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Physicochemical pretreatment of pine needle biomass by design of experiments approach for efficient enzymatic saccharification

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Abstract
Lignocellulosic biomass (LB) has emerged as one of the most suitable environmentally-friendly and renewable resources of energy in view of fast depleting fossil fuel energy resources, and growing environmental concerns. Most of the pretreatment approaches available for reducing recalcitrance of LB are cost/energy intensive, cause loss of sugars and production of inhibitors. Also there is research thrust on search for novel and potential biomass resources for energy. Pine needles may serve as one of such biomass resource that can be exploited. Dried, fallen, pine needles cause detrimental changes in fauna and flora, and reduce soil productivity, and may pose threat for fire in coniferous forests. Current study investigates the pretreatment of pine needle biomass (PNB) using design of experiments approach based on response surface methodology (RSM). Effect of three variables i.e. dilutes sulphuric acid concentration, time and temperature for pretreatment of PNB was studied using RSM. The solid residual PNB was subjected to alkali pretreatment (calcium hydroxide), followed by enzymatic hydrolysis (Celluclast 1.5 L). The optimum level of variables for physico-chemical pretreatment was: 0.25% sulphuric acid, 65°C, and 5 min. Enzymatic saccharification of pretreated biomass for 48 h (@10 units of cellulase), resulted in production of total sugars of 286 mg/g of PNB. Thus, the sugars obtained after apt pretreatment and saccharification of pine needle biomass may be used for fermentative production of biofuel and/or other commercial products.
Key words: Pine needle biomass, Physicochemical pretreatment, design of experiments approach, sugars

1. Introduction
Ever rising global energy demand due to swift economic developments, depletion of fossil fuel reserves-the major energy source, and distressing climate change affects motivated policy makers, governments, and researchers world over to explore sustainable energy systems that are renewable [1]. Conversion of abundant lignocellulosic biomass to biofuel ethanol/butanol to *fuel* the transportation sector may be an important and viable option for improving energy security and reducing greenhouse gases emissions [2]. Lignocellulosic materials such as agricultural residues (e.g., wheat straw, sugarcane bagasse, corn stover) and dedicated crops (switchgrass, salix) are renewable energy sources, can be exploited after biorefining to get several products of commercial importance such as biofuel, energy, nutrient rich animal-feed, bioplastic, and other platform chemicals/materials [3-4]. These raw materials are sufficiently abundant and generate very low net greenhouse emissions. Approximately 90% of the dry weight of most plant materials is stored in the form of cellulose, hemicellulose, lignin, and pectin [5-6].

One of the major hindrances for production of biofuel from LB is its recalcitrance to hydrolysis [7]. LB needs ample pretreatments before it can be enzymatically transformed into sugars which could be subsequently fermented to bioethanol fuel. A wide range of promising pretreatment methods have been employed including dilute acid hydrolysis, steam explosion, and hot water treatment, ammonia treatment, alkaline peroxide treatment, and Organosolv process, among several others [8]. Mild pretreatment of LB causes poor sugar release upon enzymatic hydrolysis while severe pretreatments though release more sugars but cause production of inhibitors from hemicellulosic sugars [9]. Therefore, a balance need to be struck while realizing

pretreatment so that there is maximum sugar release with no or low formation of inhibitors. Therefore, varied pretreatment severities are applied, where the first stage is conducted at low severity for efficient hemicellulose hydrolysis, and another stage under more severe conditions is followed to treat the remaining residue [10]. Also several combinational pretreatment approaches have been employed by various researchers [10]. Sequential acid and alkaline pretreatment approach has been investigated in which acid pretreatment can be used to mainly hydrolyze hemicelluloses while alkaline pretreatment to efficiently modify or remove lignin [11]. Many of these pretreatment approaches proved significantly beneficial and resulted not only improved yields of both cellulose and hemicellulose sugars but required fewer enzymes for hydrolysis than single-stage pretreatments [12]. Two stage i.e. dilute acid and lime pretreatments have been used quite extensively prior to enzymatic hydrolysis of biomass [13].

Among various lignocellulosic feed stocks available, pine needles may be an important biomass resource that can be used for biofuel-ethanol production. The pines are coniferous, evergreen and resinous trees which belong to the genus *Pinus* of the family *Pinaceae*, and are native to the northern hemisphere. The needle-shaped adult green pine leaves constitute the major portion of the litter fall in coniferous forests and pose several problems [14]. The dried and fallen pine needles are hazardous to the soil environment, and cause large-scale destruction of fauna and flora. The release of tannins from pine needles in the soil may inhibit the growth of various beneficial agricultural microbes, causing delayed availability of nutrients in soil. These water-soluble poly-phenolic compounds (tannins) in pine needles affect the soil nutrient dynamics by delaying the organic matter decomposition and mineralization of nutrients [3, 15]. Furthermore, bulk clutter of fallen dried pine leaves may pose a high risk of forest fires. However, pine needle biomass contains about 75% (by weight) of polysaccharides (cellulose, hemicelluloses) which can be hydrolysed into simple sugars that may be exploited as raw materials for microbial fermentation for production of biofuel, biomaterials, energy, and numerous other valuable products of commercial importance (biorefining) [3-4, 6-7]. Thus, exploitation of pine needle biomass as a resource would not only obviate the problems due to pine needle accumulation but would also result in valorisation of waste.

Process optimization has always been instrumental in enhancing the overall process economy [16]. Conventional *one-variable-at-a-time* (OVAT) approach suffers from many limitations [17], however, statistically based Design of experiments (DoE) approach may overcome the limitations of OVAT [18]. Design of experiments (DoE) is a powerful tool which deals with planning, execution, analysis and interpretation of the controlled tests for evaluation of the factors which control the value of a parameter or group of parameters. Using DoE multiple input factors can be manipulated for determining their effect on a desired output response. A strategically planned and executed experiment may provide a greater deal of information about the effect on a response variable due to one or more factors, than the traditional OVAT experimental approach. By employing DoE multiple input factors can be manipulated for determining their effect on a desired output response. Furthermore, DoE helps identifying important interactions between the variables that is not possible with OVAT approach. In DoE all possible combinations (full factorial) or only a portion of the possible combinations (fractional factorial) can be investigated [17]. Important interactions between the variables can be identified with DoE which is not possible with OVAT approach. Response surface methodology (RSM) is a standard DoE tool that is useful for designing experiments, building models, evaluating the effect of many factors and finding optimal conditions for desirable responses and reducing the number of desired experiments [18]. In the current study, pretreatment of pine needle biomass (PNB) was attempted by using dilute sulphuric acid at varying acid concentration, treatment time, and temperature by employing RSM. The acid pretreated biomass was subjected to alkali pretreatment, and examined for enzymatic saccharification.

2. Experimental

2.1. Chemicals and media

All the chemicals, media and media components used were of analytical grade obtained from Sigma-Aldrich Chemicals Ltd, St. Louis MO, USA; HiMedia Laboratories Ltd, Mumbai, India; Qualigens Fine Chemicals Ltd, Mumbai, India; and Merck and Co. Inc., White House Station, NJ, USA.

2.2. Preparation of pine needle biomass (PNB)

Pine needles used in the study were procured from forest area near Udhampur, Jammu, India. Pine needles were thoroughly washed with tap water and then air dried at 50°C to obtain dry matter content between 91% and

94%. The dried material was grinded and fraction passing through 4-5 mm sieve was collected and used for further experiments [5]. The powdered PNB was composed of (dry weight basis) holocellulose (64.12%), pentosan (14.12%) and lignin (27.79%) [15].

2.3. Acid pretreatment of PNB

For acid pretreatment [6] of PNB three variables i.e. acid concentration (A), time (B), and temperature (C) were selected for evaluation by employing central composite design (CCD) of RSM (Design expert 6.0, Stat Ease, Inc., Minneapolis, Minnesota, USA). The experimental design for the selected process variables is presented in Table 1. The lower and higher limits of variables were set based on the literature survey. Pretreatment of PNB was carried out with sulphuric acid concentrations (0.25% to 1.93%) at a solid to liquid loading of 1:10. It was maintained for varying time period, and respective temperatures as per RSM design. For three variables (n=3) and five levels, a total number of 20 experiments were designed (Table 2).

Table 1: Experimental range and levels of the independent variables used in RSM for pretreatment of pine needle biomass

Study Type: Response Surface			Experiments: 20	
Initial Design: Central Composite				
Response	Name	Units	Design Model: Quadratic	
Y	Sugar content	Mg/g	Experimental values	
Factors	Name	Units	Lower	Higher
A	Acid concentration	%, v/v	0.25	1.5
B	Time	Min	5	35
C	Temperature	°C	65	105

Table 2: Experimental design based on RSM for pretreatment of pine needle biomass

Run No.	Pretreatment variables		
	A Acid concentration (%)	B Time (min.)	C Temperature (°C)
1	0.88	-3.52	85
2	0.88	17.50	118.64
3	0.88	17.50	85
4	0.25	30	105
5	0.88	17.50	85
6	-0.18	17.50	85
7	0.25	5	65
8	0.88	17.50	85
9	0.88	17.50	85
10	0.88	17.50	85
11	1.50	30	65
12	0.88	17.50	85
13	1.93	17.50	85
14	0.88	17.50	51.36
15	1.50	5	105
16	0.88	38.52	85
17	1.50	30	105
18	0.25	5	105
19	1.50	5	65
20	0.25	30	65

Sugar content/g PNB obtained after sequential, acid and alkali pretreatment, followed by enzymatic saccharification (described ahead), was selected as the response for the combination of independent variables. A

quadratic polynomial equation was developed to predict the response as the function of independent variables and their interaction.

Regression analysis and estimation of the coefficient were performed using Design expert software. The optimal level of the variables as predicted by the model, based on point prediction method of the design expert software, was used for executing the experiment for validation of model.

Liquid fraction was separated using Whatman filter paper and the PNB was repeatedly washed till the pH reached 7. Solid residues were air-dried at 50°C to obtain dry matter content between 91% and 94%. The reducing sugar released in the liquid fraction was estimated spectrophotometrically by dinitrosalicylic acid (DNS) method [6]. Equal volume of sample and DNS reagent (1 ml each) was mixed and incubated at 95°C for 10 min, followed by addition of 1 ml sodium potassium tartarate (40%). The reaction mixture was allowed to cool to room temperature, and absorbance was measured at 575 nm (UV-1800 Spectrophotometer, Shimadzu, Japan). A standard curve of glucose was developed, and amount of reducing sugar was analysed [7].

2.4. Alkali pretreatment

Solid residues from all the RSM designed acid pretreatment experiments were subjected to alkali treatment [6-7]. In this pretreatment 0.5 g of acid pretreated PNB from each of the experimental run was treated with calcium hydroxide at 0.01 g/g of PNB at a solid to liquid ratio of 1:10. Pretreatment was executed at 95°C for 30 min. Liquid fraction was separated using muslin cloth. The reducing sugar in the liquid fraction was estimated by DNS method [7]. The solid residues were repeatedly washed till the pH reached 7. Solid residues were air-dried at 50°C to obtain 95% dry matter content [6].

2.5. Cellulase for enzymatic hydrolysis

Cellulase employed for saccharification of sequentially acid and alkali pretreated PNB was of fungal origin i.e. from *Trichoderma reesei* ATCC 26921, procured from Sigma-Aldrich (Celluclast 1.5 L). For enzymatic hydrolysis, 0.1 g of PNB obtained after sequential acid and alkali pretreatments, was suspended in 10 ml of acetate buffer (50 mM, pH 5), and was subjected to cellulase treatment 2-14 Units [6-7]. The contents were incubated under shaking (160 rpm) at 50°C for 48 h (Innova, New Brunswick, USA). Enzymatic reaction was stopped by heating the contents in boiling water bath for 10 min. Solid and liquid fractions were separated and sugar content in the liquid fraction was estimated by DNS method [6].

3. Results and discussion

3.1 Acid pretreatment of PNB

The main objective of acid pretreatment was to increase the accessibility of the cellulolytic enzymes to the cellulosic fractions by solubilizing the hemicellulosic fraction of the biomass [19]. Acid pretreatment can be performed either with concentrated or with diluted acid, however, latter is preferred. Usage of concentrated acid for biomass pretreatment is less attractive because it is toxic, corrosive, and hazardous, and causes the formation of potential microbial inhibitors, and loss of sugar [20]. In the current study dilute acid was employed at respective concentrations, and other variables like temperature and time of treatment. Sugars (mostly hemicellulosic) released in the liquid fraction obtained after acid pretreatment were assayed. The results show that low concentration of sulphuric acid and low/moderate temperatures resulted in higher sugar yield, for instance adequate sugars were released in run number 6 (13800 µg/g), 7 (14200 µg/g) and 20 (11100 µg/g). But in the experimental runs where high temperature was used, the amount of sugar released was low. Furthermore, the sugar yield was even lower in the cases where high temperature was coupled with high acid concentration. It is due to the reason that the high temperature and high acid concentration cause degradation of sugars into inhibitors (furfurals) [20]. These degradation compounds reduce the total yield of monomeric sugars on one hand, and cause inhibition of subsequent enzymatic and/or fermentation steps. Dilute sulphuric acid pretreatment being relatively inexpensive, effective, and less hazardous, has widely been used for significantly reducing the recalcitrance of lignocellulosic biomass [10]. However, acids like hydrochloric acid and nitric acid have also been examined for pretreatment of various lignocellulosic materials [21, 22].

The solid fraction left after acid pretreatment was washed well to attain neutral pH, and subjected to alkali pretreatment [6-7].

3.2. Alkali pretreatment of PNB

Washed and dried solid fractions of acid pretreated PNB were immersed in 0.01 g calcium hydroxide at a solid to liquid loading of 1:10 for 30 min. After that the liquid fraction was separated using muslin cloth and was

assayed for sugars. The amount of sugar in the liquid fraction was negligibly small. Alkali treatment appears to be the most effective method in breaking the ester bonds between lignin, hemicellulose and cellulose, and avoiding fragmentation of the hemicellulose polymers compared with acid or oxidative reagents [19, 22]. So there is a release of majorly lignin content in the liquid fraction. Alkali pretreatment also removes acetyl and various uronic acid substitutions on hemicellulose that increase the accessibility of hemicellulose as well as cellulose to enzymes [23]. Sodium, potassium, calcium and ammonium hydroxides are the frequently used reagents for the alkaline pretreatment [19]. The solid fractions left after alkali pretreatment were washed well to attain neutral pH, and used further for saccharification with cellulose.

3.3. Saccharification of PNB after sequential acid and alkali pretreatments

For hydrolysis of the pretreated biomass the enzyme Celluclast 1.5 L from *Trichoderma reesei* ATCC 26921 (Sigma- Aldrich) was used. Acid and alkali pretreated PNB (0.1 g) was incubated with enzyme (10 FPU) at 50°C for 48 h. The sugars released were subsequently estimated. Celluclast catalyses the breakdown of the glucose polymers that comprise cellulose to glucose, cellobiose (i.e., pairs of glucose units) and longer chains of glucose units. Chandel *et al*. [24] executed enzymatically hydrolysis of ammonia pretreated bagasse by commercial enzymes (Celluclast 1.5 L and Novozym 188) using 15 FPU/g dry biomass and 17.5 Units of β-glucosidase/g dry biomass at 50°C, 150 rpm for 96 h to get maximum sugars.

3.4. Sugar generated after optimisation of pretreatment variables

Total sugar obtained after sequential acid and alkali pretreatment, and enzymatic saccharification is depicted as a response in the RSM-designed experiments. Experimental and predicted response (sugar content mg/g PNB) obtained for optimized acid-based pretreatment variables from central composite design of response surface methodology is shown in the Table 3.

Table 3: Experimental and predicted responses for sugar yield from PNB using CCD of RSM

Run order	Experimental variables*			Response Sugar content (mg/g)	
	A Acid concentration (%)	B Incubation time (min.)	C Temperature (°C)	Experimental	Predicted
1	0.88	-3.52	85	202	187.69
2	0.88	17.50	118.64	132	105.36
3	0.88	17.50	85	138	126.07
4	0.25	30	105	204	188.41
5	0.88	17.50	85	120	126.07
6	-0.18	17.50	85	244	246.17
7	0.25	5	65	286	260.04
8	0.88	17.50	85	128	126.07
9	0.88	17.50	85	128	126.07
10	0.88	17.50	85	120	126.07
11	1.50	30	65	153	129.1
12	0.88	17.50	85	120	126.07
13	1.93	17.50	85	134	117.24
14	0.88	17.50	51.36	152	164.65
15	1.50	5	105	120	118.77
16	0.88	38.52	85	206	206.32
17	1.50	30	105	123	158.8
18	0.25	5	105	126	159.79
19	1.50	5	65	110.1	135.58
20	0.25	30	65	231	242.1

* A- acid concentration, B- incubation time, and C- temperature.

A regression equation for the model was suggested as:

Response i.e. sugar content (mg/g) = $+126.07 -38.51A +5.54B -17.63C +19.78A^2 +25.08 B^2 +3.16 C^2 +2.86 AB +20.86 AC +11.64 BC$

The equation shows the released sugar content as a function of acid concentration (A), incubation time (B) and temperature (C).

Table 4: Results of ANOVA obtained for sugar yield after pretreatment and saccharification based on CCD of RSM

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model*	42970.60	9	4774.51	7.56	0.0020	Significant
A	20251.21	1	20251.21	32.80	0.0002	Significant
B	418.89	1	418.80	0.66	0.4343	
C	4243.57	1	4243.57	6.72	0.0268	Significant
A^2	5636.68	1	5636.68	8.93	0.0136	Significant
B^2	9065.01	1	9065.01	14.36	0.0035	Significant
C^2	143.90	1	143.90	0.23	0.6433	
AB	65.55	1	65.55	0.10	0.7539	
AC	3481.95	1	3481.95	5.52	0.0407	Significant
BC	1083.45	1	1083.45	1.72	0.2194	
Residual	6312.18	10	631.22			
Lack of Fit	6052.85	5	1210.57	23.34	0.0018	
Pure Error	259.33	5	51.87			
Cor Total	49282.79	19				

* A- acid concentration, B- incubation time, and C- temperature.

The model F-value of 7.56 implies the significance of this model. There is only a 0.2% chance that a model F-value this large could occur due to noise. Values of Prob>F less than 0.05 indicate model terms are significant. In this case A, C, A^2, B^2, and AC are significant model terms. Values greater than 0.1 indicate the model terms are not significant. Adequate precision measures signal to noise ratio and its value found in the present model was 8.707, which shows an adequate signal (S/N ratio > 4 is desirable).

The three-dimensional response surface plots were generated to investigate the interaction between various variables viz. acid concentration (A), time (B) and temperature (C) and to visualize their combined effects on response as shown in Figure 1. The response in Figure 1(a) depicts the interaction between acid concentration and time (AB), and it was found that acid concentration and time had different effect on the response individually. However, upon their interaction with each other, acid concentration and time showed an increase in response (sugar release) but was insignificant with much higher p-value (0.7539) than the desired value of 0.05. In Figure 1(b) the interaction between the acid concentration and temperature (AC) is shown and it was found that the interactive affect of both the variables cause a significant increment in the response, as is also depicted by its p-value (0.0407). Though acid concentration and temperature individually illustrated different effects on the response. In Figure 1(c) the interaction between time and temperature (BC) is shown and the plot illustrates that their interaction though increased response but the interaction as such was not significant. From the 3D response surface plots it was concluded that the response was influenced maximally by the interaction between acid concentration and temperature (AC) followed by time and temperature (BC), and acid concentration and time (AB).

The perturbation plot (Figure 1. d) shows the response changes as each factor moves from the chosen reference point, keeping all other factors constant at the reference value. Acid concentration (A) emerged to be the most important variable that caused maximum effect on response as a single variable, followed by pretreatment time (B) temperature (C). Validation of the statistical model was executed by using point prediction tool of RSM, an optimum value of all the 3 variables i.e. sulphuric acid concentration (A), pretreatment time (B) temperature (C) were employed and an experiment was conducted. Precise closeness of observed (286 mg/g) and predicted response (289 mg/g) indicated the vaidity of the model.

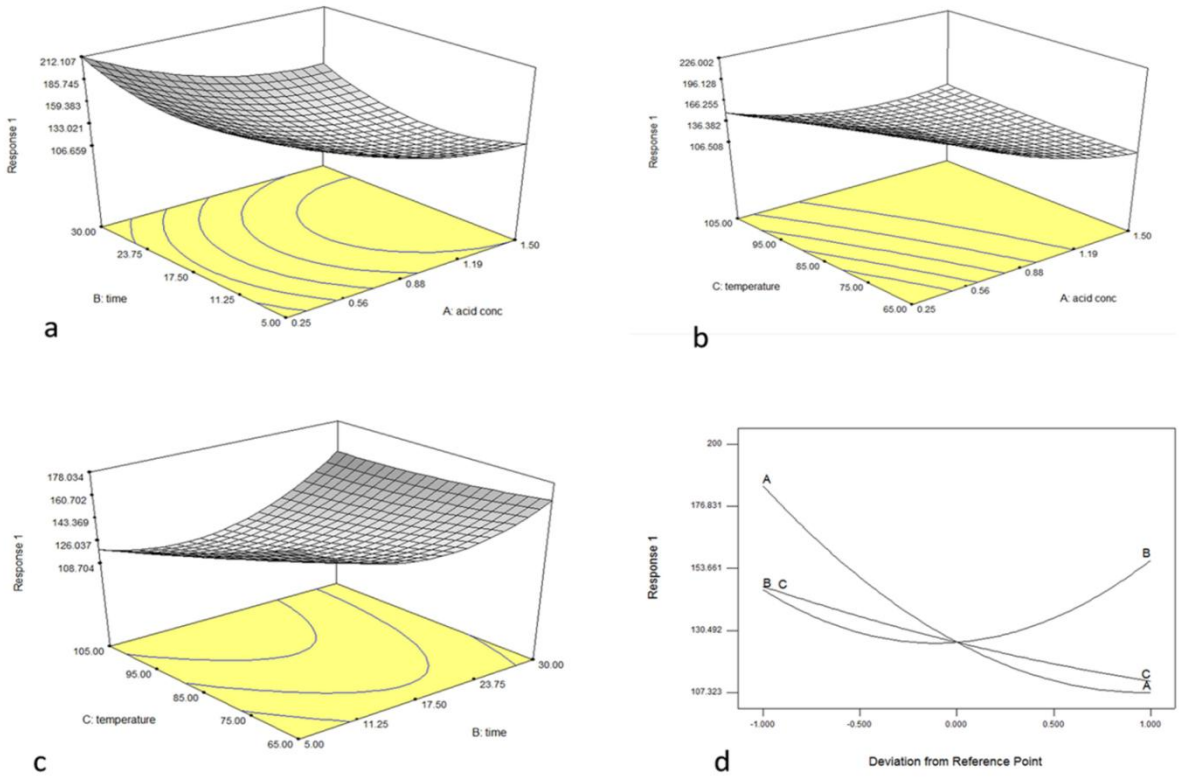

Figure 1: Response surface plots for pretreatment optimization of pine needle biomass based on physico-chemical variables. Plots show interaction between the variables *viz.*: acid concentration and time (a), acid concentration and temperature (b), time and temperature (c), and the perturbation plot (d).

Design of experiemnts approach has variously been used for pretreatment of lignocellulosic materials using dilute acids. Pretreatment optimization of the rice hulls was executed with diluted acid [25]. A central composite design (CCD) was employed to get regression equation for the functional variables: acid concentration and heating time. Optimum acid pretreatment of rice hulls with 0.3% (w/v) H_2SO_4 for 33 min was documented as the most effective pretreatment. The pretreated rice hull in those conditions was treated enzymatically for saccharification for 48 h [23]. Leenakul *et al.* [26] reported the diluted sulphuric acid for pretreatment of bamboo. The dry feedstock with solid/liquid loading at 10% (w/w) was pretreated in an autoclave at different temperatures (120 and 140°C) with different residence times (30, 60, 90 min) and different sulfuric acid concentrations (0.6, 0.9, 1.2% w/w). Maximum glucose and xylose yields were achieved under conditions: 140°C, 1.2% sulfuric acid concentration, and 90 min. Vats *et al*. [5] optimized the physico-enzymatic pretreatment of *P. roxburghii* fallen foliage (needles). The analysis of variance (ANOVA) was applied for the validation of the predicted model at 95% of confidence level. This model predicted 334 mg/g release of reducing sugars after 24 h of incubation time on treating *P. roxburghii* fallen foliage with 1.18 ml of cellulase.

Phuengjayaem *et al*. [27] applied RSM based optimization for hydrolysis of acid pretreated sweet sorghum straw and a reducing sugar yield of 0.440 g/g dry substrate was obtained under optimized condition. Pandiyan *et al*. [16] optimized the conditions for enzymatic hydrolysis of alkali pretreated biomass of *Parthenium* sp. using RSM and 574 mg/g of reducing sugar yield was obtained. Akanksha *et al*. [28] optimized the pretreatment and saccharification conditions for reducing sugar yield from sorghum by using Box Behnken design and reported a total reducing sugar yield of 0.408 g/g of pretreated sorghum biomass under optimized conditions. Thus, appropriate pretreatment and saccharification of lignocellulosic biomass may be applied for release of sugars which can be utilized for production of energy, biofuel, bioplastic, and other materials of industrial importance [3-4, 29].

Conclusion

It may be concluded from the current study that dilute sulphuric at low concentration and moderate temperature, for short time is fairly good pretreatment approach for getting maximum sugar yield from PNB. Optimal conditions for physicochemical pretreatment were 0.25% sulphuric acid, 65°C, and 5 min. Incubation time for enzymatic saccharification (10 units of Celluclast 1.5 L) of pretreated biomass was 48 h, and a total sugars of 286 mg/g of PNB was obtained. Thus, pine needles may be an important biomass resource that can potentially be exploited in biorefineries for production of various commercial products including biofuel.

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