In addition to the importance of the PHA synthase, it should also be noted that the acetyl-CoA acetyltransferase activity is mediated by the ratio of acetyl-CoA to CoA in *C. necator* (Leaf and Srienc 1998).

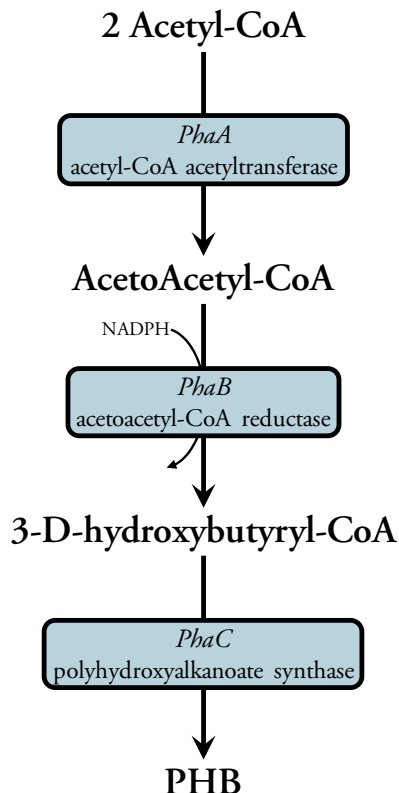


Figure 8 – PHB producing pathway native to the bacterium *C. necator*. This pathway consists of three reaction steps and has been investigated in *S. cerevisiae*, where PHB can be obtained from the intermediate metabolite acetyl-CoA.

Table 3 – Various microorganisms that have been reported as PHB producers from defined carbon sources in literature. The aeration conditions, induction of PHB production by nitrogen limitation, maximum PHB content (% PHB/cell dry weight) and carbon source utilized are also given.

Microorganism	Aeration	Nitrogen limitation	Maximum PHB content (%)	Carbon source	Reference
Natural producers					
<i>Alcaligenes latus</i>	Aerobic	Yes	88	Sucrose	(Wang and Lee 1997)
<i>Bacillus brevis</i>	Aerobic	No	42	Glucose	(Yilmaz et al. 2005)
<i>Bacillus cereus</i>	Aerobic	No	28	Glucose	(Yilmaz et al. 2005)
<i>Bacillus circulans</i>	Aerobic	No	19	Glucose	(Yilmaz et al. 2005)
<i>Bacillus coagulans</i>	Aerobic	No	24	Glucose	(Yilmaz et al. 2005)
<i>Bacillus licheniformis</i>	Aerobic	No	5	Glucose	(Yilmaz et al. 2005)
<i>Bacillus megaterium</i>	Aerobic	No	12 62	Glucose Glycerol	(Yilmaz et al. 2005) (Naranjo et al. 2013)
<i>Bacillus subtilis</i>	Aerobic	No	14	Glucose	(Yilmaz et al. 2005)
<i>Burkholderia cepacia</i>	Aerobic	Yes	47	Glucose	(Young et al. 1994)
			60	Xylose	(Ramsay et al. 1995)
			56	Lactose	(Young et al. 1994)
<i>Burkholderia sacchari</i>	Aerobic	Yes	42	Sucrose	(da Cruz Pradella et al. 2010)
		No	58	Xylose	(Lopes et al. 2011)
<i>Cupriavidus necator</i>	Aerobic	Yes	76	Glucose	(Kim et al. 1994)
			-70	Fructose	(Mothes et al. 2007)
			62	Glycerol	(Cavalheiro et al. 2009)

<i>Paracoccus denitrificans</i>	Aerobic	Yes	-70 -70	Glucose Glycerol	(Mothes et al. 2007) (Mothes et al. 2007)
<i>Pseudomonas pseudoflava</i>	Aerobic	Yes	22 22 25	Glucose Xylose Arabinose	(Bertrand et al. 1990) (Bertrand et al. 1990) (Bertrand et al. 1990)
Recombinant producers					
<i>Cupriavidus necator</i>	Aerobic	Yes	73	Xylose	(Kim et al. 2016)
<i>Escherichia coli</i>		No	77	Glucose	(Kahar et al. 2005)
		Yes	53	Glucose	(Perez-Zabaleta et al. 2016)
		No	36	Xylose	(Yup Lee 1998)
		No	60	Glycerol	(Mahishi et al. 2003)
<i>Komagataella pastoris</i> (<i>Pichia pastoris</i>)	Aerobic	No	2	Glucose	(Vijayasankaran et al. 2005)
	Oxygen-limited	No	27	Glucose	(Vijayasankaran et al. 2005)
<i>Saccharomyces cerevisiae</i>	Aerobic	No	3	Glucose	(Kocharin et al. 2013)
	Aerobic	No	5	Xylose	Paper II
	Oxygen-limited	No	15	Xylose	Paper II
	Anaerobic	No	16	Xylose	Paper III
	Aerobic	No	8	Glucose+Galactose	(Carlson and Srienc 2006)
	Anaerobic	No	4	Glucose+Galactose	(Carlson and Srienc 2006)
<i>Yarrowia lipolytica</i>	Aerobic	No	10	Acetate	(Li et al. 2016)

Under specific conditions that lead to cessation of growth, such as carbon starvation, PHB can be degraded by endogenous depolymerases to provide energy to the cell. The presence of PHB-degrading pathways is, however, not beneficial for the industrial application of natural producers, since PHB formation may be reduced (Jendrossek and Handrick 2002). Therefore, PHB accumulation was initially investigated in recombinant producers lacking the depolymerization step.

3.3.1.2 Recombinant producers

The advent of metabolic engineering triggered the investigation of PHB production in efficient cell factories such as bacteria and yeasts (Table 3). The bacterium *E. coli* proved to be a suitable host for the production of PHB from pure carbon sources, reaching PHB content levels as high as 77% from glucose (Kahar et al. 2005). In addition, *E. coli* grows faster than most of the natural producers, which is important in achieving high productivities. However, as mentioned above, this bacterium is susceptible to phage infections, which difficult its implementation in industry. Optimization of natural PHB producers has also been attempted. The bacterium *C. necator* has been engineered to expand the range of carbon sources that can be utilized. The xylose assimilation pathway native to *E. coli* was introduced in *C. necator* allowing PHB to be obtained from that sugar (Kim et al. 2016).

There are as yet no reports of eukaryotic organisms with a natural ability to produce PHB. However, the larger size of eukaryotic compared to bacterial cells may constitute a competitive advantage, since, theoretically, larger polymer granules could be formed (Li et al. 2016). Therefore, PHB production has also been attempted in transgenic plants, especially in those where acetyl-CoA is a precursor in the biosynthesis of fatty acids (see reviews by e.g. Scheller and Conrad 2005, and Snell et al. 2015). Among the yeast species, *Komagataella pastoris* (formerly known as *Pichia pastoris*) was investigated as a host for PHB production. The polymer was formed on glucose, but only at specific oxygen-limited levels (Vijayasankaran et al. 2005). More recently, a recombinant strain of *Yarrowia lipolytica* (initially named *Candida lipolytica*) was reported to produce PHB from acetate. The potential of this oleaginous yeast is based on its efficient lipid accumulation, which is linked to a high pool of acetyl-CoA, a compound that is also the precursor in PHB biosynthesis (Li et al. 2016). Finally, due to its physiological characteristics, the yeast *S. cerevisiae* was a natural candidate for engineering. *S. cerevisiae* can utilize complex carbon sources with relatively good efficiency and, as outlined in Chapter 1, the knowledge and genetic engineering techniques available for this yeast are considerable. In the following section, the key strategies and current advances in PHB production in *S. cerevisiae* are described in more detail.

3.3.2. Engineering of *S. cerevisiae* for PHB accumulation

3.3.2.1. Essential metabolic reactions

The production of PHB by *S. cerevisiae* was initially achieved from glucose through the heterologous expression of *PhaC* from *C. necator*, which encodes a PHB synthase (Leaf et al. 1996). In that study, the formation of PHB relied on the availability of cytosolic 3-hydroxybutyryl-CoA derived from the fatty acid beta-oxidation pathway. Later, the production of this polymer was increased by the addition of the two remaining enzymes involved in PHB production from acetyl-CoA in *C. necator* (Figure 8): acetyl-CoA acetyltransferase and acetoacetyl-CoA reductase (Breuer et al. 2002; Carlson and Srien 2006).

3.3.2.2. Impact of acetyl-CoA supply

Cytosolic acetyl-CoA is generated in *S. cerevisiae* from acetaldehyde in two steps:

- 1) acetaldehyde is first oxidized to acetate using a NADP⁺-dependent cytosolic aldehyde dehydrogenase, encoded by *ALD6*, and then
- 2) acetate is converted into acetyl-CoA via two isoenzymes - acetyl-coenzyme A synthetases (encoded by *ACS1* and *ACS2*), which are highly regulated. Both ACS isoenzymes are active under aerobic conditions, but only *Acs2p* (encoded by *ACS2*) is functional under anaerobic conditions. Furthermore, *ACS1* is repressed in the presence of glucose (de Jong-Gubbels et al. 1997).

When glucose was used as the sole carbon source, it was converted into ethanol, which was then channeled into the acetate node and further into acetyl-CoA and PHB (Carlson and Srien 2006; Kocharin et al. 2012). The overexpression of the genes *ADH2* (glucose-repressible alcohol dehydrogenase II) and *ALD6* successfully increased the ethanol conversion into acetate (Kocharin et al. 2012), indicating that higher levels of precursors were needed to improve PHB formation in *S. cerevisiae*. In another study that aimed at producing isoprenoids in yeast, it was shown that the addition of a mutated version of an ACS native to *Salmonella enterica* enhanced the cytosolic acetyl-CoA supply (Shiba et al. 2007). Therefore, Kocharin and coworkers applied the same strategy but for PHB production in yeast. The addition of this non-regulated and functional ACS indeed had a positive impact on PHB production (Kocharin et al. 2012). In the same study, the overexpression of *ERG10*, which encodes an alternative acetyl-CoA acetyltransferase, reduced the formation of other compounds that have acetyl-CoA as a precursor, such as free fatty acids, phospholipids and ergosterol. This modification also had a positive impact on the production of butanol in yeast, where the biosynthetic pathway, as in the case of PHB, is also dependent on the acetyl-CoA levels (Steen et al. 2008).

Another strategy employed involved removing pathways competing for acetyl-CoA utilization, which is not a trivial task since acetyl-CoA is involved in several metabolic pathways, some of which are highly associated with cell maintenance and survival. For instance, the *CIT2* and *MLS1* genes, encoding the citrate and malate synthases, respectively, were deleted in an attempt to limit acetyl-CoA utilization in the glyoxylate cycle. However, biomass formation and amino acid synthesis were severely compromised, and PHB accumulation decreased (Kocharin et al. 2012).

Elementary mode analysis of the PHB-producing pathway predicted that the addition of the enzyme ATP-citrate lyase could increase the PHB production in yeast by providing an alternative route for the formation of acetyl-CoA through cytosolic citrate at the expense of one ATP molecule (Carlson et al. 2002). Although this modification has not been evaluated *in vivo* for PHB production in yeast, it has been reported to increase both butanol and fatty acid formation in *S. cerevisiae*, for which acetyl-CoA is also the precursor (Lian et al. 2014; Tang et al. 2013).

3.3.2.3. Cofactor engineering

The enzyme AAR native to *C. necator* that is involved in the PHB-producing pathway, requires the cofactor NADPH (Figure 8). Therefore, metabolic engineering strategies to increase the pool of this cofactor, such as the addition of the phosphoketolase pathway and/or the non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase, have been attempted in yeast (Kocharin et al. 2013). The first involves xylulose-5-phosphate phosphoketolase and acetate kinase from *Aspergillus nidulans*, and this is expected to increase the intracellular pool of NADPH by increasing the carbon flux in the PPP; the main NADPH producing pathway in the cell (Stanton 2012). The second involves an NADP⁺-dependent enzyme native to *Streptococcus mutans*, which increases the volumetric ethanol yield under anaerobic conditions (Bro et al. 2006), meaning that an improvement in the conversion rate of glucose could be expected. These two alternatives were investigated both individually and simultaneously by Kocharin and coworkers, who found that the addition of the phosphoketolase pathway alone resulted in the best PHB-producing strain (Kocharin et al. 2013). The effect of replacing the AAR from *C. necator* by a novel NADH-dependent AAR native to the mesophilic purple sulfur bacterium *Allochromatium vinosum* was investigated in the present work in a xylose-utilizing strain (Paper II). This modification changed the redox state of the cell and allowed a completely anaerobic PHB formation from the pentose sugar. This constituted an important finding since anaerobic processes are generally preferred in industrial applications and PHB levels were considerably improved.

3.3.2.4. Substrate range

As highlighted in the two previous chapters, the utilization of xylose as a carbon source is essential if lignocellulosic biomass is to be used in biorefineries. Aerobic production of PHB from xylose was recently demonstrated using a recombinant *S. cerevisiae* strain

carrying the oxido-reductive xylose pathway from *S. stipitis* (Paper I). The amount of PHB produced was relatively low, although higher than the one previously reported on glucose (Table 3) (Kocharin et al. 2013).

In summary, PHB can be produced by yeast from hexose and pentose sugars, but the levels obtained are still lower than those observed with the best natural producers. Advances in metabolic engineering techniques have significantly improved PHB production in recombinant *S. cerevisiae* and this trend is expected to continue in the coming years.

3.3.3. Impact of nitrogen availability on PHB accumulation

In addition to the metabolic engineering strategies employed for PHB formation, it is also important to consider the conditions that lead to optimal polymer accumulation in the natural producers. PHB formation is optimal when there is excess carbon, while other elements, such as nitrogen or phosphorus, are limiting (Shang et al. 2003; Verlinden et al. 2007). Nitrogen limitation in particular often leads to the highest PHB levels reported in bacteria (Table 3) (Wang and Lee 1997). PHB accumulates due to its function as a carbon and energy reserve. The cells start accumulating this polymer after sensing nutrient limitation in order to increase their chances of survival when the carbon sources are fully depleted. This “survival” mechanism is tightly regulated, and it affords the organism a competitive advantage over another microbial species that do not accumulate PHB (Khanna and Srivastava 2005).

Nitrogen limitation is also related to carbon storage in wild-type *S. cerevisiae*, causing the formation of glycogen and trehalose when glucose is in excess (Lillie and Pringle 1980). The accumulation of these two carbohydrates is regulated by the level of glucose-6-phosphate (G6P), and it is directly linked to the duration of the G₁ phase of the growth cycle (François and Parrou 2001; Paalman et al. 2003). Benefits regarding cell survival and maintenance have been associated with glycogen and trehalose accumulation (François and Parrou 2001).

Bearing this in mind, it was important to evaluate the impact of the nitrogen level on PHB accumulation in recombinant yeast (Paper III). For further bioprocess optimization, it is important to understand whether polymer accumulation is related to the native carbon storage mechanisms. PHB accumulation in *S. cerevisiae* was demonstrated to be coupled to cell growth, which was in turn promoted by high levels of nitrogen. Under nitrogen starvation conditions, PHB formation decreased due to redirection of the carbon flux towards glycogen formation. This is in line with the results of previous studies on two yeast species, *S. cerevisiae* and *K. pastoris*, where the presence of limiting amounts of nitrogen did not improve PHB accumulation, and was attributed to a decrease in the production of recombinant protein (Carlson and Sreenc 2006; Vijayasankaran et al. 2005).

4. Yeast as a platform for carboxylic acid production

4.1. Carboxylic acids

Carboxylic acids are organic compounds consisting of carbonyl and hydroxyl functional groups, and can be used as building blocks for a wide range of products, including acidulants, flavor compounds and preservatives, and as a precursor for polymers and pharmaceuticals (Sandström et al. 2014). In 2015, the carboxylic acid market was estimated to be worth more than 12 billion euros, with a forecasted annual growth of 5% until 2024. This is expected to impact a variety of industrial sectors with relevance in the market, as shown in Figure 9. Asia is considered the leader in this field, accounting for approximately 50% of the worldwide production. The large potential of the carboxylic acid market is also confirmed by the considerable investments in this market being made by companies such as the Celanese Corporation, BASF, The Dow Chemical Company and the Eastman Chemical Company (TransparencyMarketResearch 2015).

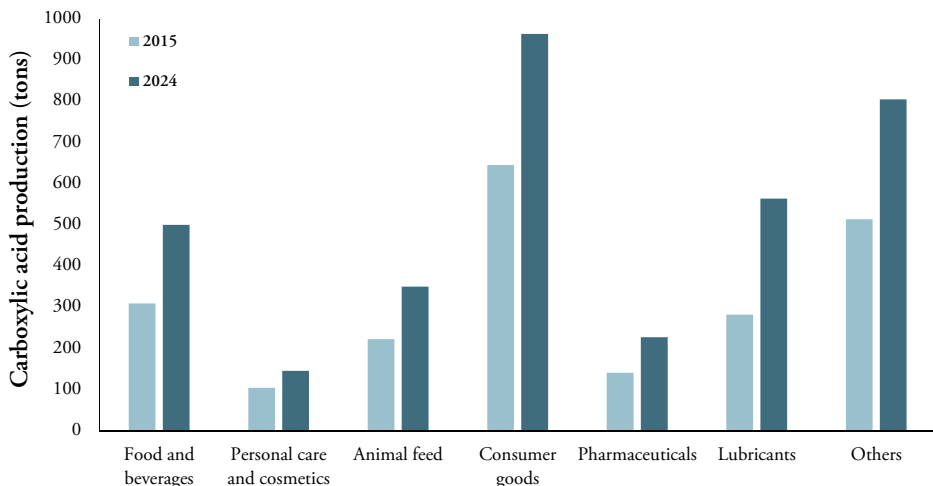


Figure 9 – Total annual production of carboxylic acids according to market sector/application, showing the amounts produced in 2015 and the predicted amounts for 2024.

(Adapted from <https://www.gminsights.com/industry-analysis/carboxylic-acid-market>).

The large-scale production of carboxylic acids is currently based on chemical synthesis processes, which are strongly dependent on fossil fuels. For instance, short-chain carboxylic acids are usually produced by the oxidation of aldehydes or hydrocarbons, by carbonylation, or through alcohol dehydrogenation (Riemenschneider 2000). In addition to environmental issues, these processes can be quite costly since they involve complex multi-step reactions. Furthermore, the chemical synthesis of some carboxylic acids is not yet possible (Werpy et al. 2004). Therefore, a biotechnological approach based on the ability of natural or recombinant microorganisms to form these compounds would be a suitable and promising alternative. A range of different carboxylic acids are intermediates in the microbial central carbon metabolism, and the number of studies carried out to improve bio-production has increased over recent years. The microbial production of carboxylic acids is described in more detail in the following section.

4.2. Microbial production of carboxylic acids

Several wild-type microorganisms secrete considerable amounts of carboxylic acids as part of their regular metabolic processes, and this can be explored for commercial applications. The secretion of compounds formed intracellularly is generally preferred for further downstream processing. However, this can be quite challenging since the transport mechanisms in most microorganisms have not yet been fully elucidated. Therefore, from a bioprocess point of view, cytosolic accumulation could also be beneficial since one less transport step would be involved in product secretion (Xu et al. 2012a; Zelle et al. 2008).

Citric and lactic acids are probably the most successful examples of the microbial production of acids, currently being produced on large-scale with the aid of *Aspergillus niger* and lactic acid bacteria, respectively (Abdel-Rahman et al. 2013; Vandenberghe et al. 1999). The development of microbial lactic acid production is particularly important since it is impossible to form an optically pure acid through chemical synthesis, and this property is essential to obtain high-quality biopolymers (Sauer et al. 2010).

Industrial production of succinic, fumaric and malic acids by microorganisms may be possible in the near future. Several bacterial species isolated from ruminants are natural succinic acid producers (Beauprez et al. 2010), but the corresponding fermentative processes are currently being scaled up using recombinant microbes. For example, the modified bacteria *Actinobacillus succinogenes* and *Corynebacterium glutamicum* have demonstrated high production levels, reaching titers of over 100 g/L (Guettler et al. 1996; Okino et al. 2008). There are also promising signs regarding the bio-production of fumaric acid by, for example, *Rhizopus* species (Cao et al. 1996). Companies such as

Dupont and Pfizer are devoting efforts to the commercialization of fumaric acid formed through fermentation by the natural producer *Rhizopus arrhizus*. Microbial production of malic acid has been reported to reach volumetric titers over 50 g/L with, for example, the fungus *Aspergillus flavus* (Battat et al. 1991; Zelle et al. 2008). Although these processes are currently being developed, significant improvements must be made to increase their competitiveness, regarding not only malic acid, but also succinic and fumaric acids, since the chemical synthesis route is still more efficient in terms of cost and yield (Xu et al. 2012c).

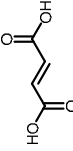
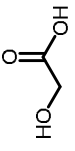
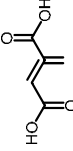
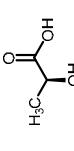
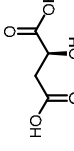

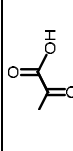
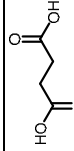
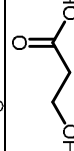
The bioprocess for itaconic acid production is a good example of the potential of microbes as cell factories since it is already considered more economically competitive than the corresponding chemical process. In fact, itaconic acid is being produced commercially with the fungus *Aspergillus terreus*, which reaches titers over 50 g/L (see review by Okabe et al. 2009). In addition to itaconic acid, acrylic, adipic, glutaric, glutaconic and 3-HP acids are examples of microbe-based processes that can offer significant advantages over the currently employed chemical routes, as they result in higher yields and productivities, offering both economic and environmental advantages (Djordjevic et al. 2011; Sandström et al. 2014; Straathof et al. 2005; Werpy et al. 2004). However, it is important to mention that some of these acids are not natural intermediates of microbial metabolism, and engineering of a microbial host is therefore required. The design of novel synthetic pathways and the development of metabolic engineering tools are thus of great importance in this field.

The unique characteristics of *S. cerevisiae* and its ability to utilize complex and cheap carbon sources are interesting for the production of carboxylic acids. The following section summarizes the main developments in the production of these compounds by recombinant yeast, focusing on the metabolic engineering strategies employed.

4.2.1. Yeast engineering towards the production of carboxylic acids

S. cerevisiae is a well-established and robust microorganism with proven suitability for large-scale bioprocesses. Its osmotolerance and its ability to grow in low-pH environments constitute significant advantages over other microbial hosts since the costs associated with neutralization and the risk of bacterial contamination are lower (Borodina et al. 2015; Xu et al. 2013). Furthermore, at low pH the free acid form, i.e. protonated, can be obtained directly without further steps (Lee et al. 2011). The detailed understanding of *S. cerevisiae* metabolism and the development of new engineering tools have resulted in several studies that aimed at the production of a variety of carboxylic acids (Table 4). The key engineering strategies are summarized below, while more detailed descriptions can be found in the literature (Abbott et al. 2009; Sandström et al. 2014).

Table 4 – Carboxylic acids whose production has been attempted in *S. cerevisiae*. The chemical structure, main industrial applications and an estimate of the annual production are also given (Adapted from Sandström et al. 2014).

Carboxylic acid	Structure	Applications	Annual production (kilotons/year)
Fumaric acid		Food additive, acidulant, polyester resins	200
Glycolic acid		Packaging, cosmetics, textiles, medical applications, precursor of polyglycolic acid	40
Itaconic acid		Bioplastics, substitute for polyacrylic acid	50
Lactic acid		Food preservative, bioplastics, polyester	450
Malic acid		Food additive, acidulant	60
Muconic acid		Precursor of adipic acid, bioplastics	NA
Pyruvic acid		Medical applications, food additive	NA
Succinic acid		Detergent, surfactant, food additive, precursor for pharmaceuticals	37
3-hydroxypropionic		Biodegradable polymers, precursor of acrylic acid	3 600

NA: Not applicable

Additionally, Figure 10 provides a schematic overview of the locations of the different metabolic intermediates that can be used as precursors for the production of the carboxylic acids listed in Table 4.

Fumaric acid is a TCA cycle intermediate, and its formation in *S. cerevisiae* has been attempted via both the oxidative and the reductive routes (Figure 10). The reductive route, involving pyruvate carboxylase, does not result in ATP formation, implying that cell maintenance and acid secretion processes may be severely compromised. Therefore, the gene encoding this endogenous enzyme was overexpressed, and the fumarase and malate dehydrogenase native to the natural producer *R. oryzae* were also integrated in *S. cerevisiae* (Xu et al. 2012a). Fumaric acid production through the oxidative route was later accomplished with the aid of *in silico* modeling (Xu et al. 2012b). Finally, the overexpression of the heterologous gene from *R. oryzae* coding for pyruvate carboxylase was shown to improve fumaric acid production (Xu et al. 2013). Under nitrogen limiting conditions, more than 5 g/L fumaric acid could be formed with the modified *S. cerevisiae*.

Cytosolic reduction of glyoxylate into **glycolic acid** has been accomplished in yeast by the expression of the gene encoding a high-affinity glyoxylate reductase from *Arabidopsis thaliana* (Koivistoinen et al. 2013). In addition, the cytosolic isocitrate dehydrogenase gene (*IDP2*) was deleted to avoid the carbon flux towards AKG and amino acid synthesis. *ICL1*, encoding isocitrate lyase, was simultaneously overexpressed to increase the flux towards glyoxylate and further into glycolic acid.

Microbial production of **itaconic acid** has been demonstrated to have tremendous potential, and its formation has been investigated in *S. cerevisiae* by engineering the *cis*-aconitic acid decarboxylase native to *Aspergillus terreus*. Genome-scale *in silico* modeling predicted that the reduction of the metabolic flux towards amino acid synthesis would significantly improve itaconic acid production, as was experimentally demonstrated by a 30% increment in the volumetric titer (Blazeck et al. 2014). The levels obtained were still low compared to *A. terreus*, indicating that the carbon flux in the TCA cycle should be increased in order to improve the production of itaconic acid.

In contrast to lactic acid bacteria, *S. cerevisiae* cannot form **lactic acid** without the aid of metabolic engineering. The functional expression of a heterologous gene encoding a lactate dehydrogenase has been found to have a considerable effect on the final lactic acid titer (Sauer et al. 2010). Among other modifications (see review by Sauer et al. 2010), it has been demonstrated that the pyruvate decarboxylase (PDC) and the alcohol dehydrogenase should be inactivated in order to redirect the carbon flux from ethanol to lactic acid formation. Later, the simultaneous inactivation of the ethanol and glycerol formation routes was coupled with the engineering of an NADH-dependent lactate dehydrogenase. This strategy showed good potential for the fully anaerobic production of lactic acid, but it was still necessary to optimize product formation (Ida et al. 2013). In addition, cytosolic NADH levels were increased by deleting the genes encoding the

cytosolic NADH dehydrogenases, resulting in the formation of 117 g/L lactic acid (Lee et al. 2015). In a more process-related study, it was found that strains with a higher intracellular pH produced more lactic acid, and cell sorting was thus used to select the most efficient producers (Valli et al. 2006). Lactic acid production from xylose has also been described recently (Turner et al. 2015).

The formation of **malic acid** by yeast was investigated by fine-tuning the carbon flux at the pyruvate node. Simultaneous reduction of PDC and increase of pyruvate carboxylase activities, together with the expression of a heterologous malate transporter resulted in the formation of malic acid in yeast (Zelle et al. 2008). The study also highlighted the importance of fine-tuning the carbon fluxes through the TCA cycle, fermentative metabolism and the glyoxylate shunt.

Yeast engineering towards **muconic acid** production has been reported based on the ability of some microbial species to utilize aromatic compounds as carbon sources by the formation of this acid from catechol, which is derived from phosphoenolpyruvate (PEP). In the first study, increasing the carbon flux through the aromatic amino acid biosynthetic pathway and blocking the conversion of 3-dehydroshikimate into shikimate were essential for the production of muconic acid by yeast (Weber et al. 2012). The formation of this acid through the aromatic biosynthetic pathway was further increased by the expression of genes native of different species in combination with, for example, a rewired PPP (Curran et al. 2013).

Elimination of PDC activity has been used as a rational strategy to block the conversion of pyruvate into ethanol while still allowing respiratory growth to maintain cell viability. However, the resulting strain was unable to grow on glucose, possibly due to the essential role of PDC in cytosolic acetyl-CoA biosynthesis (Flikweert et al. 1996). A later evolutionary engineering approach resulted in a strain not only capable of assimilating glucose, but which could produce up to 135 g/L **pyruvic acid** (van Maris et al. 2004). More recently, an alternative approach based on having different levels of PDC activity has been evaluated, in which approximately 31% of the maximum theoretical yield from glucose was obtained (Wang et al. 2015).

Oxidative production of **succinic acid** was initially achieved by elimination of the mitochondrial enzymes succinate dehydrogenase and isocitrate dehydrogenase. The first was intended to block the conversion of succinate to fumarate in the TCA cycle, while the second modification aimed at redirecting the carbon flux towards cytosolic succinate, which is linked to glyoxylate shunt activity. Succinic acid was secreted into the culture medium with relatively high efficiency, which is important in industrial applications (Raab et al. 2010). A succinic acid-producing strain was later obtained by combining systems biology and direct evolution, resulting in significantly improved volumetric titers and yields (Otero et al. 2013). The effect of inactivating the metabolic routes involved in the formation of ethanol or glycerol has also been investigated (Ito et al. 2014; Yan et al. 2014).

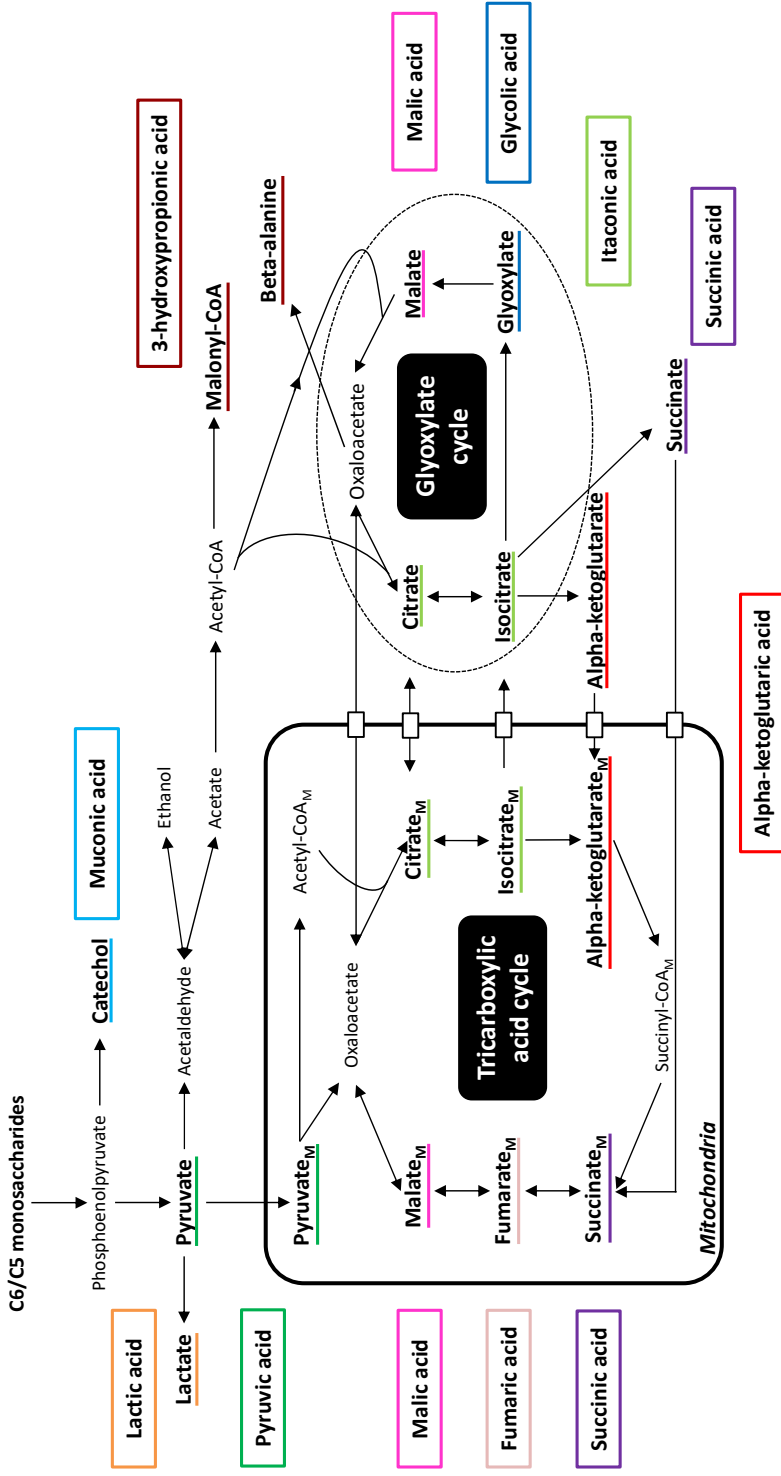


Figure 10 – Metabolic scheme highlighting the intermediates of the central carbon metabolism that can be used as precursors (underlined) for the production of carboxylic acids (boxes). The different colors indicate the main precursor(s) of each carboxylic acid.

Among the different synthetic routes developed for microbial 3-HP production, the pathways involving malonyl-CoA or beta-alanine, which are both associated with cytosolic acetyl-CoA, have recently been demonstrated to have considerable potential to reach high titers in *S. cerevisiae* (Borodina et al. 2015; Chen et al. 2014; Kildegaard et al. 2016). Considering that 3-HP formation involves a series of NADPH-dependent reactions and has acetyl-CoA as precursor, a similar strategy to the one used for isobutanol and PHB production was investigated (Chen et al. 2014). The NADPH levels were increased by the engineering of glycolysis, through the addition of an NADP⁺-dependent glyceraldehyde-3-phosphate dehydrogenase. Ethanol assimilation was improved in order to increase the flux towards acetyl-CoA and further into 3-HP (Chen et al. 2014). A more recent study focused on the optimization of 3-HP production by increasing the copy numbers of key genes involved in the biosynthetic route and fine-tuning the levels of precursors and redox cofactors. In this study, approximately 10 g/L 3-HP could be obtained from glucose (Kildegaard et al. 2016).

The engineering strategies to increase the accumulation of carboxylic acids that act as intermediates in the central carbon metabolism have some common patterns. The flux distribution at the pyruvate node has a key role, and disruption of the ethanol-producing pathway can be essential to redirect the carbon to the product of interest. In addition, a fine balance must be achieved between respiratory growth, the glyoxylate shunt and fermentative metabolism in order to maintain cell viability, as well as the intracellular levels of cofactors, during carboxylic acid formation. All these factors and engineering approaches were taken into consideration in the present work for the construction of a *S. cerevisiae* strain capable of accumulating increased levels of AKG, which is further described in the next section and in Paper IV.

4.3. The case study of AKG

4.3.1. Why AKG?

Alpha-ketoglutaric acid, also known as 2-oxoglutaric acid, is the most common ketone derivative from glutaric acid, and the corresponding salt, alpha-ketoglutarate (AKG), plays a significant biological role in cell metabolism (Pandey et al. 2015). AKG is a key metabolic intermediate involved in both the TCA and glyoxylate cycles, and it also links the central carbon to the central nitrogen metabolism, where the synthesis of several amino acids takes place (Figure 11). Besides its applications as a dietary and wound-healing supplement, AKG is also used as a building block in fine chemistry (Lininger and Wright 1998; Matzi et al. 2007; Stottmeister et al. 2005; Verseck et al. 2009). Importantly, AKG can be used as a precursor for the production of acids with novel characteristics that are not yet being produced on a large scale, such as glutaric,

glutaconic and glutamic acids (Bajaj and Singhal 2011; Djurdjevic et al. 2011; Dutta et al. 2013; Fink 2013; Sailakshmi et al. 2013; Sung et al. 2005).

Currently, AKG is usually synthesized chemically (Stottmeister et al. 2005), but this involves multi-step processes and high temperatures, the use of hazardous chemicals associated with the risk of explosion, and the formation of side products. Therefore, more attention is being directed to the biotechnological production of AKG since this constitutes an ecologically and economically attractive alternative (Aurich et al. 2012; Otto et al. 2011).

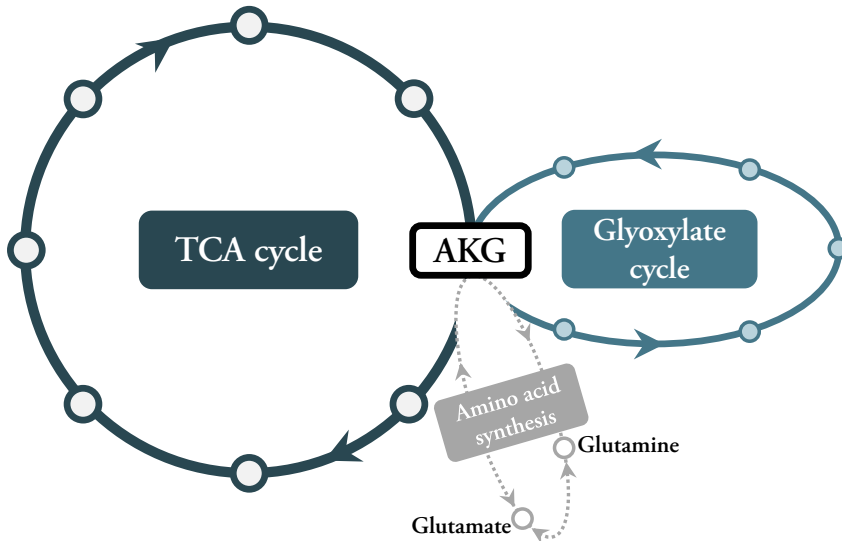


Figure 11 – AKG plays an important role in the mitochondrial metabolism, as an intermediate in the glyoxylate shunt, and also as a precursor for the synthesis of amino acids such as glutamate and glutamine.

4.3.2. Microbial production of AKG

The microbial production of AKG was first described in 1946, when the bacterium *Pseudomonas fluorescens* was found to have the ability to produce this carboxylic acid from glucose (Lockwood and Stodola 1946). Several wild-type bacteria have subsequently been described as AKG producers, mainly from glucose and/or *n*-alkanes (Asai et al. 1955; Tanaka et al. 1969). Strains of the yeast *Y. lipolytica* capable of producing AKG from *n*-alkanes were isolated from natural sources in 1969 (Tsugawa et al. 1969). Later, specific species of *Candida* and *Pichia* and also the multi-vitamin auxotrophic yeast *Torulopsis glabrata* were shown to accumulate AKG (Chernyavskaya et al. 1997; Chernyavskaya et al. 2000; Liu et al. 2003). The cytosolic production of

AKG in *S. cerevisiae* was attempted in the present work by redirecting the carbon flux towards the glyoxylate shunt (Paper IV).

4.3.2.1. Cytosolic AKG production in *S. cerevisiae*

The redirection of the carbon flux towards cytosolic AKG was attempted in this work following two strategies: (1) by blocking AKG consumption in the TCA cycle, and (2) by limiting the cytosolic NADPH regeneration pathways in the oxidative branch of the PPP (Paper IV). The AKG dehydrogenase complex, which catalyzes the mitochondrial conversion of AKG to succinate, was inactivated by deletion of the gene encoding the enzyme dihydrolipoyl transsuccinylase, *KGD2*. Deletion of *ZWF1*, encoding glucose-6-phosphate dehydrogenase (G6PD), was intended to limit NADPH formation through the PPP, and therefore force NADPH generation through the cytosolic reduction of isocitrate into AKG (Figure 4). The combined deletion of *KGD2* and *ZWF1* had severe effects on cell viability. The interruption of the TCA cycle limited the formation of ATP in the cell, resulting in impaired growth. This indicated that an intermediate activity of the TCA cycle was required for growth-coupled AKG formation, allowing biomass formation, but at a rate low enough to allow the accumulation of metabolic intermediates such as AKG. The reintegration of *KGD2* under a copper-inducible promoter was investigated to establish whether it was possible to control the carbon flux through the TCA cycle. In addition, a heterologous phosphoenolpyruvate carboxykinase (PEPCK) was introduced to offer an alternative metabolic route connecting glycolysis with the glyoxylate shunt (Figure 4). Although AKG formation was low, this study contributed to a deeper understanding of the carboxylic acid formation in *S. cerevisiae*, paving the way for future optimization studies (Paper IV).

5. Conclusions and outlook

Saccharomyces cerevisiae has tremendous potential as a microbial cell factory, and both metabolic engineering and synthetic biology are being used to broaden the range of compounds that can be obtained from this yeast. The main goal of the present work was to explore the capacity of *S. cerevisiae* to produce the biopolymer PHB and the carboxylic acid AKG. The studies were conducted mostly with xylose-rich carbon sources, bearing in mind the desire to use lignocellulosic biomass as a substrate employing the biorefinery concept. Although the levels of PHB and AKG obtained with recombinant *S. cerevisiae* were low compared with natural producers, and far from those required in industrial applications, important knowledge was obtained on the advantages and drawbacks of *S. cerevisiae* as a cell factory, as summarized below.

- The cytosolic level of acetyl-CoA is limiting, mostly due to its utilization as a biomass precursor, which reduces the accumulation of PHB in a growth-related manner.
- A change in cofactor preference in the PHB formation pathway, making it NADH-dependent, is required in yeast to match the redox state of *S. cerevisiae*. This requirement is not observed in natural producers, since PHB formation is not coupled to growth, and NADPH is thus available for PHB synthesis.
- The supply of excess nitrogen was beneficial to the accumulation of PHB, resulting in one of the highest PHB contents reported in the literature to date. In contrast to the natural producers, nitrogen limitation in yeast only affected the carbon flux towards glycogen.
- PHB could be formed from both glucose and xylose under different levels of aeration, which opens the possibility for the utilization of complex carbon sources such as lignocellulosic biomass.
- As the oxido-reduction routes for xylose assimilation have different cofactor preferences, the choice of pathway was essential to balance the redox according to the substrate and heterologous producing pathway, in contrast to the XI route.
- The cytosolic formation of AKG in a growth-coupled manner requires intermediate activity of the TCA cycle since its complete interruption by

blocking the mitochondrial utilization of AKG was so drastic that cell growth could not be sustained.

- The Weimberg pathway constitutes a promising alternative to the commonly oxido-reductive or isomerization routes engineered in yeast, bypassing glycolysis and linking directly to the mitochondrial metabolism. Although the genes encoding the Weimberg pathway could be expressed and transcribed in *S. cerevisiae*, the functionality of the proteins converting D-xylonate into AKG remains unknown. The dehydration reactions appear to be the major bottlenecks in achieving an active Weimberg pathway, since the correct assembly of [FeS] clusters in bacterial enzymes is very challenging in yeast.

The insights provided by this work pave the way for further optimization studies. For instance, targeted evolutionary engineering by the application of flow cytometry techniques is a promising way to select the best producers from the overall population, and increase volumetric yields and productivities. The evaluation of recombinant strains with different levels of TCA cycle activity, together with a deeper understanding of the transport and secretion mechanisms of carboxylic acids, can be expected to significantly improve the microbial production of carboxylic acids. Further investigations of the proteomic profile of strains carrying the Weimberg pathway should provide valuable insight into a functional route in *S. cerevisiae*. Finally, fine-tuning of the specific activity of the various enzymes involved should also be investigated to achieve a balanced flux in the upper and lower parts of the pathway, thus avoiding the accumulation of the intermediates and, possibly, their inhibitory effects.

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