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Enzymatic delignification: an attempt for lignin degradation from lignocellulosic feedstock

Rajiv Chandra Rajak[1] and Rintu Banerjee[2]

[1]Advanced Technology Development Centre, Indian Institute of Technology Kharagpur

[2]Agricultural and Food Engineering Department, Indian Institute of Technology Kharagpur

Kharagpur-721302, India

[2]**Corresponding Author**

Tel: +91-3222-283104(O)/+91-3222-283105(R).

Fax: +91-3222-282244

Email: rb@iitkgp.ac.in

Abstract

Burgeoning population growth, increased demand of transportation and industrialization urges for excessive use of fossil fuels, which in turn lead to higher emission of greenhouse gases contributing to global warming. At this juncture, biomass based biofuel production from sustainable resources like lignocellulosics acts as a better alternative for achieving zero emission. This in turn necessitates a major effort for development of an efficient biomass delignification method which is an essential prerequisite of complete biofuel production process. Lignocellulosics such as *Saccharum spontaneum* contains 17.46 % of lignin and 67 % of carbohydrate in its cell wall. To make this enormous amount of carbohydrates more accessible for hydrolysis and to be used further in fermentation, degradation of lignin through laccase has been carried out.

In the present work, Response Surface Methodology (RSM) based on Central Composite Design (CCD) has been used to investigate the effects of the different process parameters. The maximum delignification obtained was 84.67 % at 6.21 h of incubation time upon monitoring the initial lignin content of 17.46 % of the biomass. Thorough study of the biomass was carried out by elemental composition analysis and energy density measurement. Further structural characteristics of delignified substrate were analyzed by Scanning Electron Microscopy (SEM), Fourier-Transform Infra-Red Spectroscopy (FTIR) and X-Ray Diffraction Spectroscopy (XRD) which supported the efficacy of the delignification process.

Keywords: Lignocellulosic, *Saccharum spontaneum*, Response surface methodology, Laccase, Crystallinity

1. Introduction

The trend of increasing energy crisis and demand in developed as well as in developing nations has prompted worldwide interest on the production of biomass based fuels as a substitute to petro-fuels.[1,2] These issues made it imperative to find alternatives which would reduce the dependence on fossil fuels. In this context, biofuel production from biomass, specifically from lignocellulosics, is gaining global attraction owing to its low-cost, non-competitive and sustainable nature. Lignocellulosic biomass contain 40-60 % cellulose, 20-30 % hemicellulose and 15-30 % lignin.[3] Generally, lignocellulosics such as, grass species represent potential candidates for the bioethanol production because of their high regenerative capacity and reduced land requirement. *Saccharum spontaneum* (Kans or Sarkanda) is a perennial tall grass that grows up to 4m in height, has deep rhizome and root system to utilize water efficiently and occupies vast acres of land mass worldwide.[4] Its ability to quickly grow, and colonize land as well as its high content of cell wall carbohydrates (67.85 %, dry weight basis) makes it a potential candidate for bioethanol production.[5-7]

Biomass based biofuel production, necessitates dismantling of plant cell wall constituents into carbohydrate polymers for subsequent hydrolysis into monomeric sugars. One of the key aspects of biomass heterogeneity towards hydrolysis is associated with the composition and content of lignin molecule which is a large and complex aromatic structure containing phenylpropanoid subunits linked by carbon-carbon and carbon-oxygen bonds. Lignin is closely interlaced with hemicellulose molecules forming an envelope to wrap the crystalline cellulose microfibrils which hamper the accessibility of cellulase towards biomass hydrolysis.[8-12] As the breakdown or removal of lignin is an essential need for accessing the cellulose and hemicellulose components, an appropriate pretreatment process is indispensible. The environment itself is endowed with a wide variety of microbes that are capable of degrading or modifying lignin and contributes to plant biomass de-construction.[13]

Laccase (oxidoreductase, EC 1.10.3.2) is a multicopper phenol oxidase that oxidizes electron-rich phenolic and non-phenolic substrates.[14] Recently, laccases of high redox potential from basidiomycetes was used to remove lignin (with synthetic mediator 1-hydroxybenzotriazole, HBT) from lignocellulosics such as wood and non-wood biomass[15] and ensiled corn stover[16], making cellulose more accessible to hydrolysis.

The selection of appropriate delignification methods has a major impact on the yield of fermentable sugar and eventually on ethanol production from lignocellulosics. For the past two decades, several physical, chemical and physico-chemical pretreatment methods have been attempted for removal or degradation of lignin.[17,18] These modes of pretreatment generally resulted in formation of products such as furfurals, hydroxymethylfurfurals, acetic acid, formic acid and levulinic acid which acts as inhibitors[19] in the subsequent steps of hydrolysis and fermentation.[20] Enzymatic delignification is unique in nature in the sense that it selectively targets and cleaves the specific phenolic moieties of the lignin molecule. This results in formation of various phenolic intermediates which do not interfere with the hydrolysis process, but rather act as natural mediators[21] taking part in the oxidation of non-phenolic moieties of lignin molecule.[22,23] It also improves the accessibility of hydrolytic enzymes (even at lower concentration) towards depolymerized lignocellulosics for efficient hydrolysis.[24] The overall process of delignification is represented in Fig.1.

Till date, only a few reports have been cited on enzymatic delignification utilizing different types of lignocellulosics and amongst these no report are found on enzymatic delignification of *S. spontaneum*. In the present study, quantity of lignin has been monitored before and after enzymatic pretreatment via different single process parameters. RSM based on Central Composite Design (CCD) has been used to obtain optimum process conditions for enzymatic delignification of lignin. Structural, compositional and energy density measurement was performed which manifested the establishment of enzymatic delignification process.

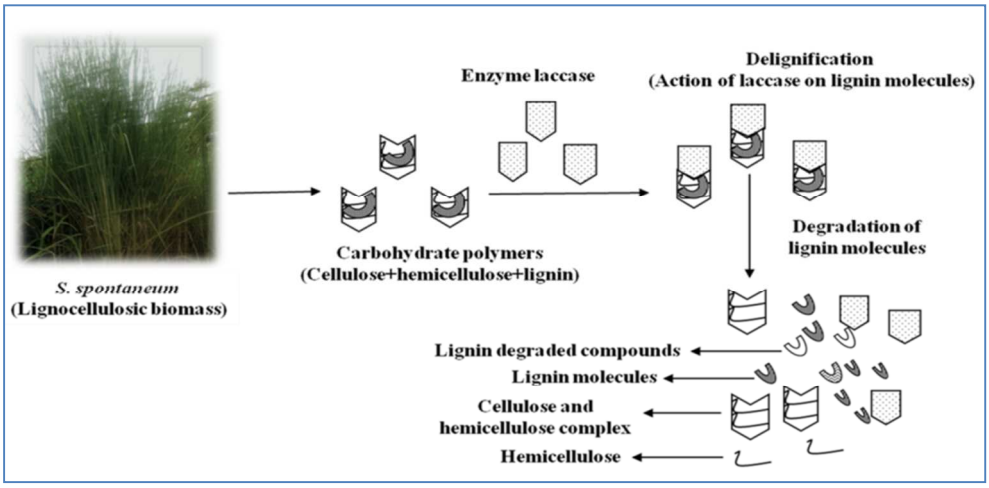

Fig. 1 Overall delignification process

2. Materials and Methods

2.1 Raw Substrate

The wasteland weed, *Saccharum spontaneum* was collected from local premises of the Indian Institute of Technology, Kharagpur, India. The whole plant, including stems and leaf sheaths, were chopped into small pieces using a chopper. The chopped pieces were then sun dried and powdered to approximately 0.2 mm particle size and used subsequently for further studies.

2.2 Biochemical composition analysis of raw substrate

Moisture content of *S. spontaneum* was determined by standard methods of Association of Analytical Communities (AOAC).[25] Lignin estimation was done by following the titrimetric method.[26] Dried powdered substrate (0.05g) was taken in a 100 mL Erlenmeyer conical flask containing 60 mL of distilled water. Potassium permanganate solution (7.5 mL) and sulphuric acid solution (7.5 mL) were mixed together. The solution was added immediately to the substrate to disintegrate the sample, followed by incubation for 10 min at 25 ^{o}C. Thereafter 1.5 mL of potassium iodide solution was added, and the free iodine was titrated with standard sodium thiosulphate solution using as starch indicator. A blank titration was carried out using the same volume of water and reagent. The amount of residual lignin (%, w/w) remaining in the solid sample was estimated by subtracting the final lignin from the initial lignin content.

Reducing sugar content was measured by following dinitrosalicylic acid method.[27] The "semi-micro determination of cellulose" method was used to measure the cellulose content[28] whereas hemicellulose was estimated by anthrone method.[29]

2.3 Elemental composition analysis of raw and delignified substrate

The carbon-hydrogen-nitrogen-sulphur (CHNS) analysis of raw and delignified substrate was carried out by using an M/s Elementar, VarioMicrocube, Germany.

2.4 Enzyme

Enzyme used for delignification was hyperactive laccase produced from *Pleurotus* sp. and its activity was measured spectrophotometrically using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as substrate.[30] One international unit (IU) of laccase activity was defined as the amount of enzyme required to oxidize 1 micro mol of ABTS per minute under the assay conditions.

2.5 Enzymatic delignification of *S. spontaneum*

Enzymatic delignification of *S. spontaneum* was carried out by incubating enzyme laccase and powdered substrate in a 50 mL Erlenmeyer conical flask with different solid loadings and reaction conditions. After, a fixed incubation time, the solid residue was separated and subsequently oven dried for residual lignin content estimation. The delignification was monitored at different conditions of solid loading, incubation time, temperature, pH, and enzyme concentration. In the beginning of the experimental work, single parameters such as solid loading (5-40 %, w/v), incubation time (1-10 h), 30-60 °C, pH (3-10), and enzyme concentration (100-1000 IU/mL) were selected to study its effects on enzymatic delignification. Further optimization was done by RSM based on Central Composite Design.

2.6 Experimental design for optimization of enzymatic delignification of *S. spontaneum*

Optimization and evaluation of enzymatic delignification of *S. spontaneum* was carried out using three-level, 2^5 full factorial central composite design (CCD) with five process parameters. The boundary parameters studied in the process of enzymatic delignification were solid loading (15-25 %), incubation time (5-7 h), 35-45 °C, pH (6-8), and enzyme concentration (300-500 IU/mL). All the experiments were performed in triplicate and the un-coded values of the process parameter was tabulated (Table 1). The resulting optimized condition was then used for delignification of *S. spontaneum* followed by residual lignin estimation.

Table 1 Experimental designs (factors and responses) for enzymatic delignification of *Saccharum spontaneum* in terms of uncoded level of variables based on central composite design

Run Order	Solid Loading (%)	Incubation Time (h)	Temperature (° C)	pH	Enzyme Concentration (IU/mL)	Delignification (%)	
						Predicted	Experimental
1	25	5	35	6	300	75.14	75.30
2	25	6	40	7	400	77.24	77.68
3	15	7	35	8	500	73.10	73.03
4	20	6	40	8	400	76.56	77.54
5	20	5	40	7	400	73.24	73.33

6	20	6	40	6	400	87.24	86.30
7	20	6	40	7	400	86.56	85.60
8	25	7	35	8	300	73.14	73.08
9	20	6	40	7	500	87.56	86.03
10	20	6	45	7	400	75.14	75.59
11	25	5	45	6	500	74.15	74.09
12	20	6	40	7	400	86.56	85.40
13	25	5	45	8	300	76.56	76.37
14	15	5	35	8	300	73.72	73.75
15	25	7	45	6	300	77.24	77.31
16	20	6	40	7	400	86.56	85.48
17	25	7	45	8	500	78.24	77.96
18	15	7	35	6	300	78.20	78.48
19	20	6	40	7	300	77.27	76.84
20	15	5	45	6	300	76.98	77.14
21	25	5	35	8	500	71.94	71.75
22	25	7	35	6	500	77.24	77.31
23	15	5	45	8	500	79.40	79.21
24	20	6	40	7	400	86.56	85.44
25	15	6	40	7	400	87.30	86.70
26	20	6	40	7	400	86.56	86.48
27	20	6	40	7	400	86.56	85.48
28	15	7	45	8	300	75.14	75.08
29	15	7	45	6	500	84.20	85.26
30	15	5	35	6	500	75.24	75.39
31	20	6	35	7	400	73.72	73.30
32	20	7	40	7	400	74.44	74.39

2.7 Response Surface Methodology (RSM)

In the present work, response surface methodology based on three levels and 2^5 factorial central composite design was adopted to explore the effects of various process parameters. The different process parameters such as solid loading (15-25 %), incubation time (5-7 h), 35-45 °C, pH (6-8), and enzyme concentration (300-500 IU/mL) were considered as factors to evaluate the response (% delignification), which was in accordance with the work carried out for optimization of wet explosion pretreatment of Douglas fir.[31] The series of experimental runs designed and conducted are tabulated in Table 1 in un-coded terms which include -1, 0, +1 as lowest, middle and highest value for five parameters respectively. The analysis of the obtained data was done by the Response Surface Regression method to fit into the 2nd order polynomial equation 1:

$$Y = \beta_{m0} + \sum_{i=1} \beta_{mi} X_i + \sum_{i=1} \beta_{mii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1} \beta k_{ij} X_i X_j \quad (1)$$

Where, Y represents the response (% delignification).Whereas, β_{m0}, β_{mi}, β_{mii} and β_{mij} stands for constant coefficients and X_i and X_j represents coded independent variables affecting the response variable Y.

2.8 Effect of mediators on enzymatic delignification

The powdered samples of *S. spontaneum* were treated with laccase in the presence of the mediators such as, ABTS, vanillic acid, and methyl syringate. The optimized process conditions of delignification, together with mediators (1-5 %) were used to explore their effects on enzymatic delignification. The treatments were performed in 50 mL Erlenmeyer conical flask placed in a water bath maintained at 40.85 °C and incubated for 6.21 h. After the treatment, the solid samples were separated, oven dried and analyzed for estimation of % delignification.

2.9 Measurement of energy density

The solid biomass samples before and after enzymatic delignification were used for energy density measurement in a standard bomb calorimeter (Oxygen Bomb Calorimeter, Eastern Instruments, Kolkata, India). The powdered samples were dried at 40 °C in an oven to remove the moisture content and then subsequently compressed to form pellets using a pelletizer before being weighed. The heat content of the samples was determined in bomb calorimeter in the

presence of excess oxygen and at high pressure (400 psi), which is considered to be a near adiabatic system.

3.0 Structural characterization of raw and delignified substrate

Scanning Electron Microscopy (SEM) images discerned the surface characteristics of both raw and delignified substrate. The procedure adopted for scanning electron microscopy included the coating of the dried substrate with gold and was subsequently observing under JEOL JSM 5800 (Jeol Ltd., Tokyo, Japan) SEM.

Fourier Transform Infrared Microscopy (FTIR) was carried out for both raw and delignified substrate to reveal the functional groups and their band intensity, stretching vibrations and absorption peaks that contribute to the lignin, cellulose and hemicellulose structure by following the KBr pellet technique. Spectra of FTIR were obtained over the range of 400-4000 cm^{-1} with a spectral resolution of 0.5 cm^{-1}.

X-Ray Diffraction was performed to analyze and calculate the degree of crystallinity for both raw and delignified substrates by using XRD1710 equipment using CoKα radiation (α = 1.79 Å) at 40 kV and 20 mA. Both the samples were examined from 2θ = 15 to 75° with scanning speed of 3°/min. Percent crystallinity was defined as $[(I_{002}-I_{am})/I_{002}]\times100$, where I_{002} stands for maximum crystalline intensity peak at 2θ between 22°and 23°for cellulose *I*, and I_{am} corresponds to minimum crystalline intensity peak at 2θ between 18° and 19° for cellulose *I*.[32]

4. Results and Discussion

4.1 Biochemical characterization of *S. spontaneum*

Biochemical compositional analysis is a pre-requisite in terms of carbohydrate content to confirm the biomass as a potential lignocellulosic substrate. The biochemical composition illustrated that the *S. spontaneum* is rich in cellulose (38.70 %, w/w) and hemicellulose (29.00 %, w/w) with moisture content of (4.95 %, w/w) which makes it a suitable candidate for bioethanol production. However, the high lignin content (17.46 %, w/w) of this substrate necessitates an effective delignification process to degrade the lignin which acts as a physical barrier for accessing cellulose and hemicelluloses of plant cell wall. Therefore, lignin degradation was necessary to utilize this substrate further. The reported composition of cellulose (45.10 %, w/w),

hemicellulose (22.75 %, w/w) and lignin (24.56 %, w/w) of *S. spontaneum*[33] were slightly different than the present study which might be due to the difference either in geographical and seasonal variations or may due to different methods used for the compositional analysis.

4.2 Elemental composition analysis of raw substrate

The elemental compositional analysis (Table 2) shows that the raw substrate contains higher percentage of carbon and hydrogen than the delignified substrate, which indicates a higher degree of cross linking and occurrence of high molecular weight compounds.[34] During enzymatic pretreatment, C-C and C-O bonds of lignin which hold together the mono-lignols or lignin precursors of lignin molecule[35,36] were cleaved selectively by the enzyme which was confirmed by the reduced percentage of carbon and hydrogen of the delignified substrate. This further indicated that the lignin precursors constituting the lignin molecule were cleaved specifically by the laccase. The higher percentage of oxygen in the delignified substrate in turn had a positive effect on the enzyme for oxidative cleavage of the electron-rich phenolic and non-phenolic moieties of lignin with a simultaneous reduction of oxygen to water.[37] The effectiveness of the delignification process was also supported by the loss of low amount of carbon (7.70 %) in terms of less reduction in energy density or better fuel properties of the substrate after enzymatic delignification. Nitrogen loss might be associated with the enzyme catalysis reaction. Increased amount of oxygen in the delignified substrate might be because of oxidation-reduction reactions carried out by laccase which comes under the family oxidoreductase. In plants, thiol (-SH) containing amino acids are buried under the hydrophobic core of proteins that might be oxidized by laccase during delignification which contributes to higher content of sulphur in the delignified substrate.

Table 2 Elemental composition analysis of raw and delignified substrate

Substrate	C (wt. %)	H (wt. %)	N (wt. %)	S (wt. %)	O (wt. %)
raw	38.69	4.712	0.7	0.218	55.68
delignified	35.71	4.175	0.62	0.345	59.15

Note: Oxygen (wt. %) was calculated from the difference of C, H, N and S.

4.3 Effect of single process parameter on enzymatic delignification

4.3.1 Effect of solid loading on enzymatic delignification

To achieve highest reaction efficiency in an enzyme mediated delignification of *S. spontaneum*, a proper solid loading has to be maintained. High substrate concentration results in inefficient interaction between enzyme and substrate molecules while the low substrate concentration reduced the affinity of the enzyme towards the substrate. In the present study, solid loading was varied from 5-40 % (Fig. 2). Solid loading of 20 % was selected as optimum with delignification (71.93 %, w/w) and 80 % (w/w, dry wt) solid recovery. At high solid loading, recovery of residual liquid was very low due to high viscous nature which could not be further used for by-product analysis. Solid recovery was approximately 80 % (w/w, dry wt) in each level of all the parameters studied during delignification.

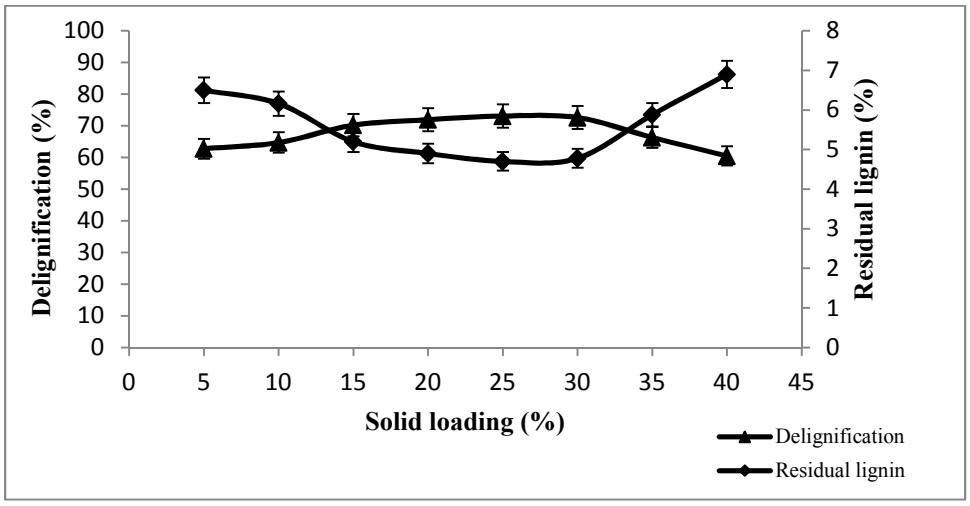

Fig. 2 Effect of solid loading on enzymatic delignification

4.3.2 Effect of incubation time on enzymatic delignification

The rate of the reaction itself defines the consumption of the substrate or formation of the product with respect to time. Hence, to study the effect of incubation time on enzymatic delignification, reaction was carried out for a time period from 1-10 h at 20 % solid loading. It was found that significant increase in delignification was observed up to 6 h of incubation (76.16 %, w/w) which might be due to saturation of all the active sites of enzyme. Fig. 3 shows the effect of incubation time on enzymatic delignification.

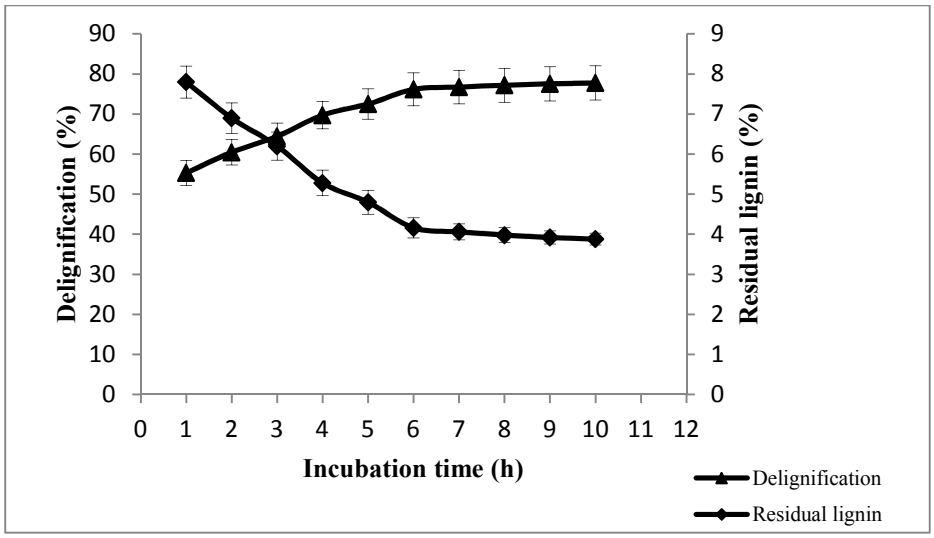

Fig. 3 Effect of incubation time on enzymatic delignification

4.3.3 Effect of temperature on enzymatic delignification

Temperature plays an important role in the disruption of lignocellulose matrix. At high temperature (110 °C) solubilization of hemicelluloses was observed whereas crystallinity of the cellulose was unaffected up to 170 °C. The present work was focused on enzyme based degradation of lignin which operates at minimal process conditions. Enzymes, being proteins, easily got denatured in terms of active site distortion while at low temperature their activity reduced because of the lack of kinetic motion between enzyme and substrate molecules.[38]

A range of temperature (30-60 °C) was selected to study its effect on enzymatic delignification process. Fig. 4 clearly demonstrated that maximum lignin degradation (74.21 %, w/w) occurred at 40 °C.

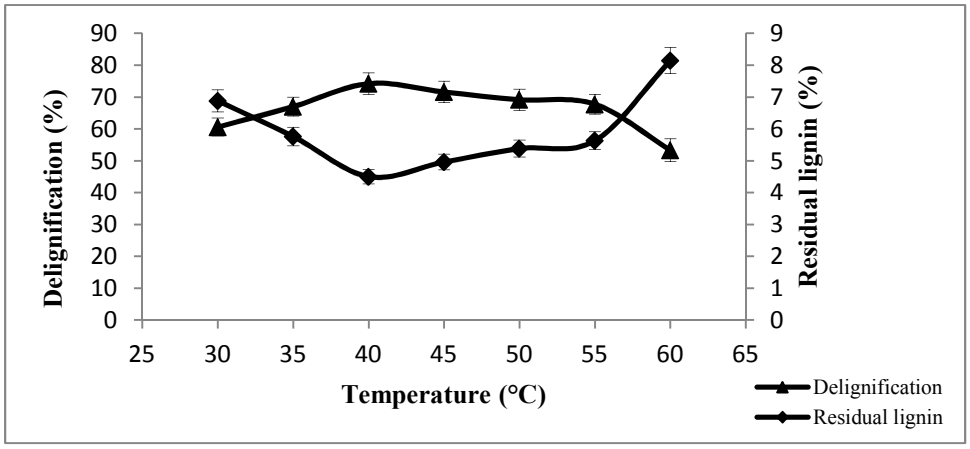

Fig. 4 Effect of temperature on enzymatic delignification

4.3.4 Effect of pH on enzymatic delignification

Enzymes are generally amphoteric molecules with respect to acid and basic groups residing on their surface. The charges on the respective groups will differ according to the pH of their surroundings. Variations in pH not only change the shape of an enzyme but also affect the surface hydrophobicity of the substrate. The surface charge of the substrate can be affected by variations in pH via surface functional groups to change surface hydrophobicity. Therefore, pH optimum of an enzyme-substrate reaction is an important decisive factor for an enzyme mediated delignification process. A broad range of pH (3-10) was selected to study its effect on delignification process (Fig. 5). It was observed that the enzyme perform best between pH 5-8 showing maximum delignification (73.08 %, w/w) at pH 7.

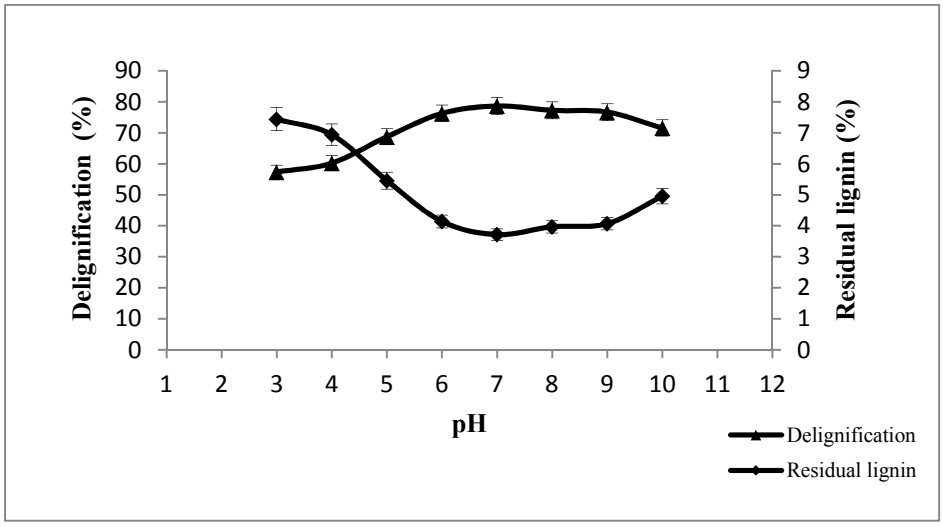

Fig. 5 Effect of pH on enzymatic delignification

4.3.5 Effect of enzyme concentration on enzymatic delignification

Enzyme concentration plays an important role in all enzyme catalyzed reactions because a small quantity of enzyme can catalyze a larger amount of substrate into products. Hence, it is necessary to maintain an optimum level of enzyme concentration to obtain maximum product.

In the present work, an enzyme concentration (100-1000 IU/mL) was used for enzymatic delignification of *S. spontaneum* (Fig. 6). It was observed that an enzyme concentration of 400 IU/mL was enough to result into maximum delignification (72.29 %, w/w) without much difference at higher enzyme concentration.

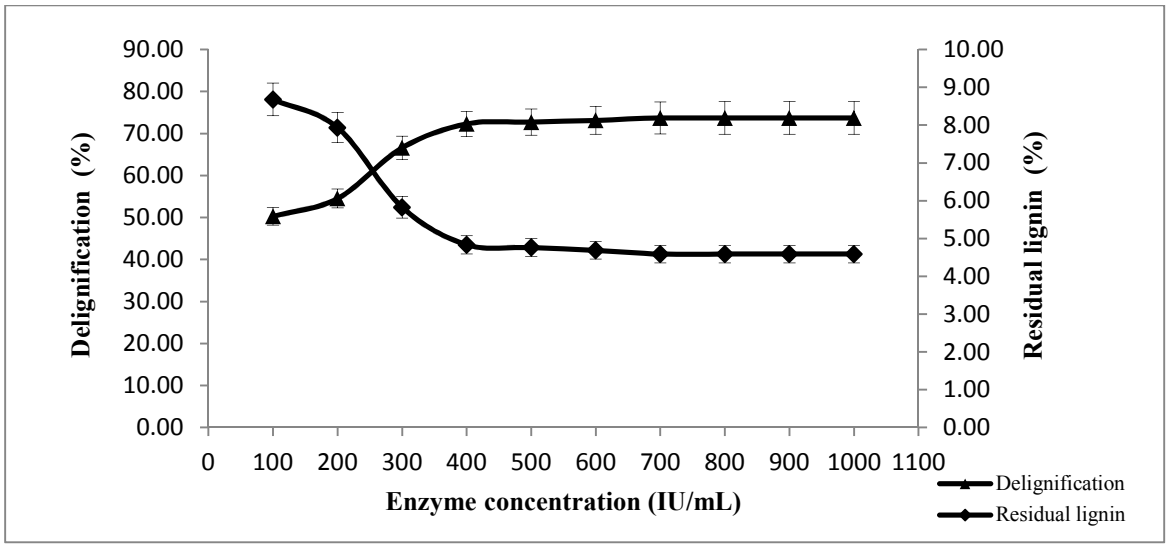

Fig. 6 Effect of enzyme concentration on enzymatic delignification

4.4 Statistical optimization of enzymatic delignification

A second order polynomial equation was developed using the experimental data of enzymatic delignification along with the term of interactions between the different experimental variables. The second order polynomial mathematical expression for per cent delignification with different variables (solid loading, incubation time, temperature, pH, and enzyme concentration) in terms of un-coded factors is represented in Equation 2:

$$Y_1 = 36.7098 - 3.4492X_1 + 35.5427X_2 + 5.2905X_3 - 33.7084X_4 - 0.1122X_5 + 0.0681X_1^2 - 2.6266X_2^2 - 0.0815X_3^2 + 1.9334X_4^2 + 0.0971X_1X_2 - 0.0043X_1X_3 + 0.0546X_1X_4 - 0.0364X_2X_3 - 0.7731X_2X_4 + 0.1961X_3X_4 + 0.0036X_3X_5 + 0.0037X_4X_5 \quad (2)$$

Where Y_1 represents percent delignification and X_1, X_2, X_3, X_4, and X_5 refers to solid loading, incubation time, temperature, pH and, enzyme concentration respectively.

Analysis of the variance (ANOVA) of the above mentioned quadratic equation for the enzymatic delignification was represented in Table 3. The ANOVA outcome is detailed as an *F*-value and its corresponding degrees of freedom (DF) and *p*-value. In an ANOVA, the *F*-value or *F*-ratio is the major statistical unit employed to test the hypothesis in order to make the effects real and significant along with the associated degrees of freedom. In addition, if the *p*-value or the probability value is found to be lower than the critical value (α), then the effect is supposed to be

significant. Usually critical value used to be set at 0.05 and hence, any value lower than this will produce significant effects, while greater value results in non significant effects.

Table 3 ANOVA analysis of quadratic model of RSM for enzymatic delignification

Source	DF[a]	Seq SS[b]	Adj SS[b]	Adj MS[c]	*F*	*p*
Regression	20	124.499	124.499	6.2249	14.18	< 0.001
Linear	5	47.457	43.102	8.6203	19.63	< 0.001
Square	5	37.737	37.737	7.5475	17.19	< 0.001
Interaction	10	39.304	39.304	3.9304	8.95	0.001
Residual error	11	4.831	4.831	0.4391		
Lack-of-fit	6	3.371	3.371	0.5619	1.93	0.245
Pure error	5	1.459	1.459	0.2918		
Total	31	129.329				

R^2 = 96.26 %, $R^2_{(adj)}$ = 89.47

a Degrees of Freedom.
b Sum of Squares.
c Mean Squares.

During ANOVA analysis, the critical *f*-value at degrees of freedom 20 and 6 is found to be 3.87 which is less than the tabulated value 14.18. Hence, it can be assumed that the regression of the quadratic polynomial equation is significant for enzymatic delignification. At degrees of freedom 5 and 6, the critical *f*-value observed to be 4.38 which is less than the tabulated values 19.63 and 17.19 and thereby indicating that the square as well as linear effects of the quadratic polynomial equation for the enzymatic delignification are significant. In addition the *f* (critical *f*-value) = 4.05 at degrees of freedom 10 and 6 which is less than the calculated value 8.95 indicated that there is a significant interaction between different parameters.[39] Moreover, the *p*-values are found to be less than 0.05 which indicated that the regression model for enzymatic delignification of *S. spontaneum* is significant. The regression coefficient R^2 was observed to be 96.26 % whereas the adj R^2 was found to be 89.47 % which is practically good for biological

system. Since there is not much difference between R^2 and adj R^2 values which indicates the adequacy of the regression model for enzymatic delignification.

The above mentioned discussion and observations from the analysis of variance (ANOVA) table are validated by the study of 3D response surface plots of the regression equation, which emphasizes the important interactions between different selected parameters and their individual effects on enzymatic delignification.

4.5 3D response surface plot analysis

Response surface plots are the graphical representation of the quadratic regression equation used to analyze the interactions and their influence on different parameters.

In the current study, different process parameters were selected and optimized for enzymatic delignification. It was clear from the response surface plot (Fig. 7) between solid loading and temperature that with increase in temperature and solid loading, percentage delignification increases up to certain extent and thereafter markedly decreases which might be due to reduced affinity of enzyme towards substrate at high substrate concentration and enzyme denaturation at high temperature. It was observed that a solid loading of 15 % and 40.85 °C was optimum for an enzymatic delignification of *S. spontaneum* (Fig. 7a). In case of *Ricinus communis*, solid loading of 36.10 % (solid: liquid 1:2.77) and 41.80 °C was found to be optimum for enzymatic delignification using laccase.[40]

The response surface plot of incubation time and temperature revealed that with increasing incubation time and temperature results into higher percentage of delignification but after a certain time interval no further increase in delignification observed which might be due to saturation of all the active sites of the enzyme (Fig. 7b). Incubation time of 6.21 h and 40.85 °C was found to be optimum for maximum delignification. In case of *Bambusa bambos*, incubation time of 8 h and 35.26 °C was reported to be optimum for enzymatic delignification using laccase.[24]

An enzyme concentration of 500 IU/mL and incubation time of 6.21 h was found to be optimum while analyzing the interaction between incubation time and enzyme concentration (Fig. 7c) whereas, an enzyme concentration of 400 IU/mL and incubation time of 8 h was reported to be optimum for enzymatic pretreatment of *Bambusa bambos*.[24]

The response surface plot between incubation time and pH revealed that the enzyme has a broad range of pH stability and the optimum pH for maximum delignification was found to be 6.0 while during single parameter selection, pH 7.0 was observed to be optimum (Fig. 7d). The optimum pH for enzymatic delignification of *Bambusa bambos* using laccase was found to be 6.87[24] which further support the above mentioned data for enzymatic delignification of *S. spontaneum*. After critical analysis of 3D surface plots, the optimum process conditions for enzymatic delignification were solid loading (15 %), incubation time (6.21 h), 40.85 °C, pH (6.0), and enzyme concentration (500 IU/mL). Following the optimum process conditions, the maximum predicted delignification obtained was 85.37 % which is very close to the obtained experimental percent delignification, 84.67 % (residual lignin, 2.67 %, w/w) with nearly 20 % (w/w) solid loss and 80 % (w/w) solid recovery which is in consistent with the solid loss of 15.6–47.5 % (w/w) during NaOH pretreatment of *S. spontaneum*.[41] The above adopted optimization process is in coherence with the optimization of hydrogel for improved swelling capacity.[42]

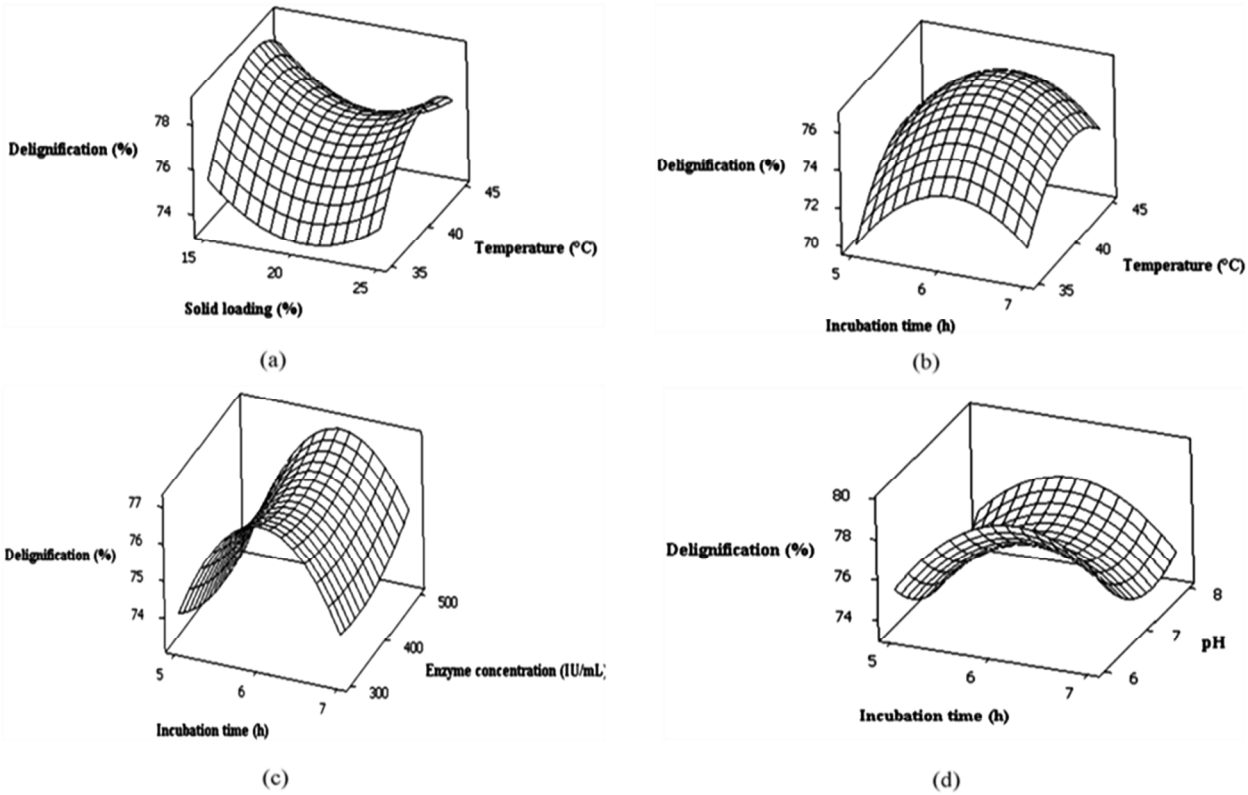

Fig. 7 Response surface plots for (a) solid loading and temperature (b) incubation time and temperature (c) incubation time and enzyme concentration (d) incubation time and pH

4.6 Delignification of *S. spontaneum* in the presence of mediators

A series of experiments were carried out to find out the effect of mediators such as, vanillic acid, ABTS, and methyl syringate for delignification of lignocellulosics. The concentrations of the mediators were varied from 1-5 %. The % delignification of delignified substrate was determined by using titrimetric method[26] where the mediator concentrations were varied from 1-5 %. The concentration of ABTS (2 %) was found to be significant having a maximum of 80.11 % delignification whereas for vanillic acid the maximum % delignification was recorded at 3 % concentration having 77.27 % delignification. In case of methyl syringate maximum delignification was recorded to be 75.85 % at 4 % concentration. Thus, mediators do not have any significant impact on the delignification process while comparing the process without mediator where 84.67 % delignification was recorded. However there are some reports which indicate the role of mediator in enhanced % delignification which was observed in the recombinant fungal laccase.[43] In the present study, though the added mediator does not have any significant role in enhanced percent delignification but it can assume that natural mediators must have present in the enzyme broth while extracting the enzyme after fermentation and thus reacted naturally without addition of any external mediator. It is worth mentioning that laccase was produced using lignin enriched biomass.

4.7 Energy density measurement

The energy density of lignocellulosics is one of the most important consideration that should be taken into account because of its prime role in overall process economy of biofuels production process. Generally, lignocellulosic feedstocks having lower energy density are considered to be less energy efficient in terms of their conversion into biofuels when compared with the high energy density feedstocks.[44-46] Initially the raw substrate contains more energy density because of higher content of lignin which carries higher energy than cellulose and hemicellulose. After pretreatment the energy density of the delignified substrate was found to be reduced (Table 4) and that might be due to degradation of lignin which is in coherence with the work where energy density of the lignocellulosic (cotton stalk) got reduced after pretreatment or delignification with ionic liquid.[47]

Table 4 Energy density of raw and delignified substrate

Biomass	Lignin (%, w/w)	Energy density (KJ/g)
raw	17.46	12.10 ± 0.33
delignified	2.67	10.48 ± 0.21

4.8 Structural characterization of *S. spontaneum*

Scanning Electron Microscopy (SEM) was done to observe the structural characteristics of *S. spontaneum* after and before enzymatic delignification. In general, lignin is a highly polymeric cross linked structure that imparts rigidity and strength to the plants. The raw substrate before pretreatment is in the form of rigid and highly ordered surface structure. However, rigidity and ordered surface structure was distorted in the enzyme mediated delignified substrate because of an enzymatic action on lignin which further enhanced the surface area of cellulose making it amenable for cellulolytic enzymes[47] (Fig. 8).

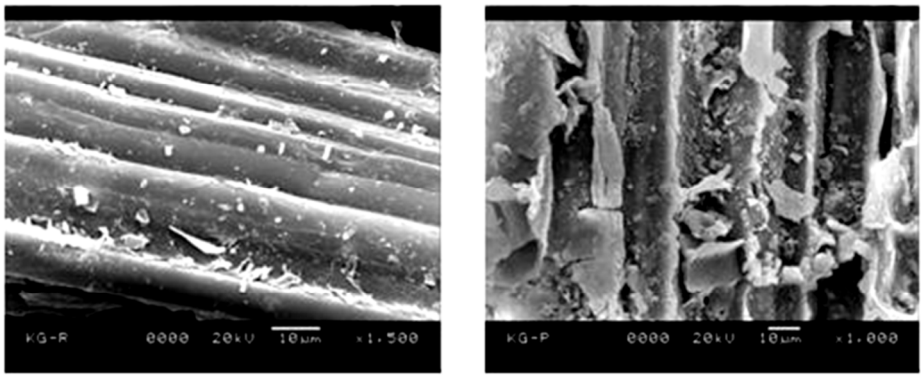

Fig. 8 SEM images (a) raw substrate (b) delignified substrate

Fourier Transform Infra-Red (FTIR) spectra show significant changes after enzymatic delignification. From Fig. 9., it was depicted that bands at 3409 cm^{-1} were because of broad O-H stretching groups and at 2919 cm^{-1} and 2854 cm^{-1}, C-H stretching in CH_3 and CH_2 groups were observed.[47] The bands at 1608 and 1637 cm^{-1} were due to >C=C< stretching and 1731 cm^{-1} were attributed to be >C=O<, respectively.[48] Absorption at 1106, 1162, 1253, and 1321 cm^{-1} could be

attributed to be acyl and O-H phenolic groups. In enzymatically delignified spectra, bands observed at 1376 cm^{-1} and 1049 cm^{-1} appears to be characteristic of C-H cellulose and hemicelluloses.[49] From the above spectral observation it is concluded that the stretching and weakening of bands with respect to its corresponding wave numbers indicates significant degradation of lignin by laccase.

Infra-red spectra of delignified sample were similar to that of raw spectra, which signifies that the delignification condition does not promote severe changes in the chemical structures of cellulose and hemicellulose.

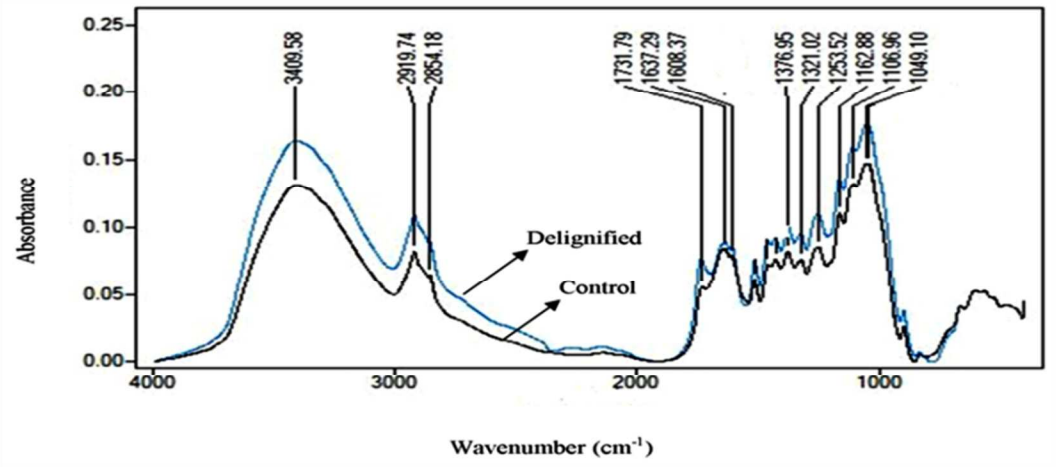

Fig. 9 FTIR spectra of raw and delignified substrate of *S. spontaneum*

Cellulose crystallinity is one of the major factors which strongly evidenced the effectiveness of the enzymatic delignification in terms of increased % crystallinity. Biodegradibility of the biomass after delignification mainly depends on the cellulose crystallinity combined with enzymes.[50] It also influences enzymatic hydrolysis of cellulose and hemicellulose after enzymatic delignification.[51, 52] Cellulose crystallinity value of raw substrate was observed to be 76.71 % which was increased to 85.26 % in the delignified substrate (Fig. 10) and is consistent with the results of increase in crystallinity of enzymatically treated *Bambusa bambos* (33 %) than the raw sample (28.44 %).[24] Crystallinity value of raw and delignified samples as well as hemicellulose and reducing sugar content of *S. spontaneum* were tabulated (Table 5). From the table it was observed that increased crystallinity (10.14 %) of the delignified substrate is due to removal of lignin and amorphous hemicellulosic fractions[53] that might expose the buried crystalline cellulose.

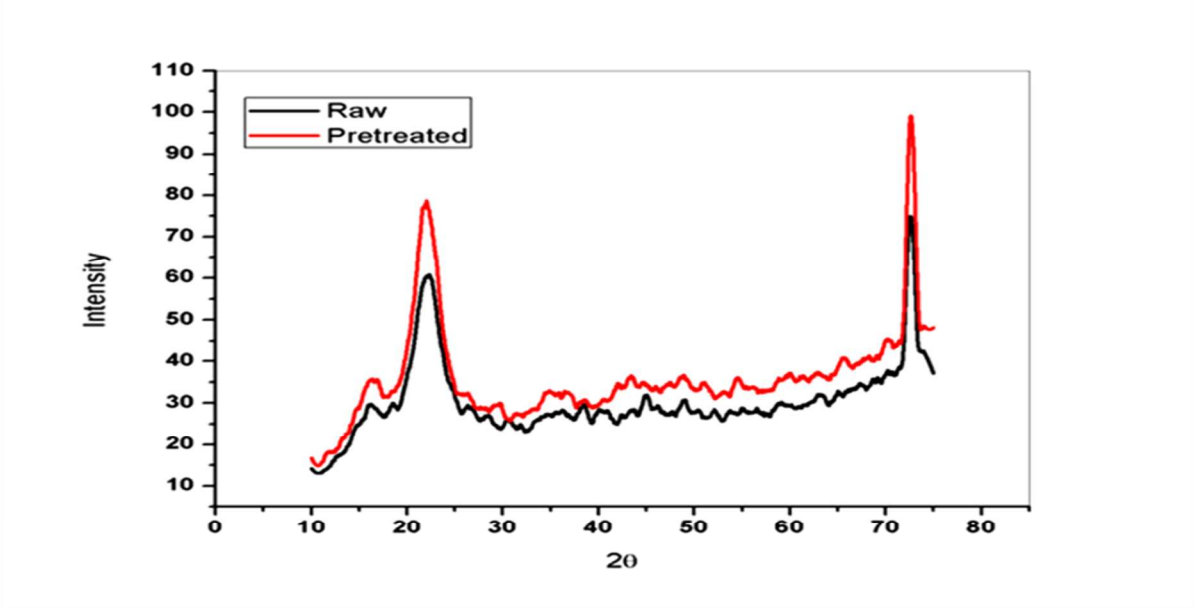

Fig. 10 XRD of raw and pretreated substrate of *S. spontaneum*

Table 5 Crystallinity and reducing sugar content of raw and delignified substrate

Incubation time (h)	% Cellulose crystallinity	% Increase crystallinity	Hemicellulose (%, w/w)	Reducing sugar (mg/g)
Raw	76.61	-	29.00	67.50
Delignified	85.26	10.14	24.48	462.18

5. Conclusion

Lignocellulose is an indispensable source for renewable biofuel production. In the present study, it has been observed that *S. spontaneum* can be a viable substrate for biofuel production owing to its richness in the content of cellulose (38.70 %) and hemicellulose (29.00 %). In order to corroborate the hypothesis, a study on enzymatic delignification of *S. spontaneum* was investigated and optimized the process conditions by RSM based on CCD design. The optimized process conditions were solid loading 15 % (w/v), incubation time 6.21 h, 40.85 °C, pH 6.0, and enzyme concentration 500 IU/mL. The maximum delignification and reducing sugar obtained were 84.67 % and 462.18 mg/g respectively, with an increased crystallinity of 10.14 % over the raw substrate. SEM analysis signifies the changes in surface characteristics of delignified biomass. FTIR shows that delignification condition does not lead to major changes in the structures of cellulose and hemicellulose. The study not only explored the potential of *S.*

spontaneum as a viable substrate but also substantiated the enzymatic delignification as one of the best methods for biofuel production, helping researchers to explore the possibility of utilizing the substrate to cater to the ever growing demand of energy.

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