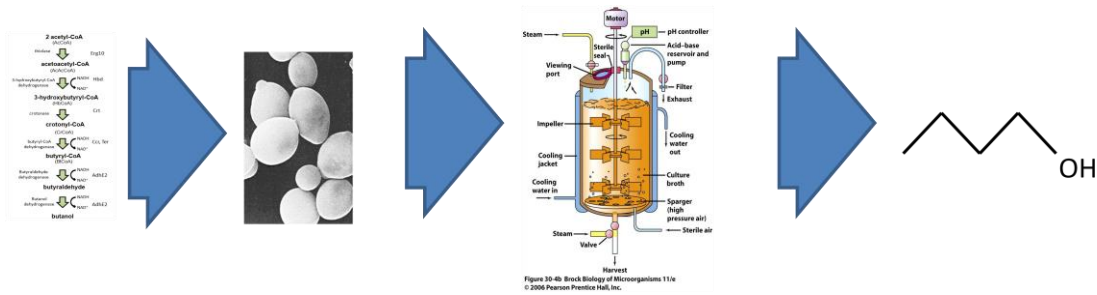


Optimization of butanol pathway in metabolically engineered *Saccharomyces cerevisiae*



Master of Science Thesis in Biotechnology

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SYSTEMS BIOLOGY GROUP

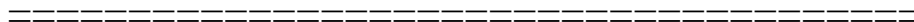
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Thesis for the Degree of Master of Science

Optimization of butanol pathway in metabolically
engineered *Saccharomyces cerevisiae*



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Göteborg, June 2012

Preface

This report presents the results of my master thesis study carried out at the Systems & Synthetic Biology Group, Department of Chemical and Biological Engineering, Chalmers University of Technology, in the period from August 2011 to June 2012.

I am indebted to various people who have made my study a highly joyful and inspiring moments. First of all, I would like to thank Professor Jens Nielsen to give me this opportunity to carry out my thesis in this group, and I wish to express my deepest gratitude to my supervisors, Dr. Verena Siewers and Dr. Anastasia Krivoruchko who were abundantly helpful and offered invaluable assistance, support and guidance. They kept me encouraged and enabled me to develop an understanding of the subject.

Various people have helped me throughout my research. I would particularly thank Dr. Il-Kwon Kim, Dr. Siavash Partow and Dr. Mahsa Rahimzadeh for invaluable discussion and assistance on my research.

Finally, I wish to thank my family for their support and encouragement throughout my work, which has been beyond words.

Göteborg, June 2012
Maryam Karimi

Abstract

Rising of energy costs and increased awareness of global warming have inspired production of renewable, biomass-derived chemicals and fuels. The reasons for producing alternatives to ethanol are multiple: ethanol suffers from low energy density, it cannot be piped, and it is costly to distill. Suitable biofuels will require minimal energy to separate them from fermentation broths, be non-toxic to the host micro-organism, and be efficiently produced from a variety of feedstocks. Butanol is a substantially better biofuel than ethanol.

In this study, *Saccharomyces cerevisiae* was chosen as a host for butanol production because it is a genetically tractable, well-characterized organism, the current industrial ethanol producer, and it has been previously manipulated to produce other heterologous metabolites.

This study has focused on optimization of the heterologous butanol pathway in *S. cerevisiae* for production of higher titers of butanol. In the first part of the present research, the butanol pathway is investigated by simultaneous overexpression of biosynthetic genes *hbd*, *crt*, *ter* and *adhE2* under regulation of different combinations of the two promoters of yeast genes *TEF1* and *PGK1* in *S. cerevisiae*. The first series of strains contains combinations of butanol synthesis genes under different promoters plus overexpression of the *ERG10* gene which resulted in the same levels of butanol production. In the second series of strains, butanol genes were cloned under control of different combinations of promoters and were expressed in yeast along with overexpression of *ALD6*, *acs_{SE}*, *ADH2*, and *ERG10* to provide the pathway with higher concentration of cytosolic acetyl-CoA. These strains resulted in different production levels of butanol. These results imply the importance of intracellular concentration of acetyl-CoA, i.e. even if the pathway becomes more efficient due to the promoter swap, it is not distinguishable probably due to the limitation in acetyl-CoA concentration, which is assumed to be lower in strains which only contain the *ERG10* plasmid. Variations in butanol titer from 7.5 to 26.8 mg/L, demonstrated that controlling different genes under different promoters can influence the microbial production titer. In the second part of the project butanol disappearance in cultivations of the engineered strains and wild type were also investigated and the probability of the usage of butanol as carbon source by yeast was further studied by shake flasks

cultivation. The decreasing trend of butanol concentration after the glucose phase could be due to the utilization of it by the cells or in lower quantity due to evaporation from the medium.

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Chapter 1

1.1 Introduction


Due to the global energy and environmental problems, synthesis of biofuels from renewable resources has been an incentive for many researchers. Biofuels have the potential to not only reduce the dependency of countries on foreign oil imports, but also dramatically decrease greenhouse gas emissions which cause global warming. Flexible fuel vehicles are designed to run on pure gasoline or a blend of gasoline and up to 85% ethanol. However, since ethanol contains less energy than gasoline, a vehicle will reach 20 to 30 % fewer miles per gallon when fuelled with ethanol.

1.1.1 Butanol

Butanol has several advantages over ethanol as a fuel. Although it can be made from the same feedstocks as ethanol, unlike ethanol, it is miscible with gasoline and petrodiesel at any ratio. It also can be used as a pure fuel and has been proposed as jet fuel by the Sir Richard Branson Group at Virgin Airlines. Butanol does not absorb water unlike ethanol, and has a higher energy content compared to ethanol. These properties provide the potential for butanol to be used in the same manner as gasoline, without modification for vehicle usage [1].

Butanol is a four carbon alcohol with the molecular formula C_4H_9OH (MW 74.12). Some of its characteristics can be seen in Table 1 [2].

Table 1. Some properties of butanol [2]

Melting point (°C)	-89.3			
Boiling point (°C)	117.7			
		Butanol structure		
				
Ignition temperature (°C)	35			
Flash point (°C)	365			
Density at 20°C (g/mL)	0.8098			
Critical pressure (hPa)	48.4			
Critical temperature (°C)	287			
		Properties of fuels		
	Butanol	Gasoline	Ethanol	Methanol
Energy density (MJ/L)	29.2	32	19.6	16
Air-fuel ratio	11.2	14.6	9	6.5
Heat of vaporization (MJ/kg)	0.43	0.36	0.92	1.2
Research octane number	96	91-99	129	136
Motor octane number	78	81-89	102	104

Donaldson et al. (2011) have reported that 10-12 billion pounds of butanol are produced each year, which is a 7-8.4 billion dollar market at its current price. The projected market expansion of butanol is estimated 3% per year. The major amount of currently produced butanol is used in the form of butyl acrylate and butyl methacrylate esters which are mostly used in enamels, latex surface coating and lacquers [3]. Other applications of butanol are diluents for brake fluid formulation and it is also used in antibiotic, hormone and vitamin manufacturing. In addition, there has been recent interest in using butanol as replacement for gasoline [4].

Butanol properties are similar to gasoline and it is superior to ethanol as a fuel (Table 1) in many aspects such as lower volatility, higher energy content, lesser corrosiveness and higher hydrophobicity [5]. Moreover, Atsumi et al. (2008) reported that the branched butanols including isobutanol, 2-methyl-1-butanol have higher octane numbers compared with butanol [6], therefore they can be good candidates as fuel additives [7].

Early industrial butanol production was based on fermentation of carbohydrates with the bacterium *Clostridium acetobutylicum* which produces mainly butanol and acetone [8]. High demands for butanol and the development of the petrochemical industry caused a replacement of biological processes by efficient chemical processes. In recent years, increases in the oil price and concerns about global warming have refreshed interest in biotechnological butanol production. Reflecting this, some companies such as BP and DuPont have announced working on commercialization and developing of butanol processes on fermentation bases [7].

1.1.2 Microbial cell factories for industrial biotechnology

Most of the consumer products, energy and major amounts of butanol which are used nowadays are produced in the chemical industry and particularly in the petrochemical industry via classical chemical synthesis.

Chemical synthesis of products has many drawbacks such as limited range of products and the accumulation of large amounts of industrial waste. On the other hand, nowadays our society is faced with the challenges of depleting fossil fuel reserves, increasing demands for energy and consumer products and growing global environmental problems. New processes using the renewable feedstock for chemical production are on the agenda to decrease the dependency on the fossil fuel based economy [9].

Industrial biotechnology, often known as white biotechnology in Europe has provided solutions to the above mentioned challenges. The European association for Bioindustries <http://www.europabio.org/> has defined industrial biotechnology as “the application of biotechnology for processing and production of materials, chemicals and energy”. This involves the use of enzymes and microorganisms to make useful products that can be used in various industries such as food, chemicals and feed, paper and pulp, textile and energy [9].

One important aspect in industrial biotechnology is the use of fermentation or enzymatic conversion processes to produce industrial products, replacing those produced in the traditional chemical industry. Early industrial biotechnology products include important products from anaerobic fermentations such as lactic acid, butanol and isopropanol [10].

In modern industrial biotechnology, a large variety of bulk and fine chemicals such as biofuels, bioplastics, pharmaceuticals and food ingredients can be produced by microbial fermentation. Biotechnological processes based on microbial cell factories have several advantages in comparison with the traditional chemical factories. For instances, they use sustainable bio-resources instead of fossil fuels; they also result in much less carbon dioxide emission and thus decrease global warming; and they are capable of producing novel chemicals with huge diversity and complex structure, which are difficult to obtain through chemical synthesis [11]. Development of genetic engineering tools in recent years has allowed the use of a variety of

microorganisms such as the filamentous fungus *Aspergillus oryzae*, the bacterium *Escherichia coli* and the yeast *S. cerevisiae* as cell factories for production of desired products.

1.1.3 Metabolic engineering

In order to optimize production and obtain desirable cellular properties, such as higher production yield, improved physiological properties, and production of novel metabolites, certain engineering tools are needed to manipulate the cell factories. One way to achieve this is through traditional mutagenesis strategies, such as random mutagenesis and screening. Although the traditional methods provided successful cases such as production of antibiotics, solvents and vitamins, they require a lot in terms of time and labor. This is partly because traditional methods are not based on a systematic understanding of the underlying mechanism for the desired cellular properties. Even in cases where the desired properties are obtained, the underlying mechanisms remain largely unknown, which does not aid further knowledge to research and development [12].

Metabolic engineering is based on rational and directed genetic modifications in contrast to the traditional random mutagenesis and screening [13]. Stephanopoulos et al. (2007) have defined metabolic engineering as “the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or introduction of new ones with the use of recombinant DNA technology”. Metabolic engineering can be used to obtain a higher yield or productivity of the target metabolite, to delete or down-regulate by-product formation, for production of novel products and to improve cellular physiology for an optimized process, etc [14].

Metabolic engineering can be described as an iterative process including three steps: synthesis, analysis and design [13]. The synthesis step utilizes genetic engineering including cloning, transformation and homologous recombination to introduce desirable genetic modification. The newly constructed strains with the modified genetic properties are then tested in the analysis step. Different analytic techniques, such as transcription analysis, protein analysis, metabolite analysis and flux analysis, can be applied. By interpreting the analyzed data, further examination of the metabolic network is made and subsequently, new genetic manipulation targets for the next round can be proposed in the design step.

Metabolic engineering can be easily carried out in yeast because of its well-defined genetic system and highly efficient heterologous recombination feature. *S. cerevisiae* has other advantages for metabolic engineering purposes such as availability of numerous markers, auxotrophic strains and efficient transformation vectors.

Moreover, there are promoters with different strength that can control the expression level of the genes of interest. For example, glycolytic promoters such as *PGK1*, the promoter of the gene encoding 3-phosphoglycerate kinase, or *TEF1* which encodes transcriptional elongation factor EF-1 α [15], can be used for strong constitutive expression of the target genes [16, 17]

1.1.4 Metabolic engineering for butanol production

As mentioned above, butanol can be biologically produced via acetone-butanol-ethanol (ABE) fermentation by the *Clostridium* species of bacteria. ABE fermentation by *Clostridium acetobutylicum* is one of the oldest methods for industrial production of butanol. However, Clostridia are not ideal for butanol production because of the lack of genetic tools to manipulate the metabolism, their slow growth in strict anaerobic condition, their intolerance to butanol above 1-2% and oxygen and their production of other byproducts such as butyrate and acetone [3]. Many attempts have been made to maximize butanol production from an ABE process by elimination of other by-products. These have however not been completely successful [3, 18].

Therefore, owing to the negative aspects described above there is an interest in producing butanol in a process where butanol is the sole product. An environmentally responsible, cost effective process for butanol production might be achieved through the development of a recombinant microbial production host expressing the butanol biosynthetic pathway [3].

Atsumi et al., in 2007 have reported that the mutant *E. coli* BW25113 ($\Delta adhE \Delta ldhA \Delta frdBC \Delta fnr \Delta pta$) strain with overexpression of the *crt*, *bcd*, *etfAB*, *hbd* and *adhE2* genes of *C. acetobutylicum*, and the *atoB* gene of *E. coli* produced 552 mg/L butanol using 2% (w/v) glycerol as carbon source. Lan et al, in 2011 successfully expressed all of the genes in the acetyl-CoA-dependent butanol pathway in *Synechococcus elongatus* 7942 and achieved butanol production from CO₂ and light. They introduced *hbd*, *crt*, and *adhE2* genes from *C. acetobutylicum*, the *ter*

gene from *Treponema denticola*, and the *atoB* gene from *E. coli* into wild-type *S. elongatus* 7942.

A recently published international patent by DuPont describes the production of butanol in *E. coli*, *Bacillus subtilis* and *S. cerevisiae* by overexpressing the *thl*, *hbd*, *crt*, *bcd*, and *bdhAB* genes of *C. acetobutylicum* which are involved in butanol biosynthesis. 0.8 mM, 0.19 mM and 0.01 mM butanol were produced by engineered *E. coli*, *B. subtilis* and *S. cerevisiae*, respectively. These low titers of heterologous butanol production can show the difficulty of transferring this pathway to nonnative hosts. To achieve high-titer production different metabolic engineering strategies should be examined.

In another study, Steen et al., (2008) have chosen *S. cerevisiae* as a host for butanol production and by choosing appropriate isozymes they improved production of butanol to 2.5 mg/L [18].

1.1.5 *Saccharomyces cerevisiae* as a cell factory

S. cerevisiae (in the following frequently referred to as yeast) is budding yeast also known as baker's and brewer's yeast, and is a unicellular eukaryote. *S. cerevisiae* which belongs to the kingdom of fungi is mostly used in the traceable historical record of mankind for preparation of alcoholic beverages and bread and is classified as a GRAS organism (generally regarded as safe) by the Food and Drug administration (FDA). Traditionally, the great benefit of yeast was based on its fermentation ability, i.e. the conversion of sugars (mono- and few di-saccharides) into carbon dioxide and alcohol (ethanol). The modern usage of it is not limited to food production and has extended into an important model organism for understanding of molecular biology and cell physiology. Despite being a unicellular organism, these cells share many common biological features (genes, organelles, etc) with higher organisms, which have made yeast an ideal model for the study of human diseases. In addition, yeast is also used for industrial production of other metabolites, proteins and chemicals [19, 20].

S. cerevisiae is chosen as a host for butanol production because it is a genetically tractable, well characterized organism, the current industrial ethanol producer, and it has been previously manipulated for production of other heterologous metabolites [20]. Moreover, since butanol and ethanol just differ by two carbons, *S. cerevisiae* may use similar mechanisms to tolerate high concentrations of butanol as it does in high concentrations of ethanol [21].

1.2 Metabolic engineering of *Saccharomyces cerevisiae* as a host for butanol production

S. cerevisiae traditionally was used in production of bioethanol. Nowadays as a cell factory, it is used to produce other important chemicals including organic acids, e.g. lactic acid [22], succinate [23], glycerol [24], and more complex natural products, e.g. isoprenoids [25], and polyketides [26]. Moreover, in biotechnology it is used in production of heterologous proteins and is an ideal host for heterologous protein production since post-translational modifications are possible in it and it can secrete proteins [27].

Saccharomyces cerevisiae was engineered introducing the butanol pathway by Steen et al. (2008); they were able to improve butanol production to 2.5 mg/L. In a recent study (Figure 3/step 1), a driving force has been created in the acetyl-CoA pathway by overexpression of the *ADH2*, *ALD6*, *acs_{se}* and *ERG10* by pIYC08 plasmid (Chen, unpublished results).

The butanol pathway transformed to yeast along with overexpression of genes in the acetyl-CoA pathway provides higher titers of butanol (Krivoruchko, unpublished results).

1.2.1 Expression of butanol pathway from a range of organisms in *Saccharomyces cerevisiae*

The reactions for butanol production and corresponding enzymes used in this project are outlined in Figure 1 and involve the following enzymes: acetyl-CoA acetyltransferase or thiolase encoded by *ERG10* which is native in *S. cerevisiae* and catalyzes the conversion of acetyl-CoA to acetoacetyl-CoA (AcAcCoA). The second enzyme is 3-hydroxy-butyryl-CoA dehydrogenase (*hbd*) which converts AcAcCoA into 3-hydroxybutyryl-CoA (HbCoA). *Hbd* uses NADH as a cofactor and is derived from *Clostridium beijerinckii*. According to Steen et al. (2008), using *ERG10* in combination with *hbd* led to a higher production of butanol in *S. cerevisiae* compared to strains which contain *phaA* and *phaB* from *Ralstonia eutropha* [18].

The third enzyme is 3-hydroxybutyryl-CoA dehydratase (crotonase; *crt*), which is derived from *C. beijerinckii* and converts 3-hydroxybutyryl-CoA (HbCoA) to crotonyl-CoA.

Shen et al., (2011) reported that using *ter* from *T. denticola* instead of Bcd-EtfAB from *C.*

acetobutylicum which encodes butyryl-CoA dehydrogenase may provide an additional driving force for butanol production since its reaction is irreversible. Therefore, *ter* is used to catalyze the conversion of crotonyl-CoA (CrCoA) to butyryl-CoA (BtCoA).

AdhE2 which is derived from *C. acetobutylicum* and encodes a bifunctional aldehyde/alcohol dehydrogenase that acts on butyryl-CoA to produce butyraldehyde, as well as on butyraldehyde to produce butanol.

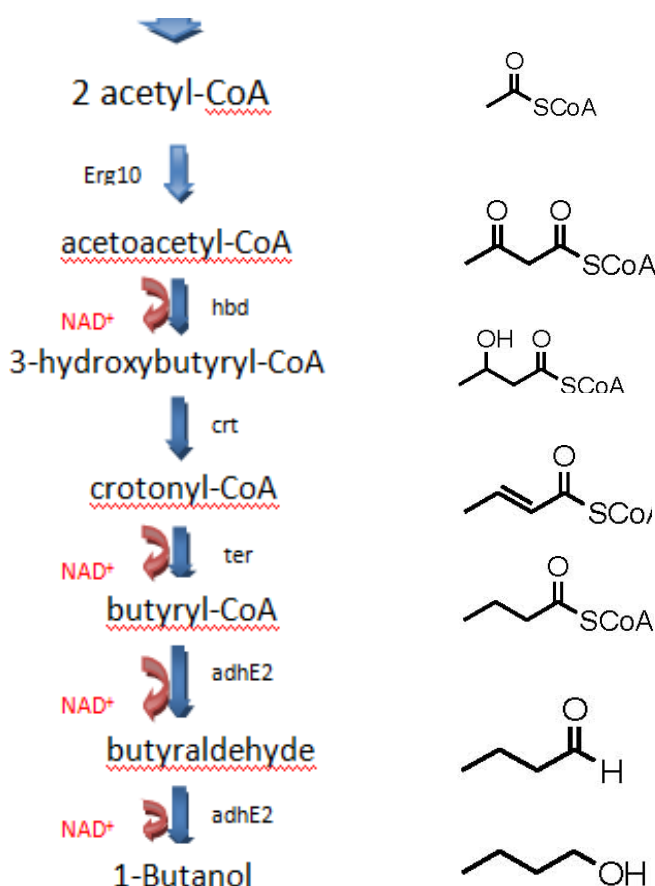


Figure 1. Butanol pathway and genes used in the current study

1.2.2 Pathway regulation and strong yeast promoters

Among strong constitutive promoters, the most commonly used ones for high-level gene expression in *S. cerevisiae* are *pTEF1* and *pPGK1* as well as promoters derived from various glycolytic pathway genes.

Since yeast prefers glucose as a carbon source, expression vectors which contain two constitutive promoters, *pTEF1* and *pPGK1* were used which provide high levels of gene expression in glucose containing medium.

In a recent experiment, the butanol pathway was expressed in a *S. cerevisiae* CEN.PK strain using the bidirectional constitutive promoter, pTEF1-pPGK1.

Two expression plasmid constructs developed in our lab, pSP-GM1 and pSP-GM2 (Figure 2 A, B) enable gene expression from this bidirectional promoter [17]. The two different promoter orientations in pSP-GM1 and pSP-GM2 allow for a variety of cloning strategies due to the different promoter-multi-cloning site combinations. In the present study, four different genes in the butanol pathway were cloned under TEF control of different promoter combinations in these vectors in order to optimize and change the expression level of the cloned genes.

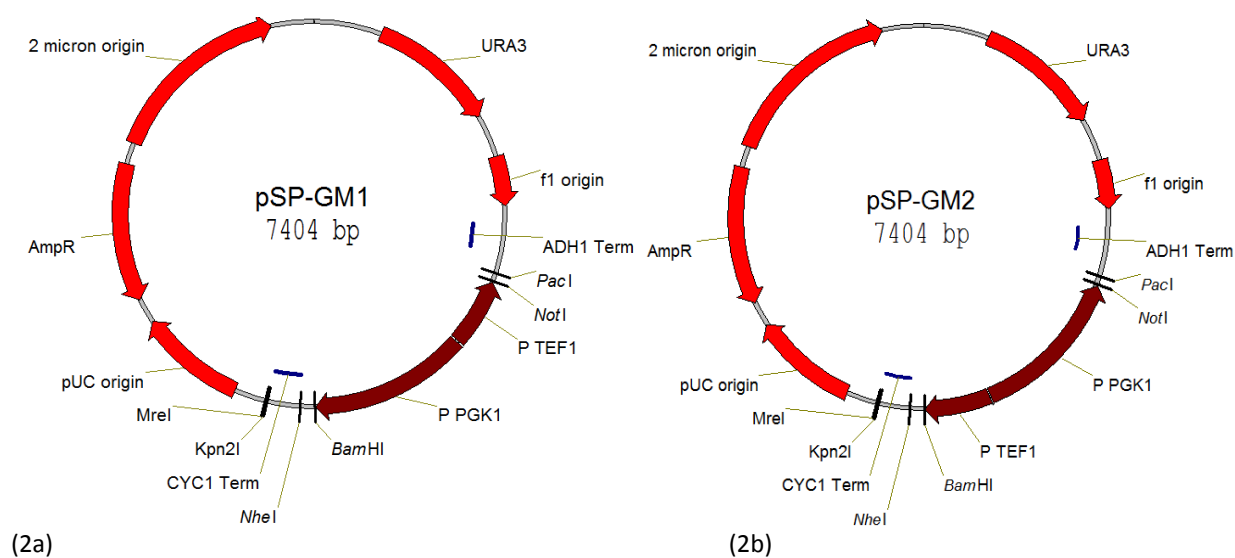


Figure 2. Schematic illustration of the plasmid maps of pSP-GM1 and pSP-GM2. The used restriction sites are presented.

Figure 3 shows the overview of the butanol pathway expressed in yeast. Since the clostridial CoA-dependent pathway for butanol synthesis requires the expression of 4 heterologous genes (step 2 in figure 3), the presence of a driving force for the acetyl-CoA pathway to channel significant amounts of acetyl-CoA towards butanol would be essential to achieve high titers.

In one of the experiments, expression of the butanol genes was coupled with overexpression of *ERG10* which catalyzes acetoacetyl-CoA formation at the start of the butanol pathway to provide a better link to the butanol pathway.

In another study, the acetyl-CoA driving force could be established by overexpression of *ALD6*, *acs_{SE}*, *ADH2* and *ERG10* (Step 1 with red arrows in fig.3), which was coupled with butanol

pathway expression. *ADH2* encodes alcohol dehydrogenase catalyzing the reaction from ethanol to acetaldehyde. Ald6 catalyzes the reaction from acetaldehyde to acetate and *acs_{SE}* encodes a mutated (acetylation-insensitive) version of acetyl-CoA synthetase from *Salmonella enterica*, which catalyzes reaction from acetate to acetyl-CoA, which further drives the flux towards acetyl-CoA. As mentioned above, Erg10 is a native enzyme catalyzing the reaction from acetyl-CoA to acetoacetyl-CoA, which represents the first step in the butanol pathway.

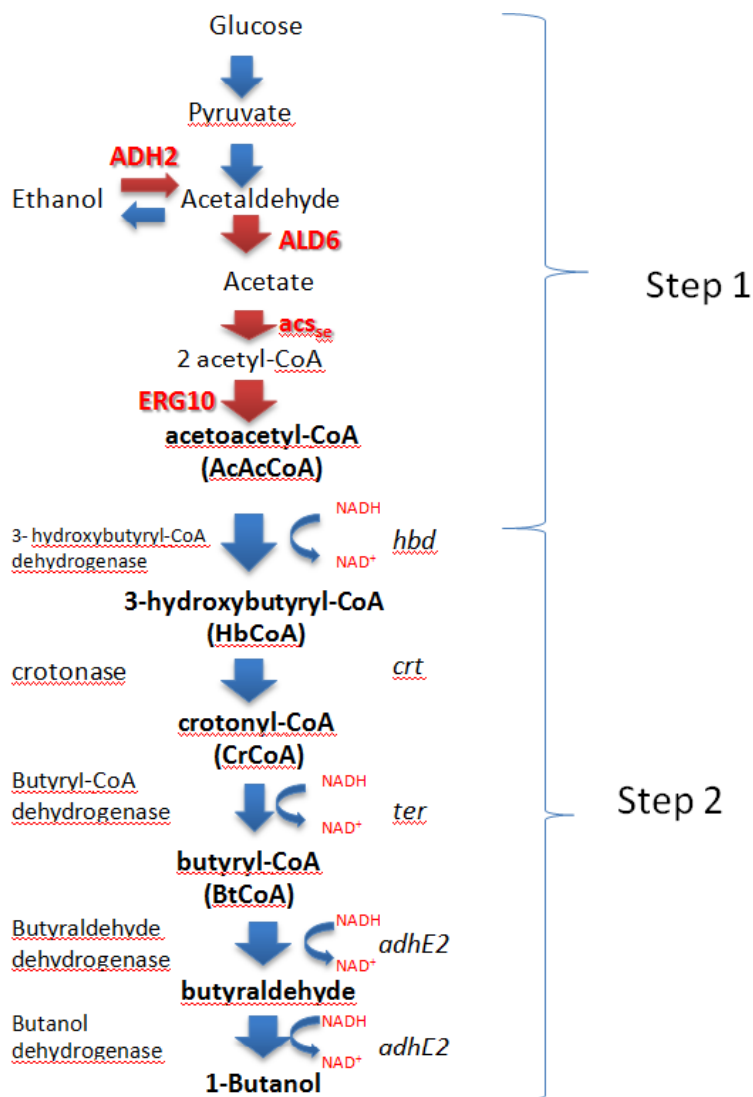


Figure 3. The butanol biosynthetic pathway re-constructed in this project. The pathway is divided into two steps: the first step of the pathway is native to yeast and an acetyl-CoA driving force was established by overexpression of *ALD6*, *acs_{se}*, *ADH2* and *ERG10* and coupled with butanol pathway expression. The 2nd step of the pathway is introduced to yeast and is comprised of genes *hbd*, *crt* from *Clostridium beijerinckii*, *adhE2* from *C. acetobutylicum* and *ter* from *Treponema denticola*. In another experiment, the butanol pathway was coupled to overexpression of *ERG10* alone.

Chapter 2

2.1 Materials and methods

2.1.1 Yeast strain

For the present study, a laboratory haploid *S. cerevisiae* strain was used, which is a derivative of the prototrophic CEN.PK 113-7D, which is referred to as the parental or wild type strain in the following.

The CEN.PK strain family was constructed in an interdisciplinary German research project by Peter Kötter, Frankfurt, Germany [28]. The auxotrophic strain CEN.PK 113-11C (*MATa his3-Δ1 ura3-52 MAL2-8c SUC2*) was used for the current study.

The haploid *S. cerevisiae* strain CEN.PK113-11C (Table 3.1) was transformed with the *URA3* based (pAK01, pMK02, pMK03, pMK04) and *HIS3* based expression plasmids (pCS01 and pCIY08).

2.1.2 Media and reagents

LB (Lysogeny broth) ampicillin liquid medium was prepared with 1 % sodium chloride (Merck, Darmstadt, Germany), 1 % tryptone (Merck) and 0.5 % yeast extract (Merck, Sigma-Aldrich) which were dissolved in de-ionized water. The pH was set to 7.0 after which the medium was autoclaved. All chemicals were purchased from Merck or Sigma-Aldrich if not mentioned otherwise. Ampicillin stock solution, 100 mg/ml, filter sterilized using a 0.2 μm filter (Sigma-Aldrich) was added to the medium after cooling to the final concentration of 0.1 mg/ml.

LB ampicillin agar plates and LB kanamycin (100 mg/l) were made in a similar fashion the only difference being the addition of 2% agar (Merck) to the above components. The mixture was poured into petri dishes after addition of antibiotics.

YPD (Yeast extract Pepton Dextrose) liquid medium was made with 1 % yeast extract, 2 % peptone from casein or peptone from meat and 2 % D-(+)-glucose–monohydrate, which was dissolved in de-ionised water after which the mixture was autoclaved.

YPD agar plates were prepared by dissolving 1 % yeast extract, 2 % peptone and 2% glucose in de-ionised water and subsequently, 2% agar was added to the mixture followed by autoclaving. The cooled mixture was poured into petri dishes.

Synthetic drop-out (SD) solid medium lacking uracil and histidine was used for selection of yeast transformants. SD-glucose –Ura –His was prepared by mixing 2 % glucose, 0.69 % yeast nitrogen base without amino acids, 0.079% complete supplement mixture lacking uracil and histidine after which the pH was adjusted to 5.5-6.0. Subsequently 2 % agar was added to the mixture followed by autoclaving. The cooled mixture was poured into petri dishes.

2.1.3 Plasmid and strain construction

For strain construction, standard molecular biology methods were used. *Escherichia coli* DH5 α were used for plasmid constructions. Primers used for PCR amplification of DNA fragments (*PGK1* and *TEF1* promoters, genes *crt*, *hbd*, *ter* and *adhE2*) are listed in Table 4. The codon optimized synthetic genes (*crt* from *Clostridium beijerinckii*, *hbd* from *Clostridium beijerinckii* and *ter* from *Troponema denticola* were cloned into pJ201, *adhE2* from *C. acetobutylicum* was cloned into pJ206 plasmids by DNA2.0) were amplified by transformation to *E. coli* DH5 α , extracted using the GeneJET™ Plasmid Miniprep Kit, and subsequently cloned downstream of the *PGK1* or *TEF1* promoter sequence in pSP-GM1 and pSP-GM2. Each plasmid scheme (pAK01, pMK02, pMK03 and pMK04) was constructed as shown in Figure 5.

Plasmid pSPGM1 +*crt* + *hbd* + *ter*+ *adhE2* is referred to as pAK01. The generated construct is shown in Figure (4). In plasmid pAK01 with 14581 bp (Table 2) (Fig.4), the *hbd* gene is under regulation of the *PGK1* promoter, *crt* under control of the *TEF1* promoter, *ter* under control of the *PGK1* promoter and *adhE2* under regulation of the *TEF1* promoter.

Table 2. Overview of different promoter-gene combinations.

Gene/Scheme	Promoter			
	pAK01	pMK02	pMK03	pMK04
<i>hbd</i>	PGK1	PGK1	PGK1	TEF1
<i>crt</i>	TEF1	PGK1	TEF1	TEF1
<i>ter</i>	PGK1	TEF1	TEF1	PGK1
<i>adhE2</i>	TEF1	TEF1	PGK1	PGK1

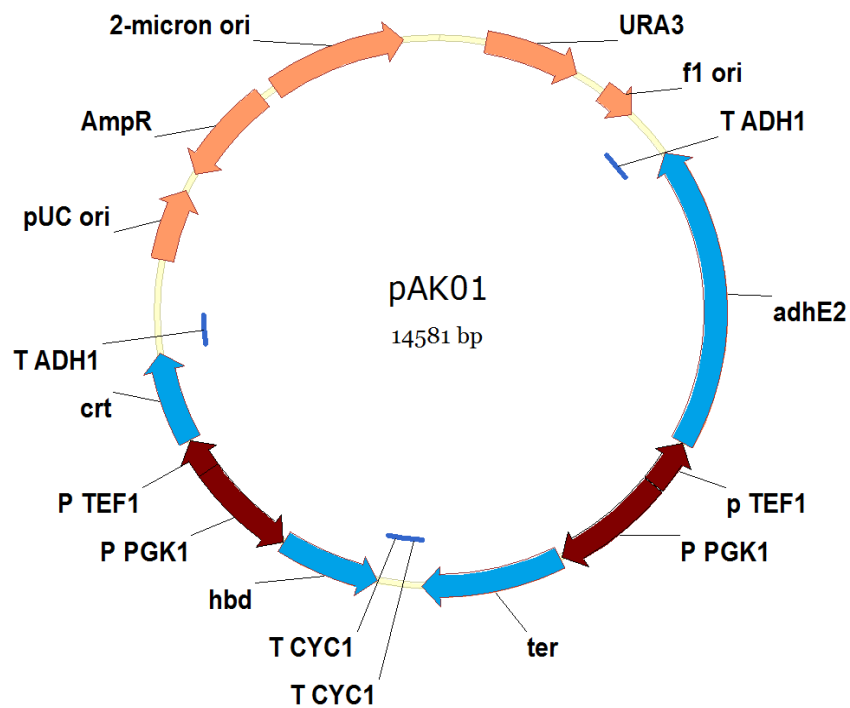


Figure 4. Schematic illustration of the plasmid pAK01



Figure 5. Schematic illustration of the plasmid schemes.

For construction of pMK02, gene *adhE2* (restricted by *NotI/PacI*) and gene *hbd* (restricted by *BamHI/NheI*) were isolated and cloned into pSP-GM1 (Figure 2a) (pSP-GM1+*adhE2+hbd* referred to as pMK06). Gene *crt* (restricted by *NotI/PacI*) and gene *ter* (restricted by *BamHI/NheI*) were isolated and cloned into pSP-GM2 (Figure 2.b) (pSPGM2+*ter+crt* referred to as pMK07).

The pMK07 plasmid cassette containing both genes *crt* and *ter* was amplified by PCR primers (Mre-TCYC and Kpn-TADH) (table 4); restricted by *Kpn2I* and *MreI* and cloned into plasmid pMK06 (pSPGM1 +*crt* + *hbd* + *ter*+ *adhE2* referred to as pMK02). The schematic design of the generated plasmid is shown in Figure 5. In plasmid pMK02 consisting of 14581 bp (Table 2) (Fig.5) the *hbd* and the *crt* gene are under regulation of the *PGK1* promoter, while *ter* and *adhE2* are under control of the *TEF1* promoter.

For construction of pMK03, gene *adhE2* (restricted by *NotI/PacI*) and gene *ter* (restricted by *BamHI/NheI*) were isolated, and cloned into pSP-GM2 (Figure 2b) (pSP-GM2+*adhE2+hbd* referred to as pMK08). Gene *crt* (restricted by *NotI/PacI*) and gene *hbd* (restricted by *BamHI/NheI*) were isolated, and cloned into pSP-GM1 (Figure 2a) (pSPGM1+*crt+ter* referred to as pMK09).

The pMK09 plasmid cassette containing both genes *crt* and *hbd* was amplified by PCR primers (Mre-TCYC and Kpn-TADH) (table 4); restricted by *Kpn2I* and *MreI* and cloned into pMK08

(pSPGM2 +*crt* + *hbd* + *ter*+ *adhE2* referred to as pMK03). The schematic design of the generated plasmid is shown in Figure 5. In plasmid pMK03 consisting of 14581 bp (Table 2) (Fig. 5) *hbd* and *adhE1* genes are under regulation of *PGK1* promoter, *crt* and *ter* are under control of the *TEF1* promoter.

For construction of pMK04, gene *adhE2* (restricted by *NotI/PacI*) and gene *hbd* (restricted by *BamHI/NheI*) were isolated, and cloned into pSP-GM2 (Figure 2b) (pSP-GM2+*adhE2*+*hbd* referred to as pMK10). Gene *crt* (restricted by *NotI/PacI*) and gene *ter* (restricted by *BamHI/NheI*) were isolated, and cloned into the pSP-GM1 plasmid (pSPGM1+*crt*+*ter* referred to as pMK11).

The pMK11 plasmid cassette containing both genes *crt* and *ter* was amplified by PCR primers (Mre-TCYC, Kpn-TADH) (table 4.); restricted by *Kpn2I* and *MreI* and cloned into pMK08 plasmid (pSPGM2 +*crt* + *hbd* + *ter*+ *adhE2* referred to as pMK04). The schematic design of the generated plasmid is shown in Figure 5. In plasmid pMK04 consisting of 14581 bp (Table 2) (Fig.5) *hbd* and *crt* genes are under regulation of the *TEF1* promoter, *ter* and *adhE2* are under control of the *PGK1* promoter.

Table 3. Plasmids and strains

Plasmid or strain	Relevant characteristics	Source
pSP-GM1	p <i>PGK1</i> - p <i>TEF1</i> bidirectional promoter <i>URA3</i> marker	Siavash Partow
pSP-GM2	p <i>TEF1</i> - <i>PGK1</i> bidirectional promoter <i>URA3</i> marker	Siavash Partow
pJ201:41696	<i>hbd</i> and <i>kan^R</i> marker	DNA2.0
pJ201:41697	<i>crt</i> and <i>kan^R</i> marker	DNA2.0
pJ201:41698	<i>ter</i> and <i>kan^R</i> marker	DNA2.0
pJ206:41699	<i>adhE2</i> and <i>kan^R</i> marker	DNA2.0
pAK01	pSPGM1 + <i>crt</i> + <i>hbd</i> + <i>ter</i> + <i>adhE2</i>	Anastasia Krivoruchko
pMK02	pSPGM1 + <i>crt</i> + <i>hbd</i> + <i>ter</i> + <i>adhE2</i>	This study
pMK03	pSPGM2 + <i>crt</i> + <i>hbd</i> + <i>ter</i> + <i>adhE2</i>	This study
pMK04	pSPGM2 + <i>crt</i> + <i>hbd</i> + <i>ter</i> + <i>adhE2</i>	This study
pCS01	<i>ERG10</i> under p <i>TEF1</i> promoter, <i>HIS3</i> marker	Cristina Serrano
pCS02	pSPGM1 + <i>adhE2</i>	Cristina Serrano
pAK03	pSP-GM1+ <i>adhE2</i> + <i>ter</i>	Anastasia Krivoruchko
pAK04	pSPGM1 + <i>crt</i>	Anastasia Krivoruchko
pMK05	pSPGM1+ <i>hbd</i> + <i>crt</i>	This study
pMK06	pSP-GM1+ <i>adhE2</i> + <i>hbd</i>	This study
pMK07	pSPGM2+ <i>ter</i> + <i>crt</i>	This study
pMK08	pSP-GM2+ <i>adhE2</i> + <i>hbd</i>	This study
pMK09	pSPGM1+ <i>crt</i> + <i>ter</i>	This study
pMK10	pSP-GM2+ <i>adhE2</i> + <i>hbd</i>	This study
pMK11	pSP-GM1+ <i>crt</i> + <i>ter</i>	This study

pIYC08	<i>ADH2 + ALD6 + acs_{SE} + ERG10</i>	Yun Chen
Strains		
CEN.PK 113-7D	<i>MATa MAL2-8c SUC2</i>	P. Kötter
CEN.PK 113-11C	<i>MATa ura3-52 his3-Δ1 MAL2-8c SUC2</i>	P. Kötter
AKS02	CEN.PK 113-11C with pAK01+ pCS01	Anastasia Krivoruchko
MKS02	CEN.PK 113-11C with pMK02+pCS01	This study
MKS03	CEN.PK 113-11C with pMK03+pCS01	This study
MKS04	CEN.PK 113-11C with pMK04+ pCS01	This study
AKY01	CEN.PK 113-11C with pAK01 + pIYC08	This study
MKY02	CEN.PK 113-11C with pMK02 + pIYC08	This study
MKY03	CEN.PK 113-11C with pMK03 + pIYC08	This study
MKY04	CEN.PK 113-11C with pMK04 + pIYC08	This study
AKY02	CEN.PK 113-7D + ΔMLS1+pIYC08+pAK01	Anastasia Krivoruchko
<i>E. coli</i> DH5α	F– Φ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rK–, mK+) <i>phoA supE44 λ– thi-1 gyrA96 relA1</i>	D. Hanahan

Table4. Primer sequences

Fragment	Primer	Primer sequence	Restriction sites
<i>adhE2-ter</i>	Kpn-TCYC Mre-TADH	Forward: <i>GTTGTTTCCGGA</i> TGTTACATGCGTACACGCGTC Reverse: <i>GAAGAA</i> <i>CGCCGCG</i> GAGCGACCTCATGCTATAACCTG	<i>Kpn21/MreI</i>
<i>ter-crt</i> <i>hbd-crt</i> <i>crt-ter</i>	Mre-TCYC Kpn-TADH	Forward: <i>GAAGAA</i> <i>CGCCGCG</i> G TGTTACATGCGTACACGCGTC Reverse: <i>GTTGTTTCCGGA</i> GAGCGACCTCATGCTATAACCTG	<i>Kpn21/MreI</i>

2.1.4 Cloning

All plasmids were transformed into *E. coli* DH5α cells prepared according to Hanahan et al. (1983). The transformation was performed as follows: the competent cells were thawed for 10 minutes in ice. Up to 10 μL of DNA was added and mixed gently. The mix was incubated for 30 minutes on ice, heat shocked for 90 seconds at 42 °C and incubated for 3 minutes on ice.

1 ml of LB medium was added to the mix which was subsequently shaken for 1 hour at 37°C. The cells were centrifuged for 1 minute at 10000x g, 900 μL of the supernatant was removed and the pellet was re-suspended in the remaining 100 μL. Cells which had taken up the plasmid were selected for on LB-ampicillin or LB-kanamycin plates. The GeneJet Plasmid Miniprep kit (Fermentas) was used to purify the plasmids from the harvested cells. Isolation of the correct plasmid was verified by enzyme restriction using *Bam*HI and *Nhe*I for *ter* and *hbd*, *Not*I and *Pac*I for *adhE2* and *crt*.

2.1.5 Preparation of competent *S. cerevisiae* cells

An overnight culture was begun by inoculating 5 mL YPD medium in 14 mL culture tubes with the desired strain. Cells were cultivated at 30°C overnight. The entire culture was then added to 45 mL of YPD and incubated on a shaker at 30°C for 3-5 hours. The culture was then centrifuged, the cell pellet washed once in 10 mL LTE buffer (0.1 M LiOAc, 10 mM Tris-HCl, and 1 mM EDTA), and resuspended in 500 µL LTE buffer. Competent cells were kept at 4°C until transformation (0-3 days).

2.1.6 Transformation of *S. cerevisiae*

The plasmids were transformed into *S. cerevisiae* CEN.PK 113-11C using a standard transformation procedure [29].

Transformation of competent *S. cerevisiae* cells was done according to the protocol¹. Each transformation was done with 50 µL competent cells. The addition of the plasmids was followed by the addition of 50 µL of 2 µg/µL ssDNA.

2.1.7 Shake-flask cultivation

20 ml culture tubes were used for pre-cultures and baffled, cotton-stopped, 100 ml Erlenmeyer flasks were used for shake flask cultivation and promoter evaluation. The shake flasks contained 25 ml medium with the following composition: 7.5 g/l (NH₄)₂SO₄, 14.4 g/l KH₂PO₄, 0.5 g/l MgSO₄·7H₂O, 2 ml/l trace metal solution, 1 ml/l vitamin solution [30]. The pH of the medium was adjusted to 6.5 by adding 2 M NaOH and it was autoclaved separately from the glucose solution that was used as a carbon source solution in a final concentration of 2%. Sterile vitamin solution was added aseptically to the medium after autoclaving. The shake flasks were incubated at 30°C and 180 rpm.

¹ pESC yeast epitope tagging vectors instruction manual. Agilent.

2.1.8 Polymerase chain reaction (PCR)

Amplifying the cassettes was done using a MJ Mini Bio RAD system. All PCRs for cloning purposes was performed using the Phusion High-Fidelity DNA Polymerase in Phusion HF Buffer (Finnzymes, Vantaa, Finland). PCR reactions were executed by using conditions instructed by the supplier in 20 μ L reaction scales. Desired plasmids were added to four deoxyribonucleotides, forward and reverse primer, buffer and DNA polymerase; and were amplified by PCR in 35 cycles.

2.1.9 Molecular biology and analytical methods

DNA extract concentration was measured with a Nanodrop 3300 (Thermo Scientific). Optical density was measured at 600 nm with a Thermospectronic Genesys 200 spectrophotometer.

Measurements of pH were done using a SevenEasy pH-meter (Mettler Toledo, Greifensee, Switzerland). DNA electrophoresis was done on 1% agarose gels, which were stained with ethidium bromide. As size maker geneRuler 1kb DNA ladder (Fermentas, St.Leon-Rot, Germany) was used in all cases. Primers were synthesized by Sigma-Aldrich (St. Louis, USA). DNA restrictions were performed by using FastDigest restriction enzymes in combination with FastDigest Green Buffer (Fermentas). All ligations were performed by using T4 DNA Ligase in T4 DNA Ligase Buffer (Fermentas).

Restrictions and ligations were performed using conditions instructed by the supplier, regularly with longer incubation times. DNA purifications from agarose gel or PCR reaction mix were performed by using the GeneJet Gel Extraction Kit (Fermentas) or GeneJet PCR Purification Kit (Fermentas).

The concentrations of butanol were measured by using isocratic high-performance liquid chromatography with refractive index detection on a Dionex ultimate 3000 system using an Aminex HPX-Biorad ion exclusion column, 7.80 mm diameter, 300 mm length, 9 μ m particle size, with sulfonated di-vinyl benzene-styrene co-polymer as packing material and H₂SO₄ (5 mM) as eluent at a flow rate of 0.6 ml/min.

Standard addition was performed based on 1, 2, 5, 10, 15, 20 mg/L of butanol. The production of a calibration plot is straightforward in that serial dilutions of a known standard solution were prepared and analyzed. The resultant peak area is plotted against the concentration to

establish the linearity of the analytical technique. The butanol concentration is determined according to the defined standards.

For the higher concentration range, a linear calibration plot was utilized with higher butanol standard concentration.

Chapter 3

3.1 Results and Discussion

3.1.1 Choosing the right promoter combination for improved productivity

The purpose of this experiment was to try to deduce an optimal scheme of promoter combinations to drive the flux of the butanol pathway forward. The transcription (in the following referred to as expression) of butanol pathway genes in yeast is regulated by endogenous strong promoters of yeast. In general two types of promoters are classified, the constitutive ones mediating a steady expression of the heterologous proteins throughout the production process (*pPGK1* and *pTEF1* in this study), and inducible ones, which require the addition of an inducer (e.g. galactose for *pGAL1*) [17].

The strong constitutive promoter of *PGK1* regulates the expression of triose-phosphate isomerase and the *TEF1* promoter regulates the expression of translation elongation factor1 alpha (also referred to as EF-1 α).

Partow et al. (2010) demonstrated that yeast 'constitutive' promoter activity may vary dependent on their growth on glucose or ethanol. They showed in an overall ranking of the different promoters of yeast that when cells are in the glucose consuming phase, *pTEF1* is stronger than *pPGK1*. When glucose is exhausted and ethanol is being consumed, *pPGK1* showed lower activity compared to the glucose phase.

Lu et al. (2007) developed a multiple-gene-promoter-shuffling method in order to optimize xylose fermentation that resulted in optimal levels of expression for several genes at a time. They chose appropriate promoters on the basis of the gene expression levels and screened each transformant for the desired phenotype. The best combination of promoters was chosen. In this study, the butanol (mg/L) produced by strains containing the butanol pathway genes under the regulation of different combination of endogenous promoters, *pPGK1*, *pTEF1*, was examined.

The strains AKS02, MKS02, MKS03 and MKS04 contained two different plasmids: first, the 'butanol plasmid' containing the butanol genes under control of different promoter

combinations (pAK01, pMK02, pMK03, pMK04) (Figure 5) and the second plasmid containing *ERG10* which encodes thiolase that catalyzes the acetylation of acetyl-CoA to acetoacetyl-CoA (pCS01). The strains AKY01, MKY02, MKY03, and MKY04 contain two different plasmids, the first one being the same as for the strains above (pAK01, pMK02, pMK03, and pMK04) and second being the pLYC08 plasmid for overexpression of *ALD6*, *acs_{SE}*, *ADH2*, and *ERG10* genes.

3.1.2 Butanol plasmid construction

In order to construct the plasmids which contain the butanol genes, the basic vectors were amplified by transformation to *E. coli* and verified by gel electrophoresis. The cloning of the butanol genes was performed and verifications were done after each cloning by digestion of the plasmids with relevant enzymes. In order to verify the final constructed plasmids ready for transformation to yeast (pAK01, pMK02, pMK03, pMK04), the constructs were digested by restriction enzymes (*Kpn21/MreI*) (Figure 6). Details of the fragments can be found in table 5.

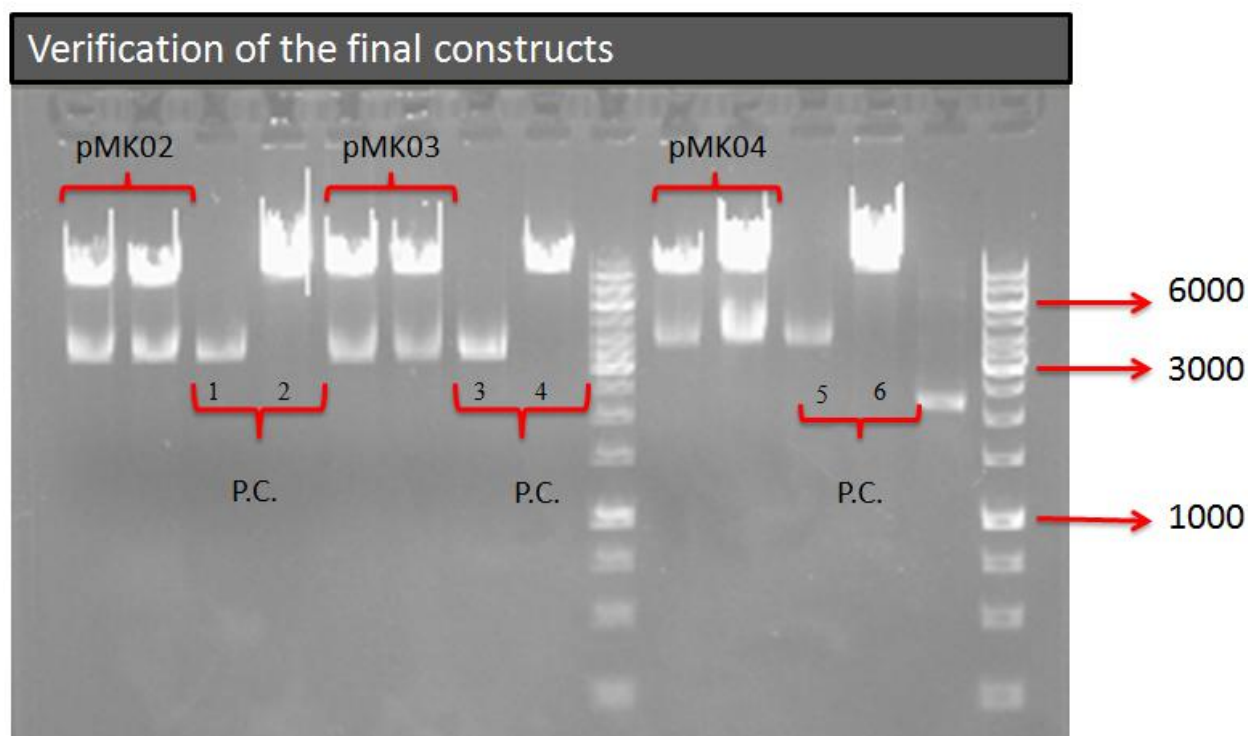


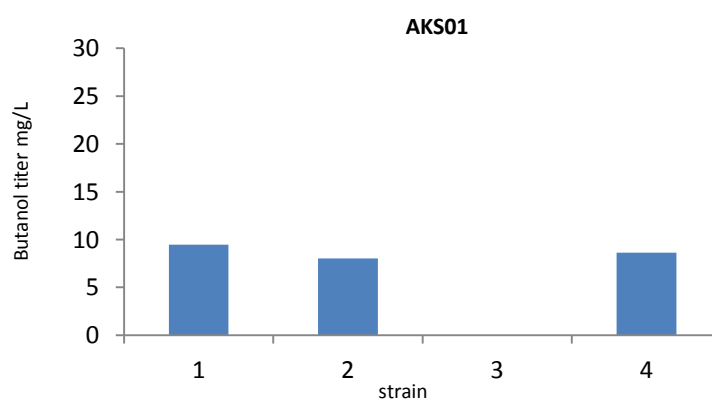
Figure 6. Gel electrophoresis for verification of the constructed final plasmids ready for transformation to yeast (1 kb ruler was used as size marker) (pMK02, pMK03, pMK04); the constructs were digested by restriction enzymes (*Kpn21/MreI*). (pMK02, P.C.1: positive control digested PCR fragment (pMK07); P.C. 2: positive control digested vector (pMK06)), (pMK03, P.C.3: positive control digested PCR fragment (pMK09); P.C. 4: positive control digested vector (pMK08)), (pMK04 P.C.5: positive control digested PCR fragment (pMK10); P.C. 6: positive control digested vector pMK08).

Table5. Details of the fragments

Item	Description Vector/Insert	Expected Size(kb)
pMK02	Cassette <i>crt+ter/adhE2+hbd</i>	10,698/3,883
1	Positive control (Digested PCR product)	3,883
2	Positive control (Digested vector)	10,698
pMK03	Cassette <i>crt+hbd/adhE2+ter</i>	3455/11,946
3	Positive control (Digested PCR product)	3,535
4	Positive control (Digested vector)	11,946
pMK04	Cassette <i>crt+ter/adhE2+hbd</i>	3883/10698
5	Positive control (Digested PCR product)	3,883
6	Positive control (Digested vector)	10,698

3.1.3. Selecting the transformants which are able to produce butanol

The outcomes of the promoter swap study are presented in Figure 7 and 8. After transformation 8 colonies were selected and cultured in test tubes containing 5 ml SD medium (section 2.1.8) for examining their ability for butanol production. The results of these test tube cultivations after 48 hours for strains AKS02, MKS02, MKS03, MKS04 and strains AKY01, MKY02, MKY03, MKY04 are plotted in figure 7(a-d) and figure 8(a-d), respectively.



(7a)

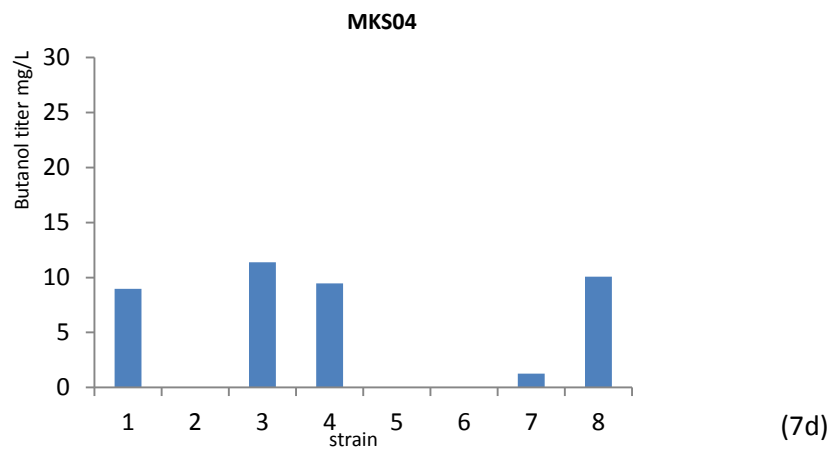
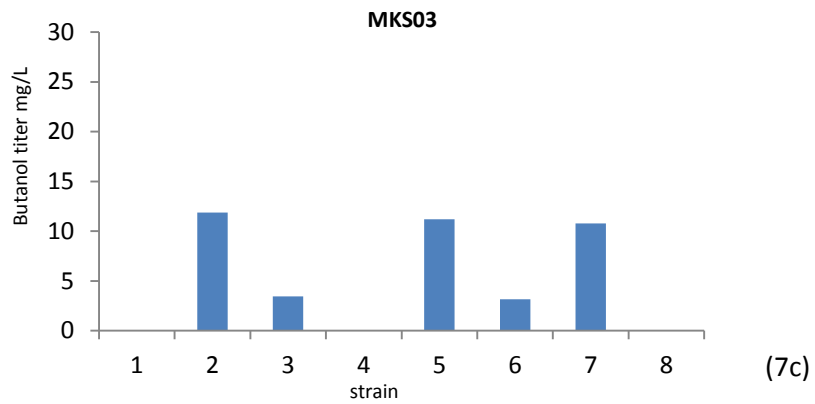
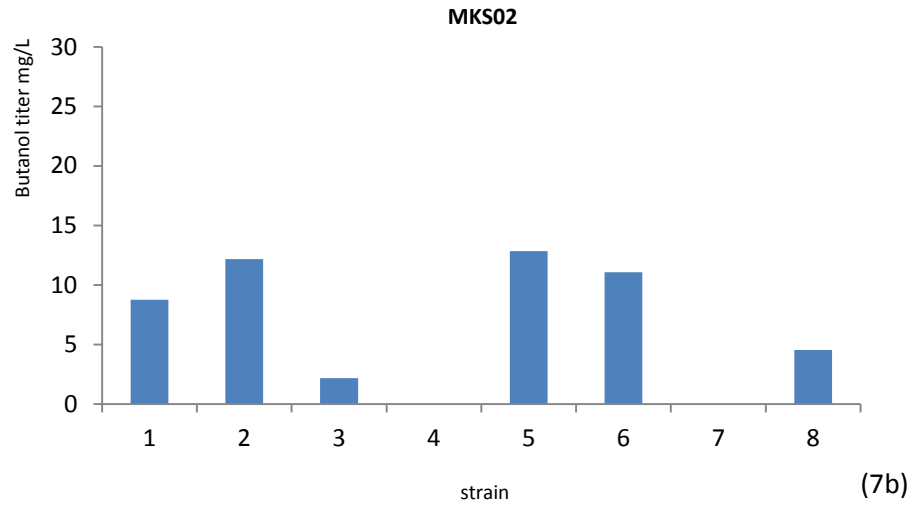
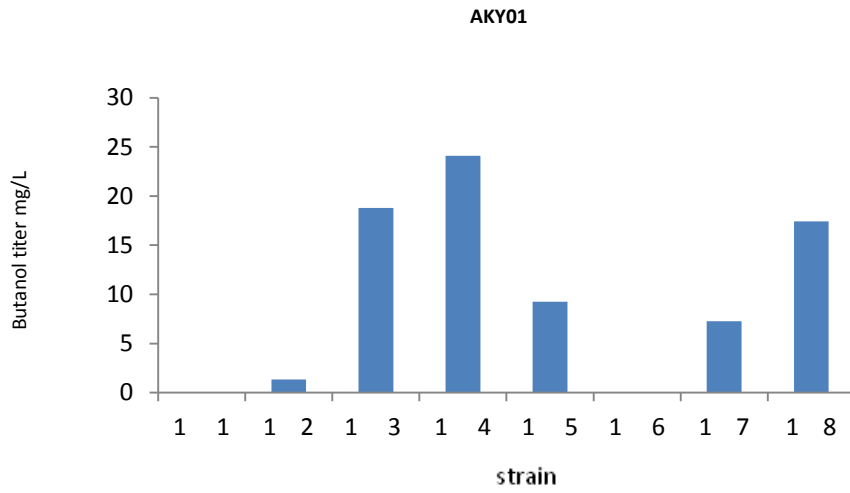
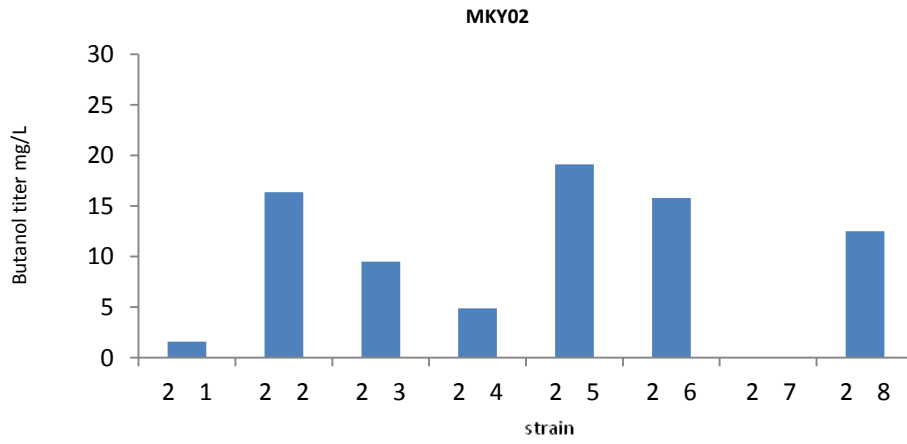


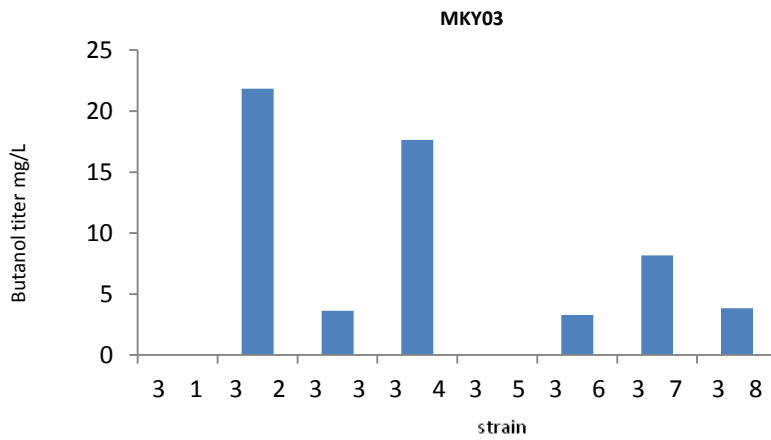
Figure 7. Butanol titers mg/L in tubes after 48 hrs for choosing the strains which are able to produce butanol. The strains AKS01, MKS02, MKS03 and MKS04 are harboring two different plasmids and were cultured in test tube containing 5 ml SD media. (7a) AKS01 (pAK01 +pCS01) ;(7b) MKS02 (pMK03+pCS01); (7c) MKS03 (pMK03+pCS01); (7d) MKS04 (pMK04+pCS01)



(8a)



(8b)



(8c)

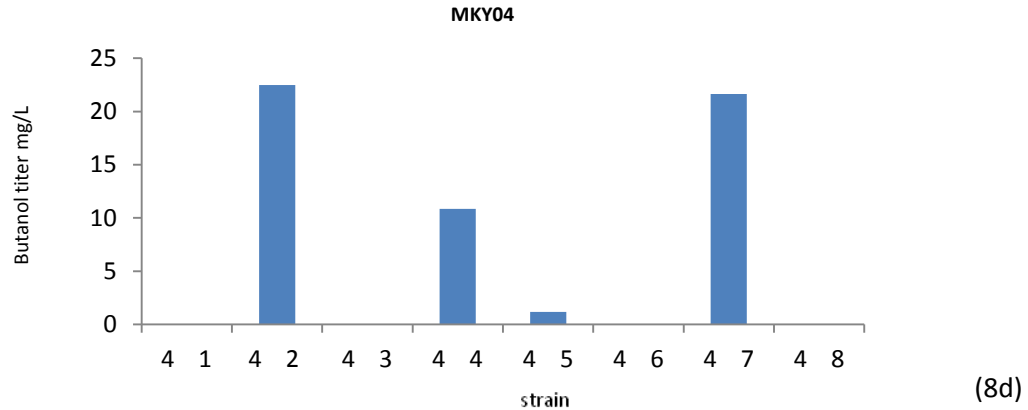
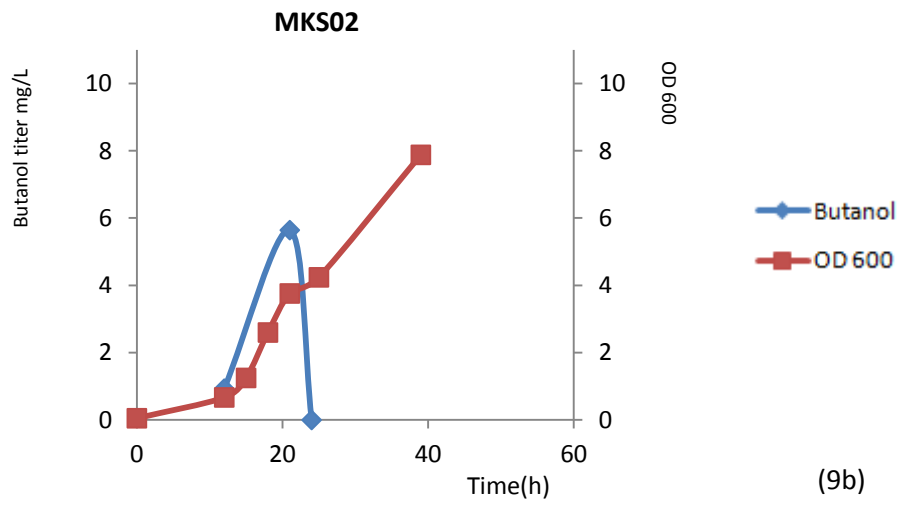
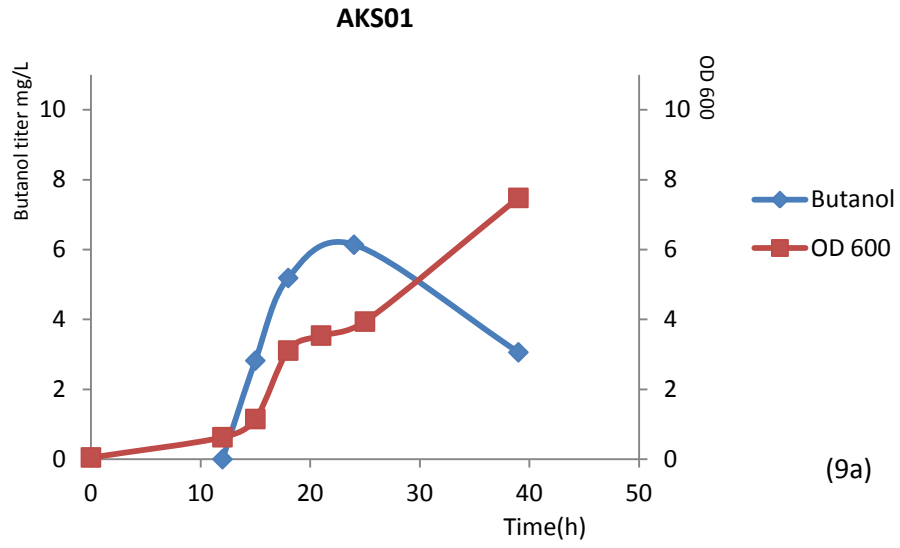


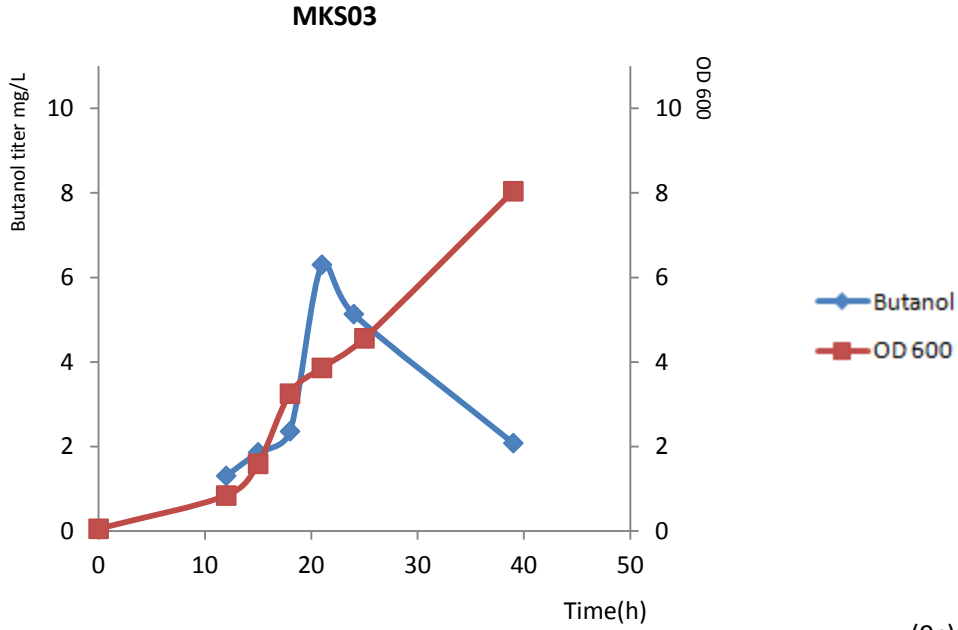
Figure 8. Butanol titers mg/L in tubes after 48 hr for choosing the strains which are able to produce butanol. The strains AKY01, MKY02, MKY03 and MKY04 are harboring two different plasmids and were cultured in test tubes containing 5 ml SD media. (8a)AKY01 (pAK01+pIYC08); (8b)MKY02 (pMK02+pIYC08);(8c)MKY03 (pMK03+pIYC08);(8d) MKY04(pMK04+pIYC08).

3 transformants, which produced higher amounts of butanol in test tubes were selected to be cultured in shake flask cultivation. The obtained results for strains AKS01, MKS02, MKS03 , MKS04 and for strains AKY01, MKY02, MKY03, MKY04 are shown in figure 11 and 12 (a, b).

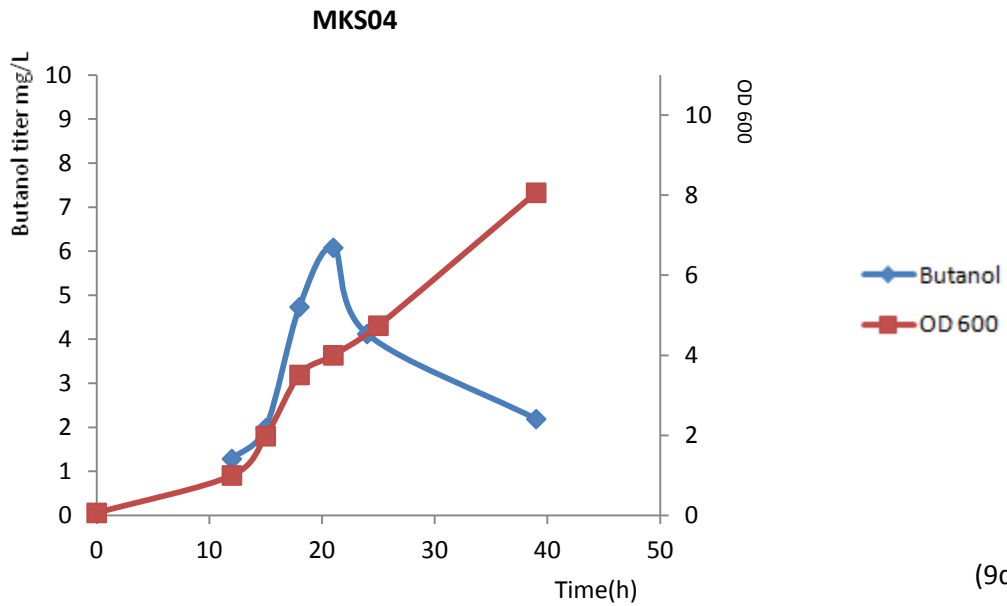
3.1.4. Butanol trend during cell growth

Fig. 9 and 10 show the OD values and butanol concentrations in AKS01, MKS02, MKS03, MKS04 and AKY01, MKY02, MKY03 and MKY04, respectively. Butanol was produced and gradually increased over time. The highest level of butanol was measured at the end of the glucose phase and was decreasing during the ethanol phase. Highest levels of butanol were obtained during the diauxic shift when *S. cerevisiae* changes metabolism from respire-fermentative growth on glucose to respiratory ethanol utilization in aerobic batch culture.



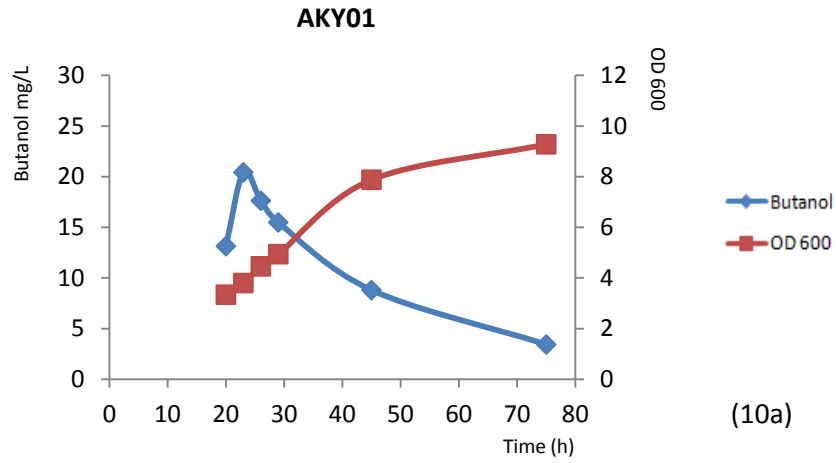


(9c)

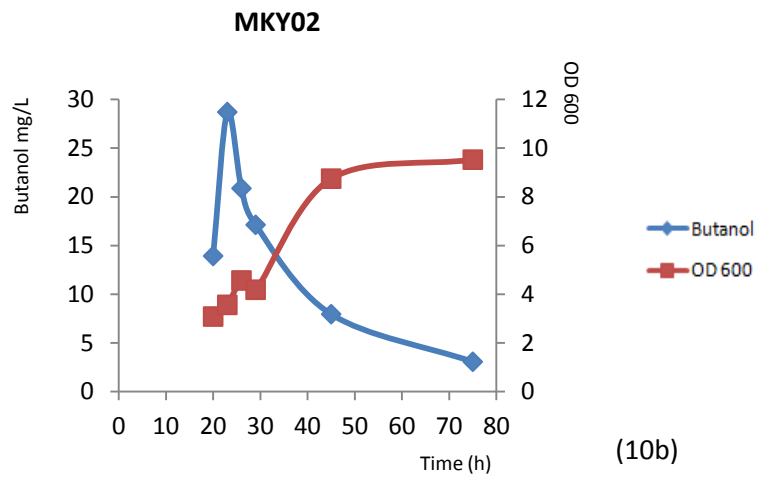


(9d)

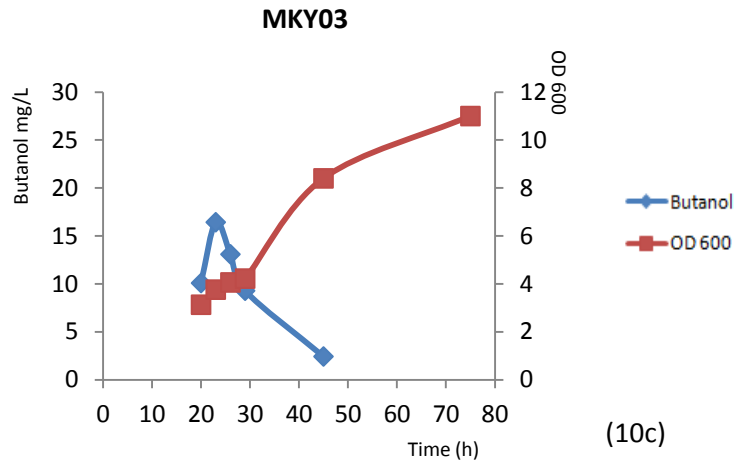
Figure 9. Butanol titer in shake flasks compared to the OD values for one replicate which produced the highest titer. The strains AKS01, MKS02, MKS03 and MKS04 are harboring two different plasmids. (9. a) AKS01 (pAK01 +pCS01) (9b) MKS02 (pMK03+pCS01) (9c) MKS03 (pMK03+pCS01) (9d) MKS04 (pMK04+pCS01).



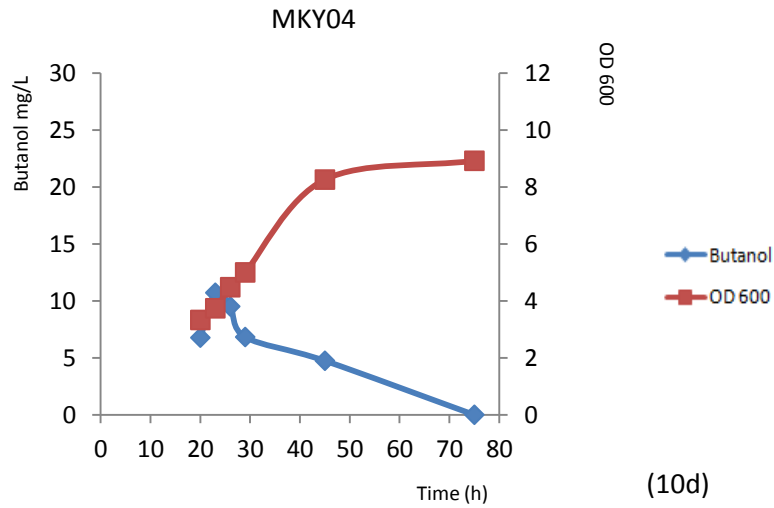
(10a)



(10b)



(10c)



(10d)

Figure 10. Butanol titer in shake flasks compared to the OD values for one replicate which produced the highest titer. The strains AKY01, MKY02, MKY03 and MKY04 are harboring two different plasmids, the first being the butanol plasmid with different promoter combinations (pAK01, pMK02, pMK03, pMK04) and the second one for overexpression of genes *ALD6*, *acs_{ser}*, *ADH2*, *ERG10* (pIYC08). (9. a) AKY01 (pAK01 +pIYC08) (9b) MKY02 (pMK03+pIYC08) (9c) MKY03 (pMK03+pIYC08) (9d) MKY04 (pMK04+pIYC08).

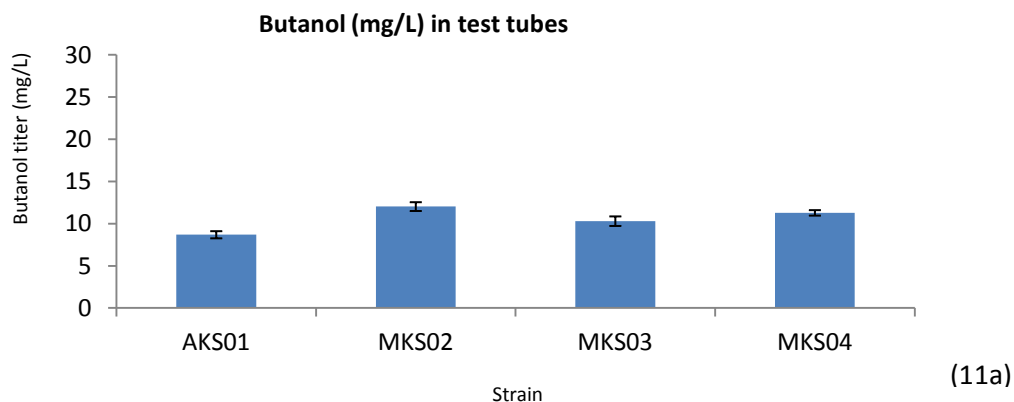
During the respiro-fermentative phase while cells are growing on glucose and glycolysis is active, acetyl-CoA is accumulated and highest level of butanol are obtained (Figure 9, 10).

After the diauxic shift during the ethanol phase, cells stopped producing butanol, presumably because the acetyl-CoA produced during glycolysis on glucose phase was used in respiratory metabolism.

The decrease in butanol observed during the ethanol phase could be due to the utilization of it by the cells or evaporation of it through the medium, which was investigated in another experiment (section 3.2, 3.3, 3.4) in this study.

3.1.5. Comparison of butanol titer in various strains

An investigation was done to determine the production of each strain containing a different plasmid combination as schematically illustrated in figure 5. Figure 11a and 12a show the outcomes of the promoter swap study using strains harboring a butanol plasmid plus pCS01 plasmid (*ERG10*) in test tubes and shake flasks, respectively. Figures 11b and 12b show the results of the combination of one of the butanol plasmids and pIYC08 (*ALD6*, *acsSE*, *ADH2*, *ERG10*) in test tubes and shake flasks, respectively.



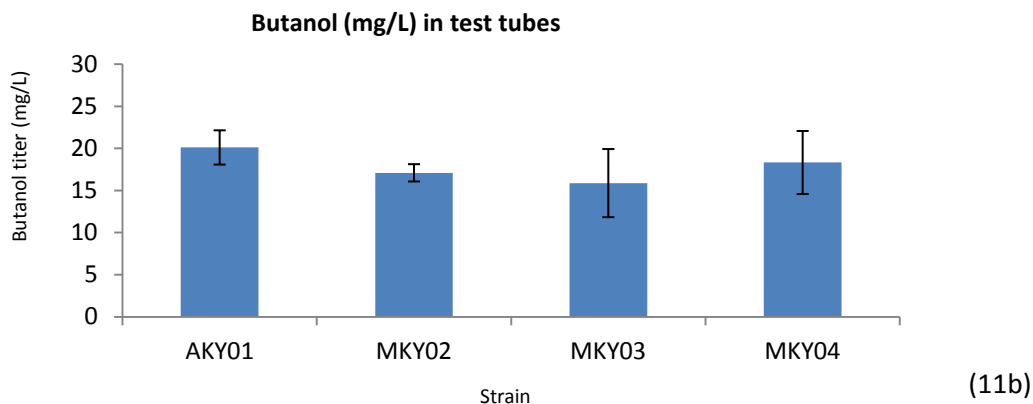


Figure 11. Butanol titers of strains with different combinations of promoters regulating butanol pathway genes in test tube culture. Presented are averaged butanol titers for 3 replicates (\pm SEM). (11a) AKS01, MKS02, MKS03 and MKS04 strains (11 b) AKY01, MKY02, MKY03 and MKY04 strains.

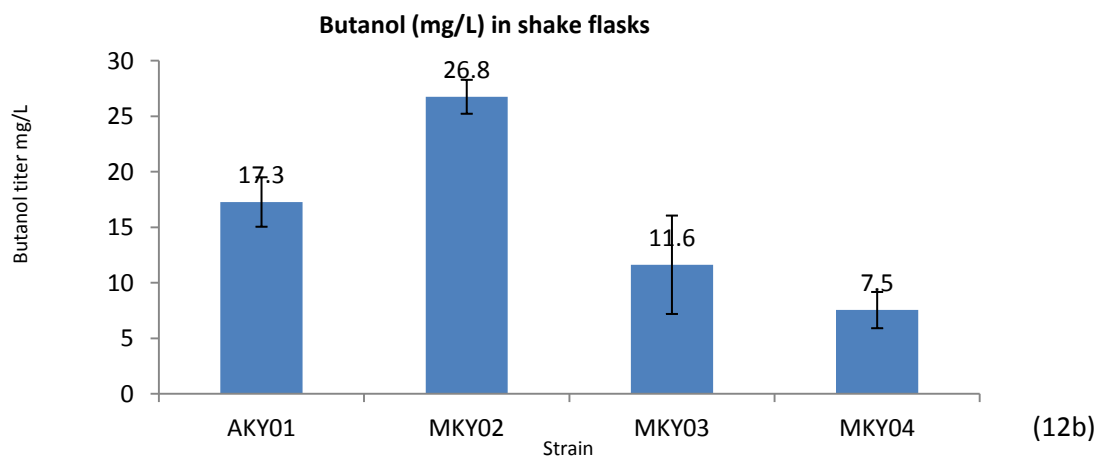
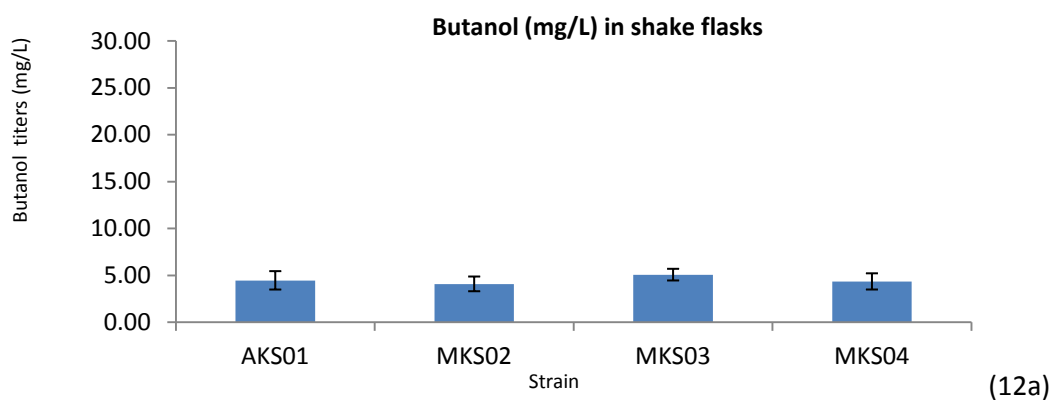


Figure 12. Butanol titer of strains with different combinations of promoters regulating butanol pathway genes in shake flask culture. Presented are averaged butanol titers for 3 replicates (\pm SEM). (11a) AKS01, MKS02, MKS03 and MKS04 strains (11 b) AKY01, MKY02, MKY03 and MKY04 strains.

The test tube results (figure 11a and 12a) show a higher titer of butanol compared to the butanol titer in shake flasks. This can be interpreted that cultivation in test tubes provided lower access to oxygen than aerobic shake flask conditions. Lan et al. (2011) indicated that AdhE2 is sensitive to oxygen, therefore higher butanol titers can be expected in low oxygen environments. The comparison of the strains in test tubes (11a, 11b) shows almost the same butanol titer in all 4 strains. It can therefore be concluded that shuffling of promoters for multiple genes of the butanol pathway to optimize the butanol level resulted in the same production titer in test tubes. In contrast, different combinations of the butanol pathway promoters in AKY01, MKY02, MKY03 and MKY04 strains revealed different butanol production titers in shake flasks (figure 12), while – as hypothesized and shown before for strains AKS01 and AKY01 (Krivoruchko, unpublished results) – the combination of a butanol pathway plasmid with pLYC08 resulted in higher titers than the combination of the butanol pathway plasmids with pCS01.

This result indicates that the intracellular concentration of acetyl-CoA in strains containing the pCS01 plasmid is a limiting factor, so even if the pathway becomes more efficient due to the promoter swap, it may not result in higher butanol concentrations due to limitations in acetyl-CoA. In contrast, strains containing pLYC08 have a driving force towards acetyl-CoA, so that the precursor is no longer limiting. Therefore the difference in the schemes becomes more obvious.

The average butanol titers in the MKY series are approximately double the amounts produced by the MKS series. In addition, the level of butanol titer in scheme No. 2 (MKY02) was the highest with approximately 26.8 mg/L and was the lowest with 7.5 mg/L in scheme No. 4 (MKY04). The only difference between scheme No.1 and scheme No.2 is that the promoters in front of *crt* and *ter* are swapped (Figure 5).

In scheme No. 2, *crt* is controlled by a weaker promoter (*pPGK1*), while *ter* is regulated by a stronger promoter (*pTEF*). Since this increases the titer by approx. 1.5-fold, this would suggest that the reaction catalyzed by *ter* was a limiting step in AKY01 and it is more important to put *ter* under a stronger promoter than *crt*. Lan et al. (2011) also observed that this step was a potential bottleneck, as insufficient Ter activity led to lower butanol production [31]

In MKY03, both *crt* and *ter* are under *pTEF1* control, whereas *adhE2* is regulated by *pPGK1* and the overall butanol titers are lower. This would suggest that *adhE2* catalyzes the limiting step in strains with scheme No. 3.

In MKY04, *ter* and *adhE2* which could represent the limiting steps of the pathway are again under regulation of the weaker promoter which resulted in titers of butanol of 7.5 mg/L, which is the lowest value and 72 % lower than for MKY02.

Further investigation and analysis of the pathway intermediates and enzyme activities would provide more information regarding rate limiting steps and bottle-necks of the system which should be optimized. In addition, testing more promoters or additional enzymes could further increase pathway flux.

3.2 Comparison of butanol disappearance in cultivations of the wild type and engineered strain

As observed in figure 9 and 10 above, butanol levels strongly decreased following the cells' shift to growth on ethanol. The purpose of this experiment, therefore, was to further investigate this decrease and to try to deduce whether it was due to cellular activity (e.g. butanol being metabolized by the cells during respiratory growth) or external factors (butanol evaporation, reaction with other medium components, etc.).

First, butanol was added to the medium without strain cultivation to investigate the evaporation (Figure 13e). The decreasing trend was not significant in medium which contained butanol without cells. It can be concluded that the butanol disappearance cannot be solely due to the evaporation through the media.

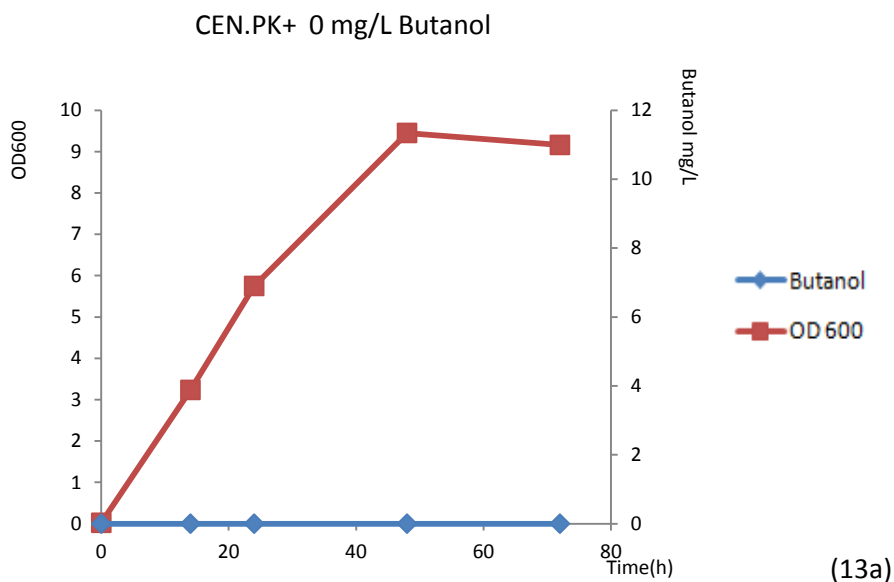
Second, for comparison of butanol disappearance in the wild type and butanol pathway engineered strain, the AKY02 ($\Delta mls1$ +pIYC08+pAK01) strain which produces butanol and the CEN.PK 113-7D reference strain were cultivated in duplicate under aerobic condition in shake flasks to investigate butanol disappearance. AKY02 is the metabolically engineered yeast which contains butanol pathway and pIYC08 plasmids along with the *MLS1* gene being deleted. *Mls1* consumes acetyl-CoA in the glyoxylate cycle and its deletion increases the flux toward the butanol pathway (Krivoruchko, unpublished results).

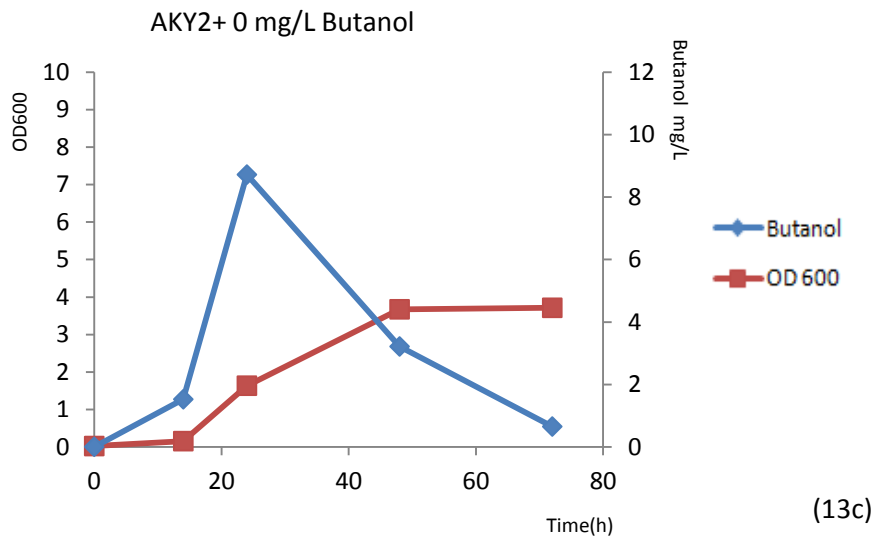
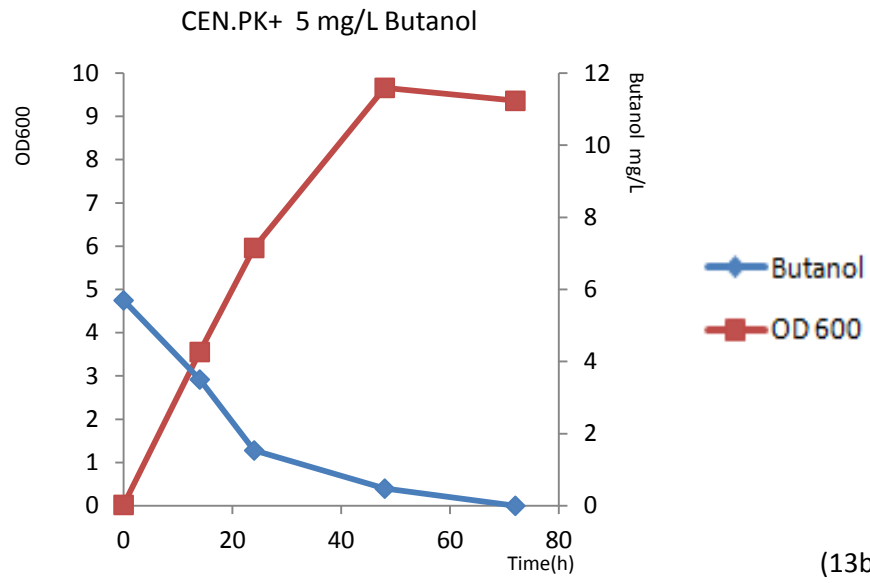
The comparisons of the growth versus butanol concentration as a function of time are plotted in figure 13(a-e). The wild type strain as a reference shows a similar growth in medium with 5 mg/L and without butanol. The AKY02 strain with the butanol pathway plasmid shows much lower cell growth than the reference strain.

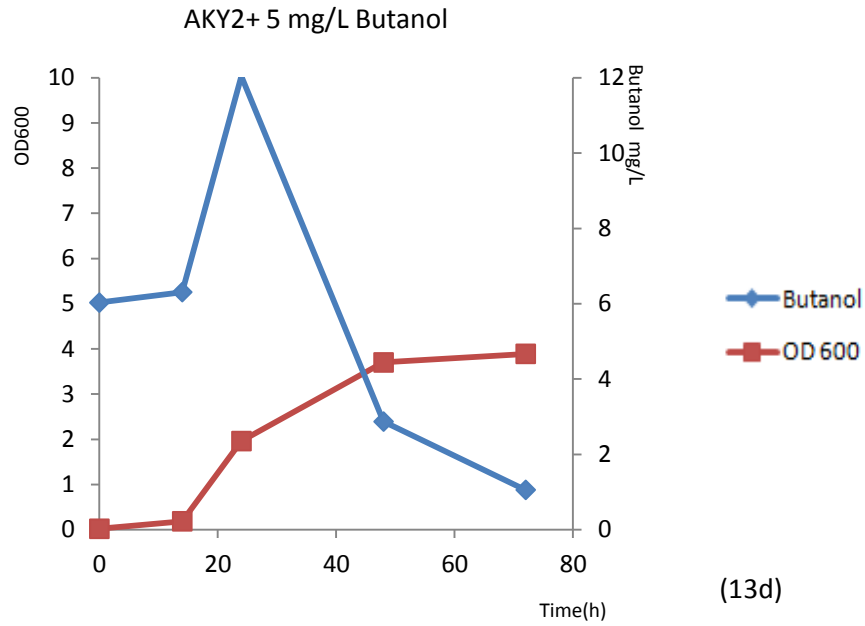
The slower growth rate may be due to a metabolic imbalance caused by deletion of *MLS1* and burden of the butanol pathway or due to insufficient expression of the selection markers which are expressed from different loci and might not work with the same efficiency as at their wild type locations.

A decrease in butanol concentration as a function of time was seen in both strains, wild type and engineered strain (Figure 13b, 13c). The results demonstrated that butanol which was produced by AKY02 decreased in the medium after production (Figure 13 c). In the reference and engineered strains with 5 mg/L butanol added to the culture also a sharp drop was detected (Figure 13b, 13d). Therefore, we realized that the sharp drop in butanol concentration is happening in both strains and is not specific to the butanol pathway expressing strains. This would suggest that the butanol decrease is not due to the pathway enzymes working in reverse direction.

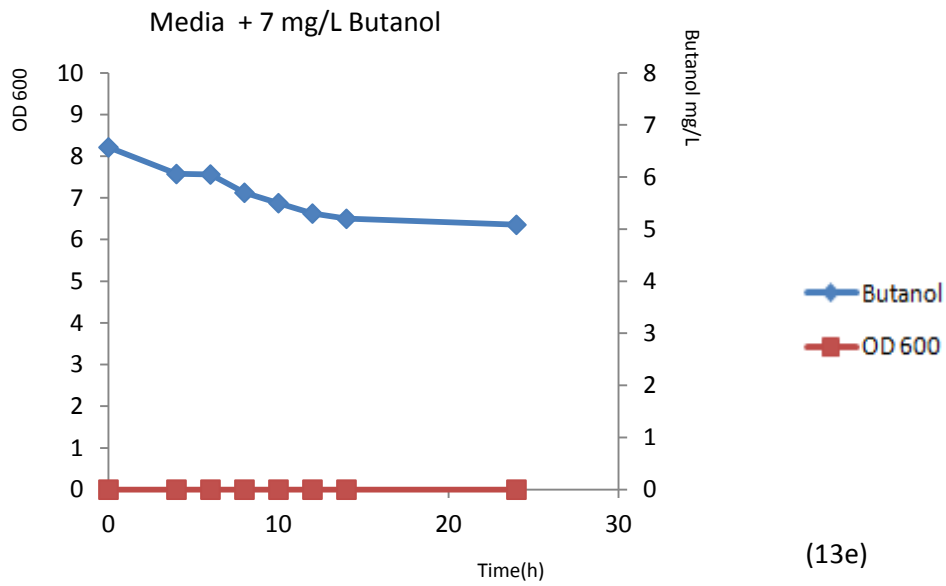
Therefore we investigated what would be the results of the wild type strain in higher concentrations of butanol.







(13d)



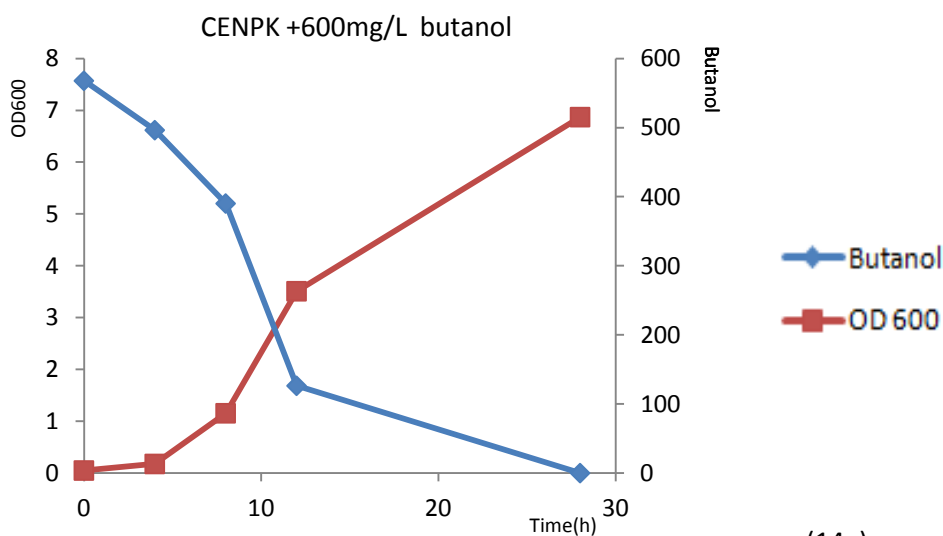
(13e)

Figure 13. (a,b,c,d,e) Comparison of butanol disappearance in the wild type, butanol pathway engineered strain and medium. (13a) Wild type strain without butanol addition to the medium (13b) Wild type strain with 5 mg/L butanol addition to the medium (13c) AKY02 the engineered strain which produces butanol without addition of butanol (13d) AKY02, the engineered strain with 5 mg/L butanol added to the medium (13e) Medium plus 7 mg/L of butanol

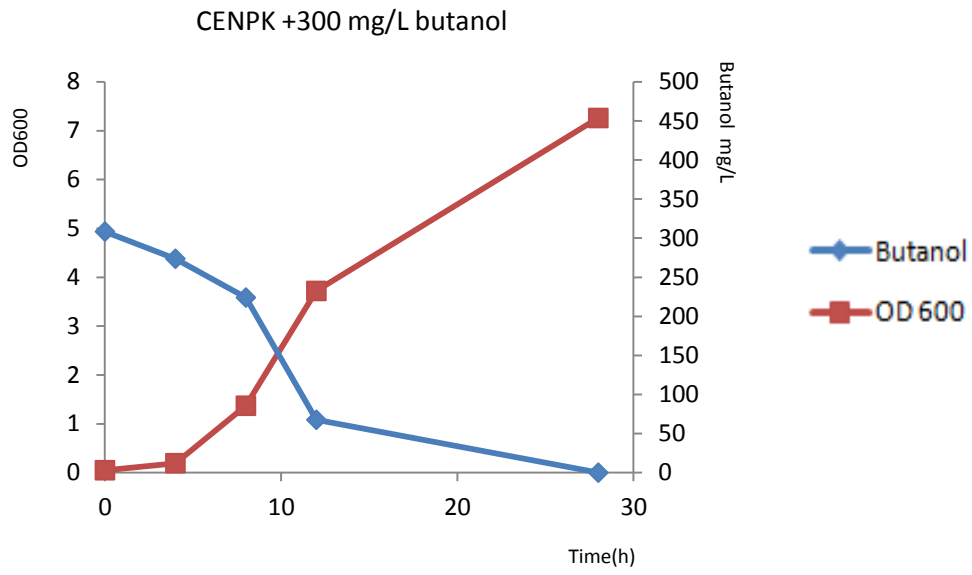
3.3 Wild type yeast in higher concentration of butanol

To investigate whether the wild type strain consumes butanol even in higher butanol concentration in the medium, CEN.PK115-7D was cultivated in duplicate under aerobic condition in shake flasks. 600, 300, 100 and 50 mg/L of butanol were added to the medium and concentrations as a function of time are plotted in figure 14 (a, b, c, d).

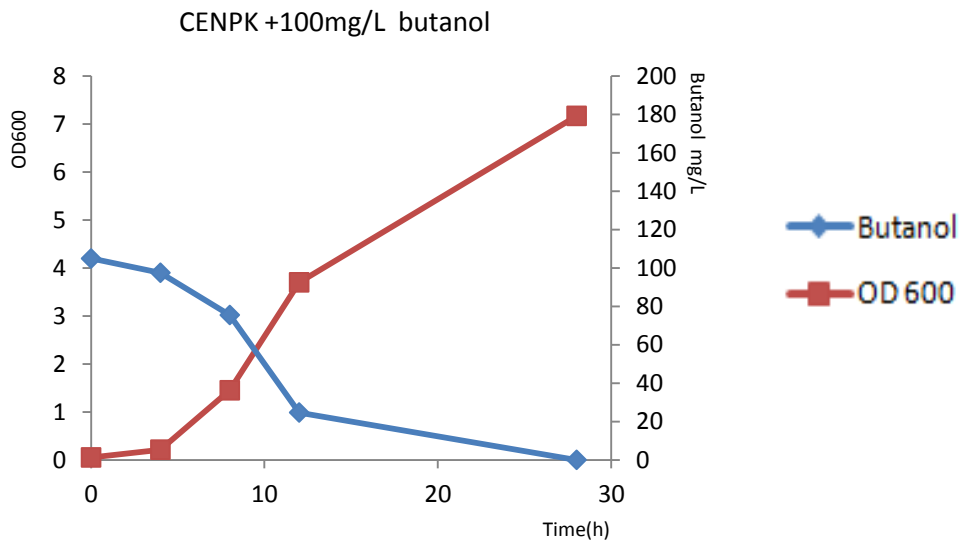
Since butanol from 1 to 2 % is toxic to the cells [32] the growth of the cells at concentrations of 50, 100, 300 and 600 mg/L of butanol was compared to the control culture (figure 13a). It can be concluded that butanol is not toxic to the cells at these levels yet. The decrease in butanol concentration is seen in all concentrations in cultures containing the wild type strain. The percentage-wise decrease looks the same in all concentration of butanol; around 80 % of butanol disappeared during the glucose phase and the remaining 20 % disappeared during ethanol phase of respiratory growth. The decreasing trend in butanol concentration with time in all cultures suggests possible butanol consumption as a carbon source in yeast. Therefore, the following experiment investigated whether butanol can be utilized as a carbon source.



(14a)



(14b)



(14c)

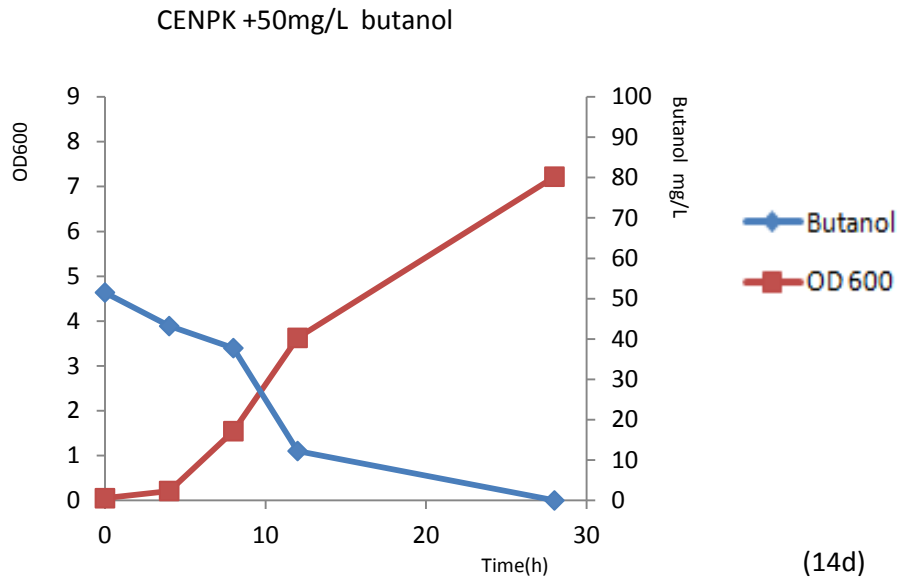


Figure 14. Graphs (a,b,c,d) shows CEN.PK115-7D cultivated under aerobic conditions in shake flasks. 600, 300, 100 and 50 mg/L of butanol were added to the medium and OD as well as butanol concentration are plotted as a function of time. a) CENPK +600 mg/L butanol addition to the medium; b) CENPK + 300 mg/L butanol addition to the medium, c) CENPK + 100 mg/L butanol addition to the medium, d) CENPK + 50 mg/L butanol addition to the medium.

3.4 Butanol as a carbon source for *S. cerevisiae*

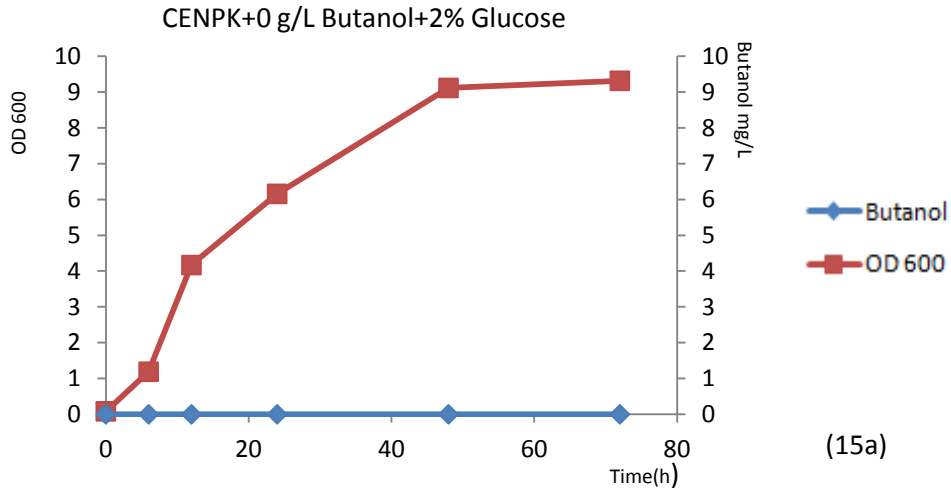
Since the previous experiment suggested that yeast cells are metabolizing butanol in some way, we decided to investigate the possibility of them using butanol as a carbon source.

In this experiment growth of yeast was studied on higher concentrations of butanol, under aerobic condition and different glucose concentration.

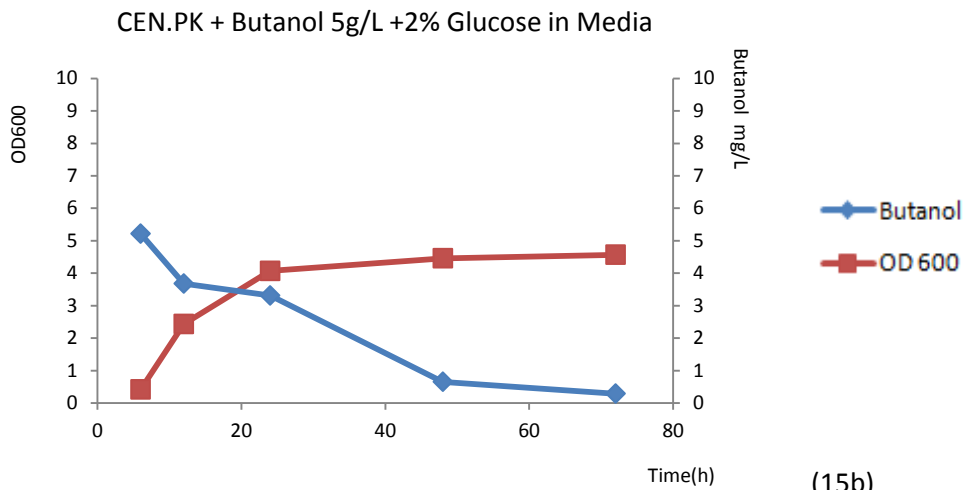
In the first duplicate series the glucose concentration in the medium was 2 % containing 10 and 5 g/L of butanol, respectively. In the second series, the glucose was decreased to 0.5 % containing 5 g/L butanol and in the last series; yeast was cultured in medium without glucose, which contained 10 g/L and 5 g/L of butanol, respectively.

The cell growth and butanol concentration as a function of time are shown in Figure 15 (a,b,c) and 16 (a, b, c). In medium with 2% glucose, the growth of the cells reached up to the final OD₆₀₀ of around 9. However, in medium with 2% glucose with 5 g/L butanol a lower growth of the cells up to a final OD₆₀₀ of 5 was observed, which was even more decreased in 10 g/L up to

a final OD₆₀₀ of 3. Butanol at 1 to 2% concentrations is known to be toxic for several strains of *S. cerevisiae* [32] which could account for this decreased growth (Figure 17). The butanol concentration showed a decrease over time in all cultures.



(15a)



(15b)

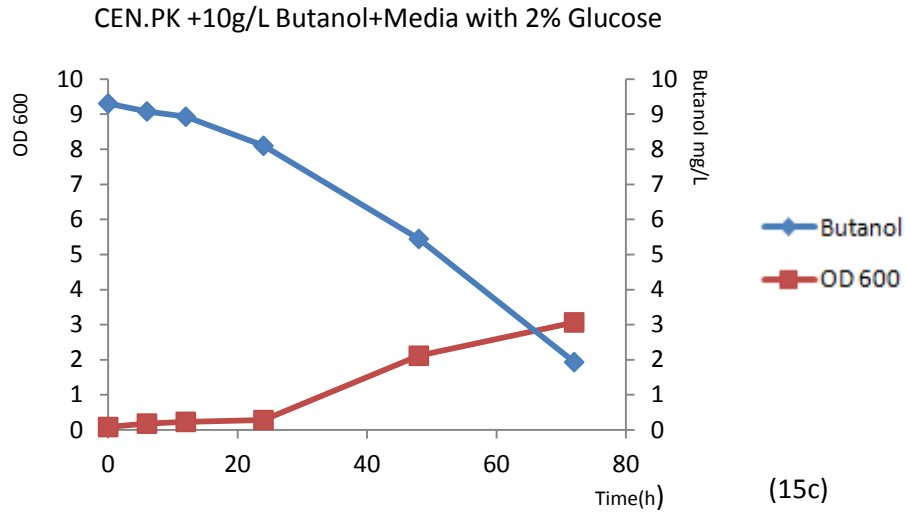


Figure 15. Butanol concentration and OD600 (as a function of time) in wild type strain cultures. Medium contained a) 0 g/L butanol plus 2% glucose; b) 5 g/L butanol plus 2% glucose; c) 10 g/L butanol plus 2% glucose.

Decreased growth was observed in 0.5 % glucose and no growth was observed when cells were cultured in medium without glucose. (Figure 16 a, b, c)

Figure (15 b) demonstrates the results of wild type yeast in 5 g/L butanol. Figure (15c) shows the growth of the wild type strain in 10 g/L butanol. In this cultivation, the butanol decreased by 10% during 0 to 24 hours after inoculation. However 61 % of the butanol was lost from 24 to 70 hours when cells started growing slowly in this high concentration of butanol.

In the cultivation shown in figure (16a), the glucose concentration of medium was reduced to 0.5 %. Slow cell growth is seen as long as glucose is available; the sharp drop of butanol is observable only during the later phase of cultivation.

Although the decrease in butanol concentration was seen in all cultures in different quantity, no growth was observed in medium lacking glucose (16b and 16c), suggesting that butanol cannot be used as a sole carbon source by *S. cerevisiae*. These results suggest that the disappearance of butanol from the medium could be due to non-specific activity of certain metabolic enzymes in yeast such as alcohol dehydrogenase Adh2.

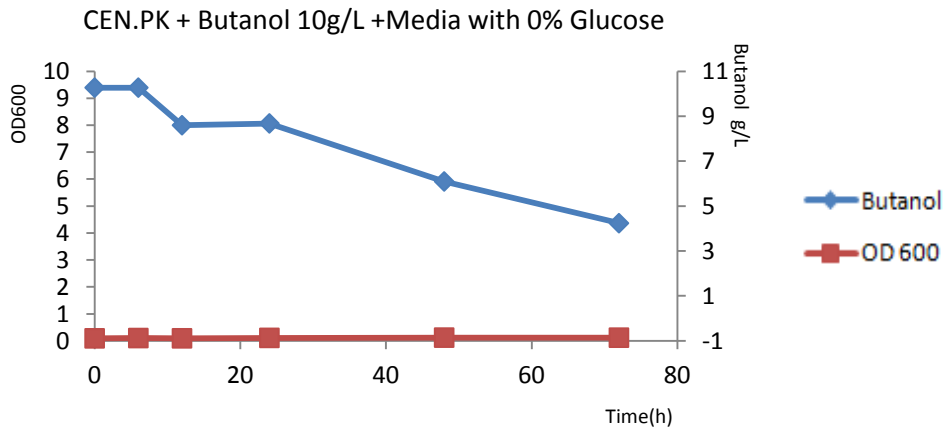
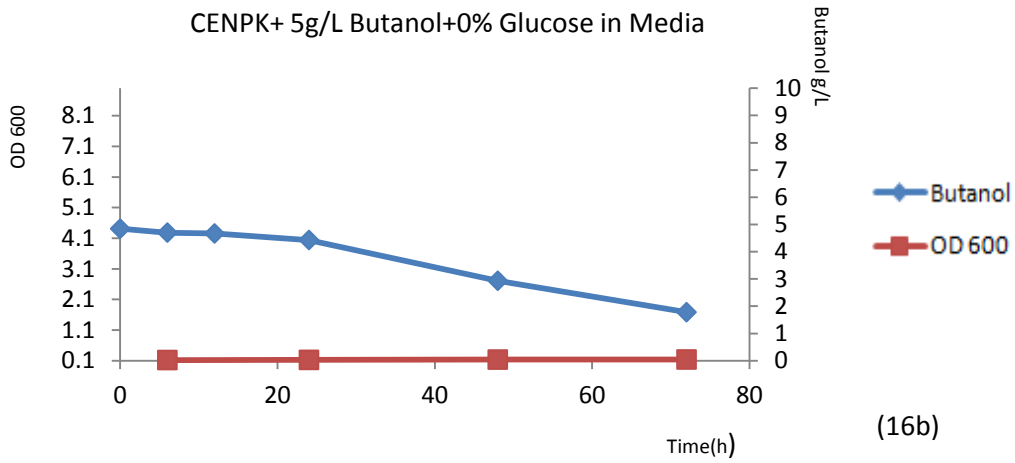
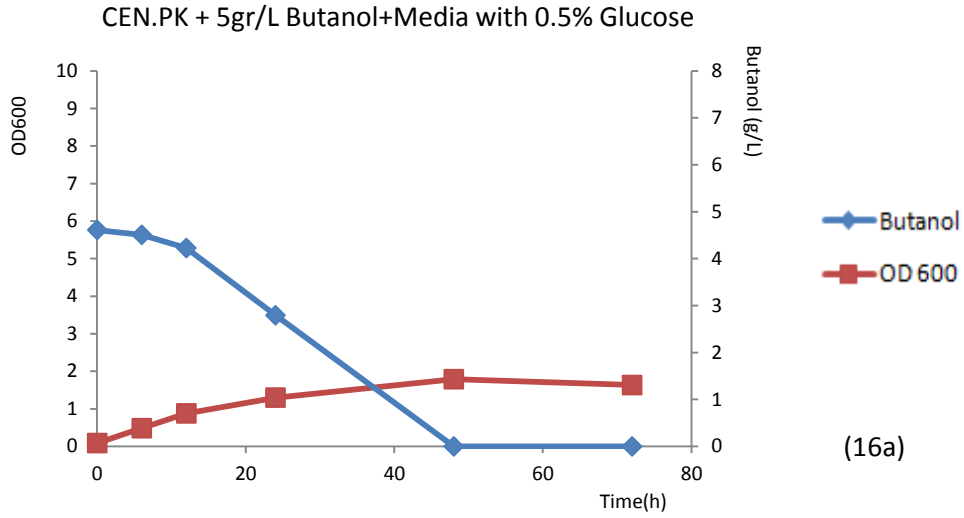


Figure 16. Butanol concentration changes and OD values of wild type strain as a function of time in shake flask culture. Cells were cultivated in medium containing a) 5 g/L butanol plus 0.5% glucose; b) 5 g/L butanol plus 0% glucose; c) 10 g/L butanol plus 0% glucose.

Comparison of OD values CENPK 2% Glu+ Diferent Butanol Concentration

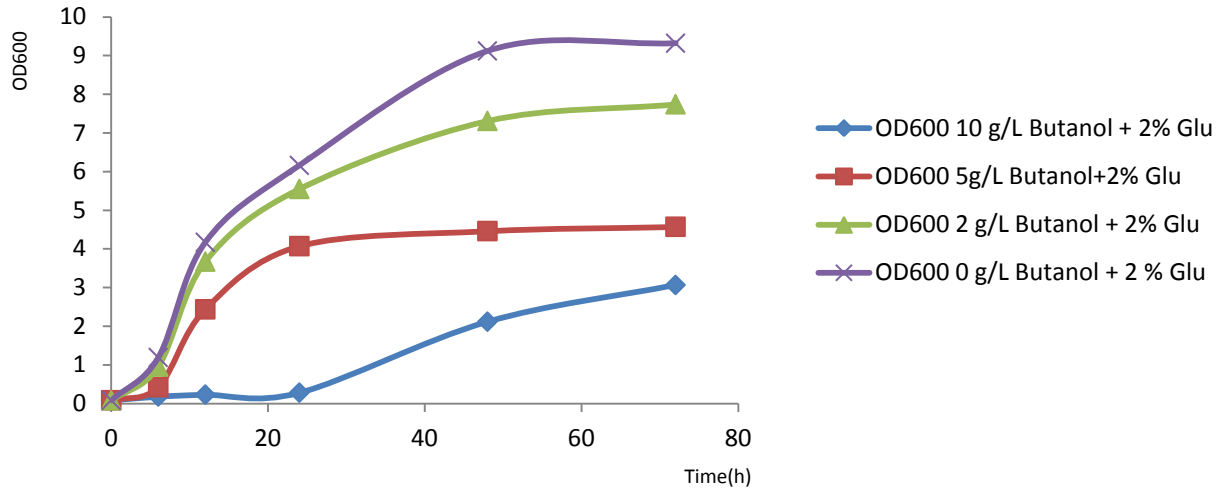


Figure 17. Growth of wild type strain in different concentrations of butanol (10-5-2-0 g/L). In medium with 2% glucose normal growth can be seen but in medium with 2% glucose with 2, 5 and 10 g/L butanol the growth is much slower.

Chapter 4

4.1 Conclusions and future perspectives

This report represents an effort for further optimization of the butanol pathway which has previously been introduced into yeast. By simultaneous overexpression of butanol genes under regulation of different combinations of two strong promoters of yeast, *pPGK1* and *pTEF1*, the relative levels of gene expression are assumed to be different.

With one of the schemes of combination of butanol genes with different promoters the butanol titer was improved to 26.8 mg/L and in another combination of promoters (No. 4) the production was the lowest with 7.5 mg/L.

The difference between the highest and the lowest butanol titer was approximately 4 fold. The highest level of production occurred with scheme No. 2, where *hbd* and *crt* are under regulation of the *PGK1* promoter, while *ter* and *adhE2* are under control of the *TEF1* promoter.

The lowest level of production was seen with scheme No. 4 where *hbd* and *crt* are under regulation of the *TEF1* promoter, while *ter* and *adhe2* are under control of the *PGK1* promoter.

According to these results, it was hypothesized that balancing of gene expression can increase the efficiency of the pathways. Among the CoA-dependent butanol biosynthesis pathway, the crotonyl-CoA reduction has been identified as t)[limiting step by Atsumi et al., (2008) [6]. In this work, we observed that this step was a potential bottleneck, since putting *ter* under regulation of a stronger promoter led to higher butanol production. Oxygen availability is an important factor in the production of butanol in yeast. Fortaine et al., (2002), who purified AdhE2 and assayed only in anoxic atmosphere, demonstrated its oxygen sensitivity [34]. We were able to compare butanol production both under oxic and lower oxygen availability conditions. Therefore, it can be suggested that the potential oxygen-sensitivity of AdhE2 could serve as a limitation for oxygenic production of butanol.

A drastic drop in butanol levels was observed during the diauxic shift when *S. cerevisiae* changes metabolism from respiro-fermentative growth on glucose to respiratory ethanol

utilization in aerobic batch culture. After the diauxic shift, cells stopped producing butanol. Instead, a trend of butanol decrease is observed during the ethanol phase.

The major butanol drop through all cultures was due to the presence of cells. However, cells did not use butanol as carbon source but its disappearance could be due other types of utilization by the cells or - in lower quantity - possible evaporation through the medium.

Some other experiments could be considered to figure out what the reason for the butanol disappearance is. It could be hypothesized that some of the alcohol dehydrogenases (ADHs) present in yeast could consume butanol instead of ethanol. The genes encoding ADHs could be tested by deletion to investigate whether the butanol concentration decreases or not.

The involvement of ADHs could also be tested by either measuring the accumulation of butyraldehyde or replacement of the last enzyme of the butanol pathway to see if butanol is disappearing. The integration of different metabolic strategies may result in the termination of butanol disappearance after its production by the cells.

A butanol-tolerant microorganism is important as a potential host for butanol production. Several studies to select butanol-tolerant strains have been conducted by using several microorganisms such as yeast, *E. coli*, *Zymomonas*, and *Lactobacillus*. Several strains of *S. cerevisiae* demonstrated limited growth in the presence of 16.2 g/L butanol, whereas two strains of *Lactobacillus* were able to grow in the presence of up to 24.3 g/L butanol [32]. In this study, it was demonstrated that the presence of butanol from 5 to 10 g/L led to a major decrease of cell growth.

However, neither strain could produce more than 30 mg/L of butanol. These results suggest that the introduction of a clostridial fermentative pathway into a heterologous host does not allow efficient butanol production *per se* and requires much more metabolic engineering and optimization efforts. More detailed energy and redox balance analysis, analyses of metabolic fluxes, the expression and activity characteristics of enzymes, and interactions between the host metabolism and the heterologous butanol pathway need to be performed. For the future prospective, the pathway should be provided with more driving forces to direct the flux towards butanol production, such as NADH and acetyl-CoA driving forces which were created in *E. coli* by Shen and co-workers (2011) and resulted in 30 g/L of butanol. Further systems-level

metabolic engineering can be performed for the development of non-native strains capable of efficiently producing butanol.

Integration of all of these strategies for systems-level metabolic engineering of the clostridial acetyl-CoA dependent pathway in yeast will allow the development of a superior strain capable of efficiently producing butanol.

Chapter 5

5. References

- [1] Gunawardena et al., "Butanol production by metabolically engineered yeast." Patent application publication, p. 1-15, Mar. 2010.
- [2] J. K. Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, "Fermentative butanol production by Clostridia.," *Biotechnology and bioengineering*, vol. 101, no. 2, p. 209-28, Oct. 2008.
- [3] V. N. Donaldson, Gail K., Andrew C. Eliot, Dennis Flint, Ann Maggio-Hall, "Fermentative production of four carbone alcohols." Patent application publication, p. 1-326, 2011.
- [4] M. Kirschner, "n-Butanol. Chemical Market Reporter," *ABI/INFORM Global*, Trade Publication, Vol. 262 Issue 15, p35, Oct. 2006.
- [5] P. Dürre, "Biobutanol: An attractive biofuel," *Biotechnol J. 2*, vol. 2, p. 1525–1534, 2007.
- [6] T. H. & J. C. L. Shota Atsumi, "Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels.," *Nature*, vol. 451, no. 7174, p. 86-9, Jan. 2008.
- [7] J. K. Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, "Fermentative butanol production by clostridia." *Biotechnology and bioengineering*, vol. 101, p. 209-28, 2008.
- [8] D. T. Jones and D. R. Woods, "Acetone-Butanol Fermentation Revisited," *Microbiological reviews*, vol. 50, no. 4, p. 484-524, 1986.
- [9] X. Chen, Metabolic engineering of *Saccharomyces cerevisiae* amino acid metabolism for production of chemicals of industrial interest, Ph.D. Thesis, Technical University of Denmark, June 2011.
- [10] H. KW., "Lignochemicals. Experientia," *Industrial biotechnology*, vol. 38, p. 176-189, 1982.
- [11] V. Shastri, *Industrial Biotechnology*. Book published in India by Isha books, ISBN: 81-8205376-5, p. 102-110, 2006.
- [12] S. G. Koffas M, Roberge C, Lee K, "Metabolic engineering," *Annu Rev Biomed Eng*, vol. 58(2–3), p. 119-20, 1999.

- [13] Jens Nielsen, "Advances in biochemical engineering biotechnology," *Appl Microbiol Biotechnol*, vol. 55(3), p. 263-83., 2001.
- [14] G. Stephanopoulos, "Challenges in engineering microbes for biofuels production." *Science (New York, N.Y.)*, vol. 315, no. 5813, p. 801-4, Feb. 2007.
- [15] T. G. Gatignol A, Dassain M, "Cloning of *Saccharomyces cerevisiae* promoters using a probe vector based on phleomycin resistance." *Gene* 91, vol. 91, p. 35-41, 1990.
- [16] C. C. F. B. and D. W. Rice, "Phosphoglycerate Kinase," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 293, no. 1063, p. 93-104, Jun. 1981.
- [17] Siavash Partow, Verena Siewers, Sara Bjørn, Jens Nielsen, "Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*," *Yeast*, vol. 27, p. 955-964, July 2010.
- [18] S. M. Eric J Steen, Rossana Chan, Nilu Prasad, A. Christopher J Petzold, Alyssa Redding, Mario Ouellet, and J. D. Keasling, "Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol.," *Microbial cell factories*, vol. 7, p. 36, Jan. 2008.
- [19] D. R. Steinmetz LM, Scharfe C, Deutschbauer AM, Mokranjac D, Herman ZS, Jones T, Chu AM, Giaever G, Prokisch H, Oefner PJ, "Systematic screen for human disease genes in yeast," *Nature genetics*, vol. 31(4), p.400-4, 2002.
- [20] R. S. and J. D. K. ae-Kyun Ro, Eric M. Paradise, Mario Ouellet, Karl J. Fisher, Karyn L. Newman¹, John M. Ndungu, Kimberly A. Ho, Rachel A. Eachus, Timothy S. Ham, James Kirby, Michelle C. Y. Chang, Sydnor T. Withers, Yoichiro Shiba, "Production of the antimalarial drug precursor artemisinic acid in engineered yeast.," *Nature*, vol. 440, no. 7086, p. 940-3, Apr. 2006.
- [21] S. G. Fischer CR, Klein-Marcuschamer D, "Selection and optimization of microbial hosts for biofuels production.," *Metab Eng* 2008, vol. 10, no. 6, p. 295-304, 2008.
- [22] L. A. Danilo Porro, Michele M. Bianchi, Luca Brambilla, Rossella Menghini, Davide Bolzani, Vittorio Carrera, Jefferson Lievense, Chi-Li Liu, Bianca Maria Ranzi, Laura Frontali, "Replacement of a metabolic pathway for large-scale production of lactic acid from engineered yeasts," *Appl Environ Microbiol*, vol. 65, p. 94211-4215, Sep. 1999.
- [23] C. L. Andreas M. Raaba, b, Gabi Gebhardt^c, Natalia Bolotin^a, Dirk Weuster-Botz^c, "Metabolic engineering of *Saccharomyces cerevisiae* for the biotechnological production of succinic acid.," *Metab Eng* 12, vol. 12(6), p. 518-25, 2010.
- [24] P. J. Geertman JM, van Maris AJ, van Dijken JP, "Physiological and genetic engineering of cytosolic redox metabolism in *Saccharomyces cerevisiae* for improved glycerol production.," *Metab Eng.*, vol. 8, p. 532-542, Nov. 2006.

- [25] K. J. Shiba Y, Paradise EM, Kirby J, Ro DK, "Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids." *Metabolic Eng.*, vol. 100, p. 19-51, Mar. 2007.
- [26] J. T. K. Sarah C. Mutka¹, Shana M. Bondi¹, John R. Carney¹, Nancy A. Da Silva², "Metabolic pathway engineering for complex polyketide biosynthesis in *Saccharomyces cerevisiae*," *FEMS Yeast Res*, vol. 6(1), no. 1, p. 40-47, 2006.
- [27] P. Novick, "The secretory process and yeast cell-surface assembly." *N. Y.: Cold Spring Harbor*, p. 361-393, 1982.
- [28] van Dijken JP et al., "An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains." *Enzyme and microbial technology*, vol. 26, no. 9–10, p. 706-714, Jun. 2000.
- [29] W. R. Gietz RD, "Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method." *Meth Enzymol*, vol. 350, p. 87–96, 2002.
- [30] J. P. V. D. Cornelis Verduyn, Erik Postma, W. Alexander Scheffers, "Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on regulation of respiration and alcoholic fermentation," *Yeast*, vol. 8, p. 8: 501–517, 1992.
- [31] L. J. Lan EI, "Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide," *Metabolic Engineering*, vol. 13, no. 4, p. 353-363, 2011.
- [32] Z. M. Knoshaug EP, "Butanol Tolerance in a Selection of Microorganisms," *Applied Biochemistry and Biotechnology*, vol. 153, pp. 13-20, Nov. 2008.
- [33] H. P. B. and T. L. S. Shirley H. Baer, "Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol- tolerant *Clostridium acetobutylicum*," *Appl. Environ. Microbiol.* vol. 53, p. 2854-2861, Dec. 1987.
- [34] Fontaine, L., Meynial-Salles, I., Girbal, L., Yang, X.H., Croux, C., Soucaille, P. Molecular characterization and transcription analysis of adhE2, the gene encoding the NADH-dependent aldehyde/alcohol dehydrogenase responsible for butanol production in alcohologenic cultures of *Clostridium acetobutylicum* ATCC 824. *J. Bacteriol.* vol. 184, p. 821–830, 2002.
- [35] Claire R. Shen, Ethan I. Lan, Yasumasa Dekishima, Antonino Baez, Kwang Myung Cho, and James Driving Forces Enable High-Titer Anaerobic 1-Butanol Synthesis in *Escherichia coli*. *Applied and environmental Mic.*, p. 2905–2915, May 2011

