Investigating the influence of temperature and glucose concentration on ethanol production in *Saccharomyces cerevisiae*

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Yeast is a well-known and common fungus naturally found in warm and moist environments. When placed in less favourable surroundings, such as those depleted of oxygen this small fungus can alter the way it generates energy allowing it to survive and grow. In doing so the fungus readily produces by-products of carbon dioxide and ethanol. This is the process of fermentation and the reason why yeast is so famous. Throughout human history this adaptation has been applied to produce food, alcoholic drinks and now biofuels. As an indication to the value of this technology the UK beer market alone is currently worth $8 billion and while the demand for renewable energy sources grows, ethanol-based biofuels are becoming a more viable source of power. Therefore, there is great interest in improving the efficiency and productivity of this process. This study looked to achieve just that; through altering the temperature and sugar concentrations of yeast's environment we hoped to deduce the optimal conditions required to produce maximum ethanol yields. To achieve this a sample of yeast was incubated over 2 days at temperatures of either 25 °C, 30 °C and 35 °C and in a sugar concentration of either 150 g/L, 180 g/L or 210 g/L before the ethanol concentration for each condition was measured. The results of this study suggest from the conditions we tested a combination of 35 °C in a sugar concentration of 180 g/L produced the largest yields of ethanol (3.09 mg/ml). These findings agree with various other studies, however other studies have also achieved much greater yields by also manipulating a range of other factors e.g. pH, nitrogen concentration and source of sugar.

Abstract

The production of ethanol using *Saccharomyces cerevisiae* is one of the oldest and most commonly used examples of biotechnology. In nature, the capability of yeast to replace oxygen with glucose as a terminal electron accepter allows this microorganism to continue to grow even in anoxic environments producing ethanol as a by-product. However, when applied to an industrial context this metabolic survival strategy has important applications in the production of alcoholic drinks, food and bioethanol. In order to investigate the influence of incubation temperature and glucose concentration on ethanol yield by *S. cerevisiae,* we prepared 9 unique batch fermentation conditions. Each sample of *S. cerevisiae* was incubated for 48 hours at a temperature ranging from 25 °C to 35 °C while inoculated in a glucose solution ranging from 150 g/L to 210 g/L. After this time the ethanol concentration for each sample was determined using an ethanol assay. The greatest mean yield of ethanol (3.09 mg/ml) was obtained when samples were incubated at a temperature of 35 °C in a glucose concentration of 180 g/L. While an increase of incubation temperature from 25 °C to 30 °C at all glucose concentrations produced a statistically significant increase in ethanol yield, no significant increase was generated when temperature was raised from 30 °C to 35 °C at each glucose concentration tested. Additionally, no significant increase in ethanol yield was found when glucose concentration increased. Therefore, while these conditions generate the highest mean ethanol yield we cannot conclude this was due to increasing incubation temperature from 30 °C to 35 °C or by variation of glucose concentration between 150 g/L and 210 g/L.

Introduction

S. cerevisiae, also known as Baker's yeast is a single-celled eukaryotic fungus typically found in warm and moist environments such as the skin of grapes (1). Its ease of handling and molecular similarities to humans, makes it a popular model organism for research. Typically, yeast plays a number of roles in industry acting as a protein supplement, animal feed and vector for the production of recombinant proteins (2). However, most notably the fermentation of sugars and production of ethanol using *S. cerevisiae* is one of the oldest and most commonly used examples of biotechnology. This microbial anaerobic oxidation of sugars has been exploited for millennia in the production of alcoholic drinks, food and more recently in the production of bioethanol (3). As an indication of the importance of this technology the UK beer market is now worth an estimated $8 Billion (4).

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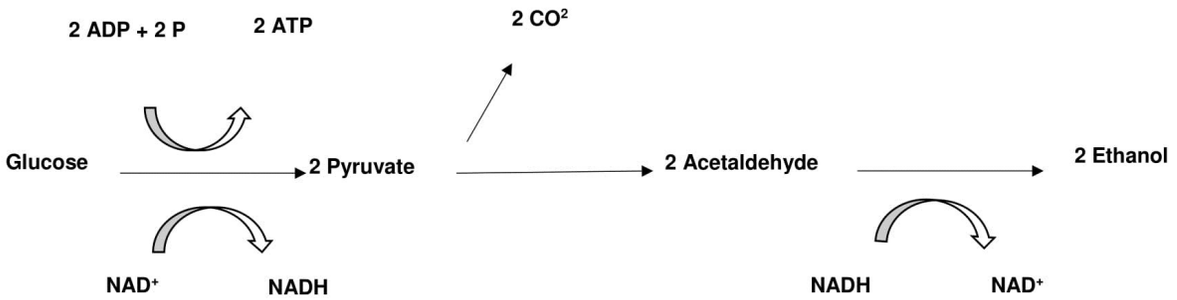

Figure 1 (above). Anaerobic fermentation of glucose sugar resulting in the production of ethanol and NAD^+ by *S. cerevisiae*.

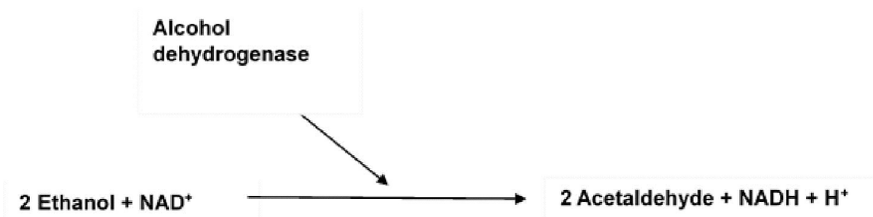

Figure 2 (left). Dehydrogenation of ethanol catalysed by alcohol dehydrogenase resulting in the production of acetaldehyde, hydrogen and NADH that can be assayed to give an indication of ethanol concentration.

Bioethanol production has become the largest consumer of corn in America. Naturally, the ability of *S. cerevisiae* to anaerobically ferment a range of hexose sugar substrates allows the fungi to survive in anoxic environments. However, it is this characteristic that makes the microorganism so attractive to industry. In the absence of oxygen *S. cerevisiae* can utilise sugars as a carbon source via glycolysis generating pyruvate and ATP. For this process to continue there must be a steady supply of the coenzyme NAD^+. To achieve this pyruvate is decarboxylated to acetaldehyde that is subsequently reduced to ethanol by NADH, regenerating NAD^+ and allowing glycolysis to continue (Figure 1) (5). Similarly, the decarboxylation of pyruvate releases CO_2 during the baking process creating air pockets within dough. *S. cerevisiae* is preferred to bacteria and other fungi in the industrial production of ethanol due to its various physiological advantages. It can operate in a large pH range with an acidic optimum meaning the process can naturally inhibit contaminating bacteria. Furthermore, the fungus has a higher ethanol tolerance (17% v/v) and is generally regarded as safe for human consumption enhancing its suitability for use in the consumables industry (6). The ability of *S. cerevisiae* to produce ethanol is heavily predicated on its fermentation conditions. The influence of factors such as carbon source, substrate concentration, temperature and pH have been heavily explored and the use of genetic manipulation is now being implemented to allow *S. cerevisiae* to ferment a greater range of substrates (7). Our study hypothesised that an increase in temperature and glucose concentration would increase the rate of fermentation by *S. cerevisiae* and therefore increase the concentration of ethanol produced. We aimed to deduce the optimal combination of glucose concentration and incubation temperature required to optimise batch production of ethanol using *S. cerevisiae*.

Materials and Methods

Broth preparation

A broth culture of *S. cerevisiae* was initially provided by the University of Liverpool (School of Life Sciences) and from this 1 ml of broth culture was plated onto several agar mediums including yeast extract-malt extract, mannitol salt agar, MacConkey agar and triple sugar iron agar that were all incubated at 37 °C overnight. Examination of the plates revealed the fungi had failed to grow on all

Table 1. Volumes of YEME broth and 300 g/L glucose stock solution required to produce 20 ml of mixed solutions with glucose concentrations of 150 g/L, 180 g/L and 210 g/L.

Volume of glucose 300 g/L stock (ml)	Volume of YEME broth (ml)	Final glucose concentration (g/L)
10	10	150
12	8	180
14	6	210

Table 2. Specific combinations of incubation temperature and substrate concentrations for batch glucose fermentation of *S. cerevisiae.*

	Glucose Concentration			
Temperature		150 g/L	180 g/L	210 g/L
	25 °C	25 °C & 150 g/L	25 °C & 180 g/L	25 °C & 210 g/L
	30 °C	30 °C & 150 g/L	30 °C & 180 g/L	30 °C & 210 g/L
	35 °C	35 °C & 150 g/L	35 °C & 180 g/L	35 °C & 210 g/L

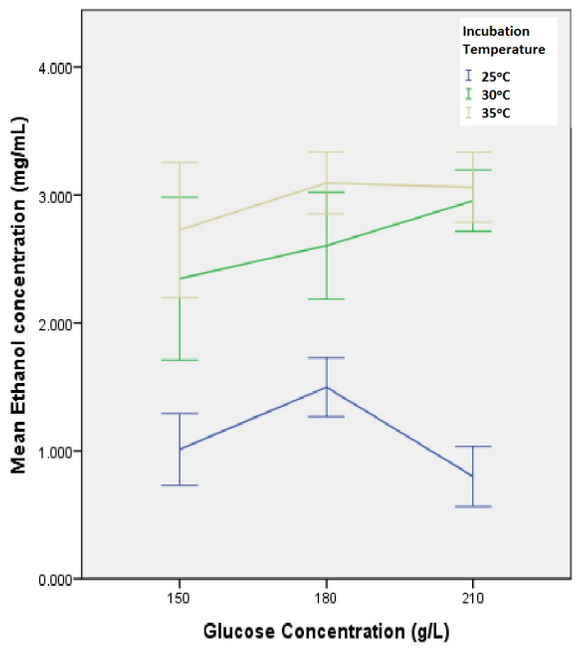

Figure 3. Ethanol yield obtained through batch glucose fermentation using *S. cerevisiae* at glucose concentrations of 150 g/L, 180 g/L and 210 g/L incubated for 48 hours at temperatures of 25 °C, 30 °C and 35 °C. The presence of overlapping error bars (± SE) between ethanol yields generated at incubation temperatures of 30 °C and 35 °C at all glucose concentrations suggests this difference is not significant. The error bars of ethanol yields generated at 25 °C at all glucose concentrations do not overlap with other incubation temperatures suggesting these produce significantly different ethanol yields.

excluding the yeast extract-malt extract (YEME) agar pate; because of this it was decided throughout the experiment *S. cerevisiae* would be inoculated into YEME broth. A 600 ml stock solution of YEME broth was mixed and from this 3 different volumes of broth (Table 1) were transferred to 20 ml glass bijoux bottles to be autoclaved. A 300 g/L glucose stock solution was purified using 0.22 µm filters removing any microbial contamination. From the stock, 3 separate volumes of solution (Table 1) were added to 20 ml glass bijoux bottles so that each one contained a combined total of 20 ml of YEME broth and glucose stock solution. The final proportions of glucose stock to YEME broth were calculated to produce solutions with glucose concentrations of 150 g/L, 180 g/L and 210 g/L (Table 1).

Incubation procedure

Each glass bijoux containing a YEME broth/glucose solution mix was inoculated with a single colony of *S. cerevisiae* approximately 1 mm in diameter using a sterile wire loop and incubated under static conditions for 48 hours at a temperature of either 25 °C, 30 °C or 35 °C in glucose concentrations of 150 g/L, 180 g/L and 210 g/L. This created 9 unique fermentation conditions (Table 2). Each fermentation condition was tested 9 times and a mean average ethanol yield was calculated.

Determination of ethanol concentration

After incubation each fermentation condition was individually assayed to determine its ethanol concentration. 0.1 ml of YEME broth now containing ethanol was added to 4.75 ml pyro buffer, 0.1 ml B-NAD and 0.05 ml alcohol dehydrogenase. After mixing and incubation at 37 °C for 25 minutes the level of NADH in each solution was measured at 340 nm using a spectrophotometer. The assay relies on the dehydrogenation of ethanol, converting NAD^+ to NADH catalysed by alcohol dehydrogenase (Figure 2). Using known ethanol concentrations of 5 mg/ml, 4 mg/ml, 3 mg/ml, 2 mg/ml and 1 mg/ml a calibration curve was constructed. Values of ethanol yield were deduced via regression analysis using the linear equation y= mx + b derived from the calibration curve where y= A_{340} reading, m= gradient of the calibration curve, x= amount of ethanol present in the sample and b= the y intercept.

Data analysis

A repeated measures two-way ANOVA with a Greenhouse-Geisser correction was used to statistically analyse data obtained throughout the study. A pairwise comparison using a Bonferroni correction was later conducted to further analyse the results of the previous statistical test.

Results

By conducting an ethanol assay on the samples, we were able to deduce the ethanol concentration and make comparisons between the ethanol yields generated at different incubation temperatures and glucose concentrations. Higher ethanol yields indicate higher rates of fermentation. The greatest mean yield of ethanol (3.09 mg/ml) was obtained when samples were incubated at a temperature of 35 °C in a glucose concentration of 180 g/L. Incubation of samples at 35 °C and glucose concentrations of 210 g/L or 150 g/L produced decreased mean ethanol yields (Figure 3). Figure 3 demonstrates that when incubated at temperatures of 25 °C, 30 °C or 35 °C, increasing glucose concentration from 150 g/L to 180 g/L resulted in an increase of mean ethanol yield from 1.01 mg/ml to 1.44 mg/ml, 2.34 mg/ml to 2.62 mg/ml and 2.73 mg/ml to 3.09 mg/ml respectively. When samples were incubated at a temperature of 30 °C and glucose concentration was further increased to 210 g/L this trend was repeated producing a higher mean ethanol yield of 2.95 mg/ml. However, increasing glucose concentration from 180 g/L to 210 g/L in samples incubated at 25 °C and 35 °C resulted in decreased mean ethanol yields of 0.642 mg/ml and 3.06 mg/ml respectively. A repeated measures two-way ANOVA with a Greenhouse-Geisser correction showed that the mean ethanol concentration was not significantly different between glucose concentrations: $P>0.05$. A further ANOVA under the same conditions showed a significant difference in ethanol concentration between incubation temperatures: $P=0.00$ ($P<0.05$). Pairwise comparison using a Bonferroni correction showed that the ethanol concentration produced at 30 °C was increased

on average by 1.555 mg/ml compared to 25 °C ($P<0.05$). Between 30 °C and 35 °C, there was not a significant increase in ethanol production ($P>0.05$). Therefore, while a combination of 35 °C incubation in a glucose medium of 180 g/L produced the highest mean ethanol yield we cannot conclude this was a result of increasing incubation temperature from 30 °C to 35 °C or by our variations of glucose concentration between 150 g/L and 210 g/L.

Discussion

Fermentation plays a central role in many of the biotechnological processes that we rely on in various aspects of our lives e.g. food, alcoholic drinks and biofuels. By conducting research into this process, we can create specific fermentation conditions that maximise its efficiency and yield, subsequently leading to developments that both improve quality and reduce costs. Our study hypothesised that by increasing both glucose concentration and incubation temperature we would achieve higher ethanol concentrations, however the absence of a significant difference between ethanol yields at the tested glucose concentrations suggests variation between 150 g/L and 210 g/L has no influence on ethanol production during batch fermentation.

The presence of a significant difference between ethanol yields when incubated at temperatures ranging from 25 °C to 30 °C demonstrates increasing the incubation temperature of batch fermentations in this range will result in larger ethanol yields. However ethanol production at temperatures ranging from 30 °C to 35 °C are not significantly different indicating that any increase above 30 °C to 35 °C would have no influence on ethanol yield during batch fermentation. This finding can be explained by enzyme kinetics and the temperature dependent nature of enzyme activity. Increasing the incubation temperature from 25 °C to 30 °C during the fermentation process provides a greater level of kinetic energy to the reactants and enzymes involved in glucose fermentation and ethanol production. This higher level of kinetic energy increases the frequency of collisions between the enzymes and reactants increasing the rate of reaction leading to a greater ethanol yield. The conclusions drawn from these results remain consistent with the findings of previous studies that suggest achieving the greatest yield of ethanol during batch fermentation with *S. cerevisiae* incubation temperatures should range from 30 °C to 35 °C (6). Therefore, it is most likely that no significant difference was observed between ethanol yields at temperatures of 30 °C and 35 °C as the optimal temperature lies within this range.

Previous literature indicates a glucose concentration of 200 g/L is optimal for ethanol production, this is consistent with our findings (8). However, our results indicated variation of glucose concentration from 150 g/L to 210 g/L produced no significant change in ethanol yield during batch fermentation contradicting findings of previous studies. Past investigations of growth and fermentation characteristics of *S. cerevisiae* indicate significant differences are observed in ethanol yield between glucose concentrations of 20 g/L and 300 g/L when increased in intervals (8). It is possible the range of glucose concentrations tested throughout our study was too small to produce a significant change in ethanol yield and by testing a greater range of glucose concentrations the study would be improved.

Further investigation of previous studies highlights that when incubated at 30 °C for 24 hours with a glucose concentration of 200 g/L (pH 6.0) ethanol yield can reach 85.56 ± 1.13 mg/ml (8), much higher than our optimal ethanol yield of 3.09 mg/ml (35 °C with 180 g/L glucose). This finding indicates that while we were able to optimise ethanol production from our limited range of incubation temperatures and glucose concentrations there is massive potential for further optimisation. Based on a theoretical maximum yield of 0.51 g ethanol/g sugars (9) through variation of incubation temperature and glucose concentration we were able to achieve an ethanol yield of 2.9% of the theoretical maximum yield.

Numerous different strategies have been employed to improve ethanol yields from batch fermentation such as optimisation of pH, nitrogen supplementation, immobilisation, carbon source and inoculum size. These are all factors that could be further investigated in later studies to provide an insight into improving ethanol yields. A key limitation of our study is derived from the fact only 2 variables were manipulated, whereas to optimise ethanol yields a study would have to consider all of the factors previously listed. Furthermore, during incubation the inoculum of *S. cerevisiae* would settle to the bottom of the universal. Consequently, it is unlikely that all glucose substrate would be utilised during fermentation producing lower yields of ethanol. As a resolution to this problem in future investigations higher yields of ethanol may be obtained by regularly shaking incubation vessels to prevent the sedimentation of *S. cerevisiae.* Our findings indicate optimal ethanol yields are produced when incubated at temperatures ranging from 30 °C to 35 °C; In order to overcome this limitation and improve the specificity of the study it would be more appropriate to test incubation temperatures that increase in 1 °C intervals rather than 5 °C intervals.

Recently the use of genetic manipulation and synthetic biology is driving the future of ethanol production using *S. cerevisiae*. These techniques have enabled *S. cerevisiae* to utilise xylose, the second most abundant sugar present in plant biomass behind glucose. Xylose fermentation using *S. cerevisiae* has become a possibility due to the introduction heterologous xylose assimilation pathways into *S. cerevisiae*. Genetically engineered *S. cerevisiae* are capable of fermenting both glucose and xylose present in hemicellulose during ethanol production greatly improving utilisation of carbon sources during

ethanol production. This technology has massive applications in the microbial conversion of biomass to bioethanol as efficient xylose utilisation is seen as a key prerequisite for developing economic sources of biofuel (10). Alternative branches of research into optimisation of ethanol production focus on improving ethanol tolerance of *S. cerevisiae*. To achieve this a number of different approaches have been used however, via directed mutagenesis researchers have been able to modify RNA polymerase II subunit Rpb7 altering the transcription of its genome resulting in improved ethanol yields (11). Studies such as these pave the way for developments in ethanol fermentation using *S. cerevisiae*.

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