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DIPLOMA THESIS

Screening of a Construct Library and Characterization of Cultivation Conditions for Isobutanol Production in *E. coli*

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2 Declaration of Authorship

I hereby declare that I am the sole author of this work. No assistance other than that which is permitted has been used. Ideas and quotes taken directly or indirectly from other sources are identified as such. This written work has not yet been submitted in any part.

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Signature Date

3 Abstract

Climate change is a serious topic, which is intensely discussed currently. Everybody is talking about environment and sustainability. The quest for carbon-neutrality is higher than ever before. Subsequently, the Bio ABC-project deals with removing carbon dioxide from industrial off-gas streams and converting it into a powerful, gasoline-like fuel to drive combustion engines.

This work deals with the very last part of this project, the stage of converting isobutanol from glucose in *E. coli*. At a later stage, the plan is to use acetate as substrate, which is produced by a CO_2-fixating organism. The whole isobutanol pathway, consisting of five genes had been transformed into *E. coli* with the GoldenMOCS-technique, because isobutanol is not a native metabolite. Only three of these five genes are native to this bacterium. Each is separately driven by promotors from the Anderson-library. Four E. coli strains serve as hosts: the wild type, the adapted wild type and two different knockout strains.

During this study the strain and construct screening were performed under different conditions in serum bottles and bioreactors. The processes were driven as continuous chemostat processes. By varying the aeration and temperature the most promising candidate was detected.

The behaviour of the clones was similar, the lower the oxygen amount in the in-gas stream, the higher the isobutanol production, but the lower the cell density. For this reason, driving a chemostat is a balancing act. In serum bottles, it was noticeable that most isobutanol was produced after the growth phase at microaerobic conditions.

Also, different temperatures, 30°C and 37°C were tested. This topic needs further analysis, because the results were not clear. One experiment showed a higher production of isobutanol at 30°C while there was no alteration in another experiment at all.

In serum bottles the most promising candidate was the knockout strain with ΔldhA, ΔadhE, Δpta, ΔfrdA and ΔwpykA. It grew as fast as the wild type and produced as much isobutanol as the other knockout strain, which grew slower. The chemostat process, where both knockout strains were compared, must be repeated due to mutations of the clones during the process.

4 Abstract (German)

Der Klimawandel ist ein ernstes Thema, das zurzeit in aller Munde ist. Das Streben nach CO_2-Neutralität ist größer als jemals zuvor. Deshalb beschäftigt sich das Bio ABC-Projekt mit der Entfernung des Kohlendioxids aus industriellen Abgasen, um es dann zu Treibstoff umzuwandeln.

Diese Arbeit behandelt den letzten Teil des Prozesses, der Produktion von Isobutanol aus Glukose in *E. coli*. Zu einem späteren Zeitpunkt soll Acetat als Substrat dienen, welches von einem CO_2-fixierendem Organismus erzeugt wurde. Da Isobutanol kein natürlicher Metabolit von *E. coli* ist, wurde der ganze Isobutanolstoffwechselweg, bestehend aus fünf Genen, mittels GoldenMOCS transformiert. Nur drei dieser fünf Gene kommen natürlich in diesem Bakterium vor. Jedes Einzelne wird von einem Promotor der Anderson Library gesteuert. Als Hostzellen dienen vier *E. coli*-Stämme: Wildtyp, adaptierter Wildtyp und zwei verschiedene Knockoutstämme.

In dieser Studie wurde das Stamm- und Konstruktscreening unter verschiedenen Bedingungen in Serumflaschen und Bioreaktoren durchgeführt. Der Prozess wurde als kontinuierlicher Chemostatprozess durchgeführt. Durch Veränderung der Begasung und der Temperatur wurde der vielversprechendste Kandidat gefunden.

Das Verhalten der Klone war ähnlich. Je geringer der Sauerstoffgehalt im Ingas-Strom war, desto besser war die Isobutanolproduktion, aber desto niedriger war die Zelldichte. Aus diesem Grund ist die Steuerung eines Chemostats eine Gratwanderung. In Serumflaschen wurde das meiste Isobutanol nach der Wachstumsphase unter mikroaerophilen Bedingungen produziert.

Es wurden auch verschiedene Temperaturen, 30°C und 37°C, getestet. Um eine konkrete Aussage machen zu können, müssen weitere Versuche durchgeführt werden, da bei einem Experiment deutlich mehr Isobutanol bei 30°C produziert wurde, und bei einem anderen kein Unterschied erkennbar war.

Der vielversprechendste Kandidat in Serumflaschen war der Knockoutstamm mit ΔldhA, ΔadhE, Δpta, ΔfrdA und ΔwpykA. Er wuchs so schnell wie der Wildtyp und produzierte so viel Isobutanol wie der andere Knockoutstamm, der langsamer wuchs. Aufgrund von Mutationen während des Prozesses, muss der Chemostatprozess, bei dem die beiden Knockoutstämme verglichen wurden, wiederholt werden.

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8 List of Abbreviations

ack	cytoplasmic tyrosine kinase
acrA	aerobic respiration control protein gene
ADH	alcohol dehydrogenase
Adh2	alcohol dehydrogenase 2
AdhA	alcohol dehydrogenase A
alsS	acetolactate synthase
ArcA	aerobic respiration control protein
AD/AW	adapted *E. coli* wild type
BB	backbone
cAMP	cyclic adenosine monophosphate
CDW	cell dry weight
cfa	cyclopropane fatty acid gene
Crp	cAMP receptor protein
D	dilution rate [h^{-1}]
E. coli	*Escherichia coli*
Fur	ferric uptake regulation protein
gatY	tagatose-bisphosphate aldolase
Glc	glucose
GOI	gene of interest
GoldenMOCS	Golden Gate derived Multiple Organism Cloning System
groESL	chaperonin
hsdSB	hydroxysteroid beta dehydrogenase
HPLC	high-performance liquid chromatography
ilvC	ketol-acid reductoisomerase
ilvD	dihydroxy-acid dehydratase
KOW4	*E. coli* wild type with four knockouts
KOW5	*E. coli* wild type with five knockouts
LB	lysogeny broth
KDC	2-keto-acid decarboxylase
kivD	= kdcA
lon	lon protease
MoClo	modular cloning system
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
OD_{600}	optical density at 600 nm
OmpT	protease 7
nt	nucleotides
PhoB	phosphate regulon transcriptional regulatory protein
PntAB	pyridine nucleotide transhydrogenase
pox	pyruvate oxydase
pta	phosphate acetyltransferase
Q/QH_2	Ubiquinon/Ubiquinol
recA	DNA repair protein
SOC	Super Optimal Broth with catabolite repression
TCA	tricarboxylic acid cycle
tnaA	tryptophanase
vvm	volume of gas per volume of media per minute
W	*E. coli* wild type
yqhD	Alcohol dehydrogenase

9 List of Symbols

Symbol	Definition	Unit
c_i	concentration of component i	mol L^{-1} or g L^{-1}
V_i	volume of i	mL or L
m_i	mass of component i	g
μ	growth rate	h^{-1}
X	biomass	g
t	time	h
D	dilution rate	h^{-1}
F	flow	L h^{-1}
x	concentration of biomass	g L^{-1}
M_i	molar mass of component i	G mol^{-1}
N_c	amount of carbon atoms of one molecule of component i	-
ex_{H_2O}	Ratio of vapor content in the off-gas stream	%
$y_{O_2}^{wet}$	Content of oxygen in the off-gas stream without cells	%
y_i^{in}	Content of component i in the in-gas strem	%
y_i^{off}	Content of component i in the off-gas strem	%
Ra_{inert}	Inert gas ratio	-
$\dot{F}$	Total gas flow	sL h^{-1}
$\dot{V}_i$	Gas stream of component i	sL h^{-1}
n_i	Molar amount of component i	mol
$\dot{n}_i$	Molar amount flow of component i	mol h^{-1}
$\dot{n}_{C_i}$	Cmol flow of component i	Cmol h^{-1}
p	Pressure	Pa
r	production	Cmol L^{-1} h^{-1}
q	specific production	Cmol $Cmol^{-1}$ h^{-1}
R	Gas constant	J mol^{-1} K^{-1}
T	Temperature	K
$Y_{i/j}$	Yield of component i to j	Cmol $Cmol^{-1}$

10 Introduction

10.1 Bio-ABC project

Since the 1950s the climate has dramatically changed because of the industrialisation and the growth of greenhouse gas emissions associated with it (Pachauri and Mayer 2015). As an idea to counteract that problem, the Bio-ABC-project was brought to life. For that, a two-step biotechnological process is being developed. The goal of this project is the fixation of CO_2 from real flue gas streams of industrial processes in the first step. It is performed by *Acetobacterium woodii*. The anaerobic bacteria fixate CO_2 by co-utilisation of H_2. Acetate acts as a metabolic intermediate. In the second step, acetate is taken up by *Escherichia coli* and is metabolized to a fuel alcohol (Novak et al. 2018). In Addition to the acetate, biological waste is also processed. CO_2 which is emitted during fermentation can be recycled as feed again (seen in Figure 1).

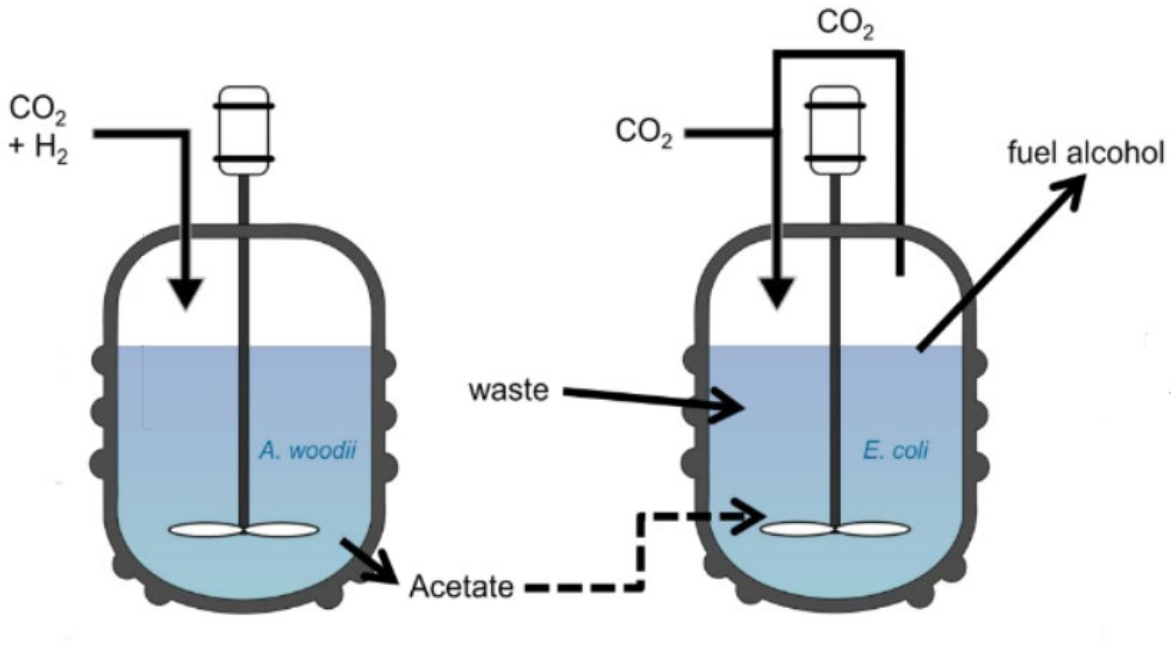

Figure 1 Graphical overview of the Bio-ABC project

(https://www.vt.tuwien.ac.at/biochemical_engineering/bioprocess_technology/projekte/aktuelle_projekte/bio_abc_development_of_a_two_step_biological_co2_fixing_process_for_the_production_of_fuel_chemicals/)

10.2 Environment

Climate change is a topic that concerns the whole planet. The human influence is the highest in history due to the enormous amounts of emissions of greenhouse gases. The changes with the most impact are the warming up of the atmosphere and ocean, the continuous decrease of snow and ice and the rise of the sea level (Pachauri and Mayer 2015).

The most popular problem is the rising temperature of the atmosphere, but it absorbs only 1% of the heat energy. 90% of the energy is absorbed by the ocean (Pachauri and Mayer 2015).

Because of the existing equilibrium of the atmosphere and the ocean, the rising CO_2-level in the atmosphere leads to a rising CO_2-level in the ocean. This phenomenon effects a drop of pH-value of the water. Due to this acidification, the solubility of $CaCO_3$ decreases. This leads to degradation of seabed substance and therefore to an increase of CO_2, which causes a decrease in the pH-value (Caldeira K 2003). Because of the enhanced solubility of CO_2 in cold water, this effect appears especially in colder areas of the sea (Doney et al. 2009).

The rising temperature causes the melting of ice sheets and massive glaciers in Greenland and Antarctica resulting in a rising sea level. Its current rate is higher than the average rate in the last two thousand years (Pachauri and Mayer 2015).

Nowadays, extreme weather and climate events are becoming more and more frequent. Cold periods are getting rare and the number of warm periods is increasing. This leads to a destruction of the fragile climate system. The frequency of heat waves, droughts, floods, cyclones and wildfires are rising. Further emission of greenhouse gases, such as CO_2, will cause more and more problems (Pachauri and Mayer 2015).

10.3 Biofuels

Biofuels from renewable sources could solve both problems, the increasing emission of greenhouse gas and the shrinking deposit of fossil fuels (Zhou and Thomson 2009). There is a large variety of biofuels. One of the most common biofuels is biodiesel. This term describes diesel which is produced from vegetable oil, animal fats or cooking fat. For the production of biodiesel, the fatty acids are transformed to fatty acid methyl esters. Glycerine emerges as a by-product (Dürre 2007).

10.3.1 Generations of biofuels

Bioethanol is another popular biofuel. It is mainly produced by yeast during cultivation of raw materials containing sugar (Dürre 2007). These sugar-rich raw materials can be made directly from crops like cane, sweet sorghum juice or molasses. Other sources are starch-rich farming products like corn, cassava, potatoes or root crops. Starch-rich raw materials need to be hydrolysed before they can be converted by yeast. All these feedstocks compete with food production. Cultivation of these plants needs more farmland, which has a bad effect on biodiversity and deforestation. If biofuels are produced in the described ways, they are called first-generation biofuels. In order to avoid negative side effects there is a strong demand for new technologies for biofuel production. New processes generate second-generation biofuels from lignocellulosic biomass as feedstock. Agricultural residues, wood, paper and energy crops, like miscanthus or switchgrass, can serve as raw material for non-food biofuels. To be competitive, there are still a few hurdles to overcome, such as developing a cost-efficient pre-treatment technology to achieve efficient depolymerization of cellulose and hemicellulose to generate fermentation compatible sugars (Saini et al. 2015).

10.3.2 Biofuels by fermentation

The microbial fermentation to generate biofuels is becoming more interesting than ever. There are some requirements that have to be met for the production of biofuels. The processes should be fast, cheap with high productivity and as non-toxic as possible (Koppolu and Vasigala 2016; Sheridan 2009). Due to the high costs of inducers they are often avoided to design a cheap process (Akita et al. 2015). The usage of alcohols that have been processed efficiently by using metabolically engineered microorganisms, presents another opportunity (Yong Jun Choi, Joungmin Lee, Yu-Sin Jang, Sang Yup Lee 2014). However, it is difficult to be competitive in this sector. Several requirements must be fulfilled by industrial fermentation. The theoretical yield should be around 95 %, approximately 100 g/L product should be achieved with a productivity of 2 g L^{-1} h^{-1} (Sheridan 2009).

Butanol is one of the first fermentative produced biofuels by using *Clostridium acetobutylicum* (Jones D. and Woods D. 1986). Nowadays, biobutanol can be competitive to chemical produced butanol. Among acetone and ethanol, butanol is produced at the ABE-fermentation (Green 2011).

E. coli is a well-known organism and offers a broad range of opportunities to produce biofuels by metabolic engineering (Koppolu and Vasigala 2016).

10.3.3 Bioethanol vs. higher alcohols

Bioethanol is often mentioned in terms of biofuels, but it only acts as an additive for petrol because it brings along some disadvantages (Koppolu and Vasigala 2016). Compared to other biofuels, bioethanol must be blended for usage. This is possible up to 85 %. The high vapor pressure is a big issue for storage and distribution. At that reason blending must occur shortly before use (Dürre 2007). Higher alcohols, C4 and bigger, are less volatile (Atsumi et al. 2008b). The low energy density represents another problem of ethanol. Because of its high hygroscopicity it leads to corrosiveness. There are other biofuels, such as biobutanol which has better properties (Dürre 2007; Felpeto-Santero et al. 2015; Koppolu and Vasigala 2016; Lamsen and Atsumi 2012). However, most higher-chain alcohols are not economically produced by natural organisms. n-Butanol is an exception (Lamsen and Atsumi 2012). Isobutanol is a similar biofuel that shares the same advantages (Koppolu and Vasigala 2016). Isobutanol, as a brunched-chain alcohol, has a higher octane number compared to butanol, as a straight-chain molecule (Atsumi et al. 2008b).

10.4 E. coli

E. coli is a well-known microorganism, which offers a wide range of applications. Its genome and metabolism are well-studied. Therefore, it presents an excellent candidate for metabolic engineering to produce biofuels. It is possible to use parts of natural pathways to create new pathways for production (Koppolu and Vasigala 2016). For industrial scale production, a robust organism is necessary (Sheridan 2009).

For several decades, ethanol, lactate, succinate and acetate have been generated by engineered *E. coli. E. coli* can act in aerobic or anaerobic environment. Under fermentation conditions with or without oxygen, different metabolic routes can be activated over its regulatory machinery (Förster and Gescher 2014). For this reason it is the organism of choice for recombinant protein production (Rosano and Ceccarelli 2014). Another big advantage of *E. coli* is its high growth rate. It doubles up to 3 times an hour (Sezonov et al. 2007). Additionally, high biomass concentrations can be achieved. In fed-batch processes cell densities up to 200 g/L CDM were obtained (Lee 1996).

E. coli is suitable for metabolic engineering for the production of Isobutanol (Koppolu and Vasigala 2016). The last two steps of the Ehrlich pathway can utilise a broad range of substrates and is compatible to 2-keto acid degradation from other organisms (Atsumi et al. 2008b).

10.4.1 Strains

Thousands of strains of *E. coli* have been isolated and can be suitable for different experiments. Each candidate brings along advantages and disadvantages (Rosano and Ceccarelli 2014). There are only five of them related to Risk Group 1 organisms and therefore classified as safe. These five strains are B, C, Crooks, K12 and W. Each has different approaches (Archer et al. 2011). BL21(DE3), a B-strain, and derivations of the K-12 lineage are often used for the first screening (Rosano and Ceccarelli 2014).

10.4.1.1 W-strain

The *E. coli* W-strain was discovered in the soil of a cemetery by Selman A. Waksman in 1943. The term W is derived from Waksman and is also called ‘Waksman’s strain’, because it has the highest streptomycin sensitivity in his *E. coli* strain collection. Waksman and Alan Schatz discovered streptomycin together around the same time (Archer et al. 2011).

This strain has many advantages which makes it very attractive for industrial applications. It has no effect on the overflow metabolism (Archer et al. 2011). In other words, it does not produce much acetic acid under high carbon source concentrations. That property enhances the yield (Wolfe 2005). Additionally, high cell densities can be achieved during fed-batch cultivations. *E. coli* W is very resistant to environmental stress factors like low pH-values, high temperatures, osmotic pressure or high ethanol concentrations. Compared to other safe strains, W exhibits a superior growth rate. Due to the combination of these advantages Waksman’s strain is very promising for production (Archer et al. 2011). *E. coli* is able to utilize a broad spectrum of substrate (Förster and Gescher 2014), but no other safe strain can convert sucrose as carbon source. There is no difference between growing on sucrose or on glucose. This is a competitive advantage due to the fact that, on an industrial level, sucrose from sugar cane is the preferred carbon source compared to glucose from starch (Archer et al. 2011).

10.4.1.2 B-strain

An ancestor of *E. coli* B appeared in studies of d'Herelle from the Institut Pasteur in Paris in 1918 the first time. He used it for his studies of bacteriophages. From there the strain was developed and spread around the world (Daegelen et al. 2009). *E. coli* B is a classical, well-studied lab strain. REL606 and BL21(DE) are sequenced by (Jeong et al. 2009). REL606 is commonly used for long term evolution experiments. BL21(DE) is a very popular strain concerning recombinant protein expression (Jeong et al. 2009), because it lacks Lon protease, which is responsible for the degradation of foreign proteins. Even extracellular proteins are safe due to the missing outer membrane protease OmpT. Another advantage is the enhanced prevention of plasmid loss because of *hsdSB* mutation. Thus, the DNA methylation and degradation are impaired. For expression under the T7 promotor the BL21(DE3) strain is engineered by inserting the λDE3 prophage, which includes the T7 RNAP, in BL21 (Rosano and Ceccarelli 2014).

10.4.1.3 K12-strain

E. coli K-12 are famous strains for classical experiments (Jeong et al. 2009). The original K-12 strain was isolated from the faeces of a convalescent diphtheria patient in 1922 (Bachmann 1996; Daegelen et al. 2009). These strains are not only used for cloning, but also for protein expression. The K-12 strains AD494 and Origami are thioredoxin reductase mutants. Thus, disulfide bonds in the cytoplasm are strengthened. For stabil plasmids HMS174, a *recA* mutant was created. Each of these strains can be used under the T7 RNAP system because of their *λDE3* (Rosano and Ceccarelli 2014). DH10B is a suitable host for large plasmids due to its lack of recA by engineering the genome. As a result the homologous recombination system is inhibited (Durfee et al. 2008).

10.5 Isobutanol

Usually, Isobutanol is used as solvent and additive for paint, industrial cleaners, paint removers and as an ink ingredient (Hongjuan Liu, Genyu Wang and Jianan Zhang 2013).

It is an isomer of n-butanol and like n-butanol much more suitable as biofuel than ethanol, because it is not hydroscopic and therefore less corrosive. Other advantages are the higher energy and blending capability in contrast to ethanol (Atsumi et al. 2008a; Dürre 2007; Koppolu and Vasigala 2016).

10.5.1 Isobutanol vs. n-butanol

One advantage of isobutanol using the 2-keto acid pathway over commonly n-butanol production is the compatibility of its pathway to many organisms. The required enzymes for production are easily available, which facilitates the metabolic engineering of the host (Chen and Liao 2016).

10.5.2 Toxicity

E. coli is not resistant to isobutanol. At a concentration of 8 g/L it is toxic to the organism (Atsumi et al. 2008b; Koppolu and Vasigala 2016). Some mutants of *E. coli* are able to grow in suspension with up to 2 % of isobutanol (Atsumi et al. 2008b).

The toxicity of isobutanol to *E. coli* is a complex issue. To reach a tolerance, various working points are required (Atsumi et al. 2010b; Rutherford et al. 2010). The influence of isobutanol and n-butanol are similar to the cell. There is only one significant difference in the stronger repression of amino acid syntheses by n-butanol. However, the effect of ethanol response differs to both (Brynildsen and Liao 2009).

Isobutanol has an impact on quinone-membrane interaction. This failure of the function of the membrane-bound Q/QH_2 causes respiratory distress and activates *ArcA*, *Fur* and *PhoB*. This leads to a decreased function of enzymes using quinones as electron carrier, caused by decreased diffusivity, weak anchoring and leakage into the cytoplasm. However, due to the complexity a significant increase of tolerance could not be shown by knocking out *ArcA*, *Fur* and *PhoB* by Brynildsen and Liao (Brynildsen and Liao 2009). In contrast Atsumi et al. (2010b) reported higher tolerance to isobutanol by deleting *yhbJ*, *acrA*, *marCRAB*, *tnaA* and *gatY*.

Atsumi et al. (2010b) suggested, that the influence of isobutanol to *E. coli* is different in the growth phase and the stationary phase. Experiments showed that the growth is inhibited due to an isobutanol concentration greater than 6-8 g/L. Nevertheless, the isobutanol production is only 20 % of the total in the growth phase. Most product is gained in the stationary phase. That means that the growth rate is not a good base for prediction of the total isobutanol production (Atsumi et al. 2010b; Chen and Liao 2016). Similar results were archived by Baez et al. (2011). It was observed that a *groESL* overexpressed *Clostridium acetobutylicum* strain showed higher n-butanol tolerance with an enhanced final titer (Tomas et al. 2003). An improved growth was published by Zingaro and Terry Papoutsakis (2013). They performed an overexpression of *groESL* in *E. coli* and detected a 3.5-fold increase of viable growth under 1% (v/v) n-butanol in media in contrast to strains without *groESL* overexpression. The increase of viability by adding 1% (v/v) isobutanol was even 9-fold.

10.5.3 Tolerance

For being competitive to common fuels, high titers of biofuels must be achieved by fermentation. Due to the toxicity of most of them, microorganisms have to be engineered to be more tolerant (Jarboe et al. 2010). Unfortunately, the tolerance to isobutanol cannot be enhanced dramatically by simple steps like inserting or knocking out genes. The toxicity of higher alcohols is very complex including a couple of genes (Minty et al. 2011; Reyes et al. 2013).

Kanno et al. (2013) have determined the morphologic change of the properties of the cell extracellular capsule of several microorganisms under growing in the presence of 2.0 % (v/v) butanol. The thickness doubled by treating with such a high butanol concentration. There is also an impact to the stability and integrity of the cytoplasmic membrane. Solvent-tolerant microorganisms are able to adjust their membrane lipid composition to reduce solvent permeability and re-establish the membrane fluidity.

Furthermore, this report showed that the insertion of *cfa* leads to an enhanced tolerance of *E. coli* to butanol and isobutanol. CFA synthase requires no energy or carbon for modifying unsaturated fatty acids in the membrane. Thus, there is no competition between solvent tolerance and production (Kanno et al. 2013).

As shown in the report of Reyes et al. (2013) the tolerance to butanol can be antagonistic to the tolerance to isobutanol. But it is also possible that they correlate.

It was observed that higher temperature during growth leads to a decreased alcohol tolerance of *Zymomonas mobilis*. If *E. coli* behaves similarly, the cultivation should be performed at lower temperatures to lower the toxicity of alcohols to the microorganisms (Baez et al. 2011).

10.5.4 Fermentation

Different kinds of feed can be used for a successful isobutanol fermentation. Among glucose (Akita et al. 2015; Atsumi et al. 2008b; Felpeto-Santero et al. 2015) in literature it was shown that isobutanol can be produced from xylose (Akita et al. 2015; Felpeto-Santero et al. 2015), lignocellulose hydrolysate (Akita et al. 2015), sucrose (Felpeto-Santero et al. 2015), amino acids (Huo et al. 2011) and CO_2 (Li and Liao 2013).

Bioethanol has been processed from lignocellulose, but complex pre-treatment must be done beforehand. Due to the longer pathway for isobutanol, lignocellulose is a challenging feedstock for industrial fermentation (Gholamreza Salehi Jouzani and Mohammad J. Taherzadeh 2015). Another feasibility for producing biofuels is directly from CO_2 by photosynthetic or litho-autotrophic microorganisms (Li and Liao 2013). Waste protein from animal wastes or fermentation residuals could be converted to keto acids, which serves as substrate for the desired bio-alcohol. The challenge for this feedstock is the hydrolysation to amino acids and the deamination for the keto acid production (Huo et al. 2011).

In a publication of Atsumi et al. (2008b), it was reported, that a titer of 22 g/L isobutanol was achieved with the pathway *alsS*, *ilvC*, *ilcD*, *kivd* and *adh2* under reduced by-product and enhanced pyruvate production, due to delete *adhE*, *ldhA*, *frdAB*, *fnr*, *pta* and *pflB* under micro-aerobic conditions. By adding 0,5 % yeast extract 86 % of the theoretical yield was reached (Atsumi et al. 2008b), which makes it competitive to the production of 1-butanol in *Clostridium* (Jones D. and Woods D. 1986).

Akita et al. (2015) determined a higher content of isobutanol at 32°C than at 37°C. They published a pH optimum at 6.5. Also Baez et al. (2011) observed higher titers at lower temperatures. They published a bioreactor experiment where higher amounts of isobutanol are produced at 30°C than at 37°C and suggested, that this phenomenon is caused by the lower ADH activity at 37°C.

A major by-product of generating isobutanol by fermentation with *E. coli* is acetate. It is mostly determined in the growth phase at the beginning of the process (Baez et al. 2011). This is called overflow metabolism (Wolfe 2005). Baez et al. (2011) reported the acetate maximum during the growth phase. They tried to reduce acetate by metabolic engineering by deleting *ack*, *pta* and *poxB*, but the accumulation could not be stopped completely. They suggested, that acetate is generated by other pathways

as well. Acetate accumulation stands in direct relationship to the growth rate (Eiteman and Altman 2006).

10.5.5 Gas stripping

Gas stripping is a simple technique to remove solvents from culture broth. Because the gas stream goes through the broth and due to the equilibrium between gas phase and liquid phase, volatile solvents are removed. This has been done for n-butanol produced in *Clostridia* by Lee et al. (2008). This method was also tested successfully for a process performed by *E. coli* to produce isopropanol (Inokuma et al. 2010).

During isobutanol production gas stripping can constitute a problem due to the high volatility (172 mmHg at 25°C) of the intermediate isobutyraldehyde. Therefore, a strong ADH is necessary. The production of isobutyraldehyde could be an alternative to isobutanol (Rodriguez GM and Atsumi S. 2012) because it is easy to remove the toxic solvent from the culture broth by gas stripping (Atsumi et al. 2009a; Baez et al. 2011; Rodriguez GM and Atsumi S. 2012). Isobutyraldehyde can be turned to various hydrocarbons. This method is currently executed for petroleum-based products. Some products that can be gained are isobutanol, isobutyric acid, oxime and imine by current existing chemical catalysis (Atsumi et al. 2009a; Rodriguez GM and Atsumi S. 2012). But Rodriguez GM and Atsumi S. (2012) have shown that there are a couple of native isobutyraldehyde reductases, which must be deleted for a high isobutyraldehyde yield. However, the total deletion of isobutanol for further increasing the isobutyraldehyde was not possible.

10.6 Cloning

The production of biofuels requires both, engineering of native pathways and using *de novo* pathways (Dellomonaco et al 2010).

10.6.1 Pathway

Biofuels produced in *E. coli* are derived from the engineered carbon catabolism. Hexose or pentose sugar is transformed into C2 molecules. These molecules are turned into the biofuel of interest (Koppolu and Vasigala 2016). Therefore *E. coli* is very suitable concerning its existing metabolic capability. Another big advantage of this microorganism is its compatibility of the last two steps of the Ehrlich pathway with 2-keto acid degradation of other organisms. For the production of biofuel in this way only two host-foreign steps are necessary (Atsumi et al. 2008b; Felpeto-Santero et al. 2015). The Ehrlich pathway is shortly described by a transamination reaction of an amino acid producing a α-keto acid, followed by a decarboxylation. The generated fusel aldehyde can now be reduced or oxidised. To produce an alcohol the aldehyde must be reduced. In the case of valine as initial amino acid, the product would be isobutanol by reducing the butyraldehyde (Hazelwood et al. 2008).

High amounts of long chain 2-keto acids are not produced by in native organisms. That is why metabolic engineering is necessary (Chen and Liao 2016; Yong Jun Choi, Joungmin Lee, Yu-Sin Jang, Sang Yup Lee 2014). These 2-keto acids are metabolised by the Ehrlich pathway. Then a 2-keto-acid decarboxylase (KDC) reduces these acids to aldehydes, which are further reduced by an alcohol dehydrogenase (ADH) to an

alcohol. In the case of isobutanol 2-keto-isovalerate is produced by valine biosynthesis pathway as a precursor for isobutanol (Atsumi et al. 2008b). In this way the production of isobutanol via the keto acid pathway is a highly promising opportunity for industry (Felpeto-Santero et al. 2015).

KDC is wildly spread in plants, yeast and fungi, but it is not common in bacteria. There are different KDCs, while some are specific, others have a broad range of substrates (Atsumi et al. 2008b). In a report of Atsumi et al. (2009b) it is shown, that KDC is not necessary for the isobutanol production. By overexpressing *alsS* from *B. subtilis*, *ilvC* and *ilvD* from E. coli, isobutanol can be produced. *AlsS* undertakes the role of an KDC and generates isobutyraldehyde by catalysing the decarboxylation of 2-ketoisovalerate (Atsumi et al. 2009b). This intermediate is converted into isobutanol by ADH which is common in many organisms (Atsumi et al. 2008b). However, for a high titer production foreign KDC and additionally the overexpression of ADH is necessary (Atsumi et al. 2009b).

To produce isobutanol in *E. coli* both foreign genes can be used, *kivD* from *Lactobacillus lacti* and *adh2* from *Saccharomyces cerevisiae* (Atsumi et al. 2008b). *kivD* is responsible to turn ketoacids into its aldehydes (La Plaza et al. 2004). The end product, isobutanol, is converted by *adh2* (Atsumi et al. 2008b). Due to its high $K_m^{isobutyraldehyde}$ value, *adh2* is not the best choice. *YqhD* and *adhA* showed higher activity and higher titer of isobutanol (Atsumi et al. 2010a), but often the limiting factor is the substrate for *kivD*. An option to increase the isobutanol titer is to enhance the 2-ketoisovalerate biosynthesis. To achieve that, *ilvIHCD* from endogenous *E. coli* is transformed into the host organism. These genes can be overexpressed. This leads to an ~5-fold increased isobutanol titer. *AlsS* from *Bacillus subtilis* can be used instead of ilvIH. *AlsS* has a higher affinity to the substrate, pyruvate and a lower affinity to the intermediate. This change leads again to an isobutanol concentration enhancement up to ~1.7-fold (Atsumi et al. 2008b; Koppolu and Vasigala 2016). In this study not only *AlsS* was used, additionally *BudB* from *Ediss* was screened as acetolactate synthase. Since *Enterobacter cloacae* is a natural 2,3-butanediol producer, it must be equipped with a strong and reliable acetolactate synthase (Saha and Bothast 1999; Xu et al. 2012).

These discoveries rely on aerobe or microaerobic fermentation. Both ketol-acid reductoisomerase and alcohol dehydrogenase require NADPH for the isobutanol pathway, but glycolysis just provides NADH. Therefore, there is an imbalance of cofactors. This lack of NADPH cannot be filled by TCA or pentose phosphate pathway because they depend on oxygen. Bastian et al. (2011) suggested, that there are two solutions to this problem. On the one hand it is possible to perform over-expression of PntAB, which is responsible for the reversible transfer of a proton from NADH to $NADP^+$. Unfortunately, this solution is destructive, because it requires energy and additionally metabolic load. On the other hand, a NADH-depending pathway can be generated by enzyme engineering (Bastian et al. 2011). For catalysation of an aldehyde to alcohol under microaerobic conditions AdhA from *Lactococcus lactis* can be used. Thus, AdhA could be a suitable candidate for anaerobe fermentation (Atsumi et al. 2010a; Bastian et al. 2011). To replace IlvC to catalyse 2-acetolactate to 2,3-dihydroxyisovalerate another solution must be found. Due to performing a

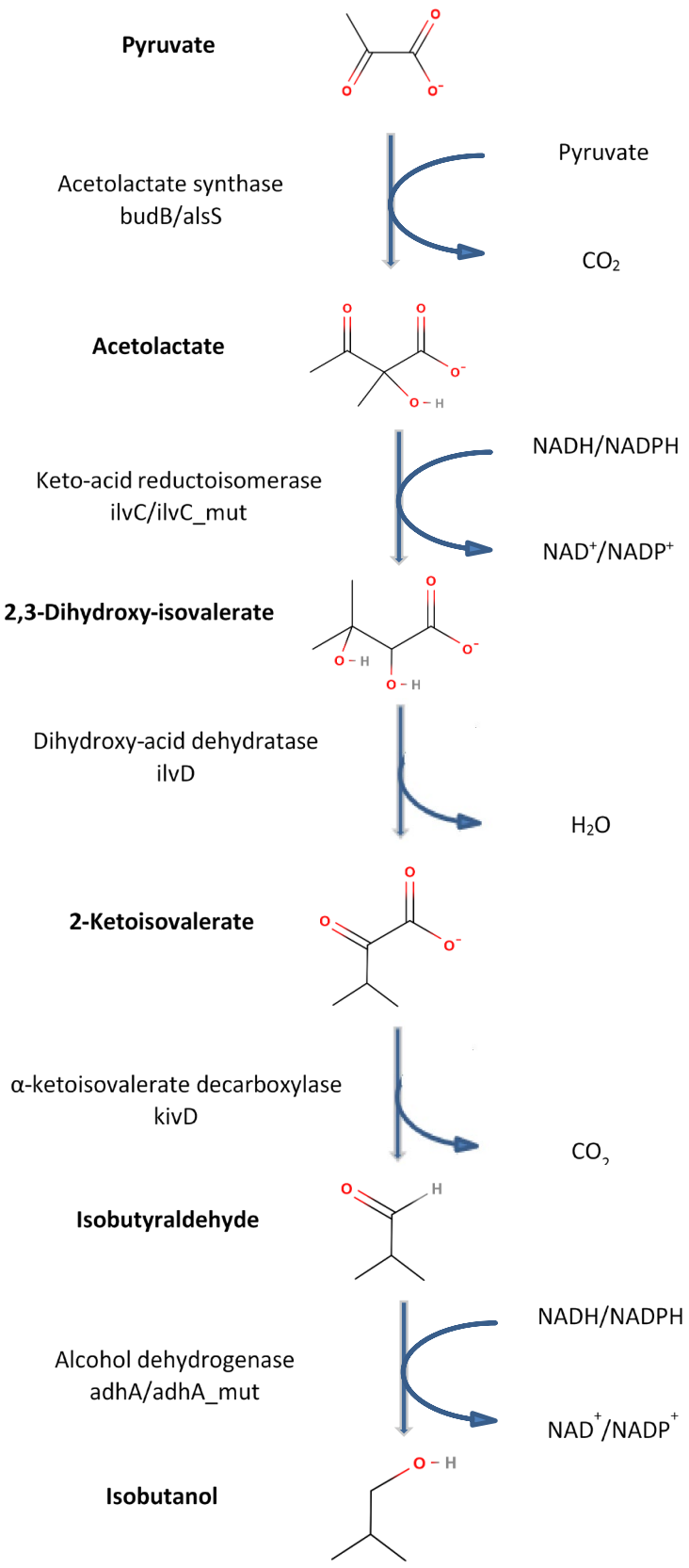

Figure 2: Isobutanol pathway with all used genes in this study; acetolactate syntase (alsS/budB), keto-acid reductoisomerase (ilvC/ilvC_mut), dihydroxy-acid dehydratase (ilvD) α-kedoisovalerate decarboxylase (kivD) and alcohol dehydrogenase (adhA(adhA_mut); IlvC_mut stands for IlvC$^{A71S, R76D, S78D, Q110V}$ and AdhA_mut for AdhA$^{Y50F, I212T, L264V}$; variants of this pathway were inserted into E. coli W, KOW4, KOW5 and AD

directed evolution of IlvC by iterative, targeted mutagenesis, the required ketol-acid reductoisomerase could be created by Bastian et al. (2011). Also AdhA was modified for a better adaption to anaerobe conditions by them.

In some reports of Atsumi et al. (2008b; 2010a), the pathway was divided into more plasmids, that require different selective markers like antibiotics, resulting in additional stress for the cells and potentially bad effects on productivity. Felpeto-Santero et al. (2015) has created the nearly full isobutanol pathway on one plasmid. They only excluded ADH because *E. coli* has sufficient opportunities for catalysing isobutyraldehyde to isobutanol (Atsumi et al. 2010a).

10.6.2 Knockouts

Further optimization can be done by performing some knockouts to minimize by-product formation. *AdhE*, *ldhA*, *frdAB*, *fnr, pta* and *pflB* are genes that can be deleted. The suggestion for these deletions is the possibility to enhance the pyruvate level for more substrate for the ilvIHCD pathway (Atsumi et al. 2008b).

Mixed acid fermentation regenerates NAD^+ to NADH, which is an important co-factor for glycolysis. By deletion of enzymes, performing this regeneration, the metabolism is forced to a heterologous pathway, which also needs NAD^+ as co-factor (Vuoristo et al. 2015). *Adh*, *ldh* and *frd* are examples for such enzymes and their deletion showed an increase of product formation in literature (Atsumi et al. 2008a).

10.6.3 Golden Gate Cloning

Common cloning techniques which use site-specific recombination are the most efficient, simplest and most flexible ones, but there is a big disadvantage. There is no way to recombine the gene of interest and the desired vector without adding 8 to 13 amino acids for the recombination sites to the target protein. An option to avoid the presence of these short sequences is to flank them with intron sequences. In this way the recombination site sequences are cut out by splicing. Unfortunately, this method is only suitable in eukaryotic hosts (Engler et al. 2008).

Engler et al. (2008) had created a new technique which combines one or more genes of interest and the target vector in one tube and one step nearly one hundred percent correct in just five minutes of restriction and ligation. They called it ‘Golden Gate’ cloning which is derived from the site-specific recombination system, ‘Gateway’. This innovative method uses the property of restriction enzymes type II which bind on a recognition sequence of the DNA, but cut outside this certain sequence. Consequently, each overhang has a specific sequence if it is designed smartly. That includes the case that the restriction enzyme binding site is cut out and is absent in the combined sequence. If a restriction enzyme is used which produces a 4 nt overhang, there are 256 different possible sequences for overhangs for recombination. A suitable endonuclease is BsaI. The sequence of the overhang can be chosen ad libitum but the 16 palindromic ones should be avoided to sustain the effectiveness of this technique (Engler et al. 2008).

Primers have to be designed in a way that the recognition site of the BsaI is outside of the sequence of the GOI. This has the big advantage that the enzyme creates the specific overhang and lacks in the final sequence. Due to this reason the possibility of re-digestion of the wanted plasmid by BsaI is excluded (Engler et al. 2008).

After the restriction digest is done, the ligation step is performed. Due to the specific sticky ends of one or more genes of interest and the vector, the recombination is only in the right way possible. Due to the unique overhangs of each DNA fragment, they can be aligned in the requested order. Moreover, there is no feasibility to produce an empty vector. However, there is a chance of failure in double or multiple combination. That means, instead of the vector-GOI junction, it is possible to create a vector-GOI-vector-GOI double sequence. But that did not occur in the report of Engler et al. (2008).

By using this method for recombination, it is important to pay attention to BsaI restriction sites inside the GOI or the vector itself. To eradicate this unwanted endonuclease recognition site, primers can be designed, which overlap the BsaI binding site and the new sequence without the binding site can be amplified by PCR. In the sequence of these primers one nucleotide is changed to induce a silent mutation. This mutation leads to the same amino acid sequence but to the loss of the binding site for the restriction enzyme (Engler et al. 2008).

This technique does not require cost intensive kits which is an additional benefit (Engler et al. 2008).

An often-used molecular tool for creating different variants of a gene is error prone PCR, but Golden Gate cloning can also be used for DNA shuffling, this is called Golden Gate Shuffling. Engler et al. (2009) demonstrated DNA shuffling in the case of three different trypsinogen genes. Each gene was divided into nine parts with BsaI restriction site tailing via PCR and inserted in a temporary vector for amplifying. The primers were designed that the first fragment of each gene has the same overhang after restriction. The same is performed in the case of the second, third, and so on. As a result, each position has a unique tailing and fits only at the desired position like a puzzle. So, there are three different puzzles with the same shape of the parts. Moreover, there are 27 fragments for the description of three genes of trypsinogen. By random ligation there are 19,683 possible combinations (Engler et al. 2009).

To perform DNA shuffling, these 27 temporary vectors and the target vector with the right overhangs after restriction, are mixed. In one pot and one step the vectors can be digested by BsaI and randomly ligated. After transforming in competent cells, the pool can be screened to figure out the most powerful clone (Engler et al. 2009).

10.6.4 Modular Cloning

Weber et al. (2011) created a modular cloning system (MoClo) for eukaryotic multigene constructs relying on Golden Gate cloning developed by Engler et al. (2008). It was shown that Golden Gate cloning is not only suitable for homologous recombination for generating a variety of a gene and assemble large fragments of DNA, it is also an ideal tool for directed assembly of desired DNA fragments, not only pieces of one gene but also pieces of a series of genes. The goal of Weber et al. (2011) was the standardization of a complex synthetic biology tool for the metabolic modelling

community worldwide for a faster and easier gene exchange strategy. They created a standardised system for gene designing within promoters, untranslated genetic models, signal peptides, coding sequences and terminators, which can be exchanged as easy as possible in one reaction (Weber et al. 2011).

10.6.5 GoldenMOCS

Based on the Golden Gate cloning strategy, developed by Engler et al. (2008; 2009) and Weber et al. (2011) the Golden Gate derived Multiple Organism Cloning System (GoldenMOCS) was created by Sarkari et al. (2017). Three steps are necessary for the GoldenMOCS. The first level is based on the single elements like promoter, coding sequence and terminator. Each of them is found on an own construct called backbone 1 (BB1). The coding sequence can be used in a variety of hosts, in contrast the promoter and terminator are host specific and can be exchanged easily. In BB2 these elements are assembled to a functional expression cassette. These cassettes can be assembled by smart predesigned fusion sites to BB3. In this way a whole metabolic pathway up to eight expression cassettes can be assembled. Two sets of fusion sites, which are generated by type II restriction endonucleases, creating a four base pair overhang outside the recognition sequence, are used. The first set is generated by restriction digestion with BpiI. The assembly of the single elements of one expression cassette into BB2 is performed by T4 ligase. For the second set BsaI is used for digestion of the BB2s. These fusion sites lead to the assembly of the single cassettes to BB3 (Sarkari et al. 2017).

In GoldenMOCS there is a switch of selective marker between BB1 and BB2 and a second one between BB2 and BB3. BB1 and BB3 contain a kanamycin resistance gene. The intermediate BB2 is equipped with an ampicillin resistance marker. As a result, the host cells with the correct assembled construct can be identified after transformation.

BB1 can be created by DNA synthesis or by performing PCR amplification of the GOI and inserted into a BB1 recipient plasmid (Sarkari et al. 2017). If the GOI contains any BpiI or BsaI restriction sites, they must be removed by overlap-extension PCR (Engler et al. 2008; Sarkari et al. 2017). That means a considerable library of genes can be applied for a quick and easy exchange of genes. This promises a directed modification instead of error prone PCR and the subsequent screening effort.

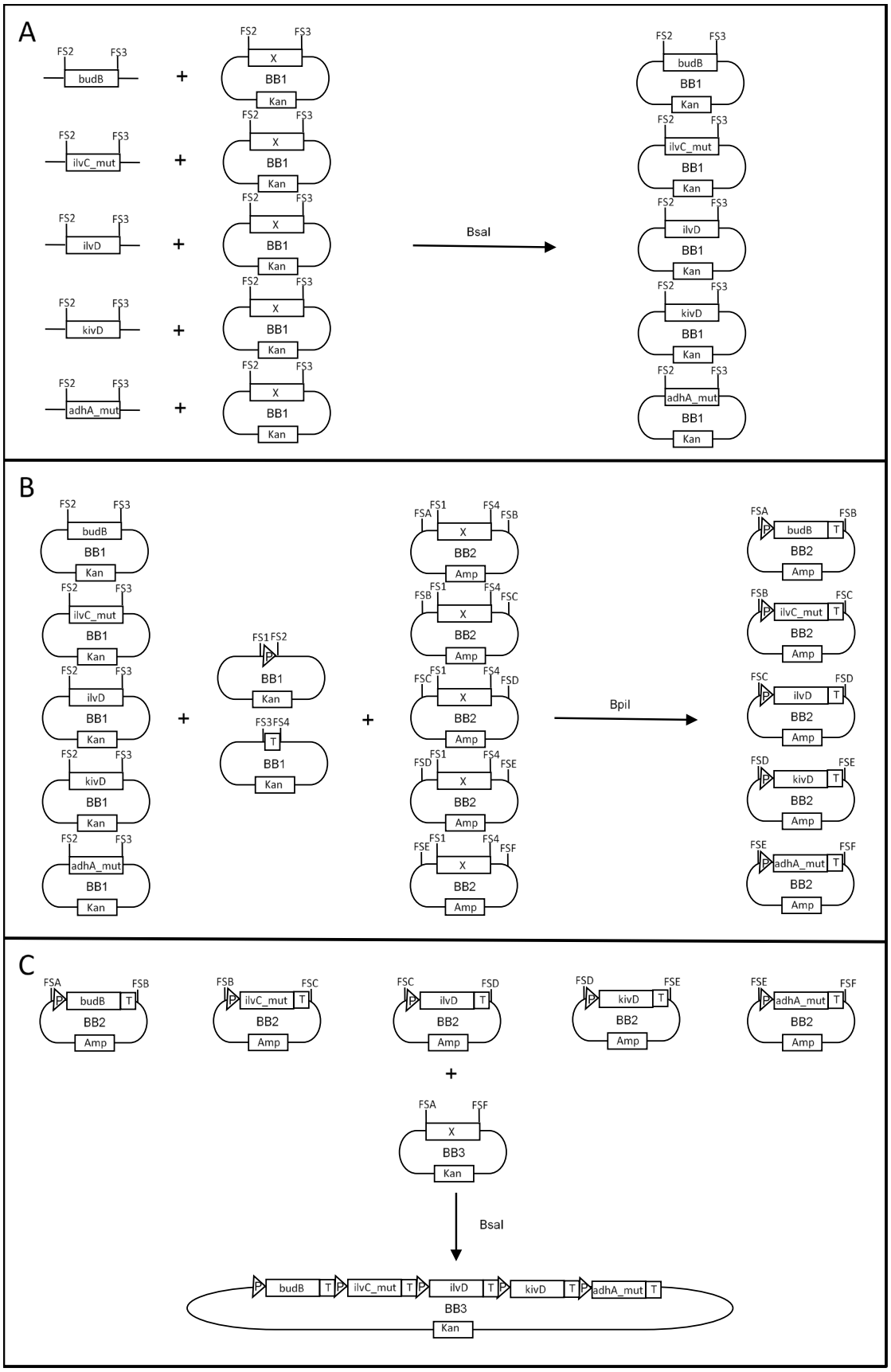

Figure 3: GoldenMOCS of the procedure of the top-construct BB3-10; at A the generation of the BB1 with kanamycin resistance after restriction digestion by BsaI is displayed, at B of BB2 with ampicillin resistance and BpiI as endonuclease and at C the assembly of BB3 with kanamycin resistance and BsaI again is shown

10.6.6 Anderson promoter library

Due to the unpredictable expression rate of the recombinant genes, fine tuning must be performed in the individual expression of each gene of the inserted pathway. This was done by testing three promoters of the Anderson promoter library shown in Figure 4. The parental sequence is J23119 and the other ones are derived from this. The activity of these promoters is given as relative fluorescens to J23119. Hence, J23112 is the weakest and equal to the parental promoter. J23100 is the strongest, which is 2547-fold stronger than J23119. In this study, J23105, J23109 and J23114 were tested.

Variant	RFP (au)
J23112	1
J23103	17
J23113	21
J23109	106
J23117	162
J23114	256
J23115	387
J23116	396
J23105	623
J23110	844
J23107	908
J23106	1185
J23108	1303
J23118	1429
J23111	1487
J23101	1791
J23104	1831
J23102	2179
J23100	2547

Figure 4: Anderson promoter library (http://parts.igem.org/Promoters/Catalog/Anderson)

10.6.7 Isobutanol adaptation

Theoretically high titers of isobutanol can be achieved in *E. coli*, up to 22 g L^{-1} (Atsumi et al. 2008b). However, this organism is not tolerant to isobutanol (Koppolu and Vasigala 2016). A simple way to enhance the tolerance of microorganisms to solvents is adaptation. Here, the cells are cultivated in presence of non-lethal concentrations of the desired solvent. (Kanno et al. 2013) Reyes et al. (2012) showed this phenomenon on n-butanol. They increased the solvent concentration over time. An evolutionary effect occurs, and the strain become more tolerant to n-butanol.

Engineering of the global transcription factor cAMP receptor protein is another opportunity to increase the isobutanol tolerance too. Performed by error-prone PCR of *crp* of *E. coli*, higher tolerance can be achieved (Chong et al. 2014).

10.7 Aims

This study deals with the very last part of the Bio-ABC project, the formation of isobutanol as a fuel alcohol. At the date of this thesis the acetate uptake (Novak et al. 2018) and the formation of isobutanol were still two separate processes. This work deals with the pathway prototyping for isobutanol. Consequently, isobutanol was generated only from glucose.

The first objective of this work was to screen a construct library for isobutanol production. The constructs were assembled from different promotor-gene combinations with different expression rates. All promotors were part of the Anderson promoter library. Also, some alternative genes were inserted into the pathway. Instead of the common keto-acid reductoisomerase and the alcohol dehyrogenase, mutations were also tested.

The second objective was to find the best producing strain, harbouring the most promising construct for isobutanol production. Here, different stains of *E. coli* were tested.

The third objective was to perform a stable chemostat process for continuously producing isobutanol under controlled aeration.

The fourth and final objective was identifying the optimal culture conditions for generating the highest yield as possible. Therefore, various aeration strategies and temperatures are tested.

11 Materials and Methods

11.1 Strains and Plasmids

Table 1: Strains

Strain	Source
W	DSMZ
Isobutanol adapted W	Other master student in this study
KOW4 (Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA*)	BOKU
KOW5 (Δl*dhA* Δ*adhE* Δ*pta* Δ*frdA* Δ*pykA*)	Other master student in this study

11.2 Media composition

11.2.1 DeLisa

DeLisa-media is a minimal defined media (Erian et al. 2018) and was used for cultivations in bioreactors but also in shake flasks and serum bottles. This media was divided into three parts. At first the desired amount glucose was dissolved in dH_2O. The volume was set on 10 % of the final volume and the final glucose concentration defined either 20 g L^{-1} or 50 g L^{-1}. The saline solution was prepared by solving KH_2PO_4, $(NH_4)_2HPO_4$ and Citric acid in dH_2O. After adjusting the pH to 7.0 with solid NaOH, the solution was filled up to the volume, final volume minus glucose solution and trace elements. These trace elements were premixed by adding $MgSO_4 * 7H_2O$, Fe(III) citrate, EDTA, $Zn(CH_3COO)_2 * 2H_2O$ and kanamycin to the trace element stock.

Both the glucose solution and the saline solution were sterilized by thermic treatment. They were autoclaved 20 min at 121°C at 1 bar. The trace element solution was sterilized by sterile filtration. After sterilization the three solutions were combined and well mixed.

Antifoam agent, Struktol J673-1 (Schill+Seilacher GmbH) was added on demand performed by a syringe sterile filtered by a syringe filter.

Table 2: DeLisa composition

Compound	Final concentration [L^{-1}]
Glucose * H_2O	22.00 g or 55.00 g
KH_2PO_4	13.30 g
$(NH_4)_2HPO_4$	4.00 g
Citric acid	1.70 g
$MgSO_4 * 7H_2O$	1.20 g
Fe(III) citrate	0.10 g
EDTA	8.4 mg
$Zn(CH_3COO)_2 * 2H_2O$	13.0 mg
Trace element stock	5 mL
Kanamycin	50 mg

Table 3: Stock solutions for DeLisa

Stock solution	Compound	Stock concentration [L^{-1}]
$MgSO_4$ x500	$MgSO_4 * 7H_2O$	600.00 g
Fe(III) citrate x100	Fe(III) citrate	10.00 g
EDTA x100	EDTA	0.84 g
$Zn(CH_3COO)_2$ x200	$Zn(CH_3COO)_2 * 2H_2O$	2.60 g
Kanamycin x1000	Kanamycin	50.00 g
Trace element stock *200	$CoCl_2 * 6H_20$	0.50 g
	$MnCl_2 * 4H_20$	3.00 g
	$CuCl_2 * 2H_20$	0.24 g
	H_3BO_3	0.60 g
	$Na_2MoO_4 * 2H_20$	0.50 g

11.2.2 Lysogeny Broth (LB)

Lysogeny Broth is a complex media and was used for molecular applications both as liquid and as solid growth media in petri dishes. The addition of an antibiotic as selective marker was optional. Either kanamycin or ampicillin was used.

Table 4: Lysogeny Broth (LB)

Compound	Final concentration [L^{-1}]
Soy peptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar agar (optional)	15 g
Kanamycin x1000 (optional)	50 mg
Ampicillin x1000 (optional)	100 mg

For precultures 2x concentrated lysogeny broth was used (LB x2) (Table 5).

Table 5: enriched Lysogeny Broth (LB x2)

Compound	Final concentration [L^{-1}]
Soy peptone	20 g
Yeast extract	10 g
NaCl	10 g
Kanamycin x1000 (optional)	50 mg

11.2.3 Super Optimal Broth with catabolite repression (SOC)

The SOC solution was used for recovery after transformation. After dissolving the compounds, the pH was adjusted to 7.0 with NaOH. The solution was sterilized by sterile filtration.

Table 6: SOC-solution

Compound	Final concentration [L^{-1}]
Glucose	3.6 g
Yeast extract	5 g
Tryptone	20 g
NaCl	10 mmol
KCl	2.5 mmol
$MgCl_2$	10 mmol
$MgSO_4$	10 mmol

11.3 Screening

After the transformation some clones were verified by gel electrophorese followed by sequencing after plasmid isolation and restriction digest.

11.3.1 Plasmid isolation

Single colonies of the transformed cells were picked with a sterile pipette tip and tipped on a LB-agar plate containing the equal antibiotic to create a master plate before inoculating 2-4 mL LB medium with antibiotic in a glass test tube with a metal lid. These cultures were incubated overnight at 37°C and 200 rpm.

2 mL of the overnight culture were centrifuged (SIGMA 1-14, Germany) for 30 s at 14,000 rpm. The supernatant was removed, and the plasmid purified by Monarch Plasmid Miniprep Kit (NEB, USA) according the manual. The plasmid was eluted in 30 µL TRIS (10 mM).

The qualification was performed by a NanoDrop 1000 (ThermoScientific, USA) after blanked with TRIS buffer. The plasmids were stored at -20°C.

11.3.2 Restriction digest

The plasmids isolated in the previous step were digested by restriction enzymes. Therefore, the following mixture (Table 7) was prepared and incubated for 1-2 h at a certain temperature (mostly 37°C).

Table 7: general restriction digest mix

component	volume
plasmid DNA	1 µL
restriction enzyme	1 µL
10x buffer (specifically to enzyme)	2 µL
TRIS buffer	16 µL

During the digestion an agarose gel could be prepared. Normally a gel with 1 % agarose was used for screening. For two gels, 1.5 g agarose and 150 mL TAE buffer were mixed and heated up in the microwave until the agarose is totally dissolved.

Afterwards the solution was cooled down to approximate 50°C and 15 µL SYBR Safe 10,000x (ThermoFisher Scientific, USA) were added and mixed. Now the liquid gel was poured into a tray with a comp. This comp could be removed after 1 h and the gel could be stored in TAE buffer at +4°C or used immediately.

Table 8: agarose gel 1 %

component	volume
TAE buffer 1x	150 mL
agarose	1.5 g
SYBR Safe 10,000x	15 µL

Therefore, the gel was put into a electrophoreses chamber. The slots had to face the cathode and the gel is submerged into TAE buffer. An aliquot of the restriction digest was mixed with a 6x loading dye (ThermoFisher Scientific, USA) and loaded gently onto the gel flanked by 6 µL of a 1 kb Gene Ladder (ThermoFisher Scientific, USA) for size detection. The lid was closed, the power supply (PowerPak Basis Power Supply; BIORAD Laboratories, Hercules, USA) was connected and the voltage was set to 120 V. After the coloured front had reached the end of the gel, that takes approximately 1h, the electrophorese was stopped and visualized by a ChemiDoc MP Imager (BIORAD, USA) and the appropriate Software (Image Lab 5.2; BIORAD, USA).

For a screening a gel with small slots (15 µL) was used to save plasmidic DNA. If a promising candidate was found, it was sent to sequencing to MicroSynth (Switzerland). Therefore, 480-1,000 ng DNA was mixed with 10 µM sequencing primer, labelled and sent.

If a DNA fragment had to be purified after digestion, the whole restriction digest solution was loaded onto an agarose gel with big slots (60 µL). The desired band was cut out after electrophoreses and treated according Plasmid extraction and purification.

11.3.3 Plasmid extraction and purification

Plasmid purification was mainly used for gaining a DNA sequence for GoldenMOCS. After an electrophoreses the band with the fitting size was cut out of the agarose gel and extracted with the QIAGEN QIAquick Gel Extraction Kit (ThermoFisher Scientific, USA). The procedure was performed according the manufacturer manual except for the elution. Instead of the attached elution buffer, 20 µL of 10 mM TRIS buffer was used. The eluate was quantified and stored at -20°C.

11.4 Cryo stocks

For culture preservation, cryo stocks was prepared. 2 mL LB medium containing the corresponding antibiotic was inoculated by a single colony on a master plate and incubated overnight at 37°C and 200 rpm. 1 mL of the cell suspension was transferred into a cryo tube. Glycerine was added to generate a 20 % solution which was vortexed thoroughly before freezing at -80°C.

11.5 Preculture and Inoculation

To gain fresh and viable cells for the inoculum, it was highly recommended to thaw fresh cells from a cryo stock. Therefore, an inoculation loop was heat sterilized by a Bunsen burner. The loop was tipped onto the frozen cells in the cryo tube to melt a part of the suspension. When the inoculation loop is cooled down, the cells were spread out onto a selective LB agar plate and incubated overnight at 37°C. Next day a shake flask filled with approximate 1/5 of 2x LB medium with 50 µL mL^{-1} kanamycin was inoculated and incubated overnight at 37°C and 200 rpm. 250 mL 2x LB medium were needed to inoculate 1 L working volume for a start cell density of OD_{600} 1.0.

Before inoculation the cells had to be washed and concentrate to remove the complex LB media and minimize the inoculum volume. The suspension was centrifuged in 300 mL beakers with a Sigma 3-18K centrifuge (Sigma, Germany) at room temperature, 4800 g and 30 min. The supernatant was removed, and the cell pellet was resuspended in 100 mL sterile NaCl 0.9 %. This washing step was performed twice and centrifuged again. Now the cells were resuspended in 20 mL sterile water to gain a high cell density. The optical density was measured at 600 nm and the volume of the inoculum was calculated according to equation *(1)*.

$$V_{Inoculum} = \frac{V_{Working\ Volume} * c_{Working\ Volume}}{c_{Inoculum}} \quad [mL] \quad (1)$$

The calculated volume was drawn up into a syringe with a needle. Now the inoculum was finished. It was highly recommended to inoculate the bioreactor, shake flask or serum bottle quickly.

11.6 Shake flask cultivations

Shake flask experiments are performed for first screenings. Strains and constructs are tested at different conditions. At first DeLisa media with usually 20 g L^{-1} glucose and 50 µg mL^{-1} kanamycin is prepared and 20 mL aliquots are filled into sterile 100 mL Erlenmeyer flask. Inocula are prepared according to Preculture and Inoculation. Shake flasks are inoculated with an initial OD_{600} of 0.5 or 1.0 and incubated at 30°C or 37°C at 200 rpm. Samples for OD_{600} measurement and HPLC analysis are taken at defined time points.

11.7 Serum bottle cultivations

To avoid evaporation of the product serum bottles are used. The procedure is similar to 11.6 Shake flask . 20 mL DeLisa media with usually 20 g L^{-1} glucose concentrations and 50 µg mL^{-1} kanamycin is inoculated at OD_{600} 0.5 or 1.0 and incubated at 30°C or 37°C and 200 rpm. The sampling was performed by a syringe with a needle and analysed equally to shake flask experiments.

11.8 Bioreactor cultivations

Different types of bioreactors were used, among a 1 L glass bioreactor (Applikon Biotechnology B.V., Netherlands) and a multi fermenter system with four bioreactors called DASGIP (Eppendorf AG, Germany). For successful process analytics, the pH electrode (Mettler-Toledo GmbH, Germany) was calibrated at pH 4.0 and 7.0. Also, the pumps were calibrated every time. The fluorescence dissolved oxygen electrode VisiFerm DO 225 (Hamilton, USA) was calibrated after autoclaving and addition all compounds of medium with air and 1 vvm for 100 % and nitrogen for the offset. All autoclavable compounds of Table 2 except glucose were autoclaved directly in the bioreactor. The other compounds were added after cooling down.

In both systems the gas composition and flow could be defined individually. At the DASGIP multireactor system a gasmixer MX4/4 (Eppendorf AG, Germany) was responsible for the aeration and for the Applikon bioreactor mass flow controllers from the series 4800 (Brooks Instrument, Germany) were used. Air, O_2 and N_2 were used. The performed experiments were started with 21 % O_2 and 1 vvm. After the batch phase, a chemostat was started and the O_2 content of the in-gas was reduced until the cells were washed out. This scheme pertained most of the experiments with small deviations. For the off-gas analytic CO_2 and O_2 were measured by GA4 (Eppendorf, Germany) for the DASGIP system and BlueSens gas sensors (Bluesens gas sensor GmbH, Germany) for the Applikon reactor.

For the dispersion of oxygen three Rushton impellers stirred at 1000 rpm constantly. At the batch phase the DO was kept above 40 % and must not get below 10 %. Due to that reason, in the end of the batch phase the agitation was increased until the maximum before the gas flow was increased. If these actions were not enough, the oxygen content of the in-gas could be increased.

To run a stable process, the pH was controlled and adjusted with NH_4OH 12.5 % if the pH droped below the setpoint because of the produced acids. Also, the temperature was controlled and kept at 37°C or 30°C. To avoid a foam-out, sterile antifoam (Struktol J673-1; Schill+Seilacher GmbH, Germany) diluted 1:10 was added on demand.

The parameters of the fermentations differed in some points and were shown at Results and Discussion for each experiment.

Samples were taken for OD_{600}, HPLC and cell dry weight immediately after inoculation, then after 3 h and further every 2 h until the batch phase is over. At the chemostat phase the culture was operated for a minimum of three volume changes to obtain a steady state. Three samples were taken at 3 h intervals before the parameters were changed. For sampling, 5 mL pre-sample was taken and discarded before 5 mL sample is taken for OD_{600} and supernatant analysis with HPLC. If cell dry weight was determined, a 15 mL sample was taken.

11.9 Off-line analytics

After inoculation a sample was taken immediately. Other samples were taken at defined time points. The volumes were dependant on the desired off-line analytical

methods and the working volume. The sample was put into pre-chilled Grainer tubes immediately to lower the metabolism of the cells. If possible, aliquots of the supernatant were frozen at -20°C as backup. For measurement of any analytes by Cedex BioHT or HPLC, the suspension was centrifuged 10 min at 4800 g and 4°C.

11.9.1 Optical Density (OD_{600})

The optical density was measured at 600 nm by a photometer, Genesiys 20 (ThermoFisher Scientific, USA). Before measuring the photometer was blanked with dH_2O. Then a 1 mL aliquot of the suspension was measured in plastic single-use cuvettes. If the measured value was out of the linear range (0.2-0.8) the sample was diluted with dH_2O. For each sample two aliquots were measured.

If no sample for cell dry weight was taken, the correlation of the OD_{600} and the CDW of other samples of the same experiment was estimated. With this factor the CDW could be calculated according to equation (2).

$$c_{CDW_2} = \frac{c_{CDW_1}}{OD_{600_1}} * OD_{600_2} \qquad [\text{g L}^{-1}] \qquad (2)$$

11.9.2 Cell Dry Weight (CDW)

For CDW measurement some preparatory work was necessary. Glass test tubes were labelled and dried in an oven at 110°C for minimum 24 h. They were cooled down to room temperature in a desiccator. Afterwards, the tubes were weighted on an analytical scale (Mettler Toledo, USA). Now they were ready to use. Three tubes were used for each sample. Each tube was filled with 4 mL suspension and centrifuged 10 min at 4800 g and 4°C. The supernatant could be used for glucose measurement by Cedex BioHT, other analytes by HPLC or frozen as a backup, the rest was discarded. The cell pellet was washed, by resuspending in 4 mL dH_2O and centrifuged again at the same conditions as before. The supernatant was discarded and the testing tubes with the cell pellet were dried in an oven at 110°C at least 72 h. The tubes were cooled down in a desiccator and weighed out. Now the CDW L^{-1} was calculated according to equation (4).

$$m_{CDM} = m_{gross\ weight} - m_{tara} \qquad [\text{g}] \qquad (3)$$

$$c_{CDW} = \frac{m_{CDM} * 1000}{V_{sample}} \qquad [\text{g L}^{-1}] \qquad (4)$$

c_{CDW} [g L^{-1}] stands for the concentration of the CDW, m_{CDW} [g] is the difference between the weight of the empty tube and the tube with the dried cell pellet and V_{sample} [mL] corresponds the sample volume.

11.9.3 Cedex BioHT

The Cedex BioHT (Roche, Germany) is a high throughput instrument for analytical measurement. At this study it was just used for glucose measurement in the batch phase to be sure that there is no more sugar in the cell broth and the batch phase was over. For this measurement, the sample was centrifuged just as previously described and diluted 1:10 with MiliQ-H_2O. This dilution was transferred or performed directly in the fitting tubes. 10-15 min after starting the measurement, the result could be read off.

11.9.4 High-performance liquid chromatography (HPLC)

HPLC was used for qualifying and quantifying produced metabolites and residual substrate in the supernatant. Therefore, an Agilent system 1100 series (Agilent Technologies, USA) with an Animex HPX87H column 300 x 7.8 mm (BIORAD, USA) was used. For detection a refractive index detector G1362A (Agilent Technologies, USA) and a UV detector G1315A (Agilent Technologies, USA) at 210 nm were integrated in the system. The injection volume was 10 µL. The separation of the analytes was performed in the column at 60°C. At a flow rate of 0.6 mL min^{-1} of 4 mM H_2SO_4 as mobile phase, one run needed 40 min. The data evaluation was performed with the Chromeleon 7 software.

For sample preparation, 450 µL supernatant (centrifuged as described previous) was mixed with 50 µL 40 mM H_2SO_4 to adapt the concentration of the mobile phase. The solution was vortexed thoroughly and centrifuged again for 10 min at 4800 g and 4°C to avoid clogging the column by solid residues in the solution. The supernatant was transferred into a HPLC vial and placed on a tray of the autosampler at 4°C.

For an accurate measurement, standards were measured at the beginning of each queue. These were prepared for glucose, acetate, succinate, lactate, formate, ethanol and isobutanol by weighting in and filled up with MiliQ-H_2O. The desired concentrations of the standards and the series dilutions were shown in Table 9.

Table 9: HPLC standards

Retention time [min]	**Analyte**	**Concentration [g L^{-1}]**				
9.33	Glucose	25	12.5	5	2.5	0.25
15.47	Acetate	10	5	2	1	0.1
12.15	Succinate	5	2.5	1	0.5	0.05
13.17	Lactate	5	2.5	1	0.5	0.05
14.26	Formate	25	12.5	5	2.5	0.25
21.64	Ethanol	5	2.5	1	0.5	0.05
30.60	Isobutanol	25	12.5	5	2.5	0.25

11.10 Data evaluation

11.10.1 Growth rate

The growth rate (μ) describes how much the biomass increases during a time frame. It was calculated according to equation (5) for a batch process. In a chemostat process the dilution rate (D) must be considered (equation (6)).The dilution rate is the ratio how much feed flows into the bioreactor with a constant working volume. Both are given in h^{-1}. During a chemostat process in steady state, the growth rate is equal to the dilution rate. If the growth rate is bigger than the dilution rate, the cell density increases but if the growth rate is smaller, cells become washed out and the cell density decreases until there are no more cells in the fermenter.

$$\mu = \frac{\ln(X_1) - \ln(X_0)}{t_1 - t_0} \quad [h^{-1}] \quad (5)$$

$$\mu = \frac{\ln(X_1) - \ln(X_0)}{t_1 - t_0} + D \quad [h^{-1}] \quad (6)$$

$$D = \frac{F_{Feed}}{V_{reactor}} \quad [h^{-1}] \quad (7)$$

$$\mu = D \quad [h^{-1}] \quad (8)$$

$$\mu > D \quad [h^{-1}] \quad (9)$$

$$\mu < D \quad [h^{-1}] \quad (10)$$

11.10.2 C-balance

The C-balance, also called carbon recovery was performed to be sure that all carbon compounds were quantified correctly. Therefore, the carbon which goes into the bioreactor is compared with the carbon which leaves or stays in the bioreactor. In an ideal case the ratio of the sum of the carbon of the products, by-products, biomass and CO_2 and the sum of the carbon of the substrate including feed and batch medium is 1 according equation (11). A chemostat is a dynamic process, there is a constant flow of substances. At that reason the C-balance must be calculated as such and the flow of carbon produced is divided by the flow of carbon is added according equation (12).

$$1 = \frac{n_{C_{product}} + n_{C_{byproduct}} + n_{C_{biomass}} + n_{C_{CO_2}}}{n_{C_{substrate}}} \quad [\frac{Cmol}{Cmol}] \quad (11)$$

$$1 = \frac{\dot{n}_{C_{product}} + \dot{n}_{C_{byproduct}} + \dot{n}_{C_{biomass}} + \dot{n}_{C_{CO_2}}}{\dot{n}_{C_{substrate}}} \quad [\frac{Cmol\ L^{-1}\ h^{-1}}{Cmol\ L^{-1}\ h^{-1}}] \quad (12)$$

For an accurate C-balance, substrate, product and by-products were measured by HPLC, the biomass was calculated based on the CDW and the CO_2 was measured by an off-gas sensor.

11.10.2.1 Substrate, product and by-product calculation

These components were measured via HPLC according High-performance liquid chromatography (HPLC) and given in g L^{-1}. To calculate a carbon balance for a continuous process, the values had to be converted into Cmol L^{-1} h^{-1}.

$$\dot{n}_{C_i} = \frac{c_i}{M_i} * N_c * D \qquad [\text{Cmol L}^{-1}\,\text{h}^{-1}] \qquad (13)$$

11.10.2.2 Biomass calculation

The CDW contains a variety of substances but for the C-balance only the carbon is relevant. Therefore, the carbon content of *E. coli* was determined externally and defined at 46.1 % (w/w) (Novak et al. 2018).

The c_{DCW} is multiplied with the carbon content dry mass and transformed into mol which is equal to Cmol.

$$\dot{n}_{CDM_C} = \frac{c_{CDM} * 0.450047}{M_C * N_C} * D \qquad [\text{Cmol L}^{-1}\,\text{h}^{-1}] \qquad (14)$$

11.10.2.3 Off-gas calculation

For analysing the off-gas, some calculations were necessary. At first there was a difference in the gas composition of the in- and off-gas without any metabolic influence. The dry in-gas takes up water by bubbling through the medium. For that reason, the off-gas had a smaller part of oxygen. To compensate that difference, the part of water in the off-gas was calculated with equation (15). This value differed from bioreactor to bioreactor and was also dependent from the flow rate, temperature and stirring speed. Normally the start conditions were used for calculations, that means 1 vvm, air, 37°C and 1000 rpm. If dramatical changes of these parameters were planned or performed, the water content of the off-gas is adjusted.

$$ex_{H_2O} = 1 - \frac{y_{O_2}^{wet}}{y_{O_2}^{in}} \qquad [\%] \qquad (15)$$

At the next step the inert gas ratio, Ra_{inert} was calculated. The fraction of the inert gas of the in-gas was divided by the fraction of the inert gas of the off-gas. The major part

of the inert gas was nitrogen but here were meant all gases except oxygen, carbon dioxide and water vapour.

$$Ra_{inert} = \frac{1-\frac{y_{O_2}^{in}+y_{CO_2}^{in}}{100}}{1-(\frac{y_{O_2}^{out}+y_{CO_2}^{out}}{100}+ex_{H_2O})} \quad [\,] \quad (16)$$

By considering the Ra_{inert} the off-gas flow, $\dot{F}_{out}$ could be calculated according equation (17).

$$\dot{F}_{out} = \dot{F}_{in} * Ra_{inert} \quad [\text{sL h}^{-1}] \quad (17)$$

Further the volume flow of consumed oxygen and produced carbon dioxide was calculated by multiply the $\dot{F}_{out}$ with the partial gas fractions (equation (18) or (19)). After, the volume flow was transformed into the mol or Cmol flow with the ideal gas equation (20).

$$\dot{V}_{O_2} = \dot{F}_{out} * \frac{y_{O_2}^{in} - y_{O_2}^{out}}{100} \quad [\text{L h}^{-1}] \quad (18)$$

$$\dot{V}_{CO_2} = \dot{F}_{out} * \frac{y_{CO_2}^{out} - y_{CO_2}^{in}}{100} \quad [\text{L h}^{-1}] \quad (19)$$

$$\dot{n}_i = \frac{p\dot{V}_i}{RT} = \frac{101325\ Pa * \frac{\dot{V}_i}{1000}}{8.3145\ \frac{J}{mol * K} * 273.15\ K} \quad [\text{mol h}^{-1}] \text{ or } [\text{Cmol h}^{-1}] \quad (20)$$

At least the $\dot{n}_{CO_2}$ was divided by the reactor volume, V_R to get the right unit for the C-balance.

$$\dot{n}_{CO_2,V} = \frac{\dot{n}_{CO_2}}{\frac{V_R}{1000}} \quad [\text{Cmol h}^{-1}\ \text{L}^{-1}] \quad (21)$$

11.10.3 Isobutanol production rate

To calculate the volumetric isobutanol production per time in a chemostat process, the isobutanol concentrations of two timepoints were subtracted und divided by the time difference, before multiplied by the dilution rate (equation (22)).

The specific isobutanol production rate was the volumetric divided by the CDW, shown in equation (23).

$$r_{Isobutanol} = \frac{c_{2_{Isobutanol}} - c_{1_{Isobutanol}}}{t_2 - t_1} * D \qquad [g\ L^{-1}\ h^{-1}] \qquad (22)$$

$$q_{Isobutanol} = \frac{r_{Isobutanol}}{c_{CDW}} \qquad [g\ L^{-1}\ h^{-1}] \qquad (23)$$

11.10.4 Substrate uptake rate

The Substrate uptake rate in the Batch phase was calculated similar to the production rate. Here the substance became less. For this reason, the later concentration was subtracted from the prior.

$$r_{Substrate} = \frac{c_{1_{Substrate}} - c_{2_{Substrate}}}{t_2 - t_1} \qquad [g\ L^{-1}\ h^{-1}] \qquad (24)$$

11.10.5 Yields

The yields were given in Cmol Cmol^{-1} and calculated with the Cmol flow ($\dot{n}_{C_i}$) in Cmol L^{-1} h^{-1} of the desired substance divided by the uptaken substrate ($\dot{n}_{C_{Substrate}}$) in Cmol L^{-1} h^{-1}. Alternative to equation (25) or (26) other yields could be calculated equally.

$$Y_{X/S} = \frac{\dot{n}_{C_{CDW}}}{\dot{n}_{C_{Substrate\ In}} - \dot{n}_{C_{Substrate\ Out}}} \qquad [Cmol\ Cmol^{-1}] \qquad (25)$$

$$Y_{P/S} = \frac{\dot{n}_{C_{Isobutanol}}}{\dot{n}_{C_{Substrate\ In}} - \dot{n}_{C_{Substrate\ Out}}} \qquad [Cmol\ Cmol^{-1}] \qquad (26)$$

12 Results and Discussion

At first the created library was screened to find the most producing construct. Therefore, a serum bottle experiment was performed. Based on this data, a batch process was run to observe the behaviour of the most promising strain-construct combination in a bioreactor.

Furthermore, four chemostat processes with a vector control and the most promising construct combined with W, KOW4 and AW, respectively were performed simultaneously. In this experiment the behaviour during a chemostat process was proved and various aeration strategies were tested.

The temperature dependence and the influence of the absence of oxygen were estimated in serum bottle experiments. In another serum bottle experiment the new knock-out strain, KOW5 was compared to the common strains. In this experiment the temperature dependence was proved again. Additionally, different initial glucose concentrations were tested.

Finally, a chemostat process was performed to compare the new KOW5 with the KOW4. During the process microaerophilic conditions were maintained and the temperature was lowered. Additionally, the evaporation of isobutanol was estimated by interposing a wash bottle in the off-gas stream.

12.1 Screening of the construct library

In Table 10 the compositions of the tested constructs including the desired promoter are shown. The promoters are part of the Anderson promoter library. BB3-6 was performed with a pool of J23109 and J23114. By sequencing the shown composition was determined.

Table 10: Construct composition with the used promoters

construct	alsS	butB	ilvC	ilvC_mut	ilvD	kdcA	adhA	adhA_mut
BB3-6	J23109	-	-	J23109	J23109	J23114	-	J23109
BB3-7	J23109	-	J23109	-	J23109	J23109	J23109	-
BB3-8	J23109	-	-	J23109	J23109	J23109	-	J23109
BB3-10	-	J23114	-	J23114	J23114	J23114	-	J23114
BB3-11	-	J23114	J23109	-	J23109	J23109	J23109	-
BB3-12	-	J23114	-	J23109	J23109	J23109	-	J23109
BB3-15	-	J23109	J23109	-	J23109	J23109	J23109	-
BB3-16	-	J23109	-	J23109	J23109	J23109	-	J23109

The construct screening was performed at a working volume of 20 mL in serum bottles. In former experiments, performed by another master student of this project in shake flasks. Evaporation was noticed by the characteristic smell of isobutanol. Due to this reason the shake flask experiments were performed in serum bottles to minimize the evaporation of the product. DeLisa with 20 g L^{-1} glucose and an addition of 50 mg L^{-1} kanamycin is used as media. The serum bottles are cultivated for 48 h at 37°C and 200 rpm in triplicates. The following results are given as an average of the triplicates. Eight constructs are screened in three different host strains (wild type, KOW4 and

adapted wild type). Additionally, an empty vector control plasmid was used as a control in each strain.

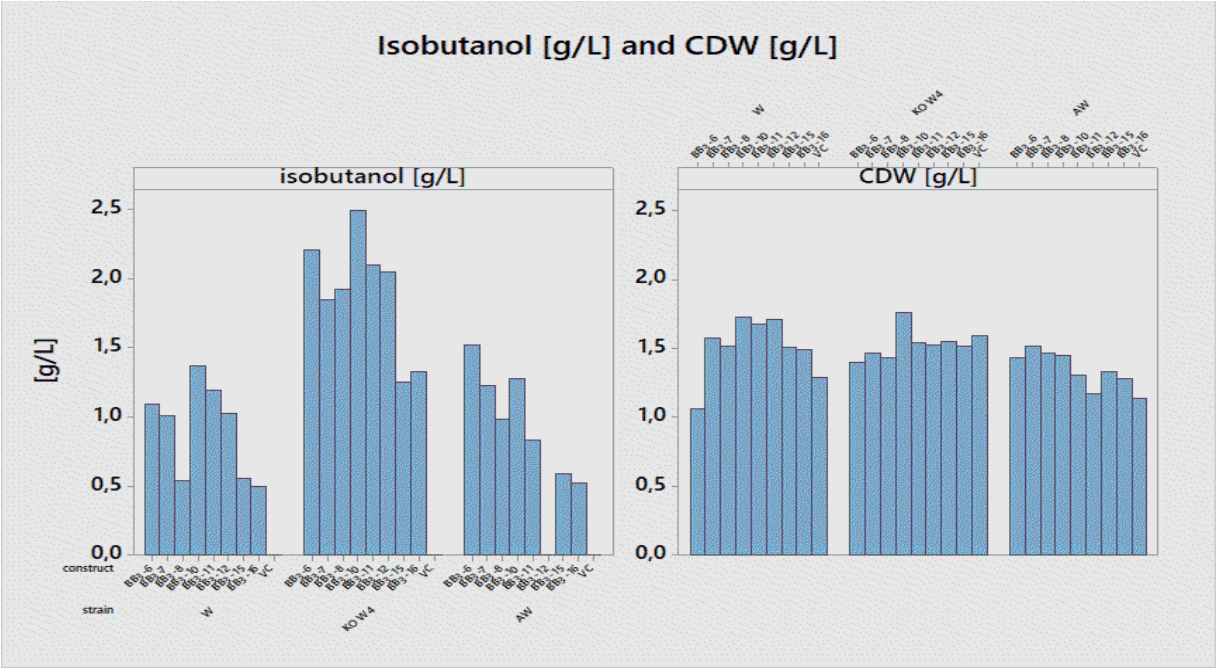

Figure 5: Screening of the construct library – Isobutanol [g L^{-1}] (left) and CDW [g L^{-1}] (right) production of each construct in W, KOW4 and AW after 48 h

In Figure 5 the isobutanol and CDW production after 48 h are shown. The CDW is around 1.5 g L^{-1} and similar to each construct and strain. But there is a massive difference in the production of isobutanol as seen in Figure 5 in the left panel. The highest titers were found in culture broths of KOW4. The best construct with the highest isobutanol concentration was BB3-10 with 2.5 g L^{-1}. This construct was also the best producer at W and had the second highest titer of AW. But nearly all constructs of KOW4 produced more isobutanol than the other strains. AW BB3-12 did not produce any isobutanol. That can be a hint that the plasmid or parts of the plasmid got lost. The VC did not produce any product as expected.

The product titers are so low that isobutanol is not toxic to the cells. Therefore, the adapted strain had not been useful at this timepoint.

If the product yields are calculated, the pattern of the graph seems similar (Figure 6). Most of the KOW4 had a higher yield than the other strains. BB3-6, BB3-8 and BB3-10 have the highest yield with 0.20 Cmol $Cmol^{-1}$. By comparing the isobutanol production in Figure 5 and the isobutanol yield in Figure 6, combinations with the highest titer, did not show the highest yield automatically. Because the VC of each strain did not produce any isobutanol, the yield also became zero. This is also held for AW BB3-12.

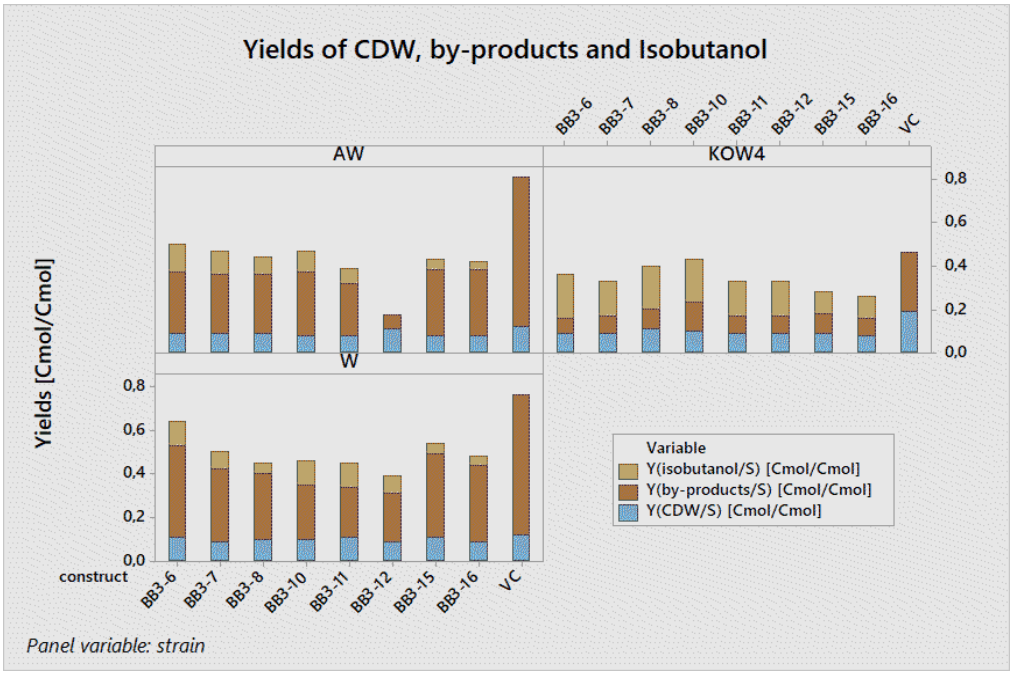

Figure 6: Screening of the construct library – Yields per Substrate of CDW, by-products and isobutanol in Cmol Cmol^{-1}

But not only isobutanol was generated, also ethanol and acids like succinate, lactate, formate and acetate were produced. In Figure 6 the yield of by-products is also shown. The VC of W and AW produced the highest amount of by-products relative to the substrate. The lowest acid to substrate yields were found at KOW4. In this strain four knockouts were performed, one of it was the lactate dehydrogenases A. For that reason, these organisms produce hardly any lactate, which reduces the by-product yield. Therefore, the VC of the KOW4 differs from the other VCs because here the construct has less influence than the strain itself. As determined in previous graphs at the AW BB3-12 something went wrong.

The theoretical yield of isobutanol from glucose in Cmol Cmol^{-1} is 61.7 %. Unfortunately, the highest product/substrate yields which were achieved, were around 20 % (Figure 6). In Figure 7 the achieved percentage of the theoretical yield is shown. Of cause the pattern looks similar to the product yield in Figure 6. KOW4 BB3-6, BB3-8 and BB3-10 achieved almost 30 % of the theoretical yield.

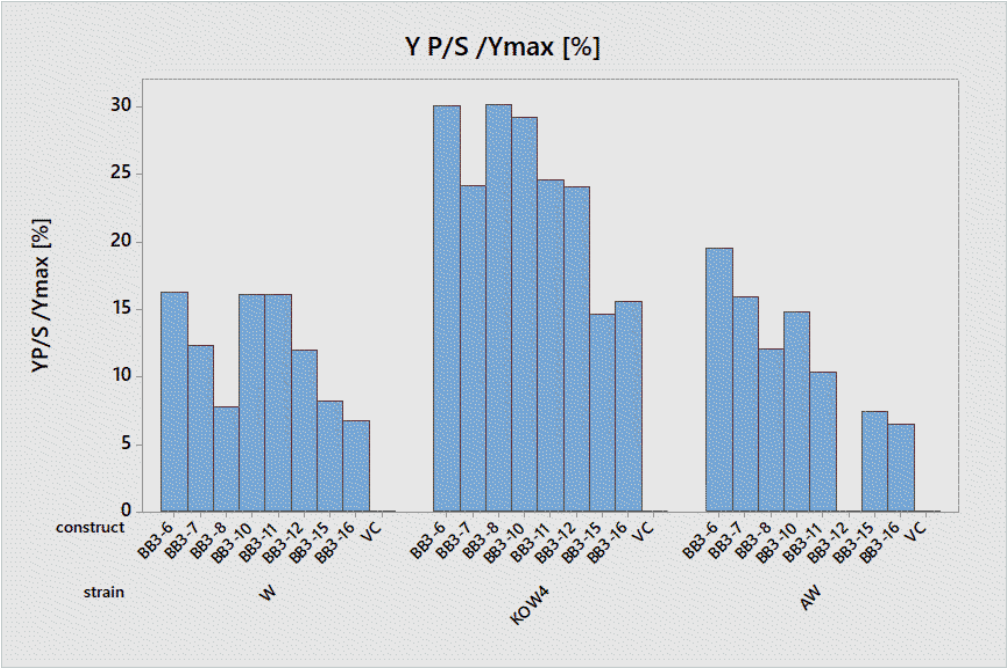

Figure 7: Screening of the construct library – percentage of the achieved Yield [Cmol Cmol^{-1}] compared to the theoretical Yield [%]

Based on this data, the most promising strain is KOW4 because the growth does not seem to be influenced by the knockouts and this strain achieved the highest product/substrate yields. The high potential constructs are BB3-6 and BB3-10. Due to the higher isobutanol concentration in culture broth of BB3-10, KOW4 BB3-10 is the highest promising clone.

12.2 Batch Bioreactor

The construct screening in serum bottles showed the strain KOW4 to be the best producer. In combination with construct BB3-10 an isobutanol concentration of 2.5 g L^{-1} was obtained. For that reason, this candidate was chosen to perform a batch cultivation in a bioreactor with 1 L working volume to further characterize the strain under defined conditions of a bioreactor.

For this first bioreactor experiment the stirrer speed was set at 400 rpm and the aeration at 0.1 vvm with air. At a temperature of 37°C the pH was kept at 7.0 with NH_3 12.5 %.

As shown in Figure 8 after 12.5 h the glucose was depleted. The isobutanol concentration in the cultivation broth was only 1.7 g L^{-1}. In comparison to the serum bottle experiment, the tier was 32 % lower and a yield of 0.20 Cmol Cmol^{-1} was obtained. On the other hand, the CDM at the end of the batch is higher than in the serum bottles. That indicates that the oxygen input is higher in the bioreactor than in the serum bottles, where the oxygen becomes limited at the end. Acetate is the metabolite with the highest concentration in the end. Succinic acid, lactic acid and ethanol are hardly produced. Only formic acid is generated considerably beside of acetic acid, as shown in Figure 8.

The values of acetic acid after 8 h and 12 h are deleted because they are not realistic (values shown in Table 12).

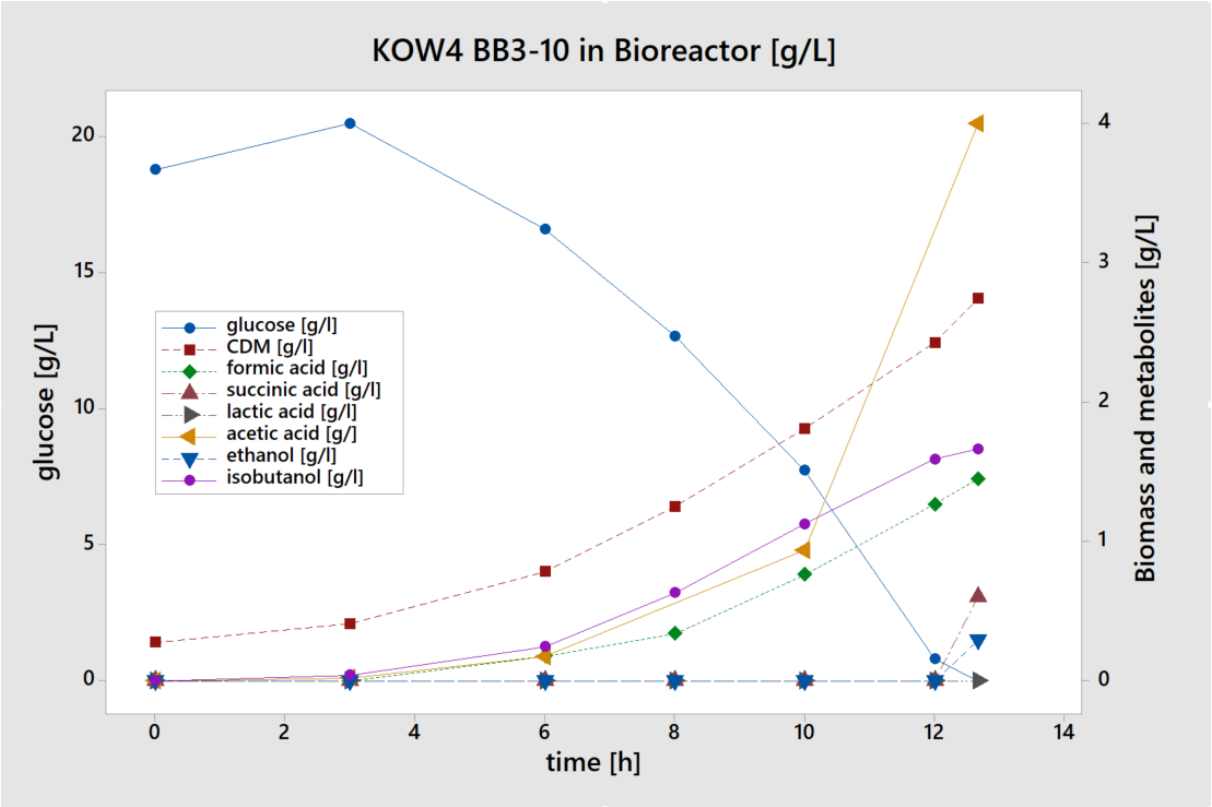

Figure 8: Batch Bioreactor KOW4 BB3-10

The isobutanol production rate is decreased in the end of cultivation (Table 11). That in combination of the characteristic smell of isobutanol indicates that the product is stripped out.

In Table 11 also the Yields of CDW, by-products and isobutanol in Cmol Cmol-1 are shown with the consequent recovery in %. The yields and the glucose uptake rate after 3 h are negative because the glucose concentration is higher than at the beginning. That indicates a measurement failure. The low recovery is generally ascribed to the lack of off-gas CO2 measurement in this experiment. Additionally, after 8 h and 12 h the acetate concentration was too low, as described above, which results in a low by-product yield and consequently a low recovery. It is suggested that also the evaporation of the isobutanol lowers the C-recovery.

Table 11: glucose uptake rate, isobutanol production rate in mmol h^{-1} and the Yields of CDW, by-products and isobutanol in Cmol $Cmol^{-1}$ with consequent Recovery in %

time [h]	r_glucose [mmol h^{-1}]	r_isobutanol [mmol h^{-1}]	Y_{CDW} [Cmol $Cmol^{-1}$]	$Y_{by\text{-}products}$ [Cmol $Cmol^{-1}$]	$Y_{isobutanol}$ [Cmol $Cmol^{-1}$]	Recovery [%]
3,00	-91,428	3,004	-0,367	-0,014	-0,049	-44,538
6,00	275,078	14,224	0,345	0,103	0,141	69,133
8,00	405,627	39,475	0,219	0,033	0,144	42,860
10,00	490,998	48,395	0,179	0,114	0,146	55,344
12,00	649,417	43,376	0,151	0,043	0,129	36,645
12,67	221,822	11,386	0,161	0,280	0,127	84,861

It was suggested that the oxygen input is the key of isobutanol production, because it becomes increased by decreasing dissolved oxygen level in the end of the batch process. In the next step defined microaerobic conditions were proven. For that reason, a chemostat process was performed.

12.3 Impact of oxygen on isobutanol and by-product production

After the last experiment it was assumed that most isobutanol is produced under microaerobic conditions. Because of the exponential increasing cell density in the batch phase, there is a lack at oxygen when the dissolved oxygen reaches zero percent. For that reason, the oxygen is the limiting factor in growth. Additionally, in the reports of Atsumi et al. (2010b) and Chen and Liao (2016) is suggested that only a minor portion of the produced isobutanol is generated in the growth phase.

As next step W, KOW4 and AW containing BB3-10 are compared by running a chemostat process with decreasing oxygen concentrations in the in-gas flow to reach defined microaerobic conditions.

In Figure 5 is seen that the construct BB3-10 achieved the highest isobutanol titer in W and KOW4 and the second highest titer in AW. Additionally, a VC in strain was used. To gain high amounts of biomass in the batch phase, the high aeration is necessary. It was suggested that strong aeration lowers the isobutanol titer because of evaporation. To be able to compare the isobutanol production under the desired conditions the aeration is fixed on 1 vvm or 0.1 vvm because of the mentioned gas stripping effect. At first all conditions with 1 vvm are tested before the gassing was changed to 0.1 vvm. In theory the lower aeration rate should yield a higher isobutanol concentration in the culture broth. The dilution rate (D) was adjusted on demand depending on the glucose concentration. If the fed glucose accumulated the dilution rate was lowered.

In Figure 9 the isobutanol productivities in mmol L^{-1} h^{-1} are shown at the different conditions. The suggestion, that isobutanol is produced by reducing the oxygen in the ingas confirmed. The strain W with the construct BB3-10 started producing isobutanol at 1 % oxygen with an in-gas flow of 1 vvm, that is equal to 0.01 vvm oxygen. But at 5 % and 0.1 vvm, equal to 0.005 vvm oxygen, there is no production of isobutanol. With an aeration of 0.1 vvm the production started at 0.5 %. The highest concentration was achieved at 0.1 % and 0.1 vvm.

The cell density became very low at conditions with low oxygen concentrations in the in-gas. But the productivity was better. This phenomenon is shown in Figure 10 where the specific productivity is shown in mmol isobutanol per g CDW per hour. That underlines the thesis of Atsumi et al. (2010b) and Chen and Liao (2016).

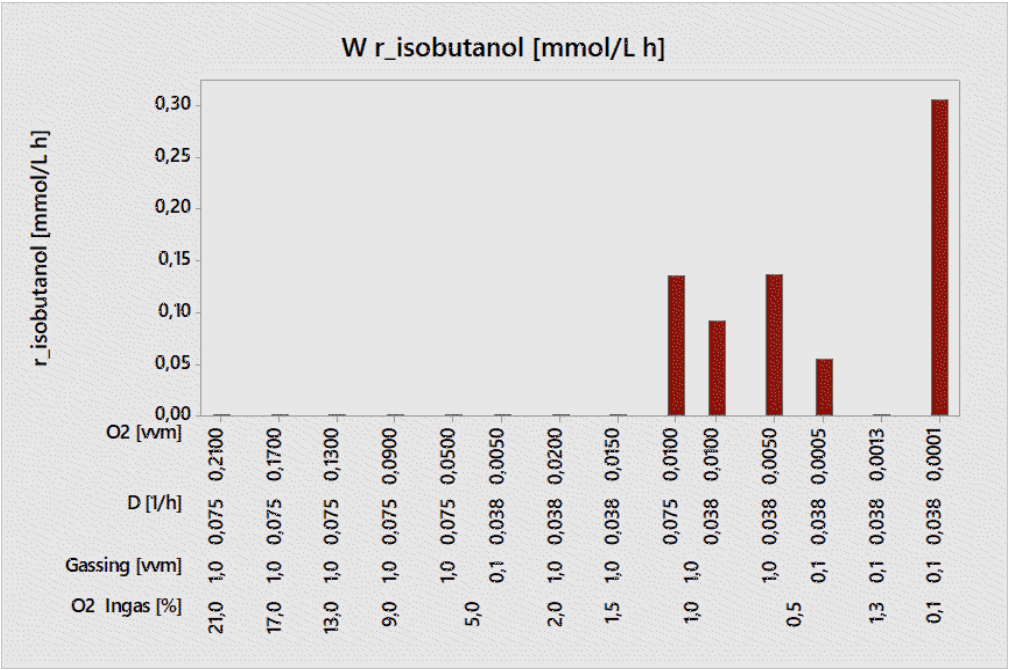

Figure 9: Impact of oxygen on isobutanol and by-product production - W r_isobutanol [mmol L^{-1} h^{-1}]

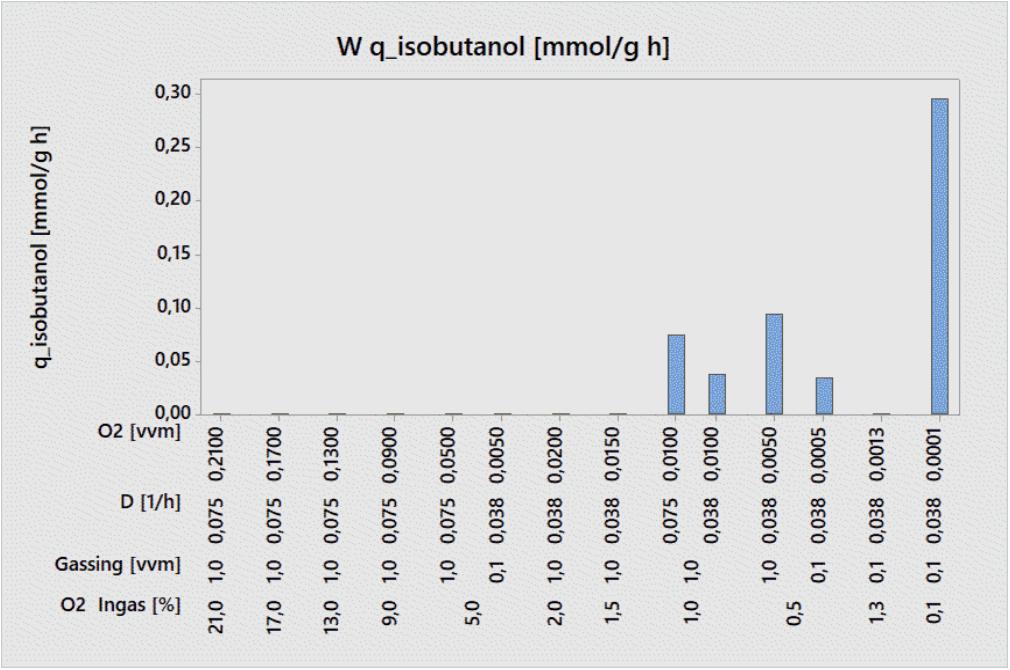

Figure 10: Impact of oxygen on isobutanol and by-product production - W q_isobutanol [mmol g^{-1} h^{-1}]

The strain KOW4 with the same construct started to produce isobutanol at an aeration of 1.0 vvm and 1.5 % (0,015 vvm oxygen) and at 0.1 vvm and 5 % (0.005 vvm oxygen), seen in Figure 11, which is earlier than W. Higher oxygen amounts at 0.1 vvm were not tested. At the condition 0.1 vvm and 1.3 % there is not any isobutanol produced, because there are troubles of getting washed out before and it is possible that the process had not been in steady state yet.

At this experiment not the lowest oxygenation achieved the highest titer, but the condition with 1 vvm, 1 % oxygen and a dilution rate of 0.075 h^{-1}. But by taking a look at Figure 12 which shows the specific production, it can be derived, that the cell density is higher at his condition than at 0.0001 vvm oxygen. Due to this reason, the specific productivity is higher at the condition with the lowest oxygen input. It was very difficult to hold the cell density constant at this setpoint, because the growth rate of KOW4 is lower than the growth rate of W and it was washed out earlier. So, on the one hand there was a higher product titer at lower oxygen levels but on the other hand the growth rate was so low at these conditions, that the cells became washed out. That is why an optimum must be found to run a chemostat. Of course, it is possible to reduce the D but then the media composition must also be changed because the glucose and maybe other components are already run out at lower dilution rate and the production would become very slow. Additionally, it was recognized, that if the oxygen reduction happens too fast, this strain cannot adapt itself to the microaerobic conditions fast enough and get thinned out. Conseuently, it was necessary to increase the oxygen amount from 1 % to 5 % at 1 vvm again. After stabilizing it was reduced to 1.5 %. This range of aeration is the crucial point for isobutanol production.

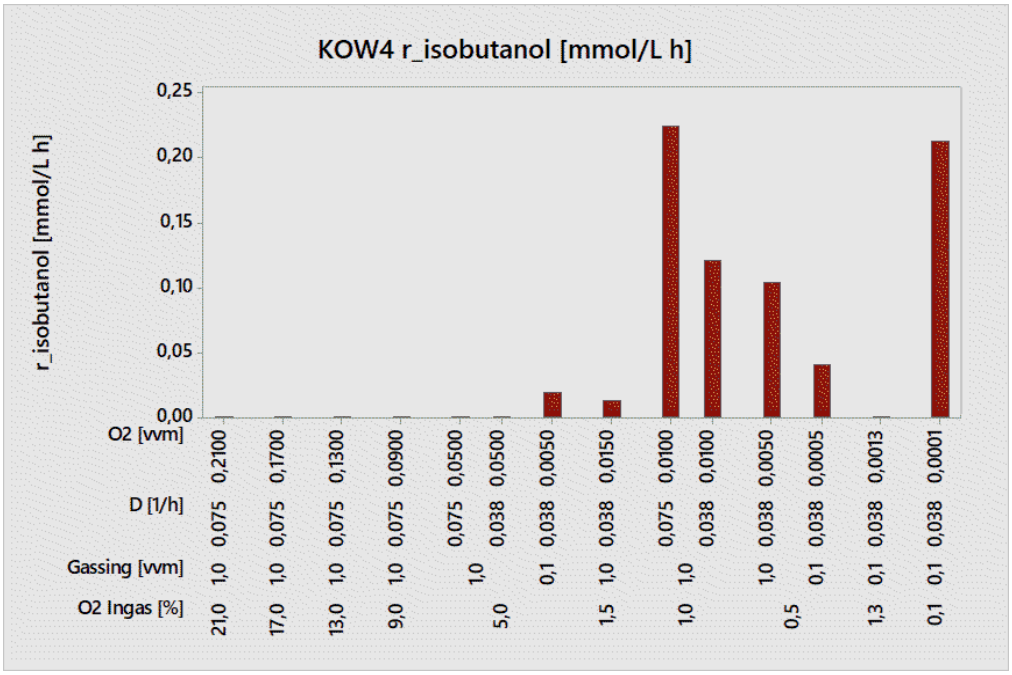

Figure 11: Impact of oxygen on isobutanol and by-product production - KOW4 r_isobutanol [mmol L^{-1} h^{-1}]

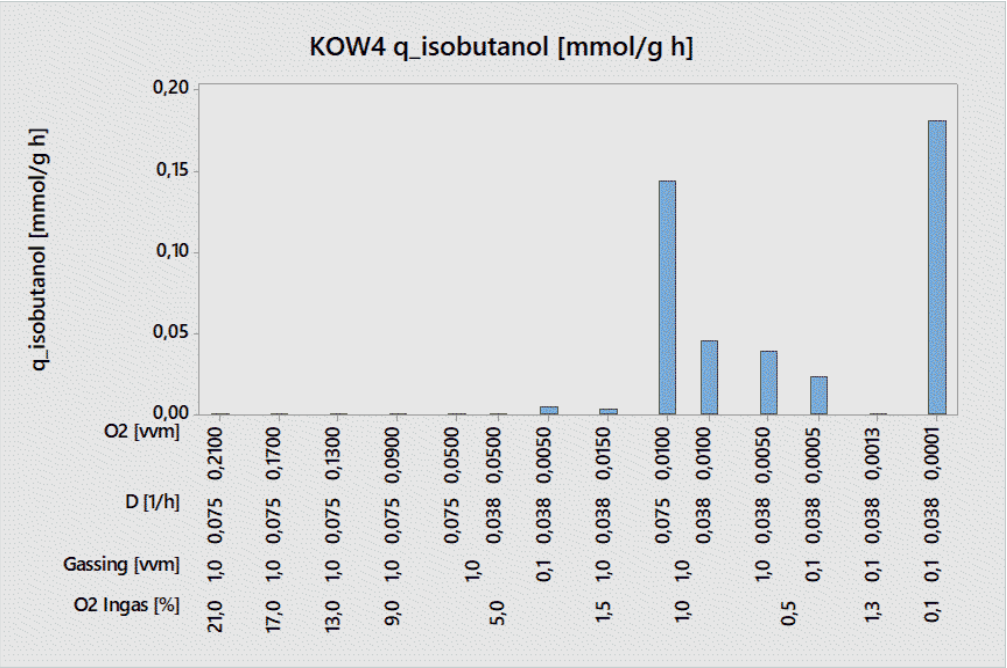

Figure 12: Impact of oxygen on isobutanol and by-product production - KOW4 q_isobutanol [mmol g^{-1} h^{-1}]

There was not any isobutanol produced by the adapted strain during the whole process. The strain could not lose the whole plasmid because there is a kanamycin resistance on it, but it obviously lost a part of it. Therefore, the isobutanol production was not possible.

Also the vector control (VC) did not produce any isobutanol, but here it was expected.

The C-Recovery of each bioreactor is the sum of the yields at a certain condition. The calculated yields are form cell dry mass, succinic acid, lactic acid, formic acid, acetic acid, ethanol, isobutanol and CO_2. The C-Recovery in this experiment is hardly achieved 100 %, equal to a sum of all yields of 1. That can indicate measurement failures, that the processes had not been in steady state yet at the timepoint of sampling or a combination of both.

At the beginning at an aeration of oxygen content of 21 % only biomass and CO_2 were produced until reaching 5 % oxygen. That phenomenon is similar at each bioreactor except the VC started to produce a small amount of acids at 9 %. Until to this point the C-Recovery is similar and a little bit too low. Maybe biomass got lost by washing the cells for CDW or the off-gas calculation is not exact enough. The ex_{H_2O} was calculated from numbers with only one decimal place.

W started to produce isobutanol at 1 % and 1 vvm and the biomass yield become smaller, shown in Figure 13. The main produced acid is acetic acid. Acid production started at 2 % and 1 vvm except lactic acid at 1 % like ethanol. The characteristic smell of isobutanol appeared simultaneously with its determination. This is a hint that isobutanol is stripped out by gassing. By reducing the gassing to 0.1 vvm, it was expected that the behaviour of the equivalent conditions like 0.5 % 1 vvm and 5 %

0.1 vvm are equal. But at condition 0.5 % 1 vvm isobutanol was produced but not at 5 % 0.1 vvm. At the time point of shifting the gas flow, the culture is already 15 d old. Therefore, mutations are possible because of the amount of generations.

In contrast to others 0.1 and 0.5 % at 0.1 vvm have a C-Recovery over 100 % because it was fed with media, containing only 13.3 g L^{-1} glucose and possibly the process had not been in steady state yet. The highest titer of W was achieved at the lowest oxygen input and reached 0.66 g L^{-1}. At this condition the CDW was very little.

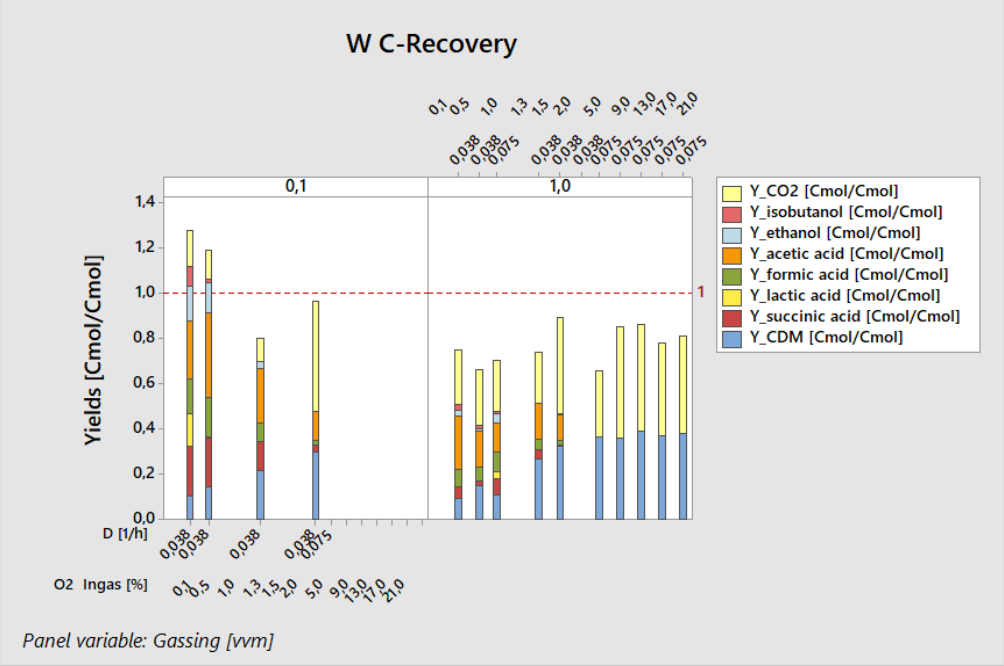

Figure 13: Impact of oxygen on isobutanol and by-product production - W C-Recovery

The pattern of the KOW4 C-Recovery, in Figure 14 looks like W. A main difference is that no lactic acid was produced except the end of the experiment at very low oxygen conditions at 0.1 vvm, there were measured concentrations about 7.4 g L^{-1}. The reason for that is obviously a mutation. This strain grows slower than W, but the production was higher in serum bottles. At 1 %, 1 vvm and a D of 0.075 h^{-1} the culture was already thinning out. When the CDW fell under 1 g L^{-1} and glucose starts to accumulate, the D was set at 0.038 h^{-1} and the oxygen amount increased to 5 % to recover the cell density. After getting in steady state, the oxygen content was lowered slower to give the bacteria time to adapt to the microaerobic conditions. In this way it worked.

Isobutanol was produced starting at 1.5 % 1 vvm and 5 % 0.1 vvm. So KOW4 starts the isobutanol production earlier than W. It was very difficult to control this process at very low oxygen amounts at 0.1 vvm because of at 1.3 % 0.1 vvm no isobutanol was generated. It was a constantly competition between cell growth and isobutanol production. Under constant conditions the process hardly reached the steady state. Additionally, the culture was 15 d old at the dilution rate shift. Therefore, the isobutanol

concentration achieved only a maximum of 0.58 g L^{-1} (not in steady state!) in the chemostat after washing out the product generated in batch.

Also, here the media of the conditions contained 0.1 and 0.5 % oxygen and 0.1 vvm a lower glucose concentration (15.8 g L^{-1}).

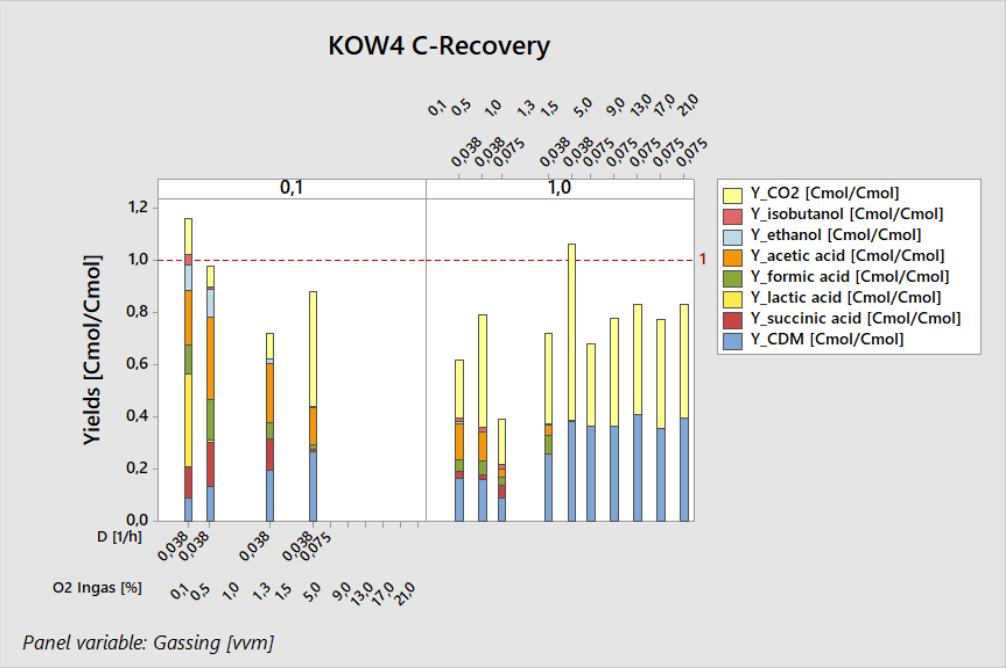

Figure 14: Impact of oxygen on isobutanol and by-product production - KOW4 C-Recovery

Unfortunately, AW did not produce any isobutanol. As shown in Figure 15, at high oxygen inputs only biomass and CO_2 are produced. The acid formation started at 2 % and 1 vvm and ethanol formation at 1 % oxygen at the same gas flow. Also, here it was very hard to keep the process constant. For this reason, the conditions with low oxygen amounts at 1 and 0.1 vvm are hardly in steady state. Here the conditions 0.1 and 0.5 % at 0.1 vvm were fed with DeLisa media which contained only 13.8 g L^{-1} glucose. The lactic acid formation in the end at 0.1 % 0.1 vvm was enormous and achieved nearly 10.0 g L^{-1}. This condition was obviously not in steady state.

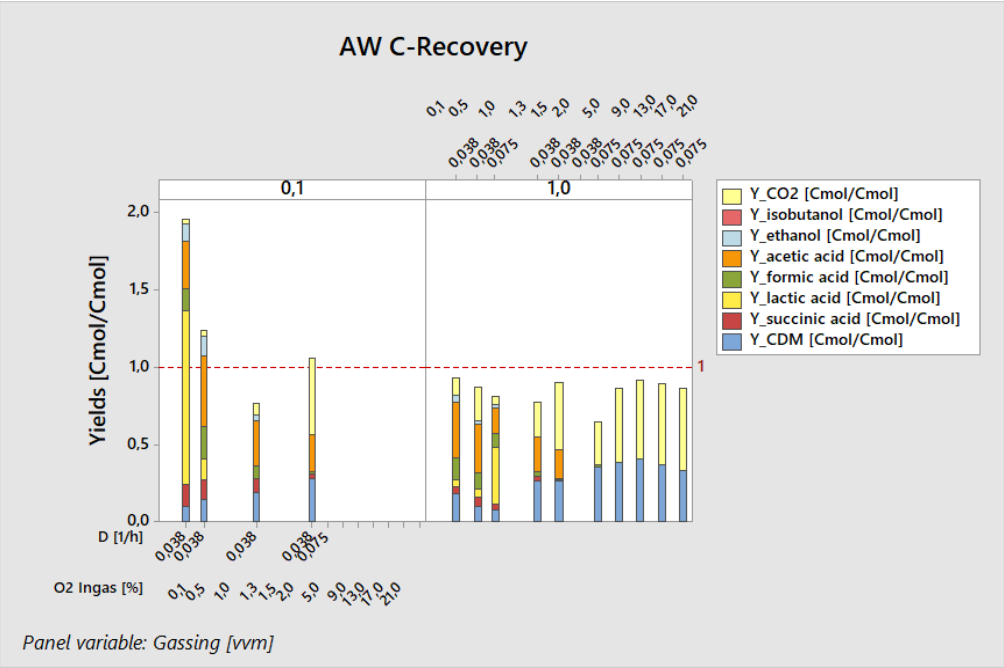

Figure 15: Impact of oxygen on isobutanol and by-product production - AW C-Recovery

The vector control did as expect, shown in Figure 16. At high oxygen rates only biomass and CO_2 were produced and at 5 % 1 vvm formic acid and acetic acid started to be produced. At 2 % succinic acid production was added and at even lower oxygen amounts lactic acid and ethanol were generated. A similar pattern was shown at 0.1 vvm. The CDW was decreasing by lowering the oxygen input and the lower the CDW the lower the CO_2 production. This is the only process were the conditions, 0.1 and 0.5 % at 0.1 vvm were run with a medium with around 20 g L^{-1} glucose.

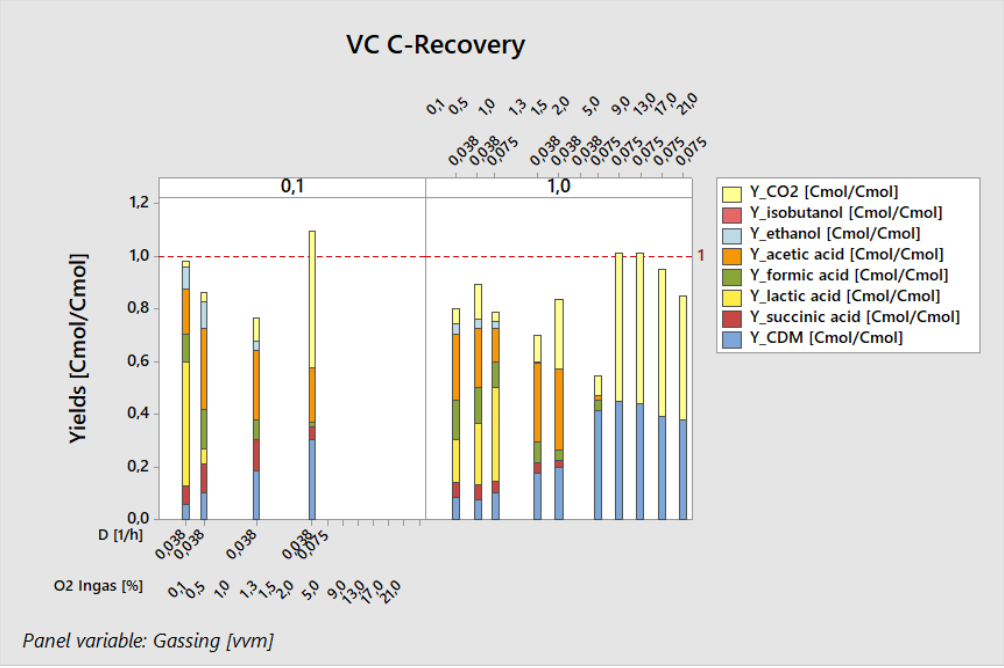

Figure 16: Impact of oxygen on isobutanol and by-product production - VC C-Recovery

In Figure 17 to Figure 20 the dependence of CDM and isobutanol is shown at both gas flows. The lower the oxygen amount in the in-gas, the lower the CDW, but the higher the isobutanol concentration. That is why a chemostat driven process is difficult to handle because the bacteria have a very little growth rate under satisfactory production conditions. On account of this, it is suggested that most isobutanol is produced after the exponential phase.

As mentioned before, AW had not produced any isobutanol. This is seen in Figure 19 again. But the dependence of oxygen on the cell density is given here too. That also applies to the vector control, which did not generate any isobutanol too of course, that can be seen in Figure 20.

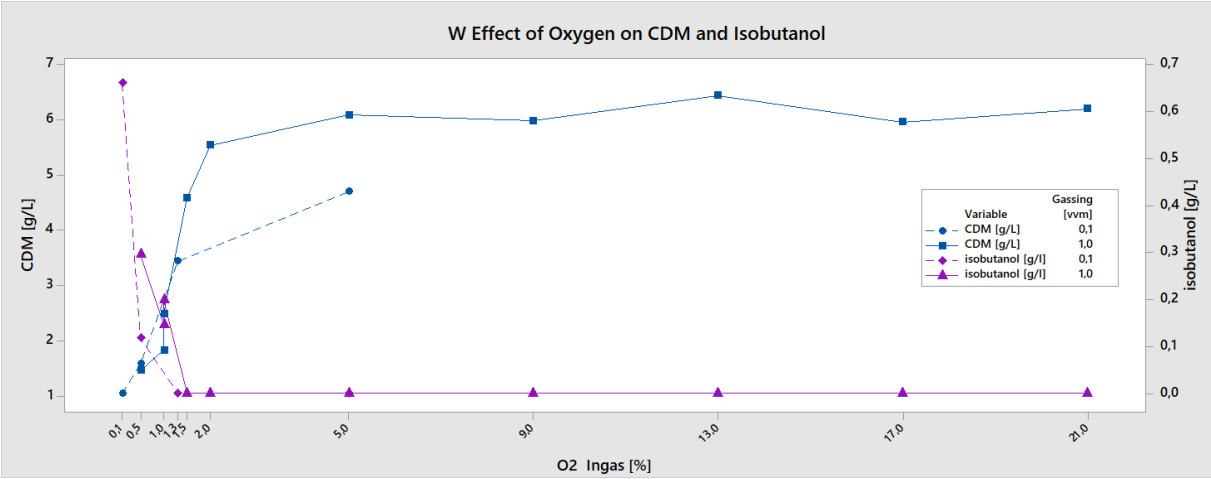

Figure 17: Impact of oxygen on isobutanol and by-product production - W Effect of Oxygen on CDM and Isobutanol

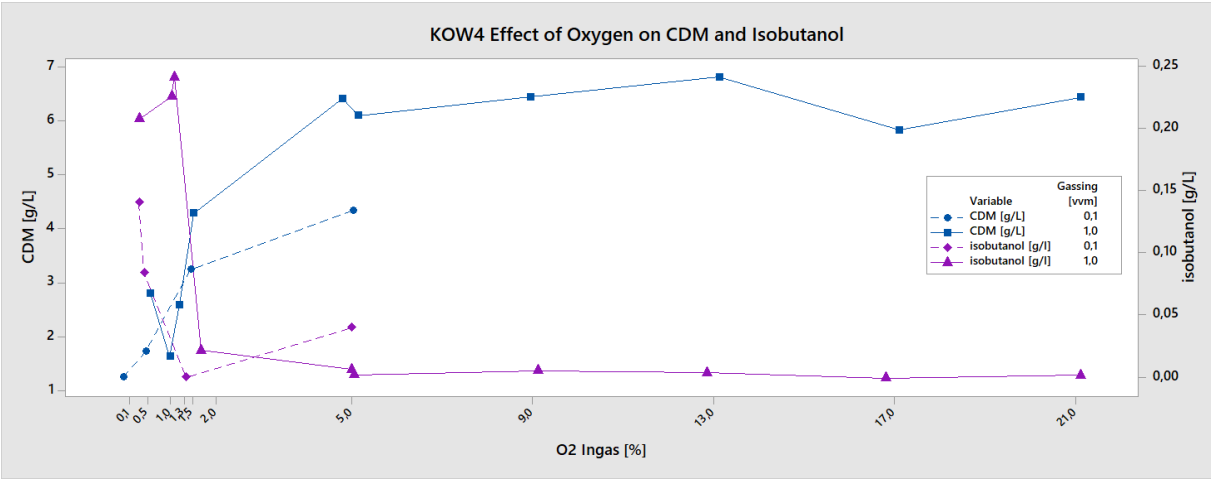

Figure 18: Impact of oxygen on isobutanol and by-product production - KOW4 Effect of Oxygen on CDM and Isobutanol

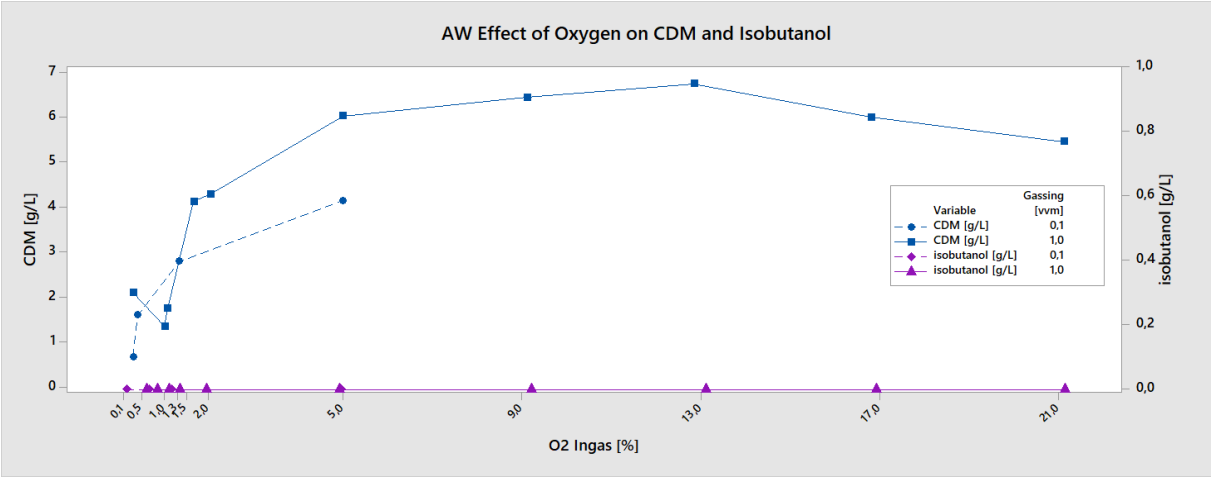

Figure 19: Impact of oxygen on isobutanol and by-product production - AW Effect of Oxygen on CDM and Isobutanol

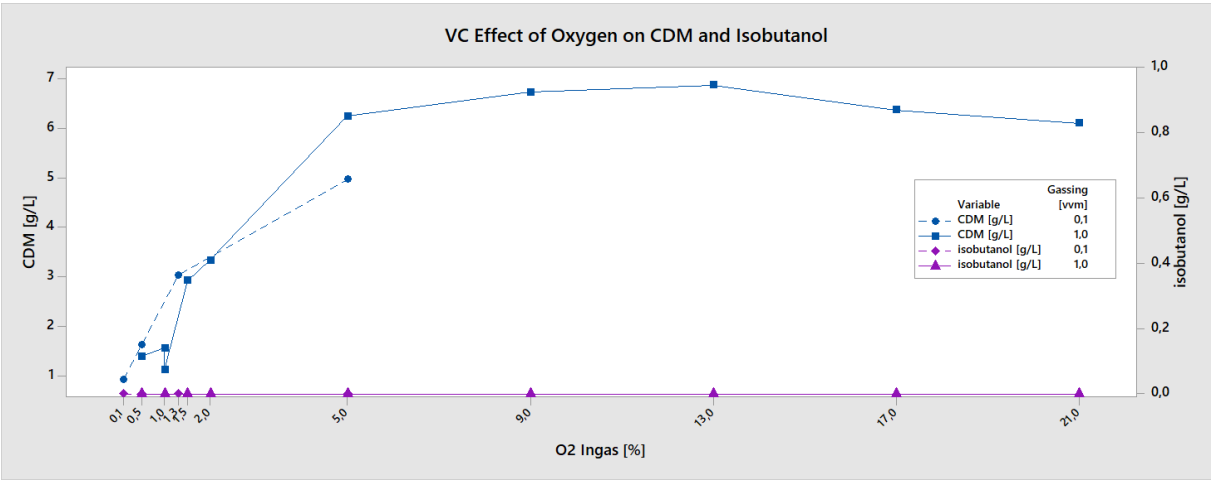

Figure 20: Impact of oxygen on isobutanol and by-product production - VC Effect of Oxygen on CDM and Isobutanol

This experiment sustains the thesis, that the isobutanol production depends on the oxygen input. But also the by-product formation increased and the growth decreased at these conditions. Unfortunately, the AW did not produce any isobutanol at all. Due to the length of this chemostat experiment it was suggested that the strains had changed during the process, which lowered the productivity.

12.4 Comparison of culture age and aerobe vs. anaerobe in serum bottles

To verify that the strains had not mutated during prolonged the chemostat cultivations, a serum bottle experiment was performed to compare the strains W and KOW4 from the original cryo well and cells from the bioreactor sampled at the end of the experiment. In this way the difference of the performance of the cultures were shown. Additionally, the same experiment was performed under aerobe as well as anaerobe conditions according the thesis, that the absence of oxygen leads to a high isobutanol production.

The atmosphere in the aerobe serum bottles consisted of air, these batches started aerobic and turned to microaerobic after using up most of the oxygen. So, at first biomass should be generated before isobutanol is produced. In the anaerobe serum bottles, air was exchanged with nitrogen. Here it is suggested that the isobutanol production starts immediately and only little amounts of glucose get lost to biomass. After 48 h a sample was taken and OD_{600} and the metabolites were measured at a HPLC.

In Figure 21 the OD_{600} measured after 48 h of cultivation are shown. The original fresh cultures, which are also used for the inoculum for the prior chemostat, are labelled as Start and the cells from the end of the chemostat are labelled as End. The fresh cells produced much more biomass at aerobic conditions. In the absence of oxygen all cultures hardly produced biomass. But most of them consumed around 40 % of the glucose, as shown in Figure 22. In contrast at aerobic conditions the fresh spread cells used more than 80 % of the glucose. The cultures from the end of the chemostat seem to be not as fit as the fresh ones. They consumed only the half of the available glucose.

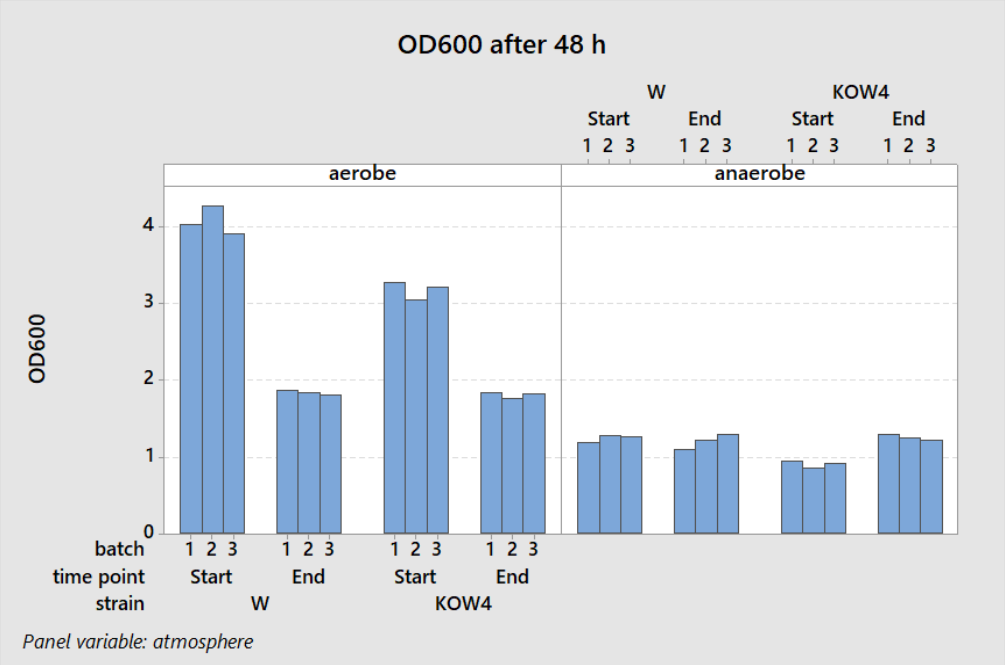

Figure 21: comparison of culture age and aerobe vs. anaerobe - OD_{600} after 48 h

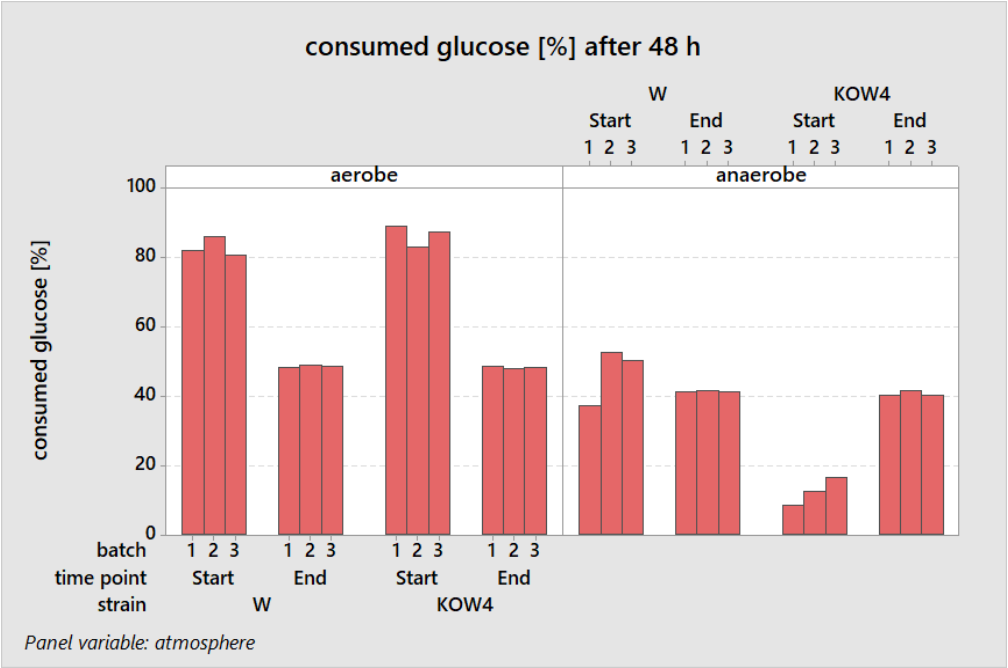

Figure 22: comparison of culture age and aerobe vs. anaerobe - consumed glucose [%] after 48 h

Because of W Start and KOW4 Start consumed nearly the same amount of glucose but the knockout strain produced only around three fourths of the biomass, so the glucose must be turned into something else. In Figure 23 is seen, that this culture produced much more isobutanol than W Start at aerobe conditions. The older cultures from the end of the chemostat did not produce any isobutanol, neither aerobe nor anaerobe. At anaerobe conditions the Start cultures both produced only small amounts of isobutanol. But the specific isobutanol production of them is higher than the specific production of W Start, shown in Figure 24. In this experiment obviously the best producer was the fresh culture of KOW4.

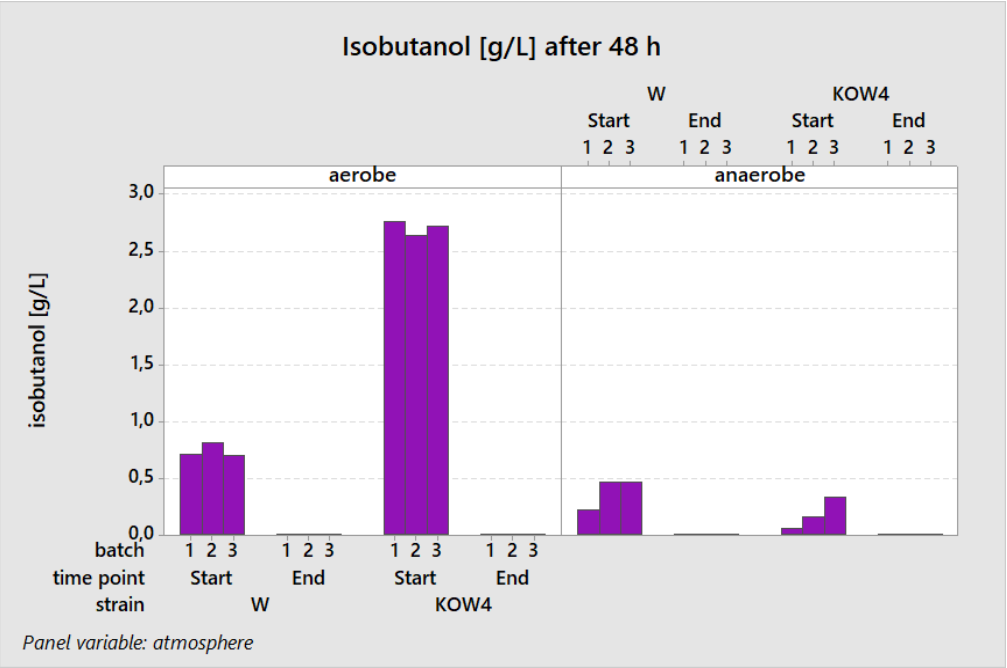

Figure 23: comparison of culture age and aerobe vs. anaerobe - isobutanol [g L^{-1}] after 48 h

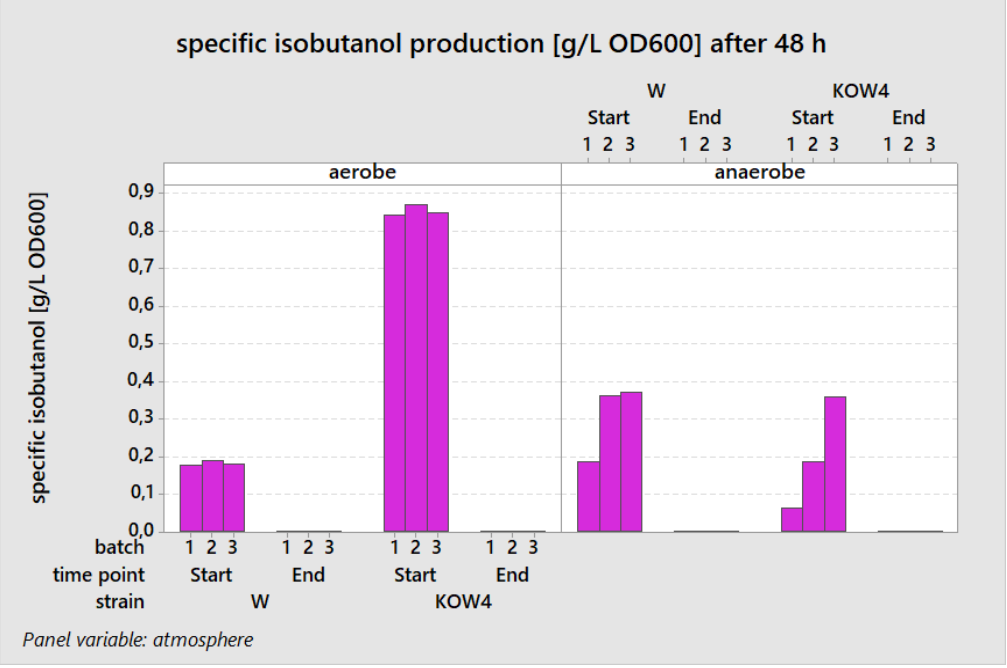

Figure 24: comparison of culture age and aerobe vs. anaerobe - specific isobutanol production [g L^{-1} OD_{600}^{-1}]

In Figure 25 the metabolic yields without CO_2 are shown. Unfortunately, the sum of the yields is above 100 %. Due to this reason there must be a mistake. By having a look at Figure 25 it can be seen, that the ethanol yield is unrealistically high. That came maybe from spraying the septum with ethanol 70 % before sampling to avoid contaminations. Therefore, the ethanol yield is deleted in Figure 26. The yields of succinic acid, formic acid and acetic acid is comparable to each strain, time point and

atmosphere. But the older cultures from the end of the chemostat produced much more lactic acid. Also, the KOW4 End which should not be able to produce any lactic acid, demonstrated on KOW4 Start, generated high amounts. The KOW4 Start primary produced isobutanol. If the biomass is considered this strain at anaerobe conditions mainly produced isobutanol. But there is a huge lack in the C-balance. The only substance containing carbon, which was not determined was CO_2. For that reason, this strain obviously had produced large amounts of it at given conditions.

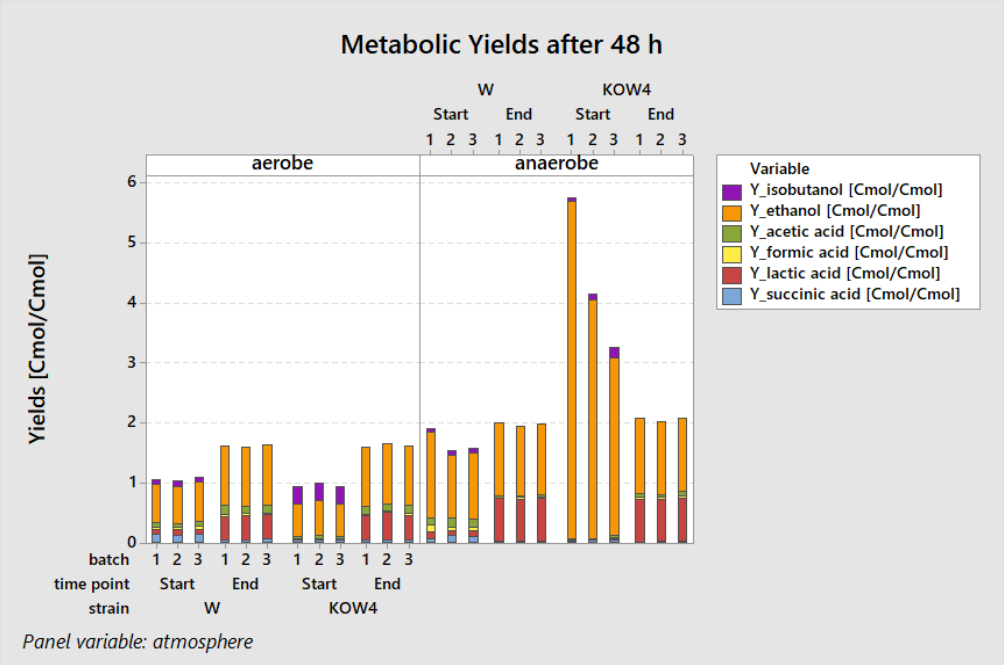

Figure 25: comparison of culture age and aerobe vs. anaerobe - metabolic yields after 48 h

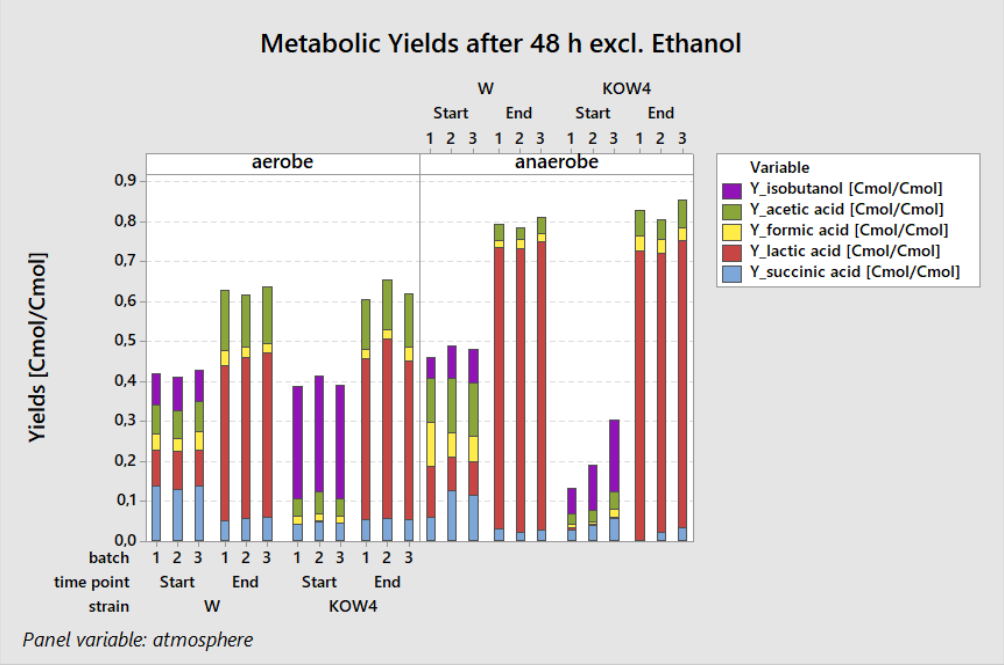

Figure 26: comparison of culture age and aerobe vs. anaerobe - metabolic yields after 48 h excl. ethanol

Summarized the KOW4 is the most promising strain at aerobe-microaerobic conditions, because of its high product yield of 0.29 Cmol Cmol^{-1}, which is nearly half of the theoretically yield, and few by-products. But it cannot grow without oxygen as shown in Figure 21.

The metabolism obviously changed over the time. A mutation cannot be excluded because no isobutanol, but high amounts of lactate was produced by the older cultures.

12.5 Comparison of 30°C vs. 37°C in serum bottles

According to the suggestion of Akita et al. (2015) and Baez et al. (2011), that the isobutanol production increases at lower temperatures, a serum bottle experiment was performed at 30°C, cultivating the strains W and KOW4 BB3-10. As reference at 37°C serve former results from the serum bottle experiment above.

Figure 27 shows the mean of triplicates with the standard deviation. The optical density, consumed glucose, produced isobutanol and the specific isobutanol after 48 h are displayed. The cell density, shown at the top on the left, was similar to the previous cultivation at 37°C. The OD_{600} of W was a little bit higher than of KOW4. But in sum the cell densities were higher at 30°C against the expectation. Nevertheless, the glucose consumption (top right) was hardly at 50 % at 30°C after 48 h, in contrast to 37°C where more than 80 % of the glucose was used up, although the glucose concentrations at the beginning were almost equal.

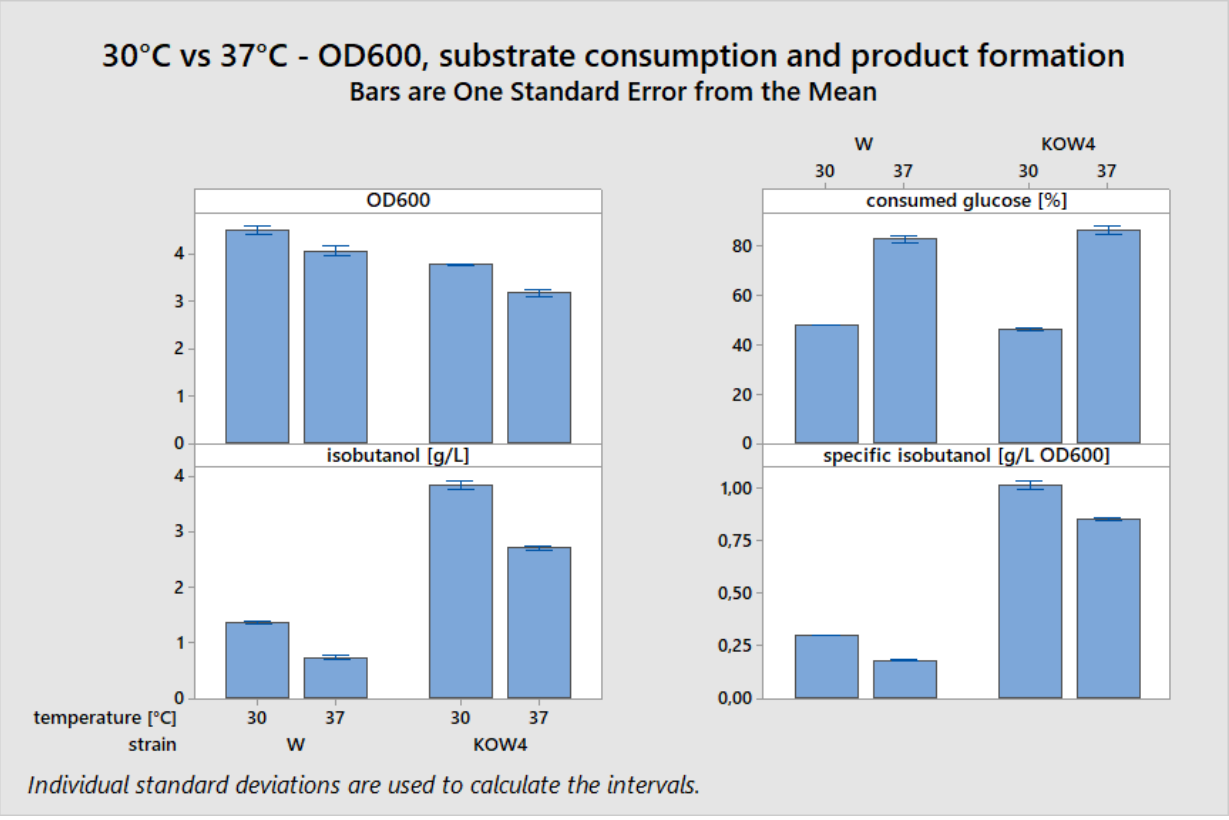

Figure 27: comparison of 30°C vs. 37°C - OD_{600}, substrate consumption [%], isobutanol [g L^{-1}] and specific isobutanol [g L^{-1} OD_{600}^{-1}] after 48 h

As predicted, the isobutanol concentration in cell broth at 30°C increased dramatically. The graph at the bottom left in Figure 27 shows an increase of 45 % by W and 30 % by KOW4. The highest concentration of nearly 4 g L^{-1}, was achieved by KOW4 at 30°C. Due to its high titer and cell density, KOW4 has a specific isobutanol production of around 1 g L^{-1} OD_{600}^{-1} at 30°C. But also the specific productivity of W was better at 30°C than at 37°C, shown in Figure 27.

In Figure 28 the yields [Cmol $Cmol^{-1}$] of the produced acids, ethanol and isobutanol are visualized. Like mentioned before, the ethanol sprayed on the septum before sampling is responsible for the high ethanol yield. For that reason, it was deleted and displayed again at Figure 29. In average the yields of the acids were a little bit higher at 30°C than at 37°C. As seen in the graph the yield of isobutanol was increased enormously. With a yield [Cmol $Cmol^{-1}$] of 75.05 %, the theoretical yield was exceeded around 20 % by KOW4 at 30°C. So there must be a mistake in measurement, probably for glucose. However, the isobutanol production was the highest we had achieved at this study yet. Also the yield of W was increased from 8.11 % to 26.53 % by lowering the temperature.

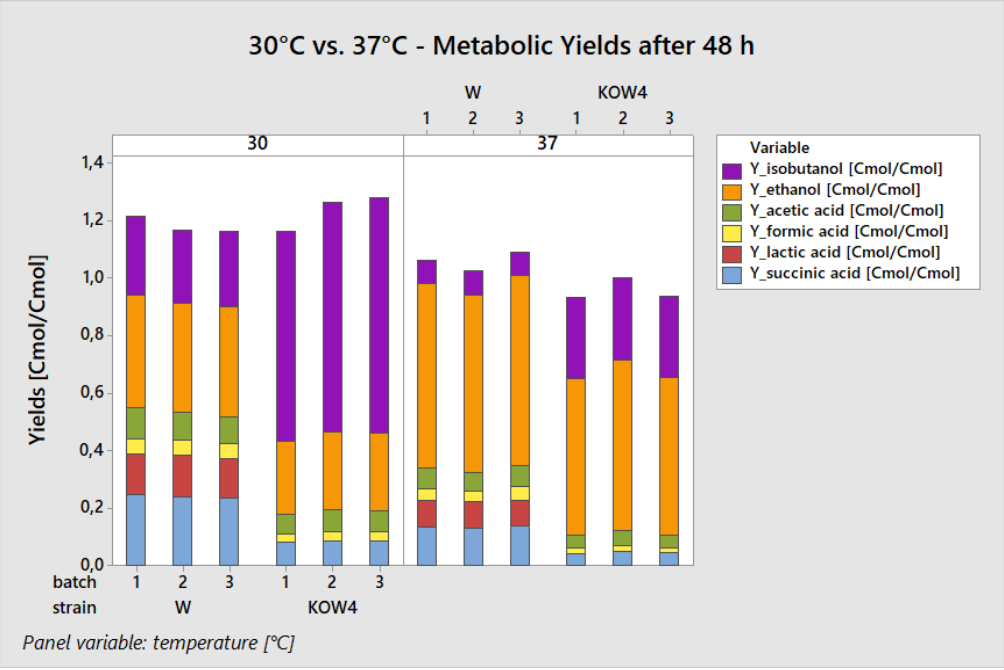

Figure 28: comparison of 30°C vs. 37°C - metabolic yields after 48 h

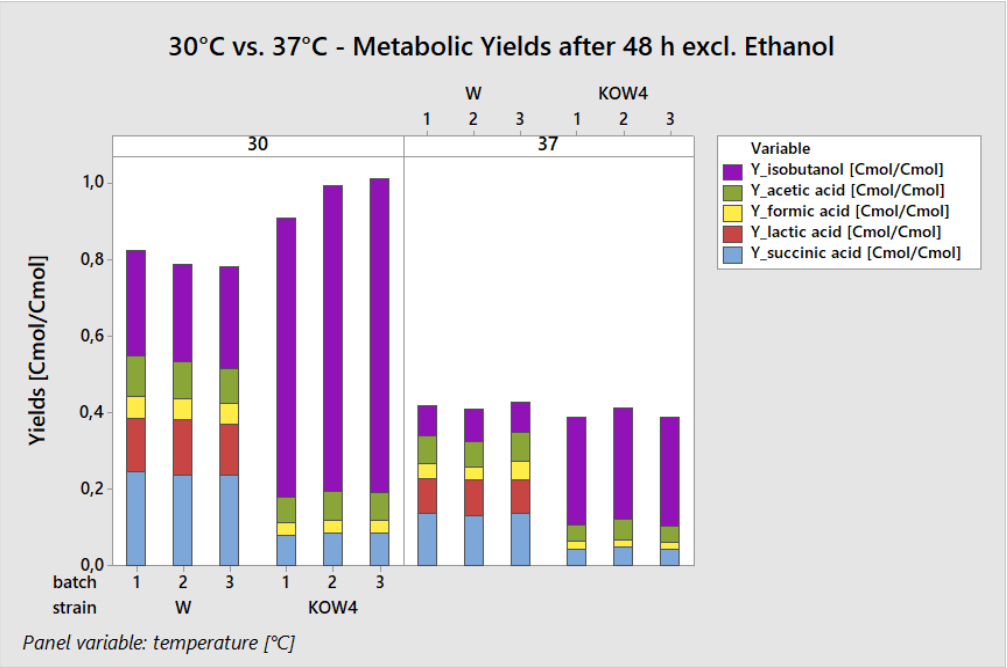

Figure 29: comparison of 30°C vs. 37°C - metabolic yields after 48 h excl. ethanol

Again, it was proven that KOW4 is the most efficient producer of isobutanol and the thesis of higher productivity at lower temperatures was verified at this experiment. There was approximately half of the glucose left, which can indicate that the metabolic activity had not been over after 48 h. The reason for this can be, that the higher isobutanol concentrations in cell broth inhibits the production, or the lower temperature lowers the metabolic activity and the batch process is very long at 30°C.

12.6 New Strain Screening

The strain with the highest production, KOW4, was modified again by knocking out a fifth gene, the pyruvate kinase *pykA*. To characterize this new candidate, a serum bottle experiment was performed again. Therefore, W BB3-10, KOW4 BB3-10 and KOW5 BB3-10 were compared. As medium DeLisa with 20 g L^{-1} or 50 g L^{-1} glucose and 50 mg L^{-1} kanamycin, adjusted to pH 6.8 was used. Batches with 20 g L^{-1} glucose were cultivated as triplets and with 50 g L^{-1} as single batch. Both were grown at 30°C and 37°C at 200 rpm. Before inoculation the amount of inoculum was calculated to start with an OD_{600} of 0.5.

Samples were taken after 0, 6, 12, 18, 24, 36, 48 and 120 h. The samples after 120 h are excluded from the graphs because after 48 h there did not occur any changes in concentrations of metabolites (data not shown). Some HPLC-samples of the batches 3 at 24, 36, 48 h from 20 g L^{-1} glucose in media disappeared and could not be measured. At this reason they are missing in the graphs.

In Figure 30 the means of the OD_{600} of the triplets with 20 g L^{-1} glucose at batch start are displayed. The final absorption was similar around 5, but KOW4 produced a little bit less biomass than the others and had a longer lag-phase. W and KOW5 started the biomass production immediately after inoculation, but KOW4 was slower. There is no

big difference between 30°C and 37°C for W and KOW5. After 12 h KOW4 reached a higher value at 37°C than at 30°C. That indicates that the growth was faster at 37°C.

In Figure 31, where the same graph is shown with 50 g L^{-1} glucose at the beginning of the process, a similar pattern can be seen. Although there was more glucose available, the biomass production was equal, except KOW4 produced even less biomass at 37°C compared with the lower glucose content. This is a hint that the biomass production is no issue of glucose, there must be another limiting factor. Due to the isolated atmosphere in the serum bottles, there is only a certain amount of oxygen in them. As seen in the chemostat experiment before, the growth rate decreases if there is less oxygen available.

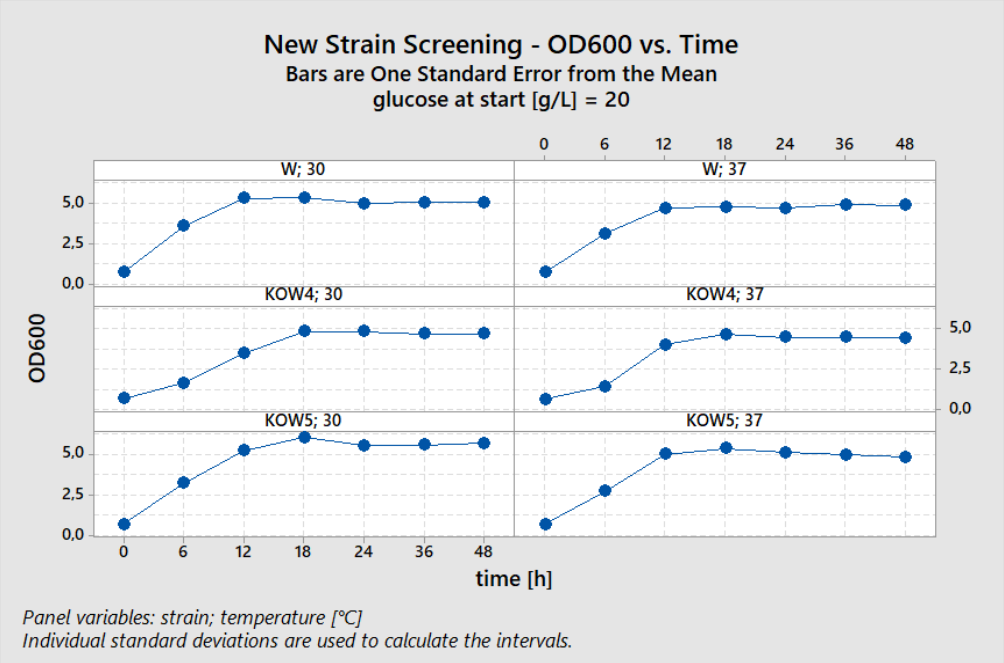

Figure 30: New Strain Screening - OD_{600} vs. Time (20 g L^{-1} Glc)

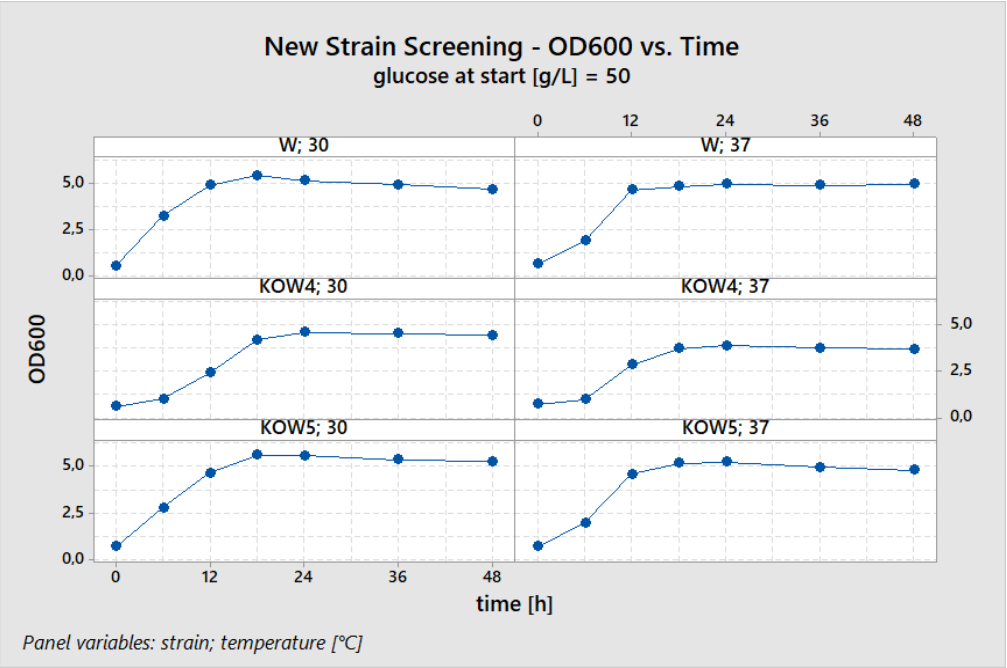

Figure 31: New Strain Screening - OD_{600} vs. Time (50 g L^{-1} Glc)

The glucose consumption, shown in Figure 32, seems like the negative pattern of the biomass production. At 37°C the glucose ran out faster than at 30°C at 20 g L^{-1} starting concentration. After 18 h the glucose was almost totally used up by KOW4 and KOW5 at 37°C. The glucose concentration of KOW5 after 18 h was around 1 g L^{-1} at 30°C, so it is the fastest strain at this temperature. After 24 h the substrate was totally used up by all strains and temperatures, except the supernatant of W at 30°C still contained 1.5 g L^{-1}. But this small amount was gone after 36 h.

In Figure 33 the glucose consumption of the high glucose started cultures are shown. There were 50 g L^{-1} glucose available, but no culture used more than 27 g L^{-1} during 48 h. After 18-24 h the maximum consumption was reached, at 37°C faster than at 30°C and then the glucose was consumed very slowly. After 24 h the KOW5 exhibited the lowest glucose concentration, but after 48 h it was comparable with the others.

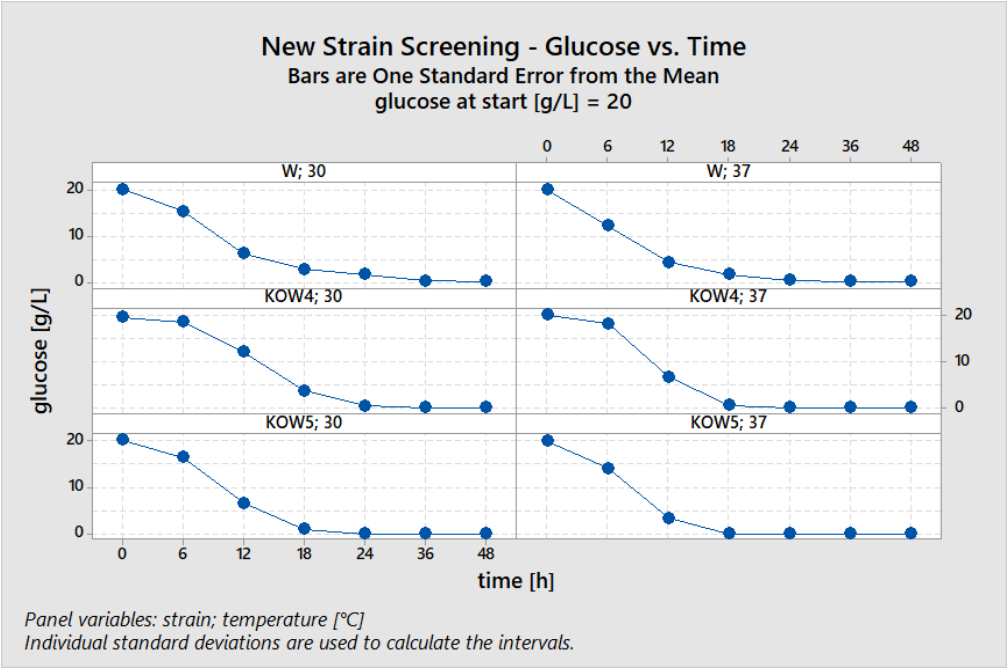

Figure 32: New Strain Screening - Glucose vs. Time (20 g L^{-1} Glc)

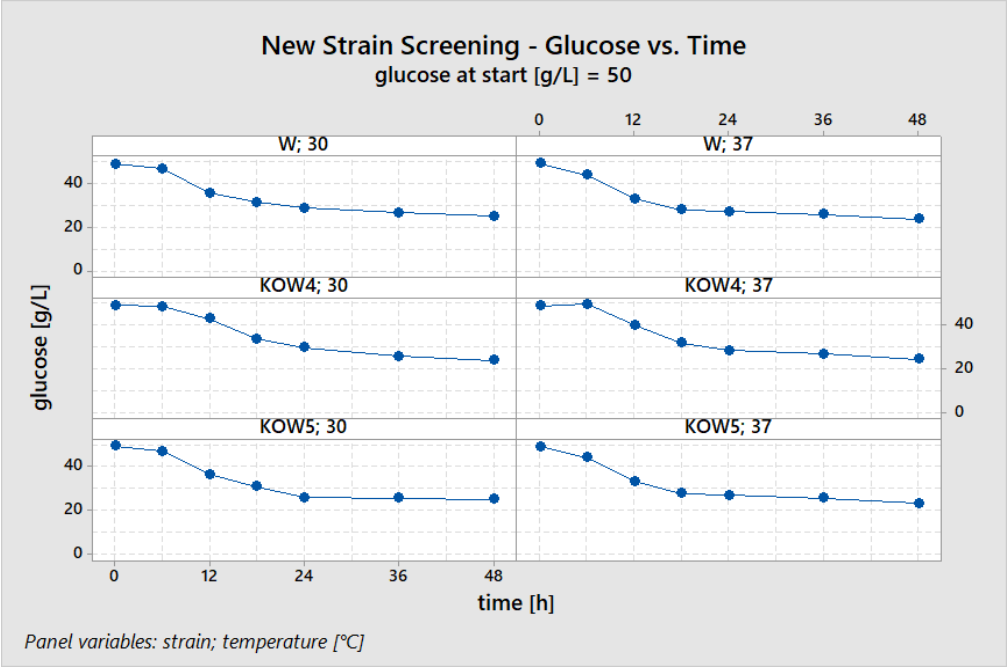

Figure 33: New Strain Screening - Glucose vs. Time (50 g L^{-1} Glc)

Shown in Figure 34, W produced fewest isobutanol, after 48 h a concentration of around 1.5 g L^{-1} was detected. In contrast, KOW4 generated 3.1 g L^{-1} and KOW5 3.3 g L^{-1} after 48 h. Unexpectedly, these values were independently of the temperature. The only difference was that the isobutanol concentration was reached quicker the maximum at 37°C. Most isobutanol was generated between 6 and 18 h, KOW4 at 30°C needed some hours more to reach the maximum. If the data is compared with the graph of consumed glucose, it can be noticed that isobutanol was

produced as long as glucose was available. But in Figure 35 there would be enough substrate, because the culture broth contained even 23-25 g L^{-1} after 48 h. The isobutanol production pattern is equal to 20 g L^{-1} glucose at start but after 24 h, when the glucose is used up in the batches with lower glucose concentration, both knockout strains produced further isobutanol but very slowly. This phenomenon leads to the question, why did they stop generating isobutanol? The suggestion is that the oxygen was used up at the same time point at each batch and these organisms cannot grow at anaerobe conditions, as seen in former experiments. But obviously they reduced producing isobutanol to a minimum or stopped the production totally like W.

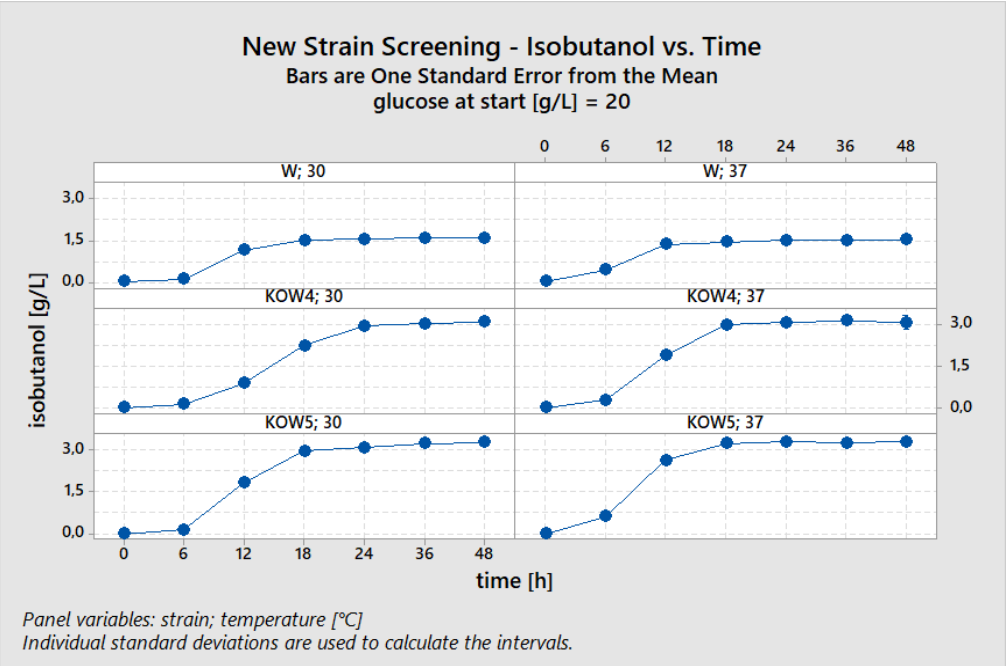

Figure 34: New Strain Screening - Isobutanol vs. Time (20 g L^{-1} Glc)

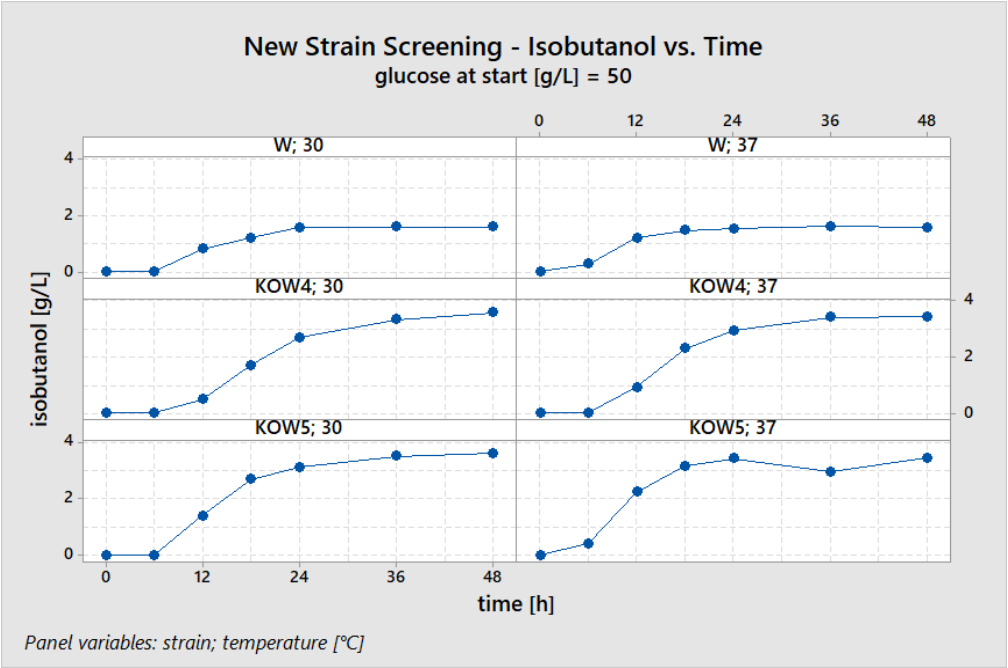

Figure 35: New Strain Screening - Isobutanol vs. Time (50 g L^{-1} Glc)

Succinic acid was produced much more by W, than by the knockout strains. W produced nearly 1.9 g L^{-1} and KOW4 and KOW5 only 0.8 g L^{-1}. In Figure 36 the data of succinic acid are shown. Between 6-12 h there was a huge increase performed by W, before increasing much smoother like the knockout strains did all the time. In Figure 37 the succinic acid production with higher glucose concentration is displayed. It looks similar and the concentrations were nearly equal after 48 h.

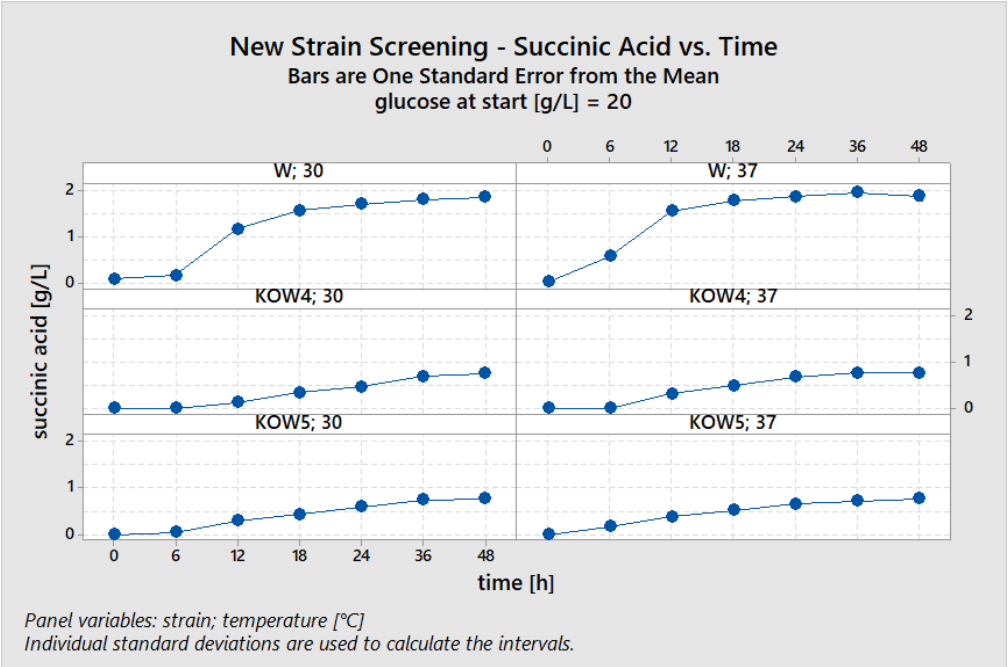

Figure 36: New Strain Screening - Succinic Acid vs. Time (20 g L^{-1} Glc)

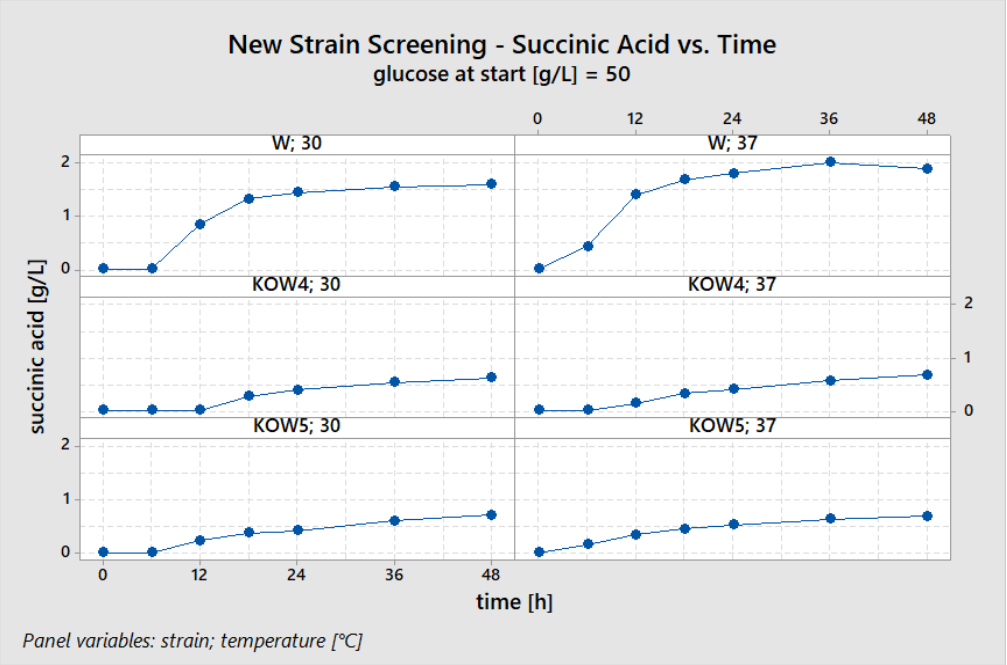

Figure 37: New Strain Screening - Succinic Acid vs. Time (50 g L^{-1} Glc)

As expected, KOW4 and KOW5 did not produce any lactic acid at any condition, shown at Figure 38. W generated similar amounts of lactic acid at both temperatures. The production started later than the other metabolites. At this time there was hardly any glucose left in cell broth and lactic acid must be produced from another source. But we expected that at this time point, when the production started, the oxygen is nearly used up too and that motivates the bacteria to produce lactic acid. In Figure 39 the lactic acid production with 50 g L^{-1} glucose is displayed. The generation started at the same time, after 18 h at 30°C and after 12 h at 37°C. But there would be enough glucose left. For this reason, the absence of oxygen was responsible for the production of lactic acid. Because there was still glucose left, the lactic acid concentration increased up to 1.7 g L^{-1} at 30°C and 1.2 g L^{-1} at 37°C until the end of the experiment after 120 h. This data is not shown in the graph below.

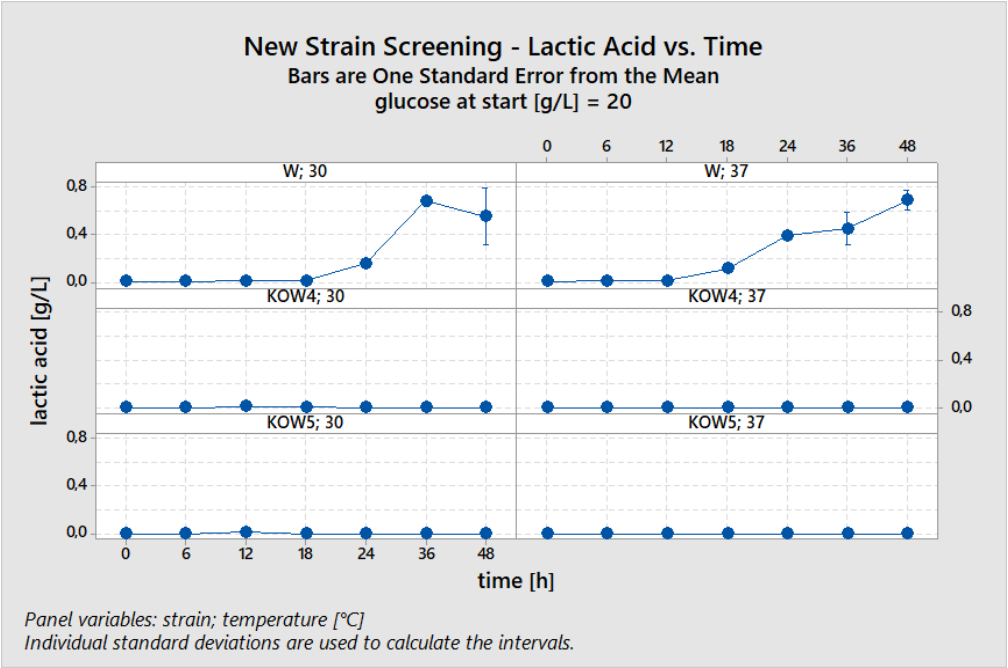

Figure 38: New Strain Screening - Lactic Acid vs. Time (20 g L^{-1} Glc)

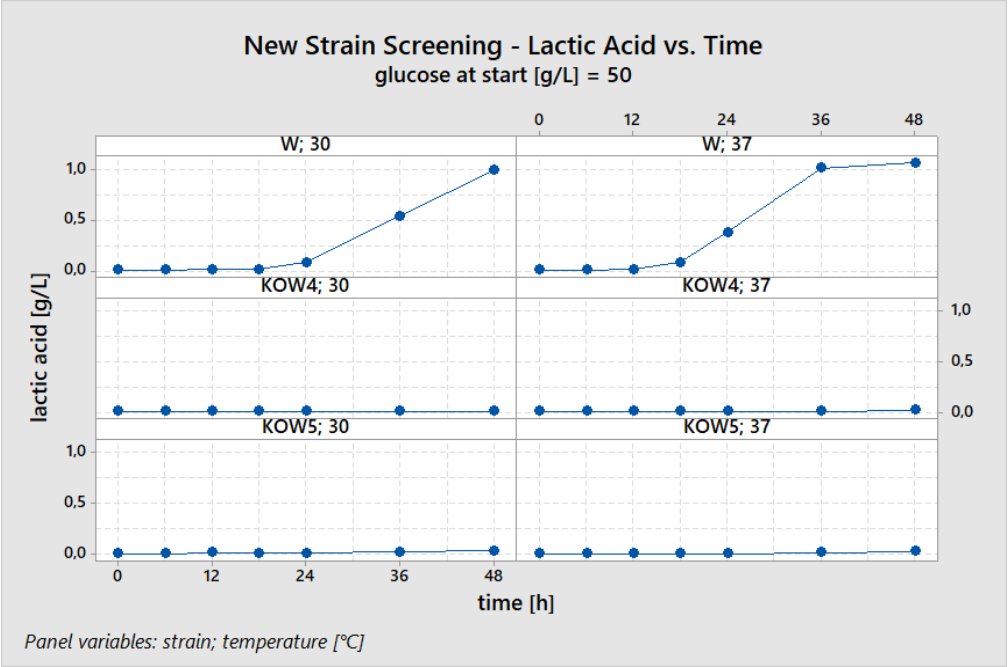

Figure 39: New Strain Screening – Lactic Acid vs. Time (50 g L^{-1} Glc)

Formic acid was produced by W in the very beginning and a part of it was degraded slowly later, seen in Figure 40. KOW4 and KOW5 need a little bit longer to start the production but the degradation occurred everywhere. At 37°C the maximum was reached earlier than at 30°C but the amounts were comparable, except KOW4. This strain achieved a higher titer at 37°C, up to 0.8 g L^{-1}, in contrast to 30°C where a concentration of 0.5 g L^{-1} were generated.

In Figure 41 the process of formic acid with high glucose is displayed. The pattern looks similar to the batches with lower glucose concentration, but there is no peak like it can be observed at low glucose conditions because of the degradation.

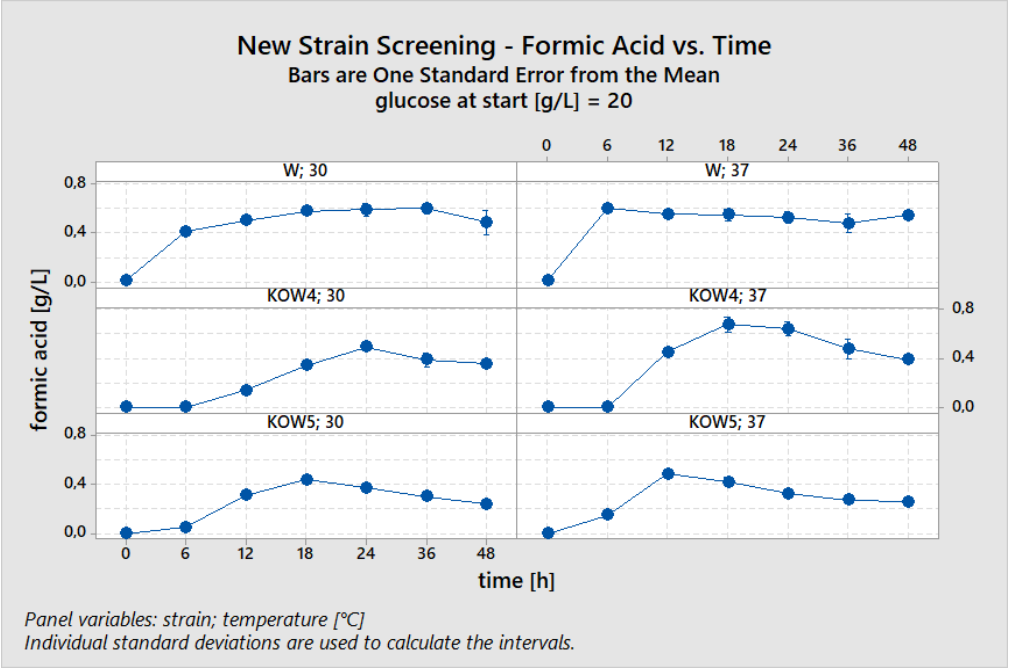

Figure 40: New Strain Screening - Formic Acid vs. Time (20 g L^{-1} Glc)

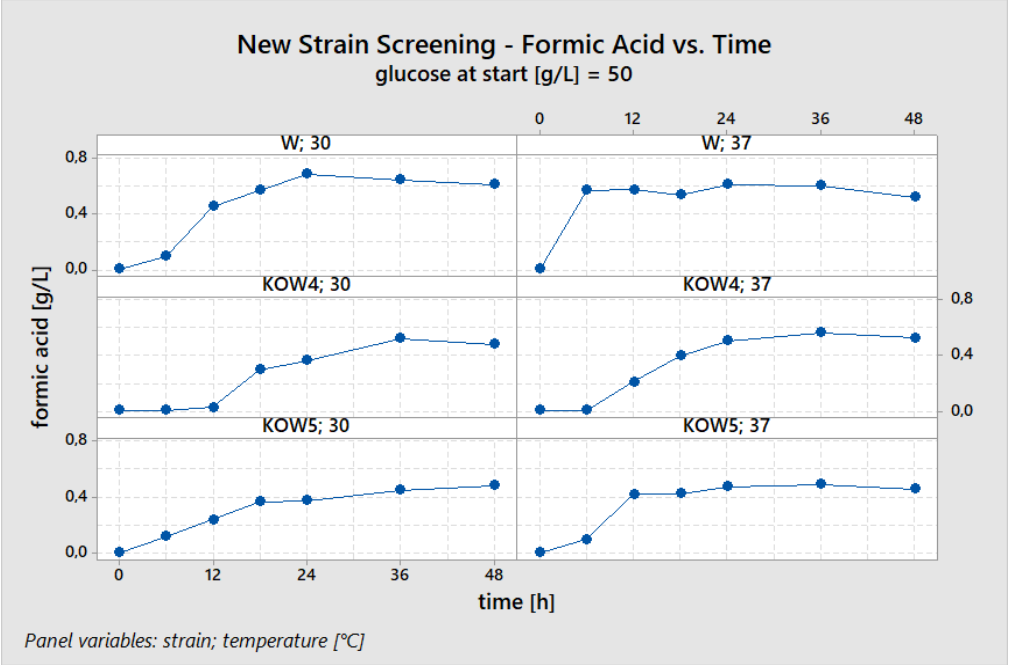

Figure 41: New Strain Screening - Formic Acid vs. Time (50 g L^{-1} Glc)

Acetic acid was produced by W at the very beginning quickly, but stopped after 6 h, shown in Figure 42. The knockout strains produce acetic acid slower, especially KOW4 started the production after 6 h. KOW5 produced the highest amount up to a concentration of nearly 1.0 g L^{-1}. The production process and the achieved concentrations were very similar to high glucose batches, seen in Figure 43.

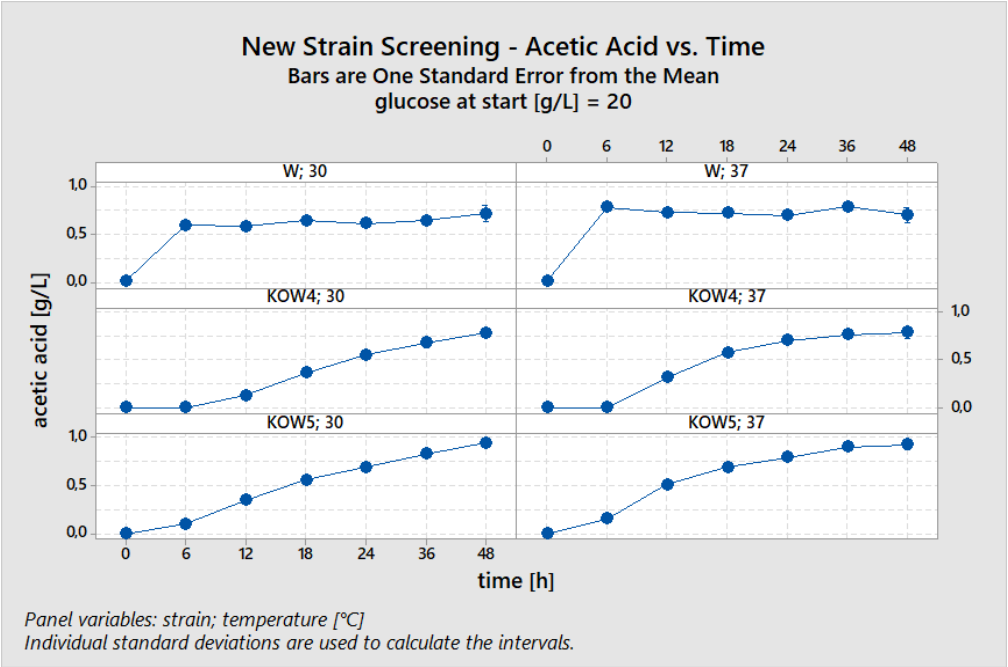

Figure 42: New Strain Screening - Acetic Acid vs. Time (20 g L^{-1} Glc)

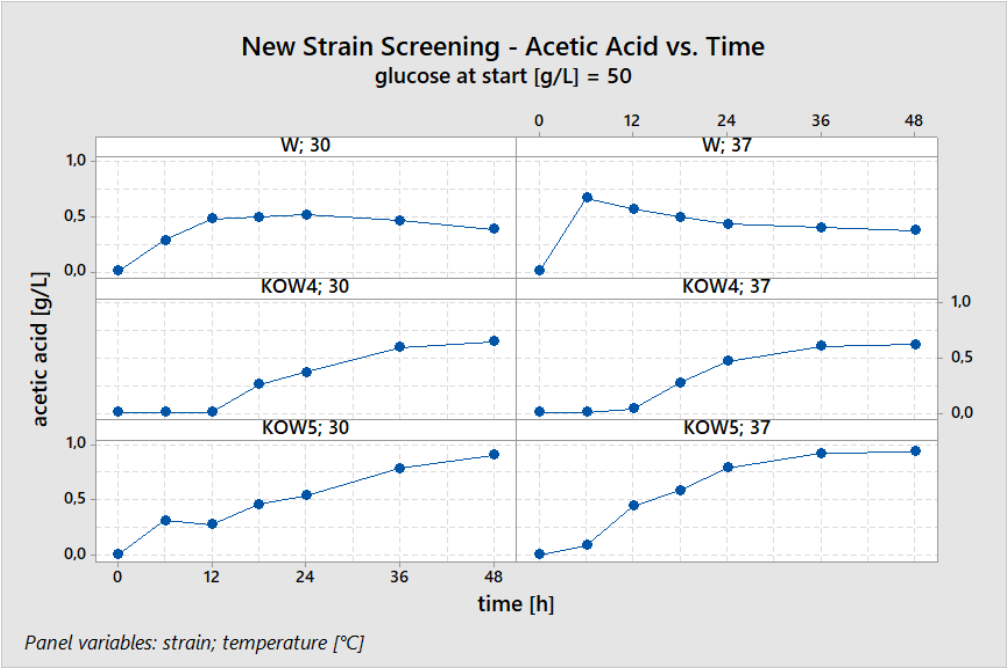

Figure 43: New Strain Screening - Acetic Acid vs. Time (50 g L^{-1} Glc)

At this experiment the septum of the serum bottles were wiped with an ethanol-soaked cloth and dried under the flame of a Bunsen burner to avoid a distortion of the ethanol values again.

In Figure 44 is shown, that W produced with 1.3 g L^{-1} the highest ethanol concentration. In contrast the knockout strains only produced around 0.5 g L^{-1} after 48 h. It is difficult to make a statement about the timepoint of the production start, because the behaviour of the batches with the same conditions was different.

The picture looks equally for high glucose batches, shown in Figure 45. There were achieved equal values after 48 h.

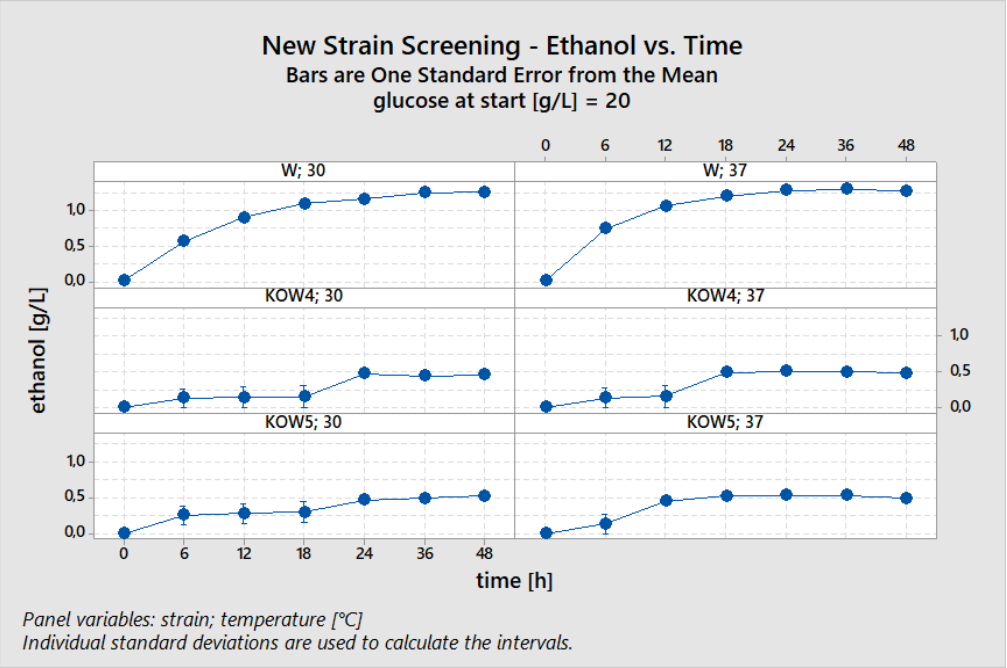

Figure 44: New Strain Screening - Ethanol vs. Time (20 g L^{-1} Glc)

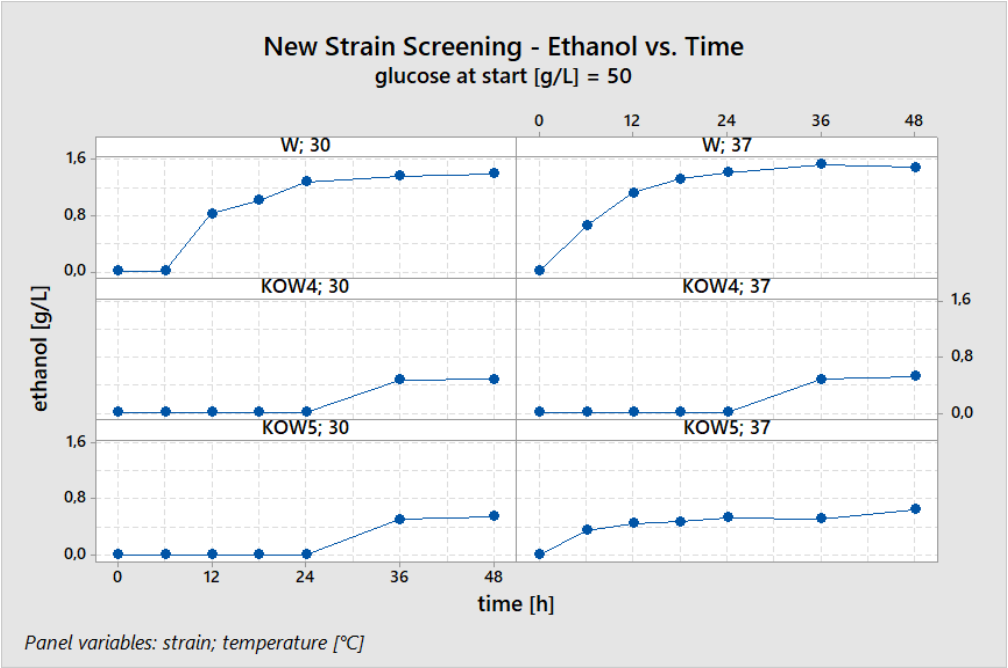

Figure 45: New Strain Screening - Ethanol vs. Time (50 g L^{-1} Glc)

The yields [Cmol $Cmol^{-1}$] were calculated too. Of course, there is a lack in the C-balance because the biomass and carbon dioxide are missing. In Figure 46 the means of the yields of the three batches with 20 g L^{-1} glucose at the beginning are displayed. The C-recovery is equal, around nearly 0.4 Cmol $Cmol^{-1}$ at both temperatures and all strains. Also, the yields of each strain look equal at both conditions. Only W produced lactic acid and generated more succinic acid, formic acid and ethanol. The yield of acetic acid is a bit smaller and the isobutanol yield is only the half of the knockout strains. W had a product yield of 0.12 Cmol $Cmol^{-1}$ and KOW4 and KOW5 around 0.26 Cmol $Cmol^{-1}$.

In Figure 47 the yields of the high glucose batches are pictured. The distribution of the product and metabolites looks equal to the low glucose batches, but the sums of the yields are a bit smaller. For that reason, the isobutanol yield of W was only 0.10 Cmol $Cmol^{-1}$ and 0.23 Cmol $Cmol^{-1}$ of the knockout strains.

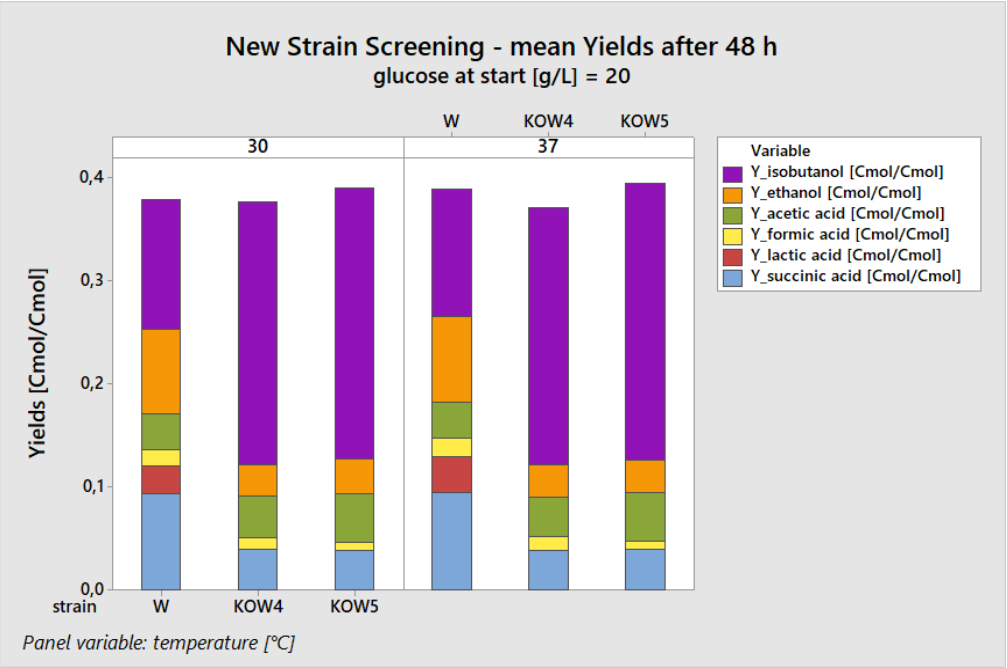

Figure 46: New Strain Screening - mean Yields after 48 h (20 g L^{-1} Glc)

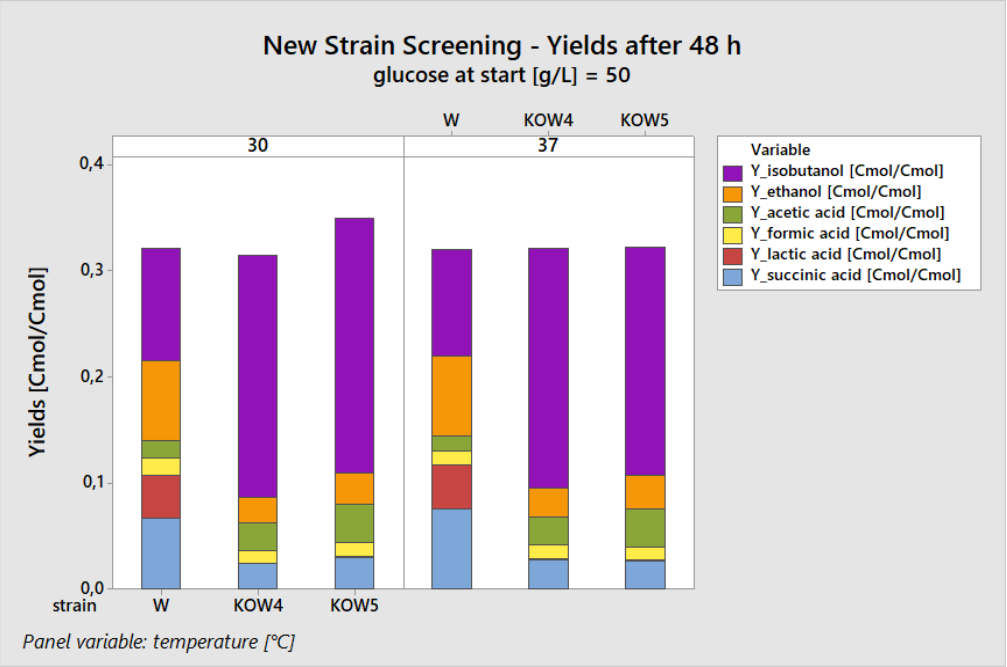

Figure 47: New Strain Screening - Yields after 48 h (50 g L^{-1} Glc)

In Figure 48 the growth rate [h^{-1}] and the isobutanol production rate [g L^{-1} h^{-1}] at 20 g L^{-1} glucose at the start are shown. The highest growth rates were found at W and KOW5, KOW4 had a smaller but broader peak. KOW5 had the highest isobutanol production rate but the rate of other knockout strain, KOW4 was only a bit smaller and W had the smallest. Because of the difference of the temperature, the peaks were shifted. At first at each temperature and strain there was a peak of the growth rate and after, the maximum of the isobutanol production rate occurs. After 6 h when there was the maximum of the growth rate reached at most conditions, there had been produced no

or only small amounts of isobutanol. After the exponential phase most isobutanol was produced. That confirms the suggestion that the cells grow very slow when producing isobutanol. In Figure 49 it can be seen exactly the same for the high glucose started batches, except W at 30°C had a very high growth rate after 6 h in contrast to the others.

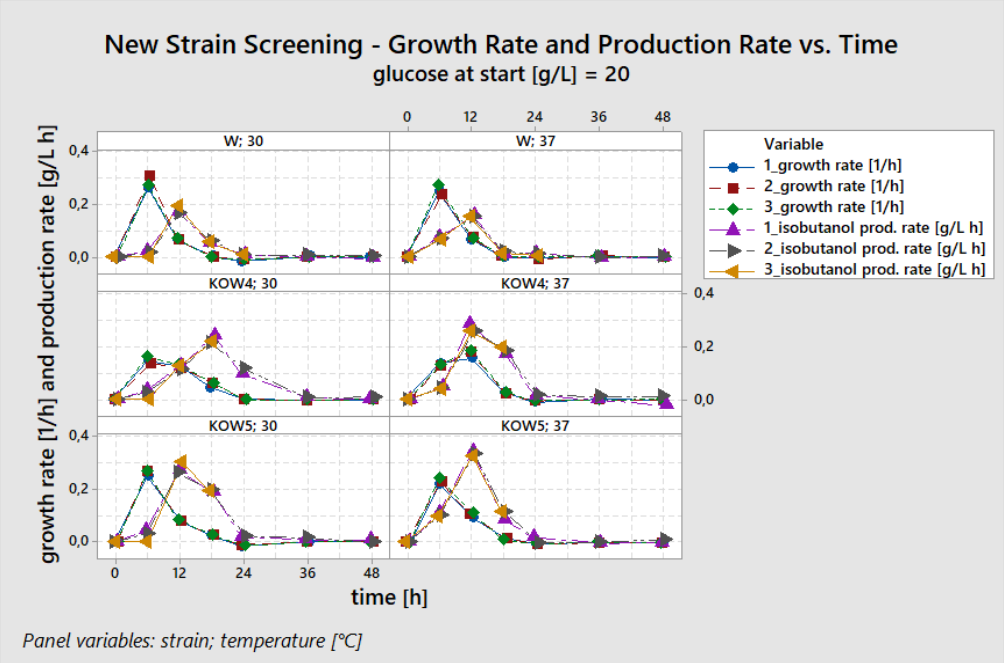

Figure 48: New Strain Screening - Growth Rate and Production Rate vs. Time (20 g L^{-1} Glc)

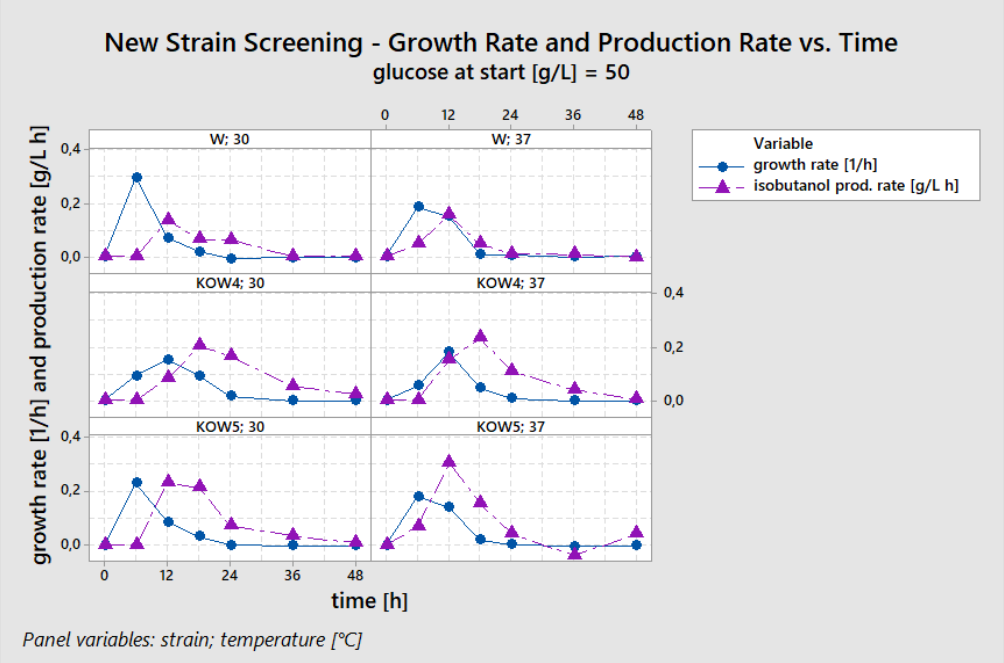

Figure 49: New Strain Screening - Growth Rate and Production Rate vs. Time (50 g L^{-1} Glc)

At this serum bottle experiment some important insights were gained but unfortunately the difference between 30°C and 37°C could not show again as seen in the previous serum bottle experiment. The maximum isobutanol yield achieved by KOW5 and was only around 44 % of the theoretical yield. But it was seen that this strain is a kind of super strain because the best properties of W and KOW4 are combined in one strain. KOW5 grew as fast as W and produced as much isobutanol as KOW4. For this reason, it is the ideal candidate for further experiments.

Another important insight that could be earned, was that more glucose does not lead to higher yields and the organisms cannot consume all glucose in a moderate time if there is a concentration of 50 g L^{-1} at the beginning. The consumption of glucose ends or is lowered to a minimum when the oxygen was used up.

Due to the fact that most isobutanol is produced after the exponential phase, the suggestion from Atsumi et al. (2010b) and Chen and Liao (2016) was underlined. It is assumed that at first when there is enough oxygen, biomass is produced. After that when the atmosphere becomes microaerobic, isobutanol is produced and when the oxygen is totally used up, the metabolic activity shrinks to a minimum and only lactic acid is produced by W.

12.7 KOW4 vs. KOW5

In prior experiments it was shown that KOW4 BB3-10 achieved the highest isobutanol titers but was limited in growth. At the chemostat experiment it could not match up to W, because of the lower growth rate at microaerobic conditions. Therefore, the dilution rate had to be reduced. But now there was a now strain, which combines good growth and high product concentration. At the next step its behaviour at microaerobic conditions were tested. To compare the most promising strain until now, KOW4 BB3-10 and the new strain, KOW5 BB3-10, a chemostat process was performed at a DASBOX multi reactor system (Eppendorf, Germany). Both strains were tested in two bioreactors. The conditions for each cultivation was equal. The batch phase was performed with a working volume of 200 mL, inoculated with an OD of 1, at 37°C and 1400 rpm. The agitation was increased on demand to keep the dissolved oxygen above 30 %. The cell broths were gassed with 1 vvm air. DeLisa adjusted to pH 6.8 was used as medium. After the glucose was used up, the chemostat process with a dilution rate of 0.1 h^{-1} was started. Therefore, the same medium was used. The stirrer speed was set back to 1400 rpm and the temperature was decreased to 30°C. The gassing was kept on 1 vvm and the amount of oxygen in the in-gas was decreased slowly, because as seen at the first chemostat, if the oxygen is decreased too fast, KOW4 gets washed out. At conditions of 5.0, 2.0 and 1.0 % oxygen in the in-gas, three samples with 3 h difference were taken. Between the conditions at least three volume changes were waited.

Because of the characteristic smell of isobutanol during fermentation, in the end of the experiment, when most isobutanol was produced, a wash bottle with a certain amount of water in it, where the off-gas bubbles through the water, was connected at the off-gas stream after the sterile filter to capture the stripped isobutanol. Unfortunately, this could be performed only at one bioreactor, only KOW4/1 showed the expected

behaviour in the end. At this condition glucose was accumulated by KOW4/2 and both KOW5 reduced producing isobutanol and only very small concentrations were found in the supernatant. Accumulation of glucose is normally a hint that the D is too high, but then the cells become washed out and the batches of the same strain would behave equally. For this reason, it was assumed, that these strains were mutated.

In Figure 50 the isobutanol production in mmol L^{-1} h^{-1} of both strains and cultures is displayed. In former cultivations no isobutanol was produced by KOW4 at 1 vvm and 5.0 % oxygen but here, isobutanol was produced under these conditions by this strain. The reason for that could be that this cultivation is performed in another bioreactor. But it is unusual that more isobutanol is produced at higher oxygen levels. Around 1.0 g L^{-1} was detected at this condition.

At the bottom on the right panel of the graph, the other KOW4 is shown. Here less isobutanol was produced at 1 vvm and 1.0 % oxygen. However, the specific production of this culture is the highest, shown in Figure 51. Nevertheless, the production pattern of these cultures of the same strain differ dramatically. Additionally, in two of three samples of the last condition around 9.0 g L^{-1} glucose was found. That corresponds nearly half of the medium concentration.

In contrast to KOW4 there was not any isobutanol detectable at 5.0 % oxygen in the supernatant of KOW5 (Figure 50). The first culture generated a higher titer, but both produced less isobutanol by decreasing the oxygen amount in the in-gas. At the first batch glucose is accumulated at the last condition, at the second batch no glucose was accumulated but the isobutanol production decreased anyway.

The isobutanol production as well as the specific isobutanol production (Figure 51) were lower than at the cultures of KOW4. At the first culture of KOW5 the specific productivity increased at 1 % oxygen in the in-gas, but at the second the specific productivity decreased.

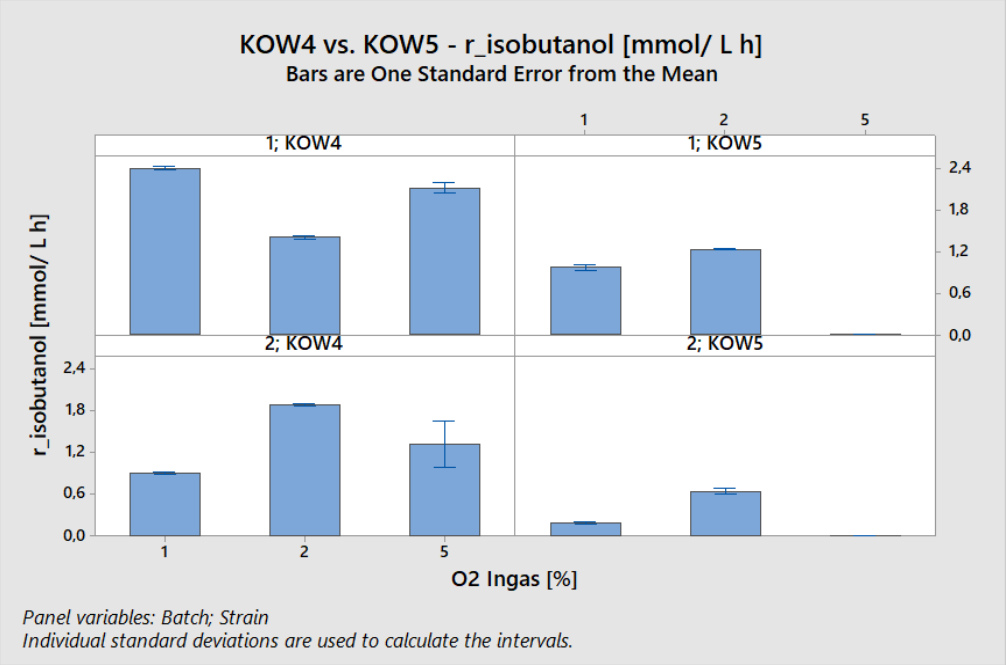

Figure 50: KOW4 vs. KOW5 - r_Isobutanol [mmol L^{-1} h^{-1}]

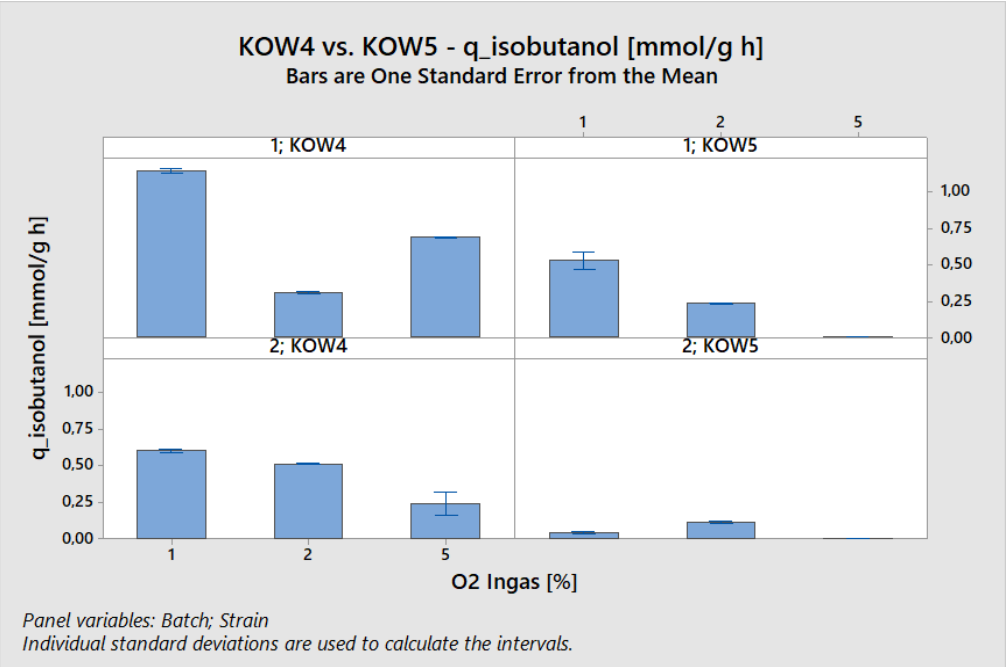

Figure 51: KOW4 vs. KOW5 - q_Isobutanol [mmol g^{-1} h^{-1}]

The carbon recovery of KOW4 is shown in Figure 52. There is a lack again. Obviously the higher the isobutanol yield, the worse the recovery was, except at the second batch at 1.0 % oxygen. At this condition at the second sample there was an error of the glucose measurement.

In Figure 53, where the yields of KOW5 are displayed, it can be seen that at 5 % oxygen only biomass and CO_2 were produced, and the C-recovery achieved the

highest level. By decreasing the amount of oxygen, the recovery decreased too and acids were formed.

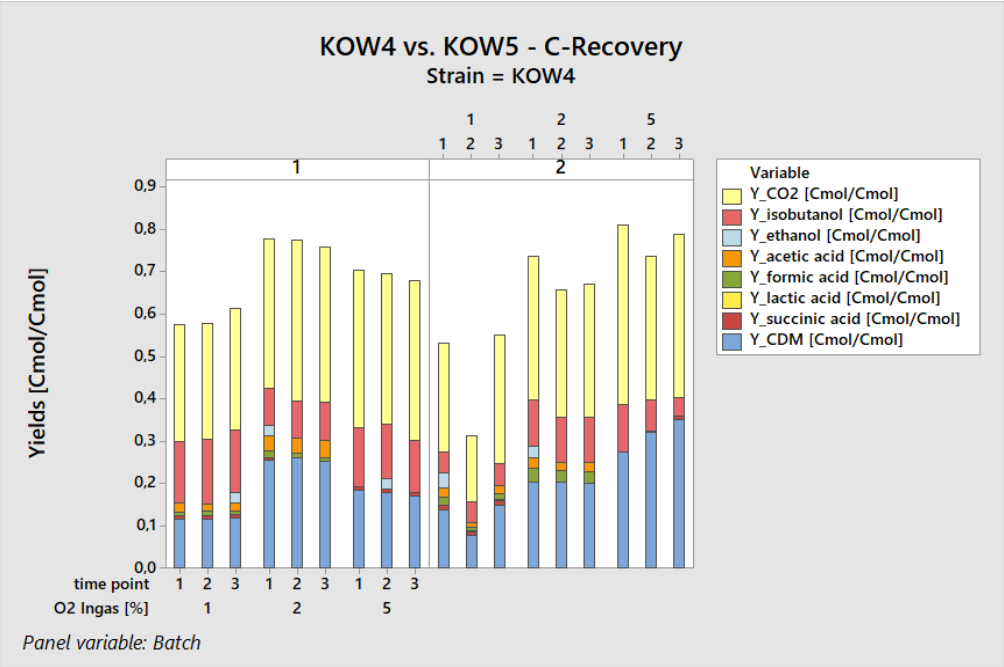

Figure 52: KOW4 vs. KOW5 - C-Recovery KOW4

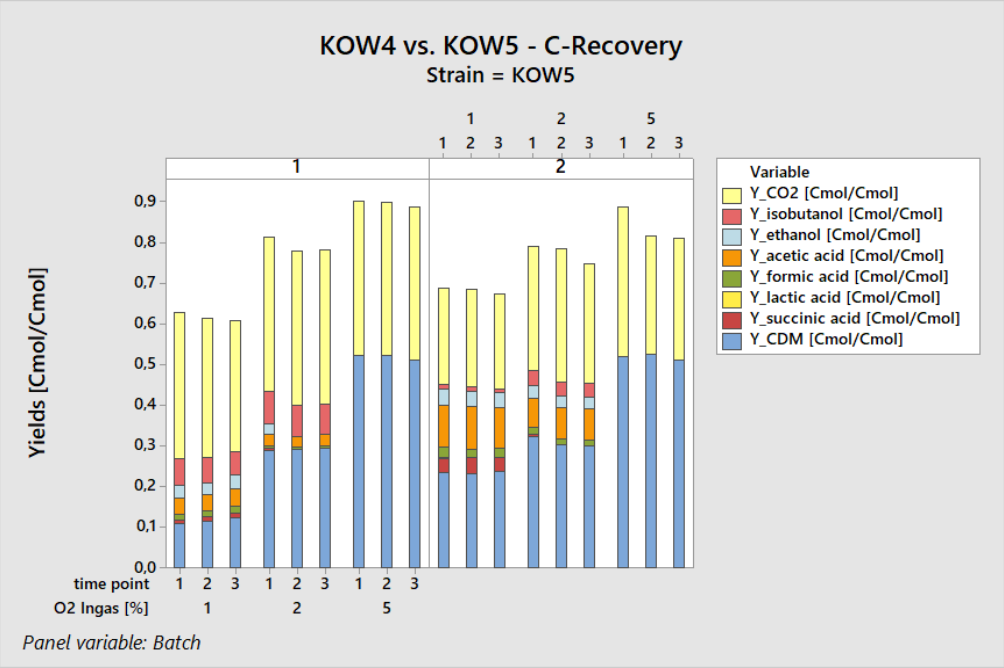

Figure 53: KOW4 vs. KOW5 - C-Recovery KOW5

It was expected that the biomass decreases and the isobutanol concentration increases by lowering the oxygen level in the in-gas. The behaviour of KOW4 concerning these parameters are shown in Figure 54. At the first culture the lower the CDM, the higher was the isobutanol titer, but that did not correlate with the amount of

oxygen. At the other cultivation the suggestion was confirmed but at the 1 % oxygen the isobutanol production was lower. This pattern is also seen for both cultures in Figure 55 where the biomass and isobutanol concentration of KOW5 are displayed.

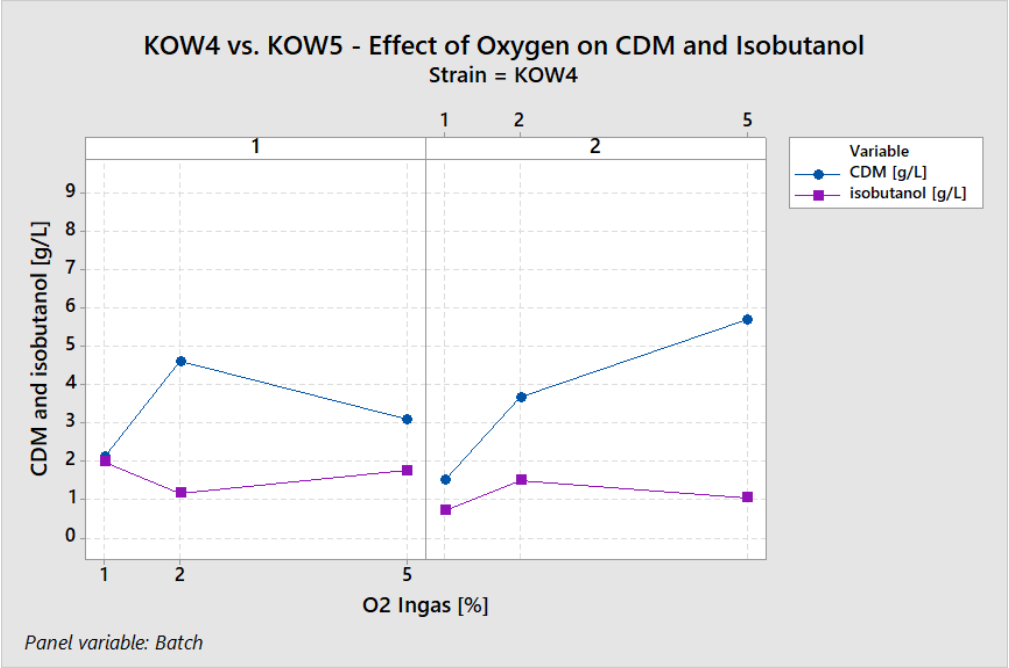

Figure 54: KOW4 vs. KOW5 - Effect of Oxygen on CDM and Isobutanol KOW4

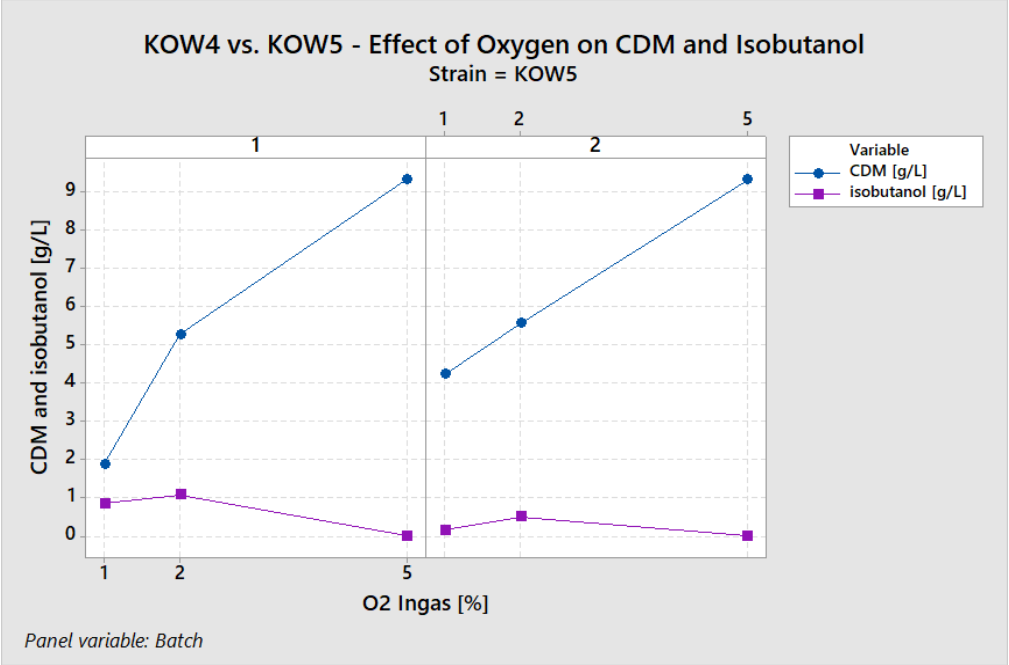

Figure 55: KOW4 vs. KOW5 - Effect of Oxygen on CDM and Isobutanol KOW5

In the wash bottle with 200 mL water in it, which was coupled to the off-gas stream of KOW4/1 at 1 %, oxygen 0.24 g L^{-1} isobutanol was detected after two hours. That is

converted into 0.0064 Cmol L^{-1} h^{-1} and is equal to 67 % of the reactor concentration. This is the reason for the characteristic smell, huge amounts of isobutanol is stripped out of the suspension during the cultivation and got lost. This could be the reason for the bad C-recovery at low oxygen amounts. But it is suggested that there was also a gas stripping effect in the wash bottle. Because of the increasing back pressure of a second wash bottle, it was not possible to connect another one without increasing the dissolved oxygen in the bioreactor and that would lead to wrong results. It is only possible to calculate the theoretical isobutanol concentration of a second wash bottle with 67 % of the concentration of the first one. If there would be five wash bottles the total isobutanol concentration would be 5.47 g L^{-1} which would correspond a product yield of 0.40 Cmol $Cmol^{-1}$. This would close the lack of the C-recovery. But these are only calculations and suggestions and further experiments concerning gas stripping are necessary.

It is hard to take a statement to this experiment because the behaviour of each single culture was too different. It is recommended to repeat this experiment to figure out the behaviour of the strains at microaerobic conditions during a chemostat process. But the knowledge about the gas stripping effect is very valuable and more attention should be given to it.

13 Conclusion

Within this work different strains of *E. coli* combined with a couple of constructs for isobutanol production were screened in serum bottles as well as in bioreactors. According to the overall aim, figuring out the best producer for the production of isobutanol, chemostat processes for a continuous production were performed to display the most promising candidate. Additionally, some parameters like aeration and temperature were tested during the processes.

At the first serum bottle screening, KOW4, which is an *E. coli* W strain with four knockouts (*ΔldhA ΔadhE Δpta ΔfrdA*) was the best producing strain with the highest yield and the lowest by-product formation. The construct BB3-10, which gained with 2.5 g L^{-1} isobutanol was the highest producing construct. It was assembled by *butB*, *ilvC_mut*, *ilvD*, *kdcA* and *adhA_mut* with the promotor J23114 for each gene.

This combination was tested in a bioreactor where only 1.7 g L^{-1} isobutanol was produced. It was suggested that isobutanol was stripped out because of the gassing. There was the characteristic smell of isobutanol noticeable in the end of the batch, when most isobutanol was produced. The phenomenon of gas stripping has been mentioned in some publications before. For example Baez et al. (2011) reported from an isobutanol removal process due to gas stripping. Although isobutanol has a lower gas pressure than water, it is easy to remove it from the culture broth. This is the reason why serum bottles were used instead of shake flasks.

By studying the W, KOW4 and AW at a chemostat process, it was noticeable, that a decrease in the amount of oxygen in the in-gas, meant an increase in the isobutanol concentration in cell broth. Unfortunately, AW did not produce any isobutanol, but since the concentrations in this experiment were quite low, there was no requirement to use an adapted strain. However, if the oxygen supply is lowered, the cell density shrinks as a side effect. Atsumi et al. (2010b) and Chen and Liao (2016) reported that most isobutanol was produced after the growth phase. Nevertheless, the Carbon was not recovered completely and it was suggested that the gas stripping effect was at least in part due to this fact. In the end of the experiment the cultures were mutated. There were too many generations after nearly 20 days of cultivation.

If the production of isobutanol increases by lowering the oxygen, most isobutanol should be generated at anaerobe conditions. But as shown, without oxygen there was hardly any cell growth and only little isobutanol production. For that reason microaerobic conditions were the solution.

As published in some reports (Akita et al. 2015; Baez et al. 2011), the isobutanol production increases by lowering the temperature. In serum bottles at 30°C the isobutanol concentration achieved nearly 3.9 g L^{-1} in contrast to 2.7 g L^{-1} at 37°C by KOW4. By calculating the C-balance this value exceeds the theoretical yield. It was assessed that there was an error at the measurement of the glucose and the estimated value of the consumed glucose was too low. But the increase of isobutanol production by decreasing the temperature was shown definitively.

The new knockout strain, KOW5 (*ΔldhA ΔadhE Δpta ΔfrdA ΔwpykA*) was also tested at both temperatures simultaneously with W and KOW4 in serum bottles. In this experiment there was no difference between the temperatures - neither at KOW5 nor at W or KOW4. It was also shown that the glucose concentration of the medium has not a big impact of the titers or yields. The limiting factor was oxygen. The lower glucose concentration could be used up totally with the available oxygen in the serum bottle. But around the same time the oxygen was used up and the rest of the glucose in the high glucose batches was not consumed.

However, KOW5 seems like having all the positive properties of W and KOW4. It grows as fast as W and produces as much isobutanol as KOW4. This strain generated 3.3 g L^{-1} isobutanol after 48 h this corresponds a yield of 0.27 Cmol $Cmol^{-1}$ and is nearly half of the theoretical yield.

In this experiment the thesis that most isobutanol is produced in the end or after the growth phase was underlined. For this reason, a chemostat process is maybe not the best choice of process design. For further experiments I would recommend a fed batch process with a temperature shift from 37°C to 30°C. At the batch phase biomass should be built up by high aeration. After starting the feeding and when the desired cell density is reached, the temperature should be shifted, and the oxygen can be lowered. Then the cells will minimize the growth and start producing isobutanol. But here, due to the toxicity of isobutanol, a product removal system, like controlled gas stripping must be included. At this point in time, the growth is not required anymore, because cells cannot be washed out. If it is absolutely necessary to develop the process continuously, I would choose a perfusion process. However, a kind of isobutanol trap in the off-gas stream must be integrated to the system to avoid product losses.

The last experiment should be repeated because it is hard to take a statement from this diffuse data. Felpeto-Santero et al. (2015) reported that it is very hard to compare and reproduce the isobutanol production. That we had seen too, it was a balancing act to figure out the ideal gassing conditions to produce isobutanol under microaerobic conditions, with low evaporation effects and generate enough biomass to drive an accurate chemostat process.

In literature, very high isobutanol concentrations, where more than 20 g L^{-1} were generated, have been reported. In this work the highest concentration was hardly 4.0 g L^{-1}. But to gain such high concentrations, more glucose was used and additives like yeast extract or L-threonine (Atsumi et al. 2008b). Here, also higher glucose concentrations were tested but the oxygen ran out before the glucose did.

The strain KOW5 combined with the construct BB3-10 seems to be the best candidate for isobutanol production in serum bottles, but further studies in bioreactors are necessary.

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15 Appendix

15.1 Fermentation raw data

Table 12: raw data of Bioreactor run KOW4 BB3-10 Batch

time [h]	CDM [g/l]	glucose [g/l]	formic acid [g/l]	succinic acid [g/l]	lactic acid [g/l]	acetic acid [g/]	ethanol [g/l]	isobutanol [g/l]
0,00	0,275	18,765	0,000	0,000	0,000	0,000	0,000	0,000
3,00	0,408	20,465	0,000	0,000	0,000	0,020	0,000	0,042
6,00	0,783	16,591	0,177	0,000	0,000	0,175	0,000	0,245
8,00	1,250	12,653	0,338	0,000	0,000	0,014	0,000	0,630
10,00	1,808	7,731	0,761	0,000	0,000	0,936	0,000	1,125
12,00	2,425	0,809	1,265	0,000	0,000	0,032	0,000	1,591
12,67	2,742	0,000	1,450	0,602	0,000	3,997	0,292	1,664