Studies on delignification and inhibitory enzyme kinetics of alkaline peroxide pre-treated pine and deodar saw dust

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ABSTRACT

Delignification of lignocellulosic biomass by alkaline peroxide pre-treatment is a preliminary important step for an overall biomass fractionation process. In the present work, saw dusts are pre-treated by aqueous alkaline peroxide solution under different temperatures over a predetermined time. It is seen that Combined Pre-treatment (CP) removes a substantially higher quantity of lignin from biomass under a particular temperature. At elevated temperatures, the extent of delignification is observed much better. The % removal is: [PR: 19.35%(30°C): 25.26%(50°C): 33.30%(100 °C)]; [CD: 14.64%(30°C): 23.64%(50°C): 28.83%(100 °C)]. Batch kinetics is investigated with certain models and corresponding parameters are estimated. As pre-treatment severity is strongly correlated to the pre-treatment

temperature, increased value of "*potential degree of delignification*" is observed at escalated temperatures. Kinetics of enzymatic hydrolysis of delignified biomass shows decreased product inhibition with increased substrate concentration under a particular enzyme loading. Starting with a combination of 50 g/L substrate concentration with an enzyme loading of 13.23 g/L, an optimum concentration of 17.2 g/L and 21.19 g/L of glucose are produced from *Pinus roxburghii* and *Cedrus deodara* respectively. Experimental data fit quite well with the competitive inhibition kinetics based theoretical models with $r^2 \geq 0.95$. It is inferred that enzymes are competitively inhibited by glucose.

Keywords: *pseudo first order kinetics; activation energy; Arrhenius constant; delignification rate constant; potential degree of delignification.*

1. Introduction

Production of fuels from lignocelluloses is one of the most promising solutions in response to the present day energy crisis [1]. Lignocelluloses are composed of lignin and sugar polymers. Upstream delignification of biomass is the most crucial and rate limiting step of the whole process of depolymerisation of sugar polymers for obtaining biofuels [2]. There are a number of pre-treatment methods available to delignify biomasses; from the perspective of economy and environment friendliness alkaline peroxide based pre-treatment can be considered the most promising chemical method to delignify a biomass effectively without producing much inhibitors for the following steps of enzymatic hydrolysis [3, 4]. NaOH and hydrogen peroxide are two major components of alkaline-peroxide based pre-treatment. As delignification is a pH dependent process, NaOH is used to make up pH of the solution till desired value is reached. If the pH of alkaline-peroxide solution is maintained below 10.5,

removal of lignin is not very effective while the same is achieved when wheat straw is treated at room temperature (25°C) using alkaline-peroxide solution at a pH of 11.5 [5]. In this process energy is also optimized as lignin can be removed from the biomass within initial 6h of pre-treatment. While pH is an important factor for delignification, presence of H_2O_2 is another crucial aspect that must be considered. In presence of H_2O_2, a 37.1% recovery of Glucan is accomplished during saccharification of alkaline peroxide treated biomass. On the other hand, only 27% glucan is recovered from NaOH treated biomass [5, 6]. Lignin removal is directly proportional to the reaction temperature for a particular substrate concentration [7]. The same research group has delignified corn stover using alkaline-peroxide solution of pH 11.5 and achieved 68.6% of total glucose using accellerase 1000 as the enzyme at a loading of 15mg/g of glucan without intermediate washing. It is claimed [8] that the ultimate glucose generation is enhanced with elevated substrate loading while pre-treated broth is neutralized with an addition of acid (instead of washing) followed by subsequent removal of water by freeze drying. Alkaline-peroxide pre-treatment of corn stover is carried out by the same research group where the pH of the pre-treatment solution is continuously monitored and maintained at 11.5 and finally 35% reduction of total insoluble biomass is achieved due to solubilization of klason lignin along with 5.5% of glucan and 10.1% of xylan [7, 8]. Supplementing alkaline-peroxide solution with Cu-(bpy) provides a yield of 80% glucose and 70% xylose [9]. Cu catalyzed alkaline peroxide pre-treatment with an upstream alkali mediated extraction of hybrid poplar improves solubilization of lignin and xylan [10]. Addition of H_2O_2 into the pre-treatment liquor (mode: fed batch) has been more effective in terms of solubilization of increased lignin and xylose. During subsequent enzyme-catalyzed hydrolysis, 85% glucose and 95% xylose yields are achieved [10]. In the present study, two biomass, namely, *Pinus roxburghii* (PR) and *Cedrus deodara* (CD) are pre-treated using alkaline-peroxide solution in order to delignify them at a pH of 11.5 and at temperatures

30°C, 50°C and 100°C.

Reaction kinetics for the pre-treatment steps is an important tool for deciding the economics of a process in terms of the capital cost for the reactor. Alkaline peroxide solution forms a pseudo homogeneous system with biomass and time-variant delignification is considered as a first-order reaction in a pseudo homogenous system. Generally two different kinetic models can be considered for delignification. In one type of model (*simultaneous model*), biomass are considered as being composed of various types of polymers with different phases of degradation being attributed to different rate-controlling mechanisms of the overall delignification process[11]. The other model (*consecutive model*) considers that the lignocellulosic biomass is composed of different polymer fractions which may be degraded consecutively or simultaneously. The consecutive model was developed based on the assumption that lignin is composed of initial, bulk and residual lignins which react successively. The simultaneous model assumes that different types of lignin dissolve simultaneously at the beginning and react concurrently with the advancement of pre-treatment [11-15]. In the present study, delignification kinetics of two different procedures has been investigated and validated using alkaline peroxide treatment of two different softwood samples.

In the current study, two different wood powders are pre-treated with 2% alkaline-peroxide pre-treatment at three different temperatures and the corresponding delignification pattern is observed in order to analyse and interpret the delignification kinetics. On the other hand, biomass is separately delignified with a specific Combined Pre-treatment (CP) comprising of autoclaving as the first step, followed by 1h probe sonication along with a subsequent 5h long alkaline peroxide pre-treatment as the final step.

2. Experimental procedure

2.1. Materials

Branches of pine (PR-*Pinus roxburghii*) and deodar (CD-*Cedrus deodara*) trees are collected from the Kullu Valley by technical helps from G. B. Pant National Institute of Himalayan Environment and Sustainable Development, Himachal Regional Centre (Mohal-Kullu, Himachal Pradesh, India) and these are chipped into small wood pieces with the help of handsaw. The wood chips are further processed into saw dust using a chain-saw (**Make:** STIHL; **Model:** Cast Iron Chain Saw, MS-180) and a circular saw (**Make:** BOSCH; **Model:** GKS190). Finely ground saw dust samples, free from impurities and larger particles, are isolated and collected after passing the saw dusts through a sieve (screen size: 85 mesh).

2.2. Chemicals used

All chemicals, like distilled water (CAS NO:7732-18-5), NaOH pellets (CAS NO: 1310-73-2), H_2SO_4 (98% CAS NO: 7664-93-9) and hydrogen peroxide (30%, CAS No: 107209), used in the experiment are purchased from Merck (Germany). Glassware used during the experiment are purchased from Borosil (India). Glass vacuum filtration device with flask, used for filtration purpose, is manufactured by Sartorius, Germany. Instruments like magnetic stirrer assisted heating mantle (Make: Remi, India; Model: Q20A) and autoclave (Make: Remi, India; Capacity: 20 L) are used. A probe sonicator (Make: PCI analytics, India; Model: PKS-750F; Probe dia: 10 mm) is used.

2.3. Experimental methods

Two different types of delignification protocols, conventional alkaline pre-treatment and combined pre-treatment, are followed in course of the present study in order to understand the delignification kinetics and the impact of various process conditions on delignification. In

conventional delignification procedure, an aqueous solution of 2% H_2O_2 is prepared and adjusted to a pH of 11.5, using NaOH pellets [7]. Afterwards, the biomass is submerged into an aqueous peroxide solution with a solid to alkaline-peroxide solution ratio of 1:40 (w/v). Three such identical experimental set up are prepared in three separate round bottom flasks fitted with glass stoppers and cook them at three different temperatures (30°C, 50°C and 100°C) for 5h. This procedure is denoted as 'AP' throughout the rest of the manuscript.

On the other hand, another pre-treatment procedure (denoted as 'CP') is followed, where three different pre-treatment procedures are followed sequentially. Primarily, saw dusts are submerged into 2% aqueous H_2O_2 solution (~pH=11.5) with a solid:alkaline-peroxide solution ratio of 1:40 (w/v) and autoclaved for 1h [16]. Afterwards, the whole broth is cooled down and the solid fraction is separated, washed with distilled water in order to neutralize it and then get them dried at 40°C. Next, the solid fraction is submerged in a 2% aqueous H_2O_2 solution (~pH=11.5) and sonicated for 1h using a probe sonicator (Make: PCI analytics, India; power used=300W; probe diameter: 10mm) [17]. On completion of sonication, the solid fraction of the broth is isolated by filtration using filter paper (Whatman no 1**,** Make: Merck) followed by drying in hot air oven at 40°C temperature. After that, three set of such dried saw dusts of identical weight are submerged into a 2% aqueous H_2O_2 solution of pH= 11.5 with a solid to alkaline peroxide solution ratio of 1:40 (w/v). Subsequently, those three set of colloidal mixtures are cooked at three different temperatures (30°C, 50°C and 100° C) for 5h. Schematic diagram of combined pre-treatment is presented in Fig. 1.

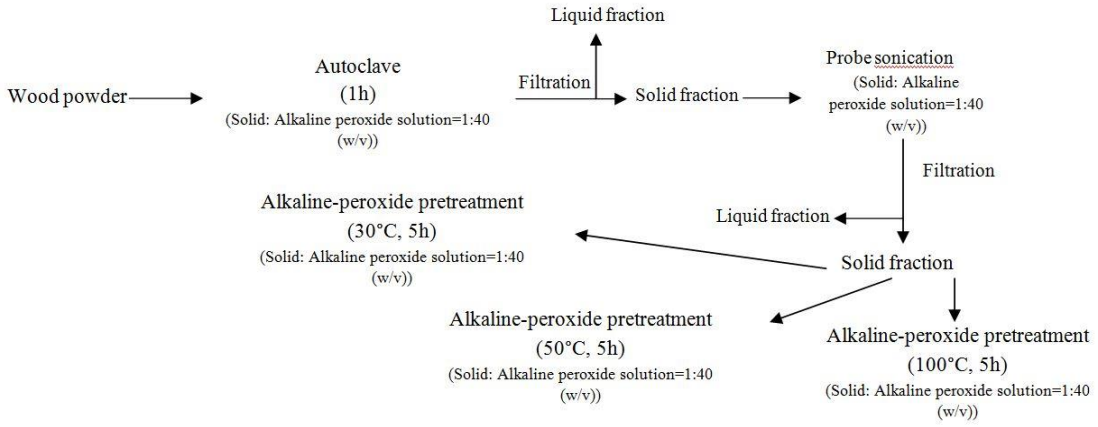


Fig.1. Schematic diagram of combined pre-treatment (CP).

2.4. Sampling and lignin quantification

During the course of delignification experiment, aliquot amount of solid fraction of samples along with aqueous peroxide solution are withdrawn at the end of each of the predetermined time intervals with an aim to understand the variation of delignification characteristics with time. While sampling, a solid (biomass) to alkaline peroxide solution ratio of 1:40 (w/v) is maintained in the reaction vessel in order to keep experimental conditions constant throughout the course of experiment. After each withdrawal, the samples are immediately washed with de-ionized water and then dried at 40°C.

Lignin is quantitatively estimated following TAPPI T222 method. 1g (±0.1) of moisture free wood sample (untreated and pre-treated) is taken and dissolved into 15 ml of 72% H_2SO_4. Afterwards, the entire broth is kept at 20°C for 2h. Thereafter, the solution is diluted using hot water and made up to a total volume of 575 ml and then boiled for four hours. Next, the mixture is filtered through a silica crucible in order to isolate acid insoluble lignin. Finally, the crucible is dried and acid insoluble lignin is estimated [18].

$$Lignin\ (\%) = \frac{Weight\ of\ lignin\ (g)}{Weight\ of\ the\ test\ specimen(g)} \times 100 \quad (1)$$

1

2 *2.4. Delignification parameters and kinetics*

3 Kinetic data for the pre-treatment of saw dusts are evaluated at different temperatures (30 °C,
4 50 °C and 100 °C) for both AP and CP. For each of these isothermals, lignin content of the
5 pre-treated sawdust is determined at reaction times 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5h.
6 Delignification kinetics is analysed by the amount of lignin removed from the pre-treated
7 solids with time at different temperatures.
8 The lignin solubilisation ratio at any time (t) can be described as follows [14, 19]:

$$L_S(t) = \frac{C_{L_o} - C_L(t)}{C_{L_o}} \quad (2)$$

9

10 Where, C_{L_o}= initial lignin content (wt %) of the biomass, $C_L(t)$ = lignin content (wt %) of the
11 biomass at any time t, $L_S(t)$= lignin ratio.
12 However, as the phenomenon of delignification is considered as a first order reaction,
13 delignification rate can be expressed as:

$$\frac{dL_S}{dt} = K_L(1 - L_S) \quad (3)$$

14 Where, K_L is the delignification rate constant. However, by solving equation (3), considering
15 the initial condition ($L_S = 0$ at t=0), a time dependant expression of lignin solubilisation
16 ratio (L_S) is found as follows:

$$L_S = 1 - \exp(-K_L.t) \quad (4)$$

Therefore, by plotting $-\ln(1-L_S)$ against time t, considering intercept '0', will provide the value of K_L from the slope.

Additionally, it has been observed for years that it is almost impossible to remove the entire lignin content from the biomass. However, a new parameter termed as 'potential degree of delignification (d_D)' is considered which stands for estimating the maximum lignin solubilisation ratio for a particular biomass under the severity of a particular pre-treatment process [14].

However, the kinetic model expressed in equation (3), can also be expressed in terms of the potential degree of delignification, as follows:

$$\frac{dL_S}{dt} = K_L(d_D - L_S) \tag{5}$$

With the limit on d_D being: $0 \leq d_D \leq 1$.

Integration of equation (5) can be expressed as given below:

$$L_S = d_D[1 - \exp(-K_L t)] \tag{6}$$

Therefore, by plotting L_S against $[1 - \exp(-K_L t)]$, the value of d_D is estimated for each of the operating pre-treatment conditions.

As delignification is a function of pre-treatment temperature, there must be a correlation between the rate constant and temperature which can be characterized by the value of activation energy. The activation energy, in this case, can be defined as the minimum energy that is required by the molecules of pre-treatment solution to initiate delignification. K_L can be related to temperature following the Arrhenius law[2]:

$$K_L = Aexp^{-E_a/RT} \tag{7}$$

Where, A = Arrhenius constant; Ea = activation energy; R = Universal gas constant = 8.314 J/mole·K; T = absolute temperature (K). Assuming the sample contains only one type of lignin and each lignin fraction is kinetically homogeneous, the activation energy (E_a) of delignification is estimated using the logarithmic form of the Arrhenius equation ($ln\ K_L = ln\ A - Ea/RT$) by plotting $ln\ K_L$ against $1/T$. The slope represents the value of Ea/R.

2.5. Physical characterization of wood powder at different stages of CP

The texture of the lignocellulosic samples are physically characterised at the end of various steps of pre-treatment, with the help of a Scanning Electron Micrograph (SEM) at ×3,500 magnification. A Field Emission Scanning Electron Microscope (FE-SEM) is used [Make: Jeol, Germany; Model: JSM-7610F] in order to determine the change of surface structure and texture of pre-treated biomass. Additionally, thermal analysis of biomass was carried out after different stages of pre-treatment using a Thermo-Gravimetric Analyzer (TGA) [Make: SHIMADZU, Japan; Model: DTG-60A] in order to understand the thermal stability of two main components of biomass: cellulose and lignin. 0.5-1.5 g of sample is placed in an alumina crucible and heated from 30°C to 900°C at a rate of 10°C/min under nitrogen atmosphere (30ml/min) [20, 21].

2.6. Enzymatic hydrolysis of delignified biomass

Hydrolysis of delignified biomass is carried out in a round-bottom flask , agitated at 115 rpm, using phosphate citrate buffer (pH 4.8) and delignified wood powder of three different concentrations (25, 50, 125 g/L) as substrate. Cellulase blend [SAE0020, 1000U/g, 1.2g/ml], cellulase [C1184, 1.3U/mg] derived from *A. niger*, hemicellulase [H2125,1.5U/mg] derived

from *A. niger* and β-glucosidase [49290, 7.7 U/mg] derived from almonds are used to depolymerise cellulosic biomass.A cocktail ofenzymes is prepared in a ratio of cellulose mix: hemicellulase: β-glucosidase = 1:1:2 [unit basis], whereas the cellulase mix is prepared using cellulase blend [SAE0020] and cellulase [C1184] in a ratio of 1:1.8 [unit basis][22]. Enzymatic hydrolysis is performed using two different enzyme concentrations (1.28 g/L and 13.23 g/L) at 50°C. Sodium Azide [0.1% (w/v)] is used as the antimicrobial agent. Sugar concentrations are determined using DNS assay as described earlier by Miller, 1959 [23]. Additionally, HPLC (Make: Waters GmbH, Germany; Model: 2489) fitted with a RI detector (Make: Waters GmbH, Germany; Model: 2414) and Brownlee amino column (Make: PerkinElmer, USA; Material: N9303501) is used to quantify individual sugars present in the hydrolysis broth.The mobile phase used at ambient temperature consisted of HPLCgrade acetonitrile and ultrapure water (70:30) at a constant flow rate of 0.6 ml/min. Sugars are finally quantified using standard curves for glucose and xylose as these two monomeric sugars are the most important substrates in a particular combination for downstream fermentation. It is well established that the enzyme activity is inhibited with an increase in product (*glucose*) concentration during enzymatic hydrolysis. Lee et al., 1983 and Andrić et al., 2010 reported that competitive inhibition is present in cellulose-cellulase systems and in between glucose and β-glucosidase [24, 25].

Assuming a pseudo-homogeneous Michaelis–Menten mechanism at the preliminary concentration of a substrate, a model is predicted corresponding to the final concentration of the product [26]:

$$\frac{dC}{dt} = \frac{K[E_0](C_{ult} - C)}{K_M\left[1 + \left(\frac{1}{K_I}\right)C\right] + 0.9(C_{ult} - C)} \quad (8)$$

Here the ultimate value of glucose concentration (C) is denoted by C_{ult} . The value 0.9 can be considered as a constant and the same is obtained by the ratio of the molecular weight of glucose units present in cellulose to that of glucose.

The apparent rate constant is denoted as *K* which determines the binding frequency between cellulase to its substrate cellulose, where K_M represents the apparent Michaelis constant which corresponds to the affinity between cellulose and cellulase. As the total sugar mostly comprises of glucose, K_I is used as the apparent competitive inhibition constant between glucose and cellulases.

In order to examine the proposed model, parameters are determined as follows:

First, in the initial stage, the sugar produced can be neglected (t→0, C→0), thus

$$\left(\frac{dC}{dt}\right)_{t\to 0} = \frac{K[E_0]C_{ult}}{K_M + 0.9C_{ult}} \tag{9}$$

Integrating the cellulose to glucose conversion with initial and final conditions gives the following equations to determine the parameter K_I:

$$\frac{t}{0.9(C - C_0)} = \beta \frac{\ln\left[\frac{C_{ult}-C_0}{C_{ult}-C}\right]}{0.9(C - C_0)} - \gamma \tag{10}$$

Where,

$$\beta = \frac{K_M C_{ult}}{K K_I [E_0]} + \frac{K_M}{K[E_0]} \tag{11}$$

$$\gamma = \frac{K_M}{0.9 K K_I [E_0]} - \frac{1}{K[E_0]} \tag{12}$$

With this, a curve is plotted considering $y \equiv \frac{t}{0.9(C-C_0)}$ against $x \equiv \frac{\ln\left[\frac{C_{ult}-C_0}{C_{ult}-C}\right]}{0.9(C-C_0)}$ and a straight line is obtained. From the slope and intercept of the straight line, the values of β and γ are inferred respectively.

3. Results and Discussion

3.1. Determination of kinetic constants

Removal of lignin is a thermally sensitive process and varies with different thermal pre-treatment conditions to a large extent [27]. Mechanism of removal of lignin and other allied components of the lignocellulosic biomass varies largely with the nature of sample. A particular delignification condition can be found to be less effective for a sample with high lignocellulosic recalcitrance whereas the same condition can be found very effective to de-lignify samples containing a more open lignocellulosic hetero-matrix. It is evident that lignin content of biomass changes significantly with every single step of AP and CP pre-treatment. Lignin content of biomass, after each of the pre-treatment steps is measured using TAPPI T222 and presented in Table1.

Table 1

Representation of lignin content in the untreated and treated (autoclaving followed by probe-sonication) samples and % removed in various methods of treatment.

Source	Lignin Content (wt %)after completion of each pre-treatment steps				
	Untreated	AP	CP		
			Autoclave	Probe sonication	Treatment with 2% peroxide solution

							(pH=11.5) for 5h		
		30°C	50°C	100°C			30°C	50°C	100°C
PR	32.1	31.39	30.07	26.9	29.71	27.32	25.89	23.99	21.41
CD	34.3	33.59	32.27	29.09	32.09	30.71	29.28	26.19	24.41
PR (% removal of lignin)		2.21	6.32	16.20	7.45	14.89	19.35	25.26	33.30
CD (% removal of lignin)		2.07	5.92	15.19	6.44	10.47	14.64	23.64	28.83

When the biomass is treated with alkaline-peroxide only, it dissolves lignin as well as hemicellulose without affecting the crystallinity of cellulose. H_2O_2 decomposes into superoxide anions and hydroxyl ions under alkaline condition which cleaves the ether and ester linkages, present in the lignin-cellulose-hemicellulose matrix by introducing carboxyl groups into the structural frame of lignin and eventually dissolve lignin and hemicelluloses [28]. AP treatment is not very effective in delignifying the biomass significantly (refer Table 1). However, as the extent of delignification is directly proportional to cooking temperature, biomass is pretreated with alkaline-peroxide only at three different temperatures (30°C, 50°C, 100 °C) [29]. Thus, at elevated temperatures delignification is more effective. Increased thermal energy increases the kinetic energy of molecules which eventually make more number of molecules to vibrate more rapidly and ultimately an increased number of bonds disrupt [30]. The increment in terms of % removal of lignin is found to be very similar and in the order: PR [1(30°C): 2.86(50°C): 7.32(100 °C)]; CD [1(30°C): 2.86(50°C): 7.33(100 °C)].

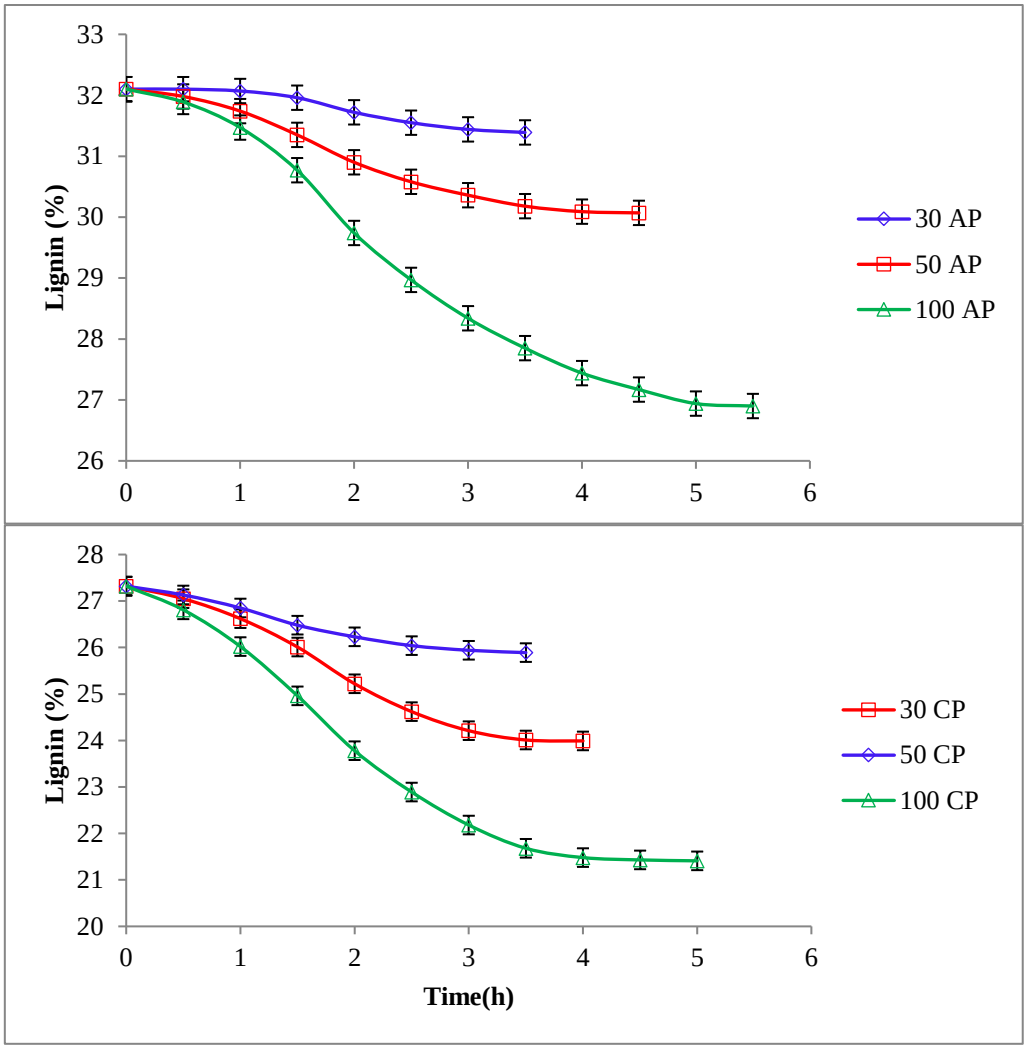
In case of CP, composed of upstream autoclaving, probe sonication and downstream alkaline peroxide pre-treatment, a better scenario of biomass delignification is observed. Each pre-treatmentstep of CP is responsible for different kind of physico-chemical modifications of the biomass.Treatment of biomass,submerged in 2% alkaline-peroxide solution, in autoclave for 1h, disrupts a considerable amount of inter-unit linkages and dissolves lignin,

along with most of the hemicellulosic sugars present in the side-chains of hemicelluloses [31]. Lignin removal is nominal [PR: 7.44%; CD: 6.44%] from the biomass following autoclave. Next, during probe sonication, numerous bubbles are formed which immediately collapse due to wave compression and forms micro-jets, thereby breaking the cell wall to a great extent and dissolve lignin. Probe sonication is also responsible for reduction of cellulose crystallinity as well as removal of amorphous cellulose [32]. When the autoclaved samples are further probe-sonicated, additional lignin is removed [PR: 14.89%; CD: 10.46%]. Ultimately the solid fractions, recovered from the sonicated sample, are treated with 2% alkaline-peroxide solution (pH=11.5) at three different temperatures (30,50,100°C) and maximum degree of delignification is achieved at 100°C temperature. The % removal with temperature is: [PR: 19.35%(30°C): 25.26%(50°C): 33.30%(100 °C)]; [CD: 14.64%(30°C): 23.64%(50°C): 28.83%(100 °C)].

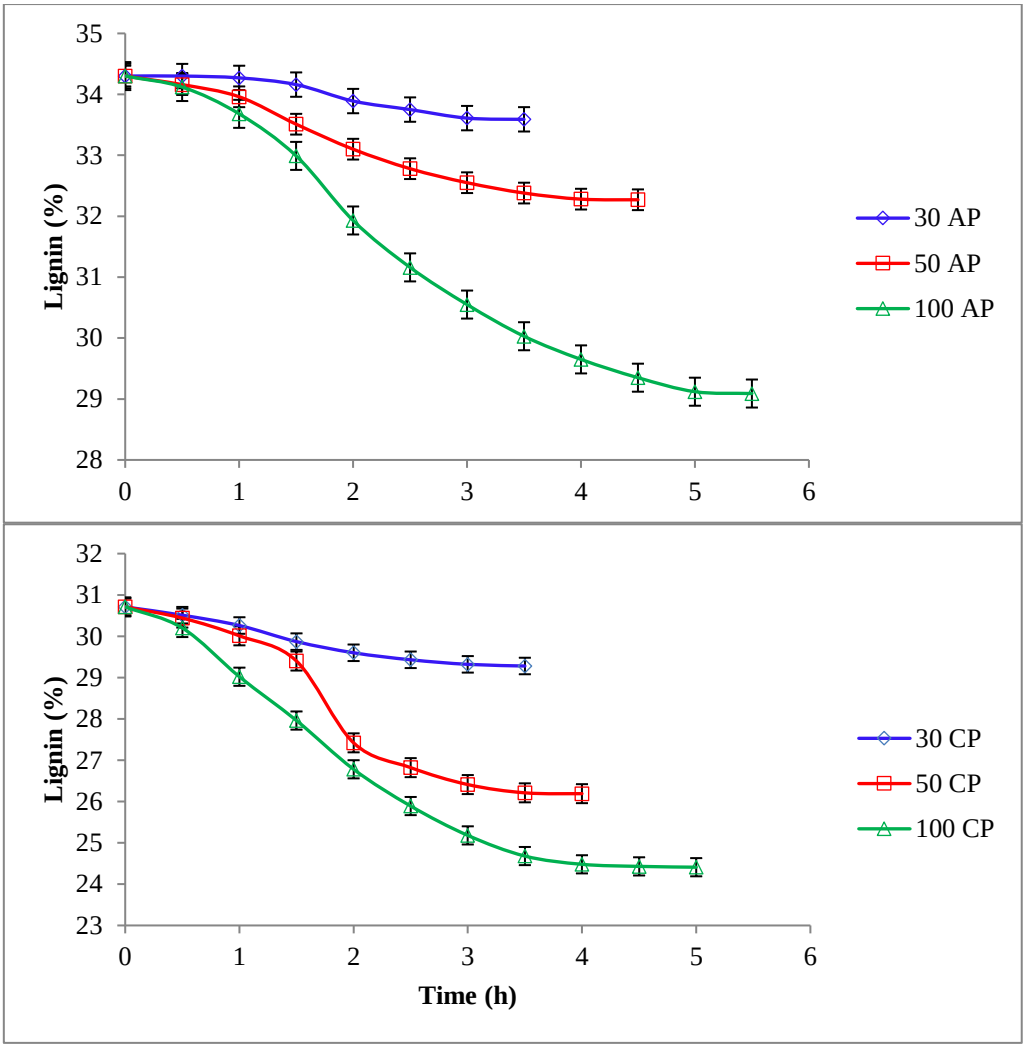
CD contains more lignin than Pinewood (PR) powder. Being a higher lignin containing biomass, CD holds a more rigid structure which is found to be more stable against delignification treatment. The same is confirmed from the % removal represented in Table 1. Cumulative effect of autoclaving and probe sonication can be effective in removing 14.89 % of lignin from PR whereas only 10.47% of lignin can be removed from CD.

Autoclaving and probe sonication are two upstream key steps of CP. During CP, time dependant variation of lignin is monitored after completion of the autoclave-probe sonication cycle. Following this, the partially pretreated samples are delignified with 2% aqueous peroxide solution (pH=11.5) for 5h and simultaneously lignin removal is monitored with time and the same is also considered for estimating parameters of delignification kinetics. In Fig.2, time dependant variation of delignification pattern of different wood samples is presented at

various pre-treatment temperatures in order to understand the impact of delignification time and temperature on these samples.



(a)



(b)

Fig. 2. Time dependant variation of delignification patterns of (a) PR and (b) CD wood samples.

It is observed that time is an important factor for an effective removal of lignin. It is inferred from these figures that removal (lignin) is directly dependent on elapsed time of pre-treatment. However, equilibrium time for removal of maximum lignin is different for different biomass and cooking temperatures. It can be found from Fig.2(a) that delignification of PR using alkaline-peroxide only at 30°C requires 3.5h to remove 2.21% of lignin. Surprisingly, with the same time frame and cooking temperature, combined pretreated

biomass loses 5.23% of its lignin. Combined pretreated biomass holds a more open and deformed matrix and therefore within a shorter period of time, more amount of hydroxyl radical and superoxide anions can reach larger number of inter and intra-chain bonds and cleave them. Similarly, at 50°C and 100°C, AP biomass requires 4.5 and 5.5h to achieve 6.32% and 16.2% delignification, respectively, whereas combined pretreated PR achieved 12.19% and 21.63% delignification within 4h and 5h at 50°C and 100°C, respectively. Similar trend of delignification pattern is observed for CD biomass also. Lignin is found primarily at the outer portion of the compound middle lamellae of plant cell and cell corner. During initial stages of the reaction, the solvent molecules and free radical ions (peroxide treatment) diffuse gradually into the cell wall and subsequently the chemical reactions start to take place. During reaction, lignin, along with other pentose sugars, dissolves in the solvent and diffuse from cell wall layers to the bulk phase. However, soluble biomolecules, primarily composed of some acidic by-products and monomeric sugars generated during de-lignification, often create a diffusional resistance in the exit pathway of lignin from the cell wall and entrance of solvent (used for alkaline pre-treatment) into the active sites of ruptured cell walls. Therefore, with an advancement of time, accumulation of reaction products in the system eventually limits further molecular diffusion. Combined effects of autoclaving and probe sonication denature lignocelluloses hetero-matrix in CP to a great extent, which eventually increase pore size and surface area of the matrix. Therefore, more amount of hydroxyl radicals come in contact with an enlarged surface area and an increased number of bonds are broken down within a limited time. For this reason, increased amount of lignin is removed from CP as compared to the same from AP biomass. Kinetic constants associated with each pre-treatment condition is estimated from the linear regression of experimental data and presented in Table 2.

The results indicate that rate constants increased with increasing temperature for both AP and CP. Increased temperature leads to more collisions of the reactants, which further increased the rate of reaction with elevated rate constant values.

From Table 2, it can be inferred that, a reduced amount of activation energy is required for CP treated samples whereas if the samples are treated with conventional alkaline pre-treatment (AP), more amount of activation energy is required to initiate the reaction. Most of the bonds, present in the biomass, are broken down with a simultaneous reduction in crystallinity during autoclave and sonication treatment. Therefore, much less amount of energy is required to break the linkages responsible for the stabilization of lignin.

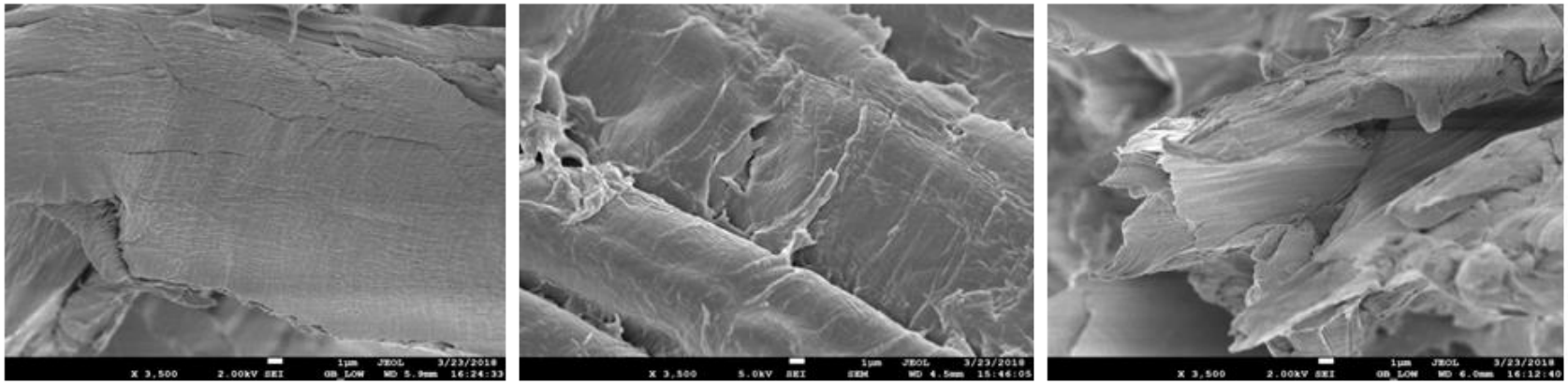
Table 2 Estimated kinetic parameters by linear regression of experimental data.

Sample Name	Temperature	AP				CP			
		K_L	d_D (h^{-1})	E_a (J/Mol)	A	K_L	d_D (h^{-1})	E_a (J/Mol)	A
PR	30	0.01	0.617	18.71	18.73	0.02	0.879	14.22	6.68
	50	0.02	0.835			0.04	0.917		
	100	0.04	0.916			0.06	0.982		
CD	30	0.01	0.590	17.87	13.37	0.02	0.785	13.05	4.54
	50	0.02	0.784			0.05	0.909		
	100	0.04	0.859			0.06	0.941		

3.2. Modification of physical structure during pre-treatment

It can be elucidated from the values of degree of delignification and activation energy (refer Table 2) that CP is the most efficient procedure for delignification of wood powders. It is essential to understand the changes of physical structure of wood powders with each step of CP. The Scanning Electron Micrograph (SEM) images obtained from the analyses are shown in Figs. 3 and 4. The SEM images snapped during three stages of pre-treatment, namely, (a) before treatment is applied, (b) after probe-sonication following autoclaving and lastly (c) combined pre-treated (CP) are presented in Fig. 3. (PR) and Fig. 4. (CD). It is evident from the SEM images that crude biomasses comprise of an undisturbed and organized hetero-matrix structure composed of lignin, cellulose and hemicelluloses [refer Fig 3(a) and 4(a)]. The smooth surface is formed due to compact organization of lignin and hemicelluloses over the cellulosic core. Autoclave treatment substantially loosens the hetero-matrix without creating much pores in the biomass structure. Therefore, autoclaving can help in surface modification only. On the contrary, it is evident from Fig.3.(b) and Fig. 4.(b) that after probe sonication, the hetero-matrix is distorted to a great extent and provides with a substantially porous matrix, which facilitates the enzymes to reach the active sites in the inner-most part of the biomass matrix. Ultrasonication assisted micro-jets break the cell wall which sufficiently distorts the heteromatrix, as shown in Figures 3(b) and 4(b). During the ultrasound treatment, a number of bonds, present in crystalline cellulose, are broken, which eventually reduce the cellulose crystallinity and make the biomass more porous. The pressure generated during autoclaving helps to loosen the crystalline part of cellulose fibres whereas amorphous part is largely affected. However, the pressurised bubbles generated during probe sonication along with the superoxide radicals, make the covalent bond very prone to breakup. After 5h treatment with alkaline peroxide, in the final stage, most of lignin get solubilised along with

hemicelluloses and the matrix gets a largely porous and distorted shape [see Fig. 3.(c) and 4(c)]. As the surface gets largely exposed for a longer duration, a substantial amount of superoxide anions and hydroxyl radicals can oxidize the lignin structure by introducing hydrophilic carboxyl groups and cleavage of some inter-unit bonds. Eventually dissolution of lignin and hemicelluloses takes place. Lignin and hemicelluloses get slowly depleted from the matrix structure during subsequent pre-treatment steps, thereby leaving the matrix more open, distorted, fragile and perfect for subsequent enzymatic hydrolysis. Moreover, continuous depletion of amorphous cellulose, in each step of combined pre-treatment, also plays a crucial role in the formation of porous matrix.



a b c

Fig.3. Scanning Electron Micrograph (SEM) images of (a) untreated, (b) probe-sonicated and autoclaved (c) CP-alkaline peroxide pre-treatment following probe-sonication and autoclaving wood powder (PR) at ×3500 magnification.

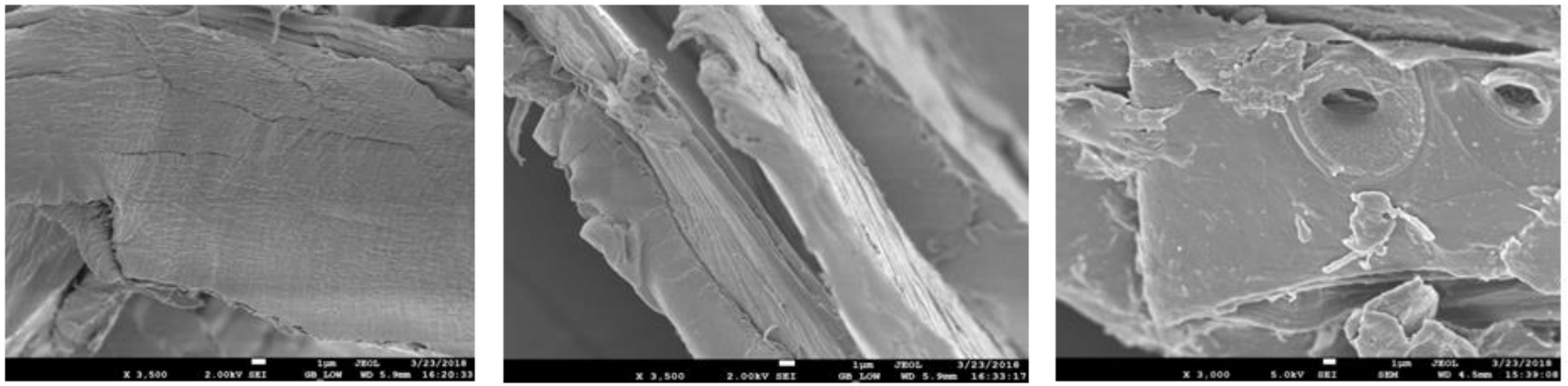


Fig.4. Scanning Electron Micrograph (SEM) images of (a) untreated, (b) probe-sonicated and autoclaved (c) CP-alkaline peroxide pre-treatment following probe-sonication and autoclaving wood powder (CD) at ×3500 magnification.

3.3. Thermal analysis of biomass at different stages of CP

Thermo-gravimetric analysis provides with an instantaneous variation of biomass weight as a function of temperature. Among the components of biomass, lignin is the most thermally stable material followed by cellulose. Additionally, structural changes and compositional variation during various steps of pre-treatment expose several moieties of biomass matrix to a great extent. Following pre-treatment, the moieties, that could have remained intact under elevated temperatures in the untreated biomass, get degraded. However, cellulose is the most important value added material of biomass as it is used to generate monomeric sugars which are eventually used as the major substrate for the downstream microbial fermentation to generate biofuels. It is thus essential to investigate the thermal tolerance of cellulose before and after pre-treatment of the parent biomass [33]. Thermal decomposition curves of cellulose in the untreated as well as pre-treated biomass (after each stage of CP) are given in Fig.5. DTG data of cellulose present in the untreated wood powders shows a single peak (*temperature*) with a peak value of 332.006°C and 335.521°C for PR and CD, respectively. The peak value represents the temperature which causes cellulose to decompose at maximum rates [20]. In case of PR biomass, the peak values of cellulose decomposition are found 331.56°C after autoclaving and 329.38°C following completion of CP respectively. Advancement of pre-treatment is found to be associated with lower peak value. With each stage of treatment, the cellulose chains of biomass matrix get depolymerised. This is also evident from Fig. 3. (a,b,c) and Fig. 4. (a,b,c). As lignin gets removed there is a simultaneous elimination of cement like structural protective cover of the cell surface. This helps in exposing and depolymerising the cellulose of the pre-treated material and the same gets easily decomposed under the same temperature. On the contrary, autoclaved deodar wood

powder and wood powder recovered after completion of CP do not show any significant differences in their peak value.

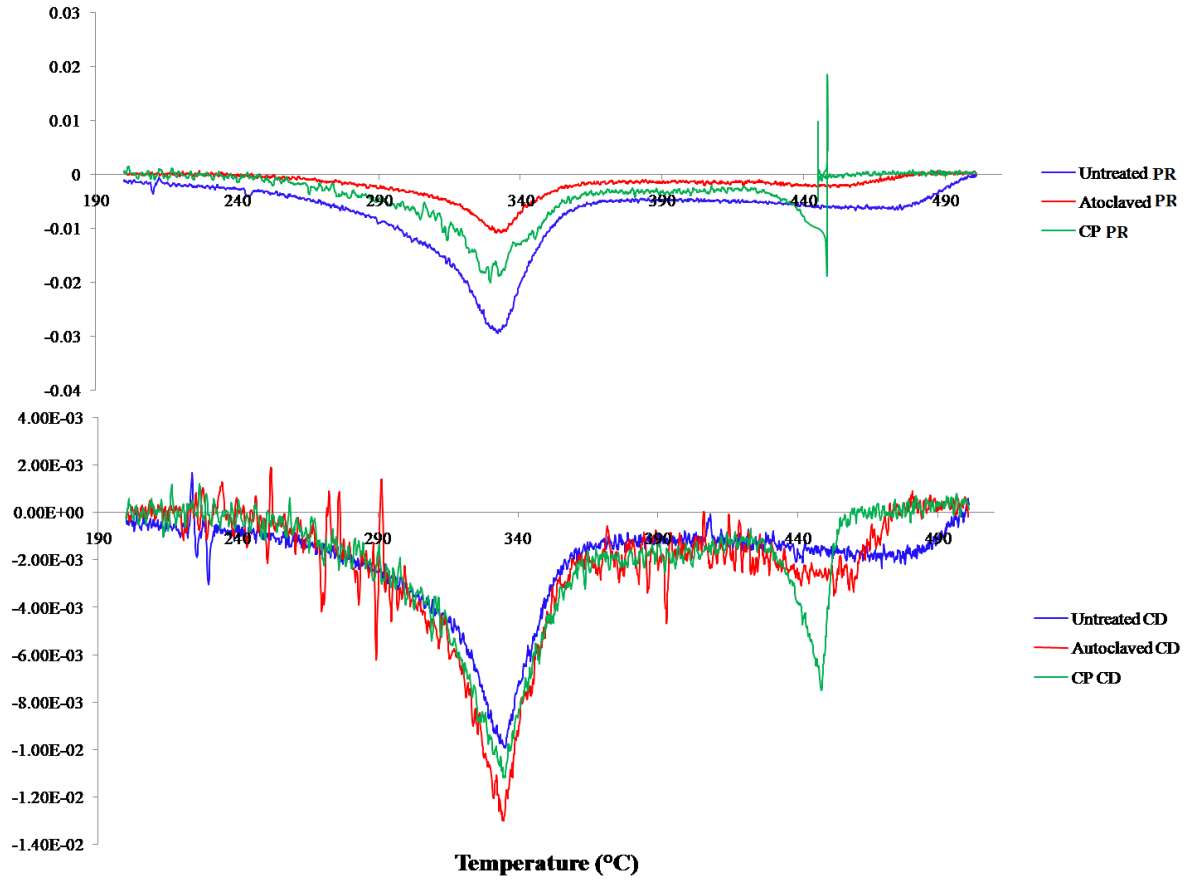


Fig.5. DTG curves of untreated, autoclaved and combined pre-treated (CP) biomass of PR (*top*) and CD (*bottom*).

3.4. Enzymatic hydrolysis of delignified PR biomass and generation of sugar

With the help of HPLC, glucose generated from delignified biomasses are estimated and the transient behavior is represented in Fig. 6.

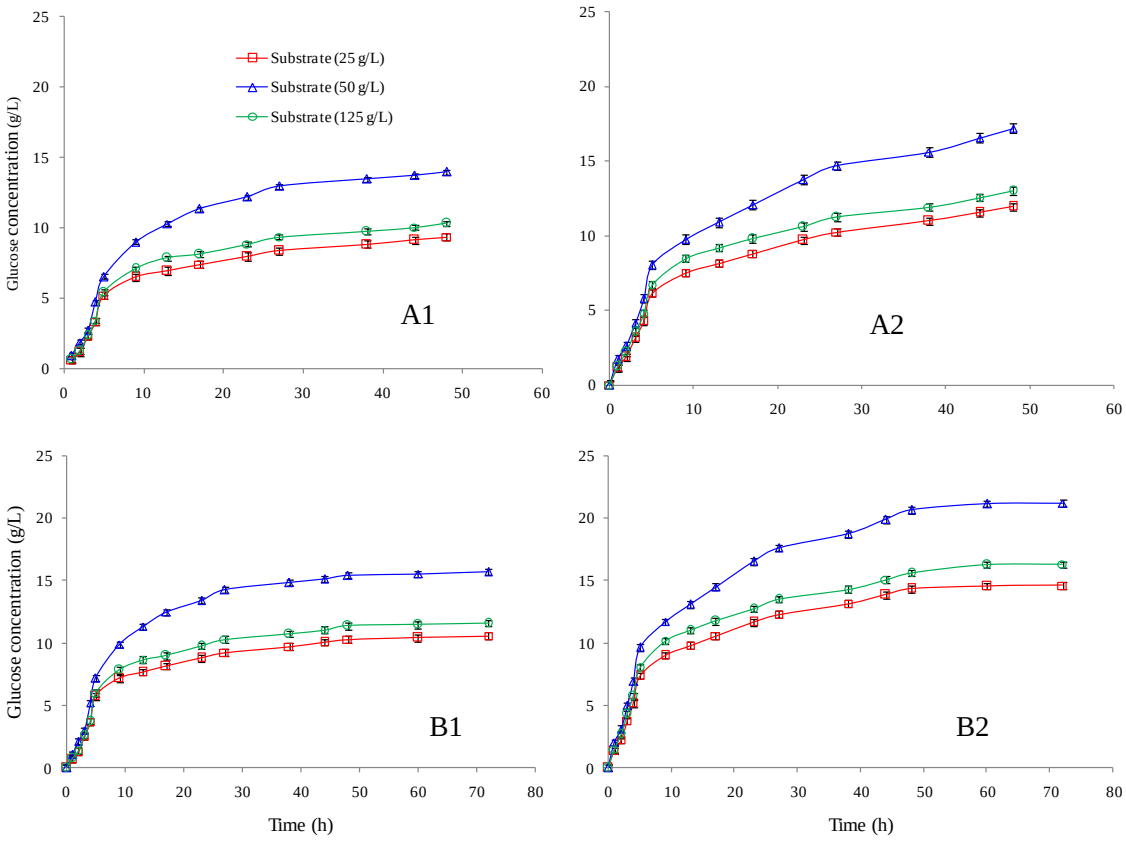


Fig. 6. Time dependent generation of glucose from delignified biomass (A: PR, B: CD; 1: 1.28 g/L enzyme concentration, 2: 13.23 g/L enzyme concentration)

It can be seen from Figure 6 that escalated production of glucose is associated with increased substrate concentration from 25 g/L to 50 g/L for a particular enzyme concentration. However, further increment of substrate often lead to decreased glucose recovery. Additionally, increased glucose generation is associated with higher concentration of enzymes with a particular substrate concentration. This can be explained with the values of inhibition kinetic constant represented in Table 3.

It can be inferred from the table that with increased enzyme concentration during hydrolysis of CD, the values of K_I gradually increases. Increased value of K_I is associated with reduced inhibition exerted primarily by glucose. The value of K_I becomes higher with increased

enzyme concentration from 1.28 g/L to 13.23 g/L for a range of substrate concentration in between 25 g/L and 50 g/L.

Table 3 Values of kinetic constants associated with product inhibition of enzymes.

Sample	Kinetic Constants	[E]=1.28 g/L			[E]=13.23 g/L		
		25	50	125	25	50	125
PR	K (h^{-1})		2.07			0.39	
	K_M(g/L)		11.87			36.12	
	K_I (g/L)	5.14	9.80	36.9	9.18	12.5	9.35
CD	K (h^{-1})		5.86			0.43	
	K_m (g/L)		70.73			51.20	
	K_I (g/L)	15.60	39.22	25.14	53.19	195.40	75.62

On the contrary, for 125 g/L substrate concentration, surprisingly, the value of K_I reduces with increased enzyme concentration. This is due to the diffusional limitation of enzymes which eventually does not allow the enzymes to penetrate into the core area of biomass [26, 34]. Moreover, it has also been inferred from the table that, for a particular enzyme loading, larger value of K_I is observed with increased substrate concentration from 25 g/L to 50 g/L. From this phenomenon, it can be concluded that with increased substrate loading (for a particular enzyme concentration), inhibition decreases and the same is a typical character of competitive inhibition. However, product generation followed by hydrolysis of substrate with

125g/L concentration leads to decreased product concentration because of limitation of free movement of enzymes and substrate particles due to high viscosity[34].

3.5. Validation of kinetics model

3.5.1. Validation of delignification kinetics model:

Two interrelated but different equations are used to fit the experimental data and the efficiency of delignification kinetics models were evaluated in terms of coefficient of determination (r^2). Comparison of experimental data with theoretical data (predicted using equations 3 and 5) are represented in Fig. 7 and Fig.8.

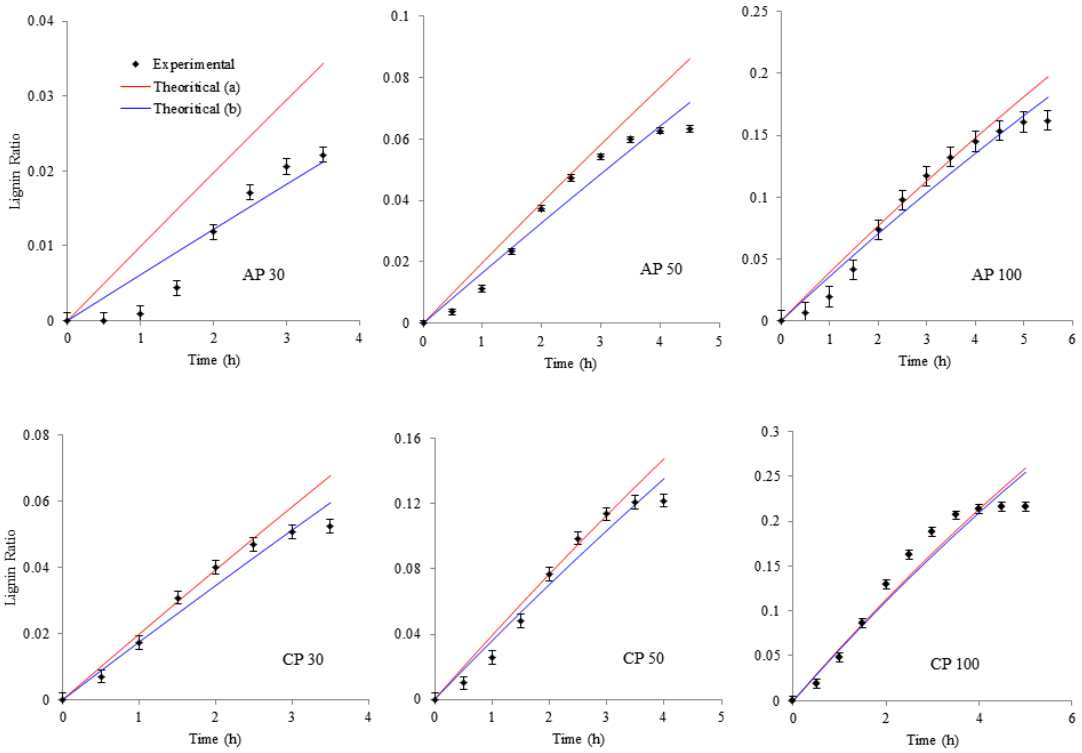


Fig.7. Validation of models for delignification kinetics with experimental data for PR wood powder [theoretical (a) represents experimental data fitted to Eq (3) and theoretical (b) represents the same fitted to Eq (5) containing 'potential degree of delignification

(d_D)].(AP30, AP50, AP100 ≡ Alkaline Pre-treatment at 30°C, 50°C, 100 °C respectively, CP30, CP50, CP100 ≡ Combined Pre-treatment at 30°C, 50°C, 100 °C respectively)

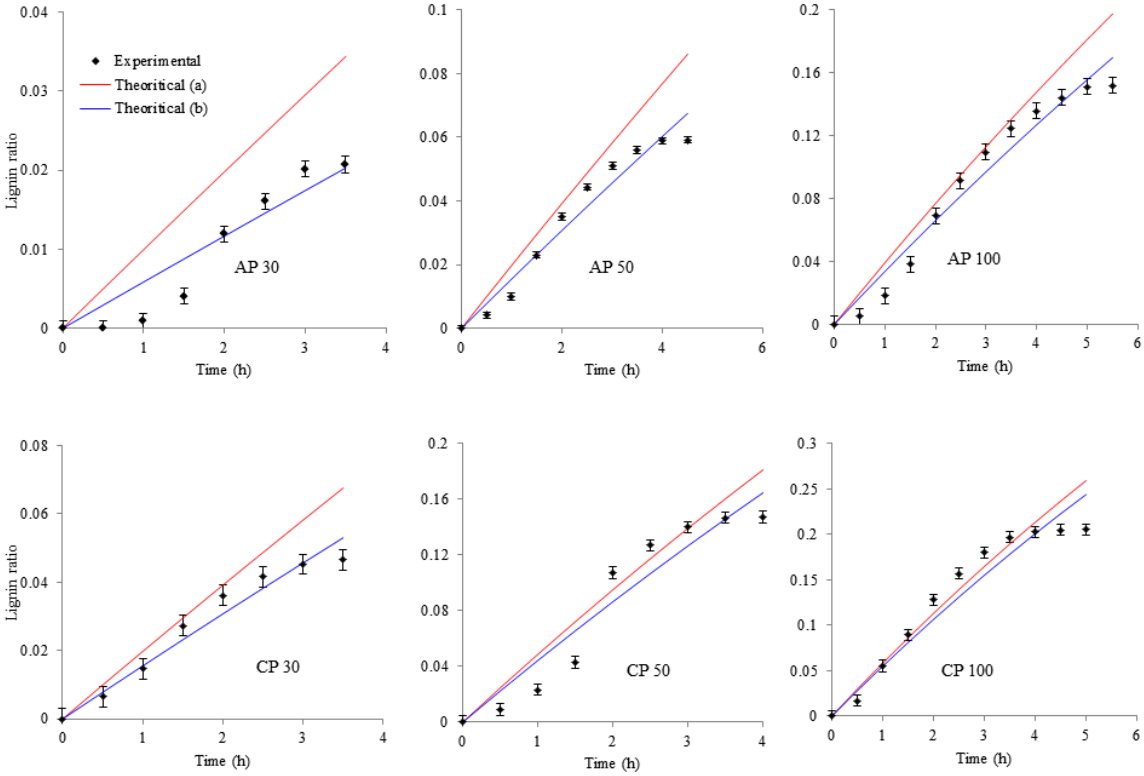


Fig.8. Validation of models for delignification kinetics with experimental data for CD wood powder [theoretical (a) represents experimental data fitted to Eq (3) and theoretical (b) represents the same fitted to Eq (5) containing 'potential degree of delignification (d_D)]. (AP30, AP50, AP100 ≡ Alkaline Pre-treatment at 30°C, 50°C, 100 °C respectively, CP30, CP50, CP100 ≡ Combined Pre-treatment at 30°C, 50°C, 100 °C respectively)

Table 4 Values of determination coefficients (r^2) obtained following estimation of parameters of two different models of delignification kinetics, represented by Eq (3) and Eq (5).

Equations	$\frac{dL_S}{dt} = K_L(1 - L_S)$				$\frac{dL_S}{dt} = K_L(d_D - L_S)$			
Temperature	PR		CD		PR		CD	
	AP	CP	AP	CP	AP	CP	AP	CP
30°C	9.09	89.17	-11.6	70.6	89.37	95.79	88.57	95.7
50°C	83.26	93.87	70.34	89.27	95.88	96.49	95.71	92.12
100°C	93.67	93.98	87.53	91.71	96.5	94.18	96.47	93.26

In this model, maximum possibility of lignin solubilization under a particular pre-treatment severity is considered believing that under a particular pre-treatment condition, it is almost impossible to remove all the lignin from biomass. Thus, a 'potential degree of delignification[1]' term is introduced. No such factor is considered in the conventional delignification kinetics model represented by Eq (3). The 'goodness of fit', given by the numerical values of r^2 [refer Table 4], is thus much better for the model represented by Eq (5) in comparison to the same represented by Eq (3).

[1]*Degree of solubilization of lignin under a specific pre-treatment condition.*

3.5.2. Validation of Enzyme Kinetics Model

Inhibition of cellulolytic enzymes by the product, glucose, is an obvious phenomena observed during enzymatic hydrolysis of lignocellulosic biomass. Therefore, it is essential to validate the kinetic models with experimental outcome in terms of determination coefficient (r^2). Comparison of theoretical data with experimental data is presented in Fig. 9 and Fig. 10.

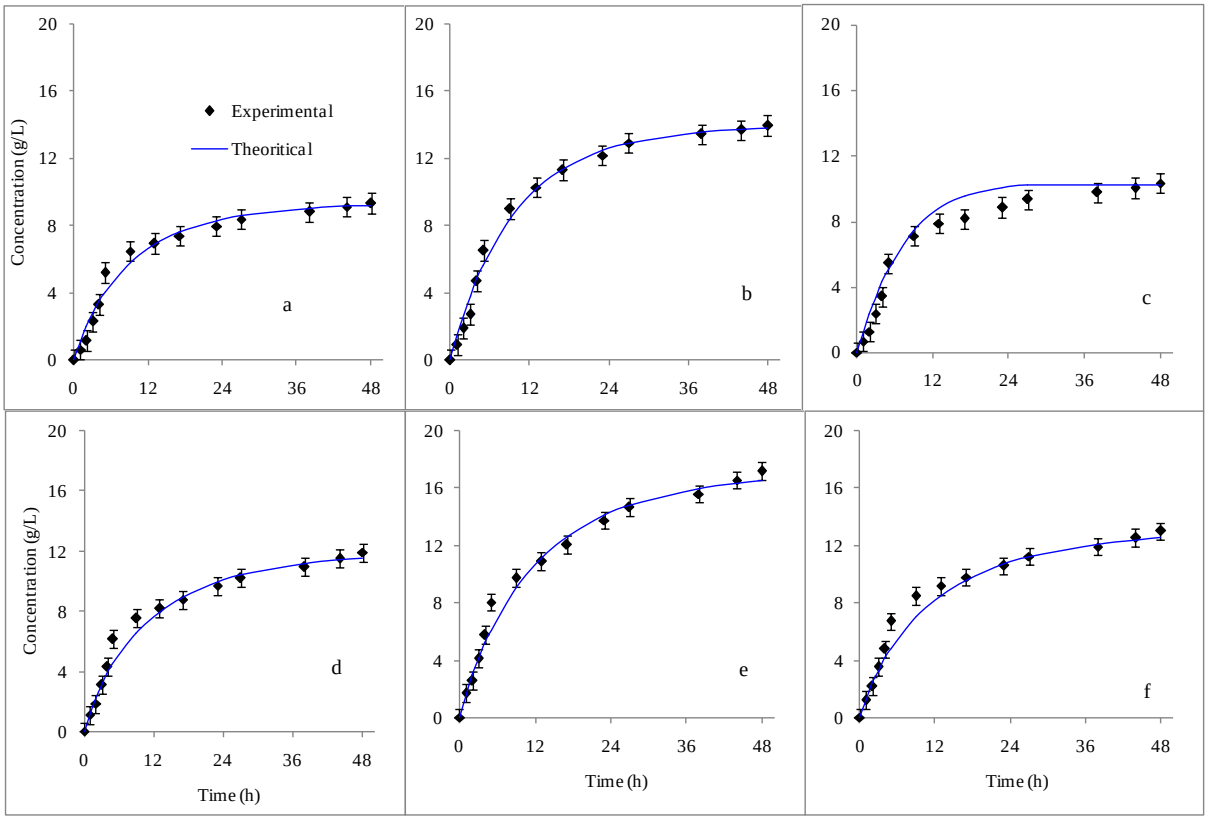


Fig. 9 Validation of theoretical data with experimental data following hydrolysis of varied loading of pretreated PR biomass with various enzyme concentrations (a: [S]=25 g/L, [E]=1.28g/L; b:[S]=50g/L, [E]=1.28 g/L; c:[S]=125g/L, [E]=1.28g/L; d: [S]=25g/L, [E]=13.23g/L; e: [S]= 50g/L, [E]=13.23 g/L; f: [S]=125 g/L, [E]=13.23g/L).

From Fig. 9 and 10, it is observed that the experimental data fit quite well to the competitive inhibition kinetics based theoretical models, represented by Eq (10) with $r^2 \geq 0.95$. Therefore, it can be inferred that the enzymes are competitively inhibited by glucose.

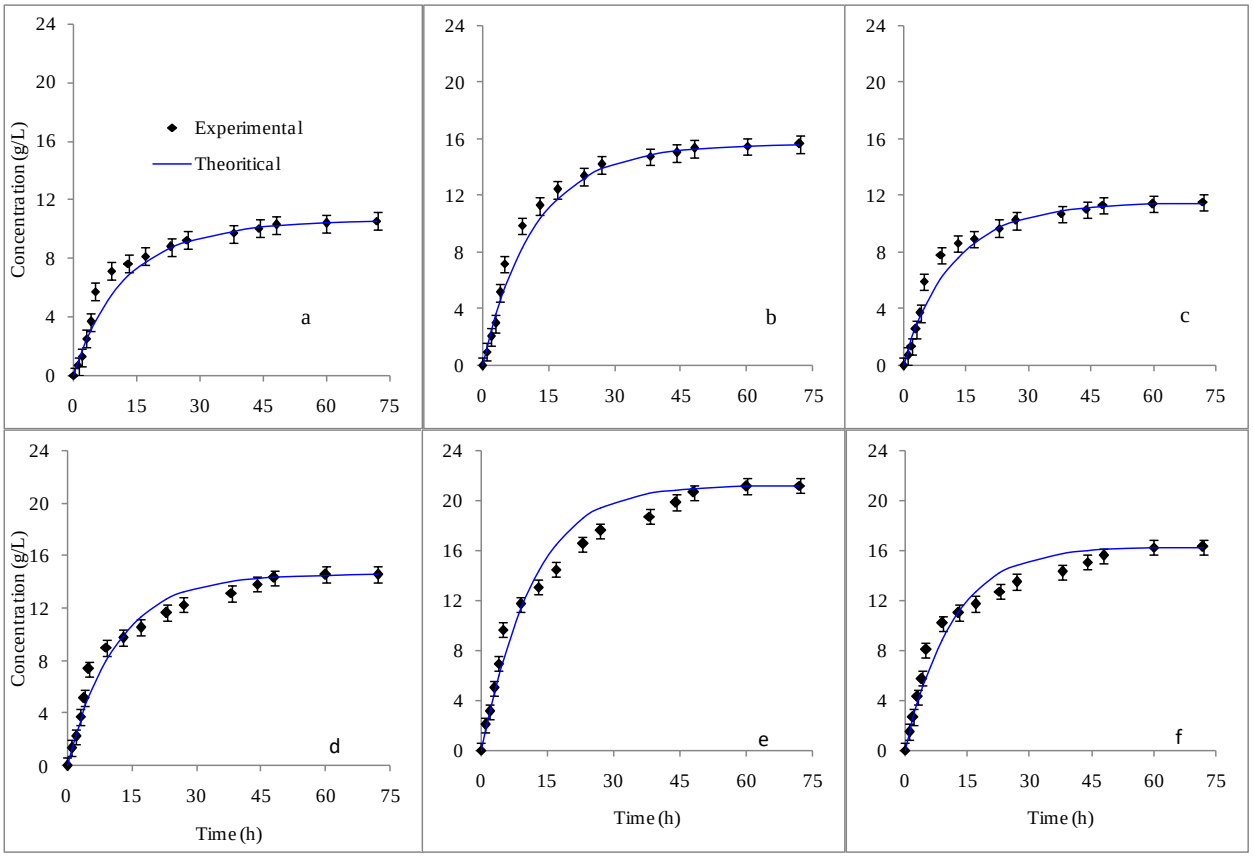


Fig10. Validation of theoretical data with experimental data following hydrolysis of varied loading of pretreated CD biomass with various enzyme concentrations (a: [S]=25 g/L, [E]=1.28g/L; b:[S]=50g/L, [E]=1.28 g/L; c:[S]=125g/L, [E]=1.28g/L; d: [S]=25g/L, [E]=13.23g/L; e: [S]= 50g/L, [E]=13.23 g/L; f: [S]=125 g/L, [E]=13.23g/L).

4. Conclusion

Pre-treatment is required for an appreciable disruption of the lignocellulosic hetero-matrix, thereby removing lignin effectively. This eventually makes cellulose more accessible to the enzyme cocktail for further extraction of hydrolysable sugars. In the present study, novel pseudo-homogeneous kinetic models, with incorporation of parameters termed as "potential degree of delignification" are developed with an aim to accurately describe the delignification kinetics while pre-treating the saw dusts. Optimum degrees of delignification are investigated under some particular pre-treatment severity. The relationship between kinetic constants and temperature could be well correlated by modified Arrhenius equations. It is also found that the first order pseudo-kinetic model can be used as a universal model in order to describe the kinetics of delignification for various methods of chemical pre-treatment using variety of feedstock. Moreover, with the help of images generated through scanning electron microscopy it can be said that the biomass matrix get delignified and deformed with increased severity of pre-treatment. The same is responsible for depolymerisation as well as thermal degradationof cellulose at a relatively lower temperature.

Enzymatic hydrolysis kinetics of delignified biomass shows decreased product inhibition with increased substrate concentration under a particular enzyme loading. Starting with a combination of 50 g/L substrate concentration with an enzyme loading of 13.23 g/L, an optimum concentration of 17.2 g/L of glucose is produced from PR. Using the same substrate concentration and enzyme loading, CD can generate 21.19 g/L of glucose. Experimental data fit quite well with the competitive inhibition kinetics based theoretical models with $r^2 \geq 0.95$. It is thus inferred that enzymes are competitively inhibited by glucose.

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List of Variables:

Notation of Variable(s)	Description of variable(s)	Unit
L_S	Lignin ratio	-
C_{L_o}	Initial lignin contentof the biomass	*(wt %)*
C_L	Lignin content of the biomass at any time t	*(wt %)*
K_L	Delignification rate constant	h^{-1}
d_D	Potential degree of delignification	-

A	Arrhenius constant	h^{-1}
E_a	Activation energy	*J/Mol*
R	Universal gas constant	*J/mole·K*
T	Absolute temperature	*K*
C	Glucose concentration at any time t	*g/l*
C_{ult}	Ultimate concentration of glucose	*g/l*
K	Apparent rate constant	h^{-1}
K_M	Apparent Michaelis constant	*g/l*
K_I	Competitive inhibition constant	*g/l*
E_o	Initial Enzyme concentration	*g/l*

1