Indian Journal of Experimental Biology
Vol.51, November 2013, pp. 944-953

Mathematical model-based optimization of physico-enzymatic hydrolysis of *Pinus roxburghii* needles for the production of reducing sugars

Siddharth Vats, Devendra Prasad Maurya, Ayushi Jain, Varija Mall & Sangeeta Negi*
Motilal Nehru National Institute of Technology, Allahabad 211004, India

Received 14 January 2013; revised 11 March 2013

The objective of this study was to optimize the physico-enzymatic pretreatment of *P.roxburghii* fallen foliage (needles) to produce reducing sugars through response surface methodology (RSM) with central composite face centered design (CCD). Under this, five parameters, i.e., concentration of laccase, cellulose and xylanase, steam explosion pressure and incubation period, at three levels with twenty six runs were taken into account. Cellulase, xylanase and laccase enzymes with activity 4.563, 38.32 and 0.05 IU/mL, respectively, were produced from locally isolated microbial strains. The analysis of variance (ANOVA) was applied for the validation of the predicted model at 95% of confidence level. This model predicted 334 mg/g release of reducing sugars on treating *P.roxburghii* fallen foliage with 1.18 mL of cellulose, 0.31 mL of xylanase and 0.01 mL of laccase, 14.39 psi steam explosion pressure and 24 h of incubation time. The experimental results obtained were in good agreement to predicted values, making it a reliable optimized model for five factors in combination to predict reducing sugar yield for ethanol production for bio-fuel industry.

Keywords: Cellulase, Enzymatic pre-treatment, Laccase, RSM, Steam explosion, Xylanase.

Energy insecurity, depleting fossil fuel reserves and rise in fuel prices are threatening the growth and economic stability throughout the world. Increasing demand for fuel and compulsion to reduce the greenhouse gas emissions has necessitated the blending of fossil fuels with biofuels. According to the article titled "Bio-fuels mandates around the world" published by "Bio-fuel Digest" dated 21st July, 2011, 60 billion gallons of bio-fuels will be required by major 52 countries including India, China, U.S. and countries of E.U. Second generation bio-fuel is seen as a promising alternative to meet this huge demand. Economics of the second generation biofuel is largely dependent on the availability, quality and cost of the biomass and the processes used. Exploration of the appropriate biomass is a major concern of this sector. Most of the second generation biofuels are obtained from biomass such as bagasse, sorghum and sugarcane tops, switch grass etc. based on the availability of the biomass in nearby area to make the process more economical[1]. But in Asian countries like India where these agricultural wastes are already in use for animal feed and other purposes[2], exploitation of these biomass for biofuel is not practically feasible. Hence, exploration of biomass which is being used neither for human consumption nor for other purposes such as animal feed etc. is necessary as a long time solution. Second generation bio-fuels obtained from non-food crops and lignocellulosic wastes like *Pinus roxburghii* fallen foliage are generating interests among the scientists for being economical and their round the year availability[3]. With this objective, in present study *P. roxburghii* fallen foliage has been explored for optimum yield of reducing sugar.

In lignocellulosic materials, lignin provides structural framework, which holds cellulose and hemicelluloses combined and embedded within it[4]. Lignin layer has to be removed or broken down to make cellulose accessible for lignocellulolytic enzymes. In order to remove or break lignin layer various chemical, physical and biological methods have been employed by various researchers[1]. A maximum ethanol titer of 47.4 g/L was achieved from lodge pole wood chips pre-treated by sulphite pre-treatment to overcome recalcitrance of lignocelluloses (SPORL) at 25% solid loading by Lan *et al.*[5]. A live tree and a beetle killed lodge pole pine tree (four years after infestation) were explored for ethanol yields of 200 and 250 L/metric ton wood by

*Correspondent author
Telephone: +919415015811
Fax: +91-532-2271200.
E-mail: sn5@mnnit.ac.in

Zhu *et al.*[6]. Similarly, Zhu *et al.*[7] achieved 37% sugar from SPORL pre-treated spruce chips and red pine with 8-10% bisulfite and 1.8-3.7% sulphuric acid on oven dry wood at 180 °C for 30 min. Biological pre-treatment generates fewer amounts of inhibitors compared to physico-chemical and chemical pre-treatment methods[8]. Secondary metabolites may also act as inhibitors in *P. roxburghii* fallen foliage[9,10]. Hence, enzymatic digestion can be a better option than microbial digestion as it can enhance the process specifically and help in release of large amount of reducing sugars. However, most of the reported works in this area are on ethanol production from pine wood or chip, whereas in the present study *P. roxburghii* fallen foliage was explored, which otherwise remains in the forest as waste and very often leads to devastating wildfire[4,11-13]. With *P. roxburghii* fallen foliage there is no issue of non-availability as *Pinus* grow wild in Himalayan ranges on a wide area, at latitudes 26˚N to 36˚N and longitudes 71˚E to 93˚E [14]. For pretreatment optimization various methods are in use. However, optimization of biological pre-treatment with approaches such as "One variable at one time" is quite tedious job and disregards the complex interactions taking place among different parameters[15]. Out of the various new statistical optimization methods, Response surface methodology (RSM) combines mathematical and statistical techniques for analysing the problems, where several independent variables have control on dependent variable[16,17].

In the present study pine forest waste i.e. *Pinus* fallen foliage was used as biomass for the yield of reducing sugar. Optimization of pre-treatment was done through step wise experimental strategy. At first, wet lab experiments were conducted and based on these data screening of most significant factors was done, followed by optimization of significant components. A mathematical model was generated with all possible relations among optimized factors like steam pressure, cellulase, laccase, xylanase in various ratios and incubation time for maximizing sugar release.

Materials and Methods

Chemicals and materials—All chemicals used in this study were of analytical grade and purchased from Hi-Media and S. D. Fine Chemicals locally. Agricultural wastes (wheat bran, rice bran etc.) were collected from local market for enzyme production. *Pinus roxburghii* fallen foliage were collected from the forests of Himalayan range in Uttranchal state of India.

Isolation of microbes for production of ligno-cellulolytic enzymes—Compost rich soil samples (5g) collected from the decomposing site around the Instiute. Soil samples collected were mixed together, crushed and 1 g of this final mixture was taken in 10 mL of distilled water and serial dilution was performed. Water (200 µL) from each test tube was taken and then spread over the plates with respective screening media[18-20]. Enzymes were produced through solid state fermentation by isolated microorganism using different agro waste residues and extraction of xylanase, cellulase and laccase was done in acetate buffer of *p*H 5-6[16] , 0.05 M citrate buffer (*p*H 4.8)[17] and 100 m*M* potassium phosphate buffer (*p*H 6.5) respectively. The crude extract of xylanase, cellulase and laccase were used for further study.

Enzyme unit—Enzyme activity was calculated in units/mL which is defined as the amount of enzyme catalysing the production of one µmole of coloured product/mL/min. Cellulase, xylanase and laccase enzymes with activity (4.563 IU/mL), (38.32 IU/mL) and (0.05 IU/mL), respectively, were produced from locally isolated microbes on agricultural wastes as substrates by solid state fermentation.

Biomass (*Pinus roxburghii fallen foliage) preparation and steam explosion*—*Pinus roxburghii* fallen foliage was cleaned manually, cut into suitable size, dried in hot air oven at 40 °C, crushed into powdered form and passed through 1 mm sieve. Fallen foliage (1 g) soaked with 1 mL of distilled water was taken in Erlenmeyer flasks of 50 mL for pre-treatment. Steam explosion was carried out at 121 °C, maintained for 8 min and then steam was allowed to release suddenly via opening the steam exit nozzle to perform explosive decompression. After pre-treatment the solid cellulosic residues were collected and washed with running water to remove inhibitor and to neutralize *p*H [17], which was followed by drying in the hot air oven at 40 °C. This dry powder was then subjected to enzymatic pre-treatment. 0.5 g dry substrate/15 mL of 100 mM sodium phosphate buffer of *p*H 6.0[21] was taken and crude enzymes were added to it according in the particular volume as predicted by RSM for the hydrolysis[22-24] in 100 mL Erlenmeyer flasks and kept in incubator shaker at 120 rpm for 12 h at 50 °C [25].

Experiment description—In the present study, optimization was done through step wise

experimental strategy. First few wet lab experiments were conducted and based on them screening of most significant factors was done followed by optimization of significant components. Based on this, a mathematical model was generated with all possible relations among optimized factors like steam pressure, cellulase, laccase, xylanase using different ratio and incubation time for maximizing sugar release. Parameters involved were enzymes in different ratios and different incubation time, ranging from 24-72 h for hydrolysis. From the experiment conducted at lab scale, maximum amount of reducing sugar released was 44.82 µmol/mL. To modulate experimental set up, ranges were set from the experimental results and fed into the software, *Design Expert® 8.0.7.1.* (Stat-Ease, Inc., Minneapolis). The ranges for different parameters were fed in the software as shown in the Table.1. Then a model has been generated through software on the basis of results of wet lab experiments (Table 2). Analysis of variance was performed based on which a mathematical model was developed, showing both coded and actual value equations (Table 3). To optimize the parameters, software was assigned priority values for each factor and based on this, software predicted values for RS release at different set of conditions. From the observations, the best values for maximum sugar released were selected for least inputs.

Developing Matrix and empirical relationship— For all evaluations software *Design expert 8.0.7.1* was used. Using response surface methodology, the five parameters i.e. concentration of laccase, cellualse, xylanase, steam pressure and incubation time for enzymatic actions were optimized. Representation of independent factors in quantitative form can be given as

$$Y= Ø (x_1, x_2, ----------x_k) \pm e_r \quad \ldots(1)$$

where, Y is response and x_1, x_2, x_3--------x_k are quantitative factors, e_r is measure of experimental error. Ø represents response functions. Representing the RS (Reducing sugars) as function of A, B, C, D and E:

$$RS=f(A, B, C, D, E).$$

The second order polynomial (regression) equation used to represent the response surface (reducing sugar) RS:

$$Y= b_0+\sum b_i x_i + \sum b_{ii} x^2_i + \sum b_{ij} x_i x_j + e_r \quad \ldots(2)$$

RS for five factors, the selected polynomial could be expressed as:

$$RS= b_0 + b_1(A) + b_2(B)+ b_3(C) + b_4(D) + b_5(E) + b_{11}(A^2) + b_{22}(B^2) + b_{33}(C^2) + b_{44}(D^2) + b_{55}(E^2) + b_{12}(AB) + b_{13}(AC) + b_{14}(AD) + b_{15}(AE) + b_{23}(BC) + b_{24}(BD) + b_{25}(BE) + b_{34}(CD) + b_{35}(CE) + b_{45}(DE) \quad \ldots(3)$$

To find out regression coefficients for reducing sugar released based on CCD (Tables 3 and 4), ANOVA was performed. All the variables were denoted with numerical factors and investigated for intervals designated as -1 for low level and +1 for high level. Table 2 shows the input values generated by applying RSM and output responses obtained from practical performed. Final equations for reducing sugar released were obtained by the use of *Design-Expert Version 8.0.7.1* software at 95% confidence level and the regression coefficients for the second order polynomial regression model were calculated from experimental data generated as shown in the Table 2.

Results

RSM results

Central Composite Design—All the coefficeints were obtained by applying central composite design, using the Design Expert Statistical software package

Table 1—Coded and actual values of parameters

Parameters	Units	Code		Factor levels			
			-2.378	-1	0	+1	+2.378
Steam explosion Pressure (A)	(Psi)	A	13.3108	14	14.5	15	15.6892
Cellulase(B)	(mL)	B	0.3107931	1	1.52	2	2.68921
laccase(C)	(mL)	C	-0.689207	0	0.52	1	1.68921
xylanase(D)	(mL)	D	-0.689207	0	0.52	1	1.68921
Incubation time (E)	(h)	E	-0.908194	24	43.85	72	105.082

*Study type = Response surface; Design type = Central composite; Runs = 26;
Blocks = 0; Design mode =linear for RS

Table 2—Experimental design matrix and results for Sugar released (RS) from pine needles.

	Input parameters				Output			
Serial no.	Standard	Run	Steam explosion	Cellulase (mL)	Laccase (mL)	Xylanase (mL)	Time (h)	Total sugar released (mg/g)
1	3	1	-1	+1	+1	-1	72	79.59
2	15	2	0	+1	0	0	48	112.86
3	18	3	0	0	0	-1	48	297
4	6	4	+1	-1	-1	+1	72	222.75
5	5	5	+1	+1	-1	-1	72	60.588
6	10	6	-1	+1	+1	+1	24	247.59
7	11	7	-1	-1	-1	-1	24	231.39
8	12	8	-1	0	0	0	48	164.241
9	8	9	-1	+1	-1	+1	72	58.80
10	7	10	-1	-1	+1	+1	72	66.96
11	14	11	0	-1	0	0	48	123.3
12	2	12	+1	-1	+1	+1	24	228.15
13	22	13	0	0	0	0	48	103.9
14	13	14	+1	0	0	0	48	118.47
15	1	15	+1	+1	-1	+1	24	202.55
16	23	16	0	0	0	0	48	121.44
17	25	17	0	0	0	0	48	94.5
18	20	18	0	0	0	0	24	242.02
19	17	19	0	0	+1	0	48	90.45
20	24	20	0	0	0	0	48	81.54
21	21	21	0	0	0	0	72	181.76
22	4	22	+1	+1	+1	-1	24	231.06
23	19	23	0	0	0	+1	48	277.56
24	16	24	0	0	-1	0	12	344.52
25	9	25	+1	-1	+1	-1	12	218.26
26	26	26	+1	0	0	0	12	199.09

(Stat Ease, Minneapolis, MN), at 95% confidence level. Significant coefficients were determined for finding final empirical relationships among various parameters to estimate reducing sugar (RS) released. While substituting the values in the quadratic term it generates results in a new quadratic coefficients and correction in the intercept was noted. In case of mixture of design, coded equations were determined first and then from these values the actual equation values were obtained by replacing each term in coded equations with the formula:

$$X\ coded = \frac{X_{actual} - \overline{X}}{(X_{high} - X_{low})/2} \quad \ldots(4)$$

Substitution in the quadratic term will generate result in a new quadratic coefficients and correction in the intercept.

Final equation in terms of coded and actual factors—The responses obtained for each experimental run performed according to RSM, CCD were analyzed by multiple regression analysis and polynomial equation was derived.

Reducing sugar released = +3595.66-31805.16 * B+16739.46 * C+20233.30* D+35302.02* E+1637.51 * B * C+202.08*B*D-33255.52*B*E-67.87*C*D+ 15016.55 *C *E +19967.35 *D*E-160.37* D^2+33279.46* E^2.

Final equation in terms of actual factors — Reducing sugar released = +277.41217 +65732.56710*cellulase-36276.60360*laccase- 9838.05968 *xylanase-1376.38172*time+ 6550.04558*cellulase* laccase+ 808.32451* cellulase* xylanase-771.29* cellulase*time-271.489*laccase*xylanase+ 1251.37* laccase*time+ 1663.94* xylanase*time-641.48730* $xylanase^2$+57.77684* $time^2$.

At first one factorial wet lab experiments were perfomed to get the ranges. The range identified were: pressure range 14-15 psi for steam explosion;

0-1 mL volume of xylanase and laccase enzyme; 0-2 mL volume of cellulase; incubation time from 24-72 h. Statistical model was evaluated by the F-test for analysis of variance (ANOVA) (Table 3). The Model F-value of 2.58 implies that there was 5.17% chance of "Model F-Value" this large could occur due to noise. *P* value for the model was 0.0498. Values of "Prob > F" less than 0.0500 indicate model or model terms were significant. In this case model and model terms B, C, D, E, BC, BD, BE, CD, CE, DE, D^2, E^2 were significant. Values greater than 0.1000 indicated that the model terms were not significant. If there were many insignificant model terms (not counting those required to support hierarchy) then model reduction would improve the model. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. This model had a ratio of 6.914 which indicates an adequate signal. This model can be used to navigate the design space. Other values were like Standard deviation (Std. Dev) of (11.35), R-Squared (0.7041), Mean (31.26), Adjusted R-Squared (0.4311), C.V. % (36.32), Pred R-Squared (NA), PRESS value (NA) and Adeq Precision (6.14).

Optimization of the parameters—Contour plots expressed different circular and distinctive shapes which were indicator of possible independence of various factors with response. Contour plot helped in finding the region of optimal factor settings and this was done by visually displaying that region. In this study the darker the colour more optimum points would be lying in that region. Contour plots played important role in studying, characterizing and identifying stationary point found either as maximum or minimum response. Like contour plots, perturbation plots also illustrated the effect of different parameters on the sugar release. Perturbation plots showed the effect on the optimized design and the effect on response of movement of each factors from a chosen saddle point or reference point. Steep slope or curvature indicated response sensitivity towards that factor. The model represented in

Table 3—ANOVA for response surface model (responses: RS, reducing sugar), quadratic model

Source	Sum of squares	D_f	Mean square	F Value	Prob> F	Significant / non-significant
Model	3987.793	12	332.3161	2.578404	0.0498*	Significant
B-cellulase	954.0333	1	954.0333	7.40224	0.0175	Significant
C-laccase	1180.243	1	1180.243	9.157379	0.0097	Significant
D-xylanase	1040.004	1	1040.004	8.069281	0.0139	Significant
E-time	1121.863	1	1121.863	8.704413	0.0113	Significant
BC	1018.216	1	1018.216	7.900229	0.0147	Significant
BD	920.7654	1	920.7654	7.144117	0.0192	Significant
BE	952.4411	1	952.4411	7.389886	0.0176	Significant
CD	1287.22	1	1287.22	9.987402	0.0075	Significant
CE	1067.237	1	1067.237	8.280576	0.0130	Significant
DE	1018.994	1	1018.994	7.906265	0.0147	Significant
D^2	1529.815	1	1529.815	11.86967	0.0043	Significant
E^2	952.5633	1	952.5633	7.390834	0.0176	Significant
Residual	1675.497	13	128.8844			
Cor Total	5663.291	25				
Std. Deviation	11.3527					
Mean	31.2567					
C.V. %	36.320					
PRESS	NA					
R-square	0.7045					
Adj. RS	0.43105					
Pred. RS	N/A					
Adeq. Pred.	6.9136					

[B]A-Steam explosion pressure, B-Cellulase enz, C-Laccase enz, D-Xylanase enz, E-incubation time, df= degree of freedom, F-fisher,s ratio P-probability.

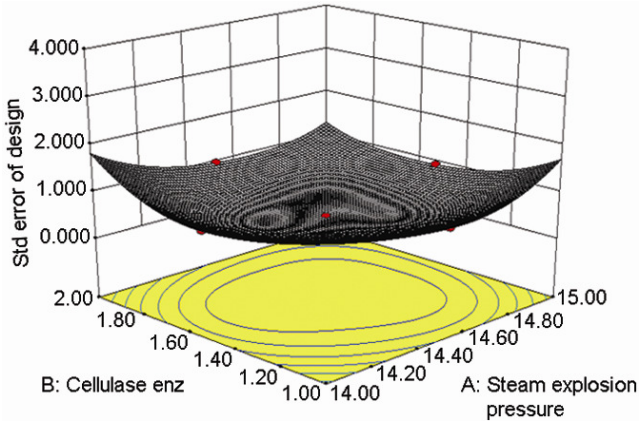

Fig .1—Final RSM model based on two sensitive parameter.

Fig. 1 was final model which has been generated after analysis of interaction of all parameters and optimization. Steam explosion pressure is on X axis, cellulase enzyme is on Z axis and standard error to design is shown on Y axis. Other impotant plots are: Fig. 2a representing normal probability plot of experimental versus predicted reducing sugar released; Fig. 2b depicting normal probability plot of reducing sugar release; Fig. 2c perturbation plot showing the effect of all factors on reducing sugar released; Fig. 2d a graphical representation showing desirability for reducing sugar released based on interaction between cellulase and laccase. Optimum factors predicted by the model were 14.39 psi steam explosion pressure for pretreatment, 1.18 mL of cellulose, 0.31 mL of xylanase and 0.01 mL of laccase for hydrolysis and 24 h of incubation time. Optimum release of reducing sugar was 334mg/g from *P. roxburghii* fallen foliage (Table 4).

Discussion

Biofuel produced from lignocellulosic and cellulosic biomass can potentially reduce the green house gas emission up to almost 90%[2]. To fully exploit the *P. roxburghii* fallen foliage for release of reducing sugar for biofuels, it's necessary to disturb the crystaline arrangement of the lignin, hemicellulose and cellulose of *Pinus roxburghii* fallen foliage just like other lignocellulosic wastes[1]. Use of chemical pretreatment strategy like dilute or concentrated acids for hydrolysis lead to production of furfural, hydroxyl methyl furfural and phenolic acids which at concentration more than 5 mM act as inhibitors and have serious negative effect on fermentation process[26,27]. Contrary to biological pre-treatment, chemical pre-treatment involve use of large amount of water and energy which adds to the cost[1]. Use of steam explosion was found much more economical for biomass like *Pinus* foliage, which are treated with high pressure steam and then suddenly the pressure was allowed to reduce leading to explosive decompression of lignocellulose. The change in the structure of the biomass might be one of the reasons for enhanced release of sugar after pre-treatment. Steam explosion disturbs the crystalline arrangement and breaks the inter and intra hydrogen bonding of cellulose fibrils and make them more prone to enzymatic hydrolysis[28,29]. It was also noted that during steam explosion disturbance in the lignin arrangement prevent enzyme from binding to the outer surface and provide enzymes access to the cellulose only[30]. Optimal hemicelluloses solubilization and pre-treatment had been achieved at either high temperature or short holding time (270 °C, 1 min) or at low temperature and high holding time (190 °C, 10 min)[1]. It was also important to note that at high temperature and high pressure (more than 1 bar) cellulose gets degraded to glucose and this glucose is lost during neutralization process. It was important to make a balance between proper pre-treatment temperatures and pressure for least loss of glucose. Based on the understanding developed from previous studies, it was found that statistical approach can be employed for optimization for hydrolysis of steam exploded fallen foliage for release of reducing sugar. Sindhu *et al*[31]. studied surfactant assisted acid pre-treatment of sugarcane tops for bioethanol production with the help of Box-Benkhen Design and 0.798 g/g reducing sugar was obtained from sugarcane tops. Phuengjayaem and Teeradakorn[32] employed RSM based optimization for saccharification of acid pretreated sweet sorghum straw by cellulase for bioethanol production and a yeild of 0.366 g/g dry substrate was obtained, at optimum conditions i.e. temperature range from 30-50 ºC for 96 h of incubation and *p*H 3-5. RSM based statistical tool was used for optimizing enzymatic hydrolysis of alkaline pretreated peroxide wheat straw by Qi *et al.* where conditions like cellulase loading 40 FPU/g, substrate concentration 22 g/L, surfactant concentration 6.676 g/L with hydrolysis time of 72 h were found optimum[33]. Janu *et al.* observed physicochemical changes during alkali pre-treatment and optimised saccharification of bagasse for improving sugar yields by Box Behnken Design[25]. In present study RSM, CCD design was

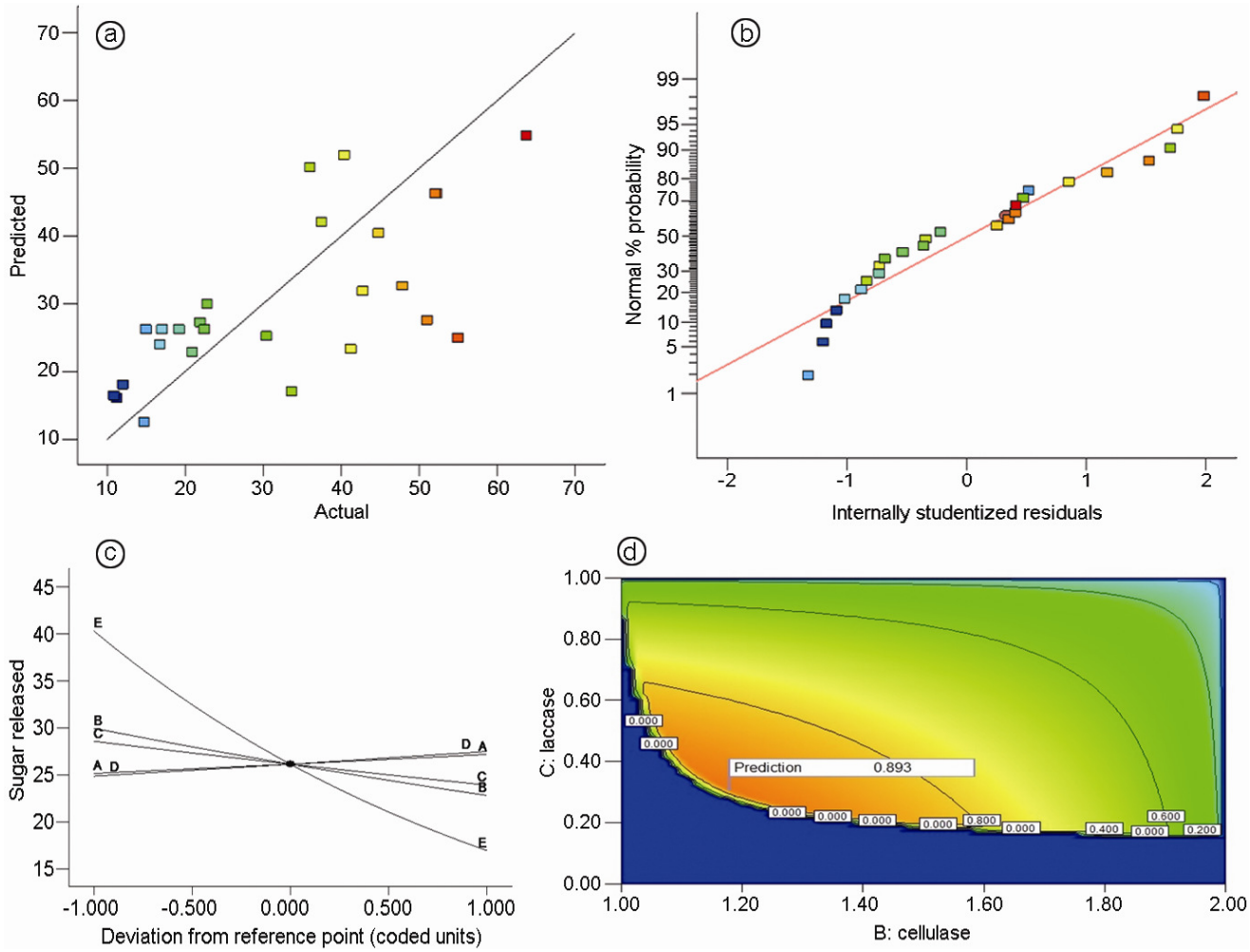

Fig. 2—a: Normal probability plot of experimental versus predicted reducing sugar release; b: normal probability plot of reducing sugar release; c: perturbation plot showing the effect of all factors on reducing sugar release; d: graph of desirability for maximum sugar release based on interaction between laccase and cellulase enzymes.

Table 4—Predicted values at different set of conditions

Pressure*	Cellulase	Laccase	Xylanase	Time	RS[c]	Desirability	
14.49	1.18	0.31	0.01	24.00	344.571	0.892661	SELECTED
14.28	1.18	0.31	0.01	24.00	344.525	0.892658	
14.77	1.18	0.31	0.02	24.00	344.556	0.892654	
14.45	1.17	0.31	0.00	24.00	344.525	0.892544	
14.00	1.21	0.29	0.03	24.00	344.514	0.891566	
15.00	1.14	0.25	0.15	24.00	344.604	0.888055	

RS[c]= Predicted values of reducing sugars (mg/g).

used to optimize the hydrolysis conditions for enhancing the release of reducing sugars from the fallen foliage. Synergistic effect of lignocellulosic degrading enzymes like cellulase, xylanase and laccase was stuided and it was found that 1.5 mL of total crude enzyme at 24 h incubation period (Table 4) sufficiently hydrolysed the substrate and attained maximum sugar release of 344 mg/g dry weight of substrate. Top five solutions provided by the software (Table. 4) have very similar levels of parameters and less error, suggesting that the model generated in this study can be applied for studying the hydrolyzing of steam exploded fallen foliage and other agricultural wastes for production of reducing sugars for bio-ethanol. Steam explosion of fallen foliage was carried out by suddenly steam release to promote hydrolysis

of hemicelluloses which lead to biotransformation of lignin. It has been reported that hemicelluloses of lignocellulose undergoes hydrolysis because of the various acids, such as acetic acids, generated during steam explosion[34-36]. Enzymes such as cellulase are costly biocatalyst and accounts for a major portion of total cost for cellulosic ethanol production (30-100 cents/gallon ethanol)[37]. Enzymes production from agro wastes can bring down the cost, hence in this study enzymes were produced from microbes reared on agricultural wastes by solid state fermentation (SSF). Kobayashi *et al.*[38] employed steam explosion for pretreatment of bamboo forest waste for methane production. Similarly, in the present study *Pinus* fallen foliage was used as substrate and it was noted that particle size of 2-5 mm undergoes better pretreatment. It can be because large particle size provides enough surface area for steam to work during pretreatment and enzymes to attach for saccharification. Ballestros *et al.*[39] observed that cellulase enzymatic hydrolysis followed by steam explosion based pre-treatment with 15 FPU/g of substrate, 150 rpm for 72 h and 2% (w/v) substrate concentration at 50 °C yielded 72% sugar. But time period of 72 h was comparatively higher for enzymatic hydrolysis[39]. Cara *et al.*[40] employed steam explosion on water and sulphuric acid soaked olive tree pruning biomass at various temperature ranging from 190-240 °C while Ruiz *et al.*[41] used only water based steam explosion for pretreatment of wheat straw[41]. But in the present study steam exploded biomass has been pretreated based on the setup suggested by the RSM. Results obtained from this study have strengthened the fact that RSM was an efficient tool to optimize agricultural waste hydrolysis with enzymes and to obtain higher sugar yields. Same approach can be applied to other substrates also. fallen foliage as substrate to produce reducing sugar from lignocellulose can be a promising preposition due to its abundance and throughout the year availability. Moreover, presence of various phytochemicals in fallen foliage can also turn this substrate as a source of pharmaceutically value added products. This model predicted minimum use of crude enzymes and optimum use of steam pressure for minimal incubation period to enhance reducing sugar release, which makes the process economical and sustainable.

Conclusions

From the present study it is concluded that physico-enzymatic pretreatment strategies are better than conventional means for being cost effective and eco-friendly. Biomass like *Pinus roxburghii* fallen foliage can be exploited instead of sugar crops without compromising with the land use and human-animal feed. Use of mathematical tools like RSM for developing an efficient strategy based on various parameters can be economical and faster. Though the yield of sugar was low (344 mg/g) the availability in abundance, cheap and throughout the year makes *P. roxburghii* fallen foliage a promising prospect. Use of reducing sugar for bioethanol production can contribute considerably to meet the growing global energy requirements.

References

1 Kumar P, Barrett DM, Delwiche MJ & Stroeve P, Methods for pre-treatment of lignocellulosic biomass for efficient hydrolysis and biofuel production, *Ind. Eng. Chem. Res*. 48(8) (2009) 3713.

2 Wang M, Wu M & Huo H, Life-cycle energy and greenhouse gas emission impacts of different corn ethanol plant types, *Environ. Res. Lett*. 2 (2007).

3 Näsholm T & Ericsson A, Seasonal changes in amino acids, protein and total nitrogen in needles of fertilized Scots pine trees, *Tree Physiol*, 6(3) (1990) 267.

4 Faulon J, Carlson GA & Hatcher PG, A three dimensional model for lignocellulosic from gymnospermous wood, *Org. Geochem*, 21(12) 1994 1169-79.

5 Lan TQ, Gleisner R, Zhu J Y, Dien BS & Hector R, High titer ethanol production from SPORL-pretreated lodgepole pine by simultaneous enzymatic saccharification and combined fermentation, *Bioresour Technol*, 127 (2013) 291.

6 Zhu JY, Zhu W, Obryan P, Dien BS, Tian S, Gleisner R & Pan XJ, Ethanol production from SPORL-pretreatedlodgepole pine: preliminary evaluation of mass balance and process energy efficiency, *App. Microbiol. Biotechnol*, 86(5) (2010) 1355.

7 Zhu JY, Pan XJ, Wang GS & Gleisner R, Sulfite pretreatment (SPORL) for robust enzymatic saccharification of spruce and red pine, *Bioresour. Technol*, 100(8) (2009) 2411.

8 Ratola N, Lacorte S, Alves A & Barceló D, Analysis of polycyclic aromatic hydrocarbons in pine needles by gas chromatography–mass spectrometry: Comparison of different extractionand clean-up procedures, *J. Chromatogr*. A 1114(2) (2006) 198.

9 Kainulainen P & Holopainen JK, Concentrations of secondary compounds in Scots pine needles at different stages of decomposition, *Soil Biol. Biochem*, 34(1) 3(2002) 7.

10 Berg B, Hannus K, Popoff T & Theander O, Changes in organic chemical components of needle litter during decomposition. Long-term decomposition in a Scots pine forest I, *Can. J. Bot*, 60(8) (1982) 1310.

11 Hamelinck CN, Hooijonk GV & Faaji APC, Ethanol from lignocellulosic biomass: Techno-economic performances in short-, middle- and long term, *Biomass Bioenerg*, 28 (2005) 384.

12 James LF, Call JW & Stevenson AH, Experimentally induced pine needle abortion in range cattle, *Cornell. Vet*, 67(2) (1997) 294.

13 Stuart LD, James LF, Panter KE, Call JW & Short RE, Pine needle abortion in cattle: Pathological observations, *Cornell. Vet*, 79 (1989) 61.

14 Gupta B, Mehta R & Mishra VK, Fire ecology of ground vegetation in *Pinus roxburghii* sergeant plantations in north-west Himalaya-floristic composition and species diversity, *Caspian J. Env. Sci*, 7(2) (2009) 71.

15 Negi S, & Banerjee R, Characterization of amylase and protease produced by Aspergillus awamori in a single bioreactor, *Food Res. Inter*, 42(4), (2009) 443.

16 Cochran WG & Cox GM, *Experimental designs*, 2nd edn. (John Wiley and sons New York,) 1900, 335.

17 Rajakumar S, Muralidharan C & Balasubramanian V, Optimization of the friction-stir-welding process and tool parameters to attain a maximum tensile strength of AA7075–T6 aluminium alloy, *Proc. Inst. Mech. Eng*. Pt. B. J, 224(8) (2010) 1175-91.

18 Buddolla V, Chandra MS, Pallavi H & Reddy BR, Screening and assessment of laccase producing fungi isolated from different environmental samples, *Afr. J. Biotechnol*, 7(8) (2008) 1129-33.

19 Kamble RD & Jadhav AR, Xylanase production under solid state and submerged fermentation conditions by bacterial strains, *Afr. J. Microbiol. Res*. 6(20) (2012) 4292.

20 Bajaj BK, Pangotra H, Wani MA, Sharma A & Sharma P, Characterization of thermo- tolerant and acid/alkali toleran β-glucosidase from bacterial isolate M+, *J. Sci. Ind. Res*, 68 (2009) 242.

21 Waeonukul, R, Kyu KL, Sakka K, & Ratanakhanokchai K. Isolation and characterization of a multienzyme complex (cellulosome) of the *Paenibacillus curdlanolyticus* B-6 grown on Avicel under aerobic conditions, *J. Biosci. Bioeng*, 107(6), (2009) 610.

22 Ghose TK & BisariaVS, Measurement of hemicellulase activities part 1: Xylanases, *Pure Appl. Chem*, 59(12) (1987) 1739.

23 Ghose TK, Measurement of cellulase activities, *Pure Appl. Chem*. 59(2) (1987) 257.

24 Jadhav A, Vamsi KK, Khairnar Y, Boraste A, Trivedi S, Patil P, Gupta G, Gupta M, Mujapara AK, Joshi B & Mishra D, Optimization of production and partial purification of laccase by *Phanerochaetechrysosporium* using submerged fermentation, *Int. J. Microbio. Res*. 1(2) (2009) 09.

25 Janu KU, Sindh R, Binod P, Kuttiraja M, Sukumaran, RK & Pandey A, Studies on physicochemical changes during alkali pre-treatment and optimization of hydrolysis conditions to improve sugar yield from bagasse *J. Sci. Ind. Res*, 70 (2011) 952.

26 Klinke HB, Thomsen AB & Ahring BK, Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass, *Appl. Microbio. Biotechnol*, 66(1), (2004) 10.

27 Martinez A, Rodriguez ME, York SW, Preston JF & Ingram LO, Use of UV absorbance to monitor furans in dilute acid hydrolysates of biomass, *Biotechnol. Prog*, 16(4) (2008) 637

28 Kim TH & Lee YY, Pre-treatment and fractionation of corn stover by ammonia recycle percolation process. Bioresour. Technol, 96(18) (2005) 2007.

29 Jeoh T, Ishizawa CI, Davis MF, Himmel ME, Adney WS & Johnson DK, Cellulase digestibility of pretreated biomass is limited by cellulose accessibility, *Biotechnol. Bioeng*. 98(1) (2007) 112.

30 Kumar R & Wyman CE, Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pre-treatment technologies, *Bioresour Technol*, 100 (18) (2009) 4203.

31 Sindhu R, Kuttiraja M, Binod P, Preeti VE, Sandhya SV, Vani S, Sukumaran RK, Pandey, A surfactant-assisted acid pre-treatment of sugarcane tops for bioethanol production, *Appl. Biochem. Biotechnol*, (2012) 1.

32 Phuengjayaem S & Teeradakorn S, *Saccharification of acid-pretreated sweet sorghum straw by cellulase for bioethanol production*. In Proceedings of International Conference on Asia Agriculture and Animal (ICAAA) 2011.

33 Qi B, Chen X, Shen F, Su Y & Wan Y, Optimization of enzymatic hydrolysis of wheat straw pretreated by alkaline peroxide using response surface methodology, *Ind. Engg. Chem. Res*, 48(15) (2009) 7346.

34 Weil JR, Sarikaya A, Rau SL, Goetz J, Ladisch CM, Brewer M, Hendrickson R & Ladisch, MR, Pretreatment of yellow poplar sawdust by pressure cooking in water, *Appl. Biochem. Biotechno*. 68(1-2) (1997) 21.

35 Baugh KD, Levy JA & McCarty PL, Thermochemical pretreatment of lignocellulose to enhance methane fermentation: I. Monosaccharide and furfurals hydrothermal decomposition and product formation Rates, *Biotechnol. Bioeng*. 31 (1998) 50.

36 Morjanoff PJ & Gray PP, Optimization of steam explosion as method for increasing susceptibility of sugarcane bagasse to enzymatic saccharification, *Biotechnol. Bioengg*. 29 (1987) 733.

37 Zhang YHP, Himmel M & Mielenz JR, Outlook for cellulose improvement: Screening and selcection strategies, *Biotechnol. Adv*, 24(5) (2006) 452.

38 Kobayashi F, Take H, Asada C & Nakamura Y, Methane production from steam-exploded bamboo, *J. Biosci. Bioeng*, 97(6) (2004). 426.

39 Ballesteros MJ, Oliva I, Negro MJ Manzanares P & Ballesteros M, Enzymic hydrolysis of steam exploded herbaceous agricultural waste (Brassica carinata) at different particle sizes, *Proc. Biochem*, 38 (2002) 187.

40 Cara C, Ruiz C, Ballesteros M, Manzanares P, Negro MJ & Castro E, Production of fuel ethanol from steam-explosion pretreated olive tree pruning, *Fuel*, 87(2008) 692.

41 Ruiz E, Cara C, Manzanares P, Ballesteros M & Castro E, Evaluation of steam explosion pre-treatment for enzymatic hydrolysis of sunflower stalks, *Enzyme Microb. Tech*, 42(2) (2008) 160.

42 Gottschalk LMF, Oliveira RO & Bon EPS, Cellulases, xylanases, bglucosidaseand ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse, *Biochem. Engg*. J, 51 (2010) 72.

43 Fang H, Zhao C & Song XY, Optimization of enzymatic hydrolysis of steam exploded corn stover by two approaches:

response surface methodology or using cellulase from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* NL02, *Bioresour. Technol*, 101 (2010) 4111.

44 Maeda R N, Serpa VI, Rocha VAL, Mesquita RAA, Anna LMMS Castro AM, Driemeier CE, Pereira N & Polikarpov I, Enzymatic hydrolysis of pretreated sugarcane bagasse using *Penicillium funiculosum* and *Trichoderma harzianum* cellulases, *Process Biochem*, 46 (2011) 1196.

45 Singh A & Bishnoi NR Enzymatic hydrolysis optimization of microwave alkali pretreated wheat straw and ethanol production by yeast, *Bioresour. Technol*, 108 (2012) 94.