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# Osmotic stress Response of *Saccharomyces cerevisiae* under HG and Elevated Temperature Environment

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**Abstract:** The High Gravity (HG) ethanol fermentation at high temperature is very attractive and promising technology for fuel ethanol production. This study was designed to improve the osmotic as well as thermal behavior of the *Saccharomyces cerevisiae* strain isolated from distillery waste. Therefore, initial pH and substrate concentrations were optimized for this strain. The *S. cerevisiae* was subjected to thermal treatment to improve its fermentation ability without significant yield losses. At pH<sub>o</sub> 5.0, 95g/L ethanol was produced with the productivity (Q<sub>p</sub>) value of 1.02. The activation energy E<sub>a</sub> value calculated at 30-40°C was 16.48kcal/mol indicating the thermal tolerance of the strain SC36. The results of glucose optimization revealed that at 250g/L glucose concentration, Q<sub>p</sub>, Y<sub>p</sub>/s and Y<sub>p</sub>/x value of 1.53g/Lh, 0.443g/g substrate and 41.4g/g biomass were obtained. The strain's potential to be able to ferment very high gravity medium is very promising for fuel ethanol production.

**Keywords:** Thermal Treatment, *Saccharomyces cerevisiae*, Monod Model, High Gravity, Thermotolerant

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## 1. Introduction

The evolutionary increased energy demand in chemical industry and automobiles has modulated the direction of research towards new renewable energy production approaches for sustainable energy supply. One among many approaches is the bioethanol production from cellulosic substrates, which is being considered an economic substitute for growing worldwide biofuel demand (Jones and Ingledew, 1994). Since 1990s, High Gravity (HG) and Very High Gravity (VHG) ethanol fermentation has revolutionized the field of biofuel industry to make the production of bioethanol an attractive alternative for sustainable energy supply. The VHG fermentation for maximum ethanol production (above 15% v/v) poses severe stresses on yeast growth, cell viability (Zhao and Bai, 2009), high osmotic pressure and substrate inhibition but the advantages do include the high ethanol yield, reduced labor, shorter ethanol recovery time, reduced capital costs and energy consumption (Casey et al., 1984). The high-temperature requirement is an essential requirement for industrial ethanol production though it can pose severe damage to *S. cerevisiae* growth and reduced fermentation rate due to glycolytic shutdown. Similarly, low pH tolerance is a fundamental requirement of ethanol fermentation to avoid the

bacterial contamination (Edgardo et al., 2008; Ortiz - Muñiz et al., 2010). Temperature tolerance is closely related to pH tolerance as both factors affect the membrane fluidity, mitochondria and cell viability. High temperature as well as high ethanol as a result of VHG media leads to the changes in membrane fluidity, and ultimately the cell death of *S. cerevisiae* (Lei et al., 2007; Piper, 1995).

The present research was attributed to study kinetics of ethanol fermentation from dextrose by *S. cerevisiae* SC36 isolated from distillery waste. The effect of fermentation parameters such as initial pH and substrate on kinetics of ethanol fermentation were investigated. The strain was then subjected to thermal treatment to improve its temperature tolerance during VHG fermentation. For microbial growth, substrate consumption and product formation, kinetic models by Monod and Leudeking-Piret were also evaluated in batch fermentation by *S. cerevisiae*.

## 2. Materials and Methods

### 2.1. Strain, Media and Culture Conditions

*S. cerevisiae* was isolated from the distillery plant and maintained on the culture medium containing (g/l): glucose, 20; yeast extract, 5; MgSO<sub>4</sub>, 5 and agar, 20 at a pH of 5.0 and

then preserved at 4°C. The fermentation media was (g/l): yeast extract, 5; MgSO<sub>4</sub>, 3; dextrose, 50-300, at 50 interval and initial pH, 4.0-5.5 at 0.5 intervals. The fermentation medium was sterilized for 15 min at 121°C. Both the pH and osmotolerance optimizations were carried out at 30°C.

## 2.2. Development and Selection of Thermotolerant Yeast Strain

To improve the thermotolerant ability of the yeast strain, two different approaches were adopted based on the methodology of Edgardo *et al.* (2008); 1) direct thermal shock and 2) progressive thermal shock. Direct thermal shock was given in four cycles, first at 45°C for 1hr and rest of all at 30°C for 30 minutes to the previously optimized strain for osmotolerance and pH<sub>0</sub>. In the progressive thermal treatment, thermal shock was given from 35 to 42°C with a 1°C rise in temperature after every 24 hr. Thermotolerance proof tests were conducted by inoculating the thermally treated yeast cells from each treatment on YMG agar plates and incubated at 36 to 42°C. After the selection of the thermotolerant strain, the ethanol fermentation profile of the selected strain at different temperatures (30 to 40°C) was conducted to confirm the fermentative ability of the isolated strain.

## 2.3. Analytical Methods

Ethanol concentration was determined spectrophotometrically by an acid-dichromate method developed by (Bennett, 1971; Pilone, 1984). Gravimetric method was used for cell mass determination by centrifuging the 50ml culture medium and drying the pellet at 60°C for 24hr. Reducing sugar content was determined following the method described elsewhere (Miller, 1959).

## 2.4. Kinetic Modeling

To describe the kinetics of fermentation process, rate equations were employed for biomass (X), glucose (S) and ethanol (P).

The equation to describe the growth rate during exponential phase of fermentation process can be described by the equation developed by Monod kinetic model.

$$dX/dt = \mu X \frac{S}{K_s + S} \quad (1)$$

Where, the specific growth rate  $\mu$  is given by the Monod as

$$\mu = \mu_{\max} \quad (2)$$

Where,  $\mu_{\max}$  is the maximum specific growth rate and  $k_s$  is the substrate saturation constant.

An unstructured model that combines both the growth-associated and non-growth associated product formation was based on the Leudeking-Piret, who originally developed it for lactic acid production (Leudeking, 1959), is as follows

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad (3)$$

Where  $\alpha$  is for growth associated and  $\beta$  is for non-growth associated product formation.

The substrate glucose is used both for the maintenance of cell growth and for the production of cell metabolites. So the substrate consumption can be described by the equation,

$$\frac{dX}{dt} = \gamma_s = -\frac{1}{Y_{x/s}} \frac{dX}{dt} - \frac{1}{Y_{p/s}} \frac{dP}{dt} - m_s X \quad (4)$$

Where  $Y_{x/s}$  and  $Y_{p/s}$  are yield constants and  $m_s$  is the maintenance coefficient. By simplifying and substituting eq 3. ineq4. we get,

$$\frac{dS}{dt} = \gamma_s = -\gamma \frac{dX}{dt} - \lambda X \quad (5)$$

Where  $\gamma$  and  $\lambda$  are constants for growth and non-growth associated substrate consumption. Both of these are represented by eq 6;

$$\gamma = \left\{ \frac{1}{Y_{p/s}} + \frac{\alpha}{Y_{p/s}} \right\} \text{ and } \lambda = \left\{ \frac{\beta}{Y_{p/s}} + m_s \right\} \quad (6)$$

## 3. Results and Discussion

The effects of pH<sub>0</sub>, dextrose and temperature were observed on the ethanol production behavior of *S. cerevisiae* SC36 strain.

### pH effect on growth and fermentation

Table 1. Kinetic analysis at various initial pH on ethanol production.

pH	$\mu_{\max}$ h <sup>-1</sup>	$Y_{p/x}$ g/g	$Y_{p/s}$ g/g	$Q_p$ g/Lh	$q_p$ g/gh
4.0	0.0255	40.875	0.4496	0.937	0.426
4.5	0.0237	38.705	0.4064	0.847	0.40
5.0	0.0341	44.72	0.489	1.019	0.466
5.5	0.0176	35.904	0.454	0.945	0.374

\*Terms used:  $\mu_{\max}$ , Growth rate;  $Y_{p/x}$ , product yield in g ethanol/g biomass;  $Y_{p/s}$ , Product Yield (g ethanol/g substrate);  $Q_p$ , volumetric productivity (g ethanol /L.hour); Specific productivity (g ethanol produced/g biomass/h). The data is presented as mean of 3 replicates.

The results of ethanol fermentation performance of the *S. cerevisiae* SC36 strain revealed that at pH<sub>0</sub> 5.0, the specific growth rate ( $\mu_{\max}$ ), yield coefficients ( $Y_{p/s}$ ,  $Y_{x/s}$ ) and volumetric productivity ( $Q_p$ ) were highest. The biomass production was higher at pH<sub>0</sub> 4.0 than pH<sub>0</sub> 5.0 but on the basis of ethanol yield and the qualitative productivity, pH<sub>0</sub> 5.0 was selected as the optimum value (Table 1). The results of our study are in agreement with the findings by Wong and Sanggari (2014) and Paramanik and Rao (2005) (Paramanik and Rao, 2005; Wong and Sanggari, 2014). The *S. cerevisiae* is acidophilic in nature and grows best at pH<sub>0</sub> range of 4.0 to 5.0 depending upon the strain. Dechant *et al.* (2014) observed that cytosolic pH acts as nutrient sensor and activates signaling pathways for promoting cell growth (Dechant *et al.*, 2014). The glycolytic and alcoholic enzyme of the yeast *S. cerevisiae* are active at neutral pH and a rapid decline in external pH triggers a rise in internal pH of the cell and the failure of which results in decline of fermentative ability of the *S. cerevisiae* (Dombek and Ingram, 1987). Paramanik and Rao reported that *S.*

*cerevisiae* was more active at pH 4.5 in grape juice fermentation (Pramanik and Rao, 2005), while various other authors reported it to be at 5.0 (Magesh et al., 2011; Oghome and Kamalu, 2012) and 3.5 (Ortiz-Muñiz et al., 2010) for *S. cerevisiae*.

#### Thermal treatment for strain improvement

**Direct Thermal Shock:** When the strain was subjected to direct thermal shock at 4°C in the first cycle for 1 hr and 30°C for the rest of cycles, no viable cell count was observed. Sudden exposure to very higher temperature may have reduced the cell's ability to adapt with the stressed environment and may have resulted in protein denaturation, loss of membrane integrity and the cell death.

In the second strategy, progressive acclimatization (35-42°C with 1°C interval) was adopted. The viable strain (isolated after treatment) which was able to grow at 41°C produced very little ethanol yield at the same temperature. This SC36 strain was further optimized for thermal fermentation profile between 30 to 40°C. Highest ethanol production was achieved at 36°C. Therefore, the strain was named as SC36 and will be referred as such hereafter in the document. Above 36°C, both the biomass and the ethanol production potential of the SC36 strain showed a sharp decline. Fermentation efficiency of SC36 decreased due to changes in membrane fluidity with increase in temperature. Any change in the fluidity of membrane is due to change in the fatty acid composition which is necessary to maintain the cell viability (Ohta et al., 1988; Suutari et al., 1990; Van Uden, 1985). It was observed that progressive increase in stress (temperature) could stimulate the modulation of enzymatic activities and other cell proteins to adjust the changing environment, hence progressive acclimatization proved the best methodology for strain development.

**Table 2.** Effect of temperature on kinetics of ethanol production.

Temp. °C	$\mu_{max}$ h <sup>-1</sup>	$Y_{p/x}$ g/g	$Y_{p/s}$ g/g	$Q_p$ g/lh	$q_p$ g/gh
30	0.0268	0.490	1.021	0.155	0.155
32	0.0274	0.492	1.026	0.156	0.156
34	0.0278	0.499	1.039	0.144	0.144
36	0.0262	0.505	0.82	0.437	0.437
38	0.0205	0.287	0.598	0.374	0.374
40	0.00172	0.036	0.074	0.149	0.149

\*Terms used:  $\mu_{max}$ , Growth rate;  $Y_{p/x}$ , product yield in g ethanol/g biomass;  $Y_{p/s}$ , Product Yield (g ethanol/g substrate);  $Q_p$ , volumetric productivity (g ethanol /L.hour); Specific productivity (g ethanol produced/g biomass/h). The data is presented as mean of 3 replicates.

#### Thermal Effect

Activation energy was used to evaluate the effect of temperature on SC36 strain. Arrhenius model is recognized very well for thermodynamic study of bioprocesses. Arrhenius equation describes the temperature dependent growth rate.

$$\mu_{max} = \mu_0 e^{-\frac{E_a}{RT}}$$

The activation energy value was calculated between 30-

40°C temperature ranges at pH 5.0. Value of activation energy determined for this strain of *S. cerevisiae* was 16.48 kcal/mol with  $3.6 \times 10^{12}$  pre-exponential factor.  $E_a$  value of this strain is little higher than *S. cerevisiae* ITV-01 (15.6 kcal/mol) indicating that it is sensitive to temperature but still less sensitive than *Schizosaccharomyces pombe* (26.2 kcal/mol) (Ortiz - Muñiz et al., 2010). This value of activation energy warrants the thermal resistance of the SC36 strain. However, our strain produced more ethanol at 36°C than the *S. cerevisiae* ITV-01 which was best at 30°C. The activation energy ( $E_a$ ) value categorizes the process in biological or diffusional range. Higher activation energy value than 12 kcal/mol mean the process is within the biological range, while below this value determines the process in diffusional range.

#### Osmotolerance Effect

The six different initial substrate concentrations (50-300g/L dextrose) were evaluated for ethanol fermentation efficiency on VHGM medium by SC36 strain at 36°C and pH 5.0. The biomass and product were increased with increase in dextrose concentration from 50 to 300 g/L. A slightly fast substrate consumption was observed at 300g/L substrate in the first 48hr with a gradual sharp decline in the residual substrate. At highest substrate concentration (300g/L), time taken to complete the fermentation was increased with a simultaneous decrease in the specific growth rate ( $\mu_{max}$ ). The  $\mu_{max}$  at 250g/L substrate was higher both above and below this concentration (Table 3). This confirms the validation of the strain SC36 to be osmotolerant strain capable of fermentation in VHGM at 250g/L substrate. The decrease in  $\mu_{max}$  at 300g/L substrate concentration indicates the appearance of substrate saturation and lower water activity caused by VHGM. Lower water activity and increased osmotic pressure can cause plasmolysis of the cell and affects the ethanol yield (Bai et al., 2008; Roukas et al., 1991). It was also observed that increase in initial substrate resulted in increase in fermentation time at the same inoculum concentration as expected. Substrate inhibition by *S. cerevisiae* has been observed even at 150 g/L initial glucose (Ortiz - Muñiz et al., 2010) and at 200g/L glucose; only 32g/L ethanol was produced in the study by Pramanik and Rao (Pramanik and Rao, 2005).

**Table 3.** Kinetics of Initial sugar concentration on specific growth rate, yield and productivity at initial pH 5, temperature 36°C.

Sugar g.L <sup>-1</sup>	$\mu_{max}$ h <sup>-1</sup>	$Y_{p/x}$ g/g	$Y_{p/s}$ g/g	$Q_p$ g/lh	$q_p$ g/gh
50	0.0343	12.250	0.480	0.500	0.170
100	0.0420	14.088	0.496	1.033	0.196
150	0.0366	17.416	0.466	0.970	0.242
200	0.0377	20.392	0.409	1.136	0.283
250	0.0420	27.873	0.443	1.533	0.3871
300	0.0311	27.654	0.443	1.383	0.3841

\*Terms used:  $\mu_{max}$ , Growth rate;  $Y_{p/x}$ , product yield in g ethanol/g biomass;  $Y_{p/s}$ , Product Yield (g ethanol/g substrate);  $Q_p$ , volumetric productivity (g ethanol /L.hour); Specific productivity (g ethanol produced/g biomass/h). The data is presented as mean of 3 replicates.

#### Kinetic Modeling for Process Optimization

### Growth Kinetics

The Monod model explains the relation between specific growth rate of microorganism and the substrate concentration. The values of  $K_s$  and  $\mu_{max}$  were determined using Monod model by plotting  $\mu$  and substrate concentration with a slope of  $\mu_{max}$  and intercept of  $K_s$  and are shown in Table 4.

### Product formation kinetics

The eq. 3 describes the product formation kinetics as described by Leudeking-Piret. The product formation rate is dependent on the instantaneous biomass ( $X$ ) and the growth rate  $dX/dt$  in a linear way.

$$\frac{dP}{dt} = \alpha \cdot \frac{dX}{dt} + \beta X$$

A plot of  $1/X \cdot dP/dt$  versus  $1/X \cdot dX/dt$  was linear plot with a slope of  $\alpha$  and intercept of  $\beta$  which may vary depending on the fermentation conditions. The values of both parameters are given in Table 4.

### Substrate consumption kinetics

The substrate consumption can be described by the eq 4. In which the constants  $\gamma$  and  $\lambda$  are dependent on fermentation. A plot of  $1/X \cdot dX/dt$  versus  $1/X \cdot dS/dt$  gave a straight line in which the slope provides the value of  $\gamma$  and intercept is the value of  $\lambda$ . The values of both constants are given in Table 4.

**Table 4.** Kinetic model parameter values for growth kinetics, product formation and substrate consumption.

Model	Parameters	
Monod	Growth Kinetics	
	$\mu_{max}$	0.4165 h <sup>-1</sup>
	$K_s$	213.81g/L
Leudeking-Piret	Product Formation kinetics	
	A	0.038
	B	11.2
	Substrate consumption kinetics	
	$\Lambda$	1.778
	$\Gamma$	0.174

## 4. Conclusion

*S.cerevisiae* SC36 is an osmotolerant and thermotolerant yeast strain which is able to grow and produce ethanol under highly stressed environment at 250 g/L dextrose concentration and 36°C fermentation temperature at pH 5.0. Kinetic parameters clearly indicate that the SC36 strain when subjected to >150g/L initial substrate concentration, exhibit higher growth rate at 250g/L. The thermal treatment of the strain and the value of activation further demonstrated that the SC36 strain is thermotolerant strain capable to produce ethanol at 36°C. The Monod model for growth kinetics and Leudeking-Piret model for product formation and substrate consumption further highlighted the kinetic characteristics of the strain SC36.

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