

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

**Metabolic Engineering**  
*of *Saccharomyces cerevisiae**  
**for Polyhydroxybutyrate Production**

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CHALMERS UNIVERSITY OF TECHNOLOGY  
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## Cover:

Schematic illustration of metabolic engineering of the central carbon and redox metabolism aiming to increase cytosolic acetyl-CoA and NADPH supply for polyhydroxybutyrate (PHB) production in *Saccharomyces cerevisiae*.

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## PREFACE

This PhD dissertation serves as a partial fulfillment of the requirements for a PhD degree at Chalmers University of Technology, Sweden. The PhD project was carried out in the Systems and Synthetic Biology group, Department of Chemical and Biological Engineering under supervision of Professor Jens Nielsen as the main supervisor, Asst. Professor Keith Tyo (January 2009 - December 2010) and Verena Siewers (January 2011- April 2013) as co-supervisors.

When I first started my PhD in January 2009, my PhD research was initiated with a challenging project to establish the novel expression system in *Saccharomyces cerevisiae*. Later on in 2011, my research was entered the field of metabolic engineering in *S. cerevisiae*. I believe the knowledge and expertise gained throughout the years during my PhD research could potentially advance the research in metabolic engineering and serve as a powerful tool for research in Systems and Synthetic Biology.

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Kanokarn Kocharin

April 2013

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## ABSTRACT

Establishing industrial biotechnology for the production of chemical compounds from the biosynthetic pathway has received a significant boost with the implementation of metabolic engineering. At present, metabolic engineering in *Saccharomyces cerevisiae* gains significant advantages of integration of knowledge acquired through a long history of use and data acquisition from novel –omics technologies hence enabling the development of a tailor-made *S. cerevisiae* with desired features for various industrial applications.

With regard to environmentally friendly (eco-friendly) materials, engineering of biodegradable polyhydroxybutyrate (PHB) producing microbes has been studied as a potential alternative to petroleum-based thermoplastics. Heterologous expression of the bacterial PHB biosynthesis pathway in *S. cerevisiae* involves the utilization of acetyl-CoA, an intermediate of the central carbon metabolism, as precursor and NADPH, a redox cofactor used during anabolic metabolism, as a required cofactor for the catalyzing enzymes in the PHB biosynthesis pathway. Provision of acetyl-CoA and NADPH by alteration of the endogenous pathways and/or implementation of a heterologous gene/pathway was investigated with the aim to improve PHB production in *S. cerevisiae*. Since the specific growth rate and the type of carbon source (fermentable/non-fermentable) influence cell physiology and affect the growth of *S. cerevisiae*, PHB production was examined at different specific growth rates on different carbon sources. Overexpression of genes in the native ethanol degradation pathway and heterologous expression of a phosphoketolase pathway from *Aspergillus nidulans* aiming to increase the production of cytosolic acetyl-coA and chromosomal integration of *gapN* from *Streptococcus mutans* to enhance the availability of NADPH were evaluated for their possibility to promote PHB production in *S. cerevisiae*. The enhancement of acetyl-CoA and NADPH either by the combined strategies of the ethanol degradation pathway and *gapN* or utilization of the phosphoketolase pathway resulted in the improved PHB content from 4 mg/gDW in the reference strain to approximately 28 mg/gDW. It is difficult for *S. cerevisiae* to compete with other natural PHB producers like *Ralstonia eutropha* which benefit from native enzymes for the biosynthesis or with the engineered *E. coli* since the metabolism in *S. cerevisiae* is more complex and involves compartmentalization and shuttle systems for precursor and redox balancing. However, the strategies employed in this study involve both engineering of the central carbon and redox metabolism and it demonstrated that it is possible to substantially improve PHB production. Furthermore, the applied strategies may well be suitable also for improving the production of other chemicals, derived from acetyl-CoA and requires NADPH for its biosynthesis.

Keywords: *S. cerevisiae*, Metabolic engineering, Polyhydroxybutyrate, Acetyl-CoA, NADPH

## LIST OF PUBLICATIONS

This thesis is based on the work contained in the following publications:

- I. Tyo KEJ, Kocharin K, Nielsen J. Toward design-based engineering of industrial microbes. *Curr Opin Microbiol* 2010. 13(3):255-262.
- II. Kocharin K, Tyo KEJ, Siewers V, Nielsen J. Chemical induced chromosomal evolution in *Saccharomyces cerevisiae*. (Manuscript in preparation)
- III. Kocharin K, Chen Y, Siewers V, Nielsen J. Engineering of acetyl-CoA metabolism for the improved production of polyhydroxybutyrate in *Saccharomyces cerevisiae*. *AMB Express*. 2012. 2(1):52.
- IV. Kocharin K, Nielsen J. Specific growth rate and substrate dependent polyhydroxybutyrate production in *Saccharomyces cerevisiae*. (Manuscript accepted for publication in *AMB Express*)
- V. Kocharin K, Siewers V, Nielsen J. Improved polyhydroxybutyrate production by *Saccharomyces cerevisiae* through the use of the phosphoketolase pathway. *Biotechnol Bioeng*. 2013. [Epub ahead of print]

## CONTRIBUTION SUMMARY

- I. KK co-wrote some parts and created some of the figures in the review with KT and JN.
- II. KT and JN conceived the study. KT and KK participated in the design of experiments. KK performed all the experiments, analyzed the data and wrote the manuscript. KT, VS and JN edited the manuscript.
- III. KK and JN designed the study. JN and VS supervised the project and edited the manuscript. YC contributed with the plasmid, pIYC08, used in this study. KK performed the experimental work, analyzed the data and wrote the manuscript.
- IV. KK and JN participated in the design of the experiment. KK performed all the experiments, analyzed the data and wrote the manuscript. JN edited the manuscript.
- V. KK, VS and JN participated in the design of the experiment. KK performed all the experiments, analyzed the data and wrote the manuscript. JN and VS edited manuscript.

## ABBREVIATIONS AND NOMENCLATURES COMMONLY USED

<i>S. cerevisiae</i> :	<i>Saccharomyces cerevisiae</i>
<i>E. coli</i> :	<i>Escherichia coli</i>
<i>R. eutropha</i> :	<i>Ralstonia eutropha</i>
<i>S. mutans</i> :	<i>Streptococcus mutans</i>
<i>S. enterica</i> :	<i>Salmonella enterica</i>
<i>A. nidulan</i> :	<i>Aspergillus nidulan</i>
CIChE:	Chemical induced chromosomal evolution
qPCR:	Quantitative real-time polymerase chain reaction
PHB	Polyhydroxybutyrate
NADPH:	Nicotinamide adenine dinucleotide phosphate hydrogen
PP pathway:	Pentose phosphate pathway
<i>phaA</i> :	Acetyl-CoA acetyltransferase
<i>phaB</i> :	NADPH-linked acetoacetyl coenzyme A (acetyl-CoA) reductase
<i>phaC</i> :	Poly-3-hydroxybutyrate polymerase
<i>ADH2</i> :	Alcohol dehydrogenase
<i>ALD6</i> :	Aldehyde dehydrogenase
<i>ERG10</i> :	Acetyl-CoA acetyltransferase
<i>acs</i> <sup>L641P</sup> :	Acetyl-CoA synthetase variant
<i>xpk</i> :	Phosphoketolase
<i>ack</i> :	Acetate kinase
<i>gapN</i> :	NADP <sup>+</sup> -dependent glyceraldehyde-3-phosphate dehydrogenase
<i>CIT2</i> :	Citrate synthase
<i>MLS1</i> :	Malate synthase

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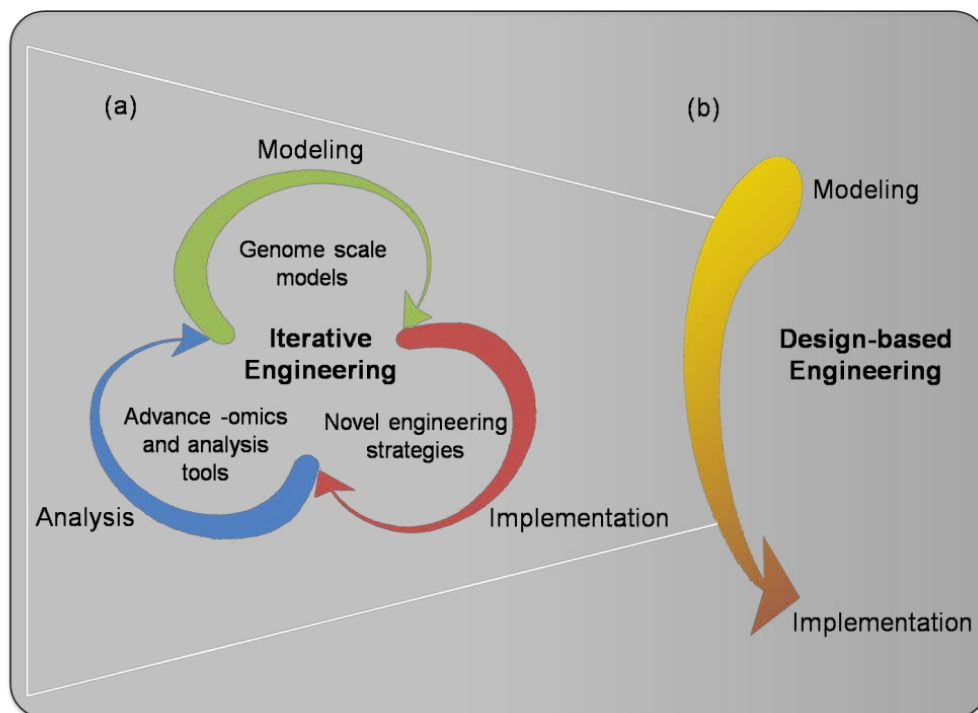
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## **CHAPTER 1. Introduction**

### **1.1. Engineering of industrial microbes**

Microbial production of chemical compounds offers several advantages over conventional chemical synthesis. Much improvement of microbial and cellular processes using industrial microbes has been achieved by improving the cell factory through an iterative cycle of modeling, implementation and data analysis (Figure 1a), often referred to as the metabolic engineering cycle. Genome-scale metabolic models (GEMs) have been used to predict changes in enzyme reaction rates (fluxes) in response to nutrient or genetic perturbations thus allowing the prediction of cellular alteration that will increase the production of a desired product. Stoichiometric models have been created through curating enzyme biochemistry literature and using comparative genomics approaches for organisms that have little biochemical evidence. The addition of regulatory information to the stoichiometric models as well as identifying the reversibility/irreversibility of enzymatic reactions by calculating the thermodynamic feasibility can improve the predictive capabilities of the models (Feist et al. 2007; Henry et al. 2007; Herrgard et al. 2006). The next step in the iterative metabolic engineering cycle is the implementation of strategies offered by the predictive model to construct the engineered microbe with the desired characteristics. Implementation generally consists of using strong plasmid-based overexpression, knockout and/or introduction of one or more heterologous gene(s). Instead of using a binary option of strong overexpression and deletion which can cause a metabolic burden in the cell (Gorgens et al. 2001), a well fine-tuned alteration of constitutive expression levels such as by engineering of a constitutive strong promoter (Nevoigt et al. 2006) and chemical induced chromosomal evolution (CIChE) is more attractive for strain improvements (Tyo et al. 2009). As models are not 100% predictive and molecular implementations may therefore not perform as expected, the engineered cells must be analyzed after construction. Technological advancements have enabled -omics measurements and hence generated a massive amount of -omics data of engineered microbes. Due to the availability of databases, a great number of mathematical models and integrative strategies have been developed and established as systems biology tools to integrate and visualize multidimensional data. Model reconstruction benefits from the integrative analysis of -omics data. Therefore, many models are significantly revised after

incorporating new biological information from the integrative –omics data analysis (Österlund et al. 2012). As much more information is gathered from each iterative cycle of modeling, implementation and analysis as well as the advancement in systems biology enable the transition from iterative engineering of industrial microbe to the design-oriented paradigm (Figure 1b).

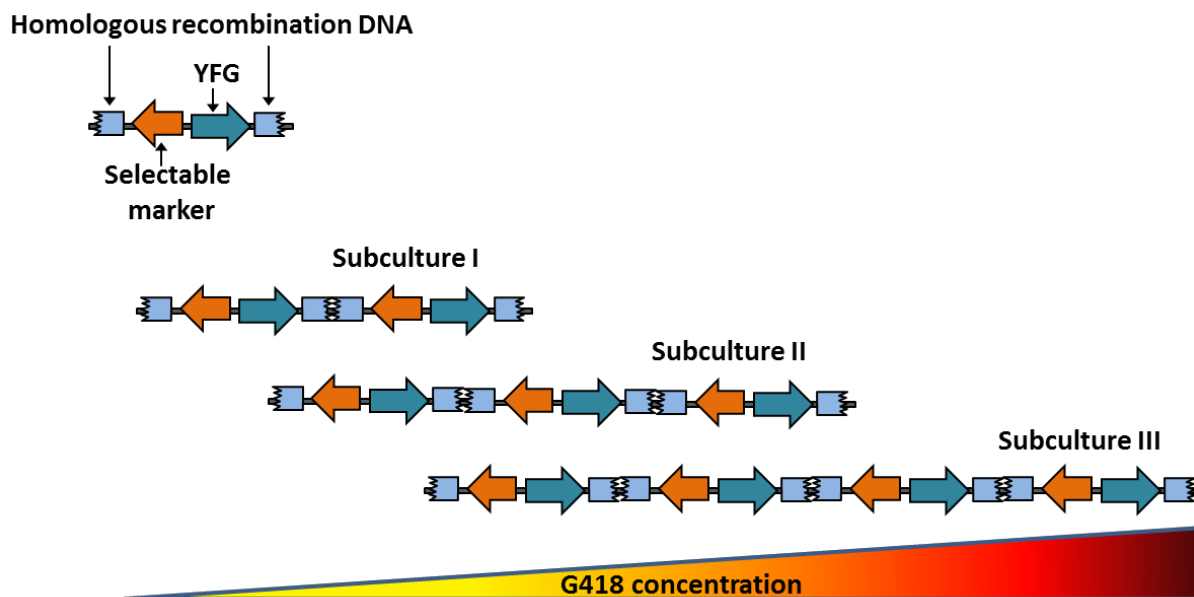


**Figure 1 Transitioning from iterative to linear design-based engineering of industrial microbes.** (a) Iterative engineering – genome-scale models describe the cellular processes under desired conditions. These models guide implementation of engineering strategies. These implementations are characterized by –omics and integrated analysis tools, which are used to revise the model and improve the predictive capability. (b) Design-based engineering – models and implementation tools are reliable enough that the expected outcome is usually achieved, as in civil and electrical engineering. The transition from an iterative cycle to a linear design-based engineering will be accomplished by better modeling and implementation aided by enhanced analysis (Tyo et al. 2010b).

## 1.2. CICHÉ as a molecular tool for implementation of desired features

Traditionally, improvement of microbial and cellular processes were achieved mainly by evolutionary (classical) breeding methods or repeated rounds of mutagenesis and selection of a desired phenotype (Kern et al. 2007). Until now, evolution engineering is still very useful for engineering of industrial microbes, since the implementation of constructive engineering from models may be hampered by the difficulty of predicting secondary responses or side effects of inter-related regulatory and metabolic processes in a cell (Conrad et al. 2011; Sauer 2001; Steen et al. 2008). In order to troubleshoot this difficulty, novel expression systems which allow fine-tuning of the expression system rather than a strong expression system without the scalability (either by overexpression or by deletion) have been developed (Alper et al. 2005; Nevoigt et al. 2006).

A novel fine-tuned expression system based on chemical induced chromosomal evolution (CICHÉ) has been successfully developed and implemented in *Escherichia coli* (Tyo et al. 2009). The CICHÉ in *E. coli* is a stabilized plasmid-free expression system which allows ~40 copies of a recombinant pathway to be implemented on the chromosome. This strategy uses an inducible promoter, antibiotic resistant marker, in combination with the *E. coli recA* homologous recombination system to introduce the genes of interest into the chromosome and evolve the strain with chromosomal integration in the increased antibiotic concentration thus resulting in an increased copy number of the integrated genes. This expression system is stabilized by the deletion of *E. coli recA* to prevent further change of the evolutionary recombinant pathway through homologous recombination. The resulting strain therefore requires no selection pressures and is unaffected by genetic instabilities. In case of *Saccharomyces cerevisiae*, most of the studies on chromosomal integration were carried out in order to implement a stable expression system for heterologous genes. However, when using the common integrative plasmids, in most cases only one copy of foreign DNA is integrated into the chromosome resulting in generally low expression levels (Plessis and Dujon 1993). Therefore, this expression system has been improved by using the long terminal repeats (LTRs) of Ty retrotransposons (e.g. delta or sigma elements) sequence and ribosomal DNA sequences to introduce multiple integrations and increased expression levels (Boeke et al. 1991; Fujii et al. 1990; Sakai et al. 1990).



**Figure 2 Chemical Induced Chromosomal Evolution (CIChE) strategy:** The expression level of the genes of interest is controlled by the copy number of tandem repeats on the chromosome which corresponds to the concentration of antibiotic used as a selection pressure during the chromosomal evolution. YFG = Your Favorite Gene (Paper 2)

In order to develop a stable fine-tuned expression system in *S. cerevisiae*, the CIChE strategy was applied and investigated for heterologous expression. The CIChE strategy was implemented in *S. cerevisiae* by using a bacterial  $neo^r$  as a selectable marker.  $Neo^r$ , an aminoglycoside-3'-phosphotransferase, inactivates G418, aminoglycoside antibiotic which block protein synthesis by inhibit at the polypeptide elongation phase (Eustice and Wilhelm 1984). Since the bacterial  $neo^r$  used in this strategy is derived from a transposon Tn5, and no eukaryotic promoter is used in the construct, therefore the expression level is relatively low. Moreover, the increased copies of  $neo^r$  are direct proportional to the concentration of G418 it inactivates, therefore using  $neo^r$  as a selectable marker enables the CIChE integrative construct to be evolved when increasing antibiotic concentration. Two different types of integration sequences for homologous recombination were investigated for their integrative capability. The delta element of the Ty1 retrotransposon was selected as site for random integration of the insulin precursor while *his3Δ1* was selected for targeted integration of *gapN* into the yeast chromosome. The CIChE constructs used for either random integration or

targeted integration share the common design where the gene of interest and the selective marker are in the middle of the construct flanked by the homologous sites used for the chromosomal integration. After the whole construct was integrated into the chromosome, the evolutionary process was performed by repeated subculturing in medium containing a stepwise increase of antibiotic concentration as illustrated in Figure 2. The results of CICH implementation in *S. cerevisiae* will be described in chapter 2.1.

### **1.3. Metabolic engineering and the industrial biotechnology process**

During the past decades, metabolic engineering has been used extensively to improve the cellular processes in industrial microbes with the purpose of improving the product yield, broaden the range of substrate utilization, reduction of by-product formation and introduction of novel pathways leading to new products. By the definition of metabolic engineering given (Bailey et al. 1996; Nielsen 1998; Stephanopoulos and Sinskey 1993), metabolic engineering comprises the analysis of metabolic pathways using metabolic flux analysis to identify targets for manipulation and directed genetic modification in order to achieve the desirable phenotype. With the introduction of recombinant DNA technology, specifically to modify the targeted gene(s) or metabolic pathways, strain improvement has gradually shifted from classical breeding or random mutagenesis to a more rational approach. At present, through the advance in systems biology and the emerging of synthetic biology, metabolic engineering has gradually evolved and becomes more successful in designing tailor-made cell factories (Keasling 2012; Nielsen and Keasling 2011; Yadav et al. 2012).

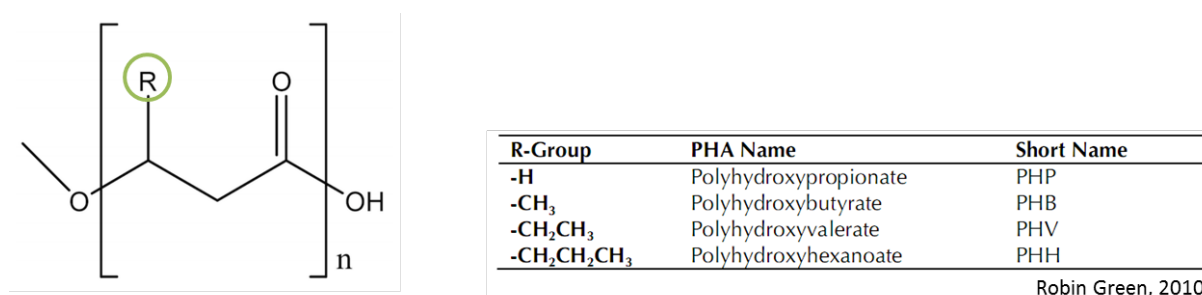
#### **1.3.1. *S. cerevisiae* as a host for heterologous expression**

*S. cerevisiae* has been used as a cell factory for the production of a wide range of industrial products due to the available knowledge obtained from a long history of its use and the ease of growth and its genetic manipulation. Several examples of industrial applications such as biofuels, biorefinery compounds and pharmaceutical proteins are reviewed (de Jong et al. 2012; Hong and Nielsen 2012; Hou et al. 2012; Martinez et al. 2012). Since the original sequence of the reference *S. cerevisiae* strain was released in 1996 (Goffeau et al. 1996) and

the *Saccharomyces* Genome Database became available (Cherry et al. 2012; Dwight et al. 2004), the genotype-phenotype relation of *S. cerevisiae* has been characterized in more detail. Furthermore, due to the extensive conservation of genes and pathways to higher eukaryotic cells, *S. cerevisiae* has also shown potential of being used as a model organism for pharmaceutical and medical application (Hou et al. 2012; Martinez et al. 2012; Munoz et al. 2012).

### 1.3.2. Polyhydroxybutyrate (PHB) as a target product

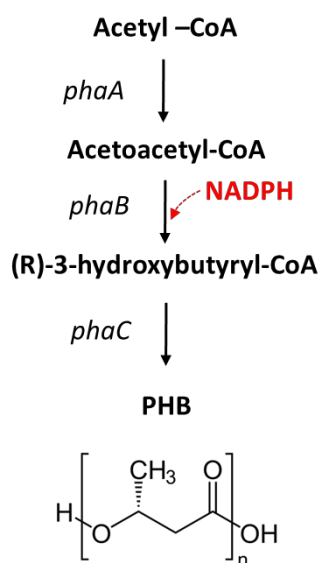
Polyhydroxyalkanoates (PHAs) are linear polyesters composed of several units of hydroxyalkanoate and produced by many microorganisms (Akaraonye et al. 2010; Green 2010; Khanna and Srivastava 2005) such as *Alcaligenes eutrophus* (Doi et al. 1992), *Pseudomonas aeruginosa* (Pham et al. 2004) and *Bacillus subtilis* (Singh et al. 2009). The chemical structure of the major PHAs is illustrated in Figure 3, where 'n' is the number of monomer units in each polymer chain, which varies between 100 and 30,000 and R is the side chain that includes alkyl groups which varies from methyl (C1) to tridecyl (C13) (Green 2010; Panchal et al.). Since the chemical properties of these biopolymers are very similar to the chemically produced conventional plastics and they are biodegradable, PHAs has become attractive targets for the production of alternative plastics.



**Figure 3 General structure of PHAs.** Structure and chemical properties can vary based on the composition of the attached R-group listed in the Table (Green 2010).



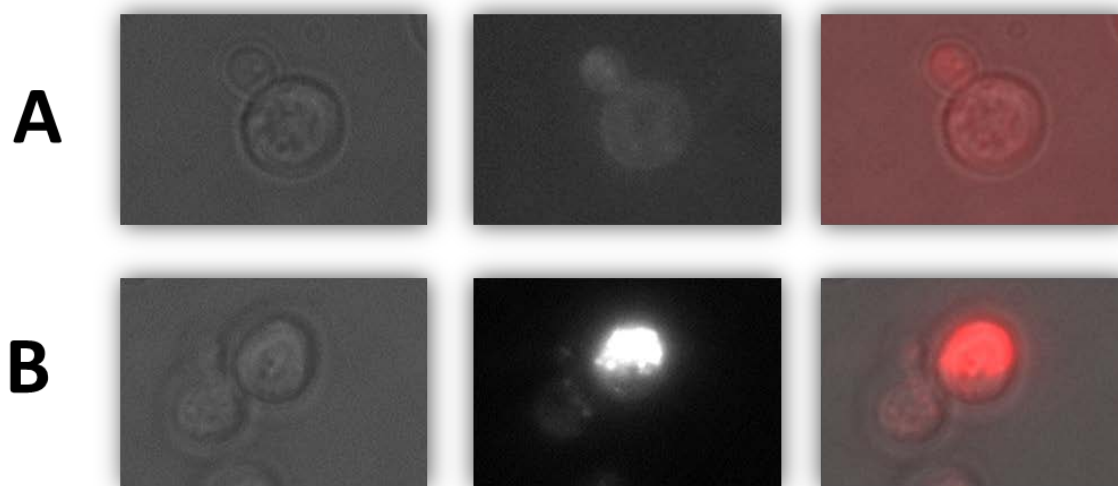
PHB is the most common type of polyhydroxyalkanoate (PHAs) synthesized and accumulated by microorganisms like *Ralstonia eutropha* (also known as *Cupriavidus necator*, *Wautersia eutropha*, *A. eutrophus*), *Bacillus megaterium* or *Pseudomonas sp.* as carbon and energy storage material in response to conditions of physiological stress (Steinbüchel et al. 1993). Biodegradable PHB is a linear polyester consisting solely of the stereospecific monomer, (R)-3-hydroxybutyric acid. It belongs to the group of short chain length PHAs consisting of C3-C5 hydroxyacid monomers. (Hankermeyer and Tjeerdema 1999; Melchioris et al. 1994).



**Figure 4 Polyhydroxybutyrate biosynthesis pathway:** *phaA* encodes acetyl-CoA acetyltransferase, *phaB* encodes NADPH dependent acetoacetyl-CoA reductase, *phaC* encodes polyhydroxyalkanoate synthase

The biosynthesis pathway of PHB illustrated in Figure 4 involves three enzymes and their sequential reactions (Carlson et al. 2002; Steinbüchel 2001; Steinbüchel and Hein 2001). The first enzyme of the pathway is acetyl-CoA-acetyltransferase [EC 2.3.1.9], encoded by *phaA*, which catalyzes the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA. The next step is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA, which is catalyzed by NADPH-dependent acetoacetyl-CoA reductase [EC 1.1.1.36] encoded by *phaB*. Finally, PHA synthase [EC 2.3.1.-] encoded by *phaC*, catalyzes the polymerization of (R)-3-hydroxybutyryl-CoA monomers to PHB (Peoples and Sinskey 1989). Several attempts have been made to evaluate *S. cerevisiae* as a cell factory for PHB production (Breuer et al. 2002;

Dimster-Denk and Rine 1996; Leaf et al. 1996; Marchesini et al. 2003; Zhang et al. 2006). Synthesis of PHB in *S. cerevisiae* has initially been demonstrated by expressing only the bacterial polyhydroxybutyrate synthase (Leaf et al. 1996). This PHB synthesis approach is successful because of the activity of native thiolase and reductase enzymes involved in the synthesis of D-3-hydroxybutyryl-CoA in *S. cerevisiae*. However, the yield obtained from employing only the polymerase activity was very low when compared with the expression of all three genes of the PHB biosynthesis pathway (Breuer et al. 2002; Carlson et al. 2002; Carlson and Srienc 2006). PHB synthesized in *S. cerevisiae* occurs and accumulates as small granules in the cytosol as revealed in Figure 5.

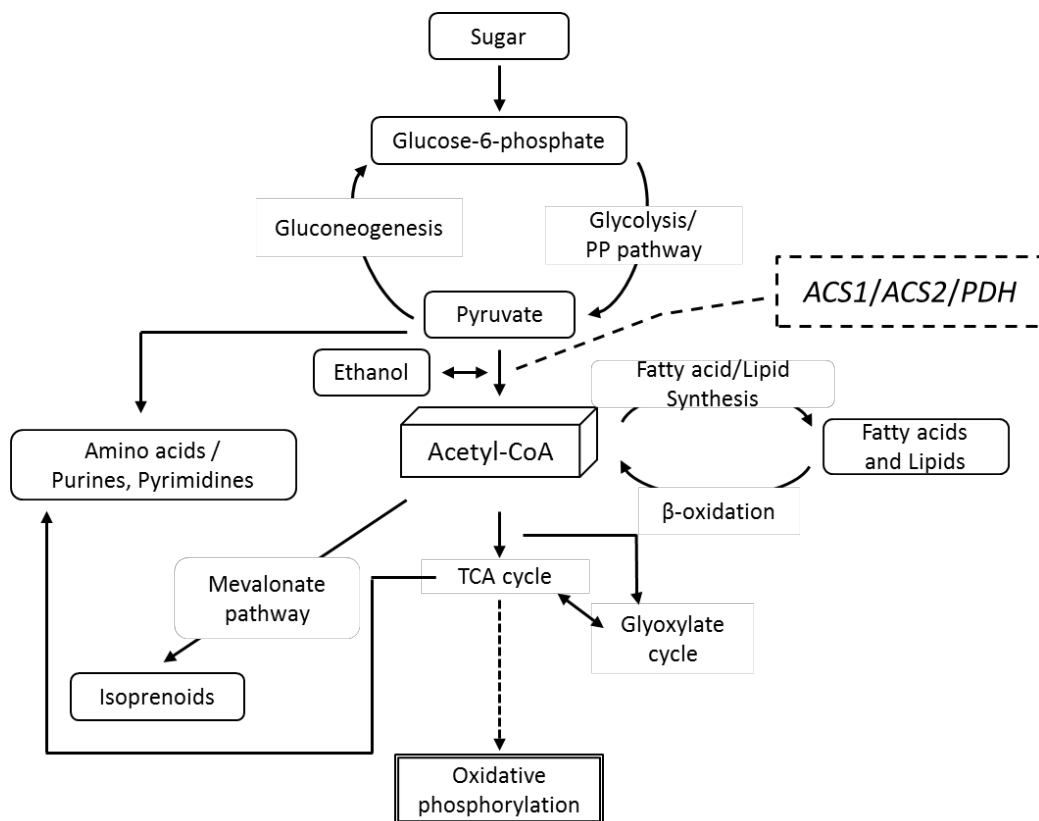


**Figure 5 Nile red staining of PHB granules accumulated in the cytosol of *S. cerevisiae*.** (A) Wild type and (B) PHB producing *S. cerevisiae* under a fluorescence microscope

In this study, PHB was selected as the model product for metabolic engineering in *S. cerevisiae* since its biosynthesis pathway utilizes acetyl-CoA, the intermediate in the central carbon metabolism, as a precursor. Moreover, the reaction in the pathway involves the consumption of NADPH as a required cofactor. Since many of the microbial-derived industrial products are directly and non-directly acetyl-CoA-derived products and associated with the redox balance in the metabolism, the platform developed for PHB biosynthesis in *S. cerevisiae* could be an advantage and applied for other industrially relevant products.

### 1.3.3. Acetyl-CoA and central carbon metabolism

Acetyl-CoA is a crucial intermediate metabolite that links anabolism and catabolism. Hence, it is involved in many central metabolic pathways, including the tricarboxylic acid cycle (TCA cycle), the glyoxylate cycle, fatty acid synthesis and  $\beta$ -oxidation, amino acid synthesis and sugar metabolism. Therefore, the availability of acetyl-CoA in the cell relies on the rate of its biosynthesis and its utilization in various metabolic pathways. The summary of acetyl-CoA metabolism is exemplified in Figure 6.



**Figure 6 Summary of acetyl-CoA metabolism.** *ACS1* and *ACS2* encode the isozymes of cytosolic acetyl-CoA synthetase and *PDH* represents the mitochondrial pyruvate dehydrogenase complex.

In *S. cerevisiae*, acetyl-CoA is synthesized by cytosolic acetyl-CoA synthetase, *Acs1p* or *Acs2p*, however these two acetyl-CoA synthetase differ in their kinetic properties and the transcriptional regulation (van den Berg et al. 1996). Since the expression of *ACS1* is

transcriptionally repressed at high concentrations of glucose or other fermentable sugars, *ACS1* contributes to acetyl-CoA synthesis only when cells undergo gluconeogenesis or in the presence of a non-fermentable carbon source (Kratzer and Schuller 1995). *ACS2* is likely to be (in)directly involved in the transcriptional regulation of *ACS1* since the absence of *ACS2* prevented the complete glucose repression of *ACS1* (van den Berg et al. 1996). Moreover, *ACS2* appears to be co-regulated with genes in the fatty acid biosynthesis pathway (Hiesinger et al. 1997). Acetyl-CoA can also be synthesized by the mitochondrial pyruvate dehydrogenase complex via oxidative decarboxylation (Pronk et al. 1996) and generated as the end-product from  $\beta$ -oxidation (Hiltunen et al. 1992). *Acs1p* is localized in mitochondria, nucleus, cytoplasm and peroxisome (Chen et al. 2012; Devirgilio et al. 1992) whereas *Acs2p* is localized in cytoplasm, nucleus and possibly endoplasmic reticulum (Chen et al. 2012; Huh et al. 2003) and is primarily responsible for extra-mitochondrial acetyl-CoA production (Takahashi et al. 2006). Therefore, the availability of acetyl-CoA is corresponding to the cellular processes in each compartment and depends on nutrient and growth conditions (Wellen and Thompson 2012).

Besides being important as an intermediate in cellular metabolism, acetyl-CoA is known for its potential as a building block for biosynthesis of industrially relevant products such as isoprenoids (Scalcinati et al. 2012), biofuels like butanol (Steen et al. 2008) and biodiesel (Shi et al. 2012). When acetyl-CoA is used as a precursor for producing these bioproducts, considering the localization of acetyl-CoA is important due to its impermeability through the membrane of the organelles. Acetyl-CoA is localized in at least four subcellular compartments: nucleus, mitochondria, cytosol and peroxisomes. The nuclear acetyl-CoA serves mainly as acetyl donor for histone acetylation (Takahashi et al. 2006), whereas acetyl-CoA localized in other compartments is influenced by the carbon source/sugar utilization and serves as a link between different metabolic pathways. The mitochondrial acetyl-CoA is generated by the pyruvate dehydrogenase complex and served as fuel for the TCA cycle. The peroxisomal acetyl-CoA is generated as the end product of  $\beta$ -oxidation and subsequently used by the glyoxylate cycle which seems to be the only possible route for carbon and energy source when *S. cerevisiae* is grown on oleate (Kunau et al. 1995) or as the endogenous turnover of intermediates leaking out of the fatty acid biosynthetic pathway (Hiltunen et al. 1992). Acetyl-CoA generated in peroxisome is supposed to be used in the same compartment since *S. cerevisiae* does not have the transport system for acetyl-CoA (van Roermund et al. 1995; van Roermund et al. 1999). However, the enzymes participating in the glyoxylate cycle

are located on both sides of the peroxisomal membrane, thus enable some of the intermediate metabolites to be transported across the peroxisomal membrane to the cytosol in the form of C4 dicarboxylic acids and hence be able to pass through the mitochondrial membrane and thus enter the TCA cycle (Kunze et al. 2006). Therefore, the partitioning of acetyl-CoA into each compartments influence the utilization of acetyl-CoA in various metabolic pathways. Citrate synthase (*CIT2*) and malate synthase (*MLS1*) are responsible for shunting acetyl-CoA into the glyoxylate cycle and supplying the C4 dicarboxylic acids to the TCA cycle, where they are subsequently used for growth and energy generation. When *S. cerevisiae* utilize ethanol as sole carbon source (Kunze et al. 2006) or grown on acetate rich medium, the glyoxylate cycle takes place in cytosol (Lee et al. 2011). The deletion of *CIT2* and *MLS1* was proposed to test their effect on the availability of cytosolic acetyl-CoA, which may influence the biosynthesis of PHB in *S. cerevisiae*. The results and discussion of the gene deletions and strategy used to improve the availability of cytosolic acetyl-CoA will be discussed in the next chapter 2.2.

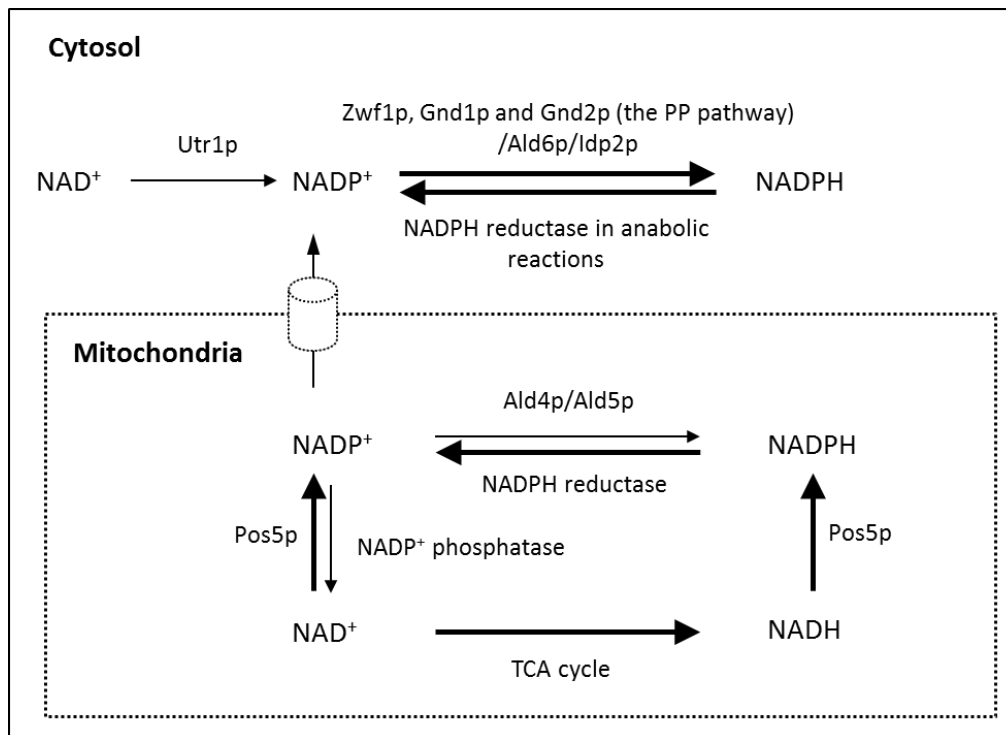
#### **1.3.4. NADPH and Redox balance**

The productivity of numerous bioproducts including primary and secondary metabolites or heterologous expression of recombinant proteins and biopolymers produced by microbial fermentation are often limited by NADPH, precursors or other intermediate metabolites. There are several investigations focusing on the strategies to alter the redox balance and study the redox regulation in *S. cerevisiae* (Bro et al. 2006; Minard and McAlister-Henn 2005; Miyagi et al. 2009; Moreira dos Santos et al. 2003; Murray et al. 2011; Shi et al. 2005).

The sources of NADPH in *S. cerevisiae* vary with the carbon source and the availability of oxygen during cultivation since the energy and carbon metabolism are highly interconnected (Madsen et al. 2011; Minard and McAlister-Henn 2005). While NADH is a reducing equivalent produced and consumed mainly in the catabolic reactions, NADPH is regarded as an anabolic reductant. These cofactor pairs, NADPH/NADP<sup>+</sup> and NADH/NAD<sup>+</sup> play a central role as electron donor/acceptors thus influencing all oxidation-reduction metabolic pathways in the cell (Murray et al. 2011). In *S. cerevisiae*, NADH cannot directly be converted to NADP<sup>+</sup>, or vice versa, due to the lack of transhydrogenase activity (Bruinenberg 1986). Moreover the compartmentalization of NADPH/NADP<sup>+</sup> and NADH/NAD<sup>+</sup> is also important

since it is believed that the mitochondrial membrane is impermeable to these cofactors (Outten and Culotta 2003; van Roermund et al. 1995). Figure 7 illustrated the proposed model for generation of cytosolic NADPH and mitochondrial NADPH. The major source of cytosolic NADPH production in *S. cerevisiae* grown on glucose has been attributed to glucose-6-phosphate dehydrogenase (Zwf1p) and 6-phosphogluconate dehydrogenase (Gnd1p/Gnd2p) in the pentose phosphate pathway (PP pathway), with the cytosolic aldehyde dehydrogenase (Ald6p) as a secondary source and isocitrate dehydrogenase (Idp2p) as a third source (Grabowska and Chelstowska 2003; Minard and McAlister-Henn 2005). A different system for generating cytosolic and mitochondrial NADPH in *S. cerevisiae* is illustrated in Figure 7 (Miyagi et al. 2009; Outten and Culotta 2003; Shi et al. 2005). In the cytosol,  $\text{NADP}^+$  is converted to NADPH by the reactions mentioned above whereas in the mitochondria, NADH serves as the substrate for NADPH generation through the NADH kinase activity of Pos5p, a mitochondrial protein required for the response to oxidative stress (Outten and Culotta 2003). The activity of mitochondrial  $\text{NADP}^+$  phosphatase (Bernofsky and Utter 1968) allows the recycling of  $\text{NADP}^+$  to  $\text{NAD}^+$  and its further use in the TCA cycle. Although there is evidence of the NADP(H) shuttle system in *S. cerevisiae* due to the viability of the mutant strain lacking ATP-NADH kinase, *utr1 $\Delta$ yef1 $\Delta$*  and the lethality in the triple mutant, *utr1 $\Delta$ yef1 $\Delta$  pos5 $\Delta$*  which implies that cytosolic  $\text{NADP}^+$  can be supplied by Pos5p from the mitochondrial  $\text{NADP}^+$  pool (Bieganowski et al. 2006; Murray et al. 2011; Shi et al. 2005), *S. cerevisiae* must carefully regulate a balance between the production and consumption of NADH and NADPH to maintain the redox balance in the cell.

In *S. cerevisiae*, glucose is metabolized via the Embden Meyerhof Parnas (EMP) pathway or the pentose phosphate (PP) pathway depending on the cellular state and culture condition, which split at the glucose-6-phosphate point (Bruinenberg et al. 1983; Gombert et al. 2001; van Winden et al. 2005). Since most NADPH-consuming processes are located in the cytoplasm, the production of cytosolic NADPH and the redox balance are crucial for the energy metabolism and the formation of NADPH-dependent products (Bruinenberg et al. 1983; Vandijken and Scheffers 1986). Several systems have been investigated with the purpose of increasing NADPH availability including the introduction of the transhydrogenase system, alteration of the ammonium assimilation pathway and the heterologous expression of *gapN*, encodes NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, to substitute the production of glycerol with the production of ethanol (Bro et al. 2006; Hou et al. 2009; Moreira dos Santos et al. 2003; Nissen et al. 2001).



**Figure 7 The proposed model for the generation of cytosolic and mitochondrial NADPH.** ATP-NADH kinase phosphorylates both NAD and NADH while NAD kinase phosphorylates only  $\text{NAD}^+$ . The thick arrows represent the favored direction of the reaction.

Zwf1p = glucose-6-phosphate dehydrogenase, Gnd1p/Gnd2p = 6-phosphogluconate dehydrogenase, Ald4p/Ald5p/Ald6p = acetaldehyde dehydrogenase, Idp2p = isocitrate dehydrogenase, Utr1p = ATP-NAD kinase, Pos5p = ATP-NADH kinase

Introducing the *E. coli* membrane bound transhydrogenase (Anderlund et al. 1999) and the cytoplasmic transhydrogenase from *Azotobacter vinelandii* (Nissen et al. 2001) into *S. cerevisiae* were carried out in order to analyze the intracellular redox and the influence on product formation during anaerobic cultivation. The introduction of *A. vinelandii* transhydrogenase resulted in an increased formation of glycerol and 2-oxoglutarate since glycerol is produced when excess NADH is formed while NADPH used to convert 2-oxoglutarate to glutamate is depleted (Nissen et al. 2001). Moreover, the increased formation of 2-oxoglutarate was accompanied with a decrease in the specific growth rate, biomass and ethanol formation (Nissen et al. 2001). Therefore, the results imply the favored direction toward NADH formation when the transhydrogenase is expressed in *S. cerevisiae*.

In *S. cerevisiae* utilizing ammonium as nitrogen source, the NADPH-dependent glutamate dehydrogenase encoded by *GDH1* accounts for the major fraction of NADPH consumption. Switching the cofactor requirement from NADPH to NADH was carried out by deletion of *GDH1* accompanied with the overexpression of *GDH2* (NADH-dependent glutamate dehydrogenase) or the GS-GOGAT system including the ATP-dependent glutamine synthetase (*GLN1*) and the NADH-dependent glutamate synthase (*GLT1*). Overexpression of *GDH2* or the GS-GOGAT system as an alternative pathway for ammonium assimilation partially restores the growth of *gdh1Δ*. The decreased flux through the PP pathway indicates a decrease in NADPH requirement when employing this redox alteration system (Moreira dos Santos et al. 2003).

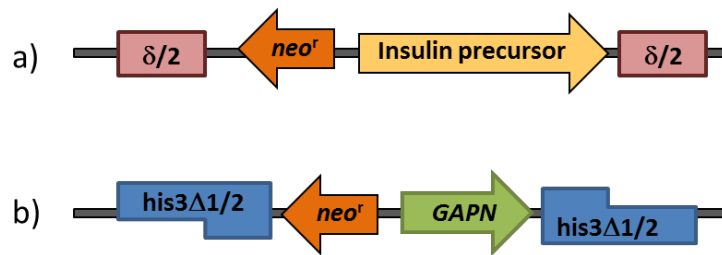
Overexpression of *gapN* from *Streptococcus mutans* in *S. cerevisiae* was investigated for its capability to decrease the flux from glucose to glycerol and improve the ethanol yield during anaerobic cultivation. Although this strategy cannot completely eliminate glycerol formation, a 40% lower glycerol yield without affecting the maximum specific growth rate was observed (Bro et al. 2006). Since NADPH is produced during the reaction catalyzed by GapN, we employ the *gapN* strategy in this study for enhancing the availability of NADPH as a required cofactor used during the biosynthesis of PHB. Besides that, establishing the phosphoketolase pathway from *Aspergillus nidulans* was also investigated for its capability to shunt carbon from glucose to the PP pathway and hence enhance NADPH availability. The result and discussion of employing GapN and the phosphoketolase pathway to increase NADPH will be discussed in chapter 2.2.3.



## CHAPTER 2. Results and Discussion

### 2.1. Case study I: CICH<sub>E</sub> implementation

For industrial application of genetically engineered microbes, it is important that the expression systems in the engineered strains remain stable throughout the production process. Therefore, we were interested in applying the CICH<sub>E</sub> strategy to the *S. cerevisiae* in order to develop a stable and tunable expression system. The implementation of the CICH<sub>E</sub> strategy in *S. cerevisiae* was investigated by using two modified CICH<sub>E</sub> constructs. The linear CICH<sub>E</sub> construct used for random integration of the insulin precursor expression cassette and one for targeted chromosomal integration of *gapN*, both illustrated in Figure 8.



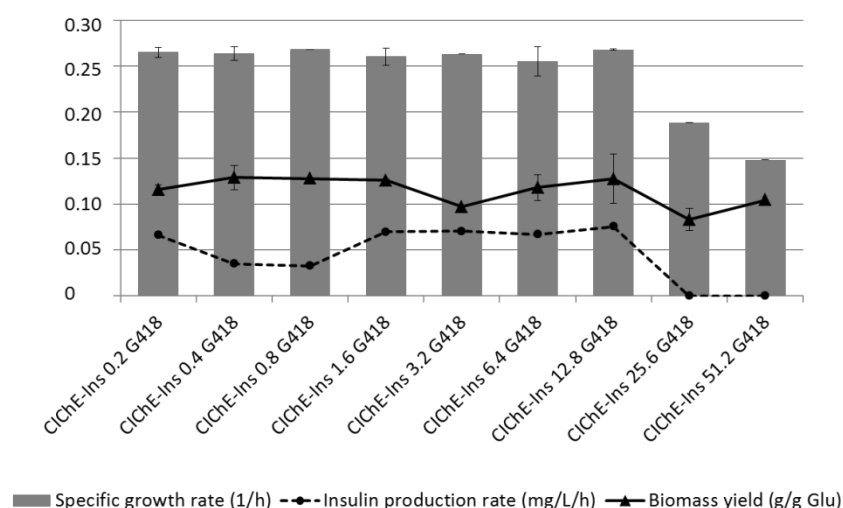
**Figure 8 Schematic structure of linear CICH<sub>E</sub> constructs.** a) Random CICH<sub>E</sub> integrative construct with the insulin precursor as the target product flanked by  $\delta$  element sequences derived from the 3' end of the yeast Ty1 retrotransposon. b) Targeted CICH<sub>E</sub> integrative construct with GapN as a target product and *his3 $\Delta$ 1* sequence for targeted homologous recombination

In both constructs, a bacterial *neo<sup>r</sup>* gene was utilized as tunable and selectable marker, the copy number of which - due to its low expression level - should correlate with the concentration of G418 used during the evolutionary steps. Firstly, PHB was used as a target product to investigate the CICH<sub>E</sub> strategy in *S. cerevisiae* for both random and targeted integration. However, the PHB biosynthesis pathway contains three different genes hence resulting in a large integrative construct. Moreover, the heterologous PHB genes were controlled by the same promoter but in opposite direction when the genes are inserted into the

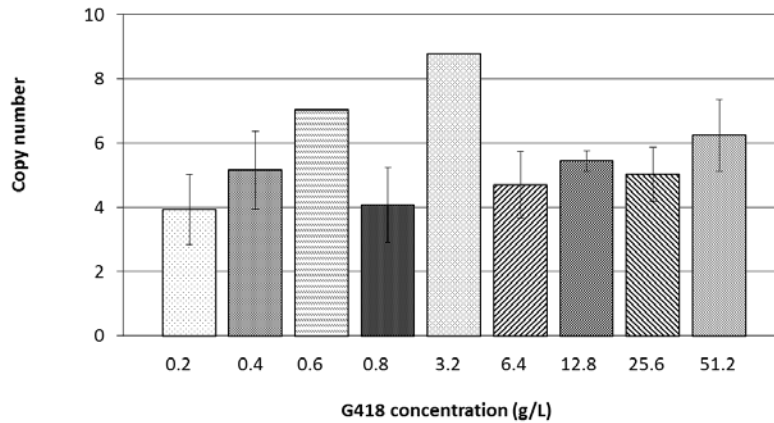
CIChE construct and this might have resulted in gene losses due to loop out events when the construct was integrated into the chromosome since no PHB was detected after the integration of CIChE-PHB. Moreover, this bulky construct (carrying 4 genes including the selectable marker) was prone to random integration rather than targeted integration. Therefore, in order to prove the concept of chemically inducible chromosomal evolution, we selected the insulin precursor as a target product for investigation of random integration and bacterial GapN for targeted integration due to their compactness, less than 1 kb, for each gene.

### 2.1.1. Random integration of CIChE construct

The evolution of strains carrying the randomly integrated CIChE-Insulin construct was accomplished by subculturing the strains in a medium containing a stepwise increasing concentration of the antibiotic, G418. Evaluation of the physiological properties of the evolved strain was carried out and the results are shown in Figure 9. When the evolved strains were grown at the respective G418 concentration, there was no significant difference in the specific growth rate, glucose consumption and insulin precursor production rate in the strains evolved from 0.2 g·L<sup>-1</sup> G418 to 12.8 g·L<sup>-1</sup> G418. However, the strains isolated from higher concentrations of G418 showed lower specific growth rates as well as lower biomass yield and insulin precursor production rate.

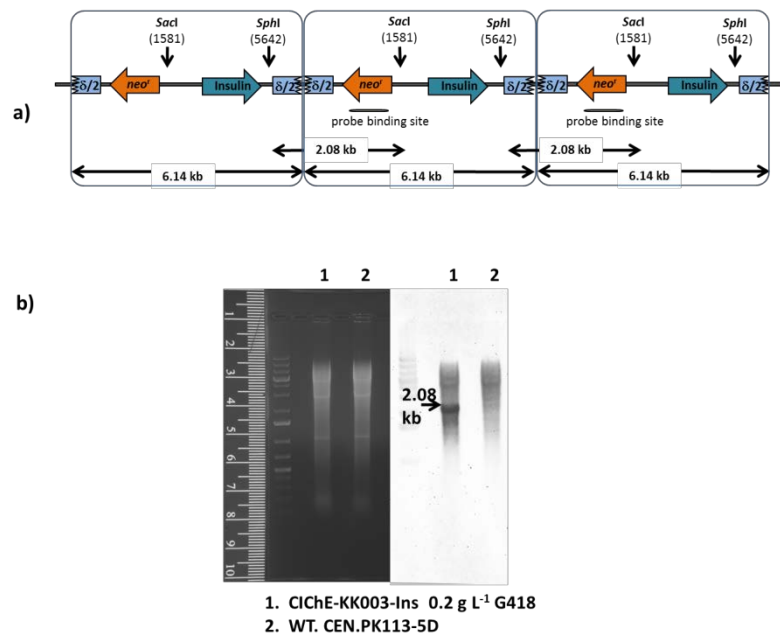


**Figure 9: Specific growth rates, insulin precursor production rates and biomass yields of the CIChE-Ins strains isolated at different concentrations of G418.**



**Figure 10: *Neo<sup>r</sup>* copy number measurement.** The copy number of *neo<sup>r</sup>* in the genome of strains isolated from different concentration of G418 was determined by qPCR using single copy gene *TAF10* for normalization.

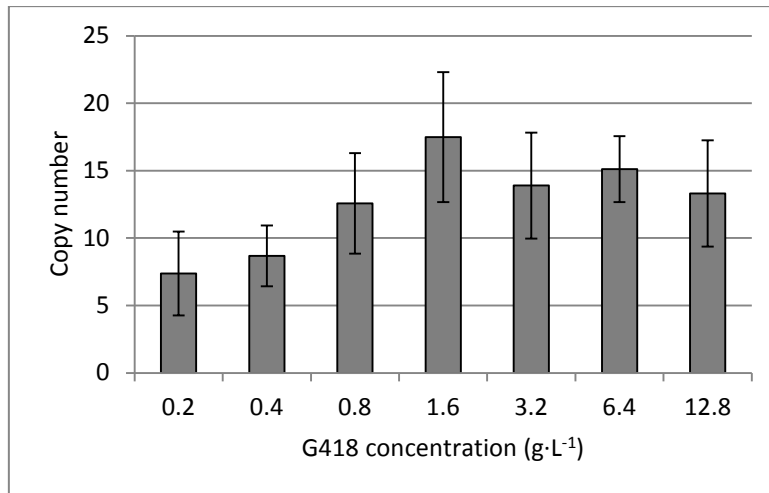
Evaluation of the *neo<sup>r</sup>* copy number using quantitative PCR (qPCR) revealed that there was no correlation between the copy number and the G418 concentration applied during the evolution process as illustrated in Figure 10. Although a general trend of increasing copy number was observed up to a G418 concentration of 0.6 g·L<sup>-1</sup>, however this trend did not continue for higher concentrations of G418. The Ty1 delta element was employed in the CICH<sub>E</sub> construct with the intention to promote homologous recombination during the evolution process because it appears in many copies in the genome thus allow the dispersed integration of the CICH<sub>E</sub> construct to occur. However, the pattern of integrated CICH<sub>E</sub> constructs was found to be in a tandem repeat manner instead of random integration into different delta sequences as shown in Figure 11. This result is consistent with findings by Wang and coworkers (Wang et al. 1996) but different from what was observed by Boeke and coworkers (Boeke et al. 1988) who found integration of delta element at multiple sites in the genome, which may lead to further lack of correlation of gene expression and copy number due to varying expression levels at different chromosomal sites.



**Figure 11: Southern blot analysis of CICHe strain.** (a) Schematic presentation of tandem integrated CICHe construct (b) Genomic DNA of strains CICHe-KK003-Ins 0.2 g·L<sup>-1</sup> G418 and CEN.PK113-5D was subjected to restriction enzyme digestion with *SacI* and *SphI* and separated by gel electrophoresis followed by hybridization with a probe containing a 1-kb *neo*<sup>r</sup> fragment.

### 2.1.2. Targeted CICHe integration

*His3ΔI* was selected as a homologous recombination site for targeted integration instead of the delta element, which leads to random integration into the chromosome. A non-phosphorylating NADP<sup>+</sup> dependent glyceraldehyde-3-phosphate dehydrogenase encoded by *gapN* from *S. mutans* was selected as a target product instead of insulin precursor due to the ease of strain characterization and further use as a redox engineered strain. Besides that, the CICHe integrated construct was redesigned in order to reduce the size of the integrated cassette but still sharing the same concept of having 2 homologous sites flanking the CICHe integration cassette. It has been shown that expression of *gapN* reduces the amount of glycerol produced by *S. cerevisiae* when grown under anaerobic conditions (Bro et al. 2006). Therefore, characterization of the evolved strain with targeted CICHe-*gapN* integration was performed under anaerobic cultivation.

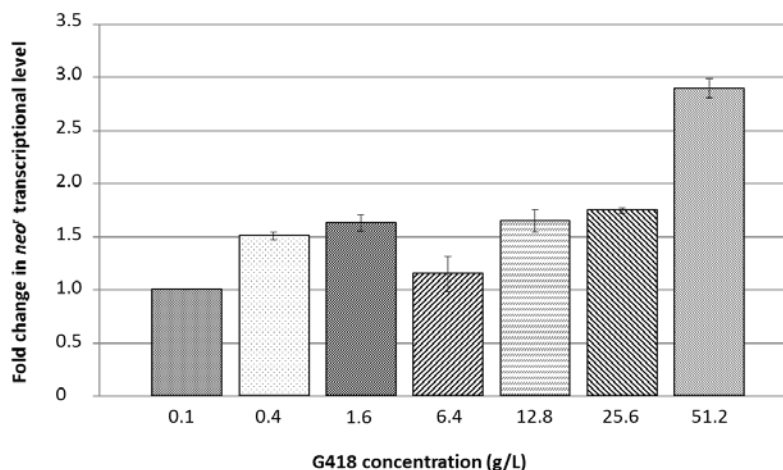


**Figure 12 *Neo<sup>r</sup>* copy number measurement.** The copy number of *neo<sup>r</sup>* in the genome of strains isolated from different concentration of G418 was determined by qPCR using single copy gene *TAF10* for normalization.

The copy number of CICH<sub>E</sub>-gapN construct tended to increase with the increasing G418 concentration (Figure 12). When the evolution was carried out in G418 concentration greater than 1.6 g·L<sup>-1</sup> however, the copy number was not proportional with the increased concentration of G418 similar to what was observed in the random CICH<sub>E</sub> integration.

### 2.1.3. Chemical induced evolution and the tunability of *neo<sup>r</sup>*

As the correlation between the increment of G418 resistance and the copy number of *neo<sup>r</sup>* was not observed during the evolution process especially in the strain evolved at higher G418 concentrations, we suspected changes in the *neo<sup>r</sup>* expression level. The transcriptional level of *neo<sup>r</sup>* in the insulin precursor construct (Figure 13) showed that although the number of *neo<sup>r</sup>* copies in the chromosome remained constant when the concentration of G418 was increased, the transcriptional level of the evolved strain increased three fold compared to the parental strain.



**Figure 13 Comparison of *neo<sup>r</sup>* transcription levels.** RNA extracted from evolved strains at different concentrations of G418 was examined by qRT-PCR. *TAF10*, *ALG9* and *ACT1* were used as reference genes to normalize the transcript level of *neo<sup>r</sup>*.

Although the CICHÉ approach is based on the assumption that the accretion in antibiotic resistance would result in an increased copy number of the CICHÉ construct, in this study such a correlation was only observed when the applied G418 concentration was low. This might be due to slower copy number evolution of the CICHÉ integrative construct in the genome compared to the acquisition of mutations leading either to higher expression of *neoR* or to G418 resistance. The dissociation between the occurrence of G418 resistance and the number of copies or integration sites of *neo<sup>r</sup>* was also observed in another study (Quinto et al. 1992). *RecA* is the main homologous recombination protein in *E. coli* whereas the *RAD52* epistasis group (consisted of *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RDH54/TID1*, *RAD55*, *RAD57*, *RAD59*, *MRE11*, and *XRS2*) in *S. cerevisiae* is responsible for the same recombination process (Symington 2002). Therefore, the more complex homologous recombination process in *S. cerevisiae* might also account for the different results in the evolution of the CICHÉ construct when integrated into the chromosome.

In order to reduce the technical difficulties when implementing the *E. coli* CICHÉ strategy to *S. cerevisiae*, two possibilities could be proposed, changing the selective marker and the homologous recombination sites. The changing of the resistance marker to another dominant marker such as metal ion resistance marker, *CUP1<sup>r</sup>* from *S. cerevisiae*, the copy number of which regulates the level of copper resistance (Fogel and Welch 1982; Fogel et al. 1983),

might improve the evolution process of the integrated CChE strain. Moreover, it will not leave an antibiotic resistance marker in the genome when applying the engineered strain in the industrial applications. Another possibility is to change the sites for chromosomal integration of the CChE construct because different integration sites can influence the expression level of the heterologous genes (Bai Flagfeldt et al. 2009). Therefore, homologous recombination sites with low to intermediate expression level would be preferable. Overall, the investigated results presented here could serve as a basis guide for implementing the process of chemical induced chromosomal evolution that could be applied as a new tunable expression system in *S. cerevisiae*.

## 2.2. Case study II: PHB production

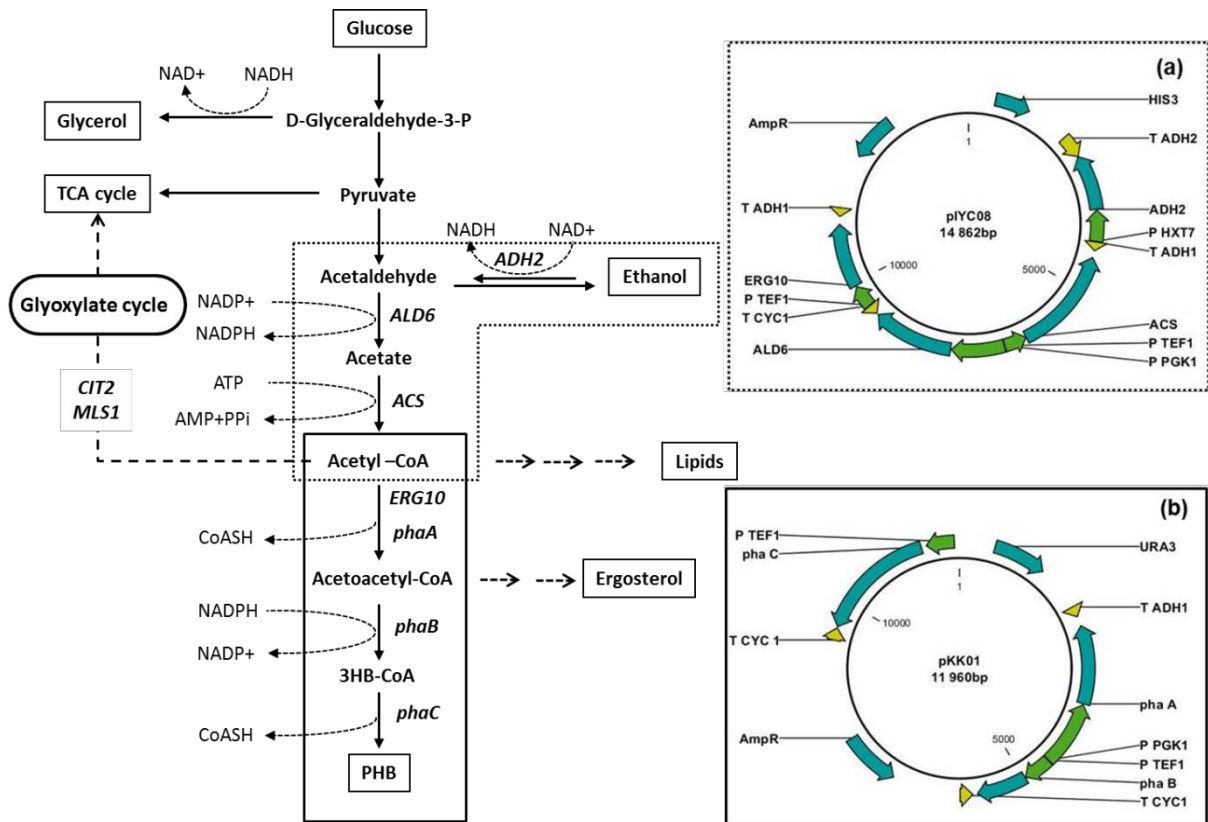
### 2.2.1. Precursor supplementation

Despite its benefits as a well-studied microorganism, elucidating *S. cerevisiae* as a model to develop a platform for producing acetyl-CoA derived molecules struggles with many constraints such as compartmentalization and transporters/shuttle systems for the heterologous target compounds. Apart from being an intermediate in several metabolic pathways, acetyl-CoA is localized in 4 subcellular compartments. In addition, acetyl-CoA cannot be transported through organelle membranes without the carnitine shuttle system, which is absent in *S. cerevisiae*. Nevertheless, acetyl-CoA can be indirectly relocated in form of acetyl-CoA derived organic acids such as succinic acid or malic acid, thus influencing the availability of acetyl-CoA and acetyl-CoA derived products formation in different compartments.

The availability of cytosolic acetyl-CoA is known to influence the production of certain compounds in *S. cerevisiae*. In this study, the biosynthesis of PHB in *S. cerevisiae* occurs in the cytoplasm and utilizes the cytosolic acetyl-CoA as a precursor, therefore the availability of cytosolic acetyl-CoA has been hypothesized to influence PHB production. Overexpression of genes from the ethanol degradation pathway including alcohol dehydrogenase (*ADH2*), acetaldehyde dehydrogenase (*ALD6*) and acetate synthetase variant (*acs<sup>L641P</sup>*) was studied in order to increase the flux from ethanol to acetyl-CoA and further improve PHB production. Moreover, to maintain the availability of cytosolic acetyl-CoA, *CIT2* and *MLS1* as the routes shunting acetyl-CoA to the glyoxylate cycle were blocked and evaluated for their effect on cytosolic PHB production. The pathway used in this strategy is presented in Figure 14 and the strains used in Case study II are listed in Table 1

Co-expression of beta-ketothiolase (*PhaA*), acetoacetyl-CoA reductase (*PhaB*) and PHA synthase (*PhaC*) results in PHB accumulation in *S. cerevisiae* as observed in previous studies (Breuer et al. 2002; Carlson et al. 2002; Carlson and Srien 2006). Although thiolase enzymes already exist in *S. cerevisiae*, they function in three different compartments, in mitochondria and peroxisomes for fatty acid  $\beta$ -oxidation and in the cytoplasm for the mevalonate pathway (Hiser et al. 1994). Therefore, we believed that overexpression of native *ERG10* together with *PhaA* would facilitate PHB biosynthesis in the cytoplasm of *S. cerevisiae*.





**Figure 14 Schematic pathway and plasmid maps for polyhydroxybutyrate production in *S. cerevisiae*.** (a) Acetyl-CoA boost plasmid (pIYC08) containing *ADH2*: alcohol dehydrogenase, *ALD6*: aldehyde dehydrogenase, *ACS*: acetyl-CoA synthetase variant (*acs*<sup>L641P</sup>) and *ERG10*: acetyl-CoA acetyltransferase. (b) PHB plasmid (pKK01) containing PHB genes from *R. eutropha*, *phaA*: acetyl-CoA acetyltransferase, *phaB*: NADPH-linked acetoacetyl coenzyme A (acetyl-CoA) reductase and *phaC*: poly(3-hydroxybutyrate) polymerase. P and T in the plasmid map represent promoter and terminator, respectively.

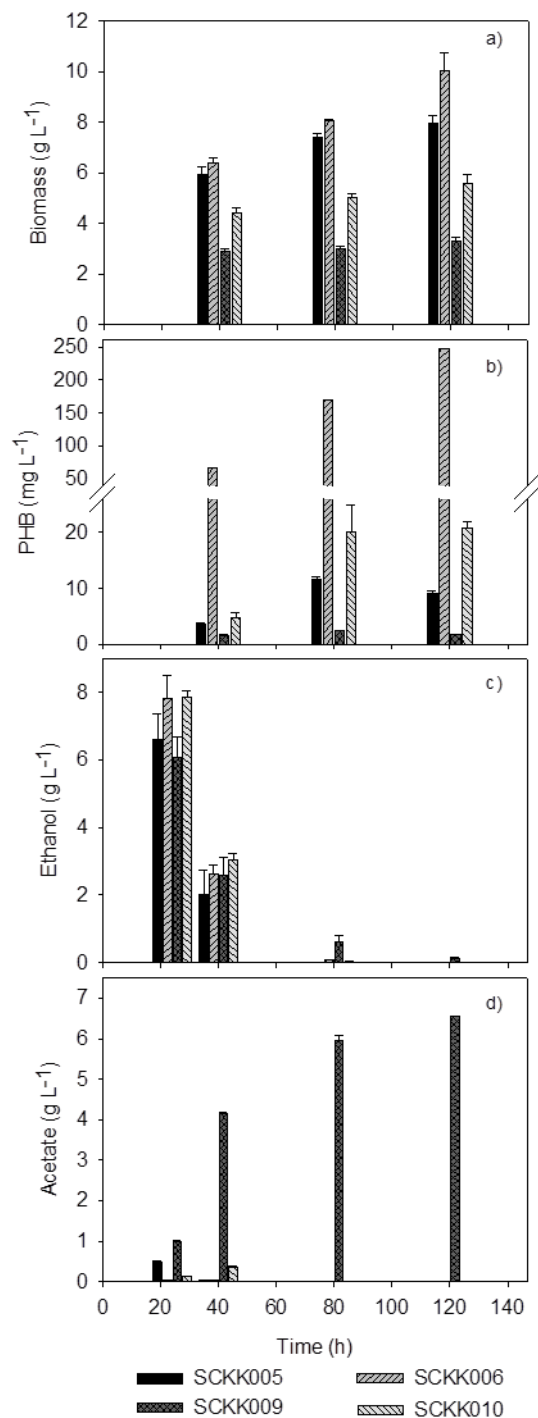
**Table 1** Yeast strains and plasmids used in Case study II.

Strain	Genotype or relevant feature(s)	Plasmid	Source
CEN.PK 113-11C	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-Δ1</i>	-	P. Kötter <sup>a</sup>
SCKK005	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-Δ1</i>	pIYC04/pKK01	Paper 3-5
SCKK006	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-Δ1</i>	pIYC08/pKK01	Paper 3-5
SCIYC32	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-Δ1</i> <i>cit2Δ</i>	-	(Chen et al. 2012)
SCIYC33	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-Δ1</i> <i>mls1Δ</i>	-	(Chen et al. 2012)
SCKK009	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-Δ1</i> <i>cit2Δ</i>	pIYC08/pKK01	Paper 3
SCKK010	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-Δ1</i> <i>mls1Δ</i>	pIYC08/pKK01	Paper 3
SCKK032	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-Δ1</i>	pJC7/pKK01	Paper 5
SCKK033	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-Δ1::gapN<sup>b</sup></i>	pIYC04/pKK01	Paper 5
SCKK034	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-Δ1::gapN</i>	pIYC08/pKK01	Paper 5
SCKK035	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-Δ1::gapN</i>	pJC7/pKK01	Paper 5
SCKK036	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-Δ1::gapN</i>		Paper 5

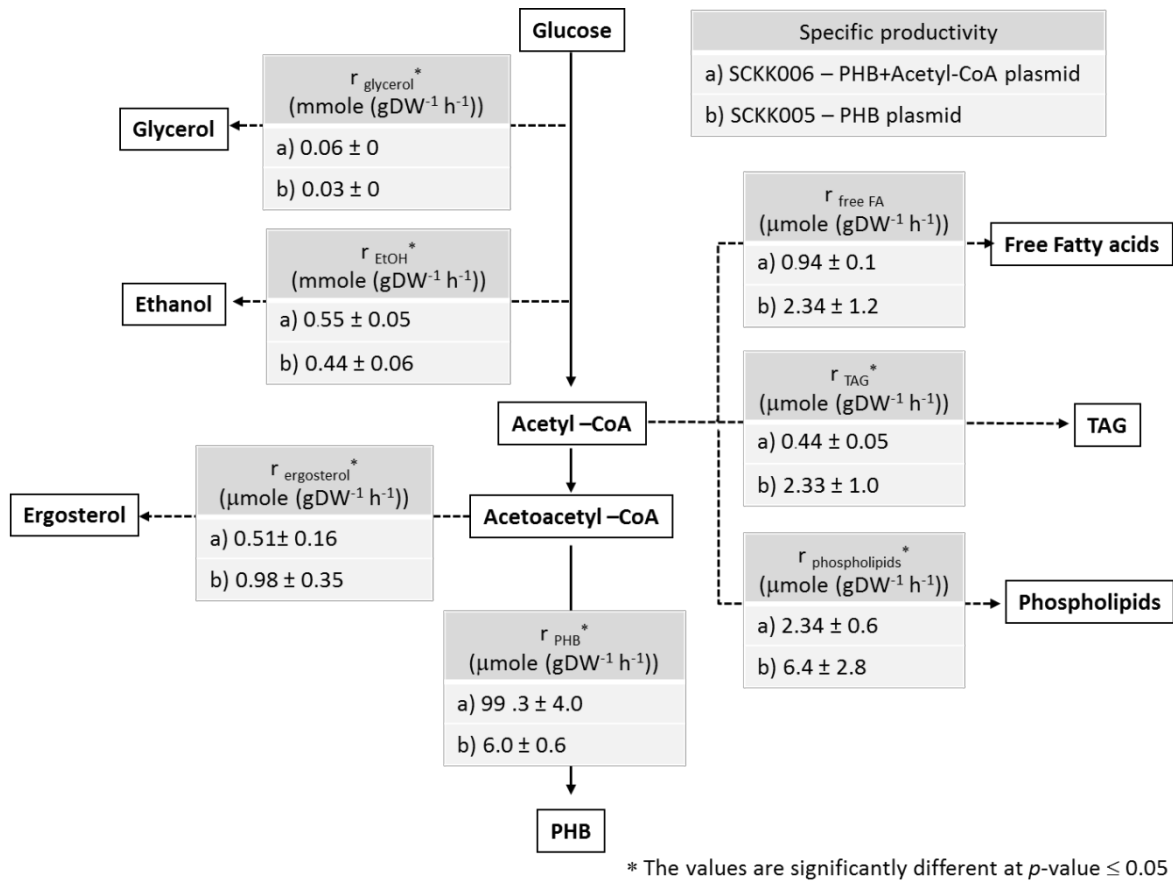
<sup>a</sup> Institute of Microbiology, J.W. Goethe Universität, Frankfurt, Germany

<sup>b</sup> *gapN* here represents the integration cassette including *neo<sup>r</sup>* and *gapN* under control of the *PGK1* promoter and *ADHI* terminator.

The acetyl-CoA boost plasmid contains 4 genes, *ADH2*, *ALD6*, *acs*<sup>L641P</sup> and *ERG10*, involved in channeling carbon from ethanol to acetyl-CoA. *ADH2* encodes a cytosolic acetyl-CoA dehydrogenase responsible for the initial step in the utilization of ethanol as carbon source. *ALD6* encodes aldehyde dehydrogenase involved in the conversion of acetaldehyde to acetate. Although, there are two acetyl-CoA synthetases present in *S. cerevisiae*, the regulation of both enzymes is complex and therefore overexpression of acetyl-CoA synthetase is believed to increase the flux towards acetyl-CoA production. Acetyl-CoA synthetase with the mutation at Leu-641 from *S. enterica* was selected in the acetyl-CoA boost plasmid to catalyze the conversion of acetate to acetyl-CoA. Mutation of acetyl-CoA synthetase at Leu-641 prevent the acetylation by acetyltransferase bypassing the need for deacetylases and thus maintaining the enzyme in an active state (Starai et al. 2005). In the acetyl-CoA boost plasmid, *ALD6*, *acs*<sup>L641P</sup> and *ERG10* are controlled by constitutive promoters,  $P_{TEF1}$  and  $P_{PGK1}$ , respectively, while *ADH2* is under control of the  $P_{HXT7}$  promoter which is strongly de-repressed under glucose depletion (Partow et al. 2010; Reifenberger et al. 1995; Sedlak and Ho 2004) hence ensuring the conversion of ethanol to acetaldehyde after glucose consumption phase. Co-transformation of acetyl-CoA boost plasmid and PHB plasmid improved the PHB production in SCKK006 to  $\sim 250 \text{ mg} \cdot \text{L}^{-1}$ , 18 times higher compared to the reference strain (SCKK005) as shown in Figure 15 b. Although the deletion of *CIT2* and *MLS1* was supposed to enhance the availability of cytosolic acetyl-CoA by reducing the drain of acetyl-CoA to the glyoxylate shunt, co-transformation of the acetyl-CoA boost plasmid and the PHB plasmid into the *cit2* $\Delta$  (SCKK009) and *mls1* $\Delta$  (SCKK010) strain resulted in lower biomass and PHB production compared to the non-deletion strains (Figure 15). Since the deletion of *CIT2* and *MLS1* cause an impaired metabolism due to the incapability to utilize C<sub>2</sub> carbon via the glyoxylate shunt, the accumulation of acetate, as high as  $6 \text{ g} \cdot \text{L}^{-1}$ , was observed in SCKK009 (*cit2* $\Delta$ ).



**Figure 15** Measurements of biomass and PHB from shake flask cultivations in a modified minimal medium with 20 g L<sup>-1</sup> glucose as carbon source. Strain SCKK005 harbors an empty plasmid (pIYC04) and the PHB plasmid (pKK01), strain SCKK006 harbors an acetyl-CoA boost plasmid (pIYC08) and the PHB plasmid (pKK01), SCKK009 and SCKK010 harbor pIYC08 and pKK01 and carry a *CIT2* and *MLS1* deletion, respectively.



**Figure 16 Comparison of specific fluxes in SCKK006 and SCKK005 during growth on glucose in aerobic batch bioreactor cultivation with 20 g L<sup>-1</sup> glucose as carbon source.** SCKK006 is *S. cerevisiae* harboring an acetyl-CoA boost plasmid (pIYC08) and the PHB plasmid (pKK01). SCKK005 is *S. cerevisiae* harboring an empty plasmid (pIYC04) and the PHB plasmid (pKK01). The fluxes towards the different lipids were calculated from measurement of the lipid composition of the biomass and the maximum specific growth rate. The mean value ± SD from at least triplicate fermentations are reported.

The specific product formation rate (flux) was calculated by using the equation:  $r_p = \mu_{\text{max}} \cdot Y_{\text{sp}} / Y_{\text{sx}}$  and the specific glucose consumption rate was calculated by using the equation:  $r_s = \mu_{\text{max}} / Y_{\text{sx}}$ .

Considering acetyl-CoA as the intermediate which can be utilized as a precursor for other metabolites in the central carbon metabolism, the specific productivities of PHB including the directly and non-directly acetyl-CoA derived products were determined. The specific fluxes are reported in Figure 16. Employing the acetyl-CoA boost plasmid helped channeling the

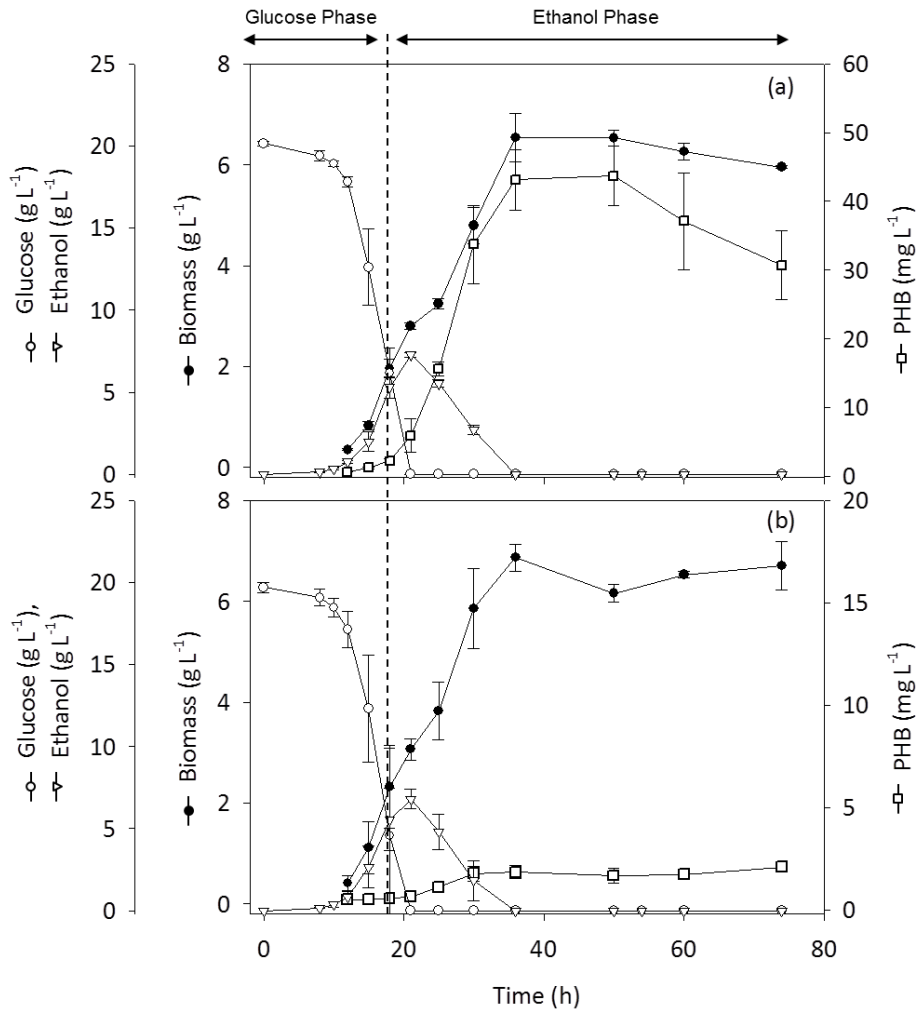
carbon flow from ethanol to acetyl-CoA and increased the specific productivity of PHB from  $6 \mu\text{mol}\cdot\text{gDW}^{-1}\cdot\text{h}^{-1}$  to  $99 \mu\text{mol}\cdot\text{gDW}^{-1}\cdot\text{h}^{-1}$ . In addition, the specific productivities of free fatty acids, triacylglycerol (TAG), phospholipids and ergosterol which utilized acetyl-CoA in the biosynthesis pathway were decreased in strain with the co-expression of acetyl-CoA boost plasmid and PHB plasmid. The higher specific productivity of glycerol in SCKK006 indicates the influence of *ADH2* overexpression and the insufficient capacity of the ethanol-acetaldehyde redox shuttle to reoxidize the cytosolic NADH (Overkamp et al. 2000) generated by the conversion reaction of ethanol to acetaldehyde thus triggering the formation of glycerol for redox balancing in the cytosol (Bakker et al. 2001). In addition, a three times higher maximum acetate concentration,  $0.42 \text{ g}\cdot\text{L}^{-1}$ , was detected in SCKK005 (Figure 4 in Paper 3). Therefore, the different concentrations of glycerol and acetate are most likely a consequence of introducing the acetyl-CoA boost plasmid in the PHB producing strain.

By comparison of the specific productivities of PHB, we can conclude that enhancement of acetyl-CoA production by overexpression of genes from the ethanol degradation pathway improved the productivity of PHB during growth on glucose and further enhanced the productivity of PHB approximately 16.5 times in bioreactor cultivations and reduce the flux from acetyl-CoA to lipids (Paper 3).

### 2.2.2. Specific growth rate and substrate dependent PHB production

PHB production in *S. cerevisiae* is mainly produced during growth in the ethanol phase as presented in Figure 17, where the specific growth rate is substantially lower compared to the exponential phase when cells grow on glucose. In addition, it was observed that the growth of PHB producing strains in aerobic shake flasks was always lower with (maximum specific growth rate of  $0.18 \text{ h}^{-1}$ - $0.20 \text{ h}^{-1}$ ) than growth in a fully aerobic bioreactor ( $0.27 \text{ h}^{-1}$ - $0.28 \text{ h}^{-1}$ ), although it resulted in higher PHB production. It is known that the specific growth rate influences the physiology of *S. cerevisiae* hence affected the fermentative capacity, TCA cycle activity and other metabolic activities (Blank and Sauer 2004; Frick and Wittmann 2005; Van Hoek et al. 1998). For this reason, we investigated the effect of specific growth rate on the PHB production in *S. cerevisiae* harboring the plasmid with overexpression of the ethanol degradation pathway (acetyl-CoA boost plasmid) and the PHB plasmid by employing the chemostat cultivation system, where the dilution rate is proportional to the specific growth

rate during batch cultivation. Besides that, we assessed the PHB production on different carbon sources - glucose, ethanol and the mixture of glucose and ethanol - with regards to their potential as a substrate for PHB production in *S. cerevisiae*.



**Figure 17: Fermentation profile of *S. cerevisiae* (SCKK006) producing PHB in aerobic batch bioreactor cultivation using a chemically defined medium with 20 g L<sup>-1</sup> glucose as carbon source. (a) SCKK006: *S. cerevisiae* harboring an acetyl-CoA boost plasmid (pIYC08) and the PHB plasmid (pKK01). (b) SCKK005: *S. cerevisiae* harboring an empty plasmid (pIYC04) and the PHB plasmid (pKK01)**

**Table 2:** Yields and kinetic parameters obtained during chemostat cultivations.

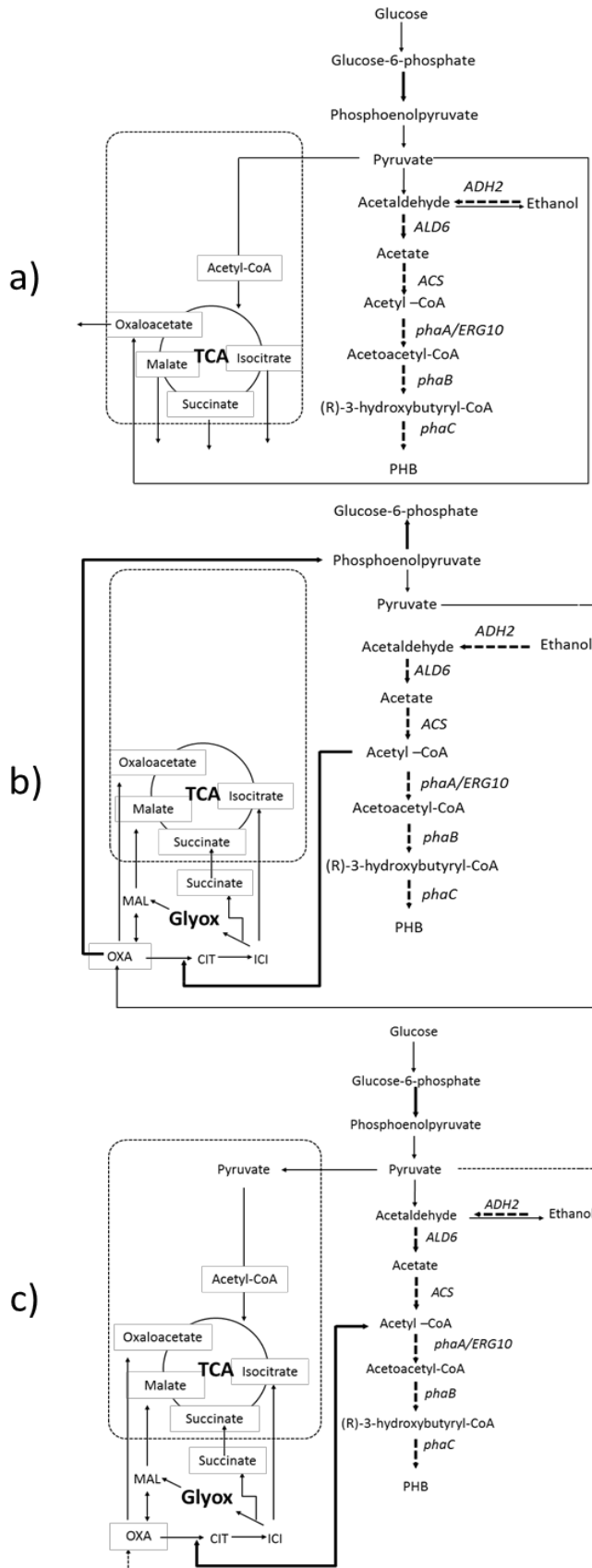
Feeding component	Dilution rate $h^{-1}$	$Y_{SX}$ Cmol/Cmol	$Y_{SEtOH}$ Cmol/C mol	$Y_{SPHB}$ Cmmol/Cmol	Ethanol accumulation Cmol/L	$Y_{xPHB}$ mg/gDW
Glucose	0.05	$0.51 \pm 0.01$	-	$2.51 \pm 0.07$	0	$4.33 \pm 0.19$
	0.10	$0.57 \pm 0$	-	$3.67 \pm 0$	0	$5.59 \pm 0$
	0.15	$0.36 \pm 0.02$	$0.02 \pm 0$	$2.44 \pm 0$	-	$5.94 \pm 0.64$
	0.20	$0.29 \pm 0$	$0.1 \pm 0$	$1.92 \pm 0$	-	$5.59 \pm 0.23$
Ethanol	0.05	$0.45 \pm 0.01$	0	$8.50 \pm 0.23$	0	$16.55 \pm 0.02$
	0.10	$0.37 \pm 0$	-	$4.94 \pm 0.58$	$0.0964 \pm 0$	$13.49 \pm 0$
	0.15	$0.12 \pm 0.01$	-	$0.46 \pm 0.05$	$0.2364 \pm 0$	$5.30 \pm 0.31$
Glucose:Ethanol (1:2)	0.05	$0.48 \pm 0.02$	0	$9.97 \pm 0.07$	0	$18.34 \pm 0.53$
	0.10	$0.38 \pm 0$	-	$7.41 \pm 0$	0.2012	$12.41 \pm 0$
	0.15	$0.30 \pm 0$	-	$2.57 \pm 0.1$	0.1637	$7.50 \pm 0.19$
	0.20	$0.22 \pm 0$	-	$1.13 \pm 0$	0.2769	$4.56 \pm 0$

The values were calculated from duplicate fermentations and are represented as mean  $\pm$  SD. The formula for biomass used in this calculation is  $CH_{1.8}O_{0.5}N_{0.2}$ .  $Y_{SX}$  = biomass yield on substrate,  $Y_{SEtOH}$  = ethanol yield on substrate,  $Y_{SPHB}$  = PHB yield on substrate,  $Y_{xPHB}$  = PHB yield per biomass



In this experiment, the chemostat cultivation was operated at different dilution rates ranging from  $0.05 \text{ h}^{-1}$  to  $0.2 \text{ h}^{-1}$  with the feeding medium being composed of different carbon sources. The biomass yields and PHB yields are reported in Table 2. Apart from growth on glucose, the biomass yield and PHB yield on substrate tended to decrease when increasing the dilution rates. The maximum PHB content,  $18.34 \text{ mg/gDW}$ , was obtained when the mixed substrate was used in the feed at a dilution rate of  $0.05 \text{ h}^{-1}$ . Comparing either glucose or ethanol as sole carbon source, ethanol alone resulted in a ~3 times higher PHB yield when the chemostat was operated at  $D = 0.05 \text{ h}^{-1}$  although it led to a lower biomass yield on substrate. The high specific growth rate and sugar concentration trigger the production of ethanol according to the respirofermentative metabolism (Duntze et al. 1969; Hanegraaf et al. 2000; Maaheimo et al. 2001). Besides that, utilizing glucose as a carbon source in the feed medium at a high specific growth rate reduces the flux to the PP pathway, which might lower the supply of NADPH (Frick and Wittmann 2005) required as a cofactor in the PHB biosynthesis pathway. Therefore, when glucose is used as a carbon source, it substantially lowers the PHB production.

The simplified schemes in Figure 18 illustrate the possible directionality of the central carbon metabolism when glucose (a), ethanol (b) and the mixture of glucose and ethanol (c) are used as carbon sources for growth and PHB biosynthesis. When the feed is composed of ethanol, the PHB yield is higher than on glucose because ethanol could replace pyruvate and serve directly as a source for acetyl-CoA used for the synthesis of PHB particularly in the tested strain with overexpression of *ADH2*. However, *S. cerevisiae* grown on ethanol requires the activity of the glyoxylate and the gluconeogenesis pathway to accomplish the synthesis of biomass and the TCA cycle intermediates. Consequently, it resulted in the lower biomass yield compared to glucose. To compensate between growth and the biosynthesis of PHB, the mixed substrate was introduced to the system and showed its potential to be used for PHB production since glucose phosphorylation still occurs while malate synthase, which converts glyoxylate to malate in the glyoxylate cycle, replaces pyruvate in the synthesis of TCA intermediates and ethanol still serves as the main source for cytosolic acetyl-CoA production (de Jong-Gubbels et al. 1995).

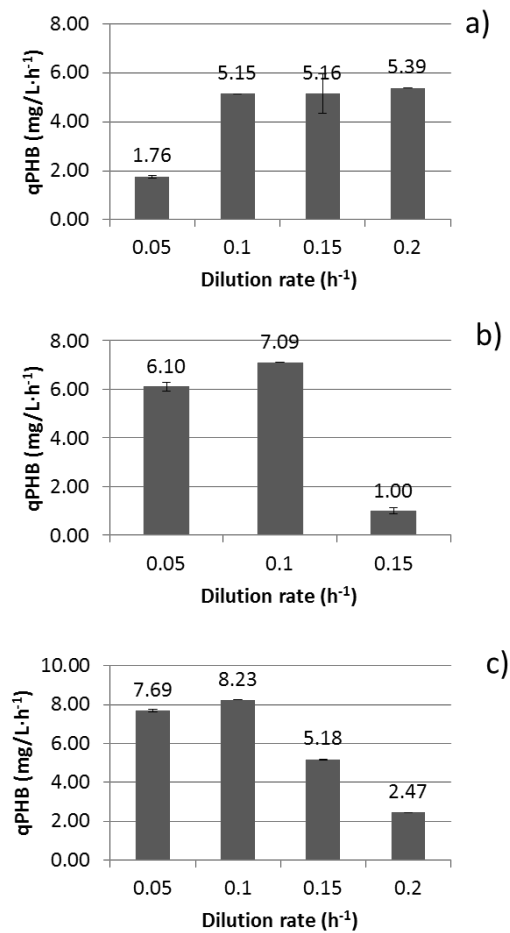


**Figure 18: Central carbon metabolism of PHB producing *S. cerevisiae* grown on different substrates. a) Glucose, b) Ethanol and c) Mixed-substrate of glucose and ethanol.**

*ADH2* = alcohol dehydrogenase; *ALD6* = aldehyde dehydrogenase; *ACS* = acetyl-CoA synthetase (*acs<sup>L641P</sup>*); *ERG10* = acetyl-CoA C-acetyltransferase; *PhaA* ( $\beta$ -ketothiolase); *PhaB* (acetoacetyl-CoA reductase); *PhaC* (polyhydroxyalkanoate synthase)

ICI = isocitrate; CIT = citrate; OXA = oxaloacetate; MAL = malate; Glyox = glyoxylate

From the industrial point of view, the overall volumetric productivity was best improved (refer to the conditions used in this study) when using the mixed substrate at the dilution rate of  $0.1 \text{ h}^{-1}$ . This operation led to a PHB productivity of  $8.23 \text{ mg/gDW}\cdot\text{h}^{-1}$  (Figure 19). The assessment of volumetric productivity confirms the potential of using a mixed-substrate for PHB production. However when mixed substrate is used, the ratio between glucose and ethanol should be considered carefully since it reflects the pattern of central carbon metabolism.



**Figure 19: PHB productivities of recombinant *S. cerevisiae* grown on different substrates at different dilution rates from the chemostat cultivation. a) Glucose, b) Ethanol, c) Mixed-substrate**

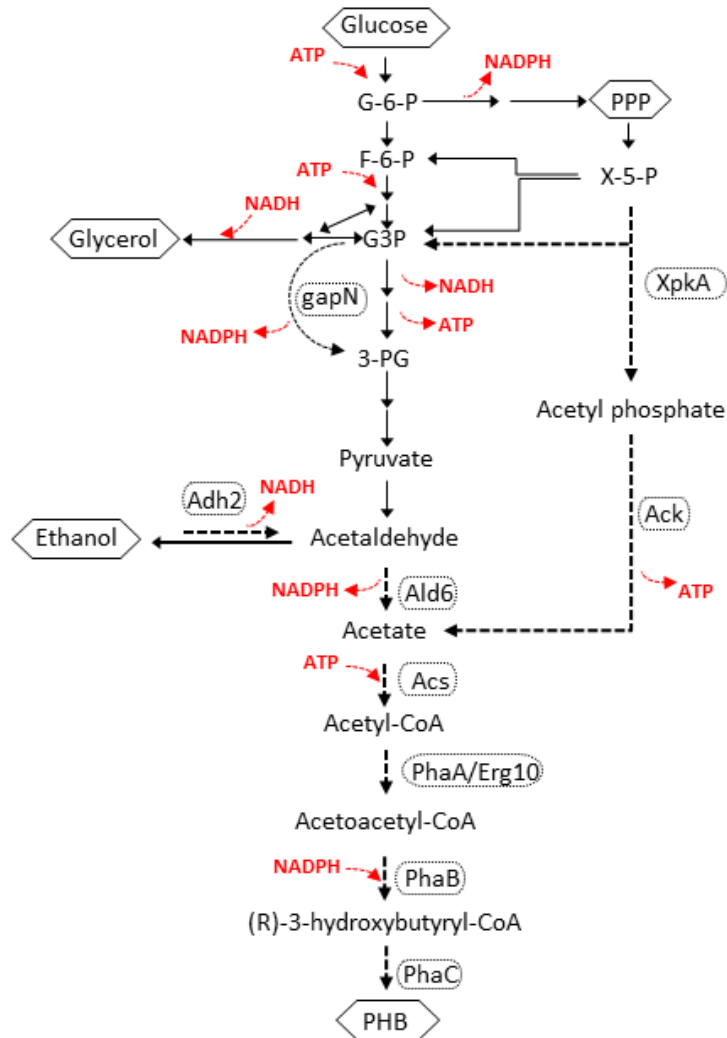
### 2.2.3. Cofactor supplementation

As the number of available databases and the reconstructed genome scale metabolic models increase, many approaches have been attempted to improve the efficiency of *S. cerevisiae* as a cell factory for the production of biocompounds. Simulations using the model of a natural PHB producer like *R. eutropha* revealed that the activities of all PHB pathway enzymes influence the overall PHB flux and that no single enzymatic step can easily be identified as rate limiting. The simulations also supported regulatory roles for both thiolase and reductase, mediated through acetyl-CoA/CoA and NADPH/NADP<sup>+</sup> ratios, respectively (Leaf and Srienc 1998). In engineered *E. coli* expressing the PHB biosynthesis pathway, instead of the availability of precursors, the expression level of acetoacetyl-CoA reductase (phaB) was suggested to be the factor controlling the flux to PHB, since the PHB flux was insensitive to the specific growth rate in a nitrogen-limited chemostat (Tyo et al. 2010a). In case of PHB producing yeast, the elementary mode analysis of recombinant *S. cerevisiae* expressing the PHB pathway was performed by Srienc and co-workers, which suggested that the introduction of the ATP citrate-lyase reaction and the transhydrogenase reaction can improve the theoretical PHB carbon yield by supplying metabolites, acetyl-CoA as precursor and NADPH as cofactor, used for PHB production (Carlson et al. 2002).

Enhancing the availability of acetyl-CoA by overexpression of genes from the ethanol degradation pathway has been shown to improve PHB production (Paper 3). Apart from acetyl-CoA supply as mentioned earlier in Chapter 2.2.1, NADPH as a required cofactor for the activity of acetoacetyl-CoA reductase (PhaB) in the PHB biosynthesis pathway is also a factor that supposed to influence PHB production in *S. cerevisiae*. Therefore, GapN from *S. mutans* was introduced into the PHB producing yeast in order to supply NADPH required during the reduction of acetoacetyl-CoA in the PHB biosynthesis pathway.

Since the major source of cytosolic NADPH production in *S. cerevisiae* grown on glucose has been attributed to glucose-6-phosphate dehydrogenase in the PP pathway, implementation of the phosphoketolase pathway from *A. nidulans* which utilizes xylulose 5-phosphate, an intermediate from the PP pathway, was proposed for channeling the flux from the EMP to the PP pathway. Overexpression of the reconstructed phosphoketolase pathway together with acetyl-CoA synthetase in *S. cerevisiae* was settled as an alternative route for providing NADPH and simultaneously contributing to increased acetyl-CoA production.

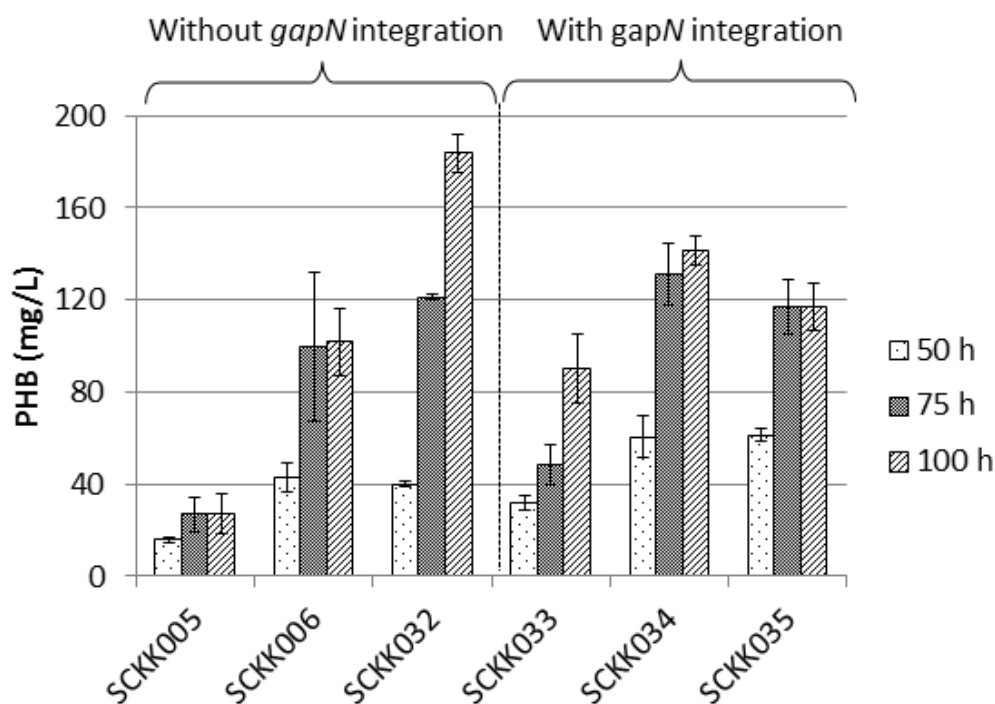
Integration of bacterial GapN and implementation of the *Aspergillus* phosphoketolase pathway were investigated for their potential to improve the PHB production in *S. cerevisiae*. The schematic pathway representing the strategies for improving the availability of NADPH is illustrated in Figure 20.



**Figure 20: Schematic pathway representing metabolic engineering strategies for improving PHB production in *S. cerevisiae*.** PPP = Pentose phosphate pathway, G-6-P = Glucose-6-phosphate, F-6-P = Fructose-6-phosphate, G3P = Glyceraldehyde-3-phosphate, 3-PG = 3-Phosphoglycerate, PHB = Polyhydroxybutyrate. Dashed arrows represent engineered pathway steps.

Besides influencing the redox metabolism by regenerating NADPH, integration of *gapN* may also result in an altered carbon flux resulting in an increased flux towards ethanol and a

reduced flux towards glycerol. When *gapN* is integrated into the chromosome of *S. cerevisiae*, a 45% decrease in glycerol formation was observed in this study (Paper.5). A comparison of PHB production in strains with and without *gapN* integration is illustrated in Figure 21. The effect of *gapN* on improving PHB production was clearly observed as the PHB yield on glucose and on ethanol of SCKK033 were substantially higher than for SCKK005 (Table 3) because GapN is expected to not only increase NADPH, but also reduce the split ratio from the glyceraldehyde-3-phosphate branch point towards glycerol. The maximum cellular PHB content obtained at 100 h in SCKK033, 14.87 mg/gDW, was 3.7 times higher than in the reference strain, SCKK005.



**Figure 21 Polyhydroxybutyrate production in *S. cerevisiae* employing different strategies to improve PHB production.** SCKK005 and SCKK033 harbor only the PHB plasmid (pKK01). SCKK032 and SCKK035 carry pJC7 (phosphoketolase pathway) whereas SCKK006 and SCKK034 carry pIYC08 (ethanol degradation pathway). Samples for PHB measurement were taken at 50 h (the glucose phase), 75 h (the ethanol phase) and 100 h (the end of fermentation where all the glucose and ethanol were depleted).

**Table 3:** Physiological parameters obtained during growth on minimal media with 20 g·L<sup>-1</sup> glucose in shake flask cultivations

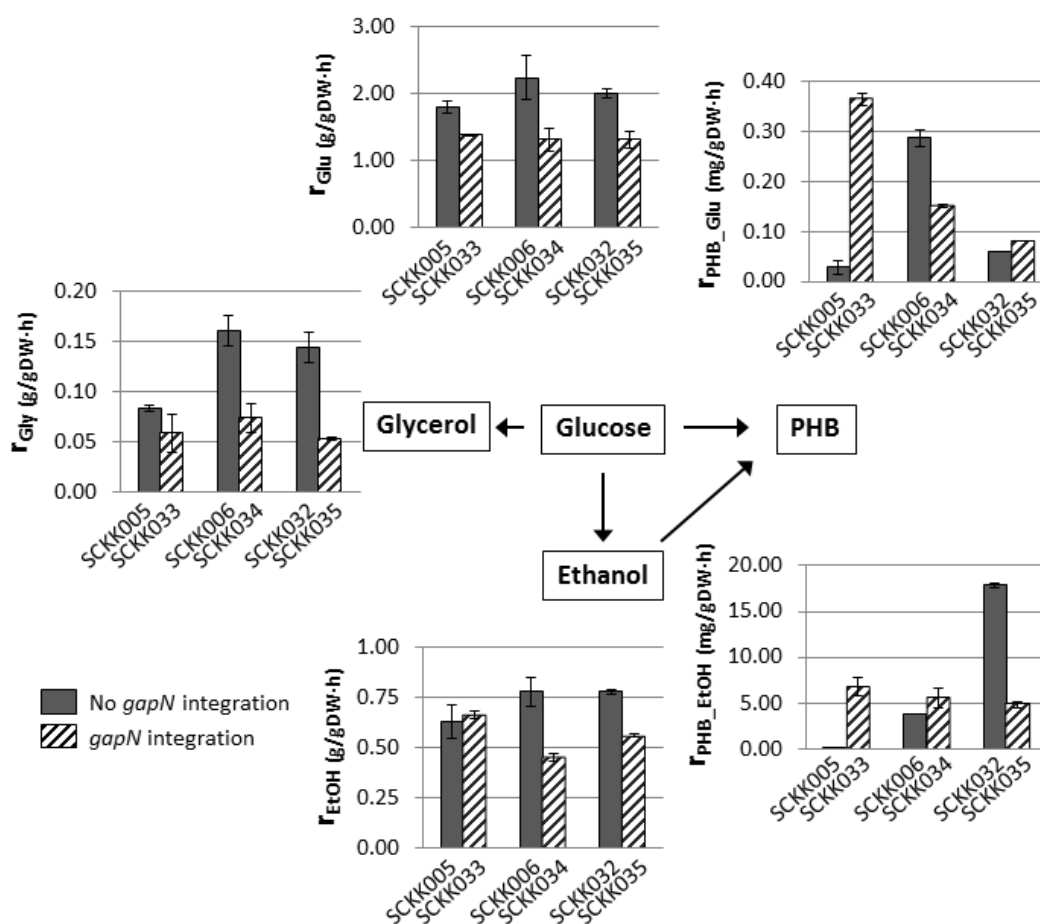
Strain	Strains without <i>gapN</i> integration			Strains with <i>gapN</i> integration		
	SCKK005	SCKK006	SCKK032	SCKK033	SCKK034	SCKK035
$\mu_{\max}$ (h <sup>-1</sup> )	0.27 ± 0.02	0.28 ± 0	0.21 ± 0.01	0.22 ± 0	0.23 ± 0.02	0.22 ± 0.02
$r_s$ (g/gDW/h)	1.80 ± 0.09	2.24 ± 0.33	2.00 ± 0	1.37 ± 0	1.31 ± 0.18	1.30 ± 0.12
$Y_{SX}$ (g/g glc)	0.15 ± 0.01	0.13 ± 0.02	0.11 ± 0	0.16 ± 0	0.18 ± 0.02	0.17 ± 0.02
$Y_{SEtOH}$ (g/ g glc)	0.35 ± 0.05	0.35 ± 0.07	0.39 ± 0.01	0.48 ± 0.01	0.35 ± 0.01	0.43 ± 0
$Y_{SGly}$ (g/g glc)	0.05 ± 0	0.07 ± 0	0.07 ± 0	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0
$Y_{SPHB}$ (mg/ g glc)	0.02 ± 0.01	0.13 ± 0.02	0.03 ± 0	0.27 ± 0.01	0.12 ± 0	0.06 ± 0
$Y_{EtOH-PHB}$ (mg/ g EtOH)	0.22 ± 0.04	6.09 ± 1.44	56.40 ± 0.77	16.11 ± 2.38	14.50 ± 2.90	12.48 ± 0.81
PHB content at 100 h (mg/gDW)	4.02 ± 0.16	15.89 ± 0	27.86 ± 0	14.87 ± 0.4	27.52 ± 4.82	21.41 ± 0.69

These values were calculated from at least triplicate shake flasks ( $n \geq 3$ ) and are represented as mean ± SD.

All strains listed in Table 3 harbor the PHB plasmid (pKK01). SCKK005 and SCKK032 carry pKK001 and pIYC04. SCKK032 and SCKK035 express the phosphoketolase pathway, SCKK006 and SCKK034 overexpress the ethanol degradation pathway.

$\mu_{\max}$  = maximum specific growth rate on glucose,  $r_s$  = specific glucose consumption,  $Y_{SX}$  = biomass yield on substrate (glucose),  $Y_{SEtOH}$  = ethanol yield on substrate (glucose),  $Y_{SGly}$  = glycerol yield on substrate (glucose)  $Y_{SPHB}$  = PHB yield on substrate (glucose),  $Y_{EtOH-PHB}$  = PHB yield on ethanol

Although integration of *gapN* involved both redox and carbon alteration, it helps increase the flux toward PHB biosynthesis pathway by supplying more NADPH rather than channeling the carbon since there was no significant difference in the specific productivity of glycerol and ethanol in SCKK005 and SCKK033 (Figure 22). Therefore, integration of *gapN* to supply NADPH supports our hypothesis that increase production of NADPH can improve PHB production in *S. cerevisiae*.



**Figure 22 Specific productivity of ethanol, glycerol and PHB obtained from shake flask cultivations.** The specific product formation rate was calculated by using the equation:  $r_p = u_{max} Y_{sp}/Y_{sx}$

$r_{EtOH}$  = specific productivity of ethanol,  $r_{Gly}$  = specific productivity of glycerol,  $r_{PHB\_Glu}$  = specific productivity of PHB in the glucose consumption phase,  $r_{PHB\_EtOH}$  = specific productivity of PHB in the ethanol consumption phase



The combined strategies to enhance the precursor, acetyl-CoA, and cofactor, NADPH, supply using *gapN* integration and over-expression of the ethanol degradation pathway is able to improve PHB production. However, there was no benefit from the provision of NADPH by GapN when combined with the reconstructed phosphoketolase pathway since no improvement on PHB production was observed. This might be due to the lack of a mitochondrial NADPH dehydrogenase in *S. cerevisiae* which otherwise couples the oxidation of cytosolic NADPH to the mitochondrial respiratory chain. Therefore, a direct oxidation of a surplus NADPH generated by the PP pathway in the respiration chain is not possible (Bruinenberg 1986; Siso et al. 1996). Furthermore, an increased production of NADPH by the EMP pathway may result in a reduced flux through the PP pathway as the glucose-6-phosphate dehydrogenase flux is very sensitive to the NADP/NADPH ratio (Vaseghi et al. 1999). When *S. cerevisiae* is grown on glucose, the PP pathway is the major source for NADPH production, specifically the dehydrogenase activity during the two first steps in the pathway (Grabowska and Chelstowska 2003; Minard and McAlister-Henn 2005). When the reconstructed phosphoketolase pathway is implemented in *S. cerevisiae*, it likely changed the split ratio of carbon from entering the lower EMP pathway to the PP pathway. The flux analysis in the strain expressing the reconstructed phosphoketolase pathway revealed a flux increase to the PP pathway (from G6P to P5P) from 32.44 to 44.55 and decreased flux to the lower EMP (from G6P to F6P) from 46.53 to 33.06 (Papini et al. 2012). Therefore, an increasing carbon flux through the PP pathway has a direct consequence for the supply of NADPH required in various anabolic reactions and of certain anabolic precursors (Frick and Wittmann 2005). For this reason, a noticeable high yield of PHB on ethanol was observed for the strain that can generate acetyl-CoA and NADPH simultaneously through the PP pathway, SCKK032. These results strongly support our hypothesis that the improved PHB production in *S. cerevisiae* required both sufficient precursor and cofactor supply.

### CHAPTER 3. Conclusion

Throughout my thesis work I have focused on metabolic engineering of *S. cerevisiae* as a model organism and the concept of metabolic engineering has been investigated for its impact on establishing *S. cerevisiae* as a platform for production of industrially relevant chemical compounds. By adapting the stable and tunable expression system from *E. coli*, the implementation of CICH<sub>E</sub> was investigated for its practicability as a novel tunable expression system in *S. cerevisiae*. Pathway engineering was examined for the potential to enhance the production of acetyl-CoA and NADPH as required precursor and cofactor for the production of PHB, the model compound in this research.

To develop a stable expression system in *S. cerevisiae*, the CICH<sub>E</sub> strategy developed in *E. coli* has shown potential to be applied in *S. cerevisiae*. However, additional studies need to be carried out. An alternative selectable marker such as the metallothionein gene would be preferable over an antibiotic resistance marker since it might improve the evolution process and be more practical for industrial applications since no antibiotic resistance marker is present in the CICH<sub>E</sub> engineered strain. It has been seen in *E. coli* that deletion of *RecA* helped stabilizing the CICH<sub>E</sub> strain. However deletion or down-regulation of genes involved homologous recombination in *S. cerevisiae*, the *RAD52* epistasis group, must be done carefully since the regulation of homologous recombination in *E. coli* and *S. cerevisiae* is different and it might affect the growth ability of the evolved strain.

Metabolic engineering of eukaryote such as *S. cerevisiae* is different from prokaryotes like *E. coli* since the cell is more complex and compartmentalized. Some metabolites can be transported or diffuse across the membrane while intermediate metabolites that interlink with many metabolic pathways such as acetyl-CoA cannot be transported across the organelles without the shuttle system. Therefore, metabolic engineering of *S. cerevisiae* that involves utilization of acetyl-CoA and NADPH should take compartmentalization into consideration.

Employing PHB as the model product in this study showed that the availability of acetyl-CoA and NADPH influences PHB production. Two alternative routes to produce cytosolic acetyl-CoA have been investigated, 1) overexpression of the ethanol degradation pathway and 2) implementation of heterologous phosphoketolase pathway to increase the split ratio of carbon to the PP pathway and simultaneously generating NADPH as required cofactor. Deletion of genes that drain cytosolic acetyl-CoA and use it in the glyoxylate cycle was not able to

promote PHB production. Moreover, it resulted in a defective growth behavior. Introduction of bacterial GapN to increase NADPH resulted in an improved PHB production. However, combined strategies did not lead to any superior effect on improving PHB production.

Since the source of acetyl-CoA varied with the type of carbon sources, ethanol seems to be the best substrate for PHB production. However in terms of productivity, the mixture of glucose and ethanol at the appropriate ratio will improve the PHB production since it compensates between biomass and product formation.

In conclusion, the papers described in the thesis demonstrate proof-of-concepts of using metabolic engineering to develop *S. cerevisiae* as a cell factory for producing industrially relevant compounds. Although the PHB production in this study could not reach the ultimate productivity compared to the natural producer, I believe that the strategies employed here could be applied to develop a platform for any product that is derived from acetyl-CoA and requires NADPH in the biosynthesis pathway.

## **CHAPTER 4. Perspectives on developing *S. cerevisiae* as a cell factory for biocompounds production**

There are many reasons supporting the utilization of *S. cerevisiae* as a model organism for expression of various heterologous pathways. Systems and synthetic biology also advance metabolic engineering in *S. cerevisiae* to meet the point where the engineered heterologous pathway can compromise with the native host metabolic pathway and ultimately be competitive with the traditional chemical synthesis pathway. Despite the success of metabolic engineering to improve PHB production in *S. cerevisiae*, there still exist areas for continued development by implementation of knowledge obtained from this study. For instance, in order to maximize the flux to product formation, pathway engineering is achieved either by removing the competitive enzyme(s) at the branch point of competing pathways or overexpressing the enzymes in the heterologous pathway, so that the substrate can be directed to the desired products instead of by-product formation. Unfortunately, a complete removal of enzymes from the competitive pathways, sometimes results in a defect in growth (as seen for the deletion of genes involved in the glyoxylate cycle- Paper 3). Therefore, implementation of CICE, which enables the tunable control of the expression level of a specific gene in a particular pathway can be applied (1) to balance the expression level with other genes in the biosynthesis pathway, (2) to control the expression level of a gene encoding an enzyme that has been identified as a factor limiting the specific productivity or the flux toward product formation, and (3) to control the expression level of an essential gene encoding a by-product formation. When a non-native pathway is employed in *S. cerevisiae* that involves intermediates such as acetyl-CoA or the redox cofactor NADPH, compartmentalization should be taken into account since *S. cerevisiae* is not capable of de novo synthesis of carnitine involved in the shuttle system for acetyl-CoA and the evidence for NADPH transport across the mitochondrial membrane still needs to be provided. Altogether, to accomplish the development of *S. cerevisiae* as a universal platform for industrial applications requires the knowledge from molecular regulation to particular pathway engineering and the overall cell physiology and metabolism.

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