Co-optimization of xyloglucanase and β-glucosidase using response surface methodology

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Xyloglucanase and β-glucosidase are important accessory enzymes required for hydrolysis of lignocellulosic biomass. In an effort to reduce the cost of production, tamarind kernel polysaccharide was used as the substrate for production of both enzymes. Further, enhancement of enzymes’ production was carried out using Plackett-Burman design and a relatively new methodology, viz. ridge analysis. The results indicate that the tamarind kernel polysaccharide supported production of both enzymes significantly. By statistical analysis, significant media components were identified and optimized. As a result of this study, there was 2.1 and 9.6 fold increase in production of xyloglucanase and β-glucosidase respectively. This study is the first report on the optimization of xyloglucanase production.

Keywords: Xyloglucanase, β-glucosidase, surface methodology

Introduction

Xyloglucanases (XG) are enzymes that break down the xyloglucan which cross links cellulose microfibrils together in lignocellulose. They are hemicellulases which are used as accessory enzymes in lignocellulose degradation. Xyloglucan is found in close association with cellulose and it has been shown that treatment with XG. Another accessory enzyme in biomass degradation is β-glucosidase which catalyses the hydrolysis of terminal non-reducing residues in beta-D-glucosides with release of glucose. Because they act on the oligosaccharides produced by the action of other cellulolytic enzymes, it plays an important role in complete saccharification of natural lignocellulosic materials. Together, these two enzymes can significantly influence degradation of lignocellulosic biomass. Interest in these enzymes is also because of their potential biotechnological applications including biofuel production, fruit juice clarification, textile processing, detergent formulations, food processes, in paper and pulp industry as also cellulose surface modification, plant growth modulation, natural polymer modifications, synthesis of biocomposites and enzyme kinetic studies²-⁵. Economization of enzyme production is the most significant interest of any industrial bio-process. Various approaches that are widely employed include use of cheaper substrates, co- cultures, improving strains of producers by mutagenesis and cloning, downstream processing etc. Enhancement of enzyme production can also be achieved by optimization of fermentation conditions using statistical design of experiments. This approach is fast growing as the preferred method for optimizing industrially useful enzymes. In this study, we attempt two strategies namely use of cheap and easily available substrate, viz. tamarind kernel polysaccharide (TKP) and statistical optimization of the production medium. Tamarind kernel polysaccharide, prepared from tamarind seed powder, is the major industrial by-product of the tamarind crop. It is abundantly produced (approx. 20,000 tons per annum) in Central and South India⁶. The major component of TKP is xyloglucan. Chemically, it is a galactoxyloglucan with glucose, galactose and xylose present in a ratio of 2.8:1:2.25⁶. It is cheap (approx. Rs. 50/kg) and easily available in local markets, making it an ideal choice of substrate for economical production of xyloglucanase. Statistical optimization, in contrast to one-factor-at-a-time approach, utilizes multifactor experiments, is less time consuming, requires fewer experimental runs and is capable of detecting the true optimum of factor(s)⁷. The biggest advantage of the

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statistical optimization method is that it helps to study multi-factor interactions which are not possible with the one-factor-at-a-time method. The most widely used statistical designs are Plackett-Burman (P-B) design and fractional factorial design. Advantages of these designs include simplicity and simultaneous assessment of a large number of factors on the relative efficiency of the production process. P-B design is one such design that can be used to screen the important variables that affect production. Here “n” variables can be screened in just “n+1” number of experiments. The factors having positive influence on the production can be subjected to further analysis for identifying their optimum concentration. Ridge analysis or ridge regression is one such analysis which is generally used to investigate second-order mixture surfaces involving many factors. It enables understanding of the typical complex interplay between the input variables and facilitates exploration of a response surface when there is a linear restriction on the factors involved. It is ideal for the present study where the factors identified by a linear model like P-B are to be extrapolated to obtain a surface response. Ridge analysis is discussed infrequently in the literature and rarely in context of enzyme optimization experiments. Use of ridge analysis for enzyme optimization has been reported by Chang et al. for lipase and Chaari et al. for lichenase. The objective of the present study was statistical optimization of medium components for production of XG and β-glucosidase using TKP as the substrate, by a locally isolated strain of Aspergillus terreus.

Materials and Methods

Organism
Aspergillus terreus NFCCI 1840, isolated from local environmental samples, was chosen for this study.

Fermentation
Czapek-Dox broth (pH 4.5±0.2), the basal medium for fermentation, was amended to contain TKP (10.0g/L) as the sole source of carbon. TKP was prepared as described by Rao and Srivastava. Media preparation and submerged fermentation was carried out as described earlier. Aliquots of the fermentation broth were drawn at intervals of 24 hrs., centrifuged at 4000 rpm for 15 mins. and the supernatant was used for all enzyme assays.

Enzyme Assays
XG and β-glucosidase activities were assayed in the culture filtrate (CF) using tamarind xyloglucan and p-nitrophenyl-β-D-glucoside as substrates, respectively. XG activity was assayed by quantifying the reducing sugars liberated from 1% (w/v) tamarind xyloglucan (Megazyme, Ireland) using DNS method as per the standard protocol described by the Commission on Biotechnology, IUPAC while β-glucosidase assay was carried out by the method of Kirchner et al. using the aliquots of CF drawn at intervals of 24 hrs. for a period of 8 days. One unit of enzyme activity was expressed as micromoles of the respective product liberated/ml of CF/minute under assay conditions.

Statistical Design of Experiments
Optimization of medium was carried out in two stages. In the first stage, 11 culture variables (in a 12–experiment run) were screened using P-B design to identify the parameters that significantly influenced enzyme production. In the second stage, the levels of positively influencing variables were optimized using ridge analysis. All experiments were carried out in triplicates.

Plackett-Burman Design to Screen for Factors Affecting Enzyme Production
P-B design was employed to determine the effect of individual variables or factors affecting enzyme production by A. terreus under submerged fermentation. A total of 11 factors including carbon sources, nitrogen sources, inducers and one physical parameter, pH, were screened. Each variable was investigated at high (+1) and low (-1) level, which represents two different parameter values. The factors are represented as F1-F11 and their (+) and (-) levels are listed in Table 1, in parenthesis, as g/L (except F2). Fermentation experiments were carried out by addition of the medium components to the basal medium, at the respective level, according to the design matrix. From the experimental trials (Run No. 1-12) CF was obtained after centrifugation and used for enzyme assays. Activity expressed as IU/ml was recorded as response value. The factorial model was fitted for the main effects using SAS 9.0 software, USA. The effect of
individual factors on production of each enzyme was calculated by the following equation:

$$Y = \beta_0 + \sum \beta_i x_i \quad (i = 1, 2, 3, \ldots, k) \quad \ldots (1)$$

where \( Y \) is the predicted response (activity in IU/ml), \( \beta_0, \beta_i \) are constant coefficients and \( x_i \) is the coded independent variables’ estimate. Regression analysis was performed to test the significance. The most significant parameters affecting the production of enzymes was identified separately. Optimum fermentation time was identified by paired student t-test.

**Ridge Analysis**

Ridge analysis was carried out to identify the optimum levels of parameters that were found to significantly increase production of the enzymes. The behavior of the system was explained by a second order polynomial equation given as

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i^2 + \sum \beta_{ij} x_i x_j \quad \ldots (2)$$

where \( X \) is the coded factors, \( Y \) is the predicted response, \( \beta_0 \) the intercept term, \( \beta_i \) the linear effect, \( \beta_{ij} \) the squared effect and \( \beta_{ij} \) the interaction effect. SAS package, version 9.0, was used for analysis of the experimental data obtained. F-test was employed to evaluate the significance of the quadratic polynomial. The determination coefficient of correlation \((R^2)\) was calculated to evaluate the performance of the regression equation. Optimal concentrations of the critical medium components were obtained by ridge analysis and also by analyzing the contour and response surface plots.

**Table 1—Plackett-Burman design matrix for screening medium components**

<table>
<thead>
<tr>
<th>Run No.</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
<th>F8</th>
<th>F9</th>
<th>F10</th>
<th>F11</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(+)</td>
<td>25.0</td>
<td>(+) 8.5</td>
<td>(+) 2.0</td>
<td>(+)</td>
<td>5.0</td>
<td>(+)</td>
<td>10.0</td>
<td>(+)</td>
<td>1.0</td>
<td>(+) 6.0</td>
</tr>
<tr>
<td>2</td>
<td>(+)</td>
<td>25.0</td>
<td>(-) 5.0</td>
<td>(+) 5.0</td>
<td>(+)</td>
<td>5.0</td>
<td>(+)</td>
<td>10.0</td>
<td>(-)</td>
<td>1.0</td>
<td>(+) 1.0</td>
</tr>
<tr>
<td>3</td>
<td>(-)</td>
<td>5.0</td>
<td>(+) 8.5</td>
<td>(+) 5.0</td>
<td>(+)</td>
<td>5.0</td>
<td>(-)</td>
<td>2.0</td>
<td>(-)</td>
<td>1.0</td>
<td>(+) 5.0</td>
</tr>
<tr>
<td>4</td>
<td>(+)</td>
<td>25.0</td>
<td>(+) 8.5</td>
<td>(+) 5.0</td>
<td>(-)</td>
<td>1.0</td>
<td>(-)</td>
<td>2.0</td>
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</tr>
<tr>
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<td>(+) 8.5</td>
<td>(-) 2.0</td>
<td>(-)</td>
<td>1.0</td>
<td>(+)</td>
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</tr>
<tr>
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<td>(-) 2.0</td>
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<td>(+)</td>
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<td>(+) 5.0</td>
</tr>
<tr>
<td>7</td>
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<td>(+) 5.0</td>
<td>(-) 2.0</td>
<td>(+)</td>
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<td>(-)</td>
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<td>10.0</td>
<td>(+) 5.0</td>
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<tr>
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<td>(+) 5.0</td>
<td>(+) 5.0</td>
<td>(-)</td>
<td>1.0</td>
<td>(+)</td>
<td>10.0</td>
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<td>1.0</td>
<td>(+) 5.0</td>
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<tr>
<td>9</td>
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<td>(+) 8.5</td>
<td>(+) 2.0</td>
<td>(+)</td>
<td>5.0</td>
<td>(+)</td>
<td>10.0</td>
<td>(-)</td>
<td>1.0</td>
<td>(+) 5.0</td>
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<tr>
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<td>25.0</td>
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<td>(+) 5.0</td>
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<tr>
<td>11</td>
<td>(-)</td>
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<td>(+) 8.5</td>
<td>(+) 5.0</td>
<td>(-)</td>
<td>1.0</td>
<td>(+)</td>
<td>10.0</td>
<td>(+)</td>
<td>1.0</td>
<td>(+) 5.0</td>
</tr>
<tr>
<td>12</td>
<td>(-)</td>
<td>5.0</td>
<td>(+) 5.0</td>
<td>(-) 2.0</td>
<td>(+)</td>
<td>1.0</td>
<td>(-)</td>
<td>2.0</td>
<td>(+)</td>
<td>1.0</td>
<td>(+) 1.0</td>
</tr>
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</table>

**Results**

**Optimization of Fermentation Time**

Means of enzyme activity from the 12 runs, on different days, were compared by paired student t-test and found to be statistically significant \((P<0.001)\) for both enzymes. Maximum XG activity \((0.33\pm 0.02\text{ IU/ml})\) was achieved on day 1 and \(\beta\)-glucosidase activity \((1.12 \pm 0.39\text{ IU/ml})\) on day 8. The activity of XG decreased with increasing number of days of fermentation. Therefore, for all further analyses, the results of the optimum day (day 1 for XG and day 8 for \(\beta\)-glucosidase) were used.

**Plackett-Burman Design to Screen for Parameters Affecting Enzyme Production**

The main effects of the eleven parameters on production of XG and \(\beta\)-glucosidase are represented in Fig. 1. Statistical analysis indicated that P-B design was significant \((P<0.001)\) and showed that TKP supported the production of both enzymes. pH had highly significant effects. For XG production, the effect of pH was positive, meaning that higher pH was favourable for enhancing XG production. On the contrary, pH was highly unfavourable for \(\beta\)-glucosidase production. The parameters with statistically significant effects were identified using regression analysis. The regression coefficients for the linear regression model are presented in Table 2.

**Regression Analysis for XG Production**

For XG, pH (F2), maltose (F8) and cellobiose (F11) had confidence level higher than 99% \((P<0.001)\) and hence were chosen. XG production increased at higher levels of pH (F2), maltose (F8) and cellobiose (F11). A linear regression equation was obtained from the regression results of P-B experiment:
\[ Y = 0.280 + 0.028F_1 + 0.318F_2 - 0.008F_3 - 0.165F_4 - 0.041F_5 - 0.011F_6 + 0.004F_7 + 0.082F_8 - 0.025F_9 - 0.121F_{10} + 0.041F_{11} \quad \cdots (3) \]

The determination coefficient \( (R^2) \) was 0.9812 and the Adj. \( R^2 \) value of 0.9727. This indicates a good agreement between the experimental and predicted values\(^{18}\). The total model was highly significant \((P<0.0001)\).

**Regression Analysis for β-glucosidase Production**

For β-glucosidase (Table 2), all parameters except sodium nitrate \((F_3)\) and ammonium sulphate \((F_4)\) were highly significant \((P<0.001)\). The regression equation obtained from P-B experiment is given below.

\[ Y = 1.210 + 0.092F_1 - 0.734F_2 - 0.02F_3 + 0.002F_4 + 0.18F_5 - 0.16F_6 + 0.34F_7 + 0.077F_8 - 0.088F_9 + 0.091F_{10} + 0.024F_{11} \quad \cdots (4) \]

Four factors with higher positive coefficients, lactose \((F_7)\), yeast extract \((F_5)\), TKP \((F_1)\) and potassium dihydrogen phosphate \((F_{10})\), were chosen. The determination coefficient \( (R^2) \) was 0.991 and the Adj. \( R^2 \) value of 0.986. The experimental and predicted goodness of fit obtained clearly indicated that the total model was highly significant \((P<0.001)\).

The above results indicate that P-B design was a suitable tool to screen medium components for maximizing XG as well as β-glucosidase production by *Aspergillus terreus* NFCCI 1840. Ridge analysis was carried out using the chosen parameters for each enzyme.

**Ridge Analysis**

Ridge regression analysis or ridge analysis was employed to optimize the levels of the chosen parameters for the individual enzymes. In the present study, ridge analysis was carried out between the coded radii of 0.0 to 1.0. The objective was to find the settings of the chosen parameters that can maximize the yield. For XG, ridge regression analysis

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Table 2—Regression results of P-B experiment

<table>
<thead>
<tr>
<th>Factors</th>
<th>Parameter co-efficients</th>
<th>Significance</th>
<th>Parameter co-efficients</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSP</td>
<td>0.028</td>
<td>0.017*</td>
<td>0.092</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>pH</td>
<td>0.318</td>
<td>&lt;0.001**</td>
<td>-0.734</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>-0.008</td>
<td>0.459</td>
<td>-0.020</td>
<td>0.255</td>
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<tr>
<td>NH₄(SO₄)₂</td>
<td>-0.165</td>
<td>&lt;0.001**</td>
<td>0.002</td>
<td>0.898</td>
</tr>
<tr>
<td>Yeast Ext.</td>
<td>-0.041</td>
<td>0.001**</td>
<td>0.180</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Urea</td>
<td>-0.011</td>
<td>0.350</td>
<td>-0.160</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.004</td>
<td>0.729</td>
<td>0.340</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.082</td>
<td>&lt;0.001**</td>
<td>0.077</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>-0.025</td>
<td>0.033*</td>
<td>-0.088</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>-0.121</td>
<td>&lt;0.001**</td>
<td>0.091</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.041</td>
<td>0.001*</td>
<td>0.024</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Constant</td>
<td>0.280</td>
<td></td>
<td>1.210</td>
<td></td>
</tr>
</tbody>
</table>

Parameter co-efficients with + sign indicate positive influence and those with – sign indicate negative influence on the enzyme activity; greater the numerical value, greater the influence. ** indicates that the effect is highly significant
* indicates that the effect is significant
No asterix indicates that the effect is not significant
Fig. 2—Surface and Contour graphs for XG optimization

analysis predicted the optimum levels of pH, maltose and cellobiose to be 8.3, 3.28 g/L and 0.85 g/L respectively, with the corresponding Y=0.5 IU/ml. The predicted values were verified by using these components at the optimized level in fermentation experiment. The practical corresponding response after fermentation was 0.51 IU/ml. The model’s goodness of fit was checked by determination coefficient \((R^2)\). \(R^2\) is a measure of the amount of reduction in the variability of response obtained. In this case, the value of the determination coefficient \((R^2 = 0.9438)\) indicated that only 5.17% of the total variations were not explained by the model. The value of the adjusted determination coefficient (Adj. \(R^2\)) was 0.9243. If adjusted \(R^2 < R^2\), it indicates that the quadratic terms are unnecessary. Hence the model is self-sufficient. This affirms the use of ridge analysis for this study. Surface and contour graphs of the predicted response surface were generated pair wise in order to visualize the response surface. The response surface graphs were generated by plotting the response along z-axis against two independent variables while keeping the other independent variable at optimum level. The graphs for XG are depicted in Fig. 2. The final composition of the optimized medium was (in g/L): NaNO_3-2.0; K_2HPO_4-1.0; MgSO_4.7H_2O-0.5; KCl-0.5; FeSO_4.7H_2O-0.05; yeast extract-0.2; TKP-10.0; maltose-3.28; cellobiose-0.85 and pH-8.3.

For β-glucosidase, the predicted optimum levels at the maximum radius were TKP-2.06 g/L; yeast extract-7.85 g/L; lactose-4.38 g/L and potassium dihydrogen phosphate-4.23 g/L with the predicted β-glucosidase activity of 1.46 IU/ml. The activity obtained from fermentation, by adding the optimized amounts of the above four components in the basal medium, was 1.74 IU/ml, \(R^2\) was 0.9835 and adj. \(R^2\) was 0.9726. The contour and surface graphs of the predicted response surface are shown in Fig. 3. The final composition of the optimized medium was (in g/L): NaNO_3-2.0; K_2HPO_4-1.0; MgSO_4.7H_2O-0.5; KCl-0.5; FeSO_4.7H_2O-0.05; yeast extract-7.85; lactose-4.38; KH_2PO_4-4.23; TKP-2.06 and pH-4.5.

Discussion

Though there are umpteen reports on improvement of XG production, predominantly by cloning and expression, optimization has not been reported so far, this being the first report on the use of statistical designs for XG optimization. The preference of alkaline pH for growth of Aspergilli is rare. Pol et al. have reported using an alkaline medium for XG production by Thermomonospora sp., though they have not attempted optimization. In the present study, the results of fermentation and optimization imply preference for alkaline pH for XG production. Damasio et al. have used 2% maltose containing medium for XG production while Master et al. have used the same medium for xyloglucan glycosyl transferase production. Apart from these, there are no reports on the requirement of maltose for production of this enzyme. The present study is an addition to the existing small list which suggests that maltose is a suitable medium component for enhancing XG production. Also, optimal fermentation time for enzyme production was 1 day which is advantageous from the cost perspective. After the first day, there is consistent decrease in activity till 8 days which is probably owing to the depletion of macro- and micronutrients in the fermentation medium which, with the passage of time, alters the fungal physiology resulting in the inactivation of secretory machinery of the enzymes. Statistical optimization of β-glucosidase using species of Aspergilli has been reported with substrates like wheat bran, bean drags, soyabean powder, ginsenosides etc. But the use of TKP, for production or statistical optimization of this enzyme, has not been reported so far. Vaithanomsat et al. have optimized cellobiose (11.5 g/L), yeast extract (2.75 g/L), ammonium...
sulphate (26 g/L) and pH (3.0) for β-glucosidase production by A. niger. Barbosa et al.\textsuperscript{24} report an optimized medium of cellobiose and ammonium ions. Results of the present study, where yeast extract had significant effect, contradict the significance of cellobiose and ammonium sulphate of the above reports. However, Elyas et al.\textsuperscript{25} have reported yeast extract as a preferred nitrogen source in comparison with ammonium sulphate, nitrates and urea. In the present study, lactose has been found to be a very good carbon source for β-glucosidase production. In comparison with cellobiose, lactose is a cheaper substrate. The optimized amount of lactose is 0.3% in the present study while Aslam et al.\textsuperscript{26} have reported 1%. Lesser amount of lactose and supplementation with cheaper TKP, as optimized here, is more economical. Enzyme production increased by 9.6 fold, which validates the statistical approach for optimization of β-glucosidase production.

**Conclusion**

Optimization led to 2.1 and 9.6 fold increase in the production of XG and β-glucosidase in the respective medium. That TKP is a suitable substrate for production of both enzymes is hence proved. From the findings of this study, the importance of choosing the right statistical tool was emphasized. Use of ridge analysis is suitable where second order mixture surfaces with many ingredients have to be investigated. Ridge analysis not only maximized the response, but also helped to provide a curved direction of steepest ascent for enzyme activity. The results obtained here have been derived by application of ridge regression analysis to the data of screening (P-B) experiments and has proved efficient in enhancing XG and β-glucosidase production.

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