Investigation of butanol tolerance in *Saccharomyces cerevisiae* and of genes linked to butanol tolerance

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The global warming on earth has been obvious since the 1950’s. Fossil fuels have a big impact on the observed warming and it is time to replace them with more environmentally friendly fuels. Biobutanol has been proven to be a preferred substitute to fossil fuels. The yeast *Saccharomyces cerevisiae* is a potential butanol producer. A problem in the biobutanol production is that the product, butanol, is toxic to the producer. In this study four *S. cerevisiae* strains were investigated for 1- and 2-butanol tolerance with spot tests and growth measurements with different concentrations of 1- and 2-butanol. One of the four strains, an ale yeast, showed a higher tolerance for 1- and 2-butanol. 2-butanol was overall more tolerated by the yeast. The gene expression for the genes TMC1, LPL1, FLR1 and RPN4 was also investigated at exposure of 3% 2-butanol. RPN4 is important in the proteasome protein degradation, which is associated with butanol tolerance. TMC1, LPL1 and FLR1 are associated to RPN4, which make them potential genes coupled to butanol tolerance. The genes TMC1 and RPN4 showed an up-regulation when exposed to 3% 2-butanol. In conclusion, 2-butanol is preferred as a biofuel produced by ale yeast and the ideal genes to use in genetic engineering to achieve a higher butanol tolerance is TMC1 and RPN4. These results contribute to the development of an effective production of biobutanol by *S. cerevisiae*.

**Keywords**
Biobutanol, Butanol tolerance, FLR1, LPL1, RPN4, *Saccharomyces cerevisiae*, TMC1
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1 Abstract

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2 Introduction

2.1 Why use biobutanol?

Anthropogenic greenhouse gas emissions and other human impacts are extremely likely the dominant cause of the observed warming since the 1950’s, according to the International Panel on Climate Changes (IPCC, 2014). The global warming is related to the usage of fossil fuels, which also is a limited fuel source. To end the increase of global warming and the emission of greenhouse gases, we need to replace the fossil fuels and move towards more environmentally friendly renewable primary energy sources (IPCC, 2014).

Biobutanol is a biofuel with numerous advantages and it could contribute to a reduction of the greenhouse gas emissions (Dürre 2007). It is environmentally friendly as it can be produced by bacteria or yeast with a plant-based feedstock (Dürre 2007, Hong and Nielsen, 2012) and is therefore a potential replacement for fossil fuels. The advantages of butanol include 1) a capability to be blended in any concentration with
gasoline, 2) a capability to be used in existing car engines, 3) a non-hygroscopic attribute (do not bind water molecules) that will prevent contamination of groundwater in case of spills. Butanol also has advantages over ethanol, which is one of the most frequently used biofuels in the world. Butanol is safer to handle, less corrosive and the energy content is higher compared to ethanol (Dürre 2007).

2.2 Butanol production by *Saccharomyces cerevisiae*

The yeast *S. cerevisiae* is on daily basis most known as a component in the production of bread and alcohols through its ability to convert sugar to ethanol, but *S. cerevisiae* also has the ability to produce butanol (Kuroda and Ueda 2016). The yeast has several advantages that makes it beneficial for butanol production; *S. cerevisiae* is a facultative anaerobe, it is easy to handle, has a tolerance to several fermentations products and an ability to ferment at low pH, which means that the microbe contamination will decrease (Kuroda and Ueda 2016, Fischer et al. 2008). Bacteria, like *Escherichia coli*, Lactobacillus species and Clostridial species, are also potential butanol producers with different advantages and disadvantages. *E. coli* has a well-studied genome but has a low tolerance to butanol. Lactobacillus species are butanol tolerant but does not have the ability to produce butanol naturally. Clostridial species are able to produce butanol naturally but have a low butanol tolerance. In current time, none of the bacteria species or yeast species is the obvious choice for biobutanol production (Liu et al. 2017).

Four isoforms of butanol exist, isobutanol, 1-butanol, 2-butanol and tert-butanol. Isobutanol and tert-butanol have higher octane numbers than 1- and 2- butanol, which is advantageous for a fuel. Although, tert-butanol has a high melting temperature that makes it unsuitable as a fuel (Hong and Nielsen 2012). *S. cerevisiae* is able to produce isobutanol naturally. This process is based on valine synthesis in the mitochondria and the Ehrlich pathway in the cytosol (Hazelwood et al. 2008, Kuroda and Ueda 2016). 1-butanol cannot be produced naturally by *S. cerevisiae*, although it can be produced by Clostridial species. Clostridia is however not the ideal butanol-producer. This is due to their slow growth, their intolerance to butanol and that their genome is not well studied. Scientists have therefore introduced the Clostridial pathway for 1-butanol production in *S. cerevisiae* (Steen et al. 2008). Other pathways have also been designed to produce 1-butanol, such as the glycine degradation pathway (Branduardi et al. 2013) and the threonine metabolic pathway (Si et al. 2014). Like 1-butanol synthesis, 2-butanol synthesis by *S. cerevisiae* is
not possible without metabolic engineering. Ghiaci et al. (2014) were able to make *S. cerevisiae* produce 2-butanol through a B$_{12}$-dependent diol dehydratase system from *Lactobacillus reuterii* where 2,3-butanediol was converted to butanone. To convert butanone to 2-butanol the authors used a secondary alcohol dehydrogenase from *Gordonia sp.*

A problem in the biobutanol-synthesis-process is that the end-product, butanol, is toxic to the producer (Fischer et al. 2008). Studies have shown that isobutanol and 2-butanol can be tolerated at higher concentrations by *S. cerevisiae* while 1-butanol seems to be the most toxic butanol isomer (González-Ramos et al. 2013, Fischer et al. 2008, Chen et al. 2011). This is probably due to that the 1-butanol molecule is a more hydrophobic molecule compared to iso- and 2-butanol, and this attribute will affect the cell membrane in a negative way (Paterson et al. 1972, Ghiaci et al. 2013).

### 2.3 Genes associated with butanol tolerance

An ability to recognize and degrade damaged proteins is essential for butanol tolerance and this is coupled to gene expression (González-Ramos et al. 2013). It has been shown that the expression of the gene *RPN4* in *S. cerevisiae* is increased during exposure of butanol (Zaki et al. 2014). The synthesis of proteasome proteins, which function is to degrade damaged proteins, is stimulated by a transcription factor encoded by *RPN4*. González-Ramos et al. (2013) suggested that this is coupled to an increased butanol tolerance. Therefore, it is interesting to investigate proteins that are associated with the RPN4 protein. Three potential proteins associated with RPN4 were found, LPL1, TMC1 and FLR1. The phospholipase B protein LPL1 is a component of lipid droplets. Lipid droplets are organelle-like structures consisting of lipids that have a function in the cell’s metabolism (Selvaraju et al. 2014). Weisshaar et al. (2017) identified LPL1 as a target of the RPN4 response and suggest that the proteasomal protein degradation is associated to the lipid droplet. TMC1 is a zinc finger protein that was found to be an effector and a substrate of the RPN4 response (Guerra-Moreno and Hanna 2016). Transcription of the gene *FLR1* was found to be activated in response to mancozeb (agricultural fungicide) in *S. cerevisiae*. When the genes *RPN4*, *YRR1* and *PDR3* were absence in the yeast during mancozeb treatment, the transcription of *FLR1* decreased (Teixeira et al. 2008).
2.4 Aim
The aim of this study was to investigate the 1- and 2-butanol tolerance for four different \textit{S. cerevisiae} strains and investigate the gene expression for \textit{LPL1}, \textit{TMC1} and \textit{FLR1} that are linked with the gene \textit{RPN4}, which is associated with butanol tolerance. The larger purpose for the study was to improve the butanol-production by \textit{S. cerevisiae}. The investigation of 1- and 2-butanol tolerance was examined with spot tests and growth measurements. The gene expression was investigated with qPCR and the hypothesis was that all examined genes would be up-regulated in exposure to butanol.

3 Material and methods

3.1 Yeast strains and growth condition
The tolerance for 1- respective 2-butanol were investigated for four different yeast strains; Red Star (active dry wine yeast), Craft Series M20 (Bavarian wheat), Saflager w-34/70 (dry lager yeast) and Safale US-05 (dry ale yeast). The yeast strains will be referred to as wine yeast, Bavarian wheat yeast, lager yeast and ale yeast.

YPD (yeast extract peptone dextrose) medium for yeast culturing was prepared as follows: 10 g Yeast extract, 20 g Peptone, 20 g Glucose and distilled water up to 1000 ml. YPD agar plates were also prepared according to the YPD medium recipe, though 20 g agar was added. The YPD medium and YPD agar were autoclaved in 121 °C for 15 minutes. Cultured yeast was kept in an incubator (TS 8136, Termaks and Ecotron, Infors HT) at 30 °C. For liquid medium, shaking at 190 rpm were included (Ecotron, Infors HT and Innova®40, New Brunswick Scientific).

Yeast from the four different strains were added to separate tubes with 5 ml YPD medium and cultured overnight at 30 °C with shaking at 190 rpm. 100 µl of the yeast cultures were then added to, and spread on YPD plates in dilutions of $10^3$ and $10^6$ with distilled water and incubated for 48 hours at 30 °C. One colony from respective strain were plated on YPD agar plates and incubated for 48 hours at 30 °C for proliferation, this will be referred to as the start plates. After the incubation, one colony from each strain were added to 5-15 ml YDP medium and incubated for 24-48 hours at 30 °C with shaking at 190 rpm. The last step, cultivation of a colony, was repeated before a new set of experiments were started.
The YPD agar start plates, with one plated colony, were after the incubation stored at 4 °C during the study.

3.2 Spot test

To investigate the butanol tolerance for the yeast strains, YPD agar plates were prepared with 1-butanol or 2-butanol respectively with concentration of 1.5 %, 3 % and 4 %. Control plates with no butanol were also prepared. One colony from each strain’s start plate was added to 5 ml YPD medium and incubated for 24 hours. The optical density (600 nm) \( \text{OD}_{600} \) of the cultures were adjusted to 1, this will be referred to as dilution 10\(^0\). A dilution series of 10\(^2\), 10\(^3\) and 10\(^4\) were prepared for every strain. 5 µl from each dilution were added to the plates. All plates (control, 1.5 %, 3 % and 4 % butanol concentration) were done in triplicate. The plates were incubated at 30 °C and analysed after 24, 48 and 72 hours (Figure 1). The experiment was repeated twice.

3.3 Growth in liquid medium with 1-butanol and 2-butanol

To further investigate the butanol tolerance, the yeast strains were grown in liquid medium with butanol. Bottles with 25 ml YPD medium were prepared. 1- or 2-butanol was added to concentrations of 2 % and 3 %, control bottles were also prepared without butanol. One colony from each strain’s start plate was added to 15 ml YPD medium and incubated for 24-72 hours. The cultures were added to respective bottle to an initial \( \text{OD}_{600} \) between 0.1-0.2. The bottles were incubated at 30 °C with shaking at 190 rpm for 48 hours and the \( \text{OD}_{600} \) was measured every 180 minutes with an interruption during the night. WPA lightwave II spectrophotometer (Biochrom) was used for OD measurements. The experiment was repeated twice.

3.4 Gene expression

The ale yeast cultured in 2-butanol was chosen for the investigation of gene expression because it showed the highest tolerance for 2 % 2-butanol and it also showed tolerance to 3 % 2-butanol. 2-butanol was chosen over 1-butanol though the strains, except lager yeast, showed a higher tolerance for 2-butanol. Two different reference genes were used, UBC6 and TAF10 though they have been proven as reliable reference genes with no up or down-regulation when exposed for different conditions (Teste et al. 2009).
3.4.1 Primers

Primers for the genes FLR1, TMC1 and LPL1 were designed using NCBI/Primer Blast (www.ncbi.nlm.nih.gov). The PCR product size were set to 150-200 and the primer melting temperature to min 57 °C, optimal 60 °C and max 63 °C. A second primer pair for FLR1 was found in a previously published study (Teixeira et al. 2008). Because of curiosity, it was decided to use both primer pairs for FLR1. The results from the two primer pairs were similar and therefore an average from the primers was used for calculation of the result. Primers for the reference gene UBC6 were selected from previous studies (Nadai et al. 2015, Teste et al. 2009). Primers for the RPN4 gene were obtained from a previous bachelor thesis (Gerebring 2016). Primers for the TAF10 gene were supplied by supervisor. Primer sequences can be found in Appendix Table 1.

3.4.2 Preparations for qPCR and implementation of qPCR

A colony from the ale yeast was cultured in YPD medium overnight. The culture was diluted to OD$_{600}$ 0.1 in YPD medium or YPD medium containing 3 % 2-butanol. The bottles were incubated until OD$_{600}$ of 1. The yeast cultured in YPD media reached OD$_{600}$ of 1 faster than the yeast cultured in 3 % 2-butanol. The yeast was therefore frozen in -80 °C in a 15 ml tube until usage. Before freezing, the yeast was centrifuged (Universal 320 R, Hettich Zentrifugen) for 15 minutes at 1500×g at 4 °C and the supernatant was discarded. The 3 % 2-butanol cultured yeast was not frozen. Two samples from each bottle, with or without butanol, was made.

The RNA was isolated using FastRNA® Pro Red Kit (MP Biomedicals) and the RNA concentration was measured on the nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific).

To remove contaminating DNA from the RNA, DNA-free™ Kit (Life technologies™) was used. 5 µg RNA, 1 µl rDNase, 5 µl DNase 1 buffer and water up to 50 µl were added to a 0.5 ml tube and mixed gently. The samples were incubated at 37 °C for 30 minutes. 5 µl DNase Inactivation Reagent were added to each sample. The samples were then incubated at room temperature for 2 minutes and mixed occasionally. After the incubation, the samples were centrifuged in Micro Star 17 (Thermo Fisher Scientific) at 10.000×g for 2 minutes at room temperature. The RNA were then transferred to a new 0.5 ml tube.
For the cDNA synthesis, High Capacity cDNA Reverse Transcription Kits Protocol (Applied Biosystems) was used, a 2× RT master mix per 20 µl reaction was prepared by mixing 2 µl 10×RT Buffer, 0.8 µl 25x dNTP, 2 µl 10× RT Random Primers, 1 µl MultiScribe™ Reverse Transcriptase and 4.2 µl Nuclease-free H₂O on ice. 10 µl of the 2× RT master mix was added to a PCR-tube and then 10 µl of the RNA sample was added to the tube. The samples were mixed and centrifuged briefly and put on ice. The samples were then loaded into the Thermal cycler S1000™ (BioRad) and were run with the program 25 °C for 10 minutes, 37 °C for 120 minutes, 85 °C for 5 minutes and 4 °C for infinity.

To prepare the qRT-PCR the cDNA samples were diluted 4-fold in 0.1 ml tubes, mixed and centrifuged briefly. Each reaction was set up in a 0.1 ml tube, one tube for every gene and condition (normal condition or 3 % 2-butanol) and in addition No Template Control (NTC) samples were set up for every primer pair. A master mix for every gene containing 2×PCR Master mix, forward and reverse primer and sterile water was prepared. 17 µl master mix was added to the 0.1 µl tubes and then 8 µl cDNA (or water for the NTCs) was added. The tubes were kept on a chilled metal racket while pipetting. The tubes were then containing 12 µl 2xPCR Master mix, 1 µl (10 pmol/µl) forward primer and 1 µl (10 pmol/µl) reverse primer for the genes UBC6, TAF10, FLR1, TMC1, LPL1 and RPN4, 8 µl template cDNA (or water for the NTCs) and 2.5 µl sterile water. The samples were then loaded in the qPCR instrument (Rotor-gene 6000, Corbett research) and were run with the program 95 °C for 10 minutes; 40 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds; and finally, a gradual increase from 60 °C to 99 °C. Melt curves for the different genes and treatments could be found in Appendix Figure 1-14. After the qPCR run Ct values (relative measure for the gene-concentration) for each gene and treatment were obtained and analysed.

The normalized target gene expression level was calculated with the Livak-method using the formula \(2^{-\Delta\Delta Ct}\) (Livak and Schmittgen 2001). The method is based on that the material in the samples will double for every PCR cycle and in that way, the relative gene expression for the target genes, in this case TMC1, LPL1, FLR1 and RPN4, can be calculated. The control sample without 2-butanol will have a \(\Delta\Delta Ct\) value equal to zero and \(2^0\) is equal to 1, therefore the value 1 determines if the gene from the butanol treated sample is up-regulated \((2^{-\Delta\Delta Ct}>1)\) or down-regulated \((2^{-\Delta\Delta Ct}<1)\) (Livak and Schmittgen 2001). To investigate significant difference between the treatments (without 2-butanol and with 3 % 2-
butanol) the ΔCt values from the Livak-method were used in statistical analyses.

3.5 Statistical analysis
2-sample t-tests were performed in Minitab 17 to investigate if there was a significant difference between the treatments, without 2-butanol and with 3 % 2-butanol, for each gene \((FLR1, LPL1, RPN4 \text{ and } TMC1)\). The t-tests were done for each reference gene, \(UBC6\) and \(TAF10\).

4 Results

4.1 Spot test
After 72 hours, all yeast strains grew at the control plate (Figure 1A, 1E). The same result was found for the 1.5 % 2-butanol plate (Figure 1F). At the 1.5 % 1-butanol plate ale yeast and Bavarian wheat yeast grew at all concentrations, wine yeast did not form a visible colony at \(10^4\) and lager yeast did grow at OD \(600\) of 1 (dilution \(10^0\)) and \(10^2\) (Figure 1B). At the 3 % 2-butanol plate ale yeast and Bavarian wheat yeast grew at all concentrations, wine yeast did not show a visible colony at \(10^4\) and lager yeast did not show a visible colony (Figure 1G). At the 4 % 2-butanol plates the yeasts wine, ale and Bavarian wheat grew at \(10^0\) (Figure 1H). At the 3 % and 4 % 1-butanol plates none of the yeast strains showed any visible colonies (Figure 1C and 1D).
Figure 1. Representative 1-butanol (A, B, C, D) and 2-butanol (E, F, G, H) spot test for the examined yeasts wine, lager, ale and Bavarian wheat after 72 hours. Plate A – control plate, B contains 1.5 % 1-butanol, C contains 3 % 1-butanol and D contains 4 % 1-butanol. Plate E – control plate, F contains 1.5 % 1-butanol, G contains 3 % 1-butanol and H contains 4 % 1-butanol. Upper row of colonies, on one plate, had an OD$_{600}$ of 1 (dilution $10^0$) at the time of addition, second row was diluted with dilution factor $10^2$, third row with $10^3$ and fourth row with $10^4$.

4.2 Growth measurements

4.2.1 1-butanol

The growth of the yeast strains was also investigated in liquid medium. The results showed a stable growth for all yeast strains in the standard YPD without butanol (Figure 2A). For the 2 % 1-butanol condition ale yeast showed the highest tolerance and percentage growth, after approximately 14-20 hours ale yeast began to grow and continued to grow during the experiment (Figure 2B and 2D). The yeasts Bavarian wheat, lager and wine showed no tolerance for 2 % -butanol (Figure 2B). For the 3 % 1-butanol condition none of the strains showed tolerance (Figure 2C and 2D). However, ale yeast was able to tolerate the 3% butanol condition for 6 hours before the OD$_{600}$ decreased (Figure 2C).
Figure 2. A-C shows representative curves of growth measurements in $OD_{600}$ of the yeasts ale, Bavarian wheat, lager and wine in a condition without 1-butanol (A), in a 2% 1-butanol condition (B) and in a 3% 1-butanol condition (C). D shows the percentage growth during 48 hours for the strains without 1-butanol and with 2 and 3% 1-butanol. Observe the different scales on the y-axis.
4.2.2 2-butanol

Overall the stains showed a higher tolerance for 2-butanol compared to 1-butanol. Further the results showed a stable growth for all yeast strains in the standard YPD without butanol (Figure 3A). For the 2 \% 1-butanol condition the ale yeast showed the highest tolerance, Bavarian wheat and wine yeast showed tolerance as well while lager yeast displayed no tolerance (Figure 3B), this is also reflected in the percentage growth during 48 hours (Figure 3D). For the 3 \% 1-butanol condition the yeasts ale, Bavarian wheat and wine showed similar tolerance, wine yeast had a slightly higher percentage growth while lager yeast showed no tolerance (Figure 3C).
Figure 3. A-C shows representative curves of growth measurements in OD<sub>600</sub> of the yeasts ale, Bavarian wheat, lager and wine in a condition without 2-butanol (A), in a 2 % 2-butanol condition (B) and in a 3 % 2-butanol condition (C). D shows the percentage growth for the strains during 48 hours without 2-butanol and with 2 and 3 % 2-butanol. Observe the different scales on the y-axis.
4.3 Gene expression

4.3.1 Relative gene expression

Relative gene expression was used to investigate the gene expression of RPN4, TMC1, LPL1 and FLR1 treated with and without butanol. Ale yeast treated with 3 % 2-butanol and without 2-butanol was used for the investigation.

The expression relative to the reference gene UBC6 (Figure 4) showed a 6.2- and 2.8-fold up-regulation for RPN4 and TMC1. FLR1 showed a 0.3-fold down-regulation and LPL1 did not seem to be obviously affected with a 0.9-fold down-regulation.

![Figure 4. The relative gene expression with UBC6 as the reference gene. The mean value was 0.3 for FLR1, 0.9 for LPL1, 6.2 for RPN4 and 2.8 for TMC1. The black dot shows the mean value with a confidence interval of 95 %, N=4. The circles are the individual values. The red dotted line (2^ΔΔCt=1) clarifies up-regulation (2^ΔΔCt>1) or down-regulation (2^ΔΔCt<1) of the gene.](image)

The expression relative to the reference gene TAF10 (Figure 5) showed an up-regulation for LPL1 (2.2-fold), RPN4 (15-fold) and for TMC1 (6.7-fold). FLR1 showed a slightly, 0.8-fold, down-regulation.
Figure 5. The relative gene expression with TAF10 as the reference gene. The mean value was 0.8 for FLR1, 2.2 for LPL1, 15 for RPN4 and 6.7 for TMC1. The black dot shows the mean value with a confidence interval of 95 %, N=4. The circles are the individual values. The red dotted line ($2^{\Delta\Delta Ct}=1$) clarifies up-regulation ($2^{\Delta\Delta Ct}>1$) or down-regulation ($2^{\Delta\Delta Ct}<1$) of the gene.

4.3.2 Comparison between treatments

When the ΔCt values for each treatment (without 2-butanol or with 3 % 2-butanol) were compared for every gene with a 2-sample t-test it could be determined if there was a significant difference between the treatments. To calculate the ΔCt values for each treatment, the Ct value from one investigated gene was subtracted with the Ct value for one reference gene. If ΔCt with 3 % butanol has a lower value than ΔCt without butanol, the relative gene expression calculated with the Livak-method (using the formula $2^{-\Delta\Delta Ct}$) will show an increase.

The results with reference gene UBC6 showed that the 3 % 2-butanol ΔCt value was significantly higher than the ΔCt without 2-butanol for FLR1 (p=0.014; t=3.73; α=0.05; N=4) (Figure 7A). The RPN4 gene and the TMC1 gene had significantly lower ΔCt values for 3 % 2-butanol (p=0.002; t=-5.79; α=0.05; N=4), (p=0.006; t=-4.50; α=0.05; N=4) (Figure 6C and 6D) and there was no difference between the ΔCt values for LPL1 (p=0.403; t=0.91; α=0.05; N=4) (Figure 7B). These results are reflecting the relative gene expression (Figure 4) were both RPN4 and TMC1 were up-regulated in 3 % 2-butanol treatment, FLR1 was down-regulated and LPL1 did not seem to be affected.
Figure 6. ΔCt values for each treatment and gene; FLR1, LPL1, RPN4 and TMC1. The reference gene was UBC6. The star indicates a significant difference between the treatments, the black dot shows the mean value with a confidence interval of 95 % (N=4) and circles show the individual values. FLR1 with 3 % 2-butanol showed a higher ΔCt value than without 2-butanol. The ΔCt values for LPL1 did not show a difference. RPN4 with 3 % 2-butanol showed a lower ΔCt value than without 2-butanol. TMC1 with 3 % 2-butanol showed lower ΔCt than without 2-butanol.

The results for the treatment comparison with the reference gene TAF10 showed a similar result as with the reference gene UBC6. RPN4 and TMC1 had a significantly lower a ΔCt value for the 3 % 2-butanol treatment (p=0.000; t=-8.10; α=0.05; N=4), (p=0.000; t=-9.69; α=0.05; N=4) (Figure 7C and 7D). There was no difference between the ΔCt values for FLR1 (p=0.214; t=1.42; α=0.05; N=4) (Figure 7A), while the 3 % 2-butanol ΔCt value for LPL1 was significantly higher than without 2-butanol (p=0.03; t=-3.01; α=0.05; N=4) (Figure 7B). This results are as well reflected in the relative gene expression with TAF10 as the reference gene (Figure 5). LPL1, RPN4 and TMC1 is up-regulated in the presence of 3 % 2-butanol and FLR1 is slightly down-regulated.
Figure 7. ΔCt values for each treatment and gene; FLR1, LPL1, RPN4 and TMC1. The reference gene was TAF10. The star indicates a significant difference between the treatments, the black dot shows the mean value with a confidence interval of 95 % (N=4) and circles show the individual values. The ΔCt values for FLR1 did not show a difference. The 3 % 2-butanol ΔCt value for LPL1 was lower than without 2-butanol. RPN4 with 3 % 2-butanol showed a lower ΔCt value. The 3 % 2-butanol ΔCt values for TMC1 was lower than without 2-butanol.

5 Discussion

In this report three main statements were concluded; the investigated yeast strains were more tolerant to 2-butanol than 1-butanol, ale yeast was most butanol tolerant of the strains investigated and the genes TMC1 and RPN4 were up-regulated in exposure of 2-butanol.

According to the performed spot tests and growth measurements the tested yeast strains were more tolerant to 2-butanol compared to 1-butanol. This has also been shown by other scientists (González-Ramos et al. 2013, Fischer et al. 2008) and is due to the different molecular character of the butanol isomers. The 1-butanol molecule is a more hydrophobic molecule that will interact with the cell membrane to a greater extent and therefore has a more toxic effect to the cell (Paterson et al. 1972).

Of the examined S. cerevisiae stains, ale yeast was the most butanol-tolerant and lager yeast was the least butanol-tolerant. Both the spot test
and growth measurement test showed that it was only in conditions without butanol that a growth rate could be seen for lager yeast and it was slow compared to the other strains. The slow growth rate is probably an explanation for the low tolerance to butanol. Ale yeast had a faster growth rate and it was only in the condition with 3 % 1-butanol that ale yeast did not grow. Without any other investigations of the two strains, like comparisons of genome or transcriptomes, this results cannot be explained further. The system for protein degradation has been shown to be essential for butanol tolerance (González-Ramos et al. 2013). A theory could therefore be that lager yeast has an ineffective or slow system for protein degradation and ale yeast has a more effective system, but this theory would need to be investigated further to be concluded. Although, when the specification sheets for the yeasts were compared, the lager yeast that was used had a recommended fermentation temperature between 9-22 °C, ideal between 12-15 °C (Fermentis 2017a), while the ale yeast had a recommended fermentation temperature between 18-28 °C (Fermentis 2017b). The yeast in this study was kept in an incubator at 30 °C, which means that it was too hot for the lager yeast. This seems to be a logical explanation for the lager yeast’s slow growth. Further investigations may therefore be needed to determine lager yeast’s butanol tolerance. It should also be mentioned that the growth measurements were hard to explain though the growth rates of the different strains were not always consistent in the repeated experiment and a third repetition of the experiment would have been preferred to secure the results.

For the gene expression investigation in ale yeast, the TMC1 gene and RPN4 showed an up-regulation at 3 % 2-butanol stress with both reference genes, UBC6 and TAF10. TMC1 up-regulation has also been shown by Guerra-Moreno and Hanna (2016) and the RPN4 up-regulation has been shown by Zaki et al (2014). Guerra-Moreno and Hanna (2016) used arsenic as a stressor for the yeast, Zaki et al (2014) used 1-butanol as stressor and the results in this study can now confirm that TMC1 and RPN4 will show an up-regulation with 2-butanol as a stressor as well. This result also indicates that TMC1 is associated to the RPN4 stress response as Guerra-Moreno and Hanna (2016) confirmed in their study.

The LPL1 gene was up-regulated 2.2-fold in exposure to 3 % 2-butanol with TAF10 as the reference gene but did not show any up- or down-regulation with UBC6 as the reference gene. Weisshaar et al. (2017) identified LPL1 as a target of the RPN4 response and this is partly shown in this study. LPL1 has different roles in the stress response. In additional to the RPN4 response it has a part in the unfolded protein response,
which function is to recognize misfolded proteins in the endoplasmic reticulum (Shamu et al. 1994). What can be said is that LPL1 has a complex role in the cell’s stress response and this needs further investigation.

The FLR1 gene also showed different results with the different reference genes, it was down-regulated with UBC6 as the reference gene but showed no up- or down-regulation with TAF10 as the reference gene. Teixeira et al (2008) showed that FLR1 was up-regulated when exposed to fungicide and in presence of the gene RPN4. Though fungicide is toxic for the yeast it must be a stressful environment. Although, the result in this study did not show any up-regulation of FLR1, it can be concluded that stress caused by fungicide do not affect the yeast in the same way as butanol-stress. Further investigation needs to conclude if FLR1 has a role in the RPN4 stress response caused by butanol.

As mentioned, the two reference genes did not show the same gene expression result for the examined genes, although the trend was similar. To prove which reference gene to trust, additional reference genes could have been included in this study.

*S. cerevisiae* has many advantages in terms of production of biobutanol (Kuroda and Ueda 2016, Fischer et al. 2008) but is not the obvious choice. Different bacterial species also have advantages in the biobutanol production. *E. coli*, for example, is a very well-known organism and is preferable in metabolic engineering. Clostridial species can produce butanol but have a higher sensitivity to butanol compared to *S. cerevisiae* (Liu et al. 2017). Although in my perspective *S. cerevisiae* seems like a preferable organism to improve with metabolic engineering to achieve a butanol-tolerant and effective butanol-producer. This opinion is based on *S. cerevisiae*’s tolerance to butanol, tolerance to several fermentations products, ability to ferment at low pH and well-studied genome (Kuroda and Ueda 2016, Fischer et al. 2008).

To achieve a *S. cerevisiae* strain that is butanol-tolerant and an effective butanol-producer, metabolic engineering is required. In theory, the best butanol-pathway and genes associated with butanol tolerance could be introduced in the yeast. An example of a successful concept is one by Steen et al. (2008). They succeeded in introducing Clostridia’s 1-butanol pathway in *S. cerevisiae* and achieved a 1-butanol production of 2.5 mg/L. Further Schadeweg and Boles (2016) increased the 1-butanol production to 130±30 mg/L when they improved the 1-butanol pathway
by overexpressing genes coupled to butanol production and inactivating genes coupled to competing pathways.

5.1 Societal and ethical aspects
In addition to the warming of the climate system, the fossil fuels are a limited fuel source and therefore we need to find a renewable environmentally friendly fuel for the future. Ethanol is currently one of the most used biofuel in the world but butanol would be a more preferable biofuel due to its higher energy content. \textit{S. cerevisiae} is a potential biobutanol producer with many advantages. For an effective biobutanol production in the future by \textit{S. cerevisiae}, research about \textit{S. cerevisiae}’s butanol tolerance and genes coupled to butanol tolerance and effective production are important.

5.2 Conclusion
In this study the usage of 2-butanol as a biofuel is preferred over 1-butanol due to \textit{S. cerevisiae}’s higher tolerance to 2-butanol. A potential yeast to use in the production of biobutanol is ale yeast due to a higher butanol tolerance and \textit{RPN4} and \textit{TMCI} is important genes to use to achieve a higher butanol tolerance in \textit{S. cerevisiae}. To achieve a butanol-tolerant \textit{S. cerevisiae} strain with effective butanol-production it is important to further investigate genes associated to butanol tolerance. To be able to replace fossil fuels with biobutanol, the final aim is to find a tolerant \textit{S. cerevisiae} strain and use molecular and cellular engineering to improve it.

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## Appendix

**Table 1.** Primers used for the gene expression investigation. FLR1 (1) is the primer pair from NBCI/Primer Blast and FLR1 (2) is the primer pair from Teixeira et al (2008).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLR1 (1)</td>
<td>TTTGCGTAGGGTGCGTACTT</td>
<td>AATGCACTCGAGCAGTCCAA</td>
</tr>
<tr>
<td>FLR1 (2)</td>
<td>CTGTGCTGCGCCAGTCTT</td>
<td>GCCAATTTTTAGCATCGACCAT</td>
</tr>
<tr>
<td>TMC1</td>
<td>GCAGTAGGGGGGTGACTAAG</td>
<td>GCTGTTCTTTGCAGCTCGTT</td>
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<td>LPL1</td>
<td>AACGCCTCATTTGGGGTTAG</td>
<td>AGGTACTCCCTGGCTCAA</td>
</tr>
<tr>
<td>RPN4</td>
<td>GCTTCTGATACCCCCACAACA</td>
<td>GGGTTTCGCTAAGCACCCTTA</td>
</tr>
<tr>
<td>UBC6</td>
<td>GATACTTTGGAATTCTGGGCTG</td>
<td>AAAGGCTTTCCTTTCATCACC TGTATTTC</td>
</tr>
<tr>
<td>TAF1</td>
<td>TCCAGGATCAGGTTTCCGTA</td>
<td>TTGTGTTGAATCTGCTGACGC</td>
</tr>
</tbody>
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**Figure 1.** Melt curves for experiment 1 FLR1 (1) control 1, FLR1 (1) control 2, FLR1 (1) with 3 % 2-butanol sample 1, FLR1 (1) with 3 % 2-butanol sample 2 and FLR1 (1) with water. FLR1 (1) control 1 was excluded from the results. FLR1 (1) is the primer pair from NBIC/Primer Blast.

![Graph](image1)

**Figure 2.** Melt curves for experiment 1 FLR1 (2) control 1, FLR1 (2) control 2, FLR1 (2) with 3 % 2-butanol sample 1, FLR1 (2) with 3 % 2-butanol sample 2 and FLR1 (2) with water. FLR1 (2) control 1 was excluded from the results. FLR1 (2) is the primer pair from Teixeira et al (2008).

![Graph](image2)
Figure 3. Melt curves for experiment 1 LPL1 control 1, LPL1 control 2, LPL1 with 3 % 2-butanol sample 1, LPL1 with 3 % 2-butanol sample 2 and LPL1 with water.

Figure 4. Melt curves for experiment 1 RPN4 control 1, RPN4 control 2, RPN4 with 3 % 2-butanol sample 1, RPN4 with 3 % 2-butanol sample 2 and RPN4 with water.
Figure 5. Melt curves for experiment 1 TAF10 control 1, TAF10 control 2, TAF10 with 3 % 2-butanol sample 1, TAF10 with 3 % 2-butanol sample 2 and TAF10 with water. TAF10 control 1 was excluded from the results.

Figure 6. Melt curves for experiment 1 TMC1 control 1, TMC1 control 2, TMC1 with 3 % 2-butanol sample 1, TMC1 with 3 % 2-butanol sample 2 and TMC1 with water.
Figure 7. Melt curves for experiment 1 UBC6 control 1, UBC6 control 2, UBC6 with 3 % 2-butanol sample 1, UBC6 with 3 % 2-butanol sample 2 and UBC6 with water.

Figure 8. Melt curves for experiment 2 FLR1 (1) control 1, FLR1 (1) control 2, FLR1 (1) with 3 % 2-butanol sample 1, FLR1 (1) with 3 % 2-butanol sample 2 and FLR1 (1) with water. FLR1 (1) is the primer pair from NBIC/Primer Blast.
Figure 9. Melt curves for experiment 2 FLR1 (2) control 1, FLR1 (2) control 2, FLR1 (2) with 3 % 2-butanol sample 1, FLR1 (2) with 3 % 2-butanol sample 2 and FLR1 (2) with water. FLR1 (2) is the primer pair from Teixeira et al (2008).

Figure 10. Melt curves for experiment 2 LPL1 control 1, LPL1 control 2, LPL1 with 3 % 2-butanol sample 1, LPL1 with 3 % 2-butanol sample 2 and LPL1 with water.
Figure 11. Melt curves for experiment 2 RPN4 control 1, RPN4 control 2, RPN4 with 3 % 2-butanol sample 1, RPN4 with 3 % 2-butanol sample 2 and RPN4 with water.

Figure 12. Melt curves for experiment 2 TAF10 control 1, TAF10 control 2, TAF10 with 3 % 2-butanol sample 1, TAF10 with 3 % 2-butanol sample 2 and TAF10 with water. TAF10 control 1 was excluded from the results.
Figure 13. Melt curves for experiment 2 TMC1 control 1, TMC1 control 2, TMC1 with 3 % 2-butanol sample 1, TMC1 with 3 % 2-butanol sample 2 and TMC1 with water.

Figure 14. Melt curves for experiment 2 UBC6 control 1, UBC6 control 2, UBC6 with 3 % 2-butanol sample 1, UBC6 with 3 % 2-butanol sample 2 and UBC6 with water.