Alkali pretreatment of rice straw and enhanced cellulase production by a locally isolated fungus Aspergillus fumigatus NITDGPKA3

Nibedita Sarkar and Kaustav Aikat

Department of Biotechnology, National Institute of Technology, Durgapur, West Bengal, India

ABSTRACT

Studies were conducted on the alkali pretreatment of rice straw by varying NaOH concentration from 0.1 M to 2.5 M. Cellulose content was maximum (62.19%) for 0.5 M NaOH. Lignin content showed a significant decrease on increase in NaOH concentration up to 0.5M NaOH. Significant morphological changes were detected in the rice straw structure by scanning electron microscopy after pretreatment. A locally isolated cellulolytic fungus Aspergillus fumigatus NITDGPKA3 was used for cellulase (CMCase and FPase) and xylanase production under submerged fermentation of rice straw pretreated with various NaOH concentrations. Maximum enzyme activities were obtained for 0.5 M NaOH. Culture conditions (incubation temperature, initial pH and rotational speed) for cellulase and xylanase production under submerged fermentation of thus pretreated rice straw by Aspergillus fumigatus NITDGPKA3 were statistically optimized by response surface methodology based on central composite design. The optimum conditions were found to be incubation temperature 30°C, rotational speed 120 rpm and initial pH 4.17 (CMCase and FPase) and 4.5 (xylanase). A significantly higher titre of xylanase was obtained compared to the available literature.

Key words: Alkali pretreatment, Cellulase, Rice straw, Submerged fermentation

INTRODUCTION

Limited storage of petroleum based fuels has increased the demand of alternative liquid fuel from renewable sources. In that aspect, lignocellulosic biomass is the only renewable carbon source available in large quantities and thus can be a solution to the problems of energy. Agricultural residues generate million tones of agricultural wastes each year such as wheat straw, corn straw, sugarcane bagasse and rice straw. Rice straw is one of the most abundant agricultural waste. According to FAO points 600-900 million tones of rice straw is produced each year globally [1]. Asia has over 90% of worldwide rice straw production. Most of the biomass remains unused, destroyed simply by burning and increases the air pollution which consequently affects public health [2]. Therefore, rice straw is believed to have potential as the preferential feedstock for fuel ethanol production in Asia [3]. Rice straw contains 25-45% cellulose, 20-30% hemicellulose and 10-15% lignin [4].

The major polysaccharide cellulose is associated with hemicelluloses and surrounded by lignin. Tightly bound lignin of the heteropolymer impedes enzymatic hydrolysis of underneath hemicellulose and crystalline cellulose [5]. Several pretreatment methods such as alkali hydrolysis, acid hydrolysis, steaming, liquid explosion are used to remove lignin and hemicellulose to make cellulose available for the microorganisms to act upon [6]. The pretreatment of lignocelluloses increases the accessibility of the surface area of cellulose for enzymatic conversion [7].
Cellulose can be hydrolyzed by acid or enzymatic treatments, for a high yield of soluble products of low molecular weight, such as hexoses and pentoses [8]. Cellulases are the enzymes primarily implicated in the saccharification process [9]. Cellulase is also used for the production of single cell protein and chemicals from renewable cellulosic resources. Due to its potential application in deinking of mixed office waste (MOW), magazines and newspapers, denim stone washing cellulase have gained significant commercial importance [10,11,12]. In addition, saccharification of lignocellulosics to fermentable sugars may be enhanced by the enzyme cocktails of cellulase and hemicellulase [14]. Large scale bioethanol production is impeded by cost of the cellulase enzyme and substrate. Thus use of microbial cellulase and cheap biomass as substrate can help to reduce the cost of bioethanol production. It is also necessary to improve the yields of the enzymes to make the process economically attractive [13]. Cellulases are produced by a variety of microorganisms including bacteria, actinomycetes and fungi where fungi are known to secrete cellulases in large amounts [14]. Cellulase is a complex of enzymes that work synergistically to attack native cellulose. It is a cocktail of the enzymes exoglucanase, endoglucanase, and β-glucosidase. At first endoglucanase (EC 3.2.1.4) acts randomly on soluble and insoluble cellulose chains; then exoglucanase (cellobiohydrolase EC 3.2.1.91) acts to liberate cellobiose from the reducing and non-reducing ends of cellulose chains and finally, β-glucosidase (EC 3.2.1.21) liberates glucose from cellobiose [15].

‘One factor at a time’ technique is a general optimization method. It is determined by varying one factor while keeping the other factors at a constant level. This type of method is simple but time consuming and laborious. Recently statistical designs for optimization has been successfully employed in enzyme production [16,9].

Response surface methodology (RSM) is a powerful strategic experimental tool that efficiently determine the optimum conditions by considering both the effect of primary factors and their mutual interactions in a multivariate system [17]. Among all nonlinear optimization techniques RSM is the most common method for process variable optimization [18, 19, 20]. RSM is an effective statistical technique for optimizing a complex processes. It reduces the number of experimental trials required to evaluate multiple parameters and their interactions. In the present study RSM was employed to identify the optimum conditions for cellulase production from rice straw by a locally isolated cellulolytic fungus Aspergillus fumigatus NITDGPKA3 under submerged fermentation.

MATERIALS AND METHODS

Microorganism
A locally isolated cellulolytic fungus Aspergillus fumigatus NITDGPKA3 was used for cellulase production. The culture was maintained on Czapek modified medium (CMM) agar slants (0.2% NaNO₃, 0.05% KCl, 0.05% MgSO₄, 0.001% FeSO₄, 0.1% K₂HPO₄, 0.2% peptone and 2% agar) at 4°C [23].

Pretreatment of Raw Material
Locally procured rice straw was washed, air dried, size fractioned to 0.5mm and stored at room temperature for further use. The main components of untreated rice straw were determined to be 40.09 % cellulose, 26.8 % hemicellulose, 18.9 % lignin and 10.76% ash. The rice straw was pretreated with NaOH of different concentrations (0.1 – 2.5 M) at 121°C and 15 psi pressure for 1 hr at the ratio of 1:10 to substrate and NaOH solution. The pretreated rice straw was washed with tap water until the pH of the filtrate reached 7. The washed straw was dried at 60°C overnight to constant weight and stored at room temperature for further use.

Estimation of cellulose, hemicellulose and lignin [21]
Cellulose estimation
For cellulose estimation, acetic/nitric reagent (150 ml of 80% acetic acid and 15 ml of concentrated nitric acid) was added to 0.5 g sample and kept in a water bath at 100°C for 30 minutes. The mixture was cooled and centrifuged for 20 minutes. The pellet was washed with distilled water, mixed with 10 ml of 67% H₂SO₄ and allowed to stand for 1 hour at room temperature. The solution (1 ml) was diluted to 100 ml and to 1 ml of this diluted solution was added 10 ml of freshly prepared anthrone reagent (0.2 % anthrone in concentrated H₂SO₄). The mixture was heated in a boiling water bath for 10 minutes, cooled and the colour was measured at 630 nm. Cellulose powder (Hi Media, India) was used as a standard.
Hemicellulose estimation

In a refluxing flask, 1 g powdered sample was mixed with 10 ml cold neutral detergent solution. The neutral detergent solution was prepared as follows.

Disodium ethylenediamine tetraacetate (18.61 g) and sodium borate decahydrate (6.81 g) were dissolved in about 200 ml of distilled water by heating and to this, 100 ml solution containing sodium lauryl sulphate (30 g) and ethoxy ethanol (10ml) was added. A solution (100 ml) of 4.5% Na₂HPO₄ was then added to the mixture. The final volume was made up to 1 litre with distilled water and pH adjusted to 7.

Decahydronaphthalene (2 ml) and sodium sulphite (0.5 g) was added to the mixture of rice straw sample and cold neutral detergent solution, which was then heated to boiling and refluxed for 1 hour. The contents were filtered through sintered glass crucible (G-2) and washed with hot water. The contents were finally washed with acetone twice and the residue transferred to a crucible. The sample was dried at 100°C for 8 hour, cooled in a desiccator and weighed. The residue was designated as neutral detergent fiber (NDF). To calculate hemicellulose content, the amount of acid detergent fiber (ADF) was subtracted from the amount of neutral detergent fiber (NDF). The acid detergent fiber (ADF) was prepared as follows.

Powdered sample (1 g) was mixed with 100 ml acid detergent solution (2% cetyl trimethyl ammonium bromide in 1 N sulphuric acid). The sample was heated to boil in 5-10 minutes and refluxed for 1 hour after the onset of boiling. The contents were filtered through a preweighed sintered glass crucible (G-2) by suction and washed with hot water twice. The contents were washed with acetone until the filtrate was colorless, dried at 100°C for overnight and weighed after cooling in a desiccator. The ADF content was expressed as a percentage i.e., W/S × 100 where W was the weight of the fiber and S was the weight of the sample.

Lignin estimation

For lignin estimation the acid detergent fiber (ADF) was mixed with 25 ml of 72% H₂SO₄ and 1 g asbestos. The mixture was kept for 3 hours at room temperature with intermittent stirring. The mixture was then diluted, filtered with preweighed Whatman No. 1 filter paper, dried at 100°C and weighed after cooling in a desiccator. Then the filter paper was transferred to a preweighed silica crucible and kept in a muffle furnace at 550°C for 3 hours. The crucible was cooled in a desiccator and weighed to calculate ash content. Asbestos was used as blank. The acid detergent lignin (% ADL) was determined using

\[
\% \text{ ADL} = \frac{\text{Weight } 72\% \text{ H}_2\text{SO}_4 (\text{Test - Asbestos blank}) - \text{Ash (Test - Asbestos blank})}{\text{Weight of the Sample}} \times 100
\]

Inoculum

Pure culture of Aspergillus fumigatus NITDGPKA3 was subcultured on Czapek modified agar medium and incubated at 30°C. Fully sporulated plates were obtained after 6 days. The sporulated plates were flooded using 20 ml of distilled water to harvest the spores and obtain the resulting spore suspension, which was used as inoculum in subsequent experiments.

Cellulase production

Rice straw samples pretreated with different alkali concentrations (0.1M- 2.5 M) were used for enzyme production to determine the maximum FPase, CMCase and xylanase activities. Enzyme production was carried out in 250 ml Erlenmeyer flasks containing 0.5 gm of alkali pretreated rice straw in 50 ml basal medium (Czapek modified medium). Tween 80 (5g/l) was added to the medium for the statistical optimization studies. The pH of the medium was adjusted to 5. The flasks were autoclaved at 121°C (15psi) for 15 minutes and thereafter cooled to room temperature and inoculated with 5% (v/v) of the inoculum containing (10⁶ spores/ml) and incubated for 5 days at 30°C, 120 rpm. After incubation the culture broths were centrifuged at 8000 rpm at 4°C for 15 minutes and the supernatants stored at 4°C for enzyme assay.

Enzyme assay

Enzyme activities were assayed by estimating total reducing sugar released. Enzyme activity was measured by the methods of International Union of Pure and Applied Chemistry (IUPAC) Commission on Biotechnology [22]. CMCase and xylanase activities were determined using 2 % (w/v) carboxymethyl cellulose (Himedia, India) and

Available online at www.scholarsresearchlibrary.com
2% (w/v) oat spelt xylan (Himedia, India) solution prepared in 0.05 M, pH 4.8 Na-citrate buffer respectively. The reaction mixture, containing suitably diluted enzyme solution (0.5 ml) and 0.5 ml of substrate solution was incubated at 50°C for 30 minutes and 10 minutes for CMCase and xylanase respectively. To determine FPase activity 0.5 ml of enzyme solution was incubated with 1 ml of 0.05 M, pH 4.8 sodium citrate buffer containing 1 cm X 6 cm (=50 mg) Whatman filter paper strip at 50°C for 60 minutes.

In all the cases, after incubation, the released reducing sugar was estimated by the DNS method with some modifications [23]. 3,5-dinitrosalycylic acid (1 ml) was added to the reaction mixture and incubated for 5 minutes in a vigorously boiling water bath. Na-K tartarate solution (1 ml) was then added to the mixtures and cooled rapidly. The reducing sugar was estimated from the absorbance measured at 540 nm using glucose and xylose as standard. Enzymatic activities were defined in International Units (IU). One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol reducing sugars/ml/minute.

Statistical optimization
Optimization of enzyme production was done using the response surface methodology with 0.5M NaOH pretreated rice straw. For statistical optimization the basal medium and enzyme production procedure used were the same as described above where initial pH, temperature and rotational speed were set according to experimental design. A two-level fractional factorial design with six centre points was employed in order to determine the optimal process conditions for the production of FPase, CMCase and xylanase. Three independent process variable including incubation temperature (A), pH (B), rotational speed (C) were chosen for statistical optimization by RSM using central composite design (CCD) and varied over five levels (Table 1).

Table 1. Experimental range and levels of independent process variables

<table>
<thead>
<tr>
<th>Factor Name</th>
<th>Range of variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Temperature</td>
<td>-α -1 0 +1 +α</td>
</tr>
<tr>
<td>B pH</td>
<td>2 3 4.5 6 7</td>
</tr>
<tr>
<td>C Rotational speed</td>
<td>100 120 150 180 200</td>
</tr>
</tbody>
</table>

The FPase, CMCase and xylanase activities were taken as the response. Both models constructed as a response function were a second order polynomial regression model.

\[ Y = b_0 + \sum b_i A_i + \sum b_{ij} A_i^2 + \sum b_{ij} A_i A_j \]  
\[ \text{Eq.1} \]

For three variable parameters, the model equation is given below (Eq. 2)

\[ Y = b_0 + b_1 A + b_2 B + b_3 C + b_{11} A^2 + b_{22} B^2 + b_{33} C^2 + b_{12} AB + b_{13} AC + b_{23} BC \]  
\[ \text{Eq. 2} \]

where \( Y \) is the predicted response (dependent variable); \( b_0 \) is a constant; \( b_{11}, b_{22} \) and \( b_{33} \) are the linear coefficients; \( b_{12}, b_{13}, b_{23} \) are the cross product coefficients and \( b_{11}, b_{22}, b_{33} \) are quadratic coefficients. A, B and C are the coded forms of selected variables i.e. incubation temperature, pH and rotational speed respectively. A total of 20 experiments including 6 centre points were generated by design of experiment using the statistical software 7.0.0 (Stat Ease, USA).

Statistical significance of respective model equations was checked by ANOVA (Analysis of Variance). 3D model graphs were obtained to analyze the effects of variables and their interactions.

RESULTS AND DISCUSSION

Alkali pretreatment of rice straw
The effect of different NaOH concentrations (0.1M – 2.5M) on chemical composition of rice straw such as cellulose, hemicellulose and lignin was analyzed. Cellulose, hemicellulose and lignin contents are shown in Table 2 where maximum cellulose content (62.19%) was obtained from 0.5 M NaOH pretreated rice straw. Further increase in alkali concentration did not show any further increase in cellulose yield. Hemicellulose content was seen to decrease

Available online at www.scholarsresearchlibrary.com
drastically with the increase at alkali concentration from 0.1M to 2.5M. The lignin content was seen to decrease at alkali concentration from 0.1M to 0.5M and was consistent after 0.5M NaOH pretreatment.

Table 2. Effect of alkali pretreatment on cellulose, hemicellulose, lignin and ash content of rice straw

<table>
<thead>
<tr>
<th>NaOH Concentration (M)</th>
<th>Cellulose (%)</th>
<th>Hemicellulose (%)</th>
<th>Lignin (%)</th>
<th>Ash (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>41.8 ± 0.01</td>
<td>23.6 ± 0.01</td>
<td>17.9 ± 0.02</td>
<td>11.24 ± 0.02</td>
</tr>
<tr>
<td>0.25</td>
<td>42.6 ± 0.02</td>
<td>24.1 ± 0.01</td>
<td>16.1 ± 0.01</td>
<td>12.12 ± 0.03</td>
</tr>
<tr>
<td>0.5</td>
<td>62.19 ± 0.02</td>
<td>14.5 ± 0.02</td>
<td>8.4 ± 0.01</td>
<td>14.54 ± 0.03</td>
</tr>
<tr>
<td>1</td>
<td>60.4 ± 0.01</td>
<td>10.21 ± 0.04</td>
<td>8.3 ± 0.01</td>
<td>16.75 ± 0.02</td>
</tr>
<tr>
<td>1.5</td>
<td>59.3 ± 0.02</td>
<td>8.34 ± 0.02</td>
<td>8.2 ± 0.02</td>
<td>17.54 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>60.6 ± 0.01</td>
<td>7.78 ± 0.01</td>
<td>8.1 ± 0.01</td>
<td>17.78 ± 0.02</td>
</tr>
<tr>
<td>2.5</td>
<td>61.8 ± 0.01</td>
<td>6.92 ± 0.02</td>
<td>8.1 ± 0.03</td>
<td>18.23 ± 0.02</td>
</tr>
</tbody>
</table>

The morphological changes of alkali pretreated rice straw with different NaOH concentration (0.1M – 2.5M) were examined by scanning electron microscopy (SEM) using S-530, SEM, Hitachi, Japan. The SEM micrographs of raw and alkali pretreated rice straw samples in Fig 1(a -h ) provide the longitudinal section. Compared with the SEM micrograph of raw straw a significant morphological change was observed in the pretreated rice straw. The untreated rice straw showed rigid and highly ordered fibrils. The rigidity and order of the fibers were gradually lost and microfibrils were fully exposed with the increase of alkali concentration. Thus external surface area and porosity increased.

In addition to SEM micrographs and determination of cellulose, hemicellulose, lignin and ash contents, all the straw samples were used for cellulase production. The FPase, CMCase and xylanase activities obtained from each sample are shown in Fig. 2. It was observed that FPase, CMCase and xylanase activities gradually increased with increase in NaOH concentration where 0.5M NaOH pretreated rice straw resulted in maximum enzyme production. An improvement of enzyme activity may be due to the increase of available cellulose surface and decrease of lignin with the gradual increase of NaOH concentration.
Figure 2 Enzyme activities obtained from pretreated rice straw samples at different NaOH concentration. Symbols: FPase (▲); CMCase (♦); xylanase (■)

Response surface approach by central composite design
The effect of three independent variables on cellulase and xylanase production was investigated and Central Composite Design (CCD) was used for optimization of production where 20 experimental runs were carried out with different combinations of the three factors. Experimental design and actual response along with the predicted response are given in Table 3. The predicted response was calculated by the regression equation. With the design conditions the CMCase, FPase and xylanase activities markedly varied in range of 1.475 – 13.9 IU/ml, 0.395 – 2.435 IU/ml and 28 – 249.11 IU/ml respectively.

Table 3. Experimental and predicted values of FPase, CMCase and xylanase obtained in experimental set up of response surface methodology

<table>
<thead>
<tr>
<th>Serial No</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>Rotational speed (rpm)</th>
<th>FPase Actual / Predicted (IU/ml)</th>
<th>CMCase Actual / Predicted (IU/ml)</th>
<th>Xylanase Actual / Predicted (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>3</td>
<td>120</td>
<td>1.375 (1.386)</td>
<td>7.865 (7.812)</td>
<td>158.100 (159.270)</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>3</td>
<td>120</td>
<td>1.811 (1.896)</td>
<td>6.752 (6.826)</td>
<td>140.000 (141.020)</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>6</td>
<td>120</td>
<td>1.182 (1.196)</td>
<td>7.591 (7.574)</td>
<td>148.900 (148.190)</td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td>3</td>
<td>120</td>
<td>1.095 (1.095)</td>
<td>8.239 (8.161)</td>
<td>157.110 (158.020)</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>3</td>
<td>180</td>
<td>1.112 (1.159)</td>
<td>7.544 (7.629)</td>
<td>150.899 (150.470)</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>3</td>
<td>180</td>
<td>1.325 (1.359)</td>
<td>4.555 (4.578)</td>
<td>90.830 (92.020)</td>
</tr>
<tr>
<td>7</td>
<td>25</td>
<td>6</td>
<td>180</td>
<td>1.520 (1.474)</td>
<td>7.861 (7.791)</td>
<td>155.980 (155.440)</td>
</tr>
<tr>
<td>8</td>
<td>35</td>
<td>6</td>
<td>180</td>
<td>1.025 (1.064)</td>
<td>6.254 (6.313)</td>
<td>125.760 (125.070)</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>4.5</td>
<td>150</td>
<td>0.395 (0.400)</td>
<td>3.467 (3.502)</td>
<td>67.120 (67.660)</td>
</tr>
<tr>
<td>10</td>
<td>38</td>
<td>4.5</td>
<td>150</td>
<td>0.561 (0.483)</td>
<td>1.476 (1.431)</td>
<td>28.000 (26.780)</td>
</tr>
<tr>
<td>11</td>
<td>30</td>
<td>2</td>
<td>150</td>
<td>2.001 (1.911)</td>
<td>10.032 (9.959)</td>
<td>197.200 (195.680)</td>
</tr>
<tr>
<td>12</td>
<td>30</td>
<td>7</td>
<td>150</td>
<td>1.485 (1.503)</td>
<td>11.151 (11.218)</td>
<td>213.310 (214.160)</td>
</tr>
<tr>
<td>13</td>
<td>30</td>
<td>4.5</td>
<td>100</td>
<td>2.181 (2.130)</td>
<td>10.132 (10.183)</td>
<td>208.000 (206.810)</td>
</tr>
<tr>
<td>14</td>
<td>30</td>
<td>4.5</td>
<td>200</td>
<td>1.935 (1.913)</td>
<td>8.530 (8.476)</td>
<td>171.191 (171.700)</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
<td>4.5</td>
<td>150</td>
<td>2.373 (2.397)</td>
<td>13.752 (13.658)</td>
<td>245.220 (245.170)</td>
</tr>
<tr>
<td>16</td>
<td>30</td>
<td>4.5</td>
<td>150</td>
<td>2.425 (2.397)</td>
<td>13.911 (13.658)</td>
<td>248.300 (245.170)</td>
</tr>
<tr>
<td>17</td>
<td>30</td>
<td>4.5</td>
<td>150</td>
<td>2.405 (2.397)</td>
<td>13.652 (13.658)</td>
<td>243.150 (245.170)</td>
</tr>
<tr>
<td>18</td>
<td>30</td>
<td>4.5</td>
<td>150</td>
<td>2.319 (2.397)</td>
<td>13.722 (13.658)</td>
<td>244.360 (245.170)</td>
</tr>
<tr>
<td>19</td>
<td>30</td>
<td>4.5</td>
<td>150</td>
<td>2.432 (2.397)</td>
<td>13.525 (13.658)</td>
<td>240.780 (245.170)</td>
</tr>
<tr>
<td>20</td>
<td>30</td>
<td>4.5</td>
<td>150</td>
<td>2.435 (2.397)</td>
<td>13.455 (13.658)</td>
<td>249.110 (245.170)</td>
</tr>
</tbody>
</table>

A comparison of predicted values with the experimental values of FPase, CMCase and xylanase activities has been shown in Fig.3(a-c) respectively. ANOVA was employed to ascertain the significant variable and their interaction effect on FPase, CMCase and xylanase production. The ANOVA of the quadratic regression model are summarized.
in Table 4. The computed R\(^2\) values for endoglucanase, FPase and xylanase were obtained between 95.3% and 99.9% which determines a good fitness of the model.

**Figure 3. Plots of predicted vs actual responses. A FPase. B CMCase. C Xylanase.**

**FPase**

FPase represents the total cellulolytic activity. FPase activity (2.435 IU/ml) was obtained at the combination of incubation temperature 30°C, initial pH 4.5 and rotational speed 150 rpm. Moreover, all the middle level runs gave higher yields compared to other combinations. Polynomial equation for FPase activity (Y) as a function of temperature (A), initial pH (B) and rotational speed (C) has given below.

\[
Y_{FPase} = 2.40 + 0.025 * A - 0.12 * B - 0.065 * C - 0.15 * A * B - 0.077 * A * C + 0.13 * B * C \\
-0.69 * A^2 - 0.24 * B^2 - 0.13 * C^2
\]  
Eq. 3a

A high Model F-value (194.21) along with high multiple correlation coefficient (R\(^2\)) value of 0.9943 and an insignificant lack of fit indicates a good effect of the parameters on FPase production. From Table 4 it is observed that the FPase production was significantly affected by temperature and rotational speed (p < 0.0001) in linear as well as in squared terms (p < 0.0001). Squared terms of initial pH also had good effect on FPase production (p<0.0001). The interaction between temperature and pH as well as initial pH and rotational speed was significant at p < 0.0001 and p<0.0004 level respectively. CV values obtained from the model was 4.06. The "Pred R-Squared"(0.9648) is in reasonable agreement with the "Adj R-Squared"(0.9892). It indicates a good correlation between the observed and predicted values.

**Table 4. Analysis of variance for the response surface quadratic model**

<table>
<thead>
<tr>
<th>Source</th>
<th>F value</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FPase</td>
<td>CMCase</td>
</tr>
<tr>
<td>Model</td>
<td>194.2065</td>
<td>1573.774</td>
</tr>
<tr>
<td>A - Temperature</td>
<td>1.821216</td>
<td>278.323</td>
</tr>
<tr>
<td>B - pH</td>
<td>43.94319</td>
<td>102.8132</td>
</tr>
<tr>
<td>C - Rotational speed</td>
<td>12.43943</td>
<td>189.1116</td>
</tr>
<tr>
<td>AB</td>
<td>40.62608</td>
<td>66.51023</td>
</tr>
<tr>
<td>AC</td>
<td>10.49225</td>
<td>114.5999</td>
</tr>
<tr>
<td>BC</td>
<td>27.84377</td>
<td>4.309912</td>
</tr>
<tr>
<td>A(^2)</td>
<td>15.0525</td>
<td>12122.26</td>
</tr>
<tr>
<td>B(^2)</td>
<td>187.6506</td>
<td>912.0597</td>
</tr>
<tr>
<td>C(^2)</td>
<td>55.6657</td>
<td>1813.496</td>
</tr>
<tr>
<td>Lack of Fit</td>
<td>2.92</td>
<td>0.36</td>
</tr>
</tbody>
</table>
The three dimensional plots (Fig. 4a and 4b) represent the interaction between temperature and initial pH as well as rotational speed and initial pH on FPase production. Fig 4a showed that FPase activity was increased with the increase of temperature and was optimum at 30°C. Any increase or decrease in temperature lowered FPase production and initial pH did not show considerable effect on enzyme activity. In Fig 4b the enzyme activity gradually increased with increase in initial pH as well as rotational speed to a certain level and then decreased, which indicates that initial pH and rotational speed were critical to obtain maximum response. The pH and rotational speed for optimum response were 4.5 and 150 rpm respectively.

Figure 4. Three dimensional plots of FPase (response). In each plot, the influence of two variables is shown while the third is set at the central level. A Temperature (A) vs initial pH (B) and their interaction effect with rotational speed (C) set at center level. B Initial pH (B) vs rotational speed (C) and their interaction effect with temperature (A) set at center level.

CMCase

From Table 3 it is observed that a maximum CMCase (13.9 IU/ml) was produced under the culture conditions similar to FPase. The experimental results were analyzed by regression analysis that reproduced the following equation in terms of linear, quadratic and interaction effect on temperature (A), initial pH (B) and rotational speed (C) on CMCase production.

\[
Y_{CMCase} = 13.66 - 0.62 * A + 0.37 * B - 0.51 * C + 0.39 * A * B - 0.52 * A * C + 0.10 * B * C -3.96 * A^2 - 1.09 * B^2 - 1.53 * C^2
\]  

Eq.3b

The Model F value (1573.774) with low probability value (p < 0.0001) indicated that the model was statistically significant. The model significance was also implied by high R2 (0.9993) value and an insignificant lack of fit determines that the model was efficient to explain the effect of three parameters on CMCase production. All of the linear and quadratic terms were significant. Interaction coefficients between temperature and initial pH as well as temperature and rotational speed were significant at p< 0.0001 where interaction of initial pH and rotational speed was significant at p < 0.5 (Table 4). CV values obtained from the model was 1.49. Like FPase the "Pred R-Squared"(0.9977) is also in reasonable agreement with the "Adj R-Squared"(0.9987). Interaction effects of temperature and initial pH as well as rotational speed and initial pH on CMCase production are depicted in Fig. 5a and 5b respectively. The maximum response in two plots was obtained under the same conditions as for FPase.
Figure 5. Three dimensional plots of CMCase (response). In each plot, the influence of two variables is shown while the third is set at the central level. A Temperature (A) vs initial pH (B) and their interaction effect with rotational speed (C) set at center level. B Initial pH (B) vs rotational speed (C) and their interaction effect with temperature (A) set at center level.

**Xylanase**

Significance of the coefficients of linear, quadratic and interaction terms were determined by the following equation.

\[
Y_{Xylanase} = 245.17 - 12.15A + 5.49B - 10.44C + 7.02AB - 10.05AC + 4.01BC - 69.99A^2 - 14.23B^2 - 19.77C^2
\]

\text{Eq.3c}

\(Y\) is the dependent variable response of xylanase production (IU/ml). The results by ANOVA showed that the model F value was 1413.67 with a probability value \(p < 0.0001\) (Table 4). The coefficient of determination \(R^2 (0.9992)\) and an insignificant lack of fit ensured satisfactory adjustment of the quadratic model to the experimental data. The model represented a reasonable agreement between the “Pred R-Squared” (0.9979) and “Adj R-Squared” (0.9985) value. All the linear as well as quadratic terms significantly affected xylanase production (\(p < 0.0001\)). Interaction effect of temperature and initial pH as well as rotational speed and temperature was more significant (\(p < 0.0001\)) compared to rotational speed and initial pH (\(p < 0.005\)).

Figure 6. Three dimensional plots of xylanase (response). In each plot, the influence of two variables is shown while the third is set at the central level. A Temperature (A) vs initial pH (B) and their interaction effect with rotational speed (C) set at center level. B Initial pH (B) vs rotational speed (C) and their interaction effect with temperature (A) set at center level.
The interaction effect of temperature and initial pH as well as rotational speed and initial pH on xylanase production has been shown in Fig. 6a and 6b respectively. Maximum xylanase activity in both plots was obtained under the similar conditions of cellulase production.

Optimization of process parameters

The optimum values of FPase, CMCase and xylanase by the adjusted model was predicted by numerical optimization step in CCD. The goals for variables of temperature and initial pH were set as “in range”. Rotational speed was set as “minimize” for low energy consumption. The goal for the response (FPase, CMCase and xylanase) was set as “maximize” because the maximum value of yield is the aim. The software Design Expert 7.0.0 produced a number of solutions and ranked according to their desirability. The solution with the highest desirability was selected and its predicted value of the response was 2.38 IU/ml, 12.55 IU/ml and 235.88 IU/ml for FPase, CMCase and xylanase respectively. The optimized conditions for optimum FPase and CMCase were temperature 30°C, initial pH 4.17 and rotational speed 120 rpm. Optimized conditions for optimum xylanase production were same except initial pH (4.5). Experimentally optimized value (FPase 2.43 IU/ml, CMCase 12.62 IU/ml and xylanase 235.84 IU/ml) with conditions of selected solution showed good agreement with the predicted value. It was ensured that the developed model was enough significant for predicting the experimental results. The optimized yield obtained in this study is a reasonable production of cellulase enzyme under submerged condition and further improvements in product yield will be achieved with the fine tuning of other parameters.

CONCLUSION

It can be concluded that the pretreated rice straw with 0.5M NaOH resulted in maximum FPase, CMCase and xylanase activities. SEM micrographs exhibited significant morphological changes in alkali pretreated rice straw compared to untreated rice straw. Locally isolated Aspergillus fumigatus NITDGPKA3 is a potential microorganism for cellulase production under submerged condition. Statistical optimization of cellulase production from alkali pretreated rice straw has been carried out using response surface methodology. The culture conditions were optimized for optimal FPase, CMCase as well as xylanase production.

REFERENCES


726

Available online at www.scholarsresearchlibrary.com