

## Optimization of cellulase synthesis by RSM and evaluation of ethanol production from enzymatically hydrolyzed sugarcane bagasse using *Saccharomyces cerevisiae*

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This study presents ethanol production from enzymatically hydrolyzed sugarcane bagasse using *Saccharomyces cerevisiae*. Response surface methodology (RSM) was used to optimize conditions for the production of endo  $\beta$ -1, 4 glucanase component of cellulase using *Aspergillus nidulans* MTCC344 under solid state condition, employing pretreated bagasse as chief substrate. Cellulase thus produced was utilized for hydrolyses of pretreated bagasse resulting in soluble sugars. FTIR and XRD of bagasse showed structural changes through pretreatment, in favor of enzymatic hydrolysis during cellulase production. Ethanol produced was found to be strongly dependent on pretreatment given, hydrolysis and fermentation conditions.

**Keywords:** *Aspergillus nidulans* MTCC344, Ethanol, Pretreated bagasse, RSM, *Saccharomyces cerevisiae*

### Introduction

Complex polyaromatic matrix of lignin containing significant amounts of hemicelluloses and crystallinity of cellulose are resistant to enzymatic hydrolysis<sup>1</sup>. Cellulase production in fungi is found to be extracellular with three components<sup>2</sup> (endo-1,4- $\beta$ -D-glucanase, EC 3.2.1.4, exo-1,4- $\beta$ -D-glucanase, EC 3.2.1.91 and 1,4- $\beta$ -D-glucosidase, EC 3.2.1.21). Cellulases are synthesized by microorganisms during their growth on cellulosic materials<sup>3</sup>. Comprehensive application of cellulase in industry is hindered by its synthesizing cost, which can be overcome by exploring new substrates for enzyme production. Bagasse, a residue remaining after extraction of sugarcane (*Saccharum officinarum*) juice, is used in manufacture of pulp and paper products, building materials, fuel and cellulosic ethanol<sup>4</sup>. Each ton of cane yield 740 kg of juice and 260 kg of moist bagasse (130 kg dry)<sup>5</sup>. Main contributors to overall costs of producing ethanol from biomass are raw materials (30-40%), capital investment (30-45%), and cellulase enzymes (10-20%). Low capital investment, low operating cost and easy availability of bagasse can be a low cost raw material for cellulase production. Solid state fermentation (SSF)

is an attractive process to produce cellulase<sup>6</sup>. Strain, *Aspergillus nidulans* MTCC344 has been used for cellulase production<sup>7,8</sup>. Optimizing methods are available for the production of cellulase possessing high activity and yield<sup>9</sup>.

This study presents formulation of a methodology that includes cellulase production and use of cellulase for hydrolyses of pretreated bagasse (PB) to yield ethanol by using *Saccharomyces cerevisiae*.

### Experimental Section

#### Microorganism

*A. nidulans* MTCC344 was used in cellulase production. Isolation was performed using a solid media containing 1.5% agar and 1% CMC to screen cellulase producing strain, followed by 0.01% congo red staining and 0.1% NaCl washing<sup>10</sup>. Isolate showed clear orange halo zone on CMC agar plate. Cultures were maintained as spore suspension in 20% (v/v) glycerol at -20°C.

#### Inoculation and Culture Conditions

Minimal medium composition (MMC) employed contained:  $\text{KH}_2\text{PO}_4$ , 0.75;  $\text{NH}_4\text{NO}_3$ , 1.5; Thiamine HCl, 0.01;  $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ , 0.5; and  $\text{CaCl}_2 \cdot 5\text{H}_2\text{O}$ , 0.05 mg/l. Bagasse was chief carbon source. Strain maintained on PDA slants at 40°C for 6 d were prepared as conidial

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suspensions by washing slant cultures with 5 ml sterilized water. Spore suspension was counted at  $10^5$  spores/ml by Haemocytometer. All trials were carried out in 250 ml Erlenmeyer flasks containing bagasse (5-25 g) [moisture content 40-80% (Moisture Analyzer-Ohaus MB35); pH 3-7] sterilized for 30 min at  $121^\circ\text{C}$ , inoculated with 3 ml of prepared spores and incubated at  $20$ - $60^\circ\text{C}$  on static incubator for 8 d in succession.

#### Feedstock Pretreatment

Bagasse was obtained from local sugar mills in Tamil Nadu. Substrates were air dried, milled by pulverizing, filtered through 60 mesh screen and washed with water at room temperature (RT) for 4 h. Initial composition of bagasse is<sup>11</sup>: cellulose, 42.7; hemicellulose, 25.4; lignin, 28.0; and ash, 0.75%. Alkaline (NaOH) pretreatment was used to delignify feedstock to isolate solid fraction, which consists mainly of cellulose. Bagasse (25 g dry wt) was subjected to pretreatment with 1N NaOH (200 ml) in a 500 ml beaker for 2, 4, 6, 8, 10 h at  $50^\circ\text{C}$ . After treatment, solution was centrifuged for 20 min at 2500 rpm and solid fraction was suspended in hot boiling water for 10 min, followed by centrifugation for 10 min at 5000 rpm. Solid was subjected to repeated washing (thrice) with distilled water and centrifugation (5000 rpm, 10 min), dried and stored in plastic containers.

#### Enzymatic Hydrolysis

NaOH PB<sup>12</sup> was layered in 250 ml Erlenmeyer flasks and sterilized. Minimal media (3 ml) containing  $10^5$  spores/ml was suspended in 2 ml sterile water and inoculated. Inoculum contributed variation (1.5-2.0%) in moisture levels. Moisture content was varied (40-80%) by addition of sterile water, incorporated with inoculum. Cultures were incubated at different temperatures (20, 30, 40, 50 and  $60^\circ\text{C}$ ). Hydrolysis was initiated at various initial pH (3-7). All experimental sequences, given out by Design expert software version 8.0.1, were performed in triplicate. Extracts were drawn at regular interval (1 d) by addition of 10 ml distilled water, allowing it to stand for 10 min and filtering through nylon cloth. This was used to determine activity and yield of cellulase at different trials. Extract was used in yeast fermentation.

#### Ethanol Production

Cellulase enzyme fermentation<sup>13</sup> (CEF) was used, with some key assumptions. *Aspergillus* strain was assumed to be able to produce cellulase in presence of monosaccharides. After complete hydrolysis of

polysaccharides, all monosaccharides are consumed in CEF. As reported<sup>14</sup>, inhibition was assumed not to occur due to furan derivatives and organic acids present in pretreated material. Extracts were supplemented with soymeal 0.2%,  $(\text{NH}_4)_2\text{SO}_4$  0.1%,  $\text{KH}_2\text{PO}_4$  0.05%,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.003%, pretreated bagasse (52% cellulose) and molasses (50% sucrose). Ordinary baker's yeast (3 g/l) was employed. Simultaneous saccharification/fermentation (SSF) took place in agitated nonsterile fermentor (3l).

#### Analytical Methods

Assay for Endo  $\beta$ -1, 4 glucanase, Exo  $\beta$ -1, 4 glucanase and  $\beta$ -glucosidase were carried out according to IUPAC<sup>15</sup>. Glucose in culture supernatant was analyzed using UV Visible spectrophotometer [Hitachi Model: 100-40 Spectrophotometer] at 540 nm. After SSF, ethanol was quantified using Quantichrom TM Ethanol assay kit (DIET-500). Resulting bluish chromic product was analyzed at 580 nm. X-ray diffraction (XRD) analysis from  $0$ - $70^\circ$ , 2 $\theta$  examined structural properties of PB and hydrolysed bagasse (HB) [Miniflex Model, Rigaku, Japan]. Fourier transform infrared spectroscopy (FTIR) spectrum of samples was recorded in the region of  $400$ - $4000\text{ cm}^{-1}$  [Nicolet 20DXB spectrometer].

#### Experimental Design

Dependency<sup>16</sup> on enzymatic hydrolysis of 4 variables [bagasse content (5-25g), moisture content (40-80%), initial pH (3-7) and growth temperature ( $20$ - $60^\circ\text{C}$ )] was chosen to obtain optimum levels. Response surface methodology (RSM) using a 4-factor, 5-level central composite design (CCD) with a total of 30 different combinations was employed to optimize levels of identified controllable factors for the production of cellulase by *A. nidulans* MTCC344. CCD, constructed by Design Expert software (Version 8.0.1, Stat-Ease Inc., Minneapolis, USA), investigated performance of response variable Y, cellulase activity (U/g). Coded values of independent variables (levels: 2, -1, 0, +1 and +2) were calculated as

$$x_i = \frac{X_i - X_0}{\Delta X}, \quad i=1,2,3,\dots,k \quad \dots(1)$$

where  $x_i$  is dimensionless value and  $X_i$  is real value of an independent variable,  $X_0$  is the value of  $X_i$  at center point and " $\Delta X$ " is step change. A second-order quadratic model was used to fit quadratic equation as

$$\begin{aligned}
Y = & b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 \\
& + b_5x_1^2 + b_6x_2^2 + b_7x_3^2 + b_8x_4^2 + b_9x_1x_2 \\
& + b_{10}x_1x_3 + b_{11}x_1x_4 + b_{12}x_2x_3 + b_{13}x_2x_4 \\
& + b_{14}x_3x_4 \quad \dots(2)
\end{aligned}$$

where  $x_1, x_2, x_3, x_4$  are coded independent input variables,  $b_0$  is intercept term,  $b_1, b_2, b_3, b_4$  are linear coefficients showing linear effects,  $b_5, b_6, b_7, b_8$  are quadratic coefficients showing squared effects and  $b_9, b_{10}, b_{11}, b_{12}, b_{13}, b_{14}$  are cross product coefficients showing interaction effects. Hydrolysis process was employed for maximum bagasse content (15 g), initial pH and growth temperatures (5.0 and 40°C respectively) at minimum moisture content (60%), so as to maximize CMCCase activity. Optimum values of factors were obtained by solving regression equation by Monte Carlo optimization. Goodness of fit of the model was evaluated by coefficient of determination ( $R^2$ ) and analysis of variance (ANOVA).

## Results and Discussion

Enzymatic hydrolysis of PB was carried out by inoculating *A. nidulans* MTCC344 spores (3 ml) suspended in sterile water containing Tween 20. RSM employing CCD was used to optimize parameters affecting hydrolysis so as to achieve cellulase of maximum activity and yield. Maximum cellulase activity (28.96 U/g) was in run no. 25. Multiple regression analysis (MRA) was applied to the data based on Eqs (1) and (2). Predicted response  $Y_{R1}$  for cellulase activity was obtained as a function of bagasse content ( $x_1$ ), moisture content ( $x_2$ ), initial pH ( $x_3$ ) and growth temperature ( $x_4$ ) as

$$\begin{aligned}
Y_{R1} = & 28.84 + 1.72x_1 + 0.82x_2 - 2.38x_3 + 0.18x_4 \\
& - 4.46x_1^2 - 4.53x_2^2 - 2.37x_3^2 - 1.80x_4^2 - 0.28x_1x_2 \\
& - 1.98x_1x_3 - 0.11x_1x_4 + 0.13x_2x_3 + 0.12x_2x_4 \\
& + 2.29x_3x_4 \quad \dots(3)
\end{aligned}$$

CCD results on experiments with different combinations for  $x_1, x_2, x_3$  and  $x_4$  are presented along with mean predicted and experimental responses for cellulase activity (Table 1). Statistical significance of model was investigated by ANOVA (Table 2). F-value (9992.21) implies its significance. Values of Prob>F (<0.05) indicate significance of the model terms. p-values

Table 1—Levels of variables tested in CCD matrix in coded terms showing levels of tested variables, cellulase activity (CMCase) in terms of experimental and predicted values

| Run | Variables and experimental values |        |       |        | Cellulase activity, U $g^{-1}$ |       |
|-----|-----------------------------------|--------|-------|--------|--------------------------------|-------|
|     | $X_1$                             | $X_2$  | $X_3$ | $X_4$  | Expt*                          | Pred  |
| 1   | -1(10)                            | -1(50) | -1(4) | -1(30) | 15.50                          | 15.53 |
| 2   | +1(20)                            | -1(50) | -1(4) | -1(30) | 23.69                          | 23.70 |
| 3   | -1(10)                            | +1(70) | -1(4) | -1(30) | 17.30                          | 17.23 |
| 4   | +1(20)                            | +1(70) | -1(4) | -1(30) | 24.30                          | 24.28 |
| 5   | -1(10)                            | -1(50) | +1(6) | -1(30) | 9.850                          | 9.880 |
| 6   | +1(20)                            | -1(50) | +1(6) | -1(30) | 10.18                          | 10.13 |
| 7   | -1(10)                            | +1(70) | +1(6) | -1(30) | 12.10                          | 12.10 |
| 8   | +1(20)                            | +1(70) | +1(6) | -1(30) | 11.18                          | 11.22 |
| 9   | -1(10)                            | -1(50) | -1(4) | +1(50) | 11.28                          | 11.29 |
| 10  | +1(20)                            | -1(50) | -1(4) | +1(50) | 19.05                          | 19.03 |
| 11  | -1(10)                            | +1(70) | -1(4) | +1(50) | 13.42                          | 13.45 |
| 12  | +1(20)                            | +1(70) | -1(4) | +1(50) | 20.04                          | 20.06 |
| 13  | -1(10)                            | -1(50) | +1(6) | +1(50) | 14.80                          | 14.80 |
| 14  | +1(20)                            | -1(50) | +1(6) | +1(50) | 14.50                          | 14.62 |
| 15  | -1(10)                            | +1(70) | +1(6) | +1(50) | 17.45                          | 17.49 |
| 16  | +1(20)                            | +1(70) | +1(6) | +1(50) | 16.23                          | 16.18 |
| 17  | -2(5)                             | 0(60)  | 0(5)  | 0(40)  | 7.600                          | 7.58  |
| 18  | +2(25)                            | 0(60)  | 0(5)  | 0(40)  | 14.45                          | 14.44 |
| 19  | 0(15)                             | -2(40) | 0(5)  | 0(40)  | 9.150                          | 9.110 |
| 20  | 0(15)                             | +2(80) | 0(5)  | 0(40)  | 12.35                          | 12.37 |
| 21  | 0(15)                             | 0(60)  | -2(3) | 0(40)  | 24.11                          | 24.13 |
| 22  | 0(15)                             | 0(60)  | +2(7) | 0(40)  | 14.65                          | 14.60 |
| 23  | 0(15)                             | 0(60)  | 0(5)  | -2(20) | 21.24                          | 21.27 |
| 24  | 0(15)                             | 0(60)  | 0(5)  | +2(60) | 22.04                          | 21.98 |
| 25  | 0(15)                             | 0(60)  | 0(5)  | 0(40)  | 28.96                          | 28.84 |
| 26  | 0(15)                             | 0(60)  | 0(5)  | 0(40)  | 28.95                          | 28.84 |
| 27  | 0(15)                             | 0(60)  | 0(5)  | 0(40)  | 28.85                          | 28.84 |
| 28  | 0(15)                             | 0(60)  | 0(5)  | 0(40)  | 28.75                          | 28.84 |
| 29  | 0(15)                             | 0(60)  | 0(5)  | 0(40)  | 28.94                          | 28.84 |
| 30  | 0(15)                             | 0(60)  | 0(5)  | 0(40)  | 28.59                          | 28.84 |

were  $p < 0.0001$  for  $X_1, X_2, X_3, X_4, X_1X_2, X_1X_3, X_3X_4, X_1^2, X_2^2, X_3^2$  and  $X_4^2$  for cellulase activity, whereas p-values were 0.0006, 0.0001 and 0.0003 for  $X_1X_4, X_2X_3$  and  $X_2X_4$  respectively. Regression equation obtained after ANOVA indicates  $R^2$  as 0.9999 for cellulase activity. The closer the  $R^2$  value to 1, stronger the model and better it predicts the response<sup>16</sup>.  $R^2$  values were in reasonable agreement with adjusted determination of coefficient (Adj  $R^2$ ) value of 0.9998. R value (0.99) indicates direct proportionality between experimental and predicted values of responses. Predicted determination of coefficient (Pred  $R^2$ ) of 0.9997 is in reasonable agreement with Adj  $R^2$  (0.9998). Adequate precision (Adeq Pre) ratio of 301.239 for the response indicates an adequate signal. A lower value (0.54% CV) indicates precision with which experiments

Table 2—Analysis of variance (ANOVA), regression coefficient estimate and test of significance for cellulase activity  $Ug^{-1}$  ( $R_1$ )

| Factor            | Sum of squares | Mean squares | df | F-value  |
|-------------------|----------------|--------------|----|----------|
| Model             | 1393.39        | 99.53        | 14 | 9992.21  |
| $X_1$             | 70.62          | 70.62        | 1  | 7090.34  |
| $X_2$             | 15.96          | 15.96        | 1  | 1602.09  |
| $X_3$             | 136.37         | 136.37       | 1  | 13691.44 |
| $X_4$             | 0.76           | 0.76         | 1  | 76.27    |
| $X_1X_2$          | 1.27           | 1.27         | 1  | 127.63   |
| $X_1X_3$          | 62.77          | 62.77        | 1  | 6301.46  |
| $X_1X_4$          | 0.19           | 0.19         | 1  | 18.78    |
| $X_2X_3$          | 0.27           | 0.27         | 1  | 27.41    |
| $X_2X_4$          | 0.21           | 0.21         | 1  | 21.48    |
| $X_3X_4$          | 84.04          | 84.04        | 1  | 8437.59  |
| $X_1^2$           | 544.86         | 544.86       | 1  | 54701.70 |
| $X_2^2$           | 561.80         | 561.80       | 1  | 56402.29 |
| $X_3^2$           | 153.83         | 153.83       | 1  | 15444.26 |
| $X_4^2$           | 89.19          | 89.19        | 1  | 8954.09  |
| Residual          | 0.15           | 0.00         | 15 |          |
| Lack of fit       | 0.042          | 0.00         | 10 | 0.20     |
| Pure error        | 0.11           | 0.021        | 5  |          |
| Correlation total | 1393.54        |              | 29 |          |

were conducted. PRESS statistic value (0.40) proves validity of model. Model predicted all variables, thereby confirming its adequacy.

Under RSM and their corresponding contour plots for cellulase activity against any two independent variables by keeping the other independent variables at zero levels, a total of six response surfaces (Fig. 1a-f) were obtained. Maximum CMCase (27.59 U/g) was found at bagasse weight (16.8 g) and moisture content (60%) for all pH ranges and temperatures studied (Fig. 1a). Similarly, maximum CMCase values were as follows: 28 U/g (Fig. 1b) was achieved at initial pH (4.25) and bagasse weight (16.8 g); 26.45U/g (Fig. 1c) was at temperature (43.8°C) and bagasse weight (16.8 g); 27.89U/mg (Fig. 1d) was at initial pH (4.25) and moisture content (60%); 26.5U/g (Fig. 1e) at 63% moisture content (when moisture content was varied from 50% to 70%) at bagasse weight (16.8 g) and initial pH (4.25); and 28 U/g (Fig. 1f) for temperature (37.5°C) and initial pH (4.25). In response plots and contour diagrams (Fig. 1), contour diagrams (Fig. 1a-e) were elliptical and tilted, indicating significant cross product interaction between the factors,  $X_1$  &  $X_2$ ,  $X_1$  &  $X_3$ ,  $X_1$  &  $X_4$  and  $X_2$  &  $X_3$ . This is also evident from coefficient estimate values (Table 2). Contour diagrams (Fig. 1f) are elliptical but not tilted, indicating a good deal of negligible interaction

between factors ( $X_3$  &  $X_4$ ). Optimum conditions were found to be at  $x_1$ (16.8 g),  $x_2$ (60%),  $x_3$ (pH 4.25) and  $x_4$ (40°C) in validation experiments. *A. nidulans* MTCC344 produced cellulase (28.80 U/g) activity on 8<sup>th</sup> day after inoculation, and this cellulase was used in ethanol production as biocatalyst.

In process scheme for ethanol production (Fig. 2), whole broth without mycelia, but with enzymes is added to SSF. In SSF cycle, processes of filling, fermentation, draining and cleaning last for 60 h. In CEF, there was a decrease in the flow of carbon (hexosans and hexoses) but in SSF feed, a majority of C6 sugars were fermented to ethanol. Yeast required by SSF slightly decreased with increasing water insoluble particles in CEF, with little variation in enzyme protein. Ethanol concentration in SSF broth varied (3.6-3.8 wt%), which corresponded to liquid phase (38.1-40.4 g/l). SSF is carried out at 30°C and pH 5 after supplementing broth with soymeal 0.2%,  $(NH_4)_2SO_4$  0.1%,  $KH_2PO_4$  0.05%,  $FeSO_4 \cdot 7H_2O$  0.003%, pretreated baggase and molasses. Extract is transferred to pressure-rated Yeast fermentor. After 42 h (Table 3), extract was separated and analyzed for ethanol using Quantichrom TM Ethanol assay kit, followed by distillation, to increase purity. Distillation is performed to produce 83.6% yield of ethanol<sup>17</sup>. Physical properties and cellulose microstructure of bagasse were

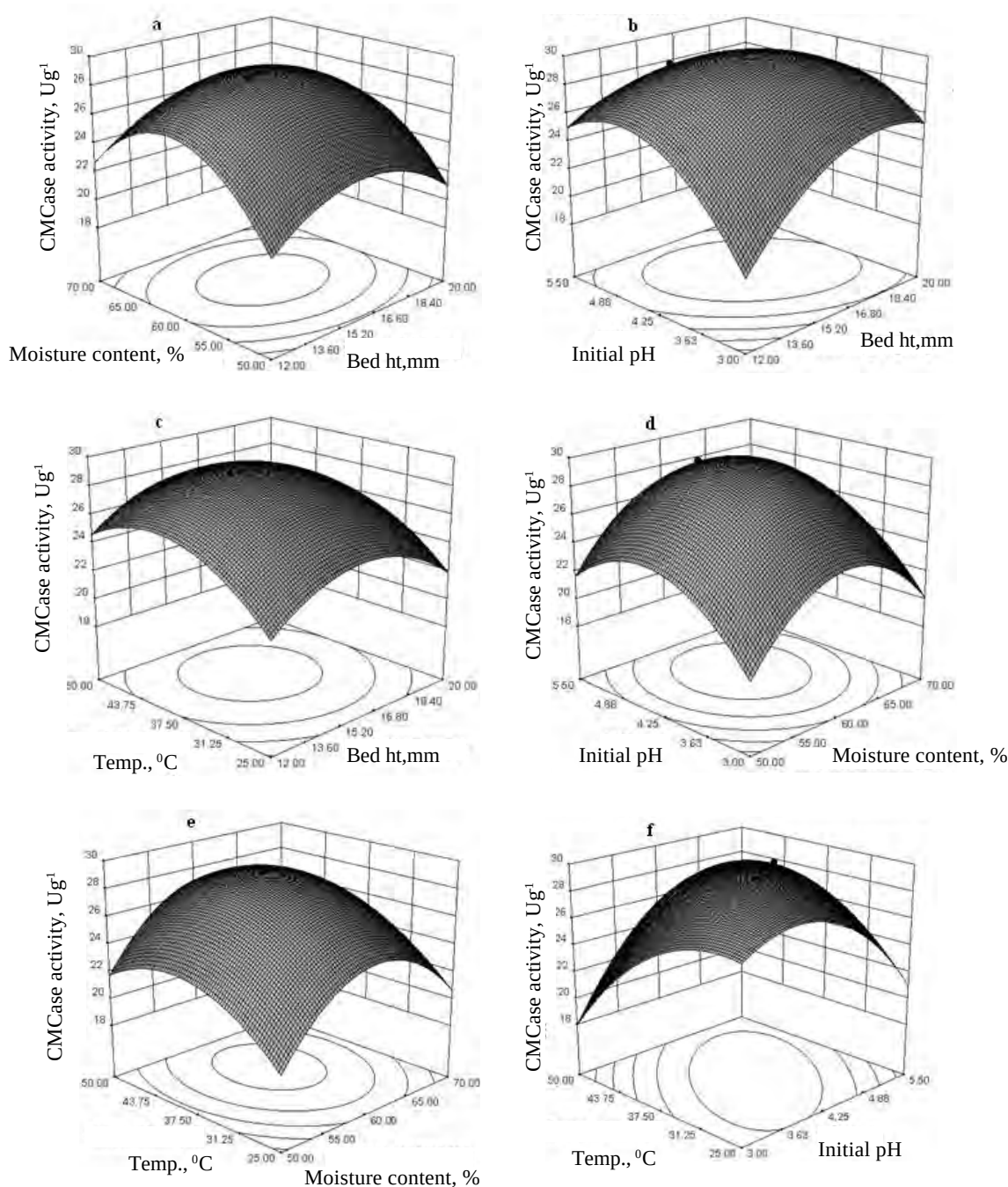


Fig.1—Response surface plots for CMCase : a) moisture content vs bagasse bed height; b) initial pH vs bagasse bed height; c) temperature vs bagasse bed height; d) initial pH vs moisture content; e) temperature vs moisture content; and f) temperature vs initial pH

incredibly modified by pretreatment and enzymatic hydrolysis of bagasse<sup>18</sup>. XRD pattern (Fig. 3) of bagasse samples indicates random orientation for crystalline nature for a cellulose, UB, PB and hydrolysed pretreated bagasse (HPB).

FTIR spectra of bagasse show peaks corresponding to cellulose<sup>19</sup> and lignin<sup>20</sup>. Lignocellulosic composition of

bagasse could be identified between 1600  $\text{cm}^{-1}$  and 1000  $\text{cm}^{-1}$ . In FTIR spectra of bagasse samples (Fig. 4), band intensities of lignin peaks were predominant in UB (1238  $\text{cm}^{-1}$ ). These peaks from 1600  $\text{cm}^{-1}$  to 1000  $\text{cm}^{-1}$  were not apparent in HPB. This region is associated to carbonyl groups including ketones, esters and aldehydes, which are cellulose related groups. The peak at 1735

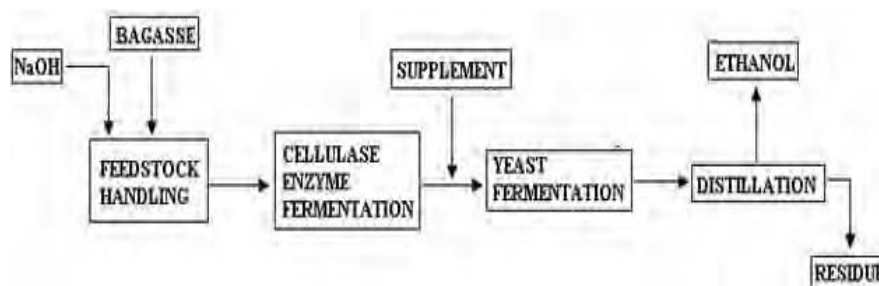


Fig.2—Overall process scheme

Table 3—Time course of ethanol production and biomass depletion

| Time, h | Ethanol, g/l | Yeast biomass, mg/ml |
|---------|--------------|----------------------|
| 4       | 2.14         | 3.15                 |
| 8       | 4.12         | 3.18                 |
| 12      | 5.63         | 3.65                 |
| 16      | 8.21         | 3.24                 |
| 20      | 12.12        | 3.62                 |
| 24      | 18.96        | 3.94                 |
| 28      | 24.51        | 3.99                 |
| 32      | 31.04        | 4.01                 |
| 36      | 38.95        | 4.08                 |
| 40      | 39.47        | 4.15                 |
| 41      | 39.97        | 4.36                 |
| 42      | 40.40        | 4.36                 |
| 43      | 40.38        | 3.21                 |
| 44      | 40.20        | 3.14                 |
| 52      | 21.48        | 2.01                 |
| 60      | 18.96        | 1.01                 |
| 61      | 5.17         | 1.01                 |

$\text{cm}^{-1}$  attributed to C=O stretching of carbonyl and acetyl groups in 4 - O - methyl - glucano acetyl xylan component of hemicellulose in bagasse, disappears on pretreatment and more gamely on hydrolysis.

Band  $1508\text{ cm}^{-1}$ , indicating presence of aromatic rings of lignin, decreases more in intensity on hydrolysis than on pretreatment. Band  $1433\text{ cm}^{-1}$  assigned to  $\text{CH}_2$  symmetrical deformation was absent in UB but appears in bagasse after hydrolysis, indicating change in environment of  $\text{C}_6$  carbon atom due to formation of hydrogen involving atom  $\text{O}_6$ . Peak at  $1267\text{ cm}^{-1}$  bandwidth attributed to C - O - C bond in cellulose chain becomes weak on pretreatment and shifts to  $1298\text{ cm}^{-1}$ , possibly due to depolmerization by alkali treatment. Lignin peak band  $1238\text{ cm}^{-1}$  was seen to shift after alkali pretreatment, proving delignification effect of NaOH and it totally disappeared in samples after hydrolysis.

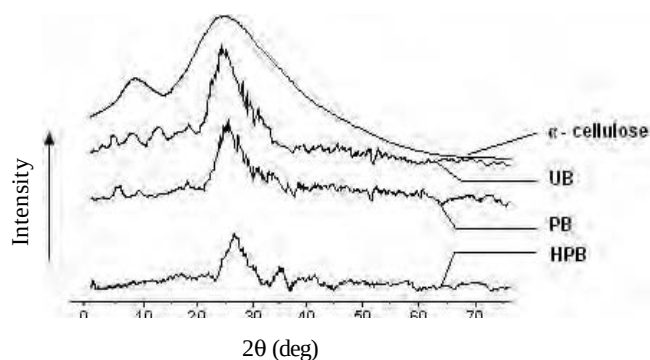


Fig. 3—X-ray diffraction pattern

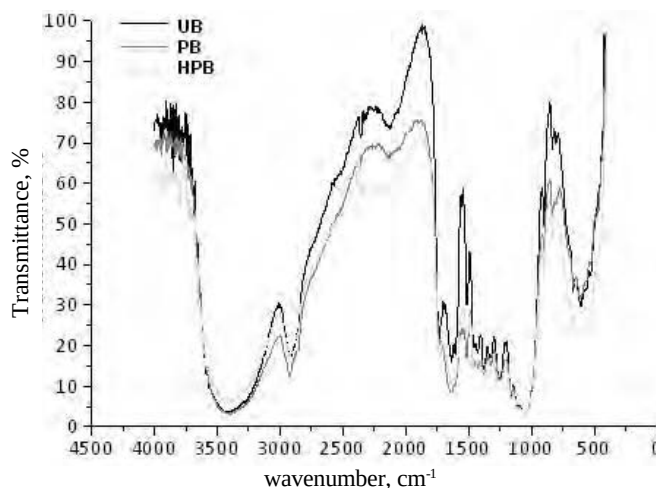


Fig.4—FTIR spectra of bagasse

There was a drastic change in band intensity of cellulose in untreated, pretreated and fungal hydrolyzed samples. A single type of cellulase preferentially endo glucanase may probably be sufficient for degradation of cellulose. Bagasse is made up of highly ordered crystalline region and less structured amorphous region. Bagasse was seen to develop smoothened surface initially (SEM images not shown) and later rough surface possibly due to

accumulation of enzyme in the matrix of pith and removal of the portion of micro fibrils<sup>14</sup>. Increase in surface roughness, however, is relatively small and is found to be localized and very superficial, as expected from long treatment time. A linear reduction in weight change was seen possibly due to roughening of bagasse surface by formation of cavities and stripping of pith wall.

## Conclusions

Validation of statistical model was carried out at  $x_1$ (16.8 g),  $x_2$ (60 %),  $x_3$ (pH 4.25) and  $x_4$ (40°C). Under these conditions, *A. nidulans* MTCC344 produced cellulase (28.80 U/g) activity on 8<sup>th</sup> day after inoculation, in agreement with the model predicted value (28.84 U/g). NaOH pretreatment on bagasse increased feasibility of cellulose exposure to cellulase. *A. nidulans* MTCC344 under solid state conditions, and *S. cerevisiae* under SSF, resulted in 83.6% yield of ethanol. Techniques (alkali pretreatment, RSM, SSF and SSF) fabricated cellulase of desired activity for ethanol production in shorter period and reduced the cost incurred in cellulase and ethanol production.

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