STATISTICAL OPTIMIZATION OF NaOH PRETREATMENT OF PINE NEEDLES USING BOX-BEHNKEN DESIGN FOR BIOETHANOL PRODUCTION

MUHAMMAD IRFAN,[*] MISBAH GHAZANFAR,[*] HAFIZ ABDULLAH SHAKIR,[**] MUHAMMAD KHAN,[**] MOHMMED AHMED A. ASIRI,[***] SHAUKAT ALI,[****] IRFAN AHMAD[***] and MARCELO FRANCO[*****]

[*]*Department of Biotechnology, University of Sargodha, Sargodha, Pakistan*
[**]*Institute of Zoology, University of the Punjab, New Campus, Lahore 54590, Pakistan*
[***]*Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, P.O. Box 61413, Abha, 9088, Saudi Arabia*
[****]*Department of Zoology, Government College University, Lahore, Pakistan*
[*****]*Department of Exact Science, State University of Santa Cruz, Ilheus, Brazil*
✉ *Corresponding author: M. Irfan, irfan.ashraf@uos.edu.pk*

Received May 31, 2023

In this study, pine needles were exploited for bioethanol production. Pretreatment is the first and foremost step towards better yield of bioethanol from lignocellulosic biomass. In this study, NaOH pretreatment of pine needles was optimized by the Box Behnken design. Substrate characterization was done by using X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). Maximum cellulose (90%) and total phenolic compounds (51.03 ± 0.002 mM) were recorded under optimized conditions, and structural analysis also revealed the significance of the pretreatment. High F and R^2 values and low P values indicated the accuracy and validity of the model. Pretreated biomass was further subjected to saccharification using commercial, as well as indigenous cellulase. Maximum saccharification (49.2%) was observed with commercial cellulase, which led to a 7% ethanol yield employing *Saccharomyces cerevisiae*. Maximum ethanol yield (7%) was observed in NaOH pretreated biomass. Results proposed that *Pinus* spp. needles could be potential cellulosic biomass for bioethanol production.

Keywords: pretreatment, pine needles, NaOH, fermentation, bioethanol

INTRODUCTION

The intense manipulation of fossil fuels, and its linked ecological risks, such as release of greenhouse gases and climate alteration, have led to exploring routes to achieve substitute and green energy.[1-4] Being abundant and cost-effective, with high sugar content, lignocellulosic biomass is an encouraging feedstock for generating biofuels.[5-8] It consists of cellulose, hemicelluloses and lignin; the strong chemical bonding among these components makes it fairly resistant, preventing its transformation into sugars that can be transformed into biofuels.[9-12]

Three steps, *i.e.* pretreatment, saccharification, and fermentation, are involved in the production of bioethanol.[13] Pretreatment is one of the most important steps in the production of bioethanol, as it reduces the resistance of biomass and cellulose crystallinity, resulting in effective saccharification, and hence improved liberation of fermentable sugars for the synthesis of bioethanol. Multiple pretreatment methods, such as chemical (acid, alkali *etc*.), physical (grinding and thermal), biological, or their combination, have been applied for this purpose.[9,14,15] Alkaline pretreatment is considered a promising approach, as it decomposes uronic acid substitutions and acetyl groups from hemicelluloses in biomass and also degrades lignin into phenolic compounds.[16] Biomass swelling during alkaline pretreatment leads to lignin breakdown and -OH breaks carbon ester bonds between hemicelluloses or cellulose and lignin.[17]

Response surface methodology (RSM) is a statistical tool applied to evaluate the impact of various parameters and their interactions on

productivity. Nowadays, this approach is being extensively employed for optimizing many biotechnological procedures.[18-20]

After pretreatment, cellulose in biomass is hydrolyzed into simple sugars by cellulase enzyme, this process is known as saccharification. Then, the third step is fermentation, in which these sugars are fermented into ethanol by *Saccharomyces cerevisiae*.[21-23] Separate hydrolysis and fermentation (SHF) is a technique in which saccharification and fermentation proceed in separate units under their optimal conditions. The process is less time-consuming, as well as cost-effective.[24,25] *S. cerevisiae* is the most commonly used microorganism among the several studied fermenting microorganisms due to its 90% theoretical yield.[26] In the present work, our research efforts have been directed towards optimizing the conditions for alkaline pretreatment of *Pinus* spp. needles for bioethanol production using the Box-Behnken design (BBD) of RSM.

EXPERIMENTAL

Substrate preparation

Pinus spp. needles were collected from the northern areas of Pakistan. These needles were rinsed with water, dried, milled to powder (2 mm particle size) and saved for later use.[27]

Pretreatment of *Pinus* spp. needles

Ten grams of pine needle substrate was soaked in NaOH solution at the ratio of 1:10 (solid: liquid) at room temperature for 2 h, followed by a steam pretreatment, as per the experimental design. Then, the substrate was filtered and washed up to neutrality.[22]

Structural characterization

The crystallinity index of the control and treated substrates was determined using a Bruker D8 Advance X-Ray Diffractometer (Germany).[13] The dried samples were scanned in the 2θ range from 5° to 60°, using steps of 0.02° in width. Cu/K radiation (1.54 Å) was generated at 40 kV and 40 mA. The estimated crystal grain sizes of (101) and (002) planes were calculated by the well-known Scherer's relation:

$$D = \frac{k\lambda}{W\mathrm{Cos}\theta} \quad (1)$$

where D is the crystallite size, θ is Bragg's angle, k = 1.78897 Å – a numerical constant denoting the wavelength of X-rays, and W denotes the width of the diffraction peak at its half-maximum intensity.

The chemical modifications in treated samples, compared to the untreated one, were examined using FTIR.[13] The sample (without any preparation) was placed in the sample holder of the FTIR spectrometer (Cary 630 FTIR Spectrometer, Agilent Technologies, USA). The spectra were recorded in the frequency range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}.

Analytical methods

Reducing sugars (RS) in the filtrate were estimated by the DNS method.[28] The method described by Dubois *et al*. was used to estimate total sugars (TS).[29] The method of Carralero *et al*. was used to estimate total phenolic (TP) compounds in the filtrate.[30] The cellulose content in the residue was estimated by the methods of Gopal and Ranjhan.[31] One gram (W1) of dried ground sample was taken into a round bottom digestion flask, followed by the addition of 15 mL of CH_3COOH (80%) and 1.5 mL of HNO_3, and the contents were then refluxed for 20 min. At the end of refluxing, the digested material was filtered by Whatman filter paper no. 1 and washed with hot water, followed by oven drying at 105 °C overnight. Then, the sample was weighed (W2) and incinerated for 5 h at 550 °C in a muffle furnace, and weighed again (W3).

$$\text{Cellulose (\%)} = \frac{W2 - W3}{W1} \times 100 \quad (2)$$

Experimental design

BBD, with three factors and three levels, was used to optimize the conditions of pretreatment in this study (Table 1). The independent variables used were: NaOH concentration (X1), substrate concentration (X2), and time (X3), as mentioned in Table 1. This design is most suitable for the quadratic response surface and generates the second-order polynomial regression model. The relation between actual and coded values was described by the following equation:

$$xi = \frac{Xi - Xo}{\Delta Xi} \quad (3)$$

where xi and Xi are the coded and actual values of the independent variable, Xo is the actual value of the independent variable at the center point, and ΔXi is the change of xi. The response was calculated from the following equation using Minitab software (17th version):[13]

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (4)$$

where Y is the response, X_1, X_2 and X_3 are independent variables, $ß_0$ is the intercept, $ß_1$, $ß_2$, $ß_3$ are linear coefficients, $ß_1^1$, $ß_2^2$, $ß_3^3$ are square coefficients, $ß_{12}$, $ß_{13}$, $ß_{23}$ are interaction coefficients.

Table 1
Parameters, their codes and levels used for Box-Behnken design

Factors	Codes	Levels		
		-1	0	1
NaOH conc. (%)	X_1	1	3	5
Temperature (°C)	X_2	110	120	130
Time (h)	X_3	15	45	75

Simultaneous saccharification and fermentation

Firstly, the pretreated pine needles were hydrolyzed using indigenous as well as commercial cellulase (CMCase activity of 2900 IU/mL and filter paper activity of 1500 FPU/mL), as described in our earlier report.[13] Saccharification (%) was calculated using the following formula:

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugars released (mg/ml)}}{\text{Substrate used (mg/ml)}} \times 100 \quad (5)$$

Ethanol was produced from the filtrate of saccharified pine needles using a suspension of *Sacchromyces cervisea* at 30 °C for four days of fermentation period. After termination of the fermentation period, the ethanol produced was estimated by HPLC.[13]

RESULTS AND DISCUSSION

For production of bioethanol from lignocellulosic biomass, the pretreatment is a primary stage, as it removes lignin from cellulose, making it suitable for subsequent steps, namely, saccharification and fermentation. In the present study, after pretreatment, the cellulose content in all experiments ranged from 34% to 90% and maximum cellulose content (90%) was observed under circumstances of 5% NaOH, 130 °C temperature and residence time of 45 min. The degradation of lignin is evaluated by release of phenolic compounds, more TP means more lignin degradation. The observed and predicted values of RS, TS, cellulose and TP are presented in Table 2. Multiple regression analysis was applied and second-order polynomial regression showed the relationship of base concentration, temperature and time on the production of RS, TS, cellulose and TP (Eqs. 6-9). Maximum TP was estimated as 51.03 ± 0.02 mM at X_1 3%, X_2 130 °C and X_3 75 min. Lignin degradation and release of TP recommended these conditions for pretreatment of pine needles.

Regression equations:

$$RS\ (mg/mL) = 0.573364+0.025104X_1 + 0.009468X_2 - 0.001674X_3+0.000982X_1^2 + 0.000039X_2^2 + 0.000004X_3^2 + 0.000201X_1X_2 + 0.000067X_1X_3 + 0.000013X_2X_3 \quad (6)$$

$$TS\ (mg/mL) = 205.92 + 4.0231X_1 - 3.3560X_2 - 0.4621X_3 - 0.0969X_1^2 + 0.0142X_2^2 - 0.0042X_3^2 - 0.0179\ X_1X_2 - 0.0328X_1X_3 + 0.0023X_2X_3 \quad (7)$$

$$TP\ (mM) = -537.861 + 17.204X_1 + 8.897X_2 + 0.157X_3 - 1.471X_1^2 - 0.035X_2^2 - 0.002X_3^2 - 0.054\ X_1X_2 - 0.030X_1X_3 + 0.002X_2X_3 \quad (8)$$

$$Cellulose\ (\%) = 478 - 53.2X_1 - 8.25X_2 + 5.225X_3 + 2.906\ X_1^2 + 0.0387\ X_2^2 - 0.00514\ X_3^2 + 0.350X_1X_2 + 0.0417X_1X_3 - 0.04083X_2X_3 \quad (9)$$

The statistical analysis (Table 3) showed that the regression model described by the equations was greatly significant. Fisher's F-test and probability P values confirmed its significance. The Fisher's F-test values of 40.43755, 6.87293, 19.52 and 15.69892 were observed for RS, TS, cellulose and TP, respectively. The P value revealed by this model was 0.000033, 0.009369, 0.002 and 0.000747 for RS, TS, cellulose and TP, respectively. The credibility of this model was tested by the coefficient of determination (R^2 value). The coefficient of determination values for RS, TS, cellulose and TP were 0.981129, 0.898339 and 0.952795, respectively. These values of the coefficient of determination revealed that only 1.8871, 10.166 and 4.7205% of the total variations were not explained by the model.

Furthermore, the adjusted R^2 values (0.956866, 0.767632, and 0.892103 for RS, TS and TP, respectively) also braced the model. The significance of the proposed model was confirmed by these values. The R^2 value of the model for cellulose was 97.23%, which described that only 2.77% variation was not described by the model. The adjusted and predicted R^2 values (92.25 and 90.93) for cellulose also braced the model, respectively. NaOH concentration (X_1) had a significant effect on cellulose content.

Table 2
BBD table for yield of RS, TS, cellulose and TP after treatment with NaOH

Run #	X_1	X_2	X_3	RS (mg/mL)		TS (mg/mL)		Cellulose (%)		TP (mM)	
				Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	3	120	45	0.023 ± 0.002	0.023	7.47 ± 0.01	7.47	52	52.00	49.53 ± 0.05	49.53
2	5	120	75	0.035 ± 0.001	0.037	9.21 ± 0.06	11.27	74	73.37	46.65 ± 0.09	46.29
3	5	130	45	0.103 ± 0.01	0.093	9.73 ± 0.04	8.69	90	89.37	45.25 ± 0.13	43.52
4	5	120	15	0.067 ± 0.05	0.075	9.31 ± 0.02	9.66	68	69.12	38.12 ± 0.02	40.38
5	5	110	45	0.094 ± 0.02	0.092	8.97 ± 0.03	7.59	70	70.12	39.26 ± 0.02	39.07
6	3	120	45	0.023 ± 0.002	0.023	7.47 ± 0.02	7.47	52	52.00	49.53 ± 0.05	49.53
7	1	130	45	0.122 ± 0.09	0.123	8.74 ± 0.01	10.11	43	42.87	43.10 ± 0.08	43.28
8	3	110	15	0.098 ± 0.00	0.090	8.61 ± 0.03	9.63	34	32.75	39.15 ± 0.11	37.06
9	1	120	75	0.035 ± 0.01	0.026	16.26 ± 0.04	15.91	37	35.87	49.72 ± 0.05	47.45
10	3	130	75	0.037 ± 0.01	0.044	18.03 ± 0.05	17.00	36	37.25	51.03 ± 0.02	53.11
11	3	120	45	0.023 ± 0.002	0.023	7.47 ± 0.01	7.47	52	52.00	49.53 ± 0.05	49.53
12	1	120	15	0.117 ± 0.01	0.114	8.50 ± 0.02	6.43	41	41.62	34.10 ± 0.12	34.45
13	1	110	45	0.081 ± 0.002	0.090	6.55 ± 0.03	7.58	51	51.62	32.81 ± 0.03	34.54
14	3	110	75	0.034 ± 0.007	0.033	14.47 ± 0.02	13.78	56	56.50	44.79 ± 0.05	45.32
15	3	120	45	0.023 ± 0.002	0.023	7.47 ± 0.01	7.47	52	52.00	49.53 ± 0.05	49.53
16	3	130	15	0.113 ± 0.007	0.113	9.38 ± 0.04	10.06	63	62.50	43.00 ± 0.03	42.46
17	3	120	45	0.023 ± 0.002	0.023	7.47 ± 0.01	7.47	52	52.00	49.53 ± 0.05	49.53

Table 3
Analysis of variance (ANOVA) for quadratic model for RS, TS and TP

Parameter	Source	DF	Adj SS	Adj MS	F value	P value
RS (mg/mL)	Model	9	0.023630	0.002626	40.43755	0.000033
	X_1	1	0.000031	0.000031	0.4790	0.511160
	X_2	1	0.005158	0.005158	79.4395	0.000045
	X_3	1	0.007000	0.007000	107.8040	0.000017
	X_1^2	1	0.007427	0.007427	114.3929	0.000014
	X_2^2	1	0.000025	0.000025	0.3812	0.556494
	X_3^2	1	0.000127	0.000127	1.9617	0.204070
	X_1X_2	1	0.000256	0.000256	3.9428	0.087451
	X_1X_3	1	0.000625	0.000625	9.6260	0.017260
	X_2X_3	1	0.000036	0.000036	0.5545	0.480749
	Error	7	0.000454	0.000065		

TS (mg/mL)	Model	9	156.7942	17.42158	6.87293	0.009369
	X_1	1	1.6675	1.66754	0.65785	0.444017
	X_2	1	0.6322	0.63224	0.24942	0.632794
	X_3	1	8.1584	8.15845	3.21856	0.115892
	X_1^2	1	8.4304	8.43042	3.32586	0.110966
	X_2^2	1	4.9505	4.95051	1.95301	0.204959
	X_3^2	1	58.8164	58.81645	23.20349	0.001928
	X_1X_2	1	0.5112	0.51122	0.20168	0.666941
	X_1X_3	1	15.4449	15.44490	6.09312	0.042932
	X_2X_3	1	1.9460	1.94602	0.76772	0.409981
	Error	7	17.7437	2.53481		
Cellulose (%)	Model	9	3644.65	404.96	19.52	0.002
	X_1	1	2112.50	2112.50	101.81	0.000
	X_2	1	55.13	55.13	2.66	0.164
	X_3	1	1.13	1.13	0.05	0.825
	X_1^2	1	498.98	498.98	24.05	0.004
	X_2^2	1	55.44	55.44	2.67	0.163
	X_3^2	1	78.89	78.98	3.81	0.109
	X_1X_2	1	196.00	196.00	9.45	0.028
	X_1X_3	1	25.00	25.00	1.20	0.322
	X_2X_3	1	600.25	600.25	28.93	0.003
	Error	5	103.75	20.75		
TP (mM)	Model	9	521.4342	57.93713	15.69892	0.000747
	X_1	1	30.4926	30.4926	8.26242	0.023840
	X_2	1	145.8241	145.8241	39.51318	0.000410
	X_3	1	57.3423	57.3423	15.53774	0.005590
	X_1^2	1	52.7646	52.7646	14.29735	0.006882
	X_2^2	1	0.5711	0.5711	0.15474	0.705749
	X_3^2	1	9.4421	9.4421	2.55848	0.153737
	X_1X_2	1	4.6225	4.6225	1.25253	0.299997
	X_1X_3	1	12.5670	12.5670	3.40522	0.107502
	X_2X_3	1	1.4280	1.4280	0.38694	0.553623
	Error	7	25.8336	3.6905		

In the present study, pine needles were pretreated with NaOH to obtain maximum cellulose content for ethanol production. Maximum cellulose obtained was 90% under certain conditions designed by BBD. In a similar study, NaOH steam pretreatment was found effective as it offered 60% cellulose and 9% lignin in *B. ceiba* seed pods. Maximum TS (259.57 mg/mL) were liberated with 5% NaOH solution during thermochemical pretreatment.[13] Maximum cellulose (73.19%) was reported at 2.5% NaOH treatment, followed by steam after 1 h of soaking in the cotton stalk.[32] Ghazanfar and others[17] reported maximum release of TS and cellulose with 10% seed pods concentration and 5% KOH concentration. On the other hand, Sarbishei and coworkers[33] treated tobacco product with 10% NaOH and recorded a decline in the cellulosic content from 44% to 27.6% because of the degradation of carbohydrates by alkali. Gunam and fellows[34] treated corn straw with 4% NaOH and obtained maximum cellulose content of 65.46%.

Figure 1 illustrates the Pareto charts of significant parameters influencing the liberation of RS, TS, cellulose and TP after NaOH pretreatment. The findings demonstrated that the concentration of NaOH and temperature were the most significant variables for the pretreatment. The most significant parameters for RS production were X_1^2, X_2, X_2^2 and X_1X_3, while X_2^2 and X_1X_3 were significant for TS. For TP, the factors X_1, X_1^2, X_2 and X_2^2 were determined to be the most significant variables. In the case of cellulose, all the parameters were found significant.

The contour plot (Fig. 2) illustrated the interaction of different parameters on the production of RS, TS, cellulose and TP after the pretreatment with NaOH of pine needles. Figure 3 depicts the experimental values *versus* predicted values of RS, TS and TP. The graph revealed a significant correlation among parameters.

The intensities of the peaks are associated with the crystallinity of the substrate, which increases as the lignin was removed, because most of the cellulose is crystalline in nature.[5] XRD analysis of pine needles (both untreated and treated) showed the effect of the pretreatment in terms of the crystallinity index. Thus, the crystallinity index of the pretreated substrate (53.7%) was higher than that of the untreated one (42.4%), which indicates the elimination of lignin and hemicelluloses by the pretreatment and uncovering of cellulose.

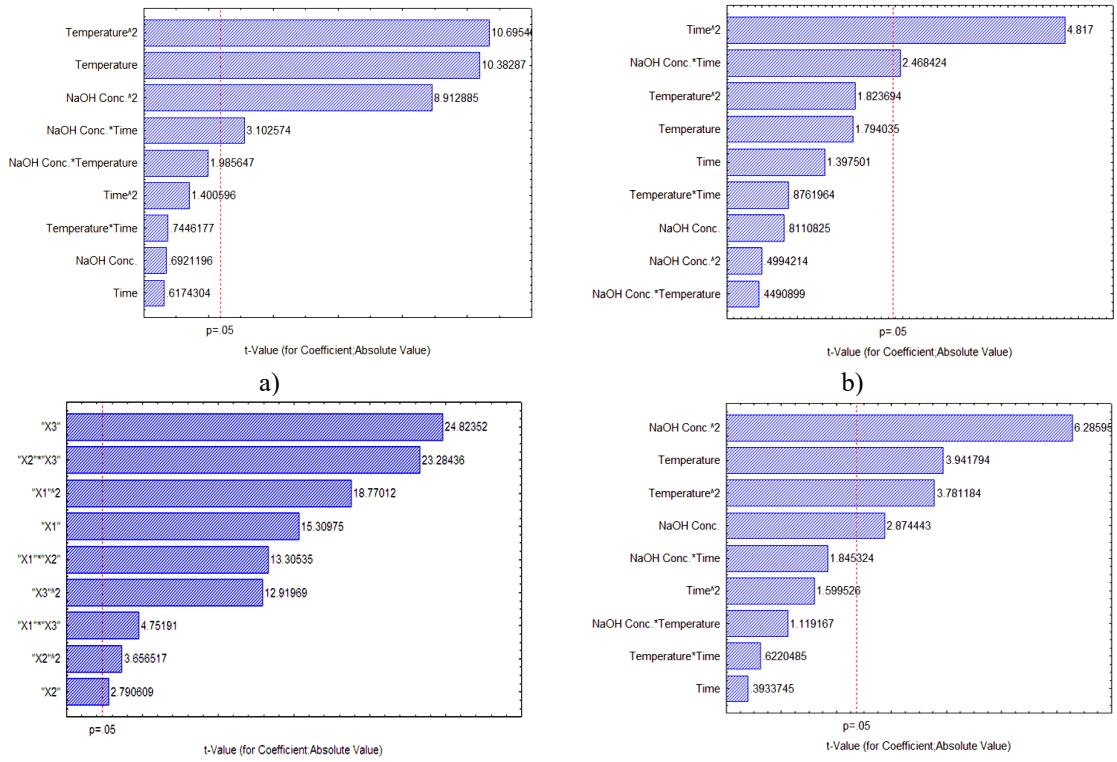

Figure 1: Pareto charts of (a) RS, (b) TS, (c) cellulose and (d) TP

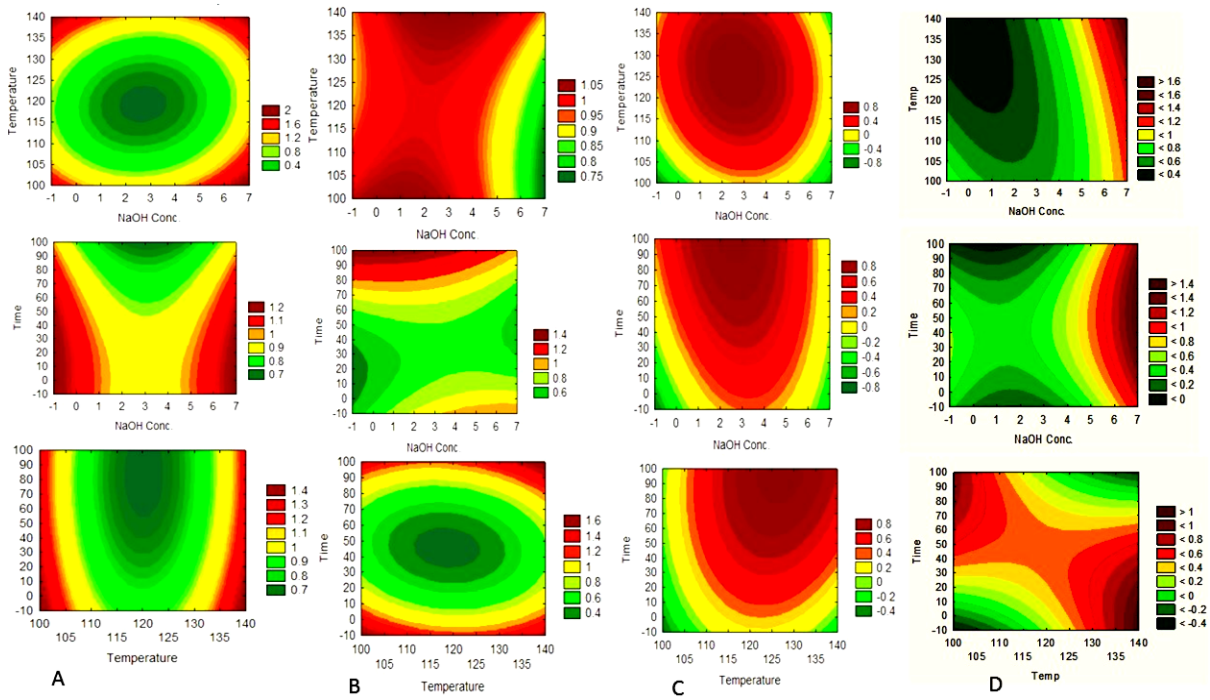

Figure 2: Countour plots showing the effect of NaOH concentration, temperature and time on A) reducing sugars (RS), B) total sugars (TS), C) cellulose, and D) total phenolic contents (TP)

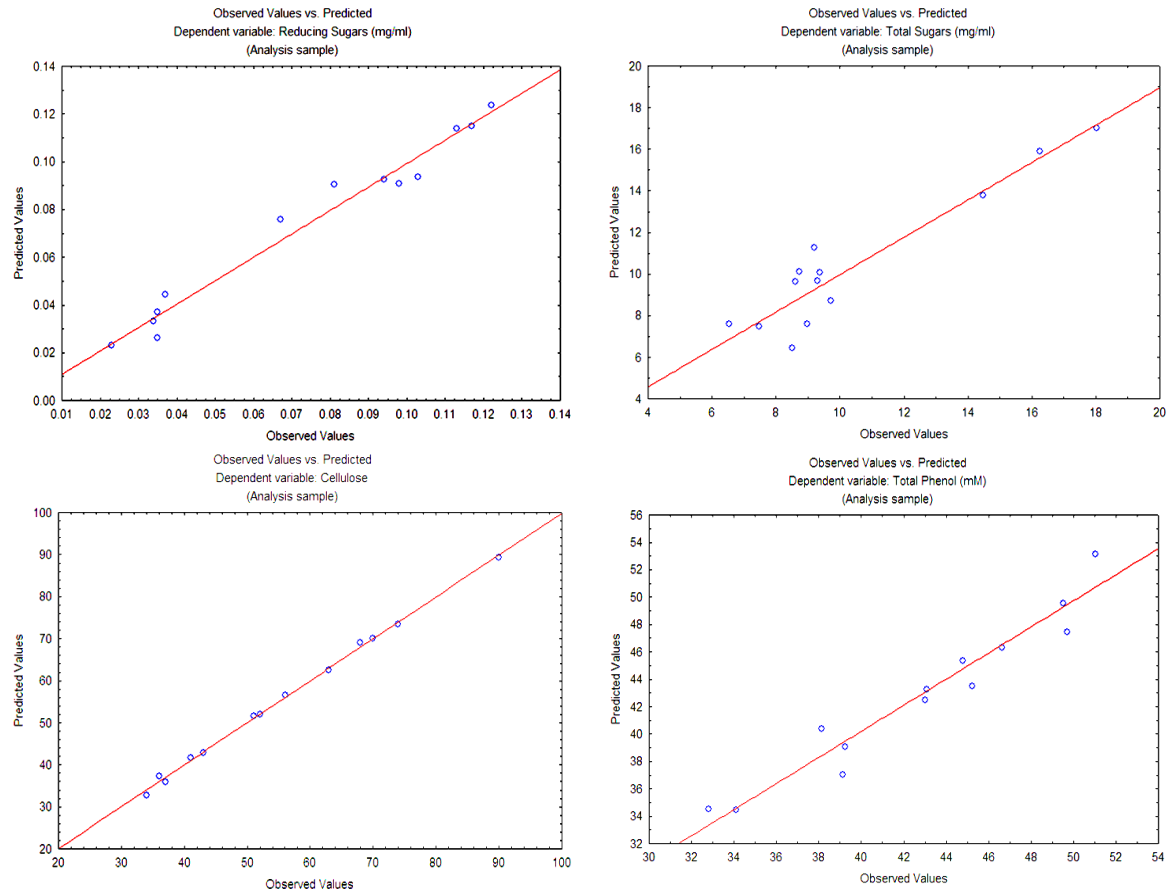

Figure 3: Observed versus predicted values of RS, TS, cellulose and TP

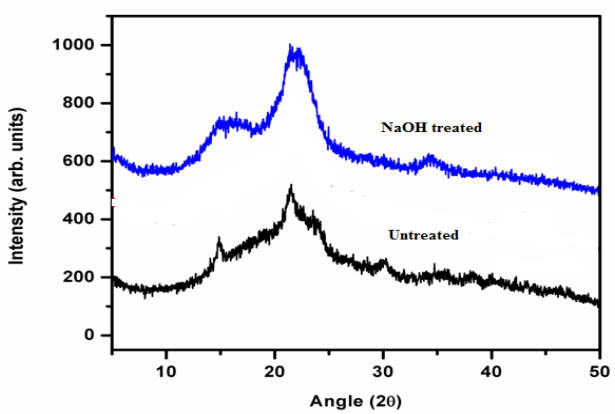

Figure 4: X-ray diffraction patterns of untreated and pretreated samples

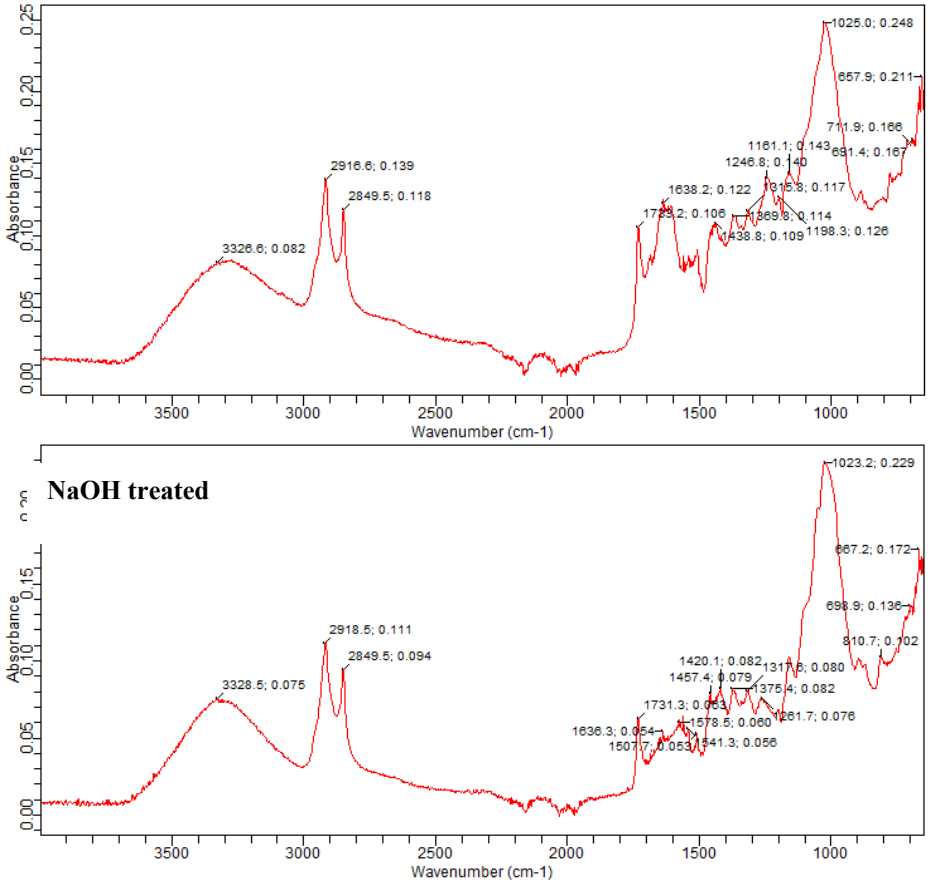

Figure 5: FTIR spectrum of untreated and NaOH treated pine needles

FTIR analysis also displayed variations in the spectra of pretreated and raw pine needles (Fig. 5). The shift of the peak from 3326.6 cm^{-1} to 3328.5 cm^{-1} indicated –OH band stretching. The intensity of –OH augmented, which illustrated the outcome of NaOH pretreatment on pine needles. The peak deviations from 1025.0 cm^{-1} to 1023.2 cm^{-1} in the samples were associated to C–O, C–H twists, which are related to cellulose breakdown. FTIR analysis showed that NaOH pretreatment successfully altered the bonds in the pretreated pine needles lignocellulosic biomass.

Maximum saccharification (49.2%) was observed in NaOH treated pine needles using commercial cellulase enzyme after 8 h of incubation at 50 °C (Fig. 6a), whereas indigenously produced cellulase also offered maximum hydrolysis (35.7%) after 8 h (Fig. 6b) in NaOH treated pine needles. The production of total sugars in the saccharification process increased with the increase in incubation time. The

saccharified biomass was further subjected for ethanol production using *Saccharomyces cervisae*. The results showed that untreated pine needles produced 4% ethanol, while NaOH treated pine needles gave 7% ethanol production after 96 h of fermentation (Fig. 7).

We examined untreated and pretreated substrates by XRD and FTIR techniques to assess the modifications caused by the pretreatment, and observed significant changes. Gunam *et al*.[34] reported alterations in the crystallinity degree of NaOH treated corn straw. A study reported that the CI (36.96%) of raw jute biomass reduced to 23.61% and 18.42% after 2% NaOH and 2% H_2SO_4 pretreatment, respectively. This decrease in CI may be due to the breakdown of intra- and inter-hydrogen bonding in the crystalline cellulose, resulting in a modified crystal structure. Awoyale and Lokhat[35] noticed peaks of reduced intensities in the treated substrate, a depiction of incomplete degradation of the cellulose upon treatment. The peak at 3334 cm^{-1} in FTIR analysis shows the absorption of –OH of alcoholic hydroxyl.[36] A study noted a peak modification from 3336 cm^{-1} (untreated) to 3315.26 cm^{-1} (treated). This alteration showed –OH band extension in the treated substrate.[5] The peak at 1315 cm^{-1} was assigned to hemicelluloses in the raw substrate. CH_2 stretching in cellulose is shown by the peaks from 1370 to 1430 cm^{-1}. The peaks around 1500 cm^{-1} are associated with the bands of C=C bonding from the lignin's aromatic ring. The peak at 1030 cm^{-1} and 1034 cm^{-1} in the raw and treated biomass, respectively, is linked with C–C–O, C=O, and C–O of cellulose. The peak at 890 cm^{-1} depicted C–O–C vibrations at glycosidic bonds in cellulose. Zhang *et al*.[37] assigned the band at 1032 cm^{-1} to polysaccharides.

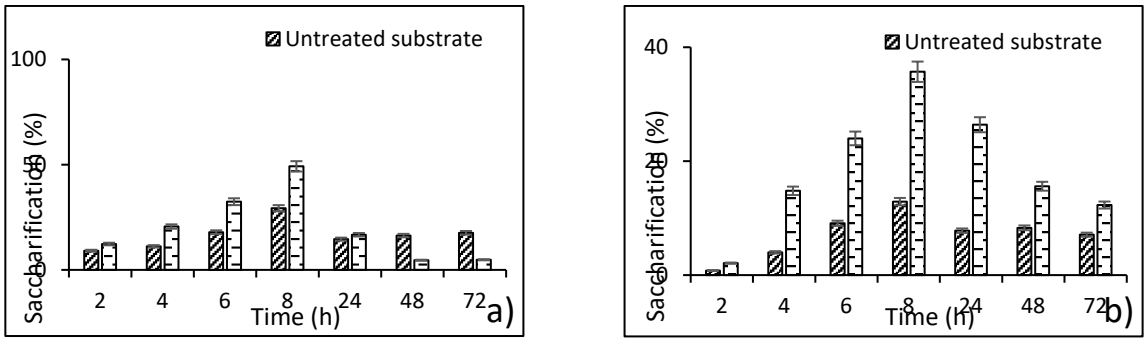

Figure 6: Saccharification of pine needles by (a) commercial cellulase and (b) indigenously produced cellulase

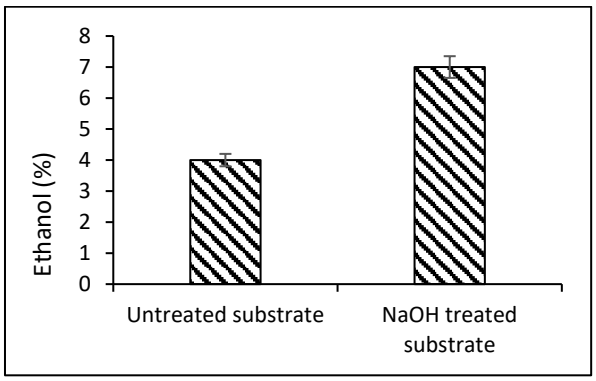

Figure 7: Ethanol production in untreated and treated pine needles

The present study found maximum saccharification when using commercial cellulase, which led to 7% ethanol yield in fermentation from the substrate, with maximum cellulose in SHF using *S. cerevisiae*. These results are in accordance with our earlier reports,[13,38] as commercial cellulase offered better saccharification, as compared to indigenous cellulase. Peace *et al*.[39] employed wood shavings for the production of bioethanol. *S. cerevisiae* converted 60.97% of the brix in wood extract into bioethanol after 72 h of fermentation period at 40 °C. Another study reported a significant ethanol titer from SHF of oil palm empty fruit bunches. Saccharification for 4 days generated 75.48% glucose, which subsequently produced 78.95% ethanol.[40] Barron *et al*.[41] used *Kluveromyces marxianus* for ethanol production and obtained 10 g/L ethanol after 2.5

days fermentation, and *Pachysolen trannophylus* produced 11.8 g/L ethanol from the hydrolysate of wheat straw. Ahmad *et al*.[42] reported that sweet sorghum and sago biomasses produced maximum ethanol titer after 3 days of fermentation.

CONCLUSION

The results of the study showed how beneficial the alkali pretreatment of *Pinus* spp. needles was for the delignification process of this biomass. We obtained the maximum cellulose contents (90%) under the optimized circumstances ascertained using a Box-Behnken design. The application of commercial cellulase further enhanced sugar production from the pretreated sample, with the resulting cellulose being successfully converted into ethanol (7%) through separate hydrolysis and fermentation (SHF). The findings of this study indicate that pine needles have great potential for use as a sustainable energy source.

ACKNOWLEDGEMENT: The authors express their gratitude to the Deanship of Scientific Research at King Khalid University for funding this work through the Small Research Group Project under grant number RGP.02/317/44.

REFERENCES

[1] K. R. Mihajlovski, M. Milić, D. Pecarski and S. Dimitrijević-Branković, *J. Serb. Chem. Soc.*, **86**, 651 (2021), https//doi.org/10.2298/JSC210308032M
[2] S. Raghavi, R. Sindhu, P. Binod, E. Gnansounou and A. Pandey, *Bioresour. Technol.*, **199**, 202 (2016), https//doi.org/10.1016/j.biortech.2015.08.062
[3] S. Maity and N. Mallick, *J. Clean. Prod.*, **345**, 131153 (2022), https://doi.org/10.1016/j.jclepro.2022.131153
[4] S. Aghaei, M. Karimi Alavijeh, M. Shafiei and K. Karimi, *Biomass Bioener.*, **161**, 106447 (2022), https://doi.org/10.1016/j.biombioe.2022.106447
[5] M. Ghazanfar, M. Irfan, M. Nadeem, H. A. Shakir, M. Khan *et al*., *Cellulose Chem. Technol.*, **55**, 821 (2021), https//doi.org/10.35812/CelluloseChemTechnol.2021.55.69
[6] H. Y. Li, X. Chen, C. Z. Wang, S. N. Sun and R. C. Sun, *Biotechnol. Biofuels*, **9**, 1 (2016), https//doi.org/10.1186/s13068-016-0578-y
[7] M. Broda, D. J. Yelle and K. Serwańska, *Molecules*, **27**, 8717 (2022), https://doi.org/10.3390/molecules27248717
[8] Z. Anwar, S. Akram and M. Zafar, in "Agroindustrial Waste for Green Fuel Application. Clean Energy Production Technologies", edited by N. Srivastava, B. Verma and P. Mishra, Springer, Singapore, 2023, pp. 313-326, https//doi.org/10.1007/978-981-19-6230-1_10
[9] S. Sun, S. Sun, X. Cao and R. Sun, *Bioresour. Technol.*, **199**, 49 (2016), https//doi.org/10.1016/j.biortech.2015.08.061
[10] P. Nargotra, V. Sharma, M. Gupta, S. Kour and B. K. Bajaj, *Bioresour. Technol.*, **267**, 560 (2018), https//doi.org/10.1016/j.biortech.2018.07.070
[11] C. E. C. Guimarães, F. S. Neto, V. de Castro Bizerra, J. G. A. do Nascimento and R. B. R. Valério, *Bioresour. Technol.*, **23**, 101543 (2023), https//doi.org/10.1016/j.biteb.2023.101543
[12] M. Jayakumar, G. T. Gindaba, K. B. Gebeyehu, S. Periyasamy and A. Jabesa, *Sci. Total Environ.*, **879**, 163158 (2023), https//doi.org/10.1016/j.scitotenv.2023.163158
[13] M. Ghazanfar, M. Nadeem, H. A. Shakir, M. Khan, I. Ahmad *et al*., *Fermentation*, **8**, 386 (2022), https//doi.org/10.3390/fermentation8080386
[14] N. Nasirpour, S. M. Mousavi and S. A. Shojaosadati, *Bioresour. Technol.*, **169**, 33 (2014), https//doi.org/10.1016/j.biortech.2014.06.023
[15] S. Singh, A. Kumar, N. Sivakumar and J. P. Verma, *Fuel*, **327**, 125109 (2022), https://doi.org/10.1016/j.fuel.2022.125109
[16] K. Karimi and M. J. Taherzadeh, *Bioresour. Technol.*, **200**, 1008 (2016), https//doi.org/10.1016/j.biortech.2015.11.022
[17] M. Ghazanfar, M. Irfan and M. Nadeem, *Energ. Sourc. A: Recov. Utiliz. Environ. Eff.*, **40**, 1114 (2018), https//doi.org/10.1080/15567036.2018.1474291
[18] L. E. N. Morando, C. X. D. Gómez, L. L. Zamora, M. Uscanga and G. Aguilar, *Biomass Convers. Bioref.*, **4**, 15 (2014), https//doi.org/10.1007/s13399-013-0091-5
[19] F. M. Ahmed, S. R. Rahman and D. J. Gomes, *Malaysian J. Microbiol.*, **8**, 97 (2012), https//doi.org/10.21161/mjm.03412
[20] W. Li, W. Du and D. Liu, *J. Molec. Catal. B: Enzym.*, **45**, 122 (2007), https//doi.org/10.1016/j.molcatb.2007.01.002
[21] R. Maceiras, V. Alfonsín, L. Seguí and J. F. González, *Energies*, **14**, 5891 (2021), https//doi.org/10.3390/en14185891
[22] M. Irfan, U. Asghar, M. Nadeem, R. Nelofer, Q. Syed *et al.*, *Waste Biomass Valor.*, **7**, 1389 (2016), https//doi.org/10.1007/s12649-016-9540-2
[23] H. Zhang, P. Zhang, T. Wu and H. Ruan, *Fermentation*, **9**, 709 (2023), https://doi.org/10.3390/fermentation9080709
[24] M. A. Kamzon, S. Abderafi and T. Bounahmidi, *Int. J. Hydr. Energ.*, **41**, 20880 (2016), https//doi.org/10.1016/j.ijhydene.2016.07.035
[25] H. Chen and X. Fu, *Renew. Sustain. Energ. Rev.*, **57**, 468 (2016), https//doi.org/10.1016/j.rser.2015.12.069
[26] A. Gupta and J. P. Verma, *Renew. Sustain. Ener. Rev.*, **41**, 550 (2015), https//doi.org/10.1016/j.rser.2014.08.032

[27] T. Zahra, M. Irfan, M. Nadeem, M. Ghazanfar and Q. Ahmad, *Punjab Univ. J. Zool.*, **35**, 223 (2020), https//doi.org/10.17582/journal.pujz/2020.35.2.223.228
[28] L. G. Miller, *Anal. Chem.*, **31**, 426 (1959)
[29] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1959)
[30] S. Carralero, M. Luz, C. Gonzalez, A. S. Yanez and P. Pingarron, *Anal. Chim. Acta*, **528**, 1 (2005), https//doi.org10.1016/j.aca.2004.10.007
[31] K. Gopal, S. K. Ranjhan, "Laboratory Manual for Nutrition Research", New Dehli, Roland Press, 1980
[32] U. Asghar, M. Irfan, M. Nadeem and Q. Syed, *Energ. Sourc. A: Recov. Utiliz. Environ. Eff.*, **38**, 1898 (2016), https//doi.org/10.1080/15567036.2015.1004386
[33] S. Sarbishei, A. Goshadrou and M. S. Hatamipour, *Biomass Convers. Biorefin.*, **11**, 2963 (2021), https//doi.org10.1007/s13399-020-00644-x
[34] I. B. W. Gunam, Y. Setiyo, N. S. Antara, I. M. M. Wijaya, I. W. Arnata *et al.*, *Rasayan J. Chem.*, **13**, 1022 (2020), https//doi.org/10.31788/RJC.2020.1325573
[35] A. A. Awoyale and D. Lokhat, *Sci. Rep.*, **11**, 557 (2021), https//doi.org/10.1038/s41598-020-78105-8
[36] M. Irfan, Q. Syed, S. Abbas, M. G. Sher, S. Baig *et al.*, *Turk. J. Biochem./Turk Biyokim. Derg.*, **36**, 322 (2011), https//doi.org/10.5505/tjb.2012.09709
[37] A. P. Zhang, C. F. Liu, R. C. Sun and J. Xie, *BioResources*, **8**, 1604 (2013), https//doi.org/10.15376/biores.8.2.1604-1614
[38] M. Ghazanfar, M. Irfan, M. Nadeem, H. A. Shakir, M. Khan *et al.*, *Fermentation*, **8**, 148 (2022), https//doi.org/10.3390/fermentation8040148
[39] A. Peace, C. Akujobi and W. Braide, *J. Taiwan Inst. Chem. Engin.*, **79**, 43 (2017), https//doi.org/10.21474/IJAR01/11061
[40] E. Triwahyuni, in *IOP Conference Series: Earth and Environmental Science*, IOP Publishing, Bristol, UK, 2020, vol. 439, 012018, https//doi.org/10.1088/1755-1315/439/1/012018
[41] N. Barron, R. Marchant, L. McHale and A. P. McHale, *Appl. Microbiol. Biotechnol.*, **43**, 518 (1995), https//doi.org/10.1007/BF00218459
[42] F. Ahmad, A. T. Jameel, M. H. Kamarudin and M. Mel, *Afr. J. Biotechnol.*, **10**, 18841 (2011), https//doi.org/10.5897/AJB11.2763