



Factors influencing the fermentation of spruce hydrolysate by *Dekkera bruxellensis* and *Saccharomyces cerevisiae*

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ABSTRACT

Bioethanol and biogas are two possible alternatives to fossil fuel resources. The second generation fermentations involving lignocellulosic material is one of the latest fields for bioethanol science. In the current work two fermentation yeasts, *Saccharomyces cerevisiae* J672 and the alternative fermentation yeast *Dekkera bruxellensis* CBS 11269, were compared in an experimental design for their ability to ferment spruce lignocelluloses. The goal was to find differences between the growth and ethanol yield of the two species. The investigation was done with a multivariate data analysis tool to examine yeast growth and ethanol yield. Four quantitative factors, pH, temperature, concentration of hydrolysate, and initial cell density were used. The analysis also included one qualitative factor with two settings, aerobic and oxygen limited. The five factors were tested in a fractional factorial design created with MODDE software. The results for the experimental design with *S. cerevisiae* showed that the main significant factor for ethanol yield was oxygen. Oxygen was the most important factor for *S. cerevisiae* ethanol yield response, oxygen limitation had positive contribution and aerobic environment had negative contribution. The oxygen factor however was not important for growth. pH was a significant factor and high pH was shown to give better response, for both yeast growth and ethanol yield. Oxygen and temperature had negative contribution while the two factors initial cell concentration and concentration of hydrolysate did not show any significant contribution to the model for *S. cerevisiae* ethanol yield. *D. bruxellensis* was adapted to spruce hydrolysate before the final experiment. This was because the non adapted *D. bruxellensis* CBS11269 did not grow well, in the first experiment, and did not build a model. The two models with *S. cerevisiae* J672 and spruce adapted *D. bruxellensis* CBS 11269 are therefore not directly comparable. The adapted *D. bruxellensis* model for ethanol yield was not complete. Analysis of the data showed a strong curvature within the model and further analysis is needed to complete the model. However the experiment resulted in a very good model for yeast growth. It showed that pH and initial cell concentration was positive for growth and that hydrolysate concentration had a strong negative contribution to growth. The temperature had a negative contribution and the oxygen factor was not significant for *D. bruxellensis* growth. When comparing the ethanol yields between both adapted and non adapted *D. bruxellensis* with *S. cerevisiae*, both *D. bruxellensis* yeasts shows a higher or equal total yield. A significant statistical difference was shown between the centre samples with access to oxygen, where *D. bruxellensis* gave a higher ethanol yield.

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1 INTRODUCTION

The major reasons for research on alternative fuels such as biofuels are: The release of carbon dioxide to the environment causing a greenhouse effect (Rodgers et al. 2008) and the limitations of fossil resources. Fossil fuels are the main source for energy in the 21st century, they supply almost 80% of the worlds total energy consumption (REN. 2010). Other problems with fossil fuels, occur when the easy accessible resources runs out. For example British petroleum (BP) caused one of the worlds biggest oil releases in history in 2010 when they tried to reach a deep oil source in the atlantic ocean (RTG. 2010). The change of main energy source from fossil fuels to recycled energy is very slow, some reasons is political but it is also low benefits and high economical risks with green energy (FRM. 2008).

Bioethanol is one promising alternative, in the field, for a future with green energy. One problem though is that the current bioethanol plants mainly use crops as substrate. It is a problem since crops may also be used for food and feed. The best alternative would be to use a substrate for bioethanol which is not competing with food sources. Lignocellulose for example is a good possible alternative source. Still most science projects in the US focus on more efficient crops per area, rather than looking for possible alternative sources. One reason is that its less economical risks to streamline an existing process than try out a new one(Rodgers et al. 2007).

Lignocellulose is a good and abundant sugar source, it exist in great amount in nature and would not compete with food productions. Lignocellulose builds up the structure of trees, bushes, crop straws etc. It should be ideal for bioethanol production, but there are problems. Microorganisms are not able to degrade or ferment untreated lignocellulose during a reasonable time. Lignocellulose is built of three main components, lignin, hemicellulose and cellulose. Cellulose is in the centre surrounded with hemicellulose, both is covered with the strong fibre, lignin. Cellulose is built of D-glucose sugar subunits (Laureano-Perez et al. 2005). Hemicellulose are polymers built of xylose, mannose and glucose. Lignin is built of amorphous polymers of phenolic compounds. The sugar molecules in hemicellulose are tightly packed, and the melting points for the sugar polymers are above 150°C. Yeast do not have capability to ferment them in this state (Fengel and Wegener. 1984). Lignin is water resistant and the binding properties of the polymers cause difficulties for the degradation of lignocellulose (Hendriks and Zeeman. 2008).

The idea of using cellulosic material as energy source is not new. A French scientist found a way to use lignocellulose material as energy source for ethanol production in the beginning of the 18th century (Braconnot H. 1819). In the end of the century Germany was first to commercialize the use of wood for ethanol production and in 1910 the United States followed by creating a big ethanol plant in Georgetown.(PDA. 1910). Unfortunately the energy efficiency of coal plants made this by then old technique almost to be forgotten. Not until the fossil fuel debate started, energy production from wood to ethanol again became a science subject and now it is one of the most important biofuel subjects.

1.1 Swedish Microdrive research program

The current work is involved in the Swedish Microdrive research program SLU and companies, it means to create an optimal use of the available biomass. Long term goals for the Microdrive program involve maximum yield of energy in the biogas and bio-ethanol processes and also to create a natural circulation for minimum environmental impact. The strategy is to raise efficiency in all steps of the process, from storage to fermentation and recirculation of plant nutrients. From other departments or industries there are currently studies on the fermentation process itself, but little is done when it comes to storage of biomass or the recirculation after ethanol and biogas production.

This program involves all steps; how to best store biomass in order to not lose yield, if it is possible to start pretreatments during storage, studies of the pretreatment and fermentation and the recirculation of end products. The main substrates that have been worked with in the Microdrive program are sugar beets and cereal grains, but lately research concerning other cellulosic or lignocellulosic material, such as straw and wood has been started. (Microdrive. 2010)

1.2 Hydrolysis

The most frequently used fermentation method is separate hydrolyse and fermentation (SHF). In this process the raw material is pretreated with heat, physical treatment or chemicals to break the molecule structure of lignocellulose. This specific step of breaking the polymers are essential for all fermentation methods. The next step is an enzyme treatment to degrade the cellulose and hemicellulose into fermentable single sugar molecules. Several chemical variants of hydrolysis exist, but they are expensive to use and unfriendly to the environment. Steam explosion is a relatively new pretreatment method, it is used for breaking the lignocellulose in the first step of the hydrolysis. Reports have shown that steam explosion with enzymatic treatment gives a higher total yield of fermentable sugars, about 80% compared to 50-60% for chemical treatments. (Palmqvist and Hahn-Hägerdal. 1999, Wyman. 1994)

1.3 Alcoholic Fermentation

The alcoholic fermentation is an incomplete oxidation of sugars. The typical end product that people are aware of, is the ethanol molecule in spirits, beer and wine. The first signs of ethanol breed is traced back to 3000 BC where a fermentation process created the ethanol in alcoholic beverages (Klieger. 2004). The fermentation process also plays a role in bakeries. The making of bread uses the carbon dioxide, a product from the fermentation which is suitable for raising the volume and ease the texture in bread. The use of ethanol as a fuel is compared to this, new. One of the first industrial plants for ethanol biofuel in modern days was built in 1975 in Brazil (Wheals. 1999). Still 35 years later only 0.2% of the total energy consumption comes from bioethanol and even less from lignocellulose material (REN. 2010).

1.3.1 Fermentation yeast

1.3.1.1 *Saccharomyces cerevisiae*

S. cerevisiae also known as the bakers yeast was the first yeast to be specified for fermentation and it is still the most common fermentation yeast (Klieger. 2004). The name of the yeast comes from greek latin and translates, sugar-fungus of beer (*Saccharo-myces cerevisiae*). *S. cerevisiae* have a long history in wine and beer production but has also been used in the making of bread. *S. cerevisiae* is also the most common yeast in bioethanol fermentations. The benefits of *S. cerevisiae* is that it grows fast, it is easy maintained, quite adaptable to new environments and produces low amounts of byproducts.

1.3.1.2 *Dekkera bruxellensis*

D. bruxellensis has long been viewed as a contaminant in beer, bioethanol and wine productions. That is because it produces unwanted byproducts that gives bad smell and taste to the beverage. A lot of research has already been done on the yeast *D. bruxellensis*. But since it has almost exclusively been viewed as a contaminant researches have been looking for ways to reduce the risk of contamination. In 2006 a fuel ethanol plant in Sweden was examined due to a noticed change of the cell shape in the continuous fermentation tank. The report showed that *D. bruxellensis* yeast together with the lactic acid bacterium *Lactobacillus vini* dominated the microbial population in the

tank. Although *S. cerevisiae* was no longer in the process there was no shown affect of the total ethanol yield (Passoth et al. 2007). The discovery started an interest for the department of microbiology at SLU Ultuna to further investigate the fermentation properties of the *D. bruxellensis* strain. An article showed that the *D. bruxellensis* strain, as *S. cerevisiae*, takes fermentation to completion, that means that all glucose in the growth medium is fermented. It also showed that *D. bruxellensis* has a good ethanol yield, which was slightly higher than that of *S. cerevisiae*. One problem though with *D. bruxellensis* is that it grows slower than *S. cerevisiae* and that might cause trouble for industrial productions. (Abbot et al. 2004, Blomqvist et al. 2010)

It has been shown that *D. bruxellensis* it is not able to compete with *S. cerevisiae* in batch fermentations (Abbot et al. 2004). However a discovery of a continuous industrial fermentation where *D. bruxellensis* was dominating the biomass, showed that the growth rate in a continuous process might not be a critical issue (Blomqvist et al. 2010). Experiments at the SLU/Dept. of Microbiology have shown adaptation skills of *D. bruxellensis*, that might be one possible reason why *D. bruxellensis* was able to take over a continuous ethanol process from *S. cerevisiae* (Blomqvist et al. 2010, South, 2010). During the last few years *D. bruxellensis* has been viewed as a possible competitor to *S. cerevisiae* in ethanol bioplants. But still there is a lot of work before it is possible to say if it will work in a large scale with different substrates.

1.4 Design of experiments

1.4.1 Multiple factors in one experiment

The classic way to solve a problem typically use a two statement solution, good or not. A hypothesis is created and it is either verified or dismissed. It will work perfectly as long as there is only one factor to consider. When several factors are involved the classic solution might miss information. The basic idea of Design of experiments (DOE) is that one will get more information about the factors by varying all factors at the same time. When using classic analysis, one factor is analysed and optimized. In one example with two, amount of sugar and juice concentrate, the best amount of sugar is found first. In the next step the best amount of juice concentrate is found and so on, if more factors are involved. With design of experiment all factors are changed at the same time in a system. Low amount of sugar and low amount of juice concentrate but also the opposite a high amount of all ingredients. In addition high concentration of sugar with a low juice concentration, low amount of sugar and high concentration of juice are analysed, finally the centre points are added which contains mid values of all factors/ingredients.

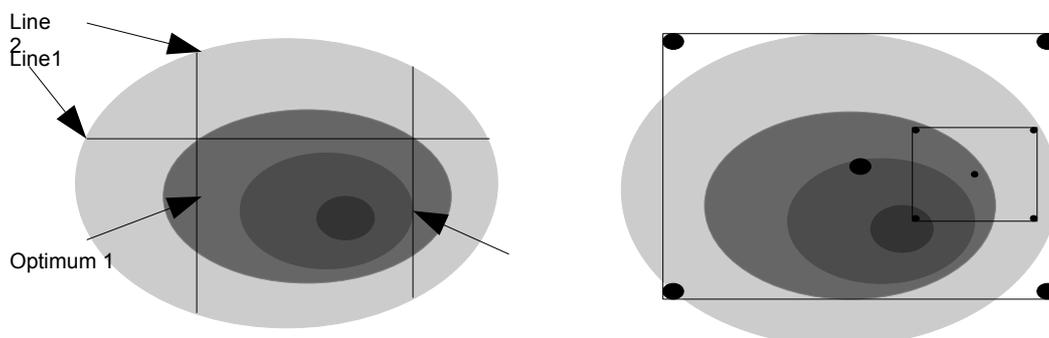


Fig 1: The two illustrations show typical true and false experiments with one factor at the time (left) and the Design of experiment strategy (right)

Figure 1 shows how the classic strategy with one factor changed at the same time could miss the

optimal area for the juice taste in a two dimensional space. With DOE it is possible to get closer to area with dark grey. If wanted the analyse system can give a optimizing model that further close up optimal area. Design of experiments can be used as both a screening and optimizing tool (2.4.2). The figure also illustrate how the DOE model will find a small area for the best blend, where the ordinary strategy will fail, no matter how many samples one take on each of the lines

Design of experiments is built on two mathematical methods depending on the raw data. Normally the multiple linear regression (MLR) method is used. Partial least square (PLS) is used when the responses are believed to be connected or dependent on each other. (Eriksson et al. 2008).

1.4.2 The Full and Fractional factorial design

To set the models in this project a computer software MODDE 9 (Umetrics AB, Umeå Sweden) was used which created a design for the factors involved in the experiment. The software has several options or designs to choose from, the one best situated for this project was the Full-Factorial Design described in 2D space (pic2). It is also shown in table 1 for three factors. The zeros represent a middle value for all involved factors. With more factors the number of experiments quickly rise, but a developed strategy to reduce the amount of experiments is built in the model design system. One of them is fractional factorial design. As shown in the table it takes away half of the number of experiments but still involves all high and low points for each factor. The fractional factorial design is a screening model to get a quick overview and possibly a direction of where to search for a optimum value. Table 1 shows the idea of the 2^3 full factorial design with three factors. A fractional factorial design that is mathematically $2^{(3-1)}$, is marked in grey. For the last factor (X3) a new setup is needed, otherwise this factor will only be tested for low values and information about this variable would be lost. The new third variable is created by multiplying the existing factors in rows. X1X2 will be (+,-,-,+) where row one is minus times minus which is plus, the second row minus times plus which is minus and so on. In total there will now be 4 experiments instead of 8, plus centre points (Eriksson et al. 2008).

Table 1: The full factorial design.

	X1	X2	X3
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+
9	0	0	0
10	0	0	0
11	0	0	0

1.4.3 MODDE software

The MODDE software is a developed computer software to help building and analysing Design of experiments. MODDE suggests models depending on what factors and how they are changing (qualitative, quantitative) and/or if they are controlled. It gives model alternatives that might fit the current experiment. MODDE is also a analysing tool, later it is possible to enter desired and measured responses and the program calculates the properties of the model shows the model quality and validity. There are also several outputs for different kind of analyses, everything from a normal ANOVA test to individual and combined components (factors and there interactions) analysis to plot analysis of individual samples response in the model (Eriksson et al. 2008)

1.4.4 Analysis with MODDE

1.4.4.1 Histogram and residuals

To evaluate the quality of the raw data, analysis starts with a look at the histogram and the residual plots which shows if the raw data have a good distribution. The histogram should have a approximate normal distribution, otherwise the data might cause a bad model and one might need to transform the raw data (ex. square, logarithmic). The residual plot shows the normal distribution of raw data against each individual sample response in a 2D plot. The residual analyses show if the

data contain outliers, extreme values that do not fit the model. If outliers are shown in the data the sample could be excluded from the model if it is reasonable. (Eriksson et al. 2008).

1.4.4.2 Summary of fit

Analysis of the models created with design of experiments can be divided into several steps. First one has to decide whether the model is valid or not. A summary of fit plot is a four bar figure that shows an overview of the model quality. The first bar R^2 shows how well the raw data fits the regression model. As all other bars in the summary of fit it varies between zero and one. One represent a perfect model while zero tells that no model could be created from the current dataset. The second bar in the summary of fit is Q^2 , it represent goodness of prediction that is, how well the model can predict new data. The Q^2 value is a better indicator than R^2 for model strength. A good predictability is needed for analysis of points in the model that is not measured. The value of Q^2 should exceed 0.5 and R^2 minus Q^2 should be smaller than 0.3 for a good model. If the model is built in the wrong way and the model shows tendency of curvature, quadratic dependency, when the model is created to measure linearity the third bar will be low. The third bar is called model validity and a value below 0.25 shows that the model should be rearranged or needs completion. The replicate plot, that shows variability within the model samples, it is represented in the last bar. It should not be lower than 0.5 for a good model. Examples of analysis tools are ANOVA tables, residual plots, R^2 and Q^2 values (Eriksson et al. 2008).

1.4.4.3 Replicate plot

The replicate plot shows the values of raw data response plotted against each experiment. Experiments with the same setup will be represented in the same bar, whereas all unique setups will be showed in a separate bar. The replicate plot gives a quick overview of the raw data and show if the response fits the model dataset. Centre points in a model should end up more or less in the middle of the response value to get a good model (Eriksson et al. 2008).

1.4.4.4 Coefficient analysis and contour plots

The coefficient plot shows a factors significance and its contribution to the model. Bars that represent each main factor, and bars that represent interaction factors are shown in the plot. When analysing, interaction factors that do not contribute are removed from the model. The remaining bars will give a view over what factors are important for the model. Interactions bars show if the factors contribute to the model in pair. When many interaction factors contribute to the model the coefficient plot will be difficult to analyse and explain. If so the model response can instead be showed in a contour plot. The contour plot is a 2 up to 4 dimensional plot where two factors, normally the strongest contributors are plotted in a 2D figure with factor 1 as x and factor 2 as y. If more than two factors are important the 3:rd and 4:th dimensions can be shown in separate 2D figures arranged next to each other, with the three values of the model setup in x and y (maximum 9 graphs). If the model is good according to the summary of fit, factors with significant contribution are important for the response from the experiment while insignificant factors can be excluded from further investigations. (Eriksson et al. 2008)

1.5 Involved factors, importance for growth/ethanol yield

1.5.1 Air supply

In the literature possible effects regarding aeration in fermentations are for example, the Custer effect, inhibition of growth in anaerobic conditions and the Pasteur effect, inhibition of fermentation

under aerobic conditions. One report suggests that *D. bruxellensis* is severely suppressed by *S. cerevisiae* in batch fermentations in aerobic conditions (Abbott & Ingledew. 2005). Another article suggests that high aeration causes a decrease in the final ethanol yield. One reason is thought to be production of acetic acid instead of alcohol and that yeast degrades ethanol in presence of air (Aguilar et al. 2003). Another reason might be evaporation of ethanol when the gas exchange is not limited. The oxygen factor is involved in the project to see if aeration induce acetic acid production and if it affects the total ethanol yield. In the industry addition of air costs money, so to be able to show that limited air is a good condition for ethanol fermentation, is desirable. In the model the factor is quantitative with two options, it will only include a non-limited aerobic and a limited aerobic value.

1.5.2 Cell concentration

It has previously been shown that a high initial cell culture concentration could increase the chance of survival in rough conditions and also effect lag-phase, yield and growth (Matsushika & Sawayama 2010). It is thought that the cells, when in high concentration, can protect each other by reducing the surface exposed to the toxic molecules and that the amount of toxics for each cell becomes lower than for a smaller population. The option will be tested for the possibility to either confirm or dismiss this suggestions for the *S. cerevisiae* and *D. bruxellensis* yeast cultures. For *S. cerevisiae* it has been shown that the end ethanol production should not be affected by initial cell concentration (Matsushika & Sawayama 2010).

1.5.3 Spruce hydrolysate

The hydrolysate which is the actual sugar supply for the fermentation is also partly toxic to the yeast. The hydrolysate is pre-treated (2.1) in several steps to release the sugar compounds from the lignocellulose material, both with high temperature and with enzymes. A problem with the pre-treatment is that it also releases toxic compounds. From lignin several phenolics are produced and some sugars are degraded to inhibitors for yeast enzymes, involved in the fermentation process (Kuhad et al. 2007, 2010). Hydrolysates from other lignocellulosic material has previously been shown to be deadly in 100% concentration (South. 2010) but, if the hydrolysate has to be diluted it will reduce the final sugar concentration in the fermentor. The highest possible concentration of hydrolysate is desired.

1.5.4 Temperature

Temperature is an important factor for yeast growth, all yeasts have a certain range where they can possibly grow. A previous research has shown that ethanol production is quicker with higher temperature (Nagodawithana et al. 1974) and it is well known that temperature effect both growth and ethanol yield . It is even possible that a higher or lower temperature have a important co-effect with some of the other factors involved.

1.5.5 pH

The pH is a factor that might be important for both cell growth and ethanol yield. Effect on yeast growth is most likely due to changes in toxicity of hydrolysate byproducts rather than a pure pH effect on growth since it is not set to extreme conditions. The ethanol yield might be effected by enzyme activity changes (Oliviero et al. 1982).

1.6 Simultaneous Saccharification and fermentation (SSF)

1.6.1 Process

Simultaneous saccharification and fermentation is like classic fermentation but it combines two

critical steps, the enzymatic polysaccharide breakdown and the actual fermentation. The idea is to shorten the process, but also it seems like the enzymes is less inhibited by sugar excess since the sugar is fermented immediately (Cot et al. 2006). This has the benefit that less enzyme is needed for a certain amount of material and since enzyme is expensive it also has an economy value. A problem is that the enzymes normally have there optimum efficiency at a higher temperature, above 45°C, than the fermentor biomass can produce ethanol in. Though it seems like SSF is just as effective or even more effective than hydrolysate fermentation. This is when the low concentration of sugar drives the equilibrium towards complete enzymatic breakdown of the sugar chains. It has been shown that SSF processes gives a higher ethanol yield (Tomás-Pejó et al. 2008).

1.6.2 Use in Microdrive and future

The SSF fermentation method could be a very useful development in the microdrive program since both the enzyme cost and the total time spent will be reduced. Also the number of different processes is kept at a minimum. SSF substrate still needs to be pretreated for breakage of lignin but future studies might be able to combine this step as well. The SSF is also possible to have running continuously, that is a huge benefit for industry production, if compared to separate hydrolysatation and fermentation (SHF). Other benefits of SSF is that the fermentation is more stable, it does not have to be stopped and cleaned and it is also proven to be more resistant to contaminations (Microdrive. 2010).

1.7 Project aims

1.7.1 DOE

The goal for the Design of experiment was to compare two yeast, *S. cerevisiae* and *D. bruxellensis* for responses, ethanol production and growth of yeast. A model was designed to look at importance and possible interactions of five factors. The factors chosen for the experiment were pH, temperature, air access, initial cell density and hydrolysate concentration. First a screening was done by using a fractional factorial design. If results builds a reliable model, the program will be able to suggest one or several fermentation options for good growth and ethanol yield. From the results it might also be possible to do further optimizations.

1.7.2 SSF

The SSF was a small pilot test of a different fermentation process than currently used in the Microdrive program. The DOE project will be used as a guide for pH and concentration settings for this experiment. It is supposed to evaluate benefits, and look for possible improvements and uses for the microdrive program. Also if possible it should compare total yield and total time needed in comparison of the SHF process. The experiment was also built to check, how high dry mass concentration that is possible to use with living organism, before released nutrients creates a deadly environment.

2 MATERIALS AND METHODS

2.1 Hydrolysate pre-treatment

The dry material of spruce (DM) provided by (UMB, Ås, Norway) had been pre-treated in a process called steam explosion where the material was kept at a temperature of 210°C and at a pressure of 22bar for 10 minutes followed by quick pressure release. Moisture analysis of the pretreated material was done to set the amount of wet material that was needed to get a final (DM) mass content of 300g/L. Steam-exploded spruce sawdust (SESS) was added in portions of 30g (DM) to

each of two separate bottles. The first day one portion of SESS was added with 40ml of buffer solution (1M Na-citrate) and 20ml of autoclaved MQ-water. 7.5ml of enzyme solution (Accellerase, A Danisco Division) was added to each flask and put on a shaker at 90rpm and at a temperature of 40°C. Portions of SESS were added with 20ml of water day 1-3 and day 5. Day four 7.5ml of enzyme is added to both flasks. At day 7 the enzyme treated material was centrifuged and the supernatant, which was the hydrolysate product, was collected. The sugar level in the hydrolysate end product was measured. The amount of enzyme was accidentally limited and the sugar level was lower than expected. The concentration was 0.1g/ml dry mass, for maximum sugar level the enzyme concentration should have been above 0.25g/ml.

2.2 Yeast

The two yeast used in the experiments were *Saccharomyces cerevisiae* J672 isolated from Agroetanol in Norrköping and *Dekkera bruxellensis* CBS 11269, isolated from an ethanol production plant in Lidköping.

2.3 Growth media

Minimal medium

KH₂PO₄ 9.375g/L, yeast extract 3g/L, MgSO₄*7H₂O 1,13g/L, Yeast nitrogen base w/o (YNB) 6.5g/L and glucose 30g/L. After H₂O addition the medium was sterile filtered.

YPD solid

glucose 20g/L, Peptone 20g/L, yeast extract 10g/L and technical agar 16g/L. After addition of H₂O the YPD was autoclaved and poured in petri dishes.

YPD solid + hydrolysate 10%

glucose 20g/L, Peptone 20g/L, yeast extract 10g/L and technical agar 16g/L and hydrolysate 100ml. After addition of H₂O the YPD was autoclaved and put in petri dishes.

TSA (Trypticase soy agar) + delvocide (to check bacterial contamination) plate

40g/L of TSA was added with 0.1g/L delvocide. After addition of H₂O the YPD was autoclaved and poured in petri dishes.

2.4 Spruce hydrolysate glucose medium

The hydrolysate used (3.1) gave a glucose concentration of 17.2g/l hydrolysate batch one (H1) and 16.5 g/l for the second (H2) For the analysis the glucose concentration was adjusted to totally 30g/l in the final sample solutions. Yeast extract was added to get a end concentration of 5g/l and ammonium sulphate 2g/l.

2.5 Culture adaption

Yeast was pre-cultured in growth medium for three days. Then they were moved into two concentrations of hydrolysate 20% and 35% respectively both enriched with yeast extract 5g/l. During two days the cells were washed and moved to new hydrolysate three times a day, each day moved to a higher concentration. From the third day the hydrolysate concentration was raised by adding 250µl of pure hydrolysate (+yeast extract 5g/l) four times a day to double the volume. After six days a concentration of about 90-100% of hydrolysate was reached. The cultures were stored on YPD+hydrolysate plates.

2.6 Culture storage

The adapted yeast was stored for future analysis. The yeast was first grown in YPD medium in a growth-tube to get a acceptable cell concentration, then the liquid was transferred to a storage tube and mixed with an equal volume of 100% glycerol. The tube was put in a -80°C storage box.

2.7 Analytical methods

2.7.1 Optical Density

Growth was measured with optical density or viable count. Optical density (OD) was measured with a spectrophotometer (Ultrospec 1100pro, Biochrom) at 600nm. A solution of NaCl was used as a reference for all measurements although differences in hydrolysate might marginally effect the OD value. The samples were continuously diluted to fit the analysis range of the machine. Viable count was performed during the SSF experiment. The samples were diluted ten fold and then put on YPD plates for count in dilution between 10^{-3} to 10^{-7} .

2.7.2 pH

All medium and plate solutions were set to a pH of 5. The pH was set by a standard solution of 9.75M HCL to get as small changes in volume as possible, if pH needed to be raised a solution of 4M or 6M NaOH was used. The pH in the design of experiment was set in the experiment start to pH 4, 5 and 6 but is not controlled. In the last experiment with *D. bruxellensis* the pH values were measured in the final solution after HPLC samples were taken.

2.7.3 HPLC

The High Performance Liquid chromatography (HPLC) was performed with a Agilent 1100 Series (Agilent technologies, Stockholm, Sweden) with quaternary pumps and a refractive index detector, the column was a Rezex-ROA-Organic Acid H+ 300x7.80 mm (Skandinaviska Genetec AB, Sweden). The ethanol custom setup measured ethanol, glucose, acetate and glycerol. Five to six references for acetic acid, ethanol and glucose were added in the beginning and the end of each run on the HPLC, (0,1), 1, 5, 10, 15 and 30g/L. All samples were sterilized with 0.2µm filter (Filtropur, Sarstedt) before analysis. The yield was calculated as [ethanol]/[glucose].

2.7.4 HPAE-PAD (Sugar analysis)

For sugar analysis of the hydrolysate a High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection was used. The column was a CarboPac PA10 4x250 mm/guard 4x50 mm, with a flow rate of 0.25 mL/min. Samples were pumped with a post-column pump with 100% sterilized MQ water as solvent at a temperature of 30°C. The analysis equipment was Chromelon 6.80 (Service pack 4).

2.7.5 T-test

The t-tests were done on an online website, <http://studentsttest.com/>. The t-test has a 95% significance level. The hypothesis H0 was, the probability that the two examined sample-sets comes from the same dataset. H1 was the opposite, they do not come from the same data set. A p-value lower than 0,05 will confirm H0 at the 95% level. A p-value above 0,05 will reject H0 and H1 will be true.

2.8 Experimental design

2.8.1 Growth experiment

Evaluation of the hydrolysate tolerance of the two yeasts was performed since in earlier experiments toxic levels of aspen sawdust hydrolysate has been observed. Another factor tested was yeast extract which was indicated as an important factor for *D. bruxellensis* growth. (South, E 2010). The spruce hydrolysate was adjusted to pH 5. It was added in 20% dilution with autoclaved deionized water or in 100% concentration to anaerobic vials. Yeast extract was added with a concentration of 5g/l to half of the vials, all samples was made in two replicates (H1 and H2).

2.8.2 Growth and pH evaluation

A second pre experiment analysed pH and hydrolysate concentration with both *D. bruxellensis* and *S. cerevisiae* to see if there were interactions between the factors. The new arrangement with only the two factors pH and hydrolysate concentration as variables was built 1:2, 1:2.86, 1:5 (0.5, 0.35, 0.2) and pH 3, 5, 7. For *D. bruxellensis* the pH range was changed since even the more tolerant *S. cerevisiae* strain had growth problems in low pH. pH 4, 5, 6 was chosen to test *D. bruxellensis*. The procedure was similar to the vial setup in 2.4.1.1.

2.8.3 *D. bruxellensis* and *S. cerevisiae* designs

All samples were mixed and prepared to fit the experimental design shown in table 2 below except for the cells (initial cell density, i.c.d).

2.8.3.1 First design

The first experiment in the project had a design as shown in table 2. It was performed in 20 ml vials for the low air (anaerobic), and 10ml well plates for the high air (oxygen access) properties. It only involved *D. bruxellensis* (*Dekkera*) and had a pH range from 3 to 7 instead of 4 to 6 as shown in the table 1. After preparations of the solutions for the design the samples were put in vials or wells. Pre culture cells were collected and washed with NaCl 0.9%. All cells were finally resuspended to a concentration of either 1 5 or 10 OD. The cells are added to the vials or wells with autoclaved deionized water. One initial OD measure was taken for each sample to get the real start concentration in each sample. I.c.d were then sampled once a day. HPLC samples were taken after cells were added (start). Cells and particles were removed from the solution by centrifugation and 0.2µm filter (Filtropour, Sarstedt) before storage in -18°C. HPLC (end) samples was taken when glucose in the solutions was consumed. This was measured with glucose paper (Nasco).

2.8.3.2 *Saccharomyces cerevisiae* vs *Dekkera bruxellensis*

The second design is shown in table 1 but the Air settings were accidentally switched for low air, 25°C and 35°C. The experiment was performed in vials 20ml for low air setting and in 96-wells high air setting, with a special lid to hinder evaporation. Each sample solution was first prepared in falcon tubes the day before experiment started. On experiment start cells were collected, washed with 0.9% NaCl and then added to the falcon tubes. HPLC and OD samples were taken and the solutions were put 2ml in well plates and 7ml in vials. The sterilization of the HPLC samples was performed up to three hours after addition of cells. The sterilized samples were stored in -18°C

A completion of the second design had to be done for low air settings and for both yeasts to get a complete design for analysis. The experiment was performed in 20ml vials. The solutions except for cells were prepared. HPLC samples were taken before adding the cells. The volume difference, 1ml was saved for addition of cells, (1/8th of total volume) was compensated with deionized water, all

samples were immediately stored in -18°C. OD samples were taken once a day. When fermentation was completed HPLC samples(end) were taken, fermentation completion was measured with glucose indicator paper.

2.8.3.3 Adapted *Dekkera bruxellensis*

The adapted *D. bruxellensis* design solutions were prepared in falcon tubes the day before experiment started, according to table 2 samples 23-44. Cells were added at the day of experiment start and then HPLC samples were taken, sterilized and stored in -18°C. OD samples were collected once a day and analysed (3.2.2). When fermentation in a well or vial was completed HPLC samples were taken, sterilized and stored in -18°C. After the experiment was ended for all experiments pH was measured in all vials and wells.

Table 2: The MODDE fractional factorial design for *S. cerevisiae* (Sacca) and *D. bruxellensis* (Dekkera)

Sacca	Dekkera	pH	i.c.d	Conc.H	temp	Air	Sacca	Dekkera	pH	i.c.d	Conc.H	temp	Air
1	23	6	1	0,2	25	High	5	31	4	1	0,2	25	Low
2	24	4	10	0,2	25	High	6	32	6	10	0,2	25	Low
3	25	4	1	0,5	25	High	7	33	6	1	0,5	25	Low
4	26	6	10	0,5	25	High	8	34	4	10	0,5	25	Low
9	27	4	1	0,2	35	High	13	35	6	1	0,2	35	Low
10	28	6	10	0,2	35	High	14	36	4	10	0,2	35	Low
11	29	6	1	0,5	35	High	15	37	4	1	0,5	35	Low
12	30	4	10	0,5	35	High	16	38	6	10	0,5	35	Low
20	42	5	5	0,35	30	High	17	39	5	5	0,35	30	Low
21	43	5	5	0,35	30	High	18	40	5	5	0,35	30	Low
22	44	5	5	0,35	30	High	19	41	5	5	0,35	30	Low

2.9 Simultaneous saccharification fermentation (SSF)

The SSF experiments were performed in 1.8L Jennie fermentors (Belach Bioteknik AB) equipped with oxygen, pH, and temperature probes. No air was added but the tank was not strictly anaerobic,

Table 3: SSF overview of the additions performed in the tanks during the experiment. Observe the small fault with water for day 1,2,3 added the first day.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Estim remaining DM g		30	60	90	90	120	120
Add spruce DM% of final	4%	4%	4%	0	4%	0	0
Add spruce g DM	60	60	60	0	60	0	0
Add spruce g	120	120	120	0	120	0	0
Added water with Spruce	60	60	60	0	60	0	0
Add water g	60	0	0	0	20	0	0
Add enzyme g	20	0	0	0	10	0	0
Total added DM (g)	60	120	180	180	240	240	240
Total enzyme (ml)	20	20	20	20	30	30	30
Total volume g	970	1090	1210	1210	1360	1360	1360
Enzyme g/g added DM	0,333		0,11	0,11	0,13	0,13	0,13
Added DM conc total vol	6,2%	11,0%	14,9%	14,9%	17,6%	17,6%	17,6%
Initial remaining DM conc		8,3%	9,9%	7,4%	11,0%	8,8%	8,8%

pH and temperature were set to pH5 and 30°C and controlled during the whole experiment. The oxygen level was measured in both tanks but not controlled. Dry mass (DM) was continuously added to give a final concentration of 20% per volume. In the actual experiment the last addition of material was cancelled since the material ran out. The actual dry mass content ended at 17,6% and total volume at 1360ml instead of 1,5L. The culture added with a approximate OD of 5 and experiment started with 4% dry mass and 0,333 ml enzyme per gram DM. The whole addition table is shown below (table 2). The enzyme was limited in both tanks with the same amount of enzyme as

for hydrolysis 2.1 that is 0.1 ml/g dry substance. The exact values for addition in tanks are shown in table 3.

3 RESULTS

3.1 Pre-experiments

Pre-experiments were done to investigate *D. bruxellensis* and *S. cerevisiae* tolerance against spruce hydrolysate (data not shown). The experiment indicated low tolerance for both yeast. In several of the samples the yeasts did not grow. Two additional experiments could confirm that *S. cerevisiae* growth was inhibited and that *D. bruxellensis* is more sensitive to extreme conditions. Especially low pH in combination with a high concentration of hydrolysate was bad for both yeast (data not shown). Several pre-experiments were made but still problems occurred with the first set for design of experiment. The results were not possible to use since in the limited air constellation up to 80% of the liquid evaporated. Due to evaporation in the wells the concentration of yeast, ethanol, sugar and toxic compounds from the hydrolysate increased. A plate that could represent air access without heavy evaporation was used in all later experiments.

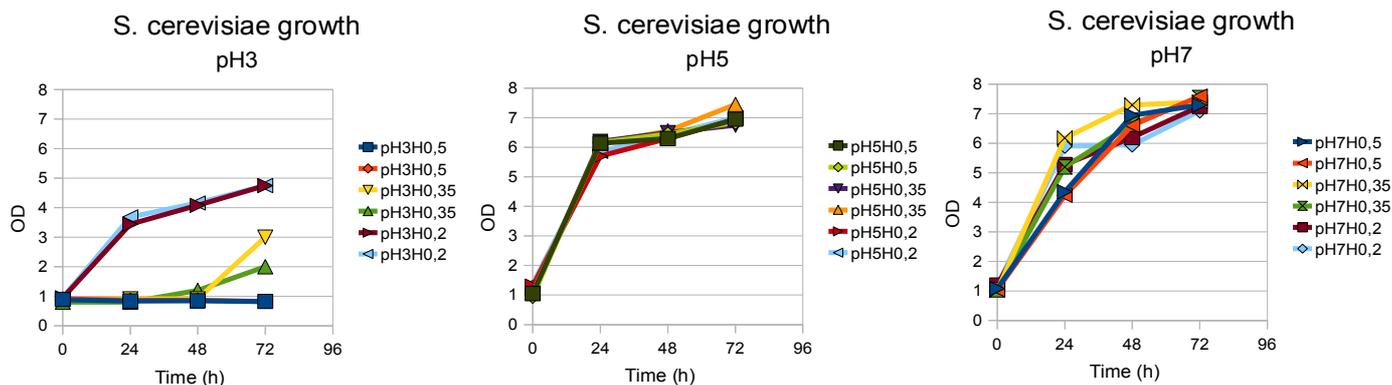


Fig 2: *S. cerevisiae* growth in different pH and hydrolysate concentrations

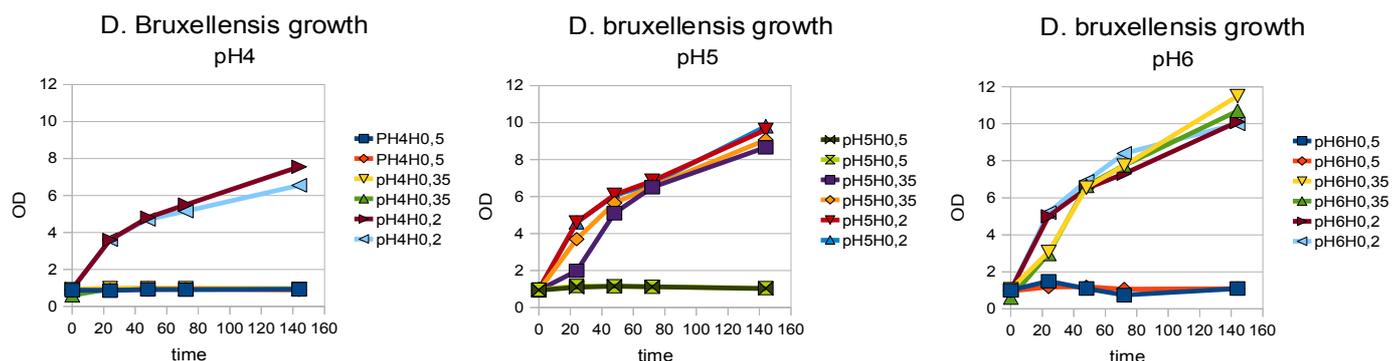


Fig 3: *D. bruxellensis* growth at different pH and hydrolysate concentrations

One pre-experiment tested different hydrolysate concentrations and pH. The result shows that *S. cerevisiae* was severely inhibited in low pH with combination of high concentration of hydrolysate (fig 2). The figure shows that the cultures in pH 3 were inhibited with raised concentration of hydrolysate and that the cultures in pH 7 were inhibited in the same way but less than in pH 3. For future experiments, it was decided to narrow the maximum values for pH, from 3 to 4 and 7 to 6. The new setup (pH 4-6) was used for *D. bruxellensis*. The yeast still showed low or no growth in wells containing 50% hydrolysate for both pH 4 and pH 6 (data not shown)

The problems found were both, due to lack of knowledge of the toxicity of spruce hydrolysate and the known sensitivity of the *D. bruxellensis* strain. Problems with growth was expected with *D. bruxellensis* but the low tolerance for *S. cerevisiae* was a bit surprising. One interesting observation from the first experiments was the pH indicator properties of the spruce hydrolysate. At pH 7 the liquid gained a dark brown colour but at pH 3 the colour was instead bright yellow.

3.2 Experimental design

3.2.1 Acetic acid

The acetic acid response in centre points for limited air, showed extreme variation compared to the general variation within the experiment. Three values 8 and 14 and one of the centre points, 20 are very high compared to the other values. The only difference between the two, 8 and 14, was the hydrolysate concentration, the other factors were, pH 4, low air, 35°C and high start cell density.

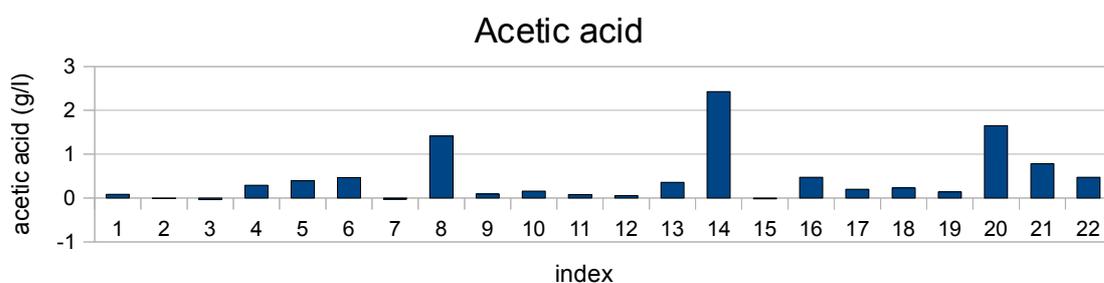


Fig 4: Acetic acid production by *S. cerevisiae* from the *D. bruxellensis* and *S. cerevisiae* experiment

The acetic acid concentrations in the experiments were generally low, but showed some high variation. It was especially difficult to analyse these results when the three centre points 20, 21 and 22, that had the same initial conditions, varied more than almost all other experiments. Initial conditions for samples 8 and 14 only differed in concentration of hydrolysate (high/low), they both showed high concentrations of acetic acid. No correlation between the amount of acetic acid could be seen from the other responses where they had similar ethanol yield and/or similar growth. With some exceptions, the majority of the values, with high acetic acid production, were in temperature of 30°C or above. Due to the great variance of acetic acid production, it was not taken to account when analysing the models.

3.2.2 A fractional factorial design with *D. bruxellensis* and *S. cerevisiae*

The second experiment with both yeasts included all experimental factors in the same run. Two sets of bottles were accidentally switched and had to be re-ran. The original setup was changed and

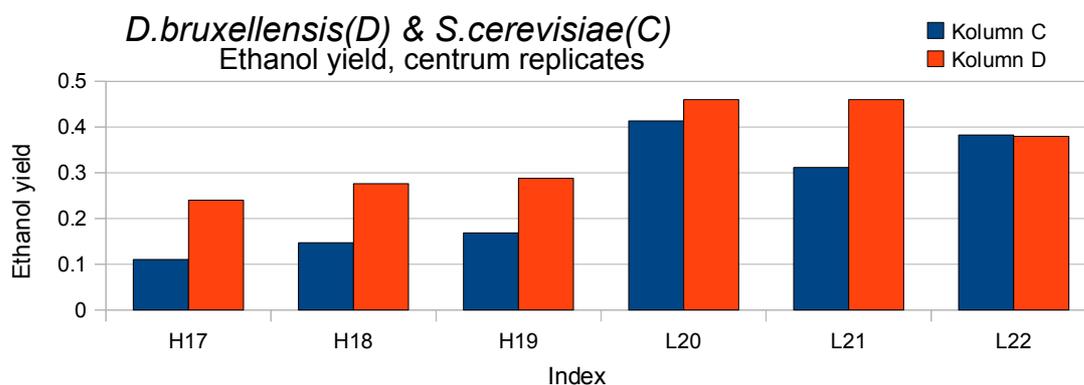


Fig 5: Blue columns are *Saccharomyces* ethanol production and red columns *D. bruxellensis* yield. High (H) and Low(L) air is shown in the index for each sample. The setup is shown in table 2.

no model could be created by MODDE, but some conclusions of growth were drawn from the first experiment concerning ethanol yield.

Fig 5 shows that the general ethanol yield for *D. bruxellensis* was higher than for *S. cerevisiae*. The differences in yield, in the samples with oxygen access, were quite high. A T-test for the ethanol yield in high and low air shows that differences between *D. bruxellensis* and *S. cerevisiae* ethanol yield with oxygen access were significant with p value = 0.0056. However the t-test did not confirm a significant difference, when oxygen was limited, p value = 0.17.

3.2.3 *D. bruxellensis* vs *S. cerevisiae* with corrected “low air” set.

There was a lot of problems to keep *D. bruxellensis* from dying in the samples and the results from those experiments did not build a meaningful model in MODDE. The results from *S. cerevisiae* however, built good models for both ethanol production and yeast growth.

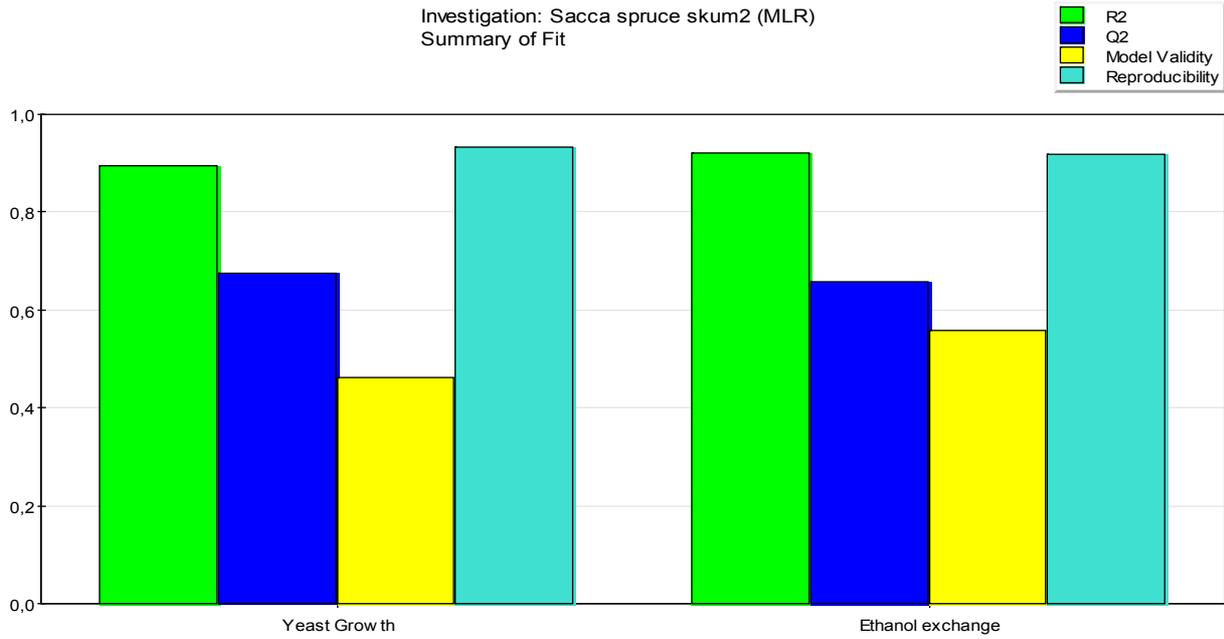


Fig 6: The summary of fit for *S. cerevisiae* growth and ethanol yield.

The summary of fit shows a good response model for yeast growth and ethanol yield.

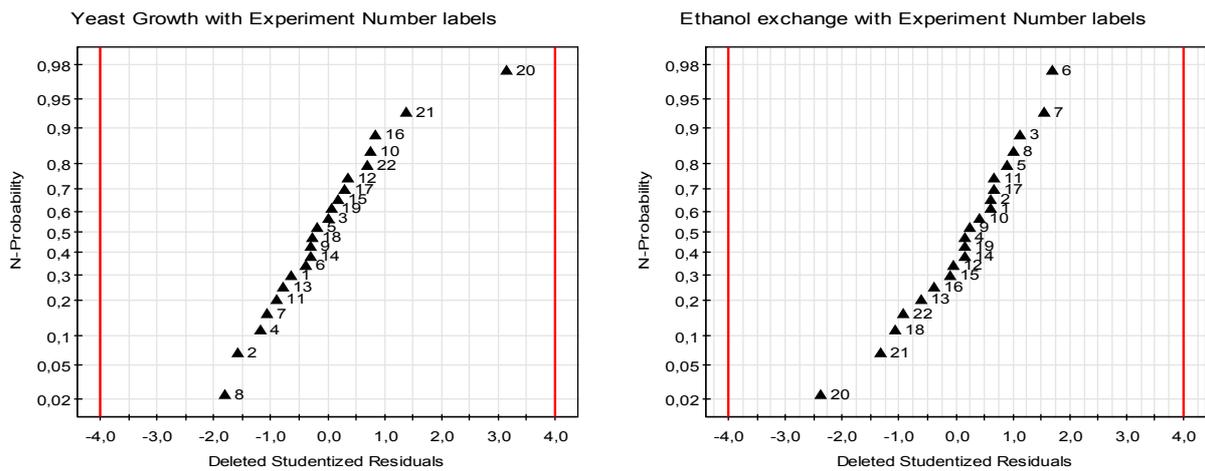


Fig 7: A N-probability plot of *S. cerevisiae* growth and ethanol yield.

The N-probability plot shows that the models do not have any outliers, one of the centre points for the yeast growth response and one for ethanol yield are deviating, but not enough to be deleted.

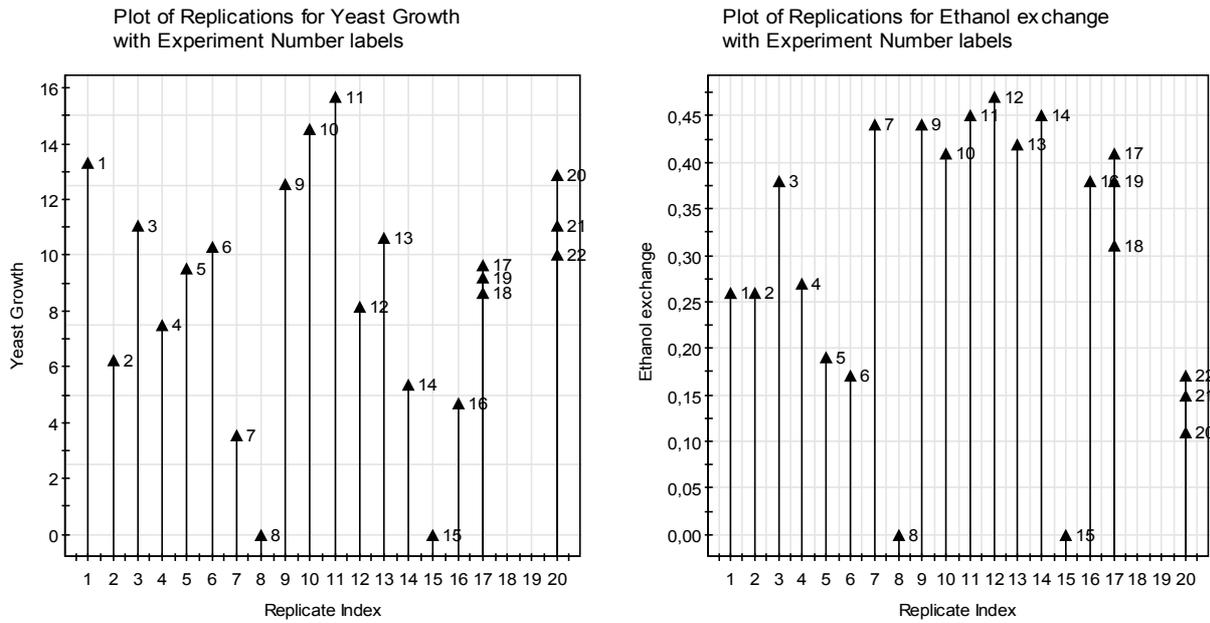


Fig 8: Replicate plot of ethanol yield and yeast growth responses.

The replicate plot indicates that no extreme values are spotted within the model. The centre points were found to be quite well centred in the model and clustered well together.

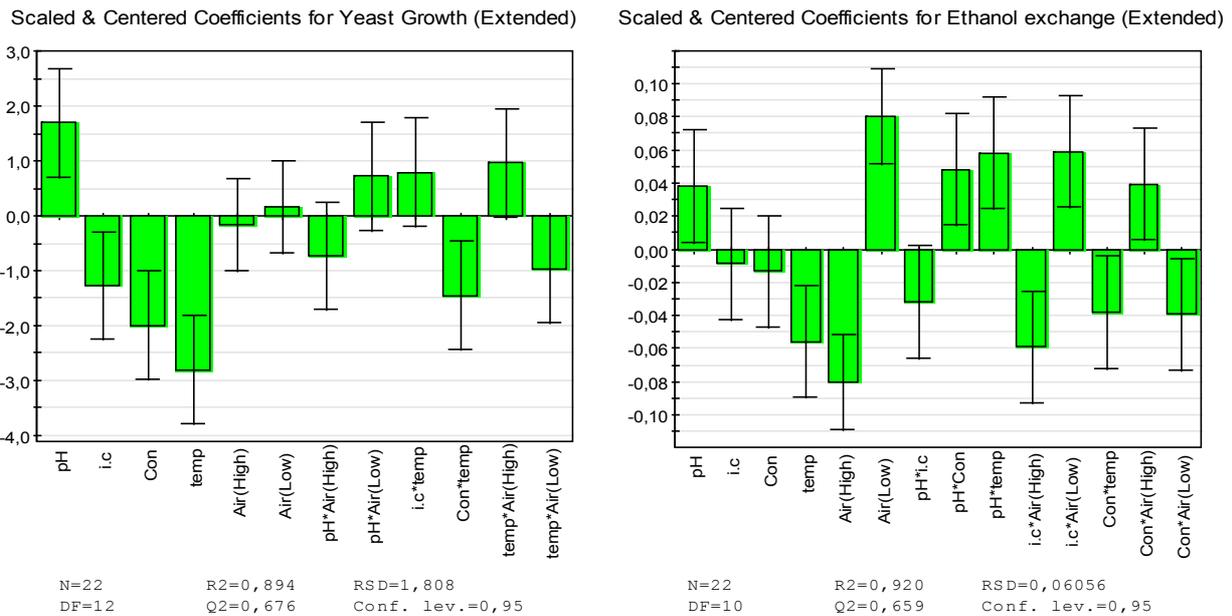


Fig 9: The coefficient plot shows the contribution, positive - more growth/yield, negative less growth/yield

The coefficient plot shows that pH was the only factor with positive contribution to growth in *S. cerevisiae*. All other factors in this experiment had negative or no contribution to growth. One significant interaction factor is concentration of hydrolysate with temperature. It had negative contribution to growth. The oxygen factor was not significant for growth. For ethanol production the factors initial cell density(i.c), and concentration(con) were not significant. For ethanol yield, low oxygen had a positive contribution to the model, temperature (and high air) had negative contribution and high pH had positive contribution to ethanol yield.

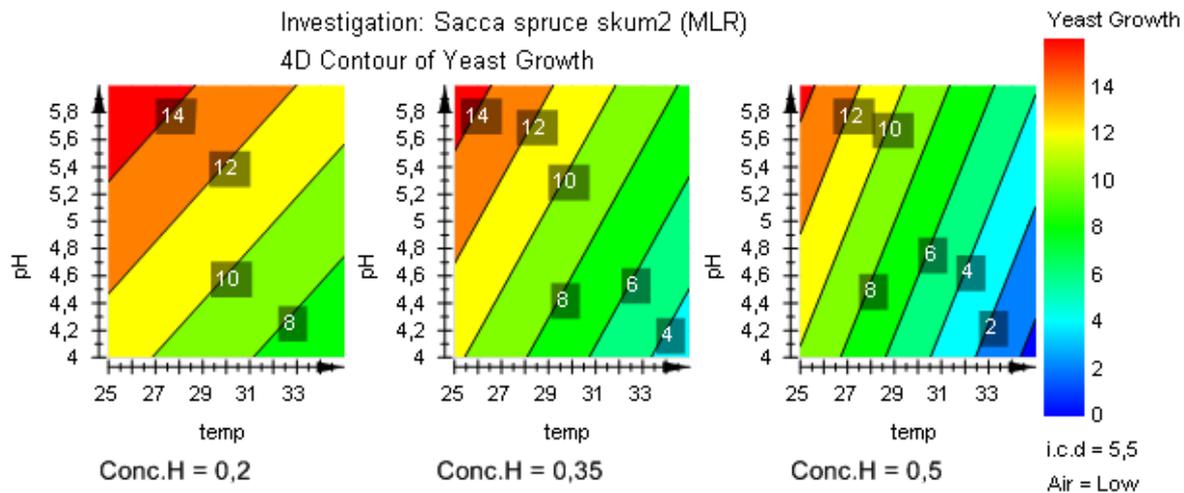


Fig 10: A 4D contour plot of yeast growth. Temp and pH are the main factors in the graph and they are shown in three different hydrolysate concentrations.

The contour plot of yeast growth shows trends and differences, for the most important factors. It shows that, low temperature and low pH gave good growth and that the difference between maximum and minimum growth, decreased with lower concentration of hydrolysate. The remaining two factors, are chosen as 5,5 i.c.d and low air. The same trends would occur with different setups of these two factors.

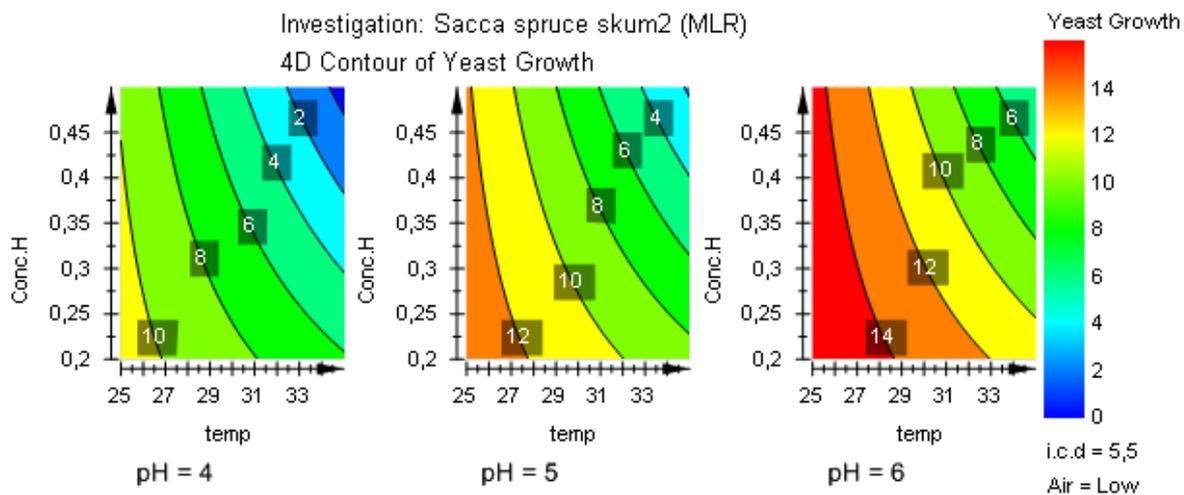


Fig 11: A contour plot with Conc.H and temp as main factors and with pH variation, i.c.d = 5,5 and low air.

Fig 11. A contour plot showed that, the combination of low temp and low concentration of hydrolysate , gave good growth. The trend was the same for all pH settings. The plot also showed, that a low temperature seemed to reduce the problems with high concentration of hydrolysate, concerning the growth of the yeast

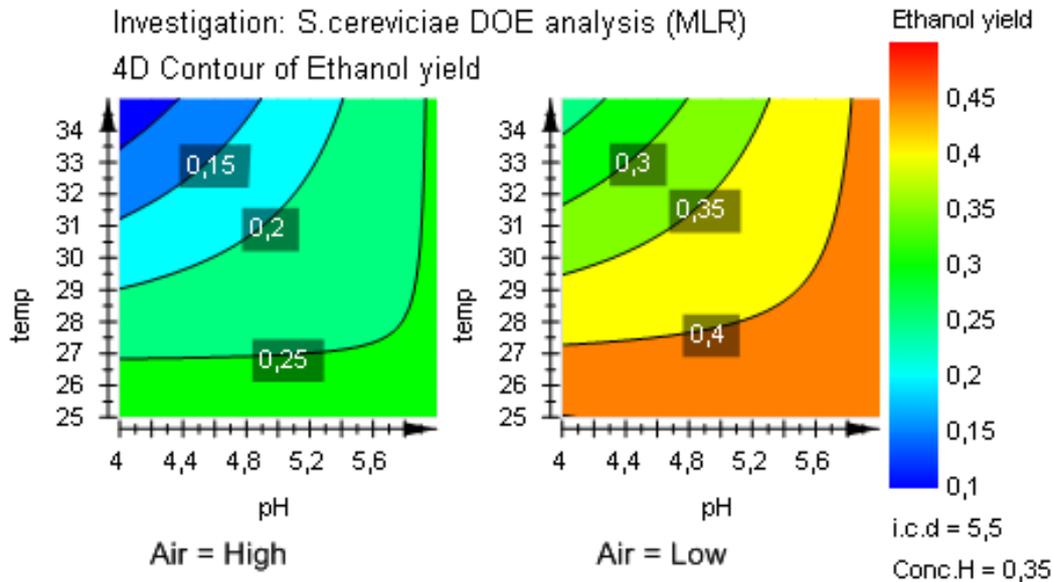


Fig 12: A contour plot that compares aerobic(high air) and anaerobic(low air) conditions initial cell concentration was 5.5 and with hydrolysate concentration 35%.

Fig 12 shows that absence of air result in higher ethanol production, this is similar for other settings of i.c.d and hydrolysate concentrations (data not shown).

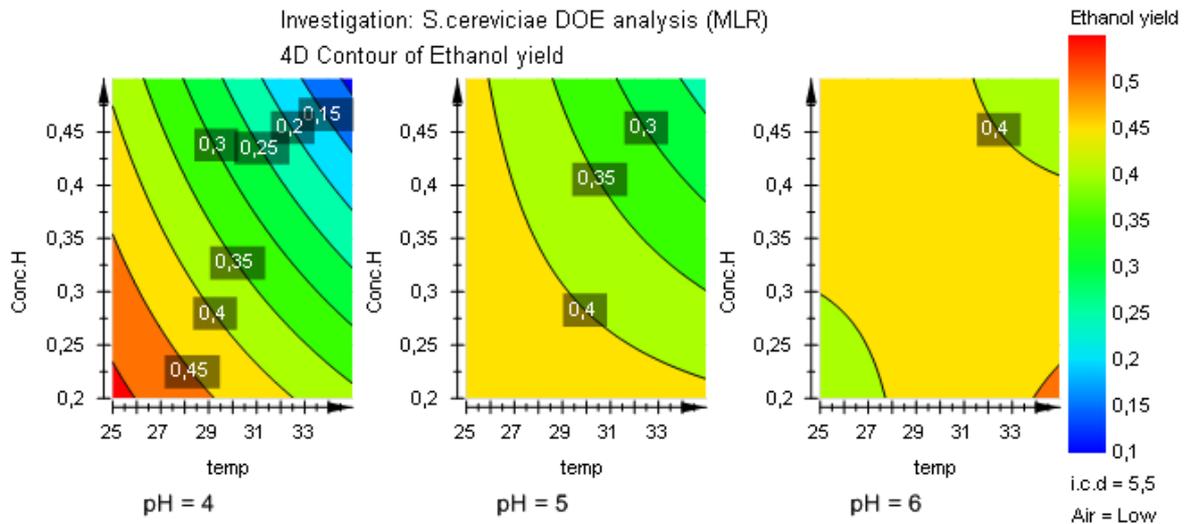


Fig 13: Contour plot of ethanol yield, i.c.d =5,5 and anaerobic conditions

Fig 13 shows that high concentrations of hydrolysate generally resulted in low ethanol yield. A higher pH seemed to reduce the this effects, but it also reduced the maximum ethanol yield in low temperatures.

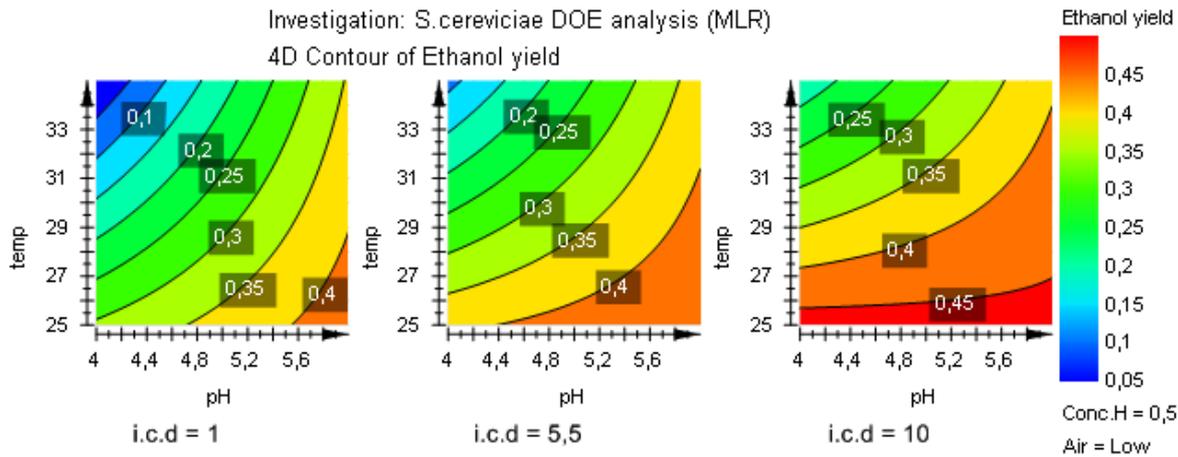


Fig 14: Contour plot of ethanol yield in 50% hydrolysate in anaerobic conditions

The hydrolysate is supposed to be the supplier of sugars. Therefore high concentration of hydrolysate is more important than maximum yield. Fig 14 shows ethanol yield, in the highest concentration of hydrolysate that was tested, 50%. The data shows that, low temperature and high pH, gave higher yield. A higher initial concentration of cells reduced the importance of high pH at low temperatures.

Table 4: Data from the MODDE optimization tool

pH	i.c.d	Conc.H	temp	Air	Yeast growth	Ethanol yield
4	10	0,2	30	Low	3,6586	0,5206
4,0645	9,9993	0,3355	25,0004	Low	5,8064	0,5361
6	1,0102	0,4966	25,101	High	7,7837	0,4606

Table 5: Contains the 3 best optimization options with hydrolysate concentration set to 50%

pH	i.c.d	Conc.H	temp	Air	Yeast growth	Ethanol yield
5,9268	1	0,5	25,0002	High	7,5665	0,4596
6	1	0,5	25	High	7,5847	0,464
4	10	0,5	25	Low	8,4086	0,4712

The two tables (4 and 5) show MODDE optimization suggestions, from the screening experiment. Table 4 show some higher yields than table 5. But since the concentration of hydrolysate is important, table 5 shows maximum yield for 50% hydrolysate. A slightly higher yield with 20% hydrolysate, will not give higher final amount of ethanol in an industrial process. The results are model suggestion for maximum yield and needs to be verify the model by empirical tests before conclusions are made.

3.2.4 Adapted *Dekkera bruxellensis* in a fractional factorial design

The results from the adapted *D. bruxellensis* experiment, built a good growth response model. The ethanol yield response, showed curvature in the model. This was demonstrated by low model validity making the model unusable for conclusions. There is a possibility to do a completion for the ethanol response to build a valid model but due to time limitation the existing model needs to be used. It is possible to look for indications of how the factors contribute.

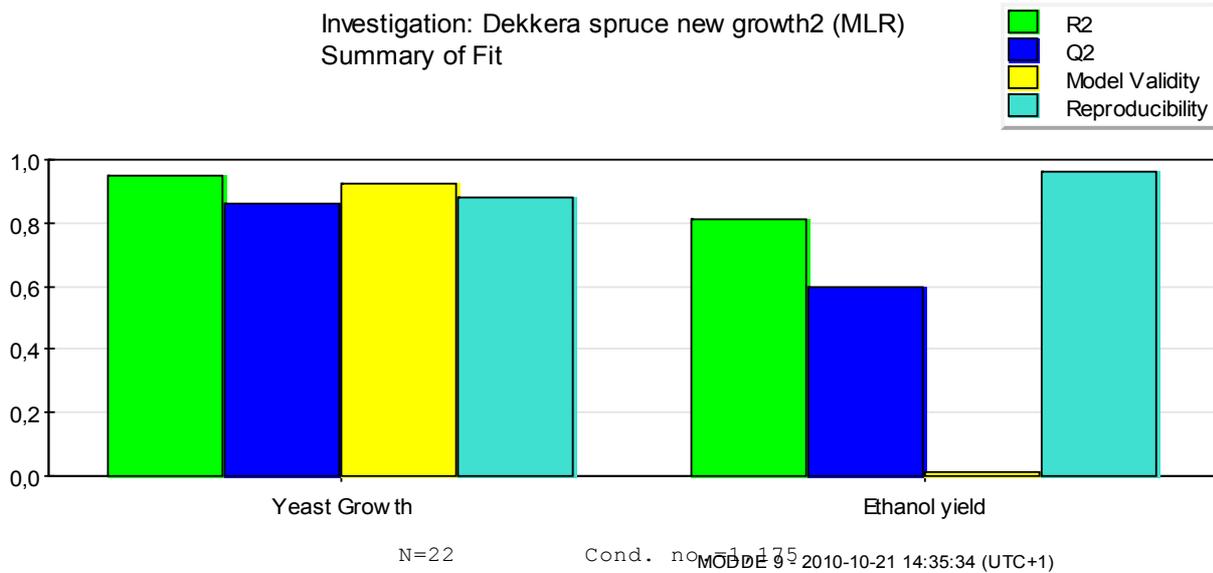


Fig 15: Summary of fit for adapted *D. bruxellensis*

The summary of fit shows that the model for *D. bruxellensis* growth is very good. The ethanol yield had a bad model validity but an acceptable Q2. This shows that the model design was bad, with a possible exponential or quadratic dependency. Further analysis showed that the problem was caused by curvature, fig 18. The curvature gave the model bad predictive skills.

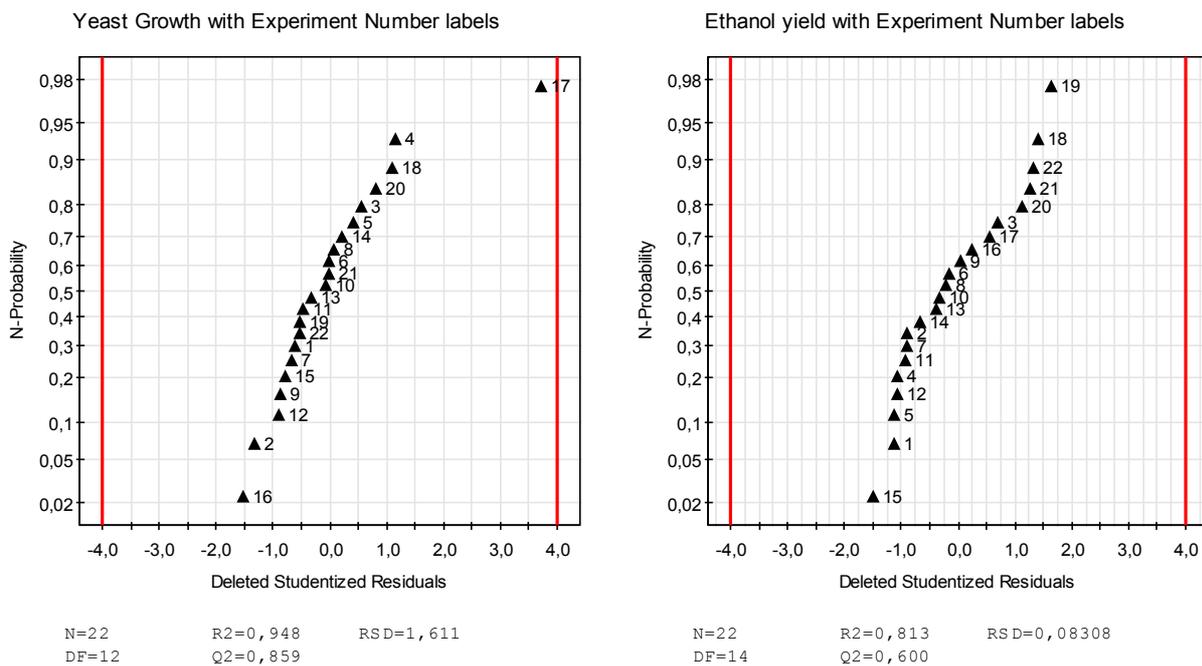


Fig 16: Normal probability plot for *D. bruxellensis* growth and ethanol yield.

The Normal probability plot for *D. bruxellensis* shows that one outlier was spotted in the growth model(17). It was not outside the boarder and no experimental faults could be found. Therefor it was kept in the model. In the ethanol yield model there was no outlier but the plot had some curvature.

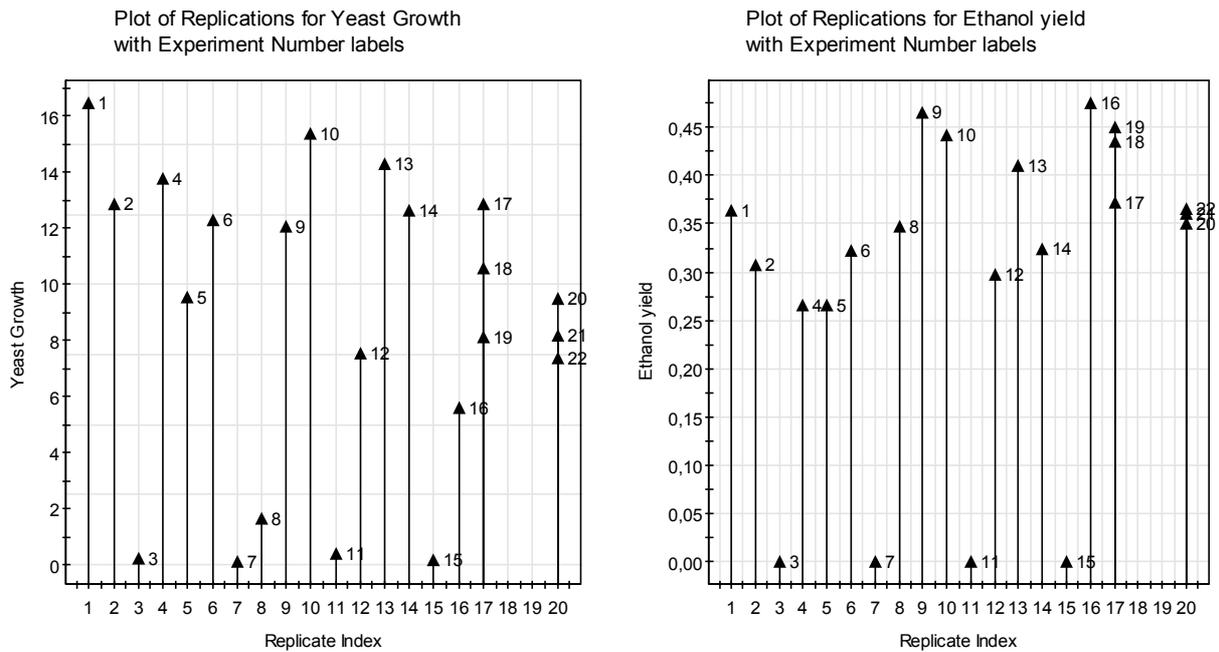


Fig 17: A replicate plot over D. bruxellensis ethanol yield and growth.
 The replicate plot shows that the centre points were quite well fitted in the middle and well clustered. Four samples 3, 7, 11 and 15 did not grow during the experiment and are therefore zero in both models.

Investigation: Adapted D.bruxellensis DOE analysis (MLR)

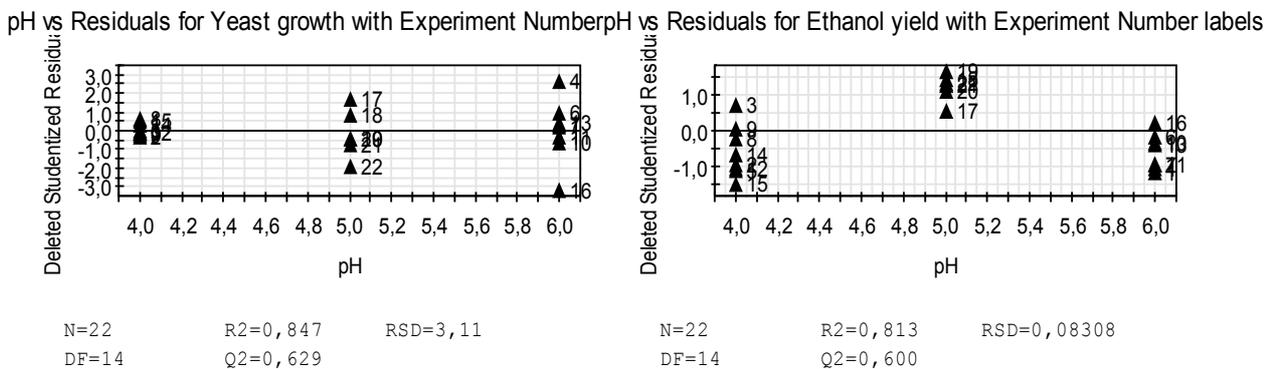
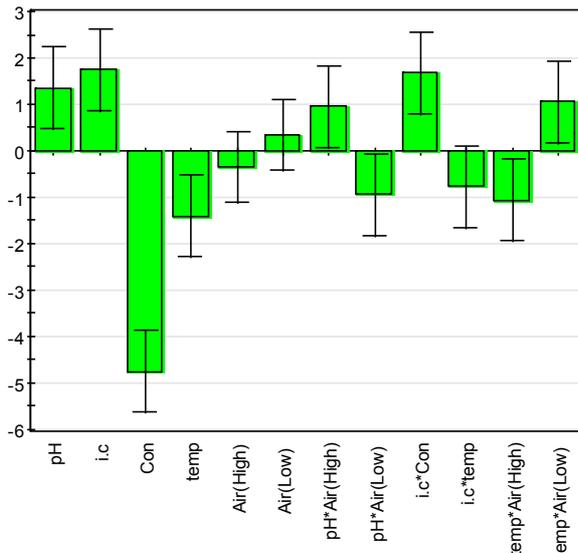


Fig 18: A residual plot of the experiment samples showing a curvature in the model for ethanol yield (figure to the right)
 This investigation of the residuals for ethanol and pH shows a strong curvature in the ethanol yield response (right figure). The curvature causes severe problems for the model and completion experiments has to be done. The yeast growth residuals (left figure) shows no curvature but a higher variation with higher pH.

Scaled & Centered Coefficients for Yeast Growth (Extended)



Scaled & Centered Coefficients for Ethanol yield (Extended)

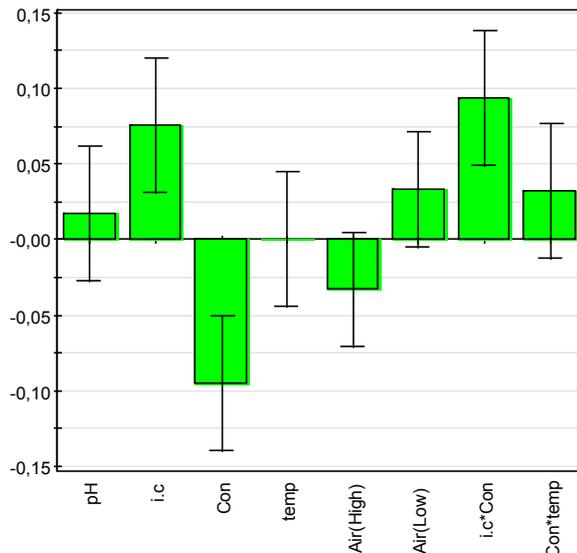


Fig 19: Coefficient plot for yeast growth and ethanol yield.

The coefficient plots show factors that contribute to the model. The ethanol response shows indications, but was not usable for conclusions. The figures show that concentration of hydrolysate (con) had a strong negative contribution to both responses. Initial cell concentration (i.c) had a positive correlation. For yeast growth, the pH factor was also significant. In both responses, it was indicated that, the interaction between initial cell density and concentration of hydrolysate was of importance and that they had positive contribution, to ethanol yield and growth.

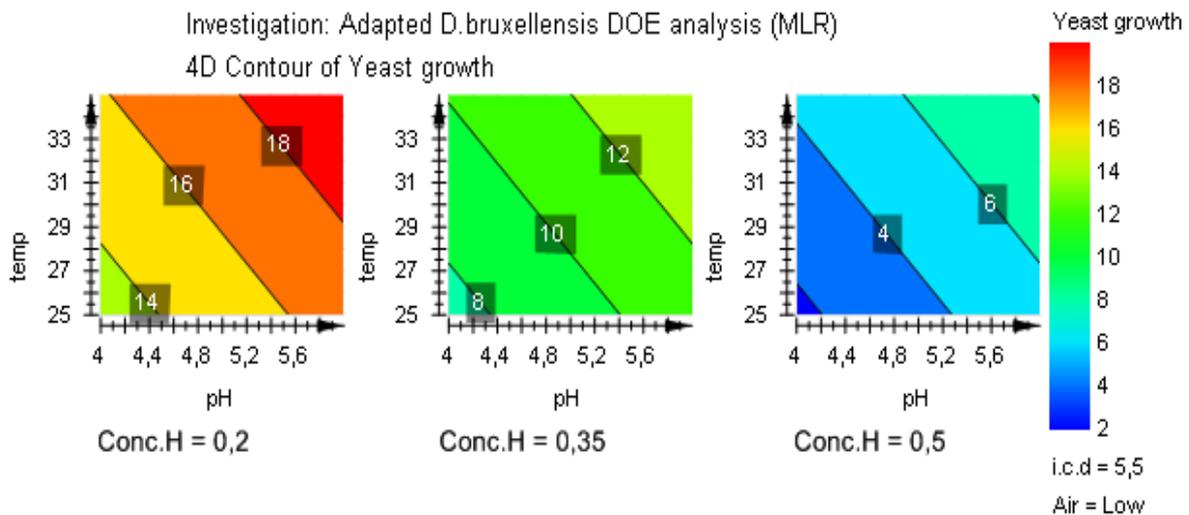


Fig 20: A contour plot of yeast growth, at low air and i.c.d 5,5

The plot shows growth of *D. bruxellensis*. In high concentration of hydrolysate growth was low. It also shows that the inhibition, by hydrolysate, was lowered with increasing pH and temperature. The low growth in 50% hydrolysate might be explained by, the fact that the samples with low cell density, did not manage to grow. From figure 20 it can be concluded that the *D. bruxellensis* growth was best at low concentration of hydrolysate. Growth was also showed to be better with higher pH and temperature.

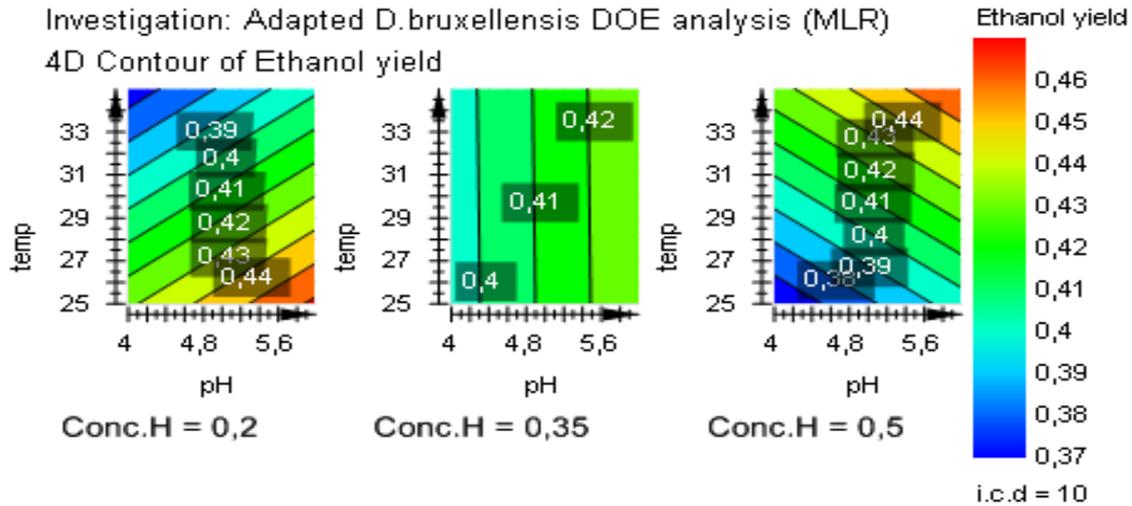


Fig 21: A 4D contour plot of ethanol yield, with i.c.d 10 and low air

A contour plot of ethanol yield, indicate that the involved factors were less important to yield, when i.c.d was high. It also shows that the trend of temperature, seems to be reversed when going from low to high concentration of hydrolysate. High pH had a positive contribution to the ethanol yield, at all concentrations and temperatures. The values are probably affected by the bad model validity and the data set should be completed, with further experiments, before any final conclusions are drawn.

3.2.5 Adapted *D. bruxellensis* strain vs the isolated *D. bruxellensis* 6F strain.

A comparison between the centre points of *D. bruxellensis* and adapted *D. bruxellensis* strains indicates that the final OD values for the adapted strain were generally higher. However a t-test, one for each of the two settings for air (H and L), did not show a statistical difference between the

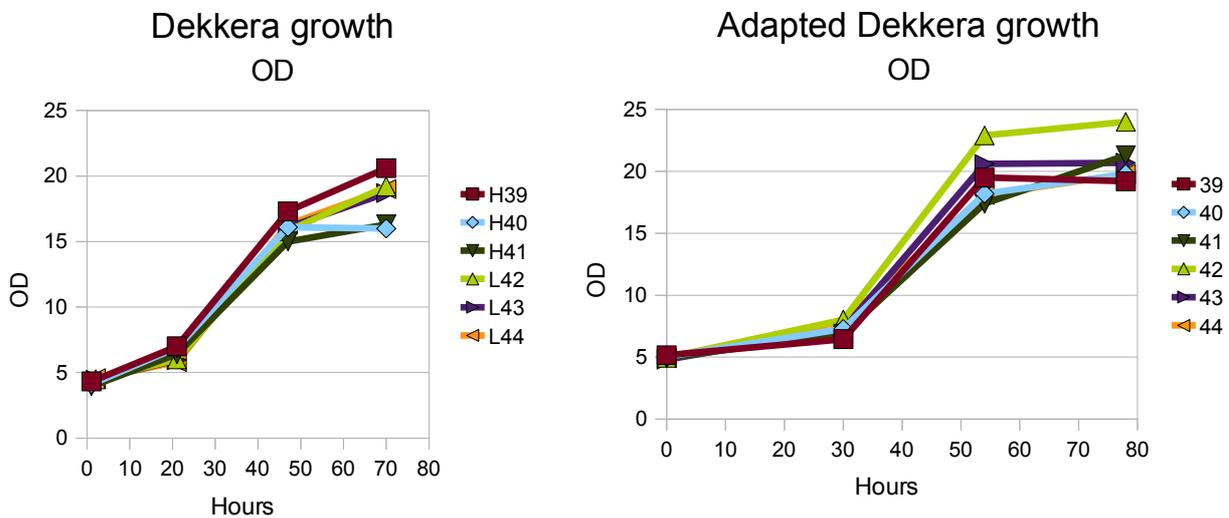


Fig 22: The two graphs show growth for centre points between the two experiments with adapted and non adapted *D. bruxellensis* strain.

centre points The first test compared samples 39-41 and gave a p-value of 0.20. The samples 42-44 gave a p-value of 0.12. The adapted strain did not grow in 50% hydrolysate, for most compositions. Even though it grew in 100% hydrolysate directly after adaptation was completed. It has to be considered, that it was not only the hydrolysate concentration, that varied in the model. PH and temperature might affect growth in combination with high concentration of hydrolysate. This was also indicated in the pre-experiment with pH and hydrolysate concentration (3.1)

3.3 SSF Simultaneous saccharification and fermentation

Table 4: SSF results show that the relative total sugar concentration level is much higher than for the normal hydrolysis (*D. bruxellensis* representing normal hydrolysis since the strain died immediately). S2.1 represent day two sample one.

Resultat SSF	start	S2.1	S2.2	S3.1	S3.2	S4.1	S5.1	S6.1	S7.1
Sacca									
glucose	0	0	0	0	0	0	0	0	0
ethanol	0	1.88	2.03	3.52	3.7	4.78	4.95	6.25	6.77
total ethanol	0	3.76	4.07	7.03	7.39	9.56	9.9	12.51	13.55
approx min glucose/l		7.52	8.13	14.07	14.79	19.11	19.8	25.02	27.1
Dekkera (Dead)									
glucose	0	1.69	1.99	3.69	3.92	5.34	6.24	7.94	8.68
total glucose		3.38	3.98	7.38	7.85	10.68	12.48	15.88	17.36

The saccharification was shown to be more efficient in SSF process, compared to hydrolysis (3.1), the same material gave almost double yield. Unfortunately in this experiment, the *D. bruxellensis* yeast strain did not grow. It was not detectable after experiment start, and no ethanol was produced. The only reasonable explanation is that, the yeast died during the first 12 hours. The fermentor with *D. bruxellensis* was instead used as a approximation of a hydrolysis. The results (table 4) show that the approximated sugar level is almost double in the SSF, compared to the hydrolysate process. Due to lack of time, the experiment was only performed once.

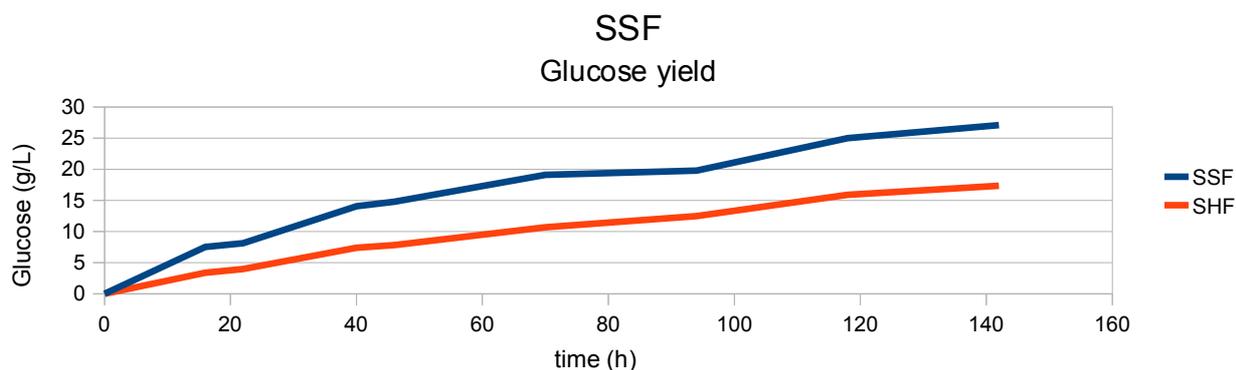


Fig 23: The minimum glucose yield for the SSF fermentation was continuously higher than for the simulated hydrolysatation (SHF)

The result of the SHF shown in this study is the actual *D. bruxellensis* SSF batch. Since the yeast died and the setup for this experiment was very similar to the hydrolysis (3.1), the result was used as a approximated hydrolysis process (SHF). The pH was continuously maintained at 5 and the temperature was 36° instead of 45° compared to the hydrolysis performed for the design of experiment (2.1). The result of final glucose concentration (17,3g/L) glucose is a good approximation of the two hydrolysates made at the BMC department that ended up with (17,2g/L) and two (16,5g/L) glucose respectively.

4 DISCUSSION

The research aimed to look for important factors and differences between the *D. bruxellensis* and *S. cerevisiae* yeasts. Results from pre-experiments showed that *D. bruxellensis* did not grow as well as *S. cerevisiae*. But *D. bruxellensis* gave a higher ethanol yield, earlier reports support that *D. bruxellensis* has higher or similar yield compared to *S. cerevisiae* (Blomqvist et al., 2010). The pre-

experiments showed weak growth for *D. bruxellensis* in samples with low pH and high concentration of the hydrolysate. It is not conclusive with previous projects, where the *D. bruxellensis* has been shown to withstand low pH (Blomqvist et al., 2010). But lignocellulosic substrate was used and different inhibitors were present, which may explain the differences.

The MODDE design results were not directly comparable between the two yeast. That is because *D. bruxellensis* had to be adapted to the hydrolysate before any model could be built. But still some differences could be noticed. Both yeast build good models for growth and ethanol yield.

4.1.1 Growth

The model for *S. cerevisiae* growth was good. It showed that several factors in the model were important for growth. Most important according to the model was temperature, where low temperature was shown to be beneficial for growth. Earlier studies have shown that *S. cerevisiae* have a lower viability, when grown in harsh conditions with high temperature (Nagodawithana et al. 1974). Two more factors correlated with low values for good growth, that was initial cell density and the concentration of hydrolysate. High concentration of hydrolysate was negative for biomass growth, but since the hydrolysate contains toxic compounds (Ekman. 1976) it was expected. A bit surprising was that high cell density seemed to be bad according to the model. The reason is likely the way growth is presented. The final OD values in high cell density samples were higher, but the calculations measure the total amount of risen OD units. High i.c.d samples were not able to raise as many OD units as low i.c.d samples. The amount of sugar limited the maximum cell density. High initial pH in the model shows better growth than low initial pH, this effect has been shown in previous studies with *S. cerevisiae* (Oliviero et al. 1982). Finally the limited air setting did not affect *S. cerevisiae* growth in the model. It is likely because *S. cerevisiae* can grow anaerobically.

The growth model for the adapted *D. bruxellensis* strain was very good, the results from the experiment could be well explained and predicted by the model. Within the tested range of the factors that was used, the results should be reliable. The model showed that *D. bruxellensis* growth was mainly dependent on the concentration of hydrolysate. That was also noted during the experiments since five of the 22 samples had no or very low growth. This was most probably caused by the toxic compounds in the hydrolysate (Ekman. 1976). High initial cell concentration had a positive contribution to the model. One explanation might be that the cells protect each other and that each cell therefore is less affected by the toxic compounds. High pH showed positive contribution to *D. bruxellensis* growth. A previous study with different pH showed that *D. bruxellensis* had a high tolerance to changes in pH (Blomqvist et al. 2010) which was not confirmed by this study. The same report showed high tolerance to temperature changes, but from the model built from the *D. bruxellensis* experiment in this study the temperature had strong negative contribution to growth. One reason for this might be the combination of factors, for example toxic compounds from the hydrolysate, change there toxicity with pH and or temperature. The maximum growth temperature for *D. bruxellensis* is limited to approximately 37-40°C (Blomqvist et al. 2010) which is quite close to the highest temperature in the model. The aeration option did not show any contribution to the model and can be ruled out as important for *D. bruxellensis* growth in this model.

A comparison of the growth response models showed that several factors have similar effects on growth. The difference is that high initial cell concentration was important for *D. bruxellensis* but not for *S. cerevisiae*. Low concentration of hydrolysate was more important for *D. bruxellensis* growth. The strong inhibition effect by hydrolysate on *D. bruxellesis* was also shown in pre experiments.

4.1.2 Ethanol yield

The model for *S. cerevisiae* ethanol yield was good. It shows that high initial pH has positive contribution to the ethanol yield. This might be related to high yeast mortality when toxic compounds, like acetic acid, easier enter the cells when pH is low (Cássio & Leáo. 1991). Initial cell density and hydrolysate concentration are not important factors for the final ethanol yield in the model in accordance with previous reports (Matsushika & Sawayama, 2010). The strongest factor for *S. cerevisiae* ethanol yield was the amount of air present. Less air gave more ethanol. This is understandable since ethanol is the endproduct of the anaerobic metabolism of *S. cerevisiae*. Another explanation concerning the aerobic samples is that *S. cerevisiae* can degrade ethanol through an aerobic mechanism, which could reduce the measured amount of ethanol. Evaporation when the gas exchange is higher might also play a role.

The model for *D. bruxellensis* ethanol yield was not possible to analyse since there was curvature in the model. Further experiments have to be done to complete the model, before any conclusions can be made.

The comparison between centre point samples proves that *D. bruxellensis* gives better, during some circumstances, or as good ethanol yield compared to *S. cerevisiae*.

4.1.3 SSF

The SSF experiment was only a small pilot project and it was not ideal for conclusions. But the total amount of sugar produced was almost double according to the experiment and it seems like fed batch SSF will speed up and reduce enzyme limitation, when a relatively small amount of enzyme is used. This is supported by earlier studies (Tomás-Pejó et al. 2008).

5 CONCLUSIONS AND FUTURE

The first objective should be to complete the *D. bruxellensis* model with additional experiments. Although the model in this project was not complete, the comparable experiments (the centre points) with *D. bruxellensis* showed the same or higher yield than *S. cerevisiae*. The adaption skills showed by *D. bruxellensis* should be analysed further and to be compared with *S. cerevisiae*. It was very unclear how and why *D. bruxellensis* was able to survive the maximum concentration of spruce hydrolysate, during the adaptation, but then suddenly died when the concentration was 50% during the experiment.

A few factors (e.g. pH) were not controlled during the experiments but still the models were strong and had good prediction skills.

Simultaneous saccharification and fermentation was shown to be good when the enzyme level was limited, the method should be further analysed.

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