Alkaline pretreatment optimization for *Pinus roxburghii* needle biomass employing response surface methodology for bioethanol production by separate hydrolysis and cofermentation

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Received 30 January 2019; revised 20 March 2019

Production of bioethanol from lignocellulosics can cater to the supply of renewable fuel to transport sector besides being environment friendly. In the present work, bioethanol production potential of *Pinus roxburghii* needle biomass (PNB) has been studied by optimizing the thermochemical pretreatment method using alkaline conditions (NaOH) firstly by one variable at a time (OVAT) approach, followed by Response Surface Methodology (RSM) with central composite design (CCD) tool. Total reducing sugar (TRS) yield was enhanced from 22.4 g/L (OVAT) to 32.4 g/L using design of experiment (DoE) approach. Effect of pretreatment on PNB was studied by FTIR, phloroglucinol staining and estimation of phenolics which indicated lignin removal. Enzymatic hydrolysis was done by the action of commercial enzymes cellulase and pectinase with loading of 5U/g biomass. The TRS yield was further enhanced to 67.95 g/L after enzymatic hydrolysis. Using separate hydrolysis and cofermentation (SHCF) approach for fermentation of PNB hydrolysate, 28.75 g/L bioethanol was obtained when combined cultures of *Saccharomyces cerevisiae* (MTCC-36) and *Pichia stipitis* (NCIM-3498) were used.

**Keywords:** Biofuel, Central composite design, Chir pine, OVAT process, Pine needles, RSM, SHCF

Sustainable economic development in the world over demands alternative energy resources. Some of the key problems that the present world economy encounters today are energy security, sustainability, pollution, economic crisis and climate alteration due to escalated green house gases (GHGs)\(^1\). Over the last few decades, energy security issues related to fast depleting oil reserves and rising energy consumption are of prime concern globally, owing to the overexploitation of fossil fuels\(^2\). Fossil fuel resources utilized at this rate are expected to be exhausted in next 50 years\(^3,4\). Exhaustion of fossil fuels along with the consequent GHG emissions has encouraged research on renewable and sustainable forms of energy.

Transportation sector utilizes an increasing amount of energy from fuels derived from fossil fuels. It is the foundation of the present globalized economy\(^5\). More efficient forms of transport and introduction of alternative fuels can significantly decrease transport sector's dependence on fossil fuels\(^6\). Bioethanol has established itself as a promising future alternative fuel to petroleum based transportation fuels\(^2,7\) and is the dominating biofuel used in transportation sector. Biomass is the solitary renewable and appropriate primary energy resource that can supply different alternative transportation fuels in a short term\(^7\). Universally, about 220 billion tones (BT) of primary biomass is produced on dry weight basis yearly, equivalent to 4500 EJ of solar energy captured annually. Using this biomass, a bioenergy market of 270 EJ is possible yearly on a sustainable basis\(^3,8\). Various feedstocks are used for bioethanol production, such as aquatic plants, algae, agricultural crops and their waste, animal wastes, wood and wood wastes, municipal solid waste, etc. Production of ethanol using lignocellulosic biomass has fascinated many researchers owing to its availability and relatively low cost\(^2,9-11\).

Lignocellulose is the most abundant biopolymer on earth and is a potential biomass for biofuel production\(^12\). Lignocellulosic biomass is primarily comprised of polysaccharides (cellulose, hemicellulose) and lignin. Polysaccharides are polymers of sugars and thus, a potential source of fermentable sugars\(^13\). The three key biopolymers are approximately in the range: cellulose (35-50%), hemicellulose (20-35%) and lignin (15-20%)\(^12,14\). However, ratio between cellulose, hemicellulose and
lignin in a particular plant varies with various factors like plant age, harvesting season and culture conditions\textsuperscript{4,15}. Lignocellulosic biomass, for instance agricultural and forest residues are the most plentiful renewable resource on earth\textsuperscript{16}. They are accessible to a considerable degree at low prices. For biofuel production, these feedstocks have been regarded as non-food based residues\textsuperscript{17}. Interestingly, agricultural residues are being produced in billions of tons every year all over the world, but a large chunk of these residues is either disposed off or burned\textsuperscript{13}.

The process of conversion of lignocellulosic biomass to bioethanol generally comprises three steps: pretreatment, hydrolysis and fermentation. Owing to the composite hierarchical framework and resistant nature of lignocellulosic biomass, pretreatment steps present the most crucial challenge to biomass use before its conversion\textsuperscript{13}. On the other hand, they resist enzymatic/microbial hydrolysis, thus a pretreatment step is essential preceding their enzymatic or biological conversion \textsuperscript{17-20}. This step helps to break open the lignin fraction and hemicellulosic part in order to let loose the masked cellulose making it amenable to enzymatic hydrolysis \textsuperscript{13,21}.

Pretreatment of lignocellulosic biomass is a vital step to convert biomass into fermentable sugars. Pretreatment technologies have developed concurrently with progress in methods used for biofuel production\textsuperscript{3,22}. A variety of pretreatment methods have been employed for the pretreatment of diverse biomass types but majority of chemical pretreatment processes utilized high amounts of costly chemicals. Chemical pretreatment using certain chemical agents (acid, alkaline or organic solvents) under different pressures and temperatures is utilized to open up the lignocellulosic matrix\textsuperscript{12}. Alkaline pretreatment using sodium hydroxide (NaOH) is one of the most efficient chemical pretreatment methods for ethanol production \textsuperscript{17,23,24}. The foremost effect of alkali pretreatment is biomass delignification, which consecutively enhances the saccharification efficiency. NaOH is generally used in chemical pretreatment of lignocellulosic biomass since it is able to delignify biomass\textsuperscript{15}. This treatment causes swelling of lignocellulosic biomass, which leads to an increase in the internal surface area, reduces cellulose crystallinity, and disrupts lignin structure, thereby enhancing the reactivity of the remaining carbohydrate\textsuperscript{16}. Response surface methodology (RSM) is a valuable tool to optimize experimental conditions and understand the interaction between different process parameters.

\textit{Pinus roxburghii} Sargent is a major tree (up to about 80\%) growing in the lower shivalik range of Himalayas in Jammu & Kashmir, Himachal Pradesh and Uttarakahal states of India. The total area under \textit{P. roxburghii} forests is estimated to be around 8,90,000 hectares and occurs between 450 m to 2300 m altitude\textsuperscript{27}. Its leaves called as ‘needles’ are rich in resins and oils and form a ubiquitous cover on the forest floor and do not allow other grasses to grow on the forest ground. In summers, these dried needles become a major source of forest fires. This needle biomass if diverted for biorefineries can serve the dual purpose of solving forest fires and production from an unutilized source.

In the present work, we studied the effect of NaOH pretreatment on pine needle biomass of \textit{P. roxburghii}, at moderate temperature (121°C) and pressure (15 psi) on total reducing sugar (TRS) yield. An experimental design was accomplished to determine the optimum conditions for enhanced TRS yield. Furthermore, hydrolysis of pine needles using commercial enzymes was performed and the resulting ethanol yield was investigated by separate hydrolysis and co-fermentation (SHCF) for conversion of both pentoses and hexoses to bioethanol\textsuperscript{17,28,29}.

\section*{Material and Methods}

\textbf{Biomass collection and physical processing}

Pine needle biomass (PNB) from \textit{Pinus roxburghii} was collected from pine forest in Tikri (Udhampur district) Jammu & Kashmir, India. PNB was dried in shade, followed by oven drying at 50°C. The dried PNB was comminuted mechanically using a blender and was sieved through a mesh of 1 mm pore size. The powdered PNB was stored in air tight containers at room temperature 25° C±2 (RT) for the subsequent experiments.

\textbf{Cultures, Chemicals and Enzymes}

The microbrial strain \textit{Saccharomyces cerevisiae} (MTCC-36) used in the study was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTech.) Chandigarh, India and was maintained by subculturing after every 15 days on YEPD (composition-g/L: yeast extract 10; peptone 20; dextrose 20; distilled water 1000 mL) agar plates. The culture of \textit{Pichia stipitis} (NCIM-3498) was obtained from National Collection of Industrial Microorganisms (NCIM), National
Chemical Laboratory (NCL) Pune, India and maintained on MGYP medium (composition-g/L: malt extract 3; glucose 10; yeast extract 3; peptone 5; agar 20). They were incubated for 24 h at 28°C and thereafter stored in a refrigerator at 4°C till further use. The chemicals used were from HiMedia Labs, Mumbai, India. All the chemicals were of highest quality and of analytical grade. Commercial enzymes cellulase (Cellulase “ONOZUKA R-10” from *Trichoderma viride*) and pectinase (polygalacturonase) were obtained from HiMedia Labs, Mumbai, India.

Thermoalkaline pretreatment using OVAT approach

The PNB was subjected to alkaline conditions followed by exposure to low temperature and pressure by one variable at a time (OVAT) method. Two alkaline liquids viz. sodium hydroxide (NaOH) and potassium hydroxide (KOH) were used in the pretreatment process. A biomass loading of 20% (w/v) was done with NaOH and KOH. 20g pine needles were soaked in different concentrations i.e. 1-5% of NaOH and KOH in 100 mL solution. The biomass was autoclaved at 121°C and 15psi pressure for 15 min. Total reducing sugar (TRS) estimation was done by DNS method.

Optimization of thermoalkaline pretreatment using RSM

NaOH provided better sugar release and hence was used as solvent for pretreatment optimization. For optimization of thermoalkaline pretreatment of PNB, effects of two independent variables i.e. biomass loading and alkali concentration on release of TRS was investigated. Lastly, after choosing the type of chemical and biomass loading to be employed in the assays, an experimental design was performed to obtain the best conditions for the release of TRS during the pretreatment step. Factors evaluated were biomass loading (g) and chemical concentration (%). The evaluated response was TRS concentration (g/L).

Software used

The independent variables of the experimental design were optimized and interpreted using Design Expert Version 6.0.10 (Stat-Ease Inc., Minneapolis, Minnesota, USA) statistical software.

Determination of total phenolics content

Total phenolics content was determined by Folin-Ciocalteu assay. The reaction consisted of adding 2 mL of 10% F-C reagent (v/v) to 1 mL of test sample (pretreated PNB hydrolysate) and vortexing it thoroughly. Subsequently, 8 mL of 700 mM Na₂CO₃ was added to the reaction mixture and the sample was incubated at room temperature for 2 h. The test sample was then read spectrophotometrically at 765 nm. Standard was prepared using aliquots of gallic acid (1 µg/µL) in 95% (v/v) methanol. Total phenolics content was determined as gallic acid equivalents by using gallic acid standard curve.

Phloroglucinol lignin staining

Phloroglucinol staining is based on staining of lignin present in the plant biomass. PNB was stained by using 1% phloroglucinol in HCl-ethanol (1:3) solution. The stain was freshly prepared as it degrades with time. Lignin containing biomass is stained red. For control, water was used as the mounting medium.

Fourier transform infrared spectra

Fourier transform infrared (FTIR) spectroscopy analysis of the PNB was carried out for elucidating the chemical changes that occur after treating the PNB with NaOH by optimized thermochemical pretreatment using RSM. FTIR spectra were recorded by Shimadzu IR-Tracer with the spectral resolution of 4 cm⁻¹. The spectrum was recorded over the spectral range of 400-4000 cm⁻¹. Sixty scans were taken per sample. 5-10 mg of sample having a particle size of ~150 µm was used for FTIR analysis.

Enzymatic hydrolysis

After the analysis of optimized pretreatment results, the experimental run providing the highest TRS yield was conducted for subsequent enzymatic hydrolysis. The pretreated biomass was hydrolyzed by using commercial enzymes cellulase and pectinase in different experimental runs. An enzyme loading of 5 U/g of biomass was used and the enzymatic treatment was carried out under shaking conditions (200 rpm). Periodic TRS estimation by DNS assay was done to assess the best time for maximum TRS release. The experiments were conducted in triplicate for cellulase + pectinase enzyme treatments. The TRS data is stated as average value of the triplicate runs.

Inoculum preparation and Fermentation

The inoculum cultures of *Saccharomyces cerevisiae* (MTCC-36) and *Pichia stipitis* (NCIM-3498) were prepared by inoculating a loopful of 24 h old grown culture on YEPD and MGYP agar plates respectively into 100 mL of broth. These cultures were incubated at 28°C and 200 rpm for 24 h. The broth was centrifuged aseptically and the pellet was suspended in sterile acetate buffer (pH 5). The optical density of the suspension was adjusted to 0.8 at
600 nm with acetate buffer which is equivalent to 1.2×10^7 cells/mL. These suspensions were used as inoculum. The inoculum at 5% (v/v) concentration was used for bioethanol production from enzymatically hydrolyzed biomass. Fermentation for ethanol production was carried out by separate hydrolysis and cofermentation (SHCF). SHCF involves the process of enzymatic hydrolysis first and thereafter, followed by fermentation to produce bioethanol. For SHCF mode, cellulase and pectinase enzymes were added to the hydrolysate of optimized thermoalkaline pretreated PNB at a loading of 5 U/g biomass per enzyme. The enzymatic hydrolysis was carried out for 72 h at 28°C under shaking conditions (200 rpm). After termination of the enzymatic treatment, the PNB was autoclaved at 121°C and 15 psi pressure and the inoculum cultures of *S. cerevisiae* (MTCC-36) and *P. stipitis* (NCIM-3498) were added. Fermentation was allowed to take place at 28°C under static conditions. Ethanol concentration and TRS yield was estimated using dichromate assay and DNS method, respectively at regular intervals of 24 h.

Results and Discussion

Physical pretreatment

There are numerous physical pretreatment techniques for example milling, chipping, grinding, freezing and radiation which are applied to pretreat the lignocellulosic waste. These methods decrease the particle size and enhance the surface area of lignocellulosic materials.

Total reducing sugar yields by one variable at a time approach

Thermal pretreatment at 150°C or above results in the formation of phenolic compounds, furfurals and hydroxymethylfurfurals (HMFs), which negatively affects the fermentation process. Therefore, thermal pretreatment of pine needles was performed at a moderate temperature i.e. 121°C and 15 psi pressure. A combined thermal and chemical method using alkali was adopted using PNB for the pretreatment step. Alkali pretreatment using dilute NaOH pretreatment is basically one of the feasible methods which yield elevated amounts of fermentable sugars with less input, in comparison to many other physical methods like ultrasound, microwave, gamma ray irradiation and steam explosion along with chemical methods such as salt, acid, wet oxidation and alkali. Before proceeding for saccharification, the powdered PNB was subjected to alkali pretreatment using NaOH. After the thermoalkaline pretreatment of PNB, the fractions were assayed for TRS. The best alkali for pretreatment was NaOH at concentration of 4% (Fig. 1). The maximum TRS yield in filtrate by 4% NaOH pretreatment was 22.4 g/L. The maximum TRS yield from KOH (5%) was low (15.0 g/L) as compared to NaOH and therefore, NaOH at 4% (1N) concentration was selected for thermoalkaline pretreatment.

Alkali pretreatment by means of NaOH is one of the methods used for chemical pretreatment of lignocellulosics. It is the most commonly used alkali in alkaline pretreatment method and has been found to be more effective than untreated biomass with regard to biohydrogen, bioethanol, biomethane and biobutanol production using diverse raw materials. The major advantage of using alkali during pretreatment is that it utilizes lower temperature and pressure compared with other pretreatment strategies. Pretreatment by means of sodium hydroxide can be carried out at severe (0.5-4% NaOH at high temperature) or moderate conditions (at least 6-8% NaOH and low temperature). In view of the high cost of sodium hydroxide, using lower concentrations is economical.

Alkali pretreatment removes inhibitors like acetyl groups, lignin and various uronic acid substitutions in order to make cellulose accessible for saccharification by enzymes. The process involved in alkaline pretreatment is cleaving linkages within lignin and the glycosidic bonds of polysaccharides. This causes a reduction in the degree of polymerization and crystallinity. It disrupts the cell wall by solubilizing...
polysaccharides, such as cellulose, hemicellulose and lignin by enzymatic hydrolysis. Also, chemical swelling of fibrous cellulose takes place which involves salvation and saponification reactions leading to the disruption of cross-links between hemicelluloses and a number of other components resulting in increased biomass porosity. In addition a high intensity of OH ions assists in quick dissolution of the cellulosic content in the biomass.

Statistical optimization of parameters/factors affecting total reducing sugar yields using Design of Experiment approach by RSM

Design of Experiment (DoE) approach was utilized to screen the physical components affecting TRS yield in the pretreatment study of PNB. To check the statistical importance of the experimental data, Design Expert Version 6.0.10 software was used. Statistical experimental design is an easier and more methodical technique to organize and understand data as compared to conventional methods. RSM is a statistical method employed for the design of experiments, model building, evaluating the effect of process variables and utilized to find optimum conditions for expected response within the defined range. Central composite design (CCD) tool of RSM was used to optimize the screened significant variables based on the preliminary experimental results attained via traditional OVAT approach and literature survey, in order to find out interaction effects of the variables on TRS yield. Using CCD, two (2) independent variables i.e. biomass loading (g) and chemical concentration (%) were screened by representing them at two levels, low (−) and high (+) in 13 trials in order to achieve maximum TRS yield. Since NaOH gave highest TRS yields by OVAT, it was selected for further pretreatment optimization studies. Pretreatment was carried out with different concentrations (1-5%) of sodium hydroxide (NaOH) at a biomass loading range of 15-20%. The design parameters and the levels chosen for each factor are as showed in Table 1. The reaction of each independent variable on the response (TRS yield) was assessed by adequate polynomial quadratic equations. Polynomial quadratic equations for the model applied were as given under:

Final equation in terms of coded factors:

Reducing sugar concentration g/L (response-X) = +31.51-2.26A+5.44B-2.39A²-4.79B²+4.03AB … (1)

where X is the reducing sugar concentration in g/L and A and B are the coded values of biomass loading and chemical concentration. ANOVA was applied to determine the “goodness of fit” of the quadratic model and validate the statistical parameters. Fig. 2 demonstrates the experimental lay out for thirteen runs with different combinations of factors and their responses (TRS yield). The experimental and predicted values were evaluated statistically in order to find out a correlation between them. The results thus attained are given in Table 2 which summarizes the statistical significance of each variable (P-value). These results indicated that the applied tests were found to verify the significance of the model using the Fisher’s F-test value ($P <0.05$). $p$-value less than 0.05 is regarded as significant and more than 0.05 is considered to be non-significant. The F-value of the quadratic model was found to be 19.89 which indicated that the developed quadratic model was significant. There was a 5.27% chance that a “Lack of Fit F-value” this large could occur due to noise. Insignificant lack of fit (6.38), proximity of $R^2$ and adjusted $R^2$ value to 1 viz., 0.9342
and 0.8872, respectively, adequate precision greater than 4 i.e. 12.141 and a low coefficient of variation (CV) value of 8.47% supports the use of RSM in optimizing the TRS yield. These results for the model recommended that the experimental data obtained was in good conformity with the model predicted value. Briefly, the experiments were accurate and reliable. These results imply that RSM is an effective mathematical tool for optimization of TRS yield using pretreatment studies.

Fig. 2 shows the interaction effects of biomass loading and NaOH concentration on TRS yield by means of three-dimensional response surface plots. It was noticed that at low level of biomass loading and low level of chemical concentration, the TRS yield was low. The yield increases on rise in both the parameters. However, the highest TRS yield i.e. 32.4 g/L was observed with middle values of biomass loading (17.5% w/v) and NaOH concentration (3.00%) by the point prediction run. The TRS yield was slightly greater than the yield predicted by point prediction run (31.514 g/L). The statistical analysis showed that both biomass loading and chemical concentration and their interaction had significant positive effect on the response (TRS yield).

Analysis of total phenols by means of Folin-Ciocalteu reagent

Phenols present in the environment are of significance from many perspectives (astringency, bitterness, antioxidants, color, protein constituents, oxidation substrates, browning reactions, etc.)47. Plants give an extraordinary range of phenolic compounds that have one or more acidic hydroxyl residues attached to an aromatic hydrocarbon ring (phenyl). Anthocyanins, hydroxycinnamic acids, tannins and flavonoids represent the major classes of phenolic compounds, which jointly account for roughly around 40% of the carbon content in the ecosphere. Structural phenolics for instance suberin, lignin and other structural polymers include much of this carbon share31. Lignin is a complex polar, phenolic polymer and imparts mechanical strength in plants. Since lignin provides resistance to the enzymatic hydrolysis of lignocellulosic biomass. Therefore, delignification of lignocellulose by pretreatment is a necessary step in the bioethanol production process to expose maximum cellulolic content48. Quantification of total phenolics content in the PNB was done by using Folin-Ciocalteu (F–C) reagent using gallic acid as a standard.

The F–C assay depends on the oxidation of phenolic compounds in the basic medium. The transfer of electrons to phosphomolybdic/phosphotungstic acid complexes results in their partial reduction from +6 to +5 valence states resulting in blue colored solution which is determined spectrosopically at 765 nm. The total phenolics content in PNB was calculated as gallic acid equivalents. After optimized thermochemical pretreatment 1418.18 µg/mL of total phenolic content was observed in the hydrolysate. Before pretreatment, total phenolics content was calculated as 357.142 µg/mL gallic acid equivalents. Thus, the total phenolics content increased approximately by 2.97 fold on pretreatment which can be attributed to the

Fig. 2 — Response surface plots for pretreatment optimization of PNB based on physicochemical variables. Plots show interaction between variables viz.: (A) biomass loading and chemical concentration; and (B) the perturbation plot.
release of phenolic compounds due to delignification of PNB by NaOH. In a study by Irfan et al., who studied the effect of H₂SO₄ concentration on the production of total phenolic compounds from pine needles by RSM reported a maximum release of 31.20 mM.

**Phloroglucinol lignin staining**

In plant tissues, the most sensitive test for lignin detection is phloroglucinol-hydrochloric acid. This method is very easy to perform and gives excellent results in no time. The lignin tissue was easily visible as bright cherry-red in color. Phloroglucinol in acidic medium (HCl) reacts with both sinapyl aldehyde and coniferyl aldehyde groups in lignin to yield an unstable pink/red color which degrades over time. The lignin content in the PNB decreased drastically after pretreatment as revealed by the phloroglucinol-HCl reagent which signifies the delignifying effect of NaOH.

**Fourier Transform Infrared Spectroscopy of Pine needle biomass**

The structural differences in PNB before and after pretreatment were evaluated by FTIR spectroscopy. In lignocellulose, lignin is a possible hindrance to efficient enzymatic hydrolysis of polysaccharides by obstructing their availability for enzymatic approach. Modification in the functional groups of biomass due to pretreatment influences their subsequent hydrolysis step. Therefore, FTIR spectrum of PNB was studied for elucidating the chemical changes that occur after pretreating it thermochemically by using NaOH. Fig. 3 shows the comparison of FTIR spectra for both untreated and pretreated PNB. NaOH is known to break the ester bonds between cellulose and lignin fraction of the lignocellulosic biomass. Band stretching and widening at 3284 cm⁻¹ in the pretreated sample resulted from vibrations due to O-H stretching of cellulose fraction. Stretching at 2917 cm⁻¹ in the spectrum indicated C-H stretching due to the presence of lignin which is prominent in untreated sample and absent in the treated sample. A sharp, well resolved peak in the untreated biomass at 2850 cm⁻¹ belongs to C-H bond stretching, characteristic of lignin and its near absence in the pretreated biomass points at the de-lignification of PNB. Ketone/aldehyde C=O stretch at 1732 cm⁻¹ in the untreated biomass belongs to hemicellulose and is absent in the treated biomass.
signifying the breakdown of hemicellulosic polymer after NaOH pretreatment\textsuperscript{51}. The band at 1607 cm\textsuperscript{-1} is because of aromatic ring vibration characteristic of lignin and its absence in the pretreated sample signifies delignification. The band at 1161 cm\textsuperscript{-1} in the untreated PNB corresponds to C-O-C asymmetrical stretching characteristic of cellulose and hemicellulose which is nearly absent in the treated biomass suggesting the deconstruction of both cellulose and hemicellulosic component\textsuperscript{52}. Thus, FTIR analysis indicated that NaOH had a significant effect on the delignification of PNB.

**Saccharification using commercial cellulase and pectinase enzymes:**

So as to attain highest substrate consumption, it is imperative to carry out the synchronous bioconversion of key polysaccharides incorporating both cellulose and hemicellulose using a combination of enzymes. The process of enzymatic hydrolysis of polysaccharides into soluble, fermentable reducing sugars occurs under the action of several enzymes acting in coherence. *Trichoderma* sp. has long been extensively used in the bioethanol industry for its highly useful and powerful source of cellulase enzyme for fragmentation of crystalline cellulose. Hemicelluloses and probably pectin are considered to limit the entry of cellulases to their pretreated lignocellulosic substrate. Adding enzymes such as pectinases can disintegrate these non-cellulosic sugars molecules and thus enhance cellulose conversion\textsuperscript{53}. In a study by Zhang *et al*.\textsuperscript{53} cellulase enzyme was employed in combination with pectinase and with xylanase for hydrolyzing the lignocellulosic biomass. Both the enzyme combinations increased the 24 h yields of glucose and xylose, but the former was more efficient. Saccharification with cellulase and pectinase enzymes carried out in different experimental runs revealed that highest TRS yields were obtained when both the enzymes were used for 48 h. For the PNB hydrolysate, the TRS yield increased to 67.95 g/L. Thus, the addition of cellulase and pectinase enzyme combination has significantly increased the TRS yield from PNB by slightly more than two fold.

**Separate Hydrolysis and co-Fermentation:**

Bioethanol production should not only give maximum amount of reducing sugars, but also attain the utmost fermentability of the hydrolysate produced through the hydrolysis process. Thus, it is essential that the pretreated biomass should be sufficiently utilized to avoid the occurrence of toxic components in the solution which hamper the enzymatic hydrolysis of the cellulosic part of lignocellulosic biomass and thus the fermentation yields\textsuperscript{52}. The cellulose and hemicellulosic reducing sugars obtained through enzymatic hydrolysis can be effectively utilized for bioethanol production either by separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) of the hydrolysate. However, in SSF, optimum growth conditions of the yeast would be unlike that of the hydrolytic enzyme and might result in lower fermentation efficiency and lower yield of bioethanol. Therefore, for higher efficiency of bioethanol production, the methodology of SHF was favoured. Since common yeast *Saccharomyces cerevisiae* cannot readily ferment pentose (C\textsubscript{5}) sugars into bioethanol, therefore, pentose utilizing yeast organisms can be used by SHCF mode. Various studies suggest that separate hydrolysis and fermentation by co-culture fermentation uses both hexose (C\textsubscript{6}) and pentose (C\textsubscript{5}) fermenting yeasts that exchange for oxygen requirement between them and utilize both the sugars. Within various pentose consuming yeasts, *P. stipitis* has depicted vast capability by having wide substrate specificity without any definite vitamin requisite for pentose consumption\textsuperscript{54}.

The pretreated biomass was hydrolyzed by cellulase and pectinase enzymes for 48 h. On establishing the optimum conditions of hydrolysis process for PNB, the fermentation of reducing sugars was recovered for the production of bioethanol using the SHCF method of fermentation with the *S. cerevisiae* (MTCC-36) and *P. stipitis* (NCIM-3498) strains. Before fermentation, the hydrolyzate obtained after enzymatic treatment was sterilized using an autoclave for 30 min at 15 psi pressure. Sample was taken out periodically after every 24 h to estimate bioethanol yield. Bioethanol thus produced was assayed by dichromate method. The combination of both the strains produced 28.75 g/L ethanol after 72 h of fermentation. Bioethanol yield of 0.423 g/g was attained from PNB using SHCF approach. Thus, the conversion efficiency for ethanol fermentation in SHCF mode was 82.96% of the theoretical yield on the basis of TRS conversion. Bioethanol yield and conversion efficiency were calculated based on the following formula:

\[
\text{Bioethanol yield (g/g biomass)} = \frac{\text{Bioethanol concentration (g/L)}}{\text{Initial glucose concentration (g/L)}} \quad \ldots (2)
\]
Conversion efficiency (g/g) = \frac{\text{Bioethanol yield (g/g)} \times 100}{0.51} \quad \ldots (3)

where 0.51 represents the maximum bioethanol yield per unit of hexose sugar from glycolytic fermentation (g/g)\textsuperscript{55}.

Residual total reducing sugar
The residual TRS was the sugar that remained unutilized in the medium after the completion of bioethanol production. For PNB the lowest residual TRS was 4.472 g/L in fermentation run in SHCF mode after 72 h. Thus, the residual TRS which remained unconverted to ethanol was in the range of 6-7% (6.58%) of the initial TRS loading.

Mass/material balance during bioethanol production
The efficiency of bioethanol production process using PNB from pretreatment to bioethanol production was found to be 82.9% based on total reducing sugar conversion efficiency. Summary of the mass/material flow of PNB is shown in the Fig. 4.

Conclusion
We have evaluated the feasibility of producing bioethanol using PNB, a non-food forestry biomass source. RSM using CCD tool enhanced the production of total reducing sugars (32.4 g/L) in comparison to the conventional OVAT process (22.4 g/L) by dilute alkali pretreatment using 4% (1N) NaOH. Making use of statistical tools such as RSM can be an inexpensive and quick technique for developing an effective strategy for enhancing TRS yield and making the biomass amenable to enzymatic hydrolysis. We found that NaOH could be an effective chemical for delignification of PNB as revealed by various tests including FTIR spectroscopy, Phloroglucinol staining test and F-C reagent test used in this study. TRS yield enhanced more than two fold (67.95 g/L) by employing enzymatic hydrolysis. The present production process provided 28.75 g/L yield of bioethanol using SHCF process with the conversion efficiency of 82.96%. Pine needles from \textit{P. roxburghii} using this strategy, thus, could be considered as a cheap and sustainable biomass for bioethanol production due to high conversion efficiency to bioethanol.

Conflict of Interest
The authors declare that there is no conflict of interest.

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