OPTIMAL GROWTH OF SACCHAROMYCES CEREVISIAE (PTCC 24860) ON PRETREATED MOLASSES FOR ETHANOL PRODUCTION: APPLICATION OF RESPONSE SURFACE METHODOLOGY

Hoda Shafaghat\textsuperscript{1}, Ghasem D. Najafpour\textsuperscript{1*}, Pouya Sirous Rezaei\textsuperscript{1}, Mazyar Sharifzadeh\textsuperscript{2}

\textsuperscript{1}School of Chemical Engineering, Noushirvani University of Technology, Babol, Iran
\textsuperscript{2}Islamic Azad University, Ayatollah Amoli Branch, Amol, Iran

Received 01.02.2010.
Accepted 08.05.2010.

\textsuperscript{*}Corresponding author email: najafpour@nit.ac.ir, najafpour8@yahoo.com.
Phone/Fax: +98 (111) 3210975
Abstract

Saccharomyces cerevisiae (PTCC 24860) growth on pretreated sugar beet molasses was optimized via statistical approach. In order to liberate all monomeric sugars, pretreated sugar beet molasses with dilute acid was obtained. The influence of process parameters such as sugar concentration, nitrogen source, pH and incubation time on cell growth were investigated by design expert software with application of central composite design (CCD) under response surface methodology (RSM). The optimal culture conditions were pH of 5.3, incubation time of 24 h and medium composition of 35 g reduced sugars, 1.5 g NH₄Cl and 1 g yeast extract per liter of the media. At optimal cell growth conditions and incubation time of 12 h, maximum ethanol production of 14.87 g/L was obtained.

Keywords: Optimization; Ethanol; Pretreated molasses; Saccharomyces cerevisiae; Fermentation; Response surface methodology
Introduction

Microorganisms as biocatalysts are widely used in fermentation processes where product and biomass are obtained from the fermentable sugars [1, 2]. These biologically active organisms have significant contributions in beverage and food industries [3]. Saccharomyces cerevisiae is a budding yeast; known as baker yeast or brewer yeast. This microorganism most often used in fermentation process and obtain energy from various carbon sources. Yeasts are the most common microorganisms for ethanol fermentation. Among the yeast kingdom, S. cerevisiae is one of the well known ethanol producers [4, 5]. Ethanol is an essential chemical which is used as a raw material for a vast range of applications including chemicals, fuel (bioethanol), beverages, pharmaceuticals and cosmetics [2, 6]. The bioconversion of glucose is resulted in ethanol production via Embden-Myerhoof-Paranas pathway [2, 7]. S. cerevisiae has short germination time and easily cultured in large scale processes [8]. The rapidly growing S. cerevisiae ensured ethanol production in batch culture with the limited substrate concentration (50 g/L); ethanol production with high substrate concentrations without substrate inhibition were reported by the immobilized cells [4].

For the economical reason, researchers have paid special attention to various sources of raw material for fermentation industries [8]. Molasses is an agro-industrial waste and a by-product of sugar industry, which has noticeable amount of monomeric and polymeric sugars [9, 10]. In fact, molasses is a dark brown, thick solution which is obtained from the final stages of crystallization. It is widely used in chemical industries to produce baker yeast and ethanol. Upgraded and pretreated molasses are the most economical source of carbohydrate for ethanol fermentation [9]. Molasses contained reduced polymeric sugars that can further be treated to form monomeric fermentable sugar by diluted acid; glucose is the predominant sugar produced from the pretreated sucrose in raw carbon resources [11].

The growth of organism is strongly influenced by medium composition and the ethanol production is cell dependent. Thus optimization of growth media composition and cultural parameters is the main task in a fermentation process [12]. To meet the optimal cell growing demands, it is necessary to improve the performance of the system and thus increase the ethanol yield without increasing the cost of production [13]. Limitations of one at a time parameter
optimization can be eliminated by employing Response surface method (RSM) which is used to explain the combined factors in a fermentation process [14, 15]. Generally, RSM defines the effect of the independent process variables, alone or in combinations and generates a mathematical model that describes the entire process [16, 17]. Also, the RSM summarizes mathematical methods and statistical inference for an approximate functional relationship between response variable and a set of design variables [18]. The most popular RSM is the Central composite design (CCD) which is an efficient and flexible technique that provides sufficient information on the effects of process variables and overall experimental error with a minimum number of experiments [19].

The purpose of present work was to maximize cell growth of *S. cerevisiae* in batch culture using pretreated molasses solution (PMS) as carbon source. Statistical analysis was applied for the optimum cell growth. In RSM, the effects of four independent variables such as PMS and NH$_4$Cl concentration, pH and incubation time were investigated. At optimum growth conditions, experiments were conduct for ethanol production.

### Experimental

**Pretreatment of molasses**

Sugar beet molasses was obtained from Shirvan (Khorasan, Iran) sugar factory. The characteristics of the molasses are summarized in Table 1. The concentrated molasses was diluted with distilled water to adjust the reduced sugar concentration to 100 g/L (220 ml of concentrated molasses diluted with 780 ml of distilled water). The sugar concentration was determined by DNS method [20]. Diluted HCl acid solution was added to molasses in a ratio of 1/400 for the purpose of pretreatment and to increase the reducing sugar content of the molasses solution [5]. After 1 day, the pretreated dilute molasses solution was autoclaved at 121 °C for 15 min and the final measured sugar concentration was 255 g/L. The pH of the pretreated molasses solution (PMS) was at acidic condition (pH = 3.78). The pH of medium was neutralized with 2M sodium hydroxide solution.

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
</table>

**Seed culture preparation**
The microorganism *S. cerevisiae* (PTCC 24860) for fermentation was obtained from Persian Culture Collection, Tehran, Science Organization of Science and Technology. The yeast was grown in a medium consisted of glucose, yeast extract, NH₄Cl, peptone, KH₂PO₄, NaCl, MgCl₂·6H₂O, CaCl₂·2H₂O: 15, 3, 2, 1, 0.1, 0.08, 0.07, and 0.01 g/L, respectively. The initial pH of the medium was adjusted to 5. In addition, the inoculated media was incubated at 30 °C in an incubator-shaker (Stuart, UK) with agitation rate of 190 rpm for duration of 24 h.

**Culture preparation and growth conditions**

The media contained sugar beets molasses as carbohydrates concentration in the range of 15-35 g/L, with increments of 5 g/L and NH₄Cl as nitrogen source in the range of 1-5 g/L with increments of 1 g/L. The initial pH of the medium in the range of 4.4 to 5.6 with increments of 0.3 was studied. The basal medium contained supplementary compounds such as yeast extract, KH₂PO₄, NaCl, MgCl₂·6H₂O, CaCl₂·2H₂O and buffer C₈H₅KO₄: 1, 0.1, 0.08, 0.07, 0.01 and 10.2 g/L, respectively. Potassium hydrogen phthalate (C₈H₅KO₄) was used as a buffer solution to adjust the pH of the media. A 50 mL of the prepared media was transferred to a series of 250 mL Erlenmeyer flasks. The sterilized and inoculated media was incubated and agitated in an incubator-shaker maintained at 190 rpm and 30 °C [4]. Samples were analyzed for cell growth, substrate and product concentrations.

**Growth measurements (Cell optical density)**

In batch fermentation, about 30-35% of carbon source is converted to cell population while ethanol yield is 50% of carbohydrates [21]. The remaining sugar source is used for ATP generation and cell maintenance. Cell optical density was monitored to determine cell growth and samples were drawn in every 2 h time interval. Sample size of 1 ml was drawn from the inoculated medium for analysis of cell growth and substrate utilization. For determination of cell growth or cell optical density (OD), the light absorbance of each sample was measured by spectrophotometer (Unico, USA) at wavelength of 620 nm. Known volume of the samples with defined OD was filtered (0.45 μm, Sartorius, Biotech, Germany) and the cell dry weight (CDW) was measured based on the developed calibration curve. The CDW was proportional to cell optical density. For initial investigation of cell growth and sugar utilization, a 100 ml medium was prepared in a 250 ml Erlenmeyer flask which consisted of: molasses with total sugar; yeast extract, NH₄Cl, peptone, KH₂PO₄, NaCl, MgCl₂·6H₂O, CaCl₂·2H₂O: 30, 3, 2, 1, 0.1, 0.08, 0.07,
0.01 g/L, respectively. The prepared media was autoclaved, and then inoculated with 5% of seed culture. The culture was incubated in an incubator-shaker maintained at 190 rpm and 30 °C.

**Ethanol analysis**

Gas chromatograph (Philips PU4400, UK) equipped with flame ionization detector (FID) and data acquisition system with computer software (Claritylite 4.2, Data Apex, Czech Republic) was used to analyze ethanol concentration. The installed column was PEG 20 M (glass column) 1.5 m and 1/8 mm (Philips, USA). Temperature programming was implemented for the liquid sample analysis. During the analysis, the column temperature was initially maintained at 120 °C, after 2 min, the oven temperature was increased at a rate of 10 °C/min until it reached to 150 °C. The injector and detector temperatures were 150 and 200 °C, respectively. The flow rate for carrier gas (Nitrogen) was set at 30 ml/min. A solution of 2-Methyl-1-Butanol (1%, v/v) was used as an internal standard with concentration of 50 μL/mL of sample. The injection sample volume was 1 μL. In each set of experiments, the data points were repeated in triplicates and the mean value was reported.

**Response surface method (RSM)**

Response surface method (RSM) was used to define the optimum condition for cell growth. The RSM consists of empirical correlations to evaluate the relations between a cluster of controlled experimental factors and measured responses according to one or more selected criteria. Effects of four parameters including sugar from the pretreated molasses, NH₄Cl, pH and incubation time were investigated.

DESIGN EXPERT 7.0 (Stat-Ease, Inc, Minneapolis, MN, USA) software was used for experimental data analysis. A design of 30 experiments was formulated for four factorial (2⁴) and six replicates at the central points. The second-order polynomial model was employed. The value of the dependent response was the mean value of three independent experiments. The optimum values of the selected variables were obtained by analyzing the response surface plots. Each of the parameters was coded at five levels from the lowest, medium low, medium, medium high and highest values: −2, −1, 0, +1 and +2, respectively [22]. In addition, statistical analysis of the model was performed by the analysis of variance (ANOVA).
Results and discussion

Ethanol fermentation is known as growth associated process, in other words, in presence of active cells the rate of ethanol production has reached to maximum value while the cell concentration has reached to stationary phase [2, 21].

Cell growth

The logistic equation for cell growth is described by the following equation [2, 21, 23, 24]:

\[
\frac{dx}{dt} = \mu_m (1 - \frac{x}{x_m})x
\]  

(1)

Where \( \mu_m \) is the maximum specific growth rate (h\(^{-1}\)) and \( x_m \) is the maximum attainable biomass concentration (g/L). The integration of biomass production rate with the use of initial condition \((at \quad t = 0, \quad x = x_0)\) gives a sigmoidal variation of \( x \) as a function of \( t \) which may represent both an exponential and a stationary phase (Eq. (2)).

\[
x = \frac{x_0 e^{\mu_m t}}{1 - (x_0 / x_m)(1 - e^{\mu_m t})}
\]  

(2)

The experimental data was described by logistic model. Matlab 7.1 was used to incorporate experimental data for curve fitting. The maximum specific growth rate, \( \mu_m \) and \( x_m \), maximum biomass concentration were 0.74h\(^{-1}\) and 2.534 g/L, respectively. The coefficient for the fitted dated with Logistic model was \( R^2 = 0.991 \). The cell growth curve and sugar concentration profiles with respect to incubation time are shown in Figure 1. The solid line represented the Logistic model well fitted with the experimental data. The last datum point may be affected by the cell death phase. Four distinct phases were defined on growth curve such as lag (2 hours), exponential (2-14 hours), stationary (14-24 hours) and death phases (> 24 hours). At stationary phase, maximum cell concentration and sugar conversion were 2.83 g/L and 90%, respectively. The death phase was developed after 24 hours of incubation. Since the growth curve is clearly shown that the death phase starts after 24 h of fermentation, then for any sample drawn from batch culture
within 24 h should contain viable cells. Therefore the assumption for the CDW data which represent the viable cells in batch fermentation is valid.

**Figure 1**

**Response surface analysis**

The process variables were investigated in order to achieve a realistic model. It was perfectly understood from experimental data that ethanol production depends on cell growth rate in the fermentation. The effects of sugar concentration, NH₄Cl, pH and incubation time (each variable in five levels) on growth of *S. cerevisiae* were investigated. The concentrations of PMS and NH₄Cl, pH and incubation time were varied according to the experiment strategy (lowest, medium low, medium, medium high and highest values). The values for sugar concentration (15-35g/L), NH₄Cl as nitrogen source (1-5g/L), pH (4.4-5.6) and incubation time (6-30h) were the corresponding five coded levels for each of the independent variables. The values of CDW (Cell Dry Weight) were obtained by conducting 30 experiments and also the data were predicted by RSM.

The F and P-values show the significance of each coefficient. The F-value is the measurement of variation of the data about the mean. The high F-value and very low probability indicate that the model is in good prediction of the experimental results. The model F-value (37.16) and P-value (<0.0001) indicated that the model terms were significant (see Table 2). The accuracy of the fit of the model was checked by the multiple correlation coefficients ($R^2$). In this case, the statistical significance of the method was confirmed by the multiple correlation coefficient of 0.972. Reasonable agreement between the predicted value of multiple correlation coefficient (pred. $R^2 = 0.842$) and the value of the adjusted multiple correlation coefficient (adj. $R^2 = 0.946$) indicated a good consistency between the experimental and predicted values for CDW [13]. A relatively low value of the coefficient of variance (CV = 6.35%) showed a significant precision and reliability of the experiments.

**Table 2**

The three dimensional response surface plots are presented in Figure 2 to illustrate the main and interactive effects of the independent variables on CDW.
Figure 2a shows the effect of sugar concentration in PMS and NH₄Cl on cell growth at pH of 5 and incubation time of 24 h. Both variables had high linear effect on cell growth. According to the obtained data, the quadratic effect of PMS was not high (P = 0.2104), but NH₄Cl had partially significant quadratic influence on CDW (P = 0.0729). The P-value (0.1914) for the interaction of these two variables (PMS and NH₄Cl) was low. The concentration of carbohydrate plays major role; as the carbohydrate concentration was increased, the cell growth rate in the fermentation broth also increased. Since the sugar concentration in the PMS in the range of 15-35 g/L was low; there was no substrate inhibition in the fermentation. With the PMS concentration of 35 g/L maximum cell growth was obtained. In a medium with the stated amount of PMS, optimum NH₄Cl concentration was 2.12 g/L. At defined optimum conditions, the maximum CDW of 3.05 g/L was obtained.

Figure 2b shows the effect of PMS and pH on cell growth in a medium contained fixed amount of nitrogen source (NH₄Cl = 1 g/L) with an incubation time of 24 h. The presented data for analysis of cell growth and ethanol production rates showed some physiological significance on ethanol fermentation. According to the experimental data, the pH range of 4.4-5.6 was selected for the cell growth and ethanol production. The pH had high linear effect on CDW (P = 0.0022), but the quadratic effect of this factor was not significant (P = 0.1505). The low P-value (0.001) showed significant interaction between pH and carbon source, each of these two parameters had the most influence on CDW when the other factor was at the lowest levels. At carbohydrate concentration of 35 g/L and pH of 5, the maximum CDW (2.94 g/L) was predicted by regression analysis.

Effect of incubation time and PMS with fixed concentration of nitrogen source (NH₄Cl = 4 g/L) and pH value of 4.7 has been investigated. As shown in Figure 2c, an increase in incubation time up to an optimum value resulted in increase of CDW, but extended period of incubation might lead to reduction of pH and toxic byproducts which cause cell death. At low incubation time, low substrate concentration was more suitable for high cell growth and increase of incubation time resulted in increase of optimum substrate concentration. When incubation time varied from 6 to 30 h, the optimum PMS concentration shifted from 20 to 35 g/L. The high interaction between these two parameters is also confirmed by P-value (0.0003). The two low P-values of linear and
quadratic terms for incubation time showed high linear and quadratic effects of this parameter on
the response. The maximum CDW (3.08 g/L) was predicted at carbohydrate concentration of 35
g/L at incubation time of 26 h.

Figure 2d depicts variation of pH and nitrogen source for maximum cell growth while the PMS
concentration (15 g/L) and incubation time (24 h) were fixed. The P-value (0.1364) for the
interaction of these variables indicated low interaction. Therefore, pH had insignificant influence
on the optimum NH4Cl concentration. The optimum NH4Cl concentration, pH and maximum
CDW were 2.8 g/L, 5.6 and 2.45 g/L, respectively.

Effect of incubation time and NH4Cl on CDW with fixed PMS concentration (30 g/L) and pH
value of 5 is illustrated in Figure 2e. At optimum conditions (NH4Cl: 2.6 g/L; incubation time:
24 h), the maximum CDW of 2.88 g/L was obtained.

Effect of pH and incubation time on CDW with fixed concentrations of PMS (30 g/L) and NH4Cl
(2 g/L) are shown in Figure 2f. The low P-value (0.0639) shows significant interaction. It has
been reported that, S. cerevisiae is able to produce organic acids [25]. Long period of incubation
may lead to higher concentrations of acids and thus reduces the media pH which has negative
impact on cell growth. Therefore, for the process with long incubation time, it is recommended
to start with a high pH media. As the incubation time was increased from 6 to 30 h, the optimum
pH slightly shifted from 4.4 to 5.6. Maximum cell concentration of 3 g/L was obtained at
optimum conditions: incubation time (30 h) and pH (5.6).

Figure 2

The estimated values of PMS, NH4Cl, pH and incubation time for maximum CDW were 35, 1.5
g/L; 5.3 and 24 h, respectively. At these optimum levels, an experiment was carried out and the
CDW of 2.92 g/L was obtained, which was very close to the predicted value (2.98 g/L) by
central composite design (CCD).

Ethanol production

At optimum growth conditions for S. cerevisiae in PMS, influential process parameters on
ethanol production were investigated. Experiments were carried out to study the effects of
various operation parameters such as: incubation time, concentration of NH₄Cl, pH and sugar concentration on ethanol production. In each set of experiment, one parameter was varied and the rest were kept constant to determine the optimum value of the investigated parameter. The achieved optimum parameter in each step was used in the next experiment. The obtained results are shown in Figure 3.

Figure 3a shows the effect of incubation time on ethanol production and CDW. When the cells are in exponential phase, these cells have maximum activity. Based on cell growth curve, 12 h incubation time showed that the ethanol concentration was progressively increased to maximum value of 14.87 g/L. The other three sets of experiments were conducted with 12 h incubation time. At the beginning of the stationary phase, the highest ethanol concentration was performed, where the cell propagation and death rates were identical. Then the ethanol concentration was gradually decreased. That was due to sugar depletion, ethanol oxidation and organic acid formation for the long incubation time (24 h) [25]. Accumulation of ethanol in fermentation broth caused deactivation of alcohol producing enzymes [26].

Figure 3b depicts the effect of NH₄Cl concentration on CDW and ethanol production. With initial NH₄Cl concentration of 1.5 g/L, maximum CDW of 2.93 g/L was obtained. Also, at initial NH₄Cl concentration of 1.5 g/L, ethanol concentration of 14.1 g/L was achieved. It was reported that molasses may contains considerable amount of minerals and nitrogen sources, additional nitrogen sources may have negative impact on ethanol production [6]. Therefore, excess amount of nitrogen sources associated with ammonium ions may retard the growth rate that might cause an inhibitory effect, which consequently resulted in decreases of CDW and ethanol production.

The effect of media pH variation for ethanol production was also investigated. When ethanol concentration in the fermentation broth has reached to the highest value, then ethanol was utilized and organic acids were formed [25]. The intermediate byproducts in ethanol fermentation pathway (most probably organic acids) may cause pH reduction in the fermentation media [21]. At pH value of 5.6, maximum ethanol production was achieved (Figure 3c).
Figure 3d presents ethanol and CDW production with respect to initial sugar concentration in the media. The sugar concentration was in the range of 35 to 60 g/L. With initial sugar concentration of 55 g/L and incubation time of 12 h, the highest value of ethanol production was 18.3 g/L.

**Figure 3**

**Conclusion**

It was concluded that the central composite design well estimated the optimum conditions for *S. cerevisiae* growth. The optimum values were 35 g/L PMS, 1.5 g/L NH₄Cl, pH 5.3 and incubation time of 24 h for maximum CDW (2.97 g/L). With additional nitrogen source, maximum amount of ammonium chloride (5 g/L), a negative impact on trend of cell growth was observed. It was also concluded that the prolonged incubation time had reduced the pH of media. When the incubation time was prolonged, the concentration of ethanol was depleted. Most probably, *S. cerevisiae* oxidized ethanol in the fermentation broth and also the other end products which caused the media pH shift to slightly acidic condition. Based growth model developed in the fermentation media, the obtained results proved that the ethanol production rate was growth associated. Finally, the design expert leads to 12 h incubation time, maximum ethanol production (14.87 g/L) was obtained.
References


Table 1. The characteristics of molasses obtained from Shirvan Sugar Factory

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Density, g/L</td>
<td>1380</td>
</tr>
<tr>
<td>Ashes, mg ashes/L</td>
<td>0.12</td>
</tr>
<tr>
<td>TSS (Total Suspended Solids), mg TSS/L</td>
<td>0.83</td>
</tr>
<tr>
<td>Initial sugar concentration, g/L</td>
<td>515</td>
</tr>
<tr>
<td>Final pretreated sugar concentration, g/L</td>
<td>1025</td>
</tr>
</tbody>
</table>
### Table 2. Analysis of variance (ANOVA) for the response surface quadratic model

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>F-value</th>
<th>Probability (P) &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>8.34</td>
<td>14</td>
<td>0.60</td>
<td>37.16</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>0.24</td>
<td>15</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure error</td>
<td>0.0059</td>
<td>5</td>
<td>0.00118</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8.58</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adequate precision as signal to noise ratio = 25.766.
List of Figures:

Figure 1. Sugar concentration profile and *Saccharomyces cerevisiae* growth curve: (▲) Cell concentration (■) Sugar concentration.

Figure 2. Response surface plots for the interaction of (a) PMS with NH₄Cl on CDW, (b) PMS with pH on CDW, (c) PMS with incubation time on CDW, (d) NH₄Cl with PH on CDW, (e) NH₄Cl with incubation time on CDW, (f) PH with incubation time on CDW.

Figure 3. Effect of incubation time, NH₄Cl, pH, initial sugar concentration on ethanol production; (▲) CDW, (■) Ethanol production.
<table>
<thead>
<tr>
<th>Incubation Time (h)</th>
<th>Sugar Concentration (g/L)</th>
<th>Cell Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*Figure 1*
Figure 2
Figure 3
### Coded levels of independent variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coded symbol</th>
<th>Actual values (g/l) of coded levels</th>
<th>-2</th>
<th>-1</th>
<th>0</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HM</td>
<td>$x_1$</td>
<td></td>
<td>15</td>
<td>20</td>
<td>25</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>$x_2$</td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>pH</td>
<td>$x_3$</td>
<td></td>
<td>4.4</td>
<td>4.7</td>
<td>5</td>
<td>5.3</td>
<td>5.6</td>
</tr>
<tr>
<td>Fermentation time</td>
<td>$x_4$</td>
<td></td>
<td>6</td>
<td>12</td>
<td>18</td>
<td>24</td>
<td>30</td>
</tr>
</tbody>
</table>