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Delignification of cotton gin waste and its optimization by using white rot fungus *Pycnoporus cinnabarinus*

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Abstract

The present study investigated the effect of fungal pretreatment on cotton gin waste by solid and submerged state of cultivation and screening potential fungus for delignification. Cotton gin waste was treated with white rot fungi namely, *Trametes pubscens, Pleurotus ostreatus, Pycnoporus cinnabarinus* and *Phanerochaete chrysosporium* to separate cellulose and hemicellulose components by degrading lignin from a complex mixture of the above three components using their secreted enzymes. In the delignification process, solid state cultivation (SSC) was found to be more effective than submerged cultivation (SMC). Among the four fungi used in the study, *Pycnoporus cinnabarinus* showed better result in achieving lignin removal of 55.2 and 40.2% in solid and submerged cultivation respectively. The corresponding cellulose and hemicellulose reduction was determined as 61.9 and 70% in SSC, whereas their value in SMC was 44% cellulose and 56.3% hemicellulose. The confirmation of delignification process with respect to fungal pretreated and untreated cotton gin waste was assessed using FTIR, XRD and SEM analysis. Optimization of parameters for *Pycnoporus cinnabarinus* further showed substantial improvement in lignin removal *i.e.*, 60% in SSC at pH, shaking speed and temperature of 4.5, 138 rpm and 32°C respectively.

Key words

Cotton gin waste, White rot fungi, Lignin, Cellulose, Hemicellulose

Introduction

Lignocellulose wastes can be classified into several categories such as wood residues, grasses, straw, waste paper, milling wood, agricultural residues e.g. straw, cobs, peelings, nutshells, stalks, non food seeds, bagasse, domestic wastes (lignocellulose sewage and garbage), food industry residues, municipal solid wastes and several other materials (Qi *et al.,* 2005; Roig *et al*., 2006; Rodríguez *et al*., 2008). Now a day efforts have been put in place to produce bioethanol, biodiesel, biohydrogen and methane from lignocellulose biomass rather than from energy crops (jatropha and switch grass) because of consumption of land and water in high demand for their growth. Furthermore, use of corn and sugarcane to produce biofuels is increasingly being discouraged due to the current worldwide rise in food prices. In order to minimize food-feed-fuel conflicts, it is important to integrate all kinds of bio-waste into biomass economy (Mahro and Timm, 2007). The technology for bioconversion of lignocellulosic waste has long been considered rather expensive. However, recent increase in grain prices leads to divert the attention towards lignocellulosic waste for the production of biofuels that will reduce competition with grain for food and feed, and allow the utilization of variety of materials. Technologies which will allow cost effective conversion of biomass into fuels and chemicals are considered as efficient, eco-friendly and cost effective pretreatment systems for environment protection (Schneider and McCarl, 2003), Cianchetta *et al.,* (2014).

A huge quantity of waste is generated during the processing of cotton in cotton industry. India is the third largest cotton producing country in the world, with a large number of cotton mills. Cotton industries are facing a lot of problem in disposal of cotton gin waste because of stringent regulation of the

environmental act. The waste generated after the ginning of cotton fibers, is ligno-cellulosic material and thus, this biomass can be utilized to produce ethanol, which is considered as promising alternative energy source for the transport sector. Moreover the abundance, low cost and non competitiveness with food stuffs of cotton gin waste ensure reliable resource of energy (Ingram, 2008). Cellulose (linear polymer of several hundred to more than ten thousand β-(1, 4) linked D-glucose units) binds tightly with lignin and hemicelluloses forming a complex material and hence, delignification is a prerequisite step to release cellulose and hemicelluloses from the waste. In recent years, efforts have been made to produce ethanol from bioconversion of cellulosic cotton gin waste with the help of different microorganisms, including fungi. The ability of fungi to degrade lignicellulosic materials is due to their highly efficient enzymatic system such as enzyme laccase (Andreu and Vidal, 2013).

White rot fungi like *Trametes pubscens, Pleurotus ostreatus, Pycnoporus cinnabarinus* and *Phanerochaete chrysosporium* have the capability to release cellulose and hemicelluloses component from lignocellulosic biomass (Sabarez *et al.,* 2014; Adenipekun and Okunlade, 2012). For any process to be successfully implemented in a commercial way, the process needs to be optimised, at least at the basic parametric level. Pretreatment is a process of conversion of lignocellulosic material to its elemental form which will reduce the total production cost of ethanol bioconversion by 1/3rd and will remain one of the major barriers in preventing commercial success (McAloon, 2000). This delignification process can provide better access to cellulose and hemicelluloses for their further conversion into sugar and subsequently ethanol.

An effort was made in the present study to screen use and different efficient white rot fungi so that it might open some new possibilities in pretreatment process of cotton gin waste. Therefore, the present study was based on optimization of key parameters like temperature, pH and shaking speed which will be investigated to determine optimum pretreatment condition, using solid state of cultivation.

Materials and Methods

Cotton gin waste was collected from Shree Ambica Agro Industries Ltd., Balangir, Orissa, India. The waste containing lengthy cotton fibers was grand to reduce its length by milling, using pulverisette-5, Fritsch Company. The impurities were removed by heating and washing with water followed by overnight drying at 60 ºC and then storing in an air tight container at room temperate till further use.

The fungal strains, *Trametes pubscens* (NCIM.No-1087), *Pleurotus ostreatus* (NCIM.No-1200*), Pycnoporus cinnabarinus* (NCIM.No-1181) and *Phanerochaete chrysosporium* (NCIM.No-1197) were collected from the National Collection of Industrial Microorganisms, Pune, India. Fungal strains were inoculated on potato dextrose agar (PDA) plates and incubated for 4-5 days at 35 ºC, and finally stored in refrigerator for further use.

Analysis of cotton gin waste for components : The moisture content of the processed cotton waste was determined by solid determination method of ASTM E 1754-95 (ASTM, 1995) and the ash fraction was also determined following ASTM E1721-95 procedure (ASTM, 1995). Lignin degradation and analysis of carbohydrate fractions of cotton waste were done following the protocol given in the book 'Laboratory Technique in Sericulture' (Goal, 2007).

Fungal pretreatment of cotton gin waste : Pretreatment of cotton gin waste was carried out by submerge (SMC) and solid state (SSC) cultivation. In SMC pretreatment, 6g of air dried cotton waste was supplemented with 108 ml of acetate buffer (20mM, pH 4.5) and 1ml spore inoculums. For SSC pretreatment, 6g of the cotton gin waste was mixed with 9.6ml of acetate buffer (20mM, pH 4.5) and 6ml spore inoculums to obtain 75% substrate moisture content (wet basis). Sample without fungal strain was used as control. Fungal pretreatment experiments were carried out in 250ml Erlenmeyer flasks capped by a silicon stopper with inlet and exit lines connected to 0.2 µm filters. Flasks with cotton waste were autoclaved for 20 min at 121 ºC and 15 psi, cooled, mixed with acetate buffer, and then inoculated with spore suspension ($5x10^6$ spores g^{-1} cotton waste). Pretreatment was performed in an air convection incubator at 39 ºC with a shaking speed 100 rpm, and the flasks were flushed with oxygen (125ml min^{-1}) for 10 min every 7day, starting from day 0. Both SMC and SSC cultivated flasks were sampled on every 8th days and stored at 4 ºC for composition analysis.

Characterization of cotton gin waste : FT-IR spectra of dried cotton gin waste samples were recorded on FTIR spectrophotometer (Perkin Elmer-Version 5.3).Two milligram of sample was mixed with 200 mg of KBr to ensure uniform dispersion of the sample. Sample spectra were obtained over the range of 400 and 4000 cm^{-1} with spectral resolution of 0.5 cm^{-1}.

The overall crystallinity of untreated and pretreated samples was determined by XRD PW 3040 equipment, using Cu Kα radiation (α = 1.54 Å) at 30 kV and 20mA. The samples were scanned and intensity was observed at 2θ range from 15° to 75° with scanning speed of 3^0/min. Crystalline (%) was calculated by the formula developed by (Segal *et al.,* 1959).

$$[(I_{002} - I_{am})/I_{002}] \times 100$$

where I_{002} represent maximum crystalline intensity peak at 2θ between 22° and 23° for cellulose. In the above equation I and I_{am} represents minimum crystalline intensity peak at 2θ between 18° and 19° for cellulose.

SEM images for both untreated and pretreated samples of cotton gin waste were obtained after drying, followed by coating

with platinum using JEOL JSM6480 LV SEM.

Optimization of pretreatment parameters : The most effective fungal strain was selected, based on the result of cellulose, hemicelluloses and lignin degradation throughout the pretreatment method of cotton gin waste. A three level RSM based central composite design (CCD) was employed for optimization of pretreatment process using Minitab 16.2v software, which is the 20 combinations with 6 center point of three variables. Statistical analysis was performed with 95% confidence level. Three different parameters (independent variables) selected for this study were as follows (i) pH at three levels 4, 4.5 and 5, (ii) temperature 30, 35 and 40 ºC and (iii) rpm 100, 120 and 140. Optimization of pretreatment process was conducted for 32 days in case of solid state cultivation, since it rendered maximum efficiency. Pretreatment experiments were carried out in triplicates. In coded terms, the lowest, central and the highest level of 3 variables were –1, 0 and +1 respectively. After 32 days of incubation, the total lignin content of untreated cotton gin waste was determined.

Results and Discussion

The cotton gin waste was found to contain 40.3%cellulose, 15% hemicelluloses, 19.8% lignin, 9% ash and 8.5% moisture content. Thus, the total carbohydrate percentage was calculated as 56.3% (holocellulose). This high percentage of carbohydrates makes cotton gin waste a potential feed stock material for bio-ethanol production.

The major limitations in the effective conversion of cellulose and hemicelluloses in reducing sugar by enzymatic hydrolysis lie in the cross-linking of their organic components with lignin in lignocellulosic biomass (Fan *et al*., 1987). This network of interaction poses an energy barrier and requires disruption. Removal of lignin from biomass by pretreatment process exposes the crystalline structure of cellulose, improves solubilization by water and thus facilitates substrate accessibility by hydrolytic enzymes (Sun and Cheng, 2002). Therefore, a suitable pretreatment method is required before enzymatic hydrolysis in order to get maximum yield of sugar. Furthermore it has been reported that washing and heating increases the cellobiose content with improved delignification after pretreatment in both SMC and SSC cultivation (Shi *et al*., 2009). *Pycnoporus cinnabarinus* has shown the best pretreatment efficiency in solid state cultivation in comparison to submerged over all four fungi. The lignin removal with this correspond to 55.2 and 40.2% by solid state and submerge state of cultivation respectively. The released cellulose and hemicelluloses were calculated as 61.9 and 70% respectively in solid state and 44 and 56.3% in SMC, as observed in Fig. 1(a). Similarly, for *Trametes pubscens, Phanerochaete chrysosporium and Pleurotus ostreatus* lignin removal in solid state and submerge state culture were 55 and 40%, 53.2 and 39 % and 52 and 38% respectively. A substantial amount of lignin removal has been reported with *Trametes*

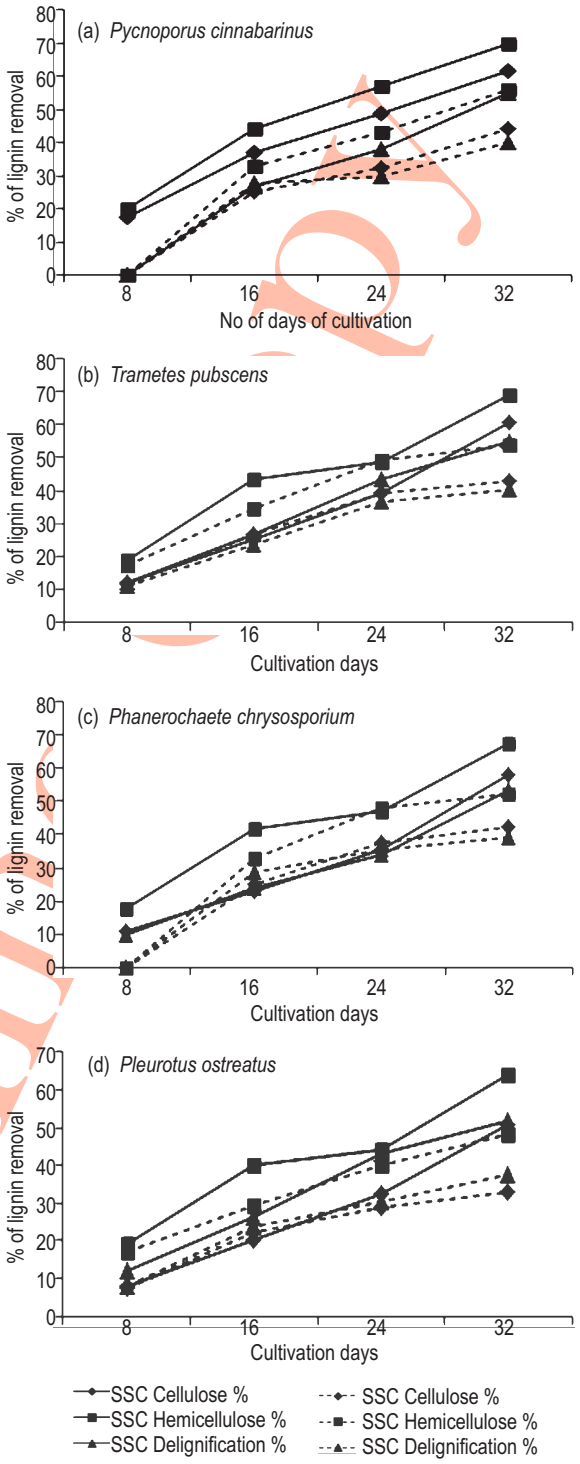

Fig. 1 : Effect of pretreatment on percentage of cellulose, hemicelluloses and delignification by using (a) *Pycnoporus cinnabarinus,* (b) *Trametes pubscens*, (c) *Phanerochaete chrysosporium* and (d) *Pleurotus ostreatus*

pubscens i.e. release of 61% of cellulose and 68.9 % hemicelllulose in solid state cultivation (Fig 1. (d). Overall, efficiency of SMC was low in all the cases.

The characteristics peak of cellulose and lignin were obtained at 1700 cm^{-1} to 1750 cm^{-1} and 1513 cm^{-1} as found in untreated sample but not in the pretreated sample (Fig. 2 a) .This may be due to reduction of compounds rich in carbonyl (C=O) *i.e.* mostly lignin, some amount of hemicelluloses, and other extractives were removed during the pretreatment process. The absorption band at 2729 cm^{-1} was attributed to the stretching vibrations of hydroxyl (OH) groups in untreated (control) sample. Furthermore, difference in the intensity of absorption at ~2500 cm^{-1} band size was due to difference in the absorbed water content between untreated and pretreated samples. This was explained with a change in the degree of inter molecular H-bonding between OH group of cellulose and water. It can be expected that there would be increase in surface area and rearrangement of cellulose microfibrils which may provide better accessibility to OH group by the enzymes in pretreated sample as a similar study supports our outcome (Suchy *et al*., 2009). The OH groups may include sorbed water, aliphatic compounds, primary and secondary alcohols found in cellulose, hemicellulose and carboxylic acids in extractives (Khan *et al*., 1993; Coates, 2000). The shoulder near the OH stretching vibrations, 2854 cm^{-1}, was attributed to CH stretching vibrations and corresponded to the aliphatic moieties in polysaccharides (cellulose and survived hemicelluloses) of treated sample. The bands in 1451-1333 cm^{-1} and 1450-1357 cm^{-1} region in untreated sample may be due to CH in-plane deformation of CH_2 groups, while peaks at 1157-1058 cm^{-1} were due to linkage present in cellulose in both the samples. The pure cellulose was obtained at the following frequencies : 1431, 1372, 1318, 1281, 1164, 1059 and 897 cm^{-1}.

Although cellulose macrofibrils have been observed

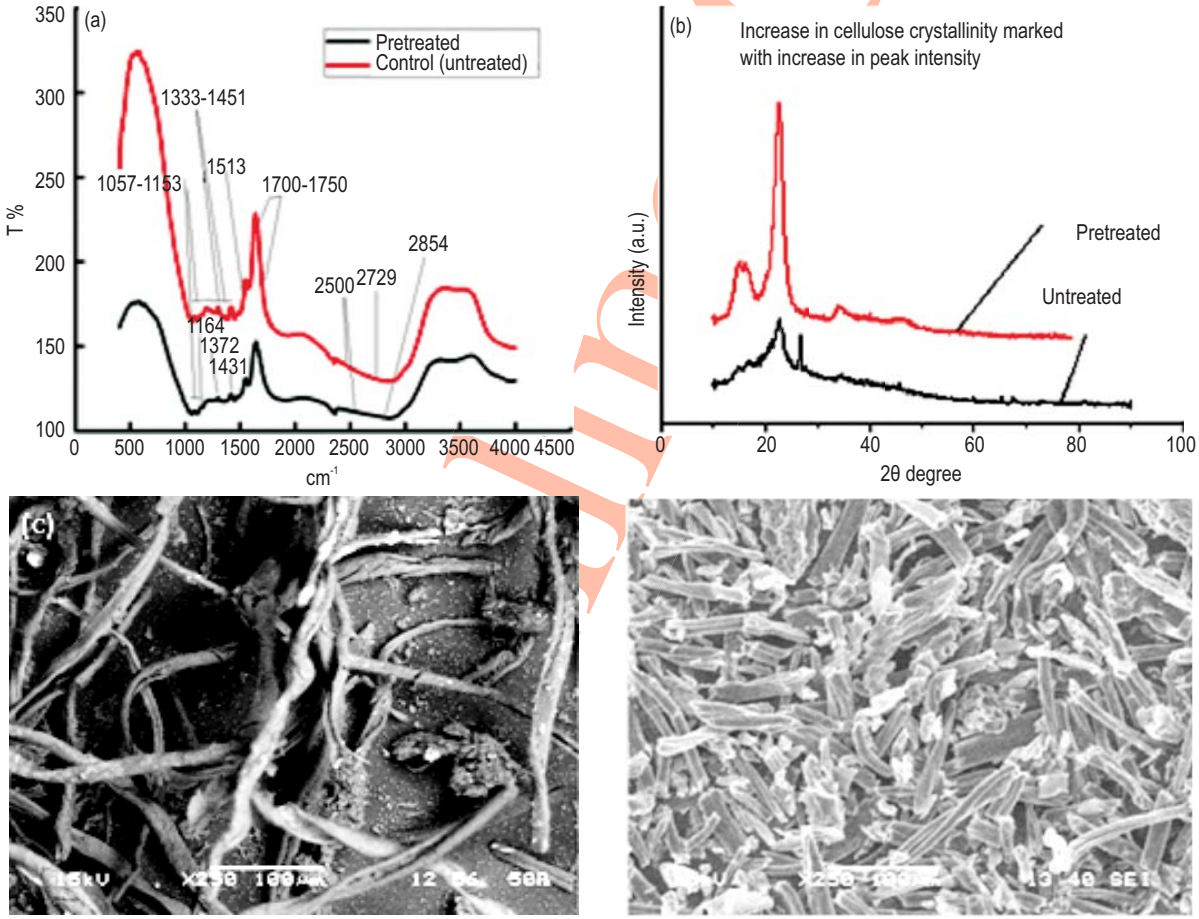

Fig 2 : Characterization of control (untreated) and pretreated cotton gin waste through (a) FTIR spectra; (b) X-Ray diffraction diagram and (c) SEM images

more profusely with increased pretreatment time, no change in XRD was detected, as shown in Fig. 2 (b). XRD for untreated and pretreated samples exhibited similar crystalline patterns. The width at half height for peaks at 2θ =17° and 26° were similar for all the samples except higher haziness in untreated sample, which suggested similarity in crystallite sizes (Ibrahim *et al.,* 2010).The cellulose crystallinity value of the untreated sample of cotton gin waste was 18.36%, while that of pretreated sample was 23.94 suggesting improvement in crystallinity of the sample. The hydrogen bonding holds the adjacent chain in place relative to one another. The crystallinity of pretreated sample was increased due to removal of lignin and hemicellulose (both of which extend amorphousness to the material) (Kumar *et al*., 2009 a, b). It is expected that amorphous region present in between the regular crystalline region is subjected to enzyme attack and after its removal is well exposed, the crystalline region with improved crystallinity was observed in the pretreated sample.

Scanning electron micrographs showed the morphology of cellulose and hemicellulose fibres with different severities (Fig 2c).The untreated sample showed compact fibres distributed over the whole region. Whereas pretreated sample showed partially degraded stretched fibres indicating the influence of enzyme treatment. The figure shows that some macrofibrils remain separated, whereas other macrofibrils agglomerated. This showed enhancement of surface area due to removal of lignin and its associated compounds such as xylan. A significant change on the surface property towards favorable interaction with enzyme had occurred due to pretreatment resulting in cleavage of the amorphous region of cellulose with retention of the crystalline fraction. Additionally, lignin removal from the pretreated sample increased the degree of crystallinity as reported by (Zhao *et al.,* 2008). Further, analysis by gravimetric analysis showed 3.4% biodegradation of cotton gin waste during pretreatment corroborating removal of lignin from the pretreated sample.

The process parameters optimization would focus on the improvement of economic feasibility of the process. The optimization study of three individual parameters *i.e.,* pH (4 to5), temperature (30 to 40°C) and rpm (100 to140) was carried out to obtain maximum percentage of lignin degradation of cotton gin waste by using central composite design. The percentage of delignification was obtained in the range of 52.9 to 60.5%. The mathematical expression relating % lignin degradation to different independent variables is expressed below in terms of coded factor:

$$Y1 = 59.38+0.24A_1-0.72A_2+0.56A_3-3.50A_1^2-0.80A_2^2-0.40A_3^2-0.86A_1A_2-0.81A_1A_3-0.28A_2A_3$$

Where, A1, A_2 and A_3 represents pH, temperature and rpm respectively. The individual action of all three parameters studied, quadratic and interaction effects between the dependent variables were found to be significant from the regression model. ANOVA of the quadratic regression for enzymatic pretreatment of pretreated cotton gin waste is summarized in Table 1. The regression model for pretreatment of cotton gin waste showed high F-value (12.67) and a very low probability value (< 0.001), revealing the significance of the model (Liu *et al*., 2010). The square and interaction effects between the variables were found to be statistically significant with a *P* –value less than 0 and 0.04 respectively. Although the square terms from the model showed more significance or were effective with high F-value 31.58. The

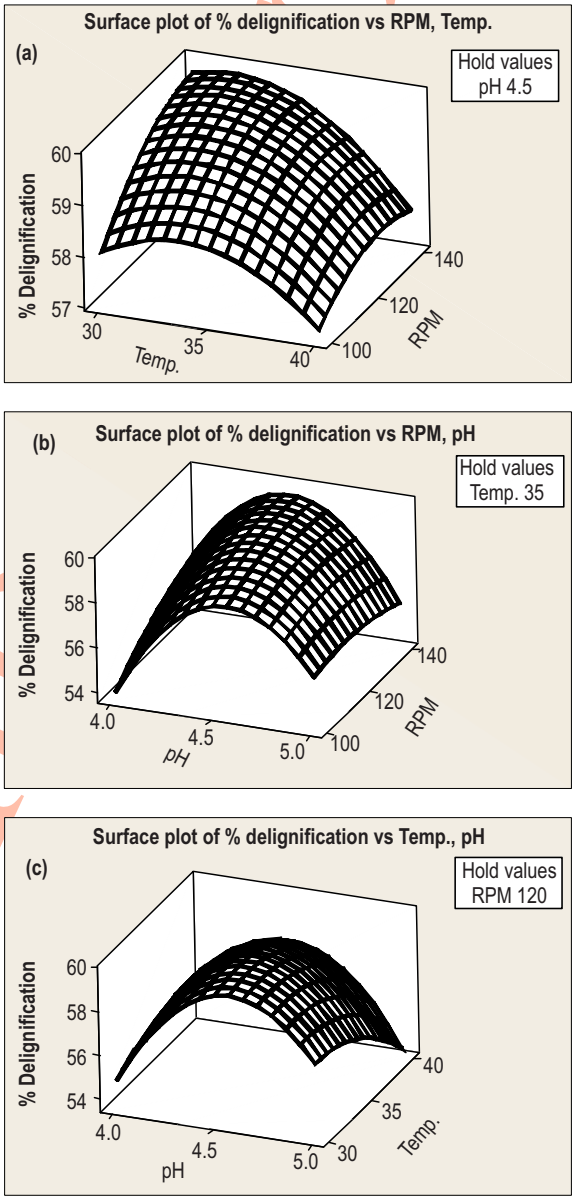

Fig. 3 : Response surface plots showing the effect of (a) temperature and shaking speed, (b) pH and shaking speed and (c) pH and temperature on pretreatment of cotton gin waste

Table 1 : ANOVA analysis of RSM model for enzymatic pretreatment of pretreated cotton gin waste

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	114.102	114.1025	12.6781	12.87	0
Linear	3	8.896	8.896	2.9653	3.01	0.081
Square	3	93.313	93.3127	31.1042	31.58	0
Interaction	3	11.894	11.8938	3.9646	4.03	0.041
Residual Error	10	9.85	9.8495	0.985		
Lack-of-Fit	5	9.336	9.3362	1.8672	18.19	0.003
Pure Error	5	0.513	0.5133	0.1027		
Total	19	123.952				
R^2 = 92.05%						

DF= degree of freedom, SS= sum of squares, MS= mean sum of squares, F= Fisher's F value(calculated by dividing the MS owing to the model by that, due to error), P= probability

quality of model fit was evaluated by coefficient (R^2) and its statistical significance was determined by F-test. For pretreated sample, R^2 values obtained was 0.9205 and hence justifies the robustness of the model. Fig. 3(a) shows interaction of temperature and RPM at a constant pH, where as Fig 3(b) shows interaction between pH and RPM at constant temperature on lignin degradation of the pretreated sample. The three dimensional plots showed that temperature at 35^{0}C and shaking speed at 120 rpm caused an increase in lignin degradation (%), yielding a maximum lignin degradation value of 59.85 % after 32 days of solid state cultivation. However, at a constant temperature interaction between shaking speed and pH gave maximum value of delignification. Fig .3(c) shows the optimization of temperature and pH by keeping the RPM constant (Manikandan *et al.,* 2010). The above statistical model suggests the optimum predicted condition of pH, shaking speed and temperature as 4.5, 138 rpm and 32 ºC to obtain high percentage of delignification. In order to check the reliability of predicted response, experiments were performed in triplicate, under optimum predicted conditions. From these experiments maximum delignification was found to be 61.2 %, which is in good agreement with the predicted value reported by Mirza, *et al.* (2013).

The present study highlighted the effectiveness of fungal pretreatment process for providing technical and economic feasibility to harness cotton gin waste in the line of intimation to cleaning up the environment. In the present study, Pycnoporus *cinnabarinus* was found to be highly efficient fungal strain in producing lignocelluloses enzyme for the delignification process in comparison to other white rot fungi. It has been further demonstrated that solid state fermentation process was more effective in providing higher delignification efficiency than submerged fermentation. Optimization of pH, temperature and shaking speed affect the percentage of delignification.

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