Optimization of saccharification conditions and sugar production by endoglucanase from Rhizopus oryzae PR7 MTCC 9642 using response surface methodology

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Abstract

The effects of saccharification time, Mn$^{2+}$ concentration and enzyme concentration on saccharification of cellulose by endoglucanase from Rhizopus oryzae were optimized by statistical analysis using response surface methodology. The response surface methodology (RSM) was used to optimize sugar production by implementing the Central Composite design. The optimal conditions for higher production of sugar from cellulose were: time 12min 30secs, Mn$^{2+}$ concentration 0.37% (w/v) and enzyme concentration 0.82% (v/v). Under these conditions, the model predicted a sugar production of 3.55mg/ml. Verification of the optimization showed that sugar production of 3.2 mg/ml was observed under the optimal experimental conditions. Value of correlation coefficient ($R^2$ 0.9973) and significant value for model $p < 0.0001$ indicated validity of model fitness and adequate for optimization.

[Key words: Endoglucanase, Response Surface Methodology, Rhizopus oryzae, Saccharification.]

Introduction

Saccharification is a process of bioconversion of polysaccharide into component sugars. Bioconversion of cellulose into glucose has attracted the attention of the investigators, as this is the first step of bioconversion of cellulose material into valuable products such as sugar, fine chemicals and biofuels [1]. Saccharification of cellulose could be accomplished by the cellulase enzymes, of which endoglucanase (EC 3.2.1.4; 1, 4-ß-D-glucan glucanohydrolase) acts on carboxy methyl cellulose (CMC), causing random endo-cleavage of the $\beta$-1, 4-
glycosidic bonds [2] of cellulose chains yielding glucose and cello-oligosaccharides. The saccharification of
cellulosic substrates were reported by many workers [3, 4, 5]

Although, bioconversion of cellulose could be accomplished by acid hydrolysis or enzyme treatments, the later
is an attractive option due to the higher yield of sugar, convenience in reaction management and lesser
harshness. The bioconversion of cellulose can be affected by the different parameters and hence optimization of
the parameters is the most important and effective aspect of the enzymatic saccharification process.

Although the stimulatory effect of manganese ions on the cellulase activity appears to be quite phenomenal, the
metal ion exhibited over 200% stimulation of the activity of cellulase enzymes of other fungal strains [6] and a
significant increase in the yield of hydrolytic products may be expected after Mn2+ treatment. Similarly,
incubation time and enzyme concentration played a pivotal role in saccharification [7,8] and these parameters
are to be optimized.

The conventional technique for the optimization is to deal with one-factor at a time. However, this type of
method is time-consuming and also does not reveal the effects between variables [9] Response surface
methodology is a powerful tool for not only optimization of the process control improvements in specific
enzyme activities but also for the additional knowledge supplied about the optimization processes in any part of
experimental domain [10,11,12]. In the present study, an attempt was made to employ response surface
methodology (RSM) to optimize the conditions (time, Mn2+ concentration, enzyme concentration) for sugar
production from carboxymethyl cellulose.

Materials and methods

Micro organism

*Rhizopus oryzae* PR7 MTCC 9642 [13], was isolated from the decaying vegetation enriched soil of India. The
strain was identified by and deposited to Microbial Type Culture Collection, India.

Chemicals

All chemicals used were of analytical grade.

Cultivation of the strain

The strain was cultivated in 100 ml Erlenmeyer flasks each containing 20 ml Basal Medium (BM) composed
of (g l-1): peptone 0.9; (NH4)2HPO4 0.4; KCl 0.1; MgSO4·H2O 0.1 and carboxy methyl cellulose 0.5 at pH 6 and
temperature 37°C for 48 hrs in static condition.

Enzyme extraction and assay

The grown culture was filtered through filter paper (Whatman No1) and filtrate was used centrifuged at 10,000
rpm for 5 min at 4°C and the supernatant was used as the enzyme. The enzyme was partially purified by
ammonium sulfate precipitation (70% w/v) followed by dialysis against 0.1(M) phosphate buffer [14] To
measure the activity of endoglucanase tubes containing the assay mixture (1ml) each containing various amount
of enzyme diluted with 0.1(M) phosphate buffer (pH-6) was incubated at 33°C with 1%(w/v) CM-cellulose for 10 minutes and the endoglucanase activity was measured [15].

Saccharification

Carboxymethyl cellulose powder (10mg) was incubated with varying concentration of enzymes (0.1 ml to 1.0ml) for various incubation time (5-15min) with different concentration of Mn²⁺ at 33°C. Blanks were prepared with inactivated enzymes. The reducing sugar released was measured by the dinitrosalicylic acid method [16] taking glucose as standard.

Central composite design

Response surface methodology (RSM) was used to optimize the fermentation parameters for enhancing saccharification and sugar production by endoglucanase that involved four steps: procedures to move into the optimum region, behavior of the response in the optimum region, estimation of the optimal condition and verification [17]. In the present study, a central composite design [18] is employed where the total number of experimental combinations is \(2^k+2k+n_0\), where \(k\) is the number of independent variables and \(n_0\) is the number of repetitions of the experiments at the centre point. For statistical calculation, the experimental variables \(X_i\) have been coded as \(x_i\) according to the following transformation equation:

\[
x_i = \frac{X_i - X_0}{\delta X}
\] (Eqn 1a)

where \(x_i\) is the dimensionless coded value of the variable \(X_i\), \(X_0\) is the value of \(X_i\) at the center point, and \(\delta X\) is the step change.

In this study, the central composite design with three factors and five levels, including six replicates at the center point, was used for fitting a second order response surface. Table 1 and Table 2 give the factors, their actual and coded values, and the experimental design, respectively. This methodology allows the modeling of a second order polynomial equation that describes the process. Sugar production was analysed by the following equation:

\[
Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{12}AB + \beta_{23}BC + \beta_{13}AC 
\] (Eqn 1b)

Where \(Y\), predicted response; \(\beta_0\), intercept; \(\beta_1, \beta_2, \beta_3\), linear coefficients; \(\beta_{11}, \beta_{22}, \beta_{33}\), squared coefficients; \(\beta_{12}, \beta_{23}, \beta_{13}\), interaction coefficients and \(A\) was time (min), \(B\) was the Mn²⁺ concentration (w/v) % and \(C\) denoted the enzyme concentration (v/v) %. The responses of the CCD design were fitted with a second-order polynomial equation.

The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination \(R^2\). Each experimental design was carried out in triplicate, and the mean values were given.

Statistical analysis

Data from the central composite design shown in Table 2 were used for determining the regression coefficients of the second-order polynomial regression models. An evaluation copy of the statistical software, Design-Expert version 7.1.5, from Stat-Ease, Inc., Minneapolis, USA was employed for regression analysis of the data and for
estimation of the coefficients of the regression equation. The statistical significance of the model was determined by the application of Fisher’s F-test.

The two-dimensional graphical representation of the system was used to describe the individual and the cumulative effects of the variables as well as the mutual interactions between the independent variables and the dependent variables.

**Result and Discussion**

Effect of variables on sugar production

On the basis of quadratic polynomial equation of response surface model (Eqn.1b), the present model and data analysis allowed not only to define optimum conditions for increased sugar production by endoglucanase but also showed combined effect of independent variables such as saccharification time, Mn\(^{2+}\) concentration and enzyme concentration in terms of coded factors in (Eqn. 2).

\[
\text{sugar produced} = +2.80 + 0.15 * A + 0.51 * B + 0.60 * C - 0.090 * A * B - 0.030 * A * C - 0.27 * B * C - 0.071 * A^2 - 0.11 * B^2 - 0.013 * C^2 \quad \text{Eqn 2}
\]

The positive value for linear coefficients (Eqn. 2) illustrated the significant, positive effect of variables on the sugar production. Linear coefficient of 0.60 indicated that sugar production was positively correlated with enzyme concentration but up to a certain extent with Mn\(^{2+}\) concentration; as after threshold concentration Mn\(^{2+}\) precipitated.

The negative quadratic coefficient for all the variable indicated the existence of maximum activity to a point; beyond that the entire variable had an inhibitory effect on saccharifying activity of endoglucanase. The response surface was selected based on the interactive effect of two independent variables with another variable being at fixed level.

Statistical testing of the model was performed with the Fisher’s statistical test for analysis of variance, ANOVA. The quadratic regression showed that the model was significant because the value of F-test was less than 0.05 and model F value 418.57 which indicated the significance of the model terms (Table 3) depicting the quadratic model was valid for the present study [19]. The value of correlation coefficient was 0.9973 (closer the R\(^2\) value to 1.0, the better is model fitness to the experimental data) which indicated that model could explain 99.73% of variability and unable to explain only 0.27% of the total variation. This indicated that the data was less variable, more uniform and homogenous. Small value of coefficient of variation 1.89 clearly indicated a very high degree of precision and a good deal of reliability of the experimental values. Values of “Prob > F” less than 0.05 for A, B, C, AB, BC, A \(^2\) and B \(^2\) indicated that model terms were significant [20]. The natural logarithm (ln) of the residual SS (sum of square) against lambda was one, dip suddenly with a minimum in the region of the best optimum value 1 (Figure.1). The data did not require a transformation, as current value of confidence interval (lambda) was to the optimum value [21]. The model showed the minimum and maximum confidence interval value of 0.58 and 1.52 respectively (Figure.1). A high correlation (R\(^2\)=0.9973) between the predicted and experimental values was found to exist (Figure 2). The clustering of the points around the diagonal line indicated a satisfactory correlation between the experimental and predicted values, thereby confirming the soundness of the model. Three dimensional response surface plots graphically represented regression equations that were generally used to demonstrate relationships between the response and experimental levels of each variable. These surface plots, therefore, allowed for visualization of the optimum levels of each variable for the
maximum production of reducing sugar due to saccharification [22]. Figure 3-5 showed the response surface plots for the present study and illustrated the interaction of two variables keeping the other variable constant at the middle value. The interactions between time (A), Mn²⁺ concentration (B) where P is 0.0005, Enzyme concentration (C) where P value is <0.0001 were significant. Surprisingly, the interaction between time (A) and enzyme concentration (C) was not significant (P = 0.1241). According to the canonical analysis, the optimal concentrations were of time 12 min 30 sec, Mn²⁺ concentration 0.37% and enzyme concentration 0.82%.

Validation

The confirmatory experiments were conducted with the parameters as suggested by the numerical modeling (suggested solutions) and keeping the time, Mn²⁺ concentration and enzyme concentration at 12 min 30 secs and 0.37% and 0.82% respectively to support the optimized data as given under optimized conditions. The data showing the effect of time, Mn²⁺ and enzyme concentration were in accordance to the results of RSM study. There were very small differences in the predicted response (3.55 mg/ml) and the observed value (3.2 mg/ml) during the experiments when carried out at above mentioned conditions. In unoptimized condition the production of reducing sugar was shown in serial number 1 of Table 2 which is 1.04 mg/ml. After optimization the maximum production of reducing sugar was 3.2 mg/ml which is around 3 fold increase in the sugar production.

Conclusion

The present study demonstrated that the saccharifying activity was affected by different length of incubation time, Mn²⁺ and enzyme concentration. The optimum response (sugar produced) 3.2 mg/ml was observed after saccharification for 12 min 30 secs at Mn²⁺ concentration of 0.37% and enzyme concentration 0.82% against the predicted value of 3.55 mg/ml indicated model accuracy. Although attempts were made to enhance cellulose saccharification by cellulase by substrate pre treatment [23], in the present study, glucose yield was maximized by optimizing few parameters only. The good saccharifying activity could make the strain an attractive source for sugar production in juice and for food industries and also as a prerequisite for bio fuel production.

Acknowledgement

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References

Table 1 Process variables and their levels.

<table>
<thead>
<tr>
<th>Variable</th>
<th>unit</th>
<th>-α</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
<th>+α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>min</td>
<td>5</td>
<td>7</td>
<td>10</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Mn$^{2+}$ conc.</td>
<td>% (w/v)</td>
<td>0.06</td>
<td>0.15</td>
<td>0.28</td>
<td>0.41</td>
<td>0.5</td>
</tr>
<tr>
<td>Enzyme conc.</td>
<td>% (v/v)</td>
<td>0.1</td>
<td>0.28</td>
<td>0.55</td>
<td>0.82</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 2 Experimental design and results of the Central Composite model.

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Time</th>
<th>Mn$^{2+}$ conc.</th>
<th>Enzyme conc.</th>
<th>Actual Value</th>
<th>Predicted Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>0.15</td>
<td>0.28</td>
<td>1.04±0.03</td>
<td>1.02</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>0.15</td>
<td>0.28</td>
<td>1.44±0.06</td>
<td>1.44</td>
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<tr>
<td>3</td>
<td>7</td>
<td>0.41</td>
<td>0.28</td>
<td>2.72±0.07</td>
<td>2.75</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>0.41</td>
<td>0.28</td>
<td>2.77±0.15</td>
<td>2.82</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>0.15</td>
<td>0.82</td>
<td>2.75±0.16</td>
<td>2.7</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>0.15</td>
<td>0.82</td>
<td>3.28±0.12</td>
<td>3.24</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>0.41</td>
<td>0.82</td>
<td>3.36±0.15</td>
<td>3.35</td>
</tr>
<tr>
<td>8</td>
<td>13</td>
<td>0.41</td>
<td>0.82</td>
<td>3.52±0.14</td>
<td>3.54</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>0.28</td>
<td>0.55</td>
<td>2.32±0.05</td>
<td>2.35</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>0.28</td>
<td>0.55</td>
<td>2.88±0.09</td>
<td>2.86</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>0.06</td>
<td>0.55</td>
<td>1.57±0.06</td>
<td>1.63</td>
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<tr>
<td>12</td>
<td>10</td>
<td>0.50</td>
<td>0.55</td>
<td>3.39±0.14</td>
<td>3.34</td>
</tr>
<tr>
<td>13</td>
<td>10</td>
<td>0.28</td>
<td>0.1</td>
<td>1.80±0.06</td>
<td>1.76</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>0.28</td>
<td>1.0</td>
<td>3.73±0.12</td>
<td>3.78</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>0.28</td>
<td>0.55</td>
<td>2.81±0.11</td>
<td>2.80</td>
</tr>
<tr>
<td>16</td>
<td>10</td>
<td>0.28</td>
<td>0.55</td>
<td>2.82±0.11</td>
<td>2.80</td>
</tr>
<tr>
<td>17</td>
<td>10</td>
<td>0.28</td>
<td>0.55</td>
<td>2.78±0.10</td>
<td>2.80</td>
</tr>
<tr>
<td>18</td>
<td>10</td>
<td>0.28</td>
<td>0.55</td>
<td>2.75±0.09</td>
<td>2.80</td>
</tr>
<tr>
<td>19</td>
<td>10</td>
<td>0.28</td>
<td>0.55</td>
<td>2.84±0.10</td>
<td>2.80</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>0.28</td>
<td>0.55</td>
<td>2.83±0.11</td>
<td>2.80</td>
</tr>
</tbody>
</table>
Table 3 Analysis of variance (ANOVA) for quadratic model for cellulase activity.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F Value</th>
<th>p-value , Prob &gt; F</th>
<th>Coefficient Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>9.624452</td>
<td>9</td>
<td>1.069384</td>
<td>418.5741</td>
<td>&lt; 0.0001 significant</td>
<td>2.804789</td>
</tr>
<tr>
<td>A-Time</td>
<td>0.317343</td>
<td>1</td>
<td>0.317343</td>
<td>124.2132</td>
<td>&lt; 0.0001</td>
<td>0.152437</td>
</tr>
<tr>
<td>B-Mn2+ conc.</td>
<td>3.507275</td>
<td>1</td>
<td>3.507275</td>
<td>1372.804</td>
<td>&lt; 0.0001</td>
<td>0.506768</td>
</tr>
<tr>
<td>C-Enz conc.</td>
<td>4.90657</td>
<td>1</td>
<td>4.90657</td>
<td>1920.511</td>
<td>&lt; 0.0001</td>
<td>0.599396</td>
</tr>
<tr>
<td>AB</td>
<td>0.0648</td>
<td>1</td>
<td>0.0648</td>
<td>25.36377</td>
<td>0.0005</td>
<td>-0.09</td>
</tr>
<tr>
<td>AC</td>
<td>0.0072</td>
<td>1</td>
<td>0.0072</td>
<td>2.818197</td>
<td>0.1241</td>
<td>0.03</td>
</tr>
<tr>
<td>BC</td>
<td>0.5832</td>
<td>1</td>
<td>0.5832</td>
<td>228.274</td>
<td>&lt; 0.0001</td>
<td>-0.27</td>
</tr>
<tr>
<td>A^2</td>
<td>0.072855</td>
<td>1</td>
<td>0.072855</td>
<td>28.51664</td>
<td>0.0003</td>
<td>-0.0711</td>
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<tr>
<td>B^2</td>
<td>0.185741</td>
<td>1</td>
<td>0.185741</td>
<td>72.702</td>
<td>&lt; 0.0001</td>
<td>-0.11353</td>
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<tr>
<td>C^2</td>
<td>0.002348</td>
<td>1</td>
<td>0.002348</td>
<td>0.91916</td>
<td>0.3603</td>
<td>-0.01277</td>
</tr>
</tbody>
</table>

R^2=0.9973, Adj R^2=0.9949, Pred R^2=0.9835, C.V. %=-1.89.

Fig. 1: Box–Cox plot for power transforms

X: Lambda
Y: Ln(ResidualSS)
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Fig. 2: Predicted Vs actual values of sugar produced

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sugar produced

- Design points above predicted value
- Design points below predicted value

X1 = A: Time
X2 = B: Mn2+ conc.

Actual Factor
C: Enz conc. = 0.55

Fig. 3: Response surface plot showing the effect of Mn²⁺ concentration and Time on sugar production with other variable constant at middle level.
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sugar produced
- Design points above predicted value
- Design points below predicted value

X1 = A: Time
X2 = C: Enz conc.

Actual Factor
B: Mn2+ conc. = 0.28

Fig. 4: Response surface plot showing the effect of enzyme concentration and Time on sugar production with other variable constant at middle level.

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sugar produced
- Design points above predicted value
- Design points below predicted value

X1 = B: Mn2+ conc.
X2 = C: Enz conc.

Actual Factor
A: Time = 10.00

Fig. 5: Response Surface plot showing the effect of enzyme concentration and Mn2+ concentration on sugar production with other variable constant at middle level.