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APPLIED  
MICROBIOLOGY

*Engineering of central-carbon metabolism in recombinant  
Saccharomyces cerevisiae for improved production of  
biopolymers.*

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## Populär vetenskaplig sammanfattning

En viktig komponent i vårt moderna samhälle är plaster och användandet av detta material ökar. Plaster skapas av komponenter som extraheras från petroleum, ett icke-förnybart fossilt bränsle. För en hållbar framtid måste användandet av petroleum minska. Alternativ till petroleum-baserade plaster är bio-plaster. Bioraffinaderier använder idag mindre än 20 % av biomassan för etanolproduktion, överskottet bränns för energiproduktion. Om detta avfall istället skulle kunna användas som substrat till mervärdeskomponenter såsom bioplaster skulle detta vara fördelaktigt.

Bioplaster består av biopolymerer som produceras i naturen och som efter användning naturligt kan brytas ner. Det finns många typer av naturligt producerade biopolymerer, t.ex poly-3- D-hydroxybutyrate (PHB). PHB kan vara den mest lovande biopolymeren då den delar många fysikaliska egenskaper med polypropylen.

Bagerijästen *Saccharomyces cerevisiae* är en av de mest välstuderade mikroorganismerna som under årtionden har blivit använd för produktion av mervärdeskemikalier, såsom etanol. Dess robusthet i storskaliga processer gör den till en attraktiv värdorganism. Att den även kan växa på billiga media såsom restavfall från bioraffinaderier bidrar likaså till detta intresse. Stammar av *S.cerevisiae*, redan designade för att konsumera olika sockerarter som naturligt finns i restavfall, samtidigt som den producerar PHB, har i detta projekt blivit optimerad ytterligare med de nyaste teknikerna inom genetisk manipulering. Målet med projektet var att minska bildandet av biprodukter samtidigt som utbytet av PHB ökade. Genom att ta bort en väsentlig gen för etanolproduktion, som i detta fallet ses som en biprodukt, lyckades en jäststam skapas med bättre kapacitet på att producera PHB.

## Abstract

Bioplastics are a promising alternative for petroleum-based plastics that today is on high demand in our contemporary lifestyles. They are made from biopolymers such as polyhydroxyalkanoates (PHAs). Producing biopolymers from waste material feedstock using microbes increases profitability and environmental benefits as well as sustainability for biorefineries. *Saccharomyces cerevisiae* is a robust microorganism that fits for this task. Engineered *S. cerevisiae* strains are already able to consume sugars present in the waste streams such as glucose and xylose, additionally also able to produce the biopolymer Poly-(R)-3-hydroxybutyrate (PHB) were in this study further engineered to improve yields, productivities and titers. In strategy one a heterologous acetylating acetaldehyde dehydrogenase (*eutE*) originally from *Escherichia coli*, was codon optimized and introduced into *S. cerevisiae*. In strategy two, an acetyl-CoA synthase originally from *Salmonella enterica* (*acs*<sup>L461P</sup>) was also introduced in combination with upregulated homogenous acetaldehyde dehydrogenase (*ALD6*). As last engineering step, to further direct the carbon flux towards PHB, alcohol dehydrogenase 1 (*ADH1*) was disrupted. The results show that  $\Delta adh1$  strains will direct the carbon flux best towards PHB compared to the other strains. Strains expressing *eutE* decreased all of their PHB production suggesting that the kinetics of the heterologous acetylating acetaldehyde dehydrogenase might not be favorable for the conditions present when using xylose as a carbon source.

## Preface

This project, my Master Thesis project, was carried out at the department of Applied Microbiology (TMB) at Lund University during the spring semester of 2016. The aim of this project was to genetically engineer different strains of *Saccharomyces cerevisiae* for improved production of the biopolymer Poly-(R)-3-hydroxybutyrate (PHB). Constructed strains were then evaluated for growth on xylose.

Throughout this project I have interacted with many people that have helped me along the way and from whom I have gotten important feedback and encouragement.

I would like to thank Daniel Brink and Yusak Budi Susilo for great feedback when struggling the most with my cloning work. Thank you both for investing time in me and my plasmid and for contributing with possible solutions and valuable advice when trying to deduce the mystery going on in my Eppendorf tubes. I would also like to thank Celina Tufvegren for the donation of the plasmid p426-GPD, which was the foundation for finally succeeding in creating the plasmid YIpAMH5.

Lisa Wasserstrom, I don't even know where to start my thanks to you. Throughout the project we have shared lab benches and have had stimulating conversations of everything from sourdough bread to pregnancies. Most importantly, a huge thank you for all the time and help you have given me when it came to genetic design of primers and other DNA material. You have taught me so much and for that I am grateful. Lisa, you are my rock in genetic design!

I would like to thank all students at TMB for the good atmosphere in the student room and for all laughs and support. This master thesis experience would not have been the same without you guys!

Diogo Portugal Nunes, thank for helping my around in lab with all the technical aspects and for being so kind and calm when I myself panicked over machines that did not work. I would also like to thank you for investing a lot of time in teaching me smart tips and how to structure data, also for reading my applications!

Alejandro Muñoz de las Heras, thank you for being my awesome supervisor and for offering this project to me. I got the feeling of us being co-workers more than student and supervisor and for that I am very thankful. You have let me work independently but always been there wherever I screwed up or got stuck. In this project I have learnt a lot, not only about science but also about myself. It has been a great experience and you should know that I appreciate everything you have done for me!

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## Introduction

A biorefinery uses natural resources to fractionate and utilize them from start to end with the ambition to do so without any waste generation. Today biorefineries has poor utilization of the biomass feedstock where less than 20 % is used for ethanol production (Taherzadeh and Karimi, 2008). Major side-streams consisting of pentose and lignin wastes are used for biogas and energy production respectively. Converting the carbon from these waste-streams into added-value compounds would increase profitability and environmental benefits of the biorefineries. One of such waste stream lignocellulosic feedstock is the spent sulfite liquor (SSL) that comes from the pulp and paper industry (Novy et al., 2013).

An essential component in modern society is petroleum-based plastics. For a sustainable future the consumption of petroleum needs to be minimized. Looking at the alternatives for petroleum-based plastics, bio-based plastics are an option. The foundation for bio-plastics is the production of bio-polymers using microorganisms. Today there are some microorganisms that naturally produce bio-polymers such as polyhydroxyalkanoates (PHAs), e.g *Cupriavidus necator*. The PHAs are considered one of the most promising biodegradable biopolymers with the capacity to compete with petroleum-based plastics in the future. Poly-(R)-3-hydroxybutyrate (PHB) is one of the most studied PHAs and is of great interest due to its physical similarity to polyethylene, the most commonly used plastic component (Philip et al., 2007). Several bacterial species naturally produce PHB as an energy storage depot, i.e. under nitrogen and phosphate starvation. Some of these natural producers are *C. necator* and *Allochromatium vinosum* and these can be found in soil (Tai et al., 2016).

Currently the bio-based plastics that enter the market are produced from sugars such as glucose. Ideally would be to convert SSL into high-value PHB. SSL is a waste product from the pulp and paper industry abundantly available to a low price, containing a combination of hexose and pentose sugars (Novy et al., 2013). The natural producers of PHB are unfortunately not suitable due to their low tolerance to the harsh conditions present in the waste streams, such as low pH and high concentration of inhibitors originating from the pretreatment process. An alternative is to apply metabolic engineering tools for the construction of an ideal microbial producer. Several engineered microorganisms have been suggested for production of PHB such as *Escherichia coli*. Engineered *E. coli* for PHB production is able to accumulate it, on the other hand further engineering needs to be done to improve tolerance to SSL conditions and the vulnerability to viruses (Lim et al., 2002, Sandstrom et al., 2015). Since the last decade, an alternative for synthesis of PHB is *Saccharomyces cerevisiae*, also known as the baker's yeast, is a well characterized and tolerant host organism (Demeke et al., 2013). This yeast also has a very efficient homologous recombination system which is favorable when doing genetic manipulations.

The pathway leading to the production of PHB consists of 3 genes; acetyl-CoA acetyltransferase (*phaA*), NAD(P)H-dependent acetoacetyl-CoA reductase (*phaB*) and poly(3)-hydroxybutyrate synthase (*phaC*), (Figure 1). The encoded genes *phaA*, *phaC* and NADPH-dependent *phaB* are obtained from *C. necator*. Alternative PHB producer strains have been constructed using an NADH-dependent *phaB* which originally from *A. vinosum*.

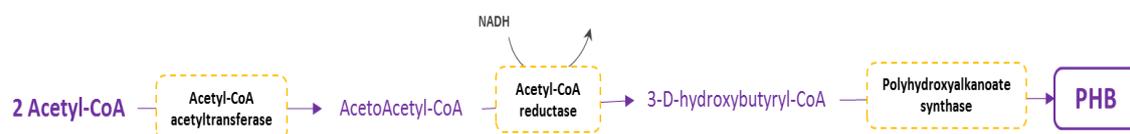


Figure 1. Metabolic pathway for PHB synthesis.

The precursor for the PHB-pathway is acetyl-CoA, which is produced in different compartments of the yeast e.g. in the cytosol or nucleus (Chen et al., 2012). Besides the presence in the other compartment the main-role for acetyl-CoA is in the cytosol where it is part of the central carbon metabolism and keeps feeding the TCA-cycle. The purpose of this study is to increase a pool of cytosolic acetyl-CoA to boost the PHB production. Naturally in *S. cerevisiae*, cytosolic acetyl-CoA is produced in two steps from acetaldehyde with the intermediate acetate. The conversion of acetaldehyde into acetate is performed by the NADP<sup>+</sup>-dependent aldehyde dehydrogenase (*ALD6*) and the acetate into acetyl-CoA by the acetyl CoA synthases (*Acs1* and *Acs2*) at expenses of 2ATP equivalents (Pronk et al., 1996). Acetaldehyde is also the precursor molecule for ethanol production. During anaerobicity and in glucose excess *S. cerevisiae* favors the carbon flux towards ethanol production and thus decreasing precursor pool of acetyl-CoA for potential PHB production. Several approaches to enhance this pool of acetyl-CoA and to reduce ethanol formation have been previously shown in other research groups, e.g. over-expression of the heterologous acetyl-CoA synthase (*acs*<sup>L641P</sup>) gene from *Salmonella enterica* and over-expression of homogenous *ALD6* has been proven to work good for increasing acetyl-CoA dependent products (Kocharin et al., 2012, Shiba et al., 2007). The most successful genetic design for increase of cytosolic acetyl-CoA was demonstrated by Song and coworkers whom also used a  $\Delta adh1 \Delta ald6$  strain in combination of up-regulation of heterologous *eutE* for improved production of lactic acid in *S. cerevisiae* (Song et al., 2016). The heterologous gene *eutE* is an acetylating acetaldehyde dehydrogenase from *Escherichia coli* that directly converts acetaldehyde into acetyl-CoA with the usage of NAD<sup>+</sup> and CoA as cofactors that successfully has been expressed in *S. cerevisiae* (Rudolph et al., 1968, Guadalupe Medina et al., 2010). Expressing *eutE* could

reduce the ATP-dependence of *Acs1* and *Acs2* in this pathway and potentially favor cell growth since the carbon flux would be directed into acetyl-CoA directly.

Previously constructed strains (TMB4425 and TMB4427) express an NADH-dependent PHB pathway constructed with the genes *phaA* and *phaC* from *C. necator* and a NADH-dependent acetoacetyl-CoA reductase (*phaB*) from *A. vinosum*. The benefits of expressing an NADH-dependent acetoacetyl-CoA reductase (AAR) are alternative specificity for NADH and higher specific activity for NADPH in comparison to the *C. necator* NADPH-AAR, offering an alternative redox sink to glycerol production under anaerobic conditions leading simultaneously with a decreased need of NADPH for biomass formation (de las Heras, *et al.*, 2016) . By-product formation under anaerobic conditions is avoided if the more co-factors are accessible (Guadalupe Medina *et al.*, 2010). The strains used also have a heterologous fungal pathway for xylose consumption; xylitol dehydrogenase (*XDH*), xylulokinase (*XK*) and a mutated xylose reductase (*XR*) from *Scheffersomyces stipitis* for enhanced preference of NADH as a cofactor. TMB4427 was further engineered expressing a mutated version of acetyl-CoA synthase ACS (Leu-642-Pro) derived from *S. enterica* (*acs*<sup>L641P</sup>). This acetyl-CoA synthase variant does not need any post-translational modification to be activated as it requires in the wild type variant, therefore this allows the enzyme to be active during growth on glucose, in contrast to the naturally present genes *Acs1* and *Acs2* that are glucose repressed (Shiba *et al.*, 2007).

In this study the CRISPR-Cas9 system was used for genome editing in *S. cerevisiae*. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) systems give bacteria and archaea an adaptive immunity against foreign nucleic acids using guided RNA. Cas9 is an enzyme that has endonuclease activity and uses a guide RNA (gRNA)

to target a directed cleavage in the foreign DNA. This system is applied for genome editing in heterologous organisms (DiCarlo et al., 2013). The gRNA is designed to target a locus of choice where editing is wanted and the insert is designed to have homologous flanks complementary to the flanks of the cut locus. Cas9 induce double strand breaks (DSB) in a designated locus and the cleavage can only be repaired by the host organism's own homologous recombination system. Genome editing is efficiently performed and generates high yields of desired mutants, since those that cannot repair the DSB by homologous recombination will die (Jakociunas et al., 2015).

The aim of this project is to first introduce the codon optimized gene *eutE* into *S. cerevisiae* and to delete the alcohol dehydrogenase 1 (*ADH1*) to further increase the flux of carbon towards the precursor acetyl-CoA for higher productivity of PHB. Next strategy is to in an alternative strain, which already has the upregulated *acs*<sup>L641P</sup>, introduce an upregulated gene cassette for the homogenous *ALD6* to push the flux towards PHB production in combination with disruption of the *ADH1* to minimize the carbon flux towards other by-products, such as ethanol. The aim is to enhance carbon flux towards acetyl-CoA in both strains with the hypothesis of further maximize the production of PHB when grown on xylose. For all metabolic pathway strategies, see Figure 2 for the metabolic map.

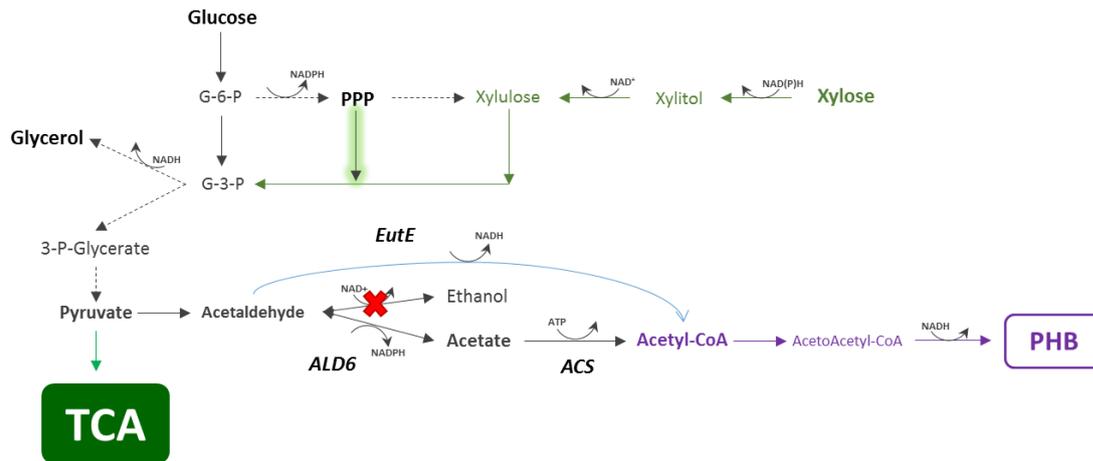


Figure 2. Metabolic map for genetic strategies going from xylose into PHB.

## Materials and methods

### Microorganisms and media

*S. cerevisiae* strains were cultivated in rich media (YPD) containing 10 g/L yeast extract, 20 g/L peptone from casein and 20 g/L glucose. YPD plates containing 10 g/L yeast extract, 20 g/L peptone from casein, 15 g/L agar-agar and 20 g/L glucose were supplemented with 200 mg/mL Geneticin (G418) or 200 mg/mL G418 and 100 mg/L Nourseothricin (CloNat). Cells were plated on 2xYNB xyl-20 plates containing 20 g/L xylose, 12 g/L agar-agar and 6.7 g/L Yeast Nitrogen Base without amino acids (Becton, Dickinson and Company, USA).

*E. coli* cells were used as host for plasmid amplification. Cells were propagated in liquid LB media containing 10g /L tryptone, 5 g/L yeast extract, 5g/L NaCl, 5 mM Tris (pH 7.5) supplemented with 50 mg/L ampicillin for growth at 37°C.

For aerobic cultivations the following media were prepared; 2x YNB xyl-50 (50 g/L xylose, 50 mM phthalic acid (pH 5.5)), YNB medium (13.4 g /L Yeast Nitrogen Base without amino acids (Becton, Dickinson and Company, USA)) and 2xYNB xyl-150 (150 g/L xylose, 50 mM phthalic acid (pH 5.5)), YNB medium (13.4 g /L Yeast Nitrogen Base without amino acids (Becton, Dickinson and Company, USA)).

Table 1 shows all strains used or constructed throughout the project. TMB4425+Cas9 and TMB 4427 have been used as background strains for all constructions.

**Table 1. Relevant *S. cerevisiae* strains that have been used or constructed throughout this project.**

Strain name	Strain background	Relevant genotype	Reference
TMB 4425 + Cas9	TMB4420 (↑PPP;mutXR;leu2-)+ TEF_Cas9_KanMX	Cas9; G418 <sup>®</sup> ; PHB pathway - <i>A. vinosum</i> PhaB (NADH-dependent); <i>S. stipitis</i> xylose pathway w XR mut	(Sandstrom et al., 2015)
TMB 4427	TMB 4425 +Cas9 (PHB- <i>A. vinosum</i> PhaB; mutXR)	G418 <sup>®</sup> (removable from Cas9 plasmid); SeACSL641P::VAC17/MRC1	Previous study, Jenny Landberg (not published)
TMB4494 #3	TMB4427 SeACSL641P::VAC17/MRC1; Cas9; PHB- <i>A. vinosum</i> PhaB; mutXR)	<i>ald6</i> ::chrXII	This study
TMB4495#1	TMB 4425 +Cas9 (PHB- <i>A. vinosum</i> PhaB; mutXR)	<i>eutE</i> ::chrXII	This study
TMB4496 #1	TMB 4425 +Cas9 (PHB- <i>A. vinosum</i> PhaB; mutXR)	<i>Δadh1</i>	This study
TMB4497 #3	TMB4494 (ALD6::chrXII)	<i>Δadh1</i> ; <i>ald6</i> ::chrXII	This study
TMB4498 #10	TMB4495 ( <i>EutE</i> ::chrXII)	<i>Δadh1</i> ; <i>eutE</i> ::chrXII	This study
TMB4499	TMB4425 +Cas9 (PHB- <i>A. vinosum</i> PhaB; mutXR)	YE <i>EpEutE</i>	This study

### Plasmid construction and DNA work

All primers and plasmids used in this study are listed in Table 2 and Table 3. All used products and reagents are purchased from Thermo Scientific if not stated otherwise. Guide RNA (gRNA) plasmid for insertion in the *ADH1* gene with the CRISPR-Cas9 method was constructed through PCR amplification using Phusion Hot Start II DNA Polymerase with primer pair 1 and 2 and plasmid LWA26, donated from Lisa Wasserstrom, as template DNA. Following PCR program was used; 98 °C for 5 min, then 25 cycles of 98 °C for 30 s, 48 °C for 30 s, 72 °C for 3 min and 30 s and a final elongation at 72 °C for 10 min. The gRNA\_*ADH1* was constructed to induce a double strand break (DSB) in the *ADH1* promoter region was constructed using primer pair 1 and 21, and plasmid LWA26 as template DNA. Following PCR

program was used; 98°C for 5 min, then 25 cycles of 98°C for 30 s, 54.3°C for 30 s, 72°C for 3 min and 30 se and a final elongation at 72°C for 10 min. Generated products were treated with *Dpn1* for 2 h followed by purification using GeneJET PCR purification kit. The generated linear gRNA\_ADH1 and gRNA\_ADH1p were ligated using 50 ng of DNA with T4 DNA ligase according to manufacturer's instructions (Thermo Scientific). Ligated products were transformed into competent *E.coli* and plated on LB ampicillin to grow at 37°C. 4 colonies from each strain were chosen and cultivated in liquid LB ampicillin prior to plasmid extraction using GeneJET Mini-prep plasmid kit. All harvested plasmids were verified through sequencing at Eurofins Genomics (Ebersberg, Germany).

**Table 2. List of all primers for PCR used throughout the project. Lower case are annealing to template, restriction sites are highlighted in bold and uppercase shows tails.**

No.	Name	Sequence (5'-3')
1.	LW103_r	gatcatttatctttcactgc
2.	ADH1_gRNA_f	TAACTTGATGGCCGGTCACTgttttagagctagaaatagcaag
3.	IF_BamHI_ALD6_f	tagaactag <b>GGATCC</b> atgactaagctacactttgacac
4.	IF_ALD6_XhoI_r	attacatga <b>CTCGAG</b> ttacaacttaattctgacagctttac
5.	ADH1::ALD6_f	CGTATTGAACAGCCAAAGAACCTAGACCACCAGCAGCACCGGAGATAGCAcaaattaaagccttcgagcgtc
6.	ADH1::ALD6_r	CCCAAGTCGCCCCATCTTGTGTGCTGGTATCACCGTCTACAAGCTTTGagtttatcattatcaatactcgccatttc
7.	LW11_f	gaacttcaccaccttagagc
8.	LW12_r	tagcaccttggagtttagcc
9.	LW248_f	cacaggaacagctatgacc
10.	LW257_r	ttgtaaacgacggccagtg
11.	ADH1p_f LW	gtttccgggtgtacaatatgg
12.	ADH1t_r LW	caataagagcgcacctatgc
13.	EutE_r LW	cagaaactgctaattcagcc
14.	LW65_f	atagcttcaaaatgtttctactcc
15.	LW66_r	tttgaattaaaacttagattagattgc
16.	TEFp_f LW XII-5 flank	AGCGTGATGAAAATTCGCCTGCTGCAAGATGAGCGGGAAACGGGCGAAATatagcttcaaaatgtttctactcc
17.	CPS1t_r LW XII-5 flank	AACCTAGCCCGTTATTGAGCTAAAAAACCTTGCAAAATTCCTATGAATAatttgacacttgattgacacttc
18.	LW261	aatagcaaacggccagtagc
19.	TDH3p_f XII-5 flank	AGCGTGATGAAAATTCGCCTGCTGCAAGATGAGCGGGAAACGGGCGAAATagtttatcattatcaatactcgccatttc
20.	CYCT1t_r XII-5 flank	AACCTAGCCCGTTATTGAGCTAAAAAACCTTGCAAAATTCCTATGAATAcaaattaaagccttcgagcgtc
21.	pADH1_NB_gRNA_f	CTAACATGTAGGTGGCGGAGgttttagagctagaaatagcaag
22.	ADH1_KO-CDS_f	GGAATATTCGGATATCCTTTTGTGTTTCCGGGTGTACAATATGGACTTgatcatgtaactcgcttgatcg
23.	ADH1_KO-CDS_r	TTTTTTATAACTTATTTAATAATAAAATCATAAATCATAAGAAATTCGCaatcagtgaggcacctatctcag

**Table 3. All plasmids used or constructed throughout this project.**

No.	Name	Features	Reference
1.	pLWA26	pSNR52p-gRNA.MRC1/VAC17-SUP4t_natMX, 2µm ori	This study
2.	p426_GDP	G3Pp-MCS-CYC1t, URA3, 2µm ori	(Knudsen et al., 2014)

3.	YIpAMH5	<i>G3Pp-ALD6-CYC1t</i> , URA3, 2 $\mu$ m ori	This study
4.	pRS42N	TEFp-Nat-ADH1t, MCS, 2 $\mu$ m ori	(Taxis and Knop, 2006)
5.	pUC57_EutE	Delivery vector, TEF1p- <i>EutE</i> -CPS1t	Genescript (Piscataway, NJ, USA)
6.	YEpEutE	TEFp-Nat- <i>eutE</i> -ADH1t, MCS, 2 $\mu$ m ori	This study
7.	gRNA_ADH1	pSNR52p-gRNA. <i>ADH1</i> -SUP4t_natMX, 2 $\mu$ m ori	This study
8.	gRNA_ADH1p	pSNR52p-gRNA. <i>ADH1</i> -SUP4t_natMX, 2 $\mu$ m ori	This study
9.	pCfB3050	pSNR52p-gRNA. <i>XII-5</i> -SUP4t_natMX, 2 $\mu$ m ori	(Stovicek et al., 2015b)

Glycerol stock with *E. coli* carrying plasmid p426\_GPD was obtained from Celina Tufvegren. Plasmids were harvested using GeneJET Mini-prep plasmid kit after overnight growth in LB media supplemented with 50 mg/L Ampicillin. 1  $\mu$ g of p426\_GPD was restricted using *Bam*HI and *Xho*I and incubated for 3 h at 37°C. Subsequently, the restricted p426\_GPD was dephosphorylated adding 1  $\mu$ l FastAP to the reaction mixture and was further incubated for 2 h at 37°C. Restricted and dephosphorylated p426\_GPD was purified using GeneJET PCR purification kit. The gene *ALD6* was amplified with Phusion Hot Start II DNA Polymerase using genomic DNA, extracted from strain TMB4425 + Cas9 using genomic DNA extraction protocol for PCR-based applications (Looke et al., 2011), as template and primer pair 3 and 4. Following PCR program was used; 98°C for 5 min, then 25 cycles of 98°C for 30 s, 60°C for 30 s, 72°C for 45 s and a final elongation at 72°C for 10 min. The PCR fragment obtained was cut with *Bam*HI and *Xho*I and cloned into corresponding sites of p426\_GPD generating the plasmid YIpAMH5. YIpAMH5 was transformed into *E. coli* and selected by colony PCR using primer pair 7 and 8 for amplification of *ALD6*. YIpAMH5 was also verified through restriction analysis using *Xba*I.

The acetylating acetaldehyde dehydrogenase (*eutE*) from *E. coli* ((GenBank accession no.YP\_001459232.1) was codon optimized for *S. cerevisiae* at Eurofins Genomics (Ebersberg,

Germany) and synthesized by Genescript (Piscataway, NJ, USA). The coding sequence was designed to be under the control of the constitutive promoter TEF1p and terminator CPS1t of the gene flanked by the restriction sites BamHI and Apal in the plasmid pUC57\_EutE. 1 µg each of plasmids pUC57\_EutE and pRS42N was restricted with *Bam*HI and *Ap*al for 2 h at 37°C. Restricted pRS42N was dephosphorylated adding 1 µl FastAP to the reaction mixture and was incubated for 2 h at 37°C. All enzymes were deactivated at 80°C for 5 min prior to purification. Restricted pUC57\_EutE was gel purified to obtain the *eutE* cassette. In 1:3 ratio the DNA fragment was cloned into corresponding sites of pRS42N with ligation and thus yielding the plasmid YEpEutE. YEpEutE was amplified using *E. coli* and validated through colony PCR using primer set 14 and 15 for amplification of the *TEF1* promoter. Following PCR program for Dreamtaq DNA polymerase was used; 95°C for 5 min, then 25 cycles of 95°C for 30 s, 45°C for 30 s, 72°C for 1 min and a final elongation at 72°C for 10 min. After plasmid extraction YEpEutE was validated through restriction analysis with *Xba*I.

Primers 5 and 6 contain flanking regions for integration in ADH1 locus, these primers were used for amplification of the ALD6 cassette integrated in YIpAMH5. Following PCR program for Phusion Hot Start II DNA Polymerase was used; 98°C for 5 min, then 25 cycles of 98°C for 30 s, 68°C for 30 s, 72°C for 1 min and 30 s and a final elongation at 72°C for 10 min. The amplified product was purified using GeneJET PCR purification kit. pUC57\_EutE was cleaved with restriction enzymes *Aat*II and *Bg*III and thus yielding the *eutE* cassette with homologous flanks included.

Gene cassettes for insertion in chromosome XII were constructed through PCR. The *eutE* cassette was amplified using primer pair 16 and 17 using pUC57\_EutE as template. Following

PCR program for Phusion Hot Start II DNA Polymerase was used; 98°C for 5 min, then 25 cycles of 98°C for 30 s, 61.9°C for 30 s, 72°C for 1 min and 30 sec and a final elongation at 72°C for 10 min. Same thing was done for the *ALD6* with primer pair 19 and 20 using YIpAMH5 as template, using following PCR program for Phusion Hot Start II DNA Polymerase; 98°C for 5 min, then 25 cycles of 98°C for 30 s, 68°C for 30 s, 72°C for 1 min and 30 sec and a final elongation at 72°C for 10 min. Amplified products were purified using GeneJET PCR purification kit and validated through gel electrophoresis.

The knock-out fragment for *ADH1* disruption was constructed by amplifying a non-coding region situated in (2005:2392) of the plasmid YIpAMH5, primers used were 22 and 23. Following PCR program for Phusion Hot Start II DNA Polymerase was used; 98°C for 5 min, then 25 cycles of 98°C for 30 s, 66.7°C for 30 s, 72°C for 30 sec and a final elongation at 72°C for 10 min. Amplified product was purified using GeneJET PCR purification kit and validated through gel electrophoresis.

### Strain construction

TMB4425 + Cas 9 was transformed with 2 µg *eutE* cassette and 1 µg gRNA\_ADH1. TMB4427 was transformed with 2 µg *ALD6* cassette and 1 µg gRNA\_ADH1. All transformations were performed using Gietz's transformation protocol (Gietz and Schiestl, 2007). The transformed strains were selected on YPD plates supplemented with Geneticin (G418) and Nourseothricin (CloNat).

TMB4427 was transformed with 2 µg of *ALD6* cassette and 1 µg pcfB3050 generating TMB4494. TMB4425 + Cas 9 was transformed with 1 µg YEpEutE thus yielding the strain TMB4499. TMB4425 + Cas 9 was transformed with 2 µg *eutE* cassette and 1 µg pcfB3050 yielding the strain TMB4495. Transformed clones were selected on YPD plates supplemented

with G418 and CloNat. Integration of the *eutE* cassette into chromosome XII-5 was confirmed by PCR using primer pair 14 and 18 and genomic DNA extracted from positive clones as template. Following PCR program for Dreamtaq DNA polymerase was used; 95°C for 5 min, then 25 cycles of 95°C for 30 s, 47°C for 30 s, 72°C for 2 min and a final elongation at 72°C for 10 min. Integration of the *ALD6* was confirmed using primer pair 7 and 18 and genomic DNA extracted from positive clones as template. Following PCR program for Dreamtaq DNA polymerase was used; 95°C for 5 min, then 25 cycles of 95°C for 30 s, 49.5°C for 30 s, 72°C for 2 min and a final elongation at 72°C for 10 min. PCR products were run on electrophoresis gel.

Constructed TMB4494 and TMB4495 were recycled for the CloNat marker by cultivation in liquid YPD supplemented with G418 prior to plating on YPD supplemented with G418. 8-10 colonies from each strain were selected and streaked on YPD + G418 + CloNat followed by streaking the same colony on YPD + G418. Plates were left at 30 °C overnight. Colonies unable to grow on YPD + G418 + CloNat but still on YPD + G418 had lost the CloNat resistance marker and were thus recycled. Recycled strains were used for further strain construction.

TMB4425 + Cas9, TMB4494 and TMB4495 was transformed separately with 2 µg knock-out cassette and 1 µg gRNA\_ADH1p thus yielding the strains TMB4496, TMB4497 and TMB4498. Positive clones were selected on plates supplemented with G418 and CloNat. Disruption of the *ADH1* gene was confirmed by amplification of the locus using genomic DNA and primers 11 and 12. Following PCR program for Dreamtaq DNA polymerase was used; 95°C for 5 min, then 25 cycles of 95°C for 30 s, 49.8°C for 30 s, 72°C for 1 min and a final elongation at 72°C for 10 min. PCR products were run on gel electrophoresis.

## Cell extract preparation

Cells were grown in YPD overnight at 30°C. Cultures were harvested at top speed (3 220 x g) for 5 min and washed twice in 10 mM potassium phosphate buffer (pH 7.5) (Shiba et al., 2007). Harvested cell pellets were resuspended in 250 µL Y-PER™ Yeast Protein Extraction Reagent (ThermoFisher scientific) and put on rocking table for 20 min at room temperature. Cell debris was removed by centrifugation at 15,000 x g for 10 min. Supernatants were collected and centrifuged at 21,000 x g for 10 min. Cell extracts were stored on ice prior to enzymatic assays and protein content determination. The protein content in the cells extracts was determined using Bradford protein assay determination kit (ThermoFisher Scientific); using BSA-standards for calibration curve (ThermoFisher Scientific).

## Enzymatic assays

### Acetaldehyde CoA dehydrogenase activity (*eutE*)

Acetaldehyde CoA dehydrogenase activity was measured spectrophotometrically using a 96-well microtiter plate Multiskan Ascent reader (Thermo Electro Corporation, Finland) at 340 nm following the absorbance change from the reduction of NAD<sup>+</sup> into NADH. The reaction was run in a total volume of 250 µL in microtiter plates. The reaction mixture contained 50 mM CHES buffer (pH 9.5, adjusted with 5 M KOH), 0.1mM Coenzyme-A, 0.8 mM NAD<sup>+</sup>, 1 mM DTT and 15 µL cell extracts. The reaction was started by the addition of 5 µL 1 M acetaldehyde (Kozak et al., 2014, Clark and Cronan, 1980).

### Acetaldehyde dehydrogenase activity (*ALD6*)

Acetaldehyde dehydrogenase activity was measured spectrophotometrically at 340 nm for the reduction of NADP<sup>+</sup> into NADPH. The reaction was run in a total volume of 250 µL in a 96-well microtiter plate. The reaction mixture contained 100 mM potassium phosphate buffer (pH 8.0, adjusted with 5 M KOH), 15 mM pyrazole, 1 mM DTT, 10 mM MgCl<sub>2</sub>, 0.4 mM

NADP<sup>+</sup> and 15 µL cell extracts. The reaction was started by the addition of 5 µL 5 mM acetaldehyde (Postma et al., 1989, Shiba et al., 2007). The addition of pyrazole inhibits the ADH's activity in the cell extracts (Young and Rafter-Tadgell, 1987).

#### **Alcohol dehydrogenase activity (ADH1)**

Alcohol dehydrogenase activity was measured spectrophotometrically at 340 nm for the reduction of NAD<sup>+</sup> into NADH. The reaction was run in a total volume of 250 µL in microtiter plates. The reaction mixture contained 100 mM Glycine (pH 9.0 adjusted with 5 M KOH), 5 mM NAD<sup>+</sup> and 15 µL cell extracts. The reaction was started by the addition of 25 µL ethanol (99,5 %) (Bernt et al., 1974).

#### **Aerobic shake flask cultivations**

Strains (TMB4425, TMB4427, TMB4494, TMB4495, TMB4496, TMB4497, TMB4498) were characterized for growth in xylose. Precultures were started by inoculating cells from 20 % glycerol stocks or started by inoculating a colony from either YPD + G418 or YNB xylose plates into 5 mL liquid 2x YNB xyl-50 media in 50 ml conical centrifuge tubes. High xylose cultivations were started by inoculating 0.05 OD<sub>620</sub> of TMB4425 in 100 mL 2x YNB xyl-150 in 1 L shake flasks with baffles in duplicates. Characterization of TMB4425, TMB4427, TMB4494, TMB4495, TMB4496, TMB4497, TMB4498 were started by inoculating at a start OD<sub>620</sub> =0.05 in 100 mL 2x YNB xyl-50 in duplicates. Samples were taken throughout the cultivations for OD<sub>620</sub>, HPLC and flow cytometry analysis. Cultures were checked for contamination throughout the cultivation by microscopy.

#### **Metabolite determination and cell dry weight analysis**

Cultures were regularly sampled for metabolite analysis. Extracellular metabolite levels were measured from the sample supernatants with HPLC using a Waters (Milford, USA) system equipped with an Aminex HPX-87H column (Bio-Rad, Richmond, USA) that operates at 60°C

with a mobile phase of 5 mM H<sub>2</sub>SO<sub>4</sub> and 0.6 mL/min flow rate. Concentrations of glucose, xylose, xylitol, glycerol, acetate and ethanol were calculated from an external standard calibration curve. Samples were analysed in technical duplicates. Cell dry weight was determined by filtering samples through a pre-weighed 0.45 µm membrane (Pall Corporation, New York, USA). The membranes were washed with ultrapure H<sub>2</sub>O and dried in a microwave oven at 350 W for 8 min. The final weight was measured after equilibration to room temperature in a desiccator. The measurements were made in technical duplicates. Biomass was measured at the end of each cultivation.

### PHB extraction

The method used relied on the quantitative conversion of PHB to crotonic acid catalysed by hot concentrated sulfuric acid (Law and Slepecky, 1961) . PHB content was analyzed by harvesting 2 mL of culture by centrifugation for 5 min at 6000 x *g*. Supernatant was carefully removed and the pellets were resuspended in ultrapure H<sub>2</sub>O, followed by centrifugation for 5 min at 6000 x *g*. This step was repeated twice, then 0.5 mL of 99% sulfuric acid was added onto the pellets that were incubated at 95°C for 1 h with open lids in a heat block (Grant QBD1, Grant Instruments, UK). The resulted solution was diluted 20 times by serial dilution and further analysed by HPLC using the same conditions as for the metabolite analysis. Commercially available PHB (#363502, Sigma-Aldrich) processed in parallel with the samples was used as control. Concentrations of crotonic acid were calculated from an external ten-point standard calibration curve. Samples were analysed in technical duplicates.

## Results

### Plasmid construction

Early attempts to construct the YIpAMH5 plasmid containing the *ALD6* over-expression cassette failed several times. The reason behind these failures was traced back to the design of the primers for the amplification of the *ALD6* gene. The included restriction sites did not have any extra bases flanking the ends as can be seen in the used primers; forwards primer (XmaI\_ALD6\_f **CCCGGG**ATGACTAAGCTACACTTTGACAC) and reverse primer (ALD6\_XhoI\_r **CTCGAG**TTACAACCTTAATTCTGACAGCTTTTAC), bold letters indicating restriction sites. This made restriction difficult and thus the gene could not be cloned into the backbone plasmid. After re-design of the primers which can be seen in Table 2, where extra bases were included to facilitate cleaving, the construction of the YIpAMH5 was achieved and verified by restriction analysis.

### Strain construction

The gRNA\_ADH1 that was targeted to cleave the open reading frame (ORF) of the *ADH1* was specific and this sequence was generated using the DNA2.0 software (Newark, California). Specificity was confirmed using BLAST to align the sequence against a *S. cerevisiae* CEN.PK113-7D genome. Despite this fact, strains transformed only with the gRNA\_ADH1 and thus working as a negative control gave the same growth ratio as strains transformed with the gRNA\_ADH1 plus the gene cassette. When running confirmative PCR all selected clones were negative. This result indicated that either the gRNA was not specific enough or that the host was performing homologous recombination with other present gene fragments in order to survive. To investigate, *ADH1* was aligned using the tool BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the multi sequence alignment tool Clustal omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Among all present alcohol

dehydrogenases *ADH1*, *ADH2* and *ADH5* showed up to 89 % homology identity, especially around the region where the Cas9 induce the DBS in *ADH1*. These results strengthen the assumption of homologous recombination took place in order for the host organism to survive.

To solve this issue a new cloning strategy was formed which included 2 steps of genome editing. In the first step the upregulated *ALD6* and the gene *eutE* were introduced separately into chromosome XII in TMB4427 and TMB4425 respectively. This region has been previously shown to generate stable gene insertions with good translation (Stovicek et al., 2015a). The insertion was confirmed by PCR using primer pair 14 and 18 for *eutE*, 7 and 18 for *ALD6* and enzymatic assays, see section below. Confirmed strains proceeded to the second step of genome editing, where the *ADH1* was disrupted by inserting a non-coding DNA sequence in the locus of the gene. To avoid homologous recombination a new gRNA was designed. This gRNA was designed to cleave in the *AHD1* promotor region using primer pair 1 and 21. Positive clones were confirmed by PCR amplification of the gene locus using primers 11 and 12, yielding different sizes if the gene was disrupted or not. PCR products were run on gel electrophoresis

### Enzymatic assays

After PCR verification of the constructed strains, enzymatic assays were performed in order to analyze the protein activity.

#### Acetaldehyde CoA dehydrogenase activity (*eutE*)

Activity of the heterologous gene *eutE* was measured for the strains TMB4495 and TMB4499 using TMB4425 as a control strain. In Figure 3 and Figure 4 shown below the activity of different clones of the same strain compared to a control is depicted. TMB4499 and TMB4495 have a significantly higher activity compared to control strain, which based on the

standard deviation could assume there is no detectable activity, indicating that the heterologous and codon optimized gene *eutE* is expressed and compatible in *S. cerevisiae*. TMB4499 has an approximate ten times higher activity compared to TMB4495. The reason for this is due the differences in copy number of the gene. When *eutE* is expressed on an episomal plasmid as TMB4499 the expression is higher and thus should also the activity be higher. TMB4495 has the integrated gene, generating a more stable construct compared to an episomal plasmid that requires an external pressure from antibiotics for the host to keep it. Adding antibiotics to the media the growth of the host could be altered. To keep consistent throughout the project, TMB4499 was not selected for continued evaluation. The reason for not continuing is due to the needed antibiotic pressure and because all the other constructs will have genome integrated genes, this despite the proven high activity of the *eutE*. Since constructed strains were to be evaluated on growth in xylose, all enzymatically tested strains were plated on 2x YNB xylose plates. For its high activity and good growth on xylose, this colony was therefore chosen to continue with for further gene manipulations throughout the project.

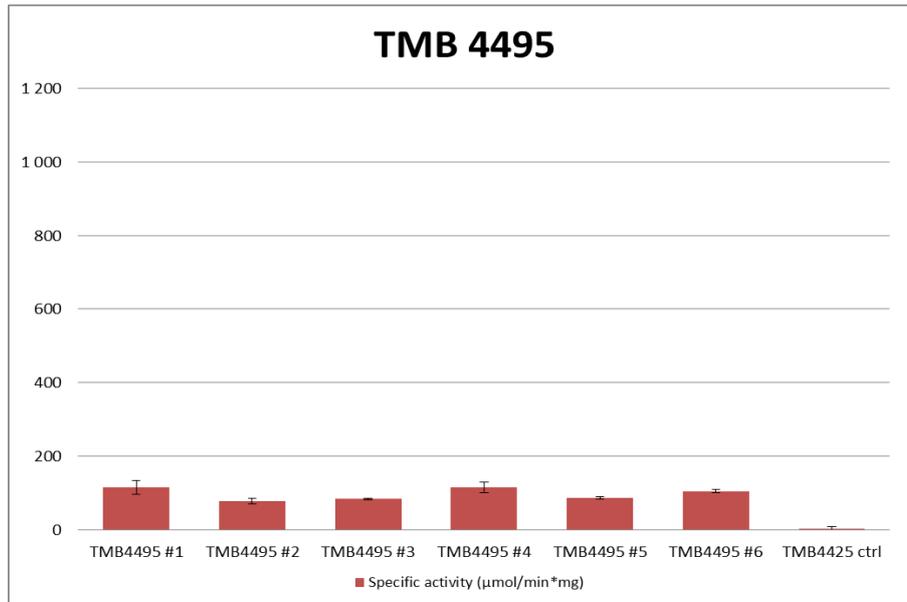


Figure 3. Acetaldehyde CoA dehydrogenase (*eutE*) activity in 6 different clones of the strain TMB4495 compared to the control strain TMB4425.

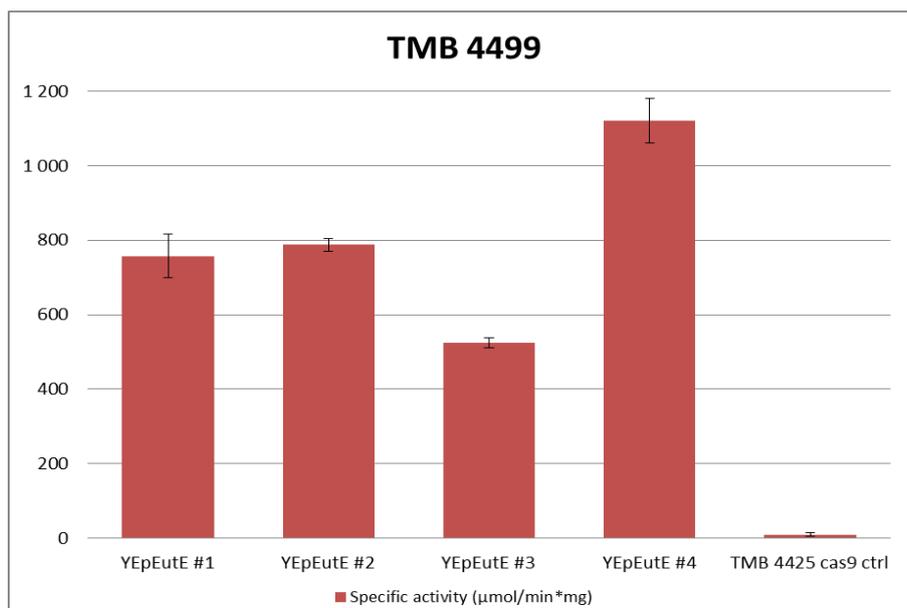


Figure 4. Acetylating aldehyde dehydrogenase (*eutE*) activity in 4 different clones of the strain TMB4499 compared to the control strain TMB4425.

#### Acetaldehyde dehydrogenase activity (*ALD6*)

Activity of the homogenous gene *ALD6* was measured for the strain TMB4494 and the background strain TMB4427. In Figure 5 the activity of *ALD6* is shown for 4 different clones compared to the control. The clones overexpressing the gene show higher activity than the reference strain. As can be seen in the figure 5, TMB4494 #3 shows the highest activity of all 4 clones, with a small standard deviation. When Student's T-test was performed (data not

shown) the result showed that the increase in activity was not significant in comparison to that of the control. Nonetheless, this clone was despite this chosen to continue with for further genetic manipulations.

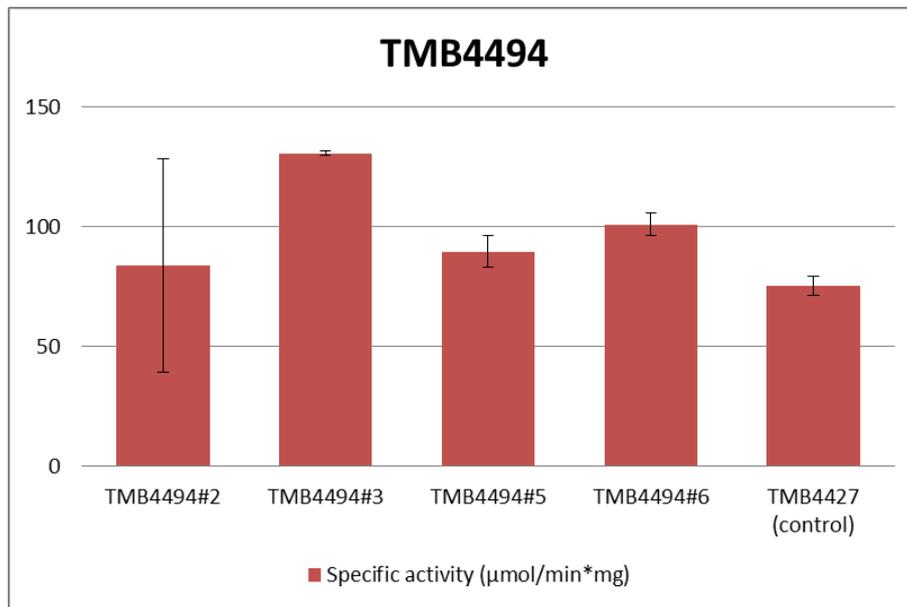


Figure 5. Acetaldehyde dehydrogenase activity in 4 different colonies of the strain TMB4494 compared to the control strain TMB4427.

#### Alcohol dehydrogenase activity (*ADH1*)

To validate that the gene *ADH1* had been completely disrupted in constructed strains TMB4496, TMB4497 and TMB4498 the alcohol dehydrogenase activity was measured in comparison to 2 control strains, TMB4425 and TMB4427. For each constructed strain, 4 colonies each were tested. The results from the assay are summarized in Figure 6. There is a significant difference in activity of all tested strains compared to the controls. These results are strong indications of complete disruption of the *ADH1* gene in all strains. Since the *ADH1* is the most active gene responsible for ethanol conversion, the remaining alcohol dehydrogenases will contribute with some alcohol conversion which can be seen in the Figure 6. All tested clones showed low activity but to be consistent, the clones from each strain that showed lowest activity were chosen for characterization. For TMB4496 clone #1, TMB4497 clone #3 and TMB4498 clone #10 were thus selected for further characterization.

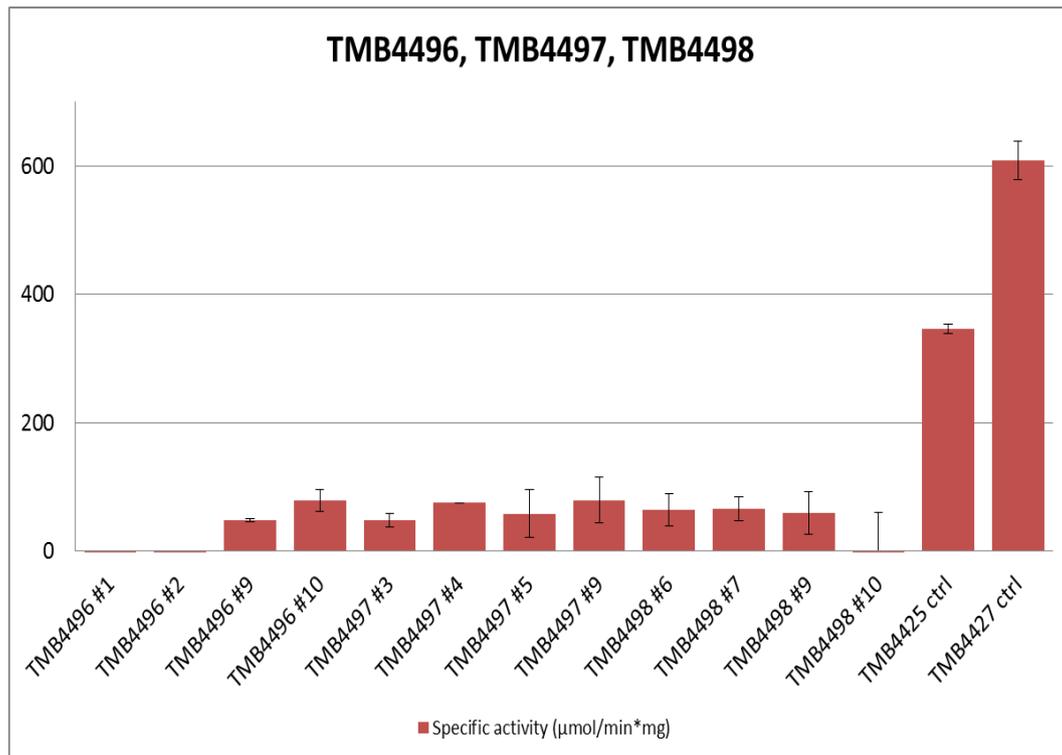


Figure 6. Alcohol dehydrogenase activity of 4 different clones each of the strains TMB 4496, TMB 4497 and TMB 4498. Constructed strains are compared to 2 control strains, TMB 4425 and TMB 4427.

## Aerobic shake flask cultivations

### Shake flask cultivations of TMB4427, TMB 4494 and TMB 4495

Strains expressing the upregulated acetyl-CoA synthetase variant (TMB4427), and the overexpressed acetaldehyde dehydrogenase (TMB4494) and the heterologous acetylating NAD<sup>+</sup>-dependent acetaldehyde dehydrogenase (TMB4495) were characterized for aerobic growth in YNB-xylose medium. All strains were compared to the control strain TMB4425. OD<sub>620</sub> and samples for metabolites were taken during the cultivation. During the growth phase the cultures were checked for bacterial contamination by microscopy. At the end of all cultivations samples for cell dry weight (CDW) were harvested. All strains were evaluated in biological duplicates and the average values are concluded the figures below (Figure 7-Figure 10).

All data corresponding to the control strain TMB4425, which can be seen in Figure 7, is based on two replicates with inconsistent sampling points for the biological duplicates, thus some points are without any standard deviation. TMB4425 reached a maximum OD<sub>620</sub> of 19.35 at 41 h. The cells entered stationary phase at approximately 48 h. After 137 h of cultivation 72 % of the xylose was consumed and a maximum 0.19 g/L of PHB and 5.04 g/L of acetate were produced. At the end of cultivation the measured value of ethanol was 0 g/L but at 96 h the observed highest concentration was 8.16 g/L. This indicates that the produced amount of ethanol is consumed while the cells were kept in stationary phase. At the end of cultivation the obtained value of CDW was 4.84 g/L.

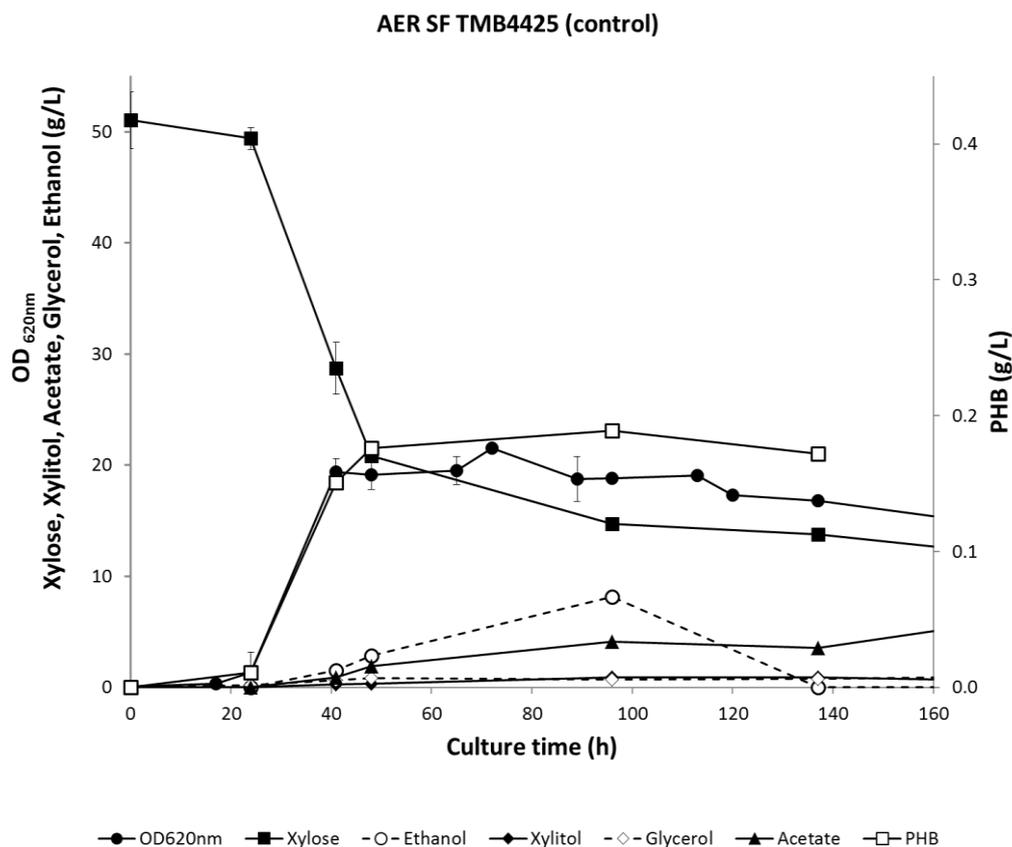


Figure 7. Aerobic cultivation of the control strain TMB4425. Graph is based on two independent cultivations with different sampling points and thus some data points do not have any standard deviation.

TMB4427 (*acs<sup>L641P</sup>*) reached a maximum OD<sub>620</sub> of 16.15 at 65 h, this is lower compared to TMB4425. Metabolite data and OD<sub>620</sub> values are summarized in Figure 8. The data shows

that xylose is consumed in higher extent compared to control strain, 86.1 % compared to 74 % respectively. At 96 h the observed maximum value of acetate was 2.94 g/L, which is lower compared to maximum 5.08 g/L of the control. The PHB was at the end of cultivation 231.91 mg/L and the ethanol 1.28 g/L. The maximum PHB titer reached was 262.31 mg/L at 72 h. At the end of cultivation the obtained average value of CDW was 5.20 g/L

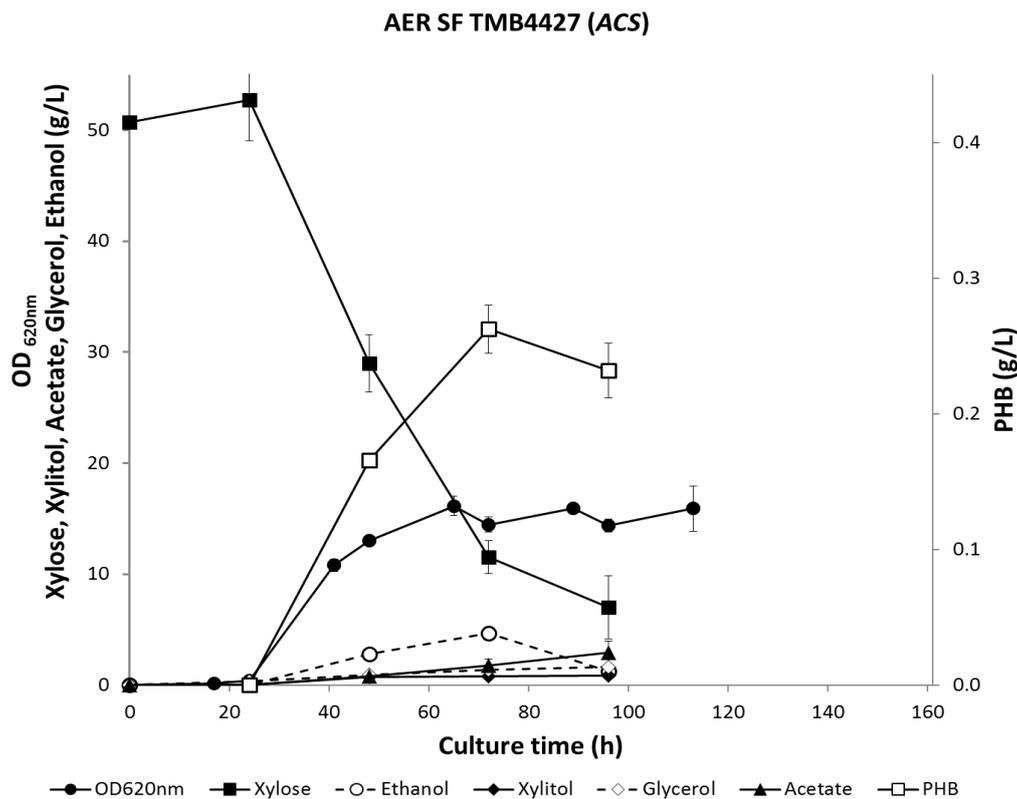


Figure 8. Aerobic shake flask cultivation in biological duplicates of the strain TMB4427.

TMB4494 reached a maximum OD<sub>620</sub> of 14.48 at 89 h. This is much lower compared to the control which has a maximum OD<sub>620</sub> of 19.35. Metabolite data and OD<sub>620</sub> values are concluded in Figure 9. Xylose consumption was determined to 59.8 %, which is less than both TMB4425 and TMB4427 that works as controls for this strain. Maximum obtained PHB concentration of 217.43 mg/L was detected at 72 h, which decreased to 185.70 mg/L to the end of cultivation which could be due to loss of cell mass since they are correlated. At the

end of cultivation the acetate was at 3.53 g/L, cell dry weight 4.48 g/L and the ethanol was completely depleted. Highest observed concentration of ethanol was at 48 h where it was determined to 0.346 g/L.

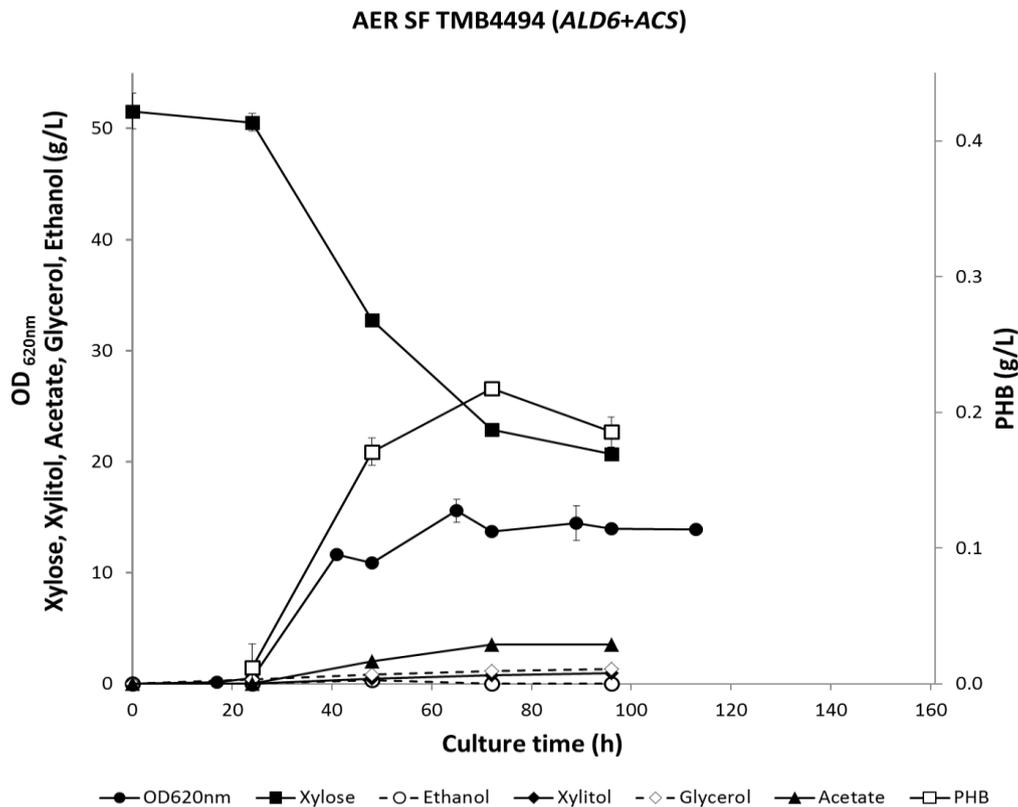


Figure 9. Aerobic shake flask cultivation in biological duplicates of the strain TMB4494.

TMB4495 (*eutE*) reached a maximum OD<sub>620</sub> of 17.6 at 65 h and had a xylose consumption of 56.4 %. Metabolite data and OD<sub>620</sub> values are concluded in Figure 10. At 96 h the acetate concentration was determined to 4.92 g/L, which was similar compared to the highest observed concentration of 5.01 g/L of the control. Highest observed concentration of ethanol was at 48 h where it was determined to 2.08 g/L and decreased thereafter to the final observed value of 0.25 g/L. No production of PHB was observed throughout the cultivation. CDW was determined to 4.39 g/L.

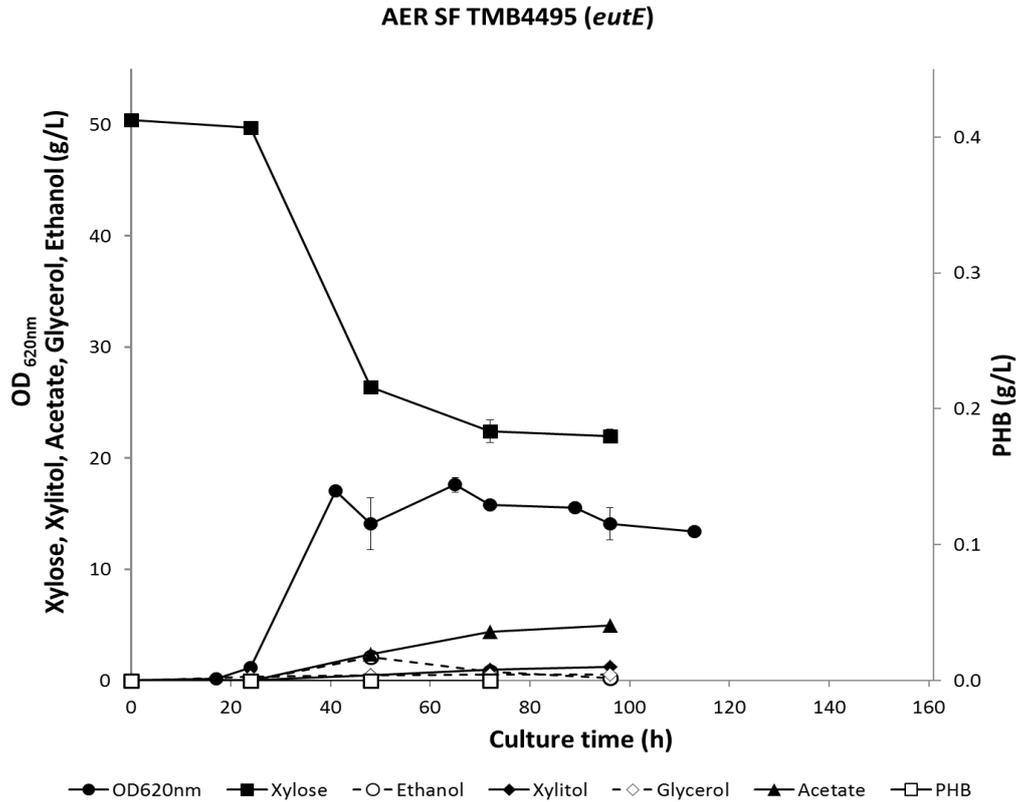


Figure 10. Aerobic shake flask cultivation in biological duplicates of the strain TMB4495.

#### Shake flask cultivations of strains lacking *ADH1*

*ADH1* was deleted in TMB4425, TMB4494 and TMB4495 and yielding the strains TMB4496, TMB4497 and TMB4498 respectively. The constructed strains were all characterized and evaluated for growth on xylose in aerobic shake flask cultivations during 161 hours. TMB4425 was used as a control for the constructed strains. OD<sub>620</sub> values and samples for metabolites were taken throughout the cultivation. At the end of cultivation, cultures were checked for bacterial contamination by microscopy. Also, at the end of all cultivations samples for CDW were harvested. All strains were evaluated in biological duplicates and the average values are concluded the figures below (Figure 11-Figure 13).

TMB4496 ( $\Delta adh1$ ) reached a maximum OD<sub>620</sub> of 20.45 at 48 h; this is similar to what the control obtained at maximum (OD<sub>620</sub> 19.35 at 41 h). All the metabolite data and OD<sub>620</sub> values are concluded in Figure 11. TMB4496 had a xylose conversion of 76.8 %, which is higher

compared to control. Highest observed ethanol concentration was 3.38 g/L at 41 h but decreased to 0.07 g/L at the end. At the end of cultivation acetate was determined to be 5.05 g/L, PHB 284.37 mg/L and CDW 5.35 g/L.

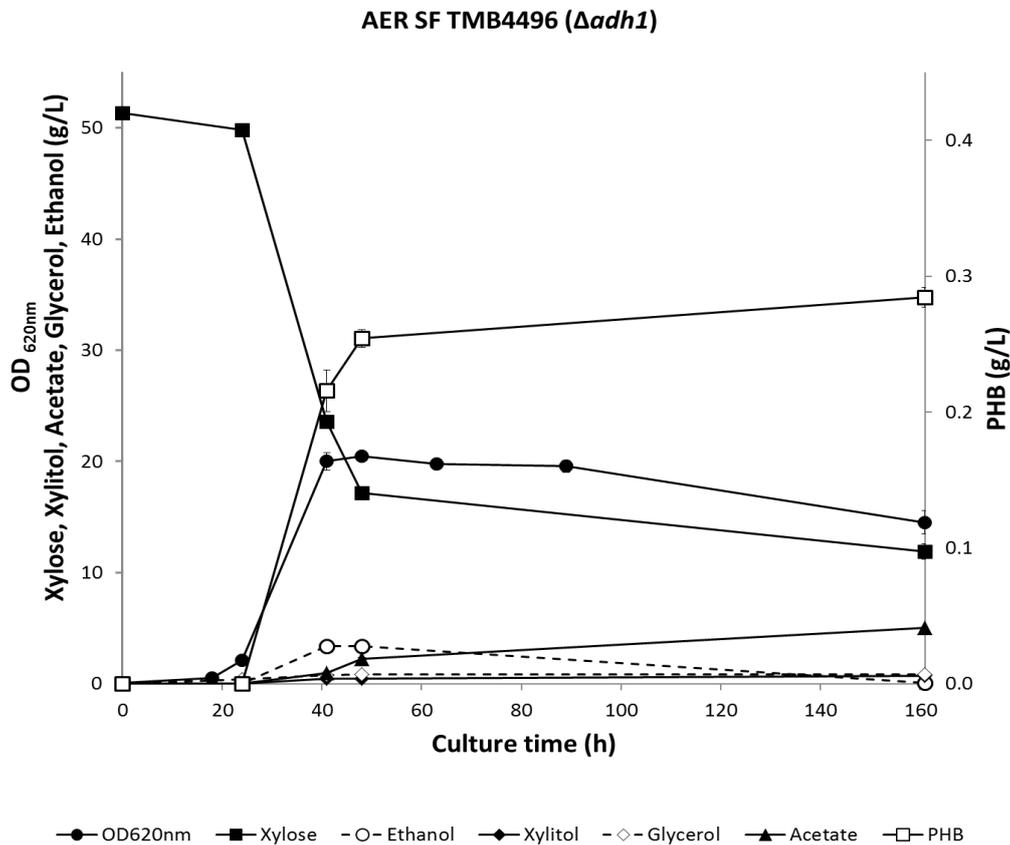


Figure 11. Aerobic shake flask cultivation in biological duplicates of the strain TMB4496.

TMB4497 ( $\Delta adh1$ ,  $acs^{L641P}$ ,  $ALD6$ ) reached a maximum OD<sub>620</sub> of 15.05 at 89 h, which is significantly lower compared to control's maximum OD<sub>620</sub>. Metabolite data and OD<sub>620</sub> values are concluded in Figure 12. The xylose consumption was determined to 60.7 % and the CDW 5.29 g/L. Highest concentration of acetate was 3.67 g/L and was observed at 89 h. This value decreased slightly to 3.5 g/L at the end of cultivation, which also could be derived to standard deviation of the two duplicates. Maximum observed of PHB was 121.38 mg/L. No ethanol was observed throughout the cultivation.

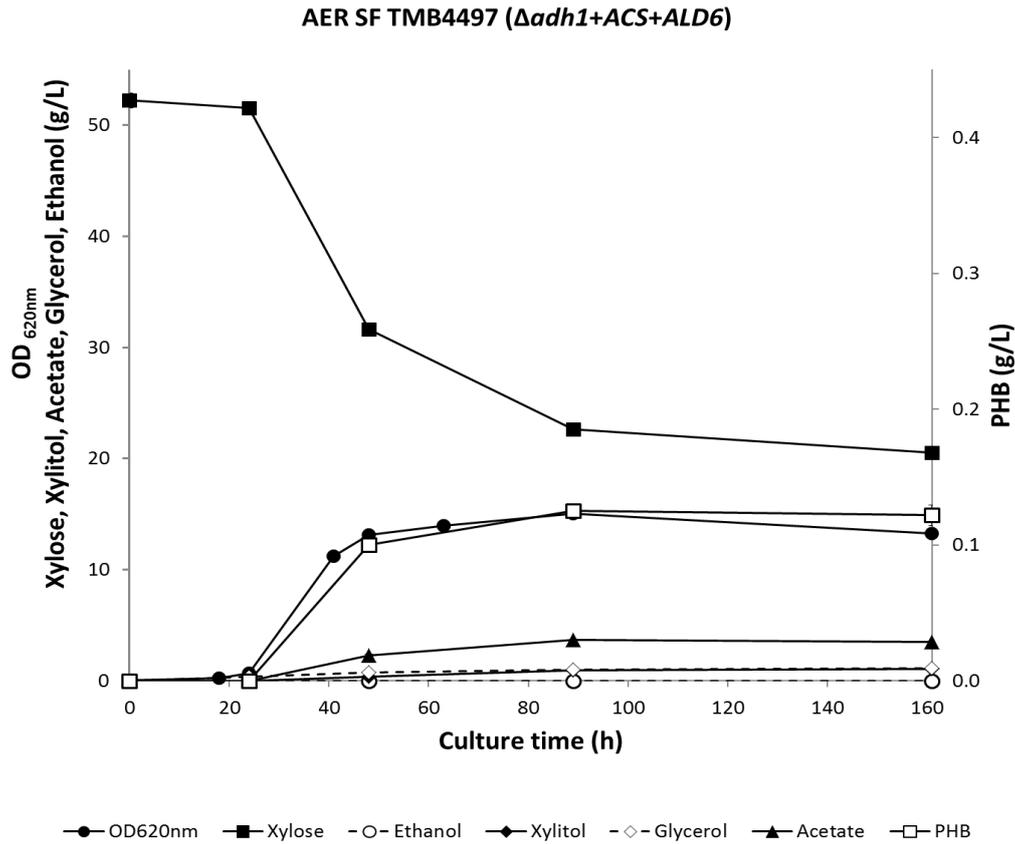


Figure 12. Aerobic shake flask cultivation in biological duplicates of the strain TMB4497.

TMB4498 ( $\Delta adh1, eutE$ ) reached a maximum OD<sub>620</sub> of 15.78 at 89 h, which is significantly lower compared to control. Metabolite data and OD<sub>620</sub> values are concluded in Figure 13. TMB4498 had a xylose consumption of 51.5 %, which is the lowest consumption rate observed during this project. Highest observed ethanol concentration was 0.92 g/L at 41 h but decreased to 0 g/L at the end. Acetate concentration was determined to 4.59 g/L and CDW to 5.09 g/L. No PHB was detected throughout the cultivation.

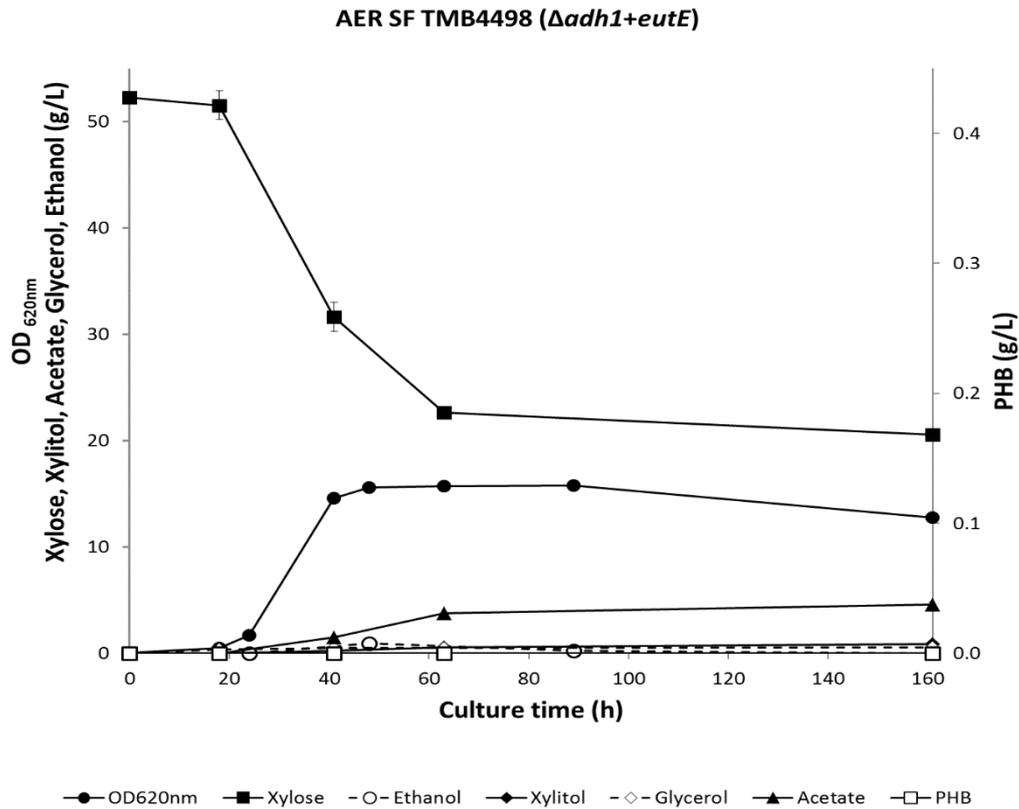


Figure 13. Aerobic shake flask cultivation in biological duplicates of the strain TMB4498.

The characterized strains and obtained results are summarized in Table 4 and Table 5. As can be seen the best producer of PHB is strain TMB4496 which still keeps a good growth and conversion of xylose compared to control strain TMB4425.

Table 4. Summary of TMB4425, TMB4427, TMB4494 and TMB4495 evaluated strains in duplicate and comparison of important data points. Ethanol and PHB titer are presented for the maximum obtained value.

Strain	TMB4425	TMB4427	TMB4494	TMB4495
	NADH-PHB; XRmut	ACS; NADH-PHB; XRmut	ALD6;ACS; NADH-PHB; XRmut	EutE; NADH-PHB; XRmut
<b>Cultivation time (h)</b>	161h**	113h	113h	113h
<b>Flask and media vol.</b>	SF 1L-0.1L media	SF 1L-0.1L media	SF 1L-0.1L media	SF 1L-0.1L media
<b>Growth condition</b>	Shake flask (Aerobic)	Shake flask (Aerobic)	Shake flask (Aerobic)	Shake flask (Aerobic)
<b>Growth rate (h<sup>-1</sup>)</b>	0.15 ± 0.01	0.19 ± 0.00	0.18 ± 0.00	0.18 ± 0.00
<b>Y<sub>sx</sub> (g/g Xyl)</b>	0.13 ± 0.01	0.12 ± 0.00	0.14 ± 0.01	0.15 ± 0.01

<b>YsEtOH (g/g Xyl)*</b>	0.16 ± 0.10***	0.12 ± 0.00	0.02 ± 0.00	0.09 ± 0.00
<b>YsAc (g/g Xyl)</b>	0.12 ± 0.01	0.07 ± 0.03	0.11 ± 0.01	0.17 ± 0.00
<b>YsXylitol (g/g Xyl)</b>	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.04 ± 0.00
<b>YsGly (g/g Xyl)</b>	0.02 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.02 ± 0.00
<b>Ethanol titer (g/L)*</b>	5.20 ± 4.19	4.67 ± 0.61	0.34 ± 0.04	2.08 ± 0.08
<b>Biomass (g/L)</b>	4.84 ± 0.92	5.2 ± 0.16	4.48 ± 0.42	4.39 ± 0.30
<b>YsPHB (mg PHB /g Xyl)</b>	5.49 ± 0.01	6.71 ± 0.76	7.60 ± 0.53	0.00 ± 0.00
<b>QsPHB (mg PHB/g Xyl · h)</b>	0.05 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.00 ± 0.00
<b>PHB titer (mg/L)</b>	226 ± 53	262.31 ± 17.87	217.43 ± 0.42	0.00 ± 0.00
<b>Final PHB content (%CDW)</b>	4.49 ± 0.49	4.47 ± 0.53	4.18 ± 0.64	0.00 ± 0.00
<b>Xylose/sugar consumed (%)</b>	74.04 ± 2.88	86.11 ± 5.71	59.81 ± 1.21	56.45 ± 1.36
<b>Reference</b>	This study	This study	This study	This study

\*Ethanol titers and yields obtained from the maximum value under aerobic conditions

\*\* TMB4425 was grown in 2 independent duplicates at different time point, but the final yields are comparable

\*\*\* Aerobic ethanol max peaks at time points not catch in the HPLC

**Table 5. Summary of TMB4425, TMB4496, TMB4497 and TMB4498 evaluated strains in duplicate and comparison of important data points. Ethanol and PHB titers are presented for the maximum obtained value.**

<b>Strain</b>	<b>TMB4425</b>	<b>TMB4496</b>	<b>TMB4497</b>	<b>TMB4498</b>
<b>Relevant genotype</b>	NADH-PHB; XRmut	$\Delta$ adh1; NADH-PHB; Xrmut	$\Delta$ adh1; ALD6; ACS; NADH-PHB; Xrmut	$\Delta$ adh1;eutE; NADH-PHB; Xrmut
<b>Cultivation time (h)</b>	161h**	161h	161h	161h
<b>Flask and media vol.</b>	SF 1L-0.1L media	SF 1L-0.1L media	SF 1L-0.1L media	SF 1L-0.1L media
<b>Growth condition</b>	Shake flask (Aerobic)	Shake flask (Aerobic)	Shake flask (Aerobic)	Shake flask (Aerobic)
<b>Growth rate (h-1)</b>	0.15 ± 0.01	0.15 ± 0.00	0.17 ± 0.00	0.15 ± 0.0
<b>Ysx (g/g Xyl)</b>	0.13 ± 0.01	0.14 ± 0.00	0.17 ± 0.00	0.2 ± 0.00
<b>YsEtOH (g/g Xyl)*</b>	0.16 ± 0.10***	0.09 ± 0.00	0.00 ± 0.00	0.04 ± 0.00
<b>YsAc (g/g Xyl)</b>	0.12 ± 0.01	0.13 ± 0.00	0.12 ± 0.00	0.18 ± 0.01
<b>YsXylitol (g/g Xyl)</b>	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00

<b>YsGly (g/g Xyl)</b>	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.02 ± 0.00
<b>Ethanol titer (g/L)*</b>	5.20 ± 4.19	3.382 ± 0.09	0.00 ± 0.00	0.92 ± 0.01
<b>Biomass (g/L)</b>	4.84 ± 0.92	5.35 ± 0.06	5.29 ± 0.04	5.09 ± 0.12
<b>YsPHB (mg PHB /g Xyl)</b>	5.49 ± 0.01	7.21 ± 0.36	3.95 ± 0.22	0.00 ± 0.00
<b>QsPHB (mg PHB/g Xyl · h)</b>	0.05 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.00 ± 0.00
<b>PHB titer (mg/L)</b>	226 ± 53	284.3 ± 7.09	124.99 ± 3.09	0.00 ± 0.00
<b>Final PHB content (%CDW)</b>	4.49 ± 0.49	5.31 ± 0.19	2.37 ± 0.04	0.00 ± 0.00
<b>Xylose/sugar consumed (%)</b>	74.04 ± 2.88	76.81 ± 1.48	60.67 ± 1.06	51.49 ± 0.71
<b>Reference</b>	This study	This study	This study	This study

\*Ethanol titers and yields obtained from the maximum value under aerobic conditions

\*\* TMB4425 was grown in 2 independent duplicates at different time point, but the final yields are comparable

\*\*\* Aerobic ethanol max peaks at time points not catch in the HPLC

### Shake flask cultivations at high xylose concentration

To study the maximum concentration of PHB that could be obtained, TMB4425 were cultivated in 150 g/L of xylose. In Figure 14 the generated data from the HPLC and OD<sub>620</sub> values are shown. The lag phase of strains growing at 150g/L of xylose was longer than the reference conditions at 50 g/L, 96 h in comparison to 15 h to reach an OD<sub>620</sub> of 0.3. At high xylose concentrations the maximum OD<sub>620</sub> of 35.2 was reached after 353 h. TMB 4425 was able to convert 50.3 % of the added xylose and accumulate 9.58 g/L of CDW. Highest acetate concentration was 2.77 g/L and PHB was 219.93 mg/L. No ethanol was detected throughout the cultivation. An interesting observation was the elevated glycerol concentrations reached in these conditions, 36.4 g/L compared with 0.9 g/L when 50 g/L of xylose is used, being increased by 40 times.

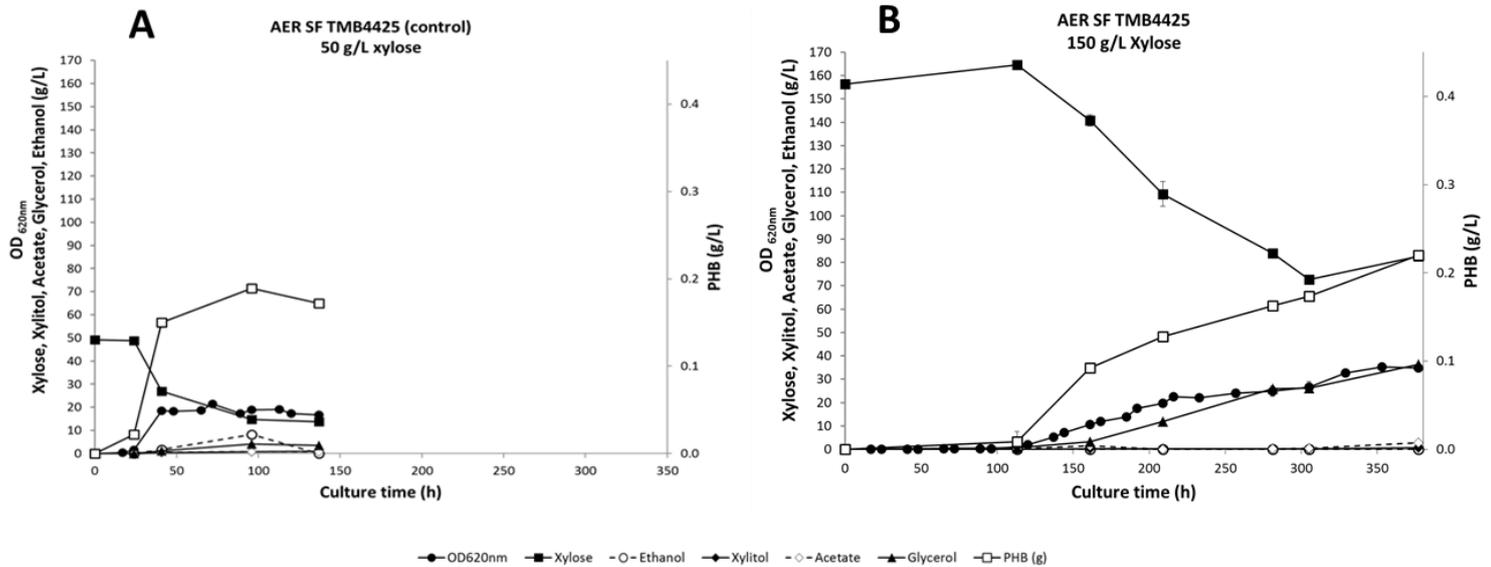


Figure 14. TMB 4425 was cultivated in 150 g/L of xylose to see which maximum concentration of PHB could be obtained without carbon source starvation. A. Control condition in 50 g/L xylose. B. Culture in 150 g/L xylose conditions. Graph is based on biological duplicates. After 281 h the graph is based on 1 culture due to low amounts of media.

## Discussion

The aim of this thesis was to metabolically engineer *S. cerevisiae* to improve the production of PHB by enhancing the cytosolic acetyl-CoA. Several strains were constructed with different combinations of genes. All strains were evaluated for aerobic growth using xylose as sole carbon source. This study presents candidate strains for further characterization in anaerobic conditions, but this was outside the scope of this master thesis project.

## Strain construction

The study was started with a first attempt to delete the *ADH1* by using the CRISPR/Cas9 system and the plasmid gRNA\_ADH1 that contains a specific target region for the *ADH1* CDS. The design of the construction was that the gene cassette of either *eutE* or *ALD6* should replace the *ADH1* by homologous recombination with the included 50 bp flanks of the gene cassettes. After several transformation attempts the results showed no positive clones suggesting the design of the construction was not properly done. Instead of reparation by external DNA cassettes for *eutE* and *ALD6* the gRNA\_ADH1 proved to be very inefficient and

generated only negative clones. What probably is happening in the genome is homologous recombination (HR) with the other alcohol dehydrogenases. The Cas9 protein induces a DSB in the *adh1* locus suggesting to be repaired by HR with the paralogues genes *ADH2* or *ADH5* due to high sequence similarity when analyzed by nBLAST. Despite that the gRNA was very specific in the cleaving site, the chosen homologous flanks of the cleaving sites showed far more identity to the paralogs *ADH2* and *ADH5* (up to 89 % identity) than the 50 bp flanks of the gene cassette and thus making it possible for the yeast to survive through recombination with homologous genes. This is confirmed in the control strain where no gene cassette was added and it was still able to survive in the same ratio, meaning that the negative control presented the same amount of CFU as the mutant. Another possible explanation could be that the target site for the DSB was mutated avoiding the recognition from the Cas9. Although this is most probably not the case since when running enzymatic assay for alcohol dehydrogenase activity, the result showed high enzymatic activity which cannot be derived from the other alcohol dehydrogenases, since *ADH1* is most active of all the paralogs (Ciriacy, 1975). When planning where to insert genes, several aspects have to be considered to eliminate homologous recombination within the host itself. It is key to target highly specific regions for the DSB that also should be surrounded by highly specific flanks. The current software used to identify target regions, DNA2.0 (Newark, California), cannot take this into consideration.

The next version of gRNA, gRNA\_ADH1p, was targeted to the promotor region of the *ADH1* since when running that full sequence in nBLAST, there was only one hit in the CEN.PK113-7D genome. This indicated high specificity and this sequence could therefore be used for designing a new gRNA. The sequence for the promotor region was added into the software

DNA2.0 (Newark, California) to find a specific cleaving site for the gRNA. When found, the sequence was double checked with nBLAST. The flanks were carefully selected to remove the full sequence of the *ADH1* and to avoid homologous recombination being able to occur on either side of the cut. As the results show, the new gRNA was highly specific and the *ADH1* was successfully deleted.

### Aerobic shake flask cultivations

The strain TMB4427 expresses a variant of the ACS that is ATP-dependent. Comparing TMB4427 to the control strain TMB4425 there was a reduction in maximum OD<sub>620</sub> that could be connected to the *acs*<sup>L641P</sup>, which suggests that the ATP-dependence of this enzyme potentially reduce the amount of ATP that could be used for growth and use it to function. However, TMB4427 present a higher biomass titer compared to the control strain TMB4425 which does not support the assumption of *acs*<sup>L641P</sup> stealing energy from growth. More xylose is consumed in TMB4427, 86.1 % compared to the control's 74 %, increasing the carbon uptake and directing it into PHB. Higher titer of PHB was observed, 262.31 mg/L compared to the control 188.97 mg/L. The yield of acetate is lower compared to control which correlate well with results demonstrated elsewhere where the ACS from *S. enterica* has been overexpressed in *S. cerevisiae* (Shiba et al., 2007). The normally produced amount of acetate is reduced due to the overexpression of ACS that converts it into acetyl-CoA. This strain also shows a lower maximum ethanol titers compared to the control strain. This could indicate that carbon is pulled from the common precursor acetaldehyde towards acetyl-CoA production due to mentioned genetic manipulation.

Looking at the strain TMB4494, which carries an over-expressed extra copy of *ALD6*, it was assumed that this alteration would push the carbons further towards PHB production, like demonstrated by Kocharin *et al.* (2012). Compared to the control and TMB4427, TMB4494

presents a higher productivity and yield of PHB but gives a lower titer compared to TMB4427 at the end of cultivation. This is because TMB4494 has a lower biomass at the end of cultivation compared to the other two strains. Based on the productivity and yield the upregulation of *ALD6* in combination with *acs*<sup>L641P</sup> has really improved specific PHB production. TMB4494 shows a higher yield of acetate, comparable to the control. This indicates that the upregulation of *ALD6* makes the acetate accumulate at a pace that the downstream *acs*<sup>L641P</sup> flux cannot match. Looking at the ethanol yield, which is low compared to TMB4427, also confirms that the upregulated *ALD6* is pulling carbon from the precursor molecule acetaldehyde into acetate as it was stated in previous studies (Shiba et al., 2007).

To bypass the ATP-dependent pathway from acetaldehyde to acetyl-CoA where 2 ATP equivalent molecules are consumed, a heterologous acetylating acetaldehyde dehydrogenase (*eutE*) was expressed into TMB4425. The constructed strain TMB4495 was evaluated for aerobic growth on xylose. Unexpectedly the results show no production of PHB. This might be due to the kinetics of *eutE* is reversible. The intended direction from acetaldehyde from acetyl-CoA was proven in this study to work by the enzymatic assays, where acetaldehyde, CoA and NAD<sup>+</sup> were used as substrates. Despite this, Medina and coworkers (2010) show in their study that the reverse direction from acetyl-CoA into acetaldehyde is possible too. Since TMB4495 still has the homogenous *Acs1* and *Acs2*, which under these circumstances are less up-regulated due to growth on xylose, that convert acetate into acetyl-CoA (Shiba et al., 2007). Since this conversion is ATP-dependent it is not really reversible. To explain the outcome of the result there are strong indications that the acetylating acetaldehyde dehydrogenase (A-ALD) *in vivo* works in reverse in TMB4495, this would mean that the amounts of acetyl-CoA synthesized by homogenous *Acs1* and *Acs2*

could be converted into acetaldehyde by the activity of A-ALD. The elevated amounts of acetaldehyde would then push the carbon towards both ethanol and acetate production correlated with the results where the yields of ethanol and acetate are both elevated in TMB4495 compared to the control. A recent publication shows that growth on acetate and glucose for the production of ethanol when introducing *eutE* was proven to increase ethanol yields and thus supports the assumption of this gene working in reverse (Papapetridis et al., 2016). If this is the case, it seems that the  $\beta$ -ketoacyl-CoA thiolase (*phaA*), the first enzyme involved in the PHB production from acetyl-CoA, cannot compete with the *eutE* when it comes to acetyl-CoA binding. Other groups have proved the conversion of acetaldehyde into acetyl-CoA to work when the homogenous genes are still present but this does not work in our case (Kozak et al., 2014, Song et al., 2016). What separates those studies from this is the carbon source. They have performed all cultivations in glucose whilst we have performed them in xylose. Using xylose as carbon source might change the distribution and amounts of precursors in the glycolytic pathway, which is highly glucose regulated, and this in turn might affect the heterologous A-ALD that previously haven't been reported. Another possible explanation for the enzyme not behaving like it is supposed to could be due to lack of adaptation of the gene. It has been proven by Kozak *et al.* (2016) that strains carrying the *eutE* through adaptation and induced evolution perform point mutations in the gene to make it function better for growth on ethanol as carbon source. Maybe this is an approach to be considered to activate and possibly improve the function of *eutE* when xylose is used as a carbon source (Kozak et al., 2016).

To further push the carbon towards PHB production next step in the genetic manipulations was to delete alcohol dehydrogenase 1 (*ADH1*), which is reported to have the highest

activity of all the alcohol dehydrogenases. The strains were further manipulated and the *adh1* gene was disrupted. It has previously been reported that growth is reduced when the carbon source is glucose for strains lacking alcohol dehydrogenase 1 (Song et al., 2016). This was also observed in this study (data not shown). When grown on xylose though,  $\Delta adh1$  strains showed no sign of growth retardation. There was no long lag-phase or major changes in the growth because there was no glucose regulation of the glycolytic pathway. TMB4496 showed similar growth to the background strain TMB4425. This strain shows the best PHB titer of 284.37 g/L and has a productivity of 7.21 (mg PHB/g Xylose), which is similar to TMB4494. It has a higher conversion of xylose and only a slight production of ethanol. The observed ethanol production could come from any of the other alcohol dehydrogenases that have lower activity (Heick et al., 1969). As can be seen in the results, there is a high yield of acetate. This is probably because the *adh1* deleted and the acetaldehyde needs to be converted into acetate by *ALD6*. The bottleneck is at the homogenous *Acs1* and *Acs2* that lead to acetate accumulation.

TMB4494 was further genetically manipulated and the *adh1* was deleted to yield the strain TMB4497. Following the same reasoning as for TMB4494 it was assumed that the *adh1* deletion would further improve the flux towards PHB, since no acetaldehyde would be pulled towards ethanol, in TMB4497. As can be seen in the results, this strain shows less PHB productivity and titers compared to the control strain TMB4425. No ethanol was formed but it accumulates more acetate. Since the flux of acetaldehyde is completely directed towards acetyl-CoA the upregulated *ALD6* cannot match this flux and acetaldehyde will accumulate. Acetaldehyde is toxic to the cell and can thus influence growth and viability of the cells (Kozak et al., 2014). Since PHB is stored intracellularly the amount of PHB is dependent on

the amount of cells, thus influencing the final PHB concentration. TMB4494 showed a poor growth compared to the already mentioned strains and this could be the reason for the low PHB titer and PHB yield.

Strain TMB4498, is based on TMB4495 with the *adh1* deleted. As the result show, no PHB is produced by this strain either since it is A-ALD based and it is probably with the same arguments as already mentioned above. When the alcohol dehydrogenase is deleted the acetyl-CoA that is sent backwards into acetaldehyde cannot be converted into ethanol in the same extent. Only a low titer of ethanol was observed that residual alcohol dehydrogenases contribute with. Comparing TMB4498 to TMB4496 where only the *eutE* is the difference, there is an increase in acetate yield, decrease in ethanol yield and lower xylose consumption in TMB4498. The residual consumed carbon is probably accumulated as acetaldehyde that is hazardous for the cells. This was also observed by another group that constructed an *adh1* null mutation in *S. cerevisiae* for lactic acid production grown on glucose (Song et al., 2016). Since  $\Delta adh1$  strains were observed to have a good growth on xylose, what is most probably affecting their maximum  $OD_{620}$  in both TMB4497 and TMB4498 is the accumulating acetaldehyde. Kozak *et al.* (2013) that constructed a  $\Delta ald \Delta acs$  strain with the *eutE* also saw an accumulation of acetaldehyde, due to the kinetics of the enzyme. This caused the cells to aggregate and loose some integrity of the plasma membrane. Observation mentioned from both these groups can be good explanations for the analyzed behaves of TMB4497 and TMB4498.

None of the evaluated strains consumed all the added xylose and this is because of the pH in the shake flasks decreased to values between 3.18-3.3 (data not shown) inhibiting growth

and carbon consumption. Only when the pH is kept at optimal level (pH 5.5) as in bioreactors, the xylose can be fully consumed.

#### Shake flask cultivations in 150 g/L Xylose

The purpose of cultivating TMB 4425 in an excess of xylose was to investigate if the normal conditions of cultivation, 50 g/L of xylose, was optimal for PHB production or if the yield could be increased if there were no carbon source limitation. As the results show, the produced amount of PHB is only slightly increased in these new conditions. What can be concluded from the results though is that the cells are stressed in this condition. The long lag-phase of 96 h shows that the cells need to adjust their metabolism to handle the high osmolarity in the media to prevent lysing (Dihazi et al., 2004). The intended carbon source excess aimed towards PHB production was rather directed towards glycerol production to cope with the high osmolarity. If looking at the actual amount of xylose consumed in mass, it is higher despite the conversion percentage only is 50.3%. To bare in mind is that the only thing differentiating high xylose conditions from normal cultivation condition is the addition of three times more xylose. Nevertheless, the aim of producing more PHB was not achieved since most consumed xylose went into glycerol and biomass production. The obtained titer of 219.93 mg/L PHB is only compared to when the same strain is cultivated in 50 g/L of xylose. This shows that 50 g/L xylose is a more optimal condition for the cells to produce PHB.

This concludes that it is not necessary to increase the concentration of xylose in the cultivation media in the aspect of PHB production. Since PHB is stored intracellularly it is correlated to the amount of biomass. This experiment clearly shows that despite more carbon source and a higher obtained cell mass, the yields of PHB could not be increased. If it is connected to the cells being more stressed in high xylose and thus down prioritizes PHB

production or if we have actually reached maximum possible concentration of PHB is hard to tell.

Worth to mention is that all precultures were cultivated in 50 g/L making the transition into 150 g/L of xylose a shock and that is the reason for the long lag-phase. What would be interesting to see is if the cells were adapted and grown in 150 g/L in the preculture, if the behavior would change when cultivated in shake flask

### Future work

A lot of interesting information has been revealed in this project but there is still more work to be done. What will be interesting to see is how these strains behave under anaerobic conditions. Since ethanol is not a highly prioritized metabolite in aerobic conditions it will be interesting to see how the *adh1* null strains behave in that condition. The natural response for *S. cerevisiae* during anaerobic conditions is to produce glycerol to restore redox balance. Now that there is a reduced ethanol production, how will the cells cope with that and will there be any difference in metabolite composition? Will they produce more or less PHB? What would be most interesting to see is what happens with the kinetics in the *eutE* strains. Maybe anaerobic conditions could up-regulate the enzyme to go from acetaldehyde into acetyl-CoA due to changed conditions and regulations in the cell.

### Conclusion

To conclude, the aim of optimizing already PHB producing strains were met. TMB4494 (*acs*<sup>L641P</sup> and *ALD6*) and TMB4496 ( $\Delta$ *adh1*) showed both higher titers and PHB yields. The best candidate of those is TMB4496 since it grows similarly to the control strain as well as presents a high PHB yield and titer. What also can be concluded is that in aerobic conditions, there is no need to increase xylose concentration in cultivations as it will only stress the cells

to produce glycerol to cope with the high osmolarity and not contribute with significantly higher PHB titers.

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