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Evaluation of different pretreatments for enhanced saccharification of *Pinus roxburghii* biomass by using mixture of polymerizing enzymes and bioreactor studies for its bioconversion into ethanol

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Abstract
This study established a novel pretreatment to overcome recalcitrance of soft wood biomass on substrate morphology, cell wall physical and chemical structures along with the subsequent enzymatic hydrolysis using mixture of cellulase and xylanase enzymes produced by bacterial and fungal cultures. Pine biomass was treated with various combinations of pretreatments viz, NaOH, NaOH+ H_2O_2, NaOH+ urea, acetone, N- methyl morpholine N-oxide (NMMO). Among all, NaOH+ urea (1:1 each) for 2 h at room temperature was found to be best for saccharification of softwood biomass. SEM micrographs indicated major increase in the wood porosity by the pretreatment, which would confirm increase in the water swelling capacity as well as enzyme adsorption. The analysis of X-ray diffraction showed considerable reduction in the cellulose crystallinity by the pretreatment, sugars so formed from best pretreated substrate was further subjected to fermentation employing separate hydrolysis and fermentation and simultaneous saccahrification and fermentation for ethanol production using mixture of ethanolgens.

Keywords: alkali pretreatment, softwood biomass, enzymatic hydrolysis, saccharification, ethanol production

Introduction
Energy insecurity, depleting fossil fuel reserves and rise in fuel prices are challenging the economic stability as well as growth throughout the world. Increasing demand for fuel and compulsion to reduce green house gas emissions necessitates the blending of fossil fuels with biofuels. Second generation biofuel is seen as a promising alternative to meet this huge demand. Exploration of the appropriate biomass is a major concern of this sector (Vats *et al.*, 2013) [24]. But in Asian countries like India, where these agricultural wastes are already in use for animal feed and other purposes, exploitation of these substrate for biofuel is not feasible. Therefore exploration of rarely used biomass for bioethanol production became a long term solution (Kumar *et al.*, 2009) [11].

Soft wood biomass consists mainly of cellulose, hemicelluloses and lignin. Cellulose is the main structure component of cell wall and is the most abundant organic compound in the world. Hemicellullose is a polymer chain composed of C5 sugars like arabinose and xylose and C-6 sugar monomers. Because of absence of crystalline structure, hemicelluloses is hydrolysed more easily as compared to cellulose. Lignin is the aromatic component giving plant their structure and strength by binding cells, fibres and vessels together (Pan *et al.*, 2012) [16].

Pine is a predominant forest species which is widely scattered throughout the world. Pine plantation though is encouraged due to its several uses but at the same time it may also lead to many serious hazards viz. forest fire and infertility of soil. Pine needles can serve as a substrate for biodegradation and for the production of economical products like biofuel and at the same time playing an important role in safe disposable of lignocellulosic biomass that will also help to solve the burning problem of global warming (Tandon *et al.*, 2012) [23].

The conversion of lignocellulosic biomass typically involves a disruptive pretreatment process followed by cellulase and xylanase catalyzed hydrolysis of the cellulose and hemicelluloses to fermentable sugars. Many factors, like lignin content, crystallinity of cellulose, and particle size, limit the digestibility of the hemicelluloses and cellulose present in the lignocellulosic biomass as reported by Hendriks (2009); Chang (2013). The pretreatments are necessary to

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improve the digestibility of the lignocellulosic biomass. Each pretreatment has its own effect (s) on the cellulose, hemicelluloses and lignin.

It is well-known that lignocellulosic hydrolysates contain fermentation inhibitors that are formed during pretreatment at high temperature and low pH. These inhibitors include furan aldehydes (furfural and 5-hydroxymethyl-furfural (HMF)), aliphatic acids (such as acetic acid, formic acid, and levulinic acid), and phenolic compounds (Jonsson LJ 2013).

Enzymatic methods of saccharification are the most preferred and use different physical and chemical pretreatment processes of biomass (Kaushal *et al.*, 2012) [9]. The three types of enzymes of cellulase involved in the process of cellulose hydrolysis are endoglucanases, cellobiohydrolases, and β-glucosidases. According to standard endo- exo energy model, these enzymes cooperate in following manner: endoglucanases act randomly along the chain, producing new attack sites for cellobiohydrolases; cellobiohydrolases act as exoenzymes, liberating cellobiose as their main product and β-glucosidases, which are not regarded as legitimate cellulases, play an important role in the process because it completes the process through hydrolysis of cellobiose and other short oligosaccharides to glucose (Berlin *et al.*, 2006).

Materials and Methods
Microorganism used
Bacillus licheniformis R2 [KJ 588781], *Bacillus mojavensis* R4 [KJ 588787], *Bacillus atropheaus* SD9 [KJ 590121] and fungal strain *Amylomyces rouxii* WF3 [KJ 588788] isolated in our laboratory were used as inoculum for enzyme production.

Production of polymerizing enzymes
Production of extracellular cellulase and xylanase enzymes
The cultures of *Bacillus mojavensis* R2 and *Bacillus licheniformis* R4 having O.D. 1.0 were inoculated in CMC broth medium (pH-5.5) @ 10.0%. *Bacillus atropheaus* SD9 was inoculated in synthetic medium @ 10%. Inoculated flasks were kept at 35 ± 2 °C at 120 rpm for 48 h. The culture contents of both organisms were centrifuged at 10,000 rpm for 15 min at 4 °C. Supernatants were collected and stored at 4 °C in refrigerator for further use.

Amylomyces rouxii WF3 grown on Sabourds dextrose agar (SDA) and used as inoculum @ 1×10^7 spores/ml. After incubation culture contents were centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant so collected was subjected to various quantititave tests.

Enzyme Assays
Carboxymethyl cellulase activity and filter paperase activity (FPase) activity in the culture supernatant were determined as described by Reese and Mandel 1963 [18].

β-glucosidase was assayed by the procedure of Berghem and Petterson 1973 [4]. Units (IU) of CMCase and FPase were defined as the μmol of glucose liberated per min of culture filtrate. One unit of β-glucosidase was defined as the amount of enzyme liberating μmol of p-nitrophenol per min. The cellulase activity was expressed in terms of complete enzyme activity i.e. CMCase + FPase + β-glucosidase. Xylanase activity in the cultural filtrate was determined according to the method of Miller. One unit of xylanase activity was defined as the μmol of xylose liberating per min.

Preparation of cocktail of enzymes
Cellulase and xylanase of potential microorganisms were used by mixing them as given below with an aim of complete hydrolysis of softwood.

Cocktail of enzymes had been mixed in the ratio of (2:2:1) i.e. 2 ml of cellulase from *Bacillus licheniformis* R2 + *Bacillus mojavensis* R4, (CMCase: 0.45 IU, FPase: 0.56 IU and β-glucosidase: 0.60 IU Cellulase: 1.61 IU)+ 2 ml of cellulase from *Amylomyces rouxii* WF3 (CMCase (0.082 IU), FPase (0.090 IU) and β- glucosidase (0.088 IU Total cellulase: 0.260 IU/ml) and 0.5 ml of xylanase from *Bacillus atropheaus*SD9 (7.09 IU/ml) + 0.5 ml of xylanase from *Amylomyces rouxii* WF3 (3.72 IU) and enzymatic dose was adjusted @ 1ml/g of biomass for hydrolysis. After 72h saccharified biomass was centrifuged at 10,000 rpm for 20 min and supernatant so collected was used for estimation of reducing sugars.

Production of Cellulase and Xylanase Enzymes
Inoculum Preparation
The bit of WF3 was placed in SDA medium and incubated at 28±2 °C for 7 days and 3 days, respectively.

Production of Cellulase and Xylanase Using various substrates of softwood biomass
To 5 g of untreated biomass, 15 ml of moistening agent *viz.* Vogel medium was added (in the ratio of 1:5 i.e. substrate: moistening agent) in 250 ml Erlenmeyer flask and autoclaved. After autoclaving, the flasks were inoculated separately with full plate growth culture scratched with 10ml of WF3 having 1×10^7 spores/ml and incubated at 30±2 °C for 10 days in static phase.

Extraction of Cellulase and Xylanase by Repeated Extraction Method (Bollag and Edelstein, 1991)
To 5 g of untreated biomass, 50 ml of phosphate buffer (0.1M, pH 6.9) with 0.1% Tween-80 was added in 250 ml Erlenmeyer flask. The contents were kept in the shaker for 1 h at 120 rpm and then filtered though muslin cloth. The process was repeated twice with 25 ml of phosphate buffer. After filtration, contents were centrifuged at 5,400 rpm for 10 min at 4 °C. The supernatant was collected for further studies.

Physical Pretreatment
Chipping of biomass were done to get small pieces. The small pieces were then grinded to mesh size of 1.5-2.0 mm. The fine particles were soaked in water for 24 h. Washed biomass was air dried for 24 h followed by drying in oven at 50 °C for overnight. The completely dried biomass was stored in air tight containers for further use.

Chemical Pretreatment (Fig.1)
1% NaOH (Sharma *et al*; 2011) [20].
5g biomass of pine wood were pretreated with 50 ml of 1% NaOH solution at room temperature for 2 h. Slurry was then neutralized to pH 7.0. Slurry was filtered thus separating supernatant and solid biomass. Supernatant was tested for sugar estimation. Biomass was subjected to enzymatic saccharification for 72h by mixture of depolymerizing enzymes from microbial isolates. Biomass was then discarded; supernatant so obtained was analyzed for sugars. In the third stage, supernatant from pretreatment hydrolysate and enzymatic hydrolysate were pooled together and sugars were estimated.

80%NMMO (Poornjead N, 2011) [17]

5g biomass of pine wood were pretreated with 50 ml of 80% NMMO and incubated for 2h at room temperature. Rest procedure is same as described earlier.

1% Acetone (Araque *et al*; 2008) [3]

5g mixed biomass of pine wood and needles in ratio of 3:1 were pretreated with 50 ml of 1% acetone and water in the ratio of 1:1 and incubated for 2h at room temperature. Rest procedure is same as described earlier.

1%H_2SO_4 (Tandon *et al*; 2011) [20]

5g biomass of pine wood were pretreated with 50 ml of 1%H_2SO_4 and incubated for 2h at room temperature. Rest procedure is same as described earlier.

NaOH+H_2O_2 (Tandon *et al*; 2011) [20]

5g mixed biomass of pine wood and needles in ratio of 3:1 were pretreated with 50 ml of 1% NaOH+ H_2O_2 (9:1) and incubated for 2h at room temperature. Rest procedure is same as described earlier.

NaOH+Urea (Modified)

5g biomass of pine wood were pretreated with 50 ml of 1% NaOH+ urea (1:1) and incubated for 2h at room temperature. Rest procedure is same as described earlier.

Scanning electron microscopy (SEM)

Micrographs were taken of untreated, NaOH+ urea pretreated and enzymatically pretreated mixed pine wood biomass samples using scanning electron microscope (Etachi S-3400 N, Japan). All samples were sputter coated with gold and imaged by SEM, as described by

X-Ray Diffraction

The crystallinity of cellulose in the enzymatically saccharified pine wood biomass, treated and untreated wood was analyzed using X-ray powder diffraction pattern of the samples.

Estimation of sugars

Reducing sugars in filtrate were estimated by using 3,5dinitrosalicylic acid method. (Miller, 1959) [13].

Quantification of inhibitors usinh HPLC

All samples were filtered sterilized and diluted appropriately by using 4mM H_2SO_4 as eluent. The quantitative analysis of sugars were performed using HPLC equipped with Ultra C18 (Restek Corp.), 250mm × 4.6 mm, 5μm and UV detector of 280 nm. Samples were run at ambient temperature with 4mM H_2SO_4 eluent at a flow rate of 1.0 ml/min. while for inhibitors Mobile phase A consist of 90: 10 water: methanol, 10mM ammonium formate and mobile phase B 10: 90 water: methanol, 10mM ammonium formate with gradient of 0-5 min at 100% A, to 100% B at 10 min, 10 min hold and injection volume of 10μl.

Bioconversion of sugar hydrolsate into ethanol

Bioethanol production was carried out by using separate hydrolysis and fermentation and simultaneous saccharification and fermentation.

Source of hydrolytic enzymes

Cellulase and xylanase of potential microorganisms were used for hydrolysis by mixing them as described earlier.

Hydrolysis

Enzymatic saccharification of the NaOH+ urea pretreated biomasscombined with treated hydrolysate was subjected to fermentation by using various ethanologens.

Fermenting microbes i.e. *Saccharomyces cerevisiae* (DSM-1334) procured from DSM- Germany, *Pichia stipitis* (NCIM 3498), *Candida shehatae* (NCIM 3500) and *Zymomonas mobilis* (NCIM 5134) were procured from NCIM- Pune, India were used for fermentation.

Fermentation was carried out in a 7.5 L stirred tank bioreactor (New Brunswick Scientific, New Jersey USA) with a 3.0 L working volume at 35.0 ºC and pH 5.5. Foaming was controlled with addition of 2-3 drops of polypropylene glycol. The bioreactor was inoculated with 15% inoculum of *S. cerevisiae* + *P. stipitis* The agitation speed 100 rpm and agitation rate 1.0 vvm respectively used for batch cultivation in bioreactor. The sample was withdrawn regularly at different intervals ranging from 2, 4,6………..58,60 h.

Simultaneous Saccharification and fermentation

Batch cultivation for the production of ethanol was carried out in a 7.5 L stirred tank bioreactor (New Brunswick Scientific, New Jersey USA) with a 3.0 L working volume at 32± 2ºC and pH 5.5. Carbon and nitrogen sources; for batch fermentations were *P. roxburghii* (biomass), yeast extract – 0.5% and 0.5% peptone. Foaming was controlled with addition of 2-3 drops of polypropylene glycol. The medium was sterilized in situ for 20 min at 121ºC. The bioreactor was inoculated with 15% inoculum of *S. cerevisiae* + *P. stipitis* and 50 ml of prepared enzyme solution under aseptic conditions. The fermentation was carried out under anaerobic conditions. The agitation speed 100 rpm and agitation rate 1.0 vvm respectively used for batch cultivation in bioreactor. The sample was withdrawn regularly at different intervals ranging from 2, 4,6………..58,60 h and ethanol estimation was done.

Ethanol Fermentation

To the pooled supernatant 0.5 % yeast extract and 0.5% peptone was added and autoclaved at 121 ⁰C, 15 lbs for 20 min. To the fermentation media 10 % (1O.D) inoculum of *Saccharomyces cerevisiae, Pichia stipitis*, *Candida shehatae, S. cerevisiae* + *P. stipitis*, *S. cerevisiae* + *C. shehatae*, was added and kept for 72 h (3 days) at 25 ºC.

Ethanol so produced was estimated by standard methods of Caputi *et al.,* 1969.

Bioethanol was estimated in terms of g/l of fermented liquor and g/g of biomass on dry weight basis. Fermentation efficiency was calculated using the following formula:

$$\text{Fermentation efficiency} = \frac{\text{Ethanol produced (g/g)}}{\text{Theoretical yield of ethanol}} \times 100$$

Theoretical yield was referred as standard value of 0.511 g/g of sugars.

Results and Discussion

Lignocellulosic biomass contains three major components i.e. cellulose, hemicellose and lignin which made the plant body rigid and hard. To convert plant biomass into valuable products cellulose and hemicellulose fractions must be easily accessible for enzymatic hydrolysis (Banerji *et al*; 2011).

Degradation of cellulosic biomass can be influenced by its constituents and their structure (Kumar *et al.*, 2009) [11]. During an alkaline pretreatment, increase in availability of

cellulose and hemicelluloses is partly due to delignification process. Alkaline conditions break ester linkage between lignin and hemicelluloses through process of saponification. Alkaline treatment increases the pore size of biomass (Cheng *et al.*, 2010). Therefore, the synergistic action of cellulolytic hydrolysis by various enzymes produced by microorganisms is essential for the achievement of high saccharification yield (Suwannarangsee *et al.*, 2012).

Mixture of enzymes of *Bacillus licheniformis* R2 and *Bacillus mojavensis* R4 (1.45 IU/ml) were used as cellulase producer while *Bacillus atropheaus* SD9 (6.24 IU/ml) showed high titres of xylanase. A fungal culture *Amylomyces roxii* WF3 showed maximum titres (0.167 IU/ml).

Degradation of cellulose to glucose involves the cooperative action of different subunits i.e. endocellulases (EC 3.2.1.4), exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91; glucanohydrolases, EC 3.2.1.74), and βglucosidases (EC 3.2.1.21). Endocellulases hydrolyze internal glycosidic linkages in a random fashion while exocellulases hydrolyze cellulose chains by removing mainly cellobiose either from the reducing or the non-reducing ends. Endocellulases and exocellulases act synergistically on cellulose to produce cello oligosaccharides and cellobiose, which are then cleaved by beta-glucosidase to glucose (Mohanram S *et al.*, 2013) [14]. Hydrolysis of hemicelluloses involve enzymes like glycoside hydrolases, carbohydrate esterases, polysaccharide lyases, endohemicellulases their concerted action hydrolyze glycosidic bonds, ester bonds and remove the chain's substituents or side chains (Sweeny *et al.*, 2012) [21]. Keeping in view, the synergistic action of three subunits of cellulose for completer hydrolysis of cellulase a mixture of enzymes from different potential enzyme producer was used in order to turn it highly robust for degradation of biomass. Therefore mixture of these depolymerizing enzymes when used yielded maximum saccharification.

The titers of cellulase of *B. licheniformis* R2 + *Bacillus mojavensis* R4 i.e. endoglucanase activity 0.45 IU, exoglucanase: 0.56 IU and β- glucosidase: 0.60 IU. Total cellulase activity: 1.60 IU/ml while xylanase activity shown by *B. atrophaeus* SD9 showed activity of 7.09IU/ml

Similarly in case of *Amylomyces rouxii* WF3 cellulase yielded as: endoglucanase activity of (0.082 IU), exoglucanase activity (0.090 IU) and β- glucosidase (0.088 IU) while xylanase yielded activity of 3.72 IU/ml.

Cost effective production of cellulase and xylanase by replacing conventional carbon source

As the major impediments to exploit the commercial potential of cellulase and xylanase are the cost of cellulase and xylanase production and yield stability. One effective approach to reduce the cost of enzyme production is to replace conventional carbon source i.e. pure cellulose and xylan by relatively cheaper substrates such as lignocellulosic forest waste.

Extracellular cellulase and xylanase production from a mixed inoculum of hypercellulolytic and hyperxylanolytic *Bacillus licheniformis* R2, *Bacillus mojavensis* R4 and *Bacillus atrophaeus* SD9 were evaluated under submerged fermentation (SmF) and solid state fermentation (SSF) by *Amylomyces rouxii WF3* utilizing untreated softwood biomass as carbon source.

Softwood biomass used in the present study includes sawdust of *Pinus roxburghii,* depending upon their local abundance, easy availability and rich cellulosic and hemicellulosic components.

Compositional analysis of pine wood

Analysis of untreated pine wood biomass yields 57% of holocellulose (cellulose+ hemicelluloses), lignin 23%, extractives (alcohol, benzene, fibres, resins, waxes) 20%. For the efficient degradation, lignin and extractives needs to knock out. After alkali pretreatment, holocellulose was found to be 87.10%, lignin 6.90% and extractives 6.00%. (Sharma *et al*; 2011) [20]. Alkali pretreatment was reported to decrease crystallinity of cellulose, remove lignin shield around cellulose and increases pore size of biomass hence increasing digestibility (Jan *et al.*, 2008).

Pretreatments

The primary obstacle using biomass lignocellulosic for ethanol conversion is the physical protection that the lignin exerts on the cellulose; for this reason, the stage of pretreatment is the most important stage as it secures a good yield of fermentable sugars. Therefore, an effective pretreatment stage aims to obtain a proper delignification of the biomass and defragmentation of the matrix of polymers in order to reduce the cellulose at grade crystalline and facilitate the enzymatic attack of the cellulases (Ruggeri and Sassi, 2003).

At three different stages, (Stage I, Stage II and stage III) saccaharification is tested. In stage I, supernatant so formed after pretreatment was subjected to saccharification. In Stage II solid biomass after enzymatic hy,drolysis with cock tail of cellulolytic and xylanolytic bacterial and fungal isolates was subjected to saccharfication. In Stage III supernatant from Ist and IInd stage were pooled together and tested for saccharification. Results were depicted in Table 1.

In stage 1, maximum sugars i.e. 11.50 mg/g were observed in NaOH+ urea pretreatment followed by H_2SO_4 pretreatment (10.24 mg/g) while minimum (3.60 mg/g) was noticed in 80% NMMO. Most of hemicelluloses (35-40%) were solubilised in the supernatant after alkali pretreatment. And residue so remains dissolves cellulose.

In second stage where solid biomass is allowed for saccharification with the addition of enzymes produced by hypercellulolytic and xylanolytic microbial isolates, highest sugars (10.50 mg/g) were observed in 80% NMMO followed by NaOH+H_2O_2 pretreatment while lowest in case of H_2SO_4 (5.50 mg/g) pretreated biomass. This might be due to the reason that as NMMO is known as cellulose solvent so the solid residue of biomass contains most of the cellulose which is the major component of any lignocellulosic biomass (Poornejad *et al*; 2011) [17].

For improved saccharification, where cellulose as well as the hemicelluloses are solubilised to greatest extent, supernantant from both the stages were pooled together. Highest sugars (18.00 mg/g) were found in NaOH+ urea pretreated biomass followed by NaOH+H_2O_2 treated biomass (15.86 mg/g). Minimum saccharification was shown by acetone treated biomass (11.31 mg). When the three stages were statistically analyzed, it was found that sugars formed in third stage was significantly different from other two stages.

Acid pretreatment at low concentration leads to limited hemicelluloses removal so higher concentration of acid is probably needed to achieve enhanced sacchraification but use of acid at higher concentration is highly corrosive so is not preferred (Zhu *et al.*, 2009). While alkaline treatment focuses on lignin removal while minimizing the sugar degradation. It is believed that the saponification of intermolecular ester bonds that cross-link xylan and lignin is the main pathway for the solubilization of lignin (Tomás-Pejó *et al*. 2011).

Taherzadeh and Karimi (2008) [22] showed that pretreatment of alkali when combined with urea or peroxide causes selective removal of lignin and xylan and decreases the crystallinity of cellulose. Apart from this, mimimum amount of furfural and hydroxymethyl furfural which are degradation products of sugars were detected after this pretreatment.

Scanning electron Micropscopy

A qualitative assessment of the substrates was conducted using scanning electron microscopy. Fig.3 a shows clear cell wall structures in untreated biomass. Vascular bundles and parenchyma were observed and closer magnification reveals a complex ordered structure that had been opened by the pretreatment process.

Pretreatments disrupted structure leaving the substrate with uneven, rough surfaces (Rahnama, A. 2013). Pretreatment resulted in a delignified structure with a different supramolecular structure than untreated material. The fibril structure is completely disrupted (Gong *et al*., 2010).

X-ray diffraction (XRD) of untreated pretreated and enzymatically saccharified pine wood biomass

The XRD patterns of untreated, alkali pretreated and after enzymatically saccharified are shown in Fig. 3b. Upon NaOH + urea pretreatment, the recovered biomass gave XRD patterns displaying significantly less ordered cellulose structures, as compared to the untreated samples. Cellulose chains within the hydrogen-bonded sheets in the pretreated samples are essentially random, with some short-range order present in the direction perpendicular to the sheets that are attributed to the cellulose II polymorph or distorted cellulose (Shafaei *et al*., 2014) [19]. Measurement of crystallinity by X-ray diffraction indicates reduction in the crystallinity of wood after pretreatment. The crystallinity reduces to a higher extent after enzymatic saccharification with depolymerizing enzymes from bacterial and fungal cultures.

Enzymatic saccharification

Sugars yields are often used as an indicator to measure the performance of enzymatic saccharification when lignocellulosic material is used (Guo *et al*., 2009). So the yields of sugars analyzed through HPLC were depicted in Fig. 2. Maximum sugars (14.80 mg/ml) were found in sample C followed by sample A (13.60 mg/ml). It is clear from the results detoxified samples (12.75 mg/ml) leads to lesser sugars as compared to non detoxified samples. The pooling of supernatant with enzyme hydrolysate and detoxification with Ca (OH)$_2$ at high pH of 10.5 was done because at high pH degradation of sugars can be minimized (Nigam *et al*., 2001).

The results revealed that maximum sugars were formed without detoxification so we recommend that when pine biomass was pretreated with NaOH+ urea no need of detoxification. Similar results were found when corn stover was alkali pretreated did not generate furfural

To our knowledge, this is the first report on NaOH+ urea pretreatment on pine biomass. A critical problem is the fermentation of acid treated hydrolysate is the inability of fermentative microorganism to withstand inhibitory compounds formed during pretreatment and usually a detoxification step is needed to improve hydrolysate fermentability (Saha *et al*., 2005).

Evaluation of fermentation inhibitors during pretreatment

The hydrolysis of biomass does not only have positive effects on the fermentation process but an inevitable part is the formation of inhibitory compounds. Hydroxymethyl furfural and acetic acid are the species that inhibit the normal fermentation processes. Evaluation of HMF and acetic acid were depicted in Fig.2 When glucose and xylose are placed under harsh conditions viz. high pressure and elevated temperatures etc during pretreatment can be degraded into furfural and hydroxymethylfurfural (5-HMF) compounds that can further be degraded into other toxic compounds. These compounds negatively affect the cells specific growth rate and cell-mass yield of microorganisms.

Maximum HMF and acetic acid (112.83, 131.57 ppm) was found in sample A then detoxification of sample A leads to decrease of inhibitors (6.11, 86.55 ppm) and in sample D (Sample B+ Sample C) 20.73, 127.20 ppm of inhibitors were observed. Oliva *et al.* (2006), who pointed out that the presence of furfural and HMF at the experimental concentration affected the respiration and oxidative phosphorylation and slowed down the electron transport system.

Larson *et al* (1992) reported that inhibitors present in pretreatment liquid can increase the ethanol concentration if not present at higher concentrations. At low concentrations, upto 100mmol/l of acetic acid concentration is permissible for ethanol yield. Inhibitors are metabolized by yeasts and leads to lag phase in ethanol production.

Therefore HMF and furfural if produced within threshold level is favourable to fermentation of cellulose and hemicelluloses sugars (Zhu *et al*., 2009). The pooled hydrolysate of pretreatment and enzymatic saccharification was directly subjected to fermentation by different ethanologens in monoculture as well as co-culture combinations. The inhibitor problem is not evident in case of alkaline pretreatment of rice hulls. Bjerre *et al*. reported that wet oxidation combined with base addition readily oxidizes lignin from wheat straw facilitates the polysaccharides for enzymatic hydrolysis

Ethanol production in shake flask experiment

Two different process configurations, simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) were compared regarding ethanol production in shake flask. Different ethanologens were used in the present study in monoculture as well as co-culture combinations for fermenting sugars produced from enzymatic saccharification of mixed pine wood biomass. Co-culture of *S. cerevisiae* + *P. stipitis* produced highest ethanol 11.08 g/l i.e. (41.11%) in terms of fermentation efficiency as compared to other co-culture and monoculture in case of separate hydrolysis and fermentation when ethanol was estimation in simultaneous seaccharification and fermentation maximum ethanol was achieved in co- culture of *S. cerevisiae* + *P. stipitis* (12.51 g/l) with fermentation efficiency of 45.00 % (Table 2).

Since *S. cerevisiae* is widely known for hexose utilization whereas *P. stipitis* can ferment pentose sugars thus this co-culture combination would have utilized most of fermentable sugars formed in the wood hydrolysate to yield highest ethanol concentration as compared to these cultures individually.Ethanol production by co-culture was studied by Verma *et al.* (2000) using *Saccharomyces diastaticus* and *S. cerevisiae* 21 in raw unhydrolysed starch which yielded ethanol of 48 per cent higher (24.8 g/l) than that obtained with monoculture of *S. diastaticus* (16.8 g/l). More than 40 yeast strains were screened by Sreenath *et al.* (2001). Among them

of *Pichia stipitis* and *Candida shehatae* to determine their fermentation rates on mixed sugars. All the tested strains fermented both glucose and xylose and attained ethanol concentration of 34.8±2.42 g/l with *Pichia stipitis* and 34.0±1.67 g/l with *Candida shehatae*.

Bioreactor studies
Separate hydrolysis and fermentation
Fermentation was carried out in a bioreactor using both modes i.e. separate hydrolysis and fermentation and simultaneous hydrolysis and fermentation. Fig.4 showed that initial content of reducing sugars were 16.60 mg/ml and after 8 h of fermentation, sugars reduced to 11.28 mg/ml and 2.05 mg/ml of ethanol had been produced which increased to 11.32 mg/ml after 32 h and sugars reduced to 7.50 mg/ml. Maximum ethanol yield was achieved at 54h of fermentation time 16.37 mg/ml of ethanol which corresponds to 60.86 % of fermentation efficiency where only 2.30 mg/ml of sugars were observed. And growth of yeast cell were found to be 0.17 mg/ml which goes on increasing to 2.07mg/ml after 32h of fermentation and after 54h it reached to 2.92mg/ml which was then become constant even after 56 h of fermentation time.

In a study conducted by Wan *et al*. (2012) an effective co-culture of *S. cerevisiae* Y5 + *P. stipitis* CBS6054 fermented lignocellulosic hydrolysate initially. The glucose was used up and furfurals and 5-HMF were completely metabolized within 12 h, xylose was used up in 96 h with ethanol concentration of 27.4 g/l ethanol. These co-culture studies match with our findings i.e. *S. cerevisiae* and *P. stipitis* had shown the maximum ethanol yield.

Enzymes used in enzymatic hydrolysis are not only inhibited by degradation products present in NaOH+ urea prêtreated hydolysate, but also avoid feed back inhibition. Therefore endo and exoglucanase were inhibited by cellobiose and βglucosidase was inhibited by glucose. It was thus concluded that inhibitory effect of pretreatment hydolysate on enzymatic hydrolysis could be attributed to sugar degradation products and other by- products produced during alkali pretreatment (Larrson *et al*., 2011) [12].

Simultaneous saccharification and fermentation
Simultaneous saccharification and fermentation process entails inoculating enzyme producing microorganisms and ethanol producing microorganisms at the same time so that the produced sugars are simultaneously converted into ethanol. (Sharma *et al*., 2012) [20].

When SSF was performed in a bioreactor, growth of yeast cells were initially 0.05mg/ml which increased to 0.58mg/ml after 24 h of fermentation time and reached upto 2.18 mg/ml in 52 h of fermentation time and found to be 2.50mg/ml in 56h of fermentation time and thereafter becomes constant to 2.10mg/ml even at 60 h of fermentation time. Sugars were estimated as 10.35mg/ml and the ethanol productivity after 24 h were 2.45 mg/ml. After 30h, sugars were reduced to 6.10 mg/ml and 4.83 mg/ml of ethanol had been produced which corresponds to fermentation efficiency of 17.95% based on theoretical ethanol production from glucose. Highest ethanol productivity was achieved at 52h of fermentation time corresponding to 18.84 mg/ml with 70.00 % of fermentation efficiency (Fig.5)

SSF process has been found to be economically viable for the conversion of substrates to fermentation products (Cao *et al*., 2009). Advantage of SSF is that a multistage process for production of ethanol is carried out in a vessel. Besides being relatively cheaper this process avoids catabolic repression of the enzymes produced during saccharification step due to higher glucose concentration (Sharma *et al*., 2012) [23]. The higher overall ethanol yield in SSF as compared to SHF is presumably due to the fact that the inhibitors present in the pretreatment liquid can increase the ethanol production if not present at too high concentration. At low concentration i.e. upto 100mmol/l acetic acid has been shown to increase ethanol yield (Larrson *et al*., 2011) [12]. Removing the inhibitors thus decreases ethanol yield. Inhibitors are metabolized by yeasts, leading to the lag phase in ethanol production.

SSF was shown to be the preferable process configuration for ethanol production. which is in accordance with results found by Soderstorm *et al.,* 2005 and Ohgren *et al.,* 2007 for softwood. This is due to lower sugars concentration and thus decreased glucose inhibition of enzyme, less inhibition of enzymes by degradation products present in pretreatment hydrolysate since fermentation seems to afford some detoxification, positive effect of fermentation inhibitors on ethanol yield. However detoxification step may constitiute up to 22% of total ethanol production cost (Von *et al*., 1994) and is therefore best avoided.

Krishna *et al*. (2012) carried out ethanol production by simultaneous saccharification and fermentation (SSF) of pretreated sugarcane leaves (with alkaline H_2O_2), using cellulytic enzyme complex from *Trichoderma reesei* (QM9414) and *Saccharomyces cerevisiae* (NRRL-Y-132). They observed 92 per cent conversion of 2.5 per cent substrate to sugar and increase in ethanol yield with addition of extra β-glucosidase.

Verma *et al*. (2011) [25] reported that the optimum temperature for maximum ethanol production using starch in co-cultures of amylolytic yeasts and *S. cerevisiae* 21 was 30⁰C, and there was no remarkable loss in ethanol yield up to 40⁰C. Yeasts, under anaerobic conditions, metabolize glucose to ethanol primarily by the Embden-Meyerhof pathway. Generally, studies comparing SSF and SHF have illustrated that determination of the better method is dependent on a number of factors. Several studies have confirmed that SHF produces higher overall yields, while SSF requires less time (Alfani *et al*. 2000; Cantarella *et al*. 2004) [2, 6]. An ideal operating process would be flexible to allow for either method to be used in accordance to feedstock availability and the desired products.

Conclusion
The overall purpose of this study is to explore a new pretreatment method to improve enzymatic hydrolysis of pine wood biomass and its conversion to valuable products i.e. bioethanol by adopting various modes of fermentation.. NaOH combined with urea at room temperature was found to be the best to dissolve cellulose/knock out lignin. The structure of *Pinus roxburghii* biomass considered as the most recalcitrant material for enzymatic hydrolysis mainly because of its highly lignified structure Further its bioconversion into ethanol using co- culture of *S. cerevisiae* + *P. stipitis* into ethanol with yield of 45.00 % with simultaneous saccharification and fermenation which is a remarkable achievement. Further it is also recommended that SSF was found to be superior than SHF as it results in low capital cost, concentration of ethanol is higher in SSF than SHF However mixing the lignin residue with yeasts makes yeast recirculation difficult. Additionally, temperature optima used for yeasts and enzyme used differ, which means that

conditions used in SSF cannot be optimal for both enzyme and yeasts. Due to reduction of glucose inhibition in enzymatic hydrolysis during SSF, the detoxifying effect of fermentation and positive effect of inhibitors present in hydolysate leads to opt SSF process configuration.
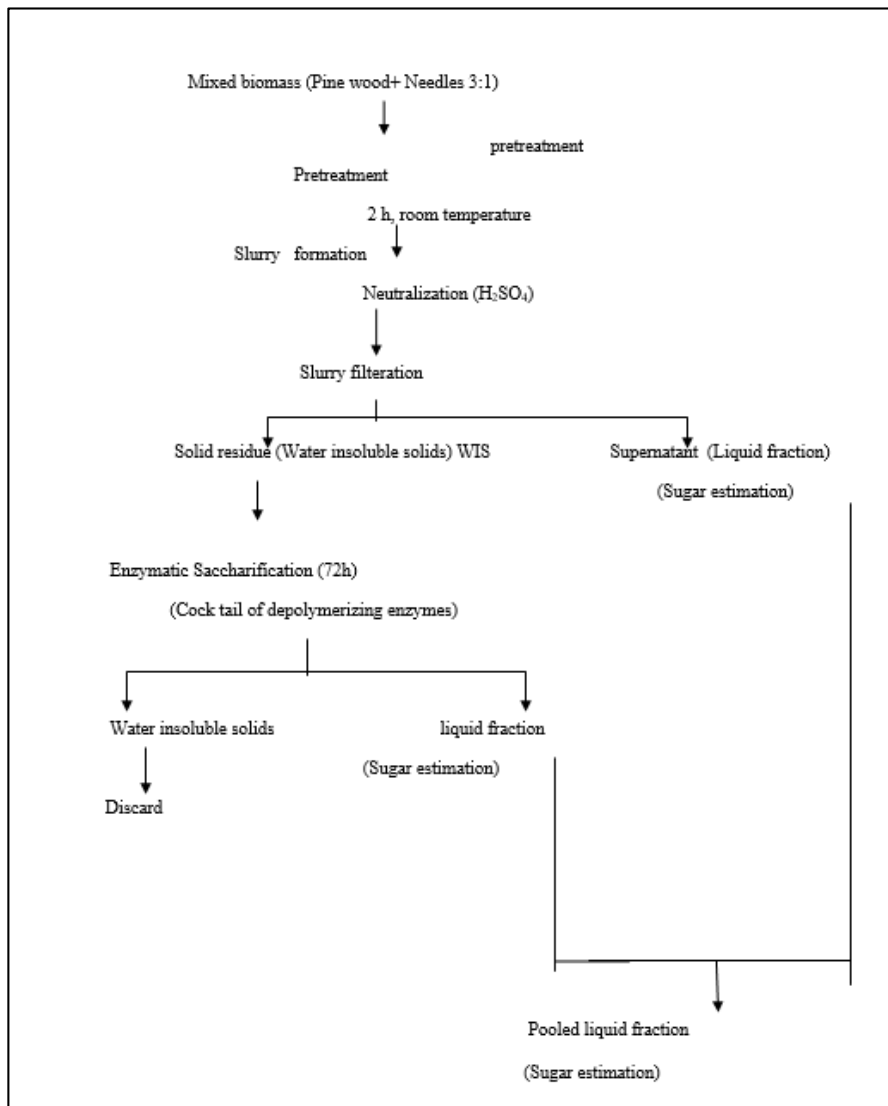

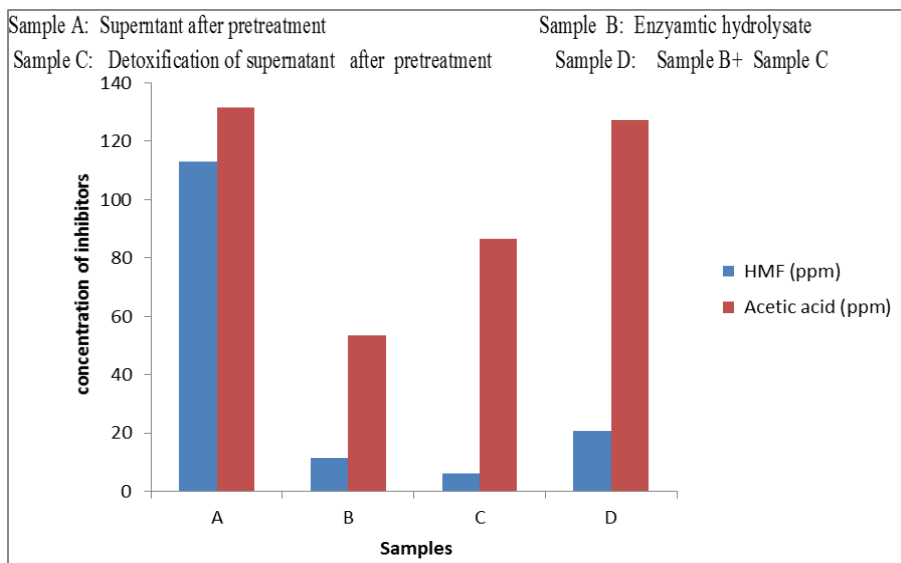**Fig 1:** Schematic flowsheet of pretreatment to pine wood biomass for maximum saccharification

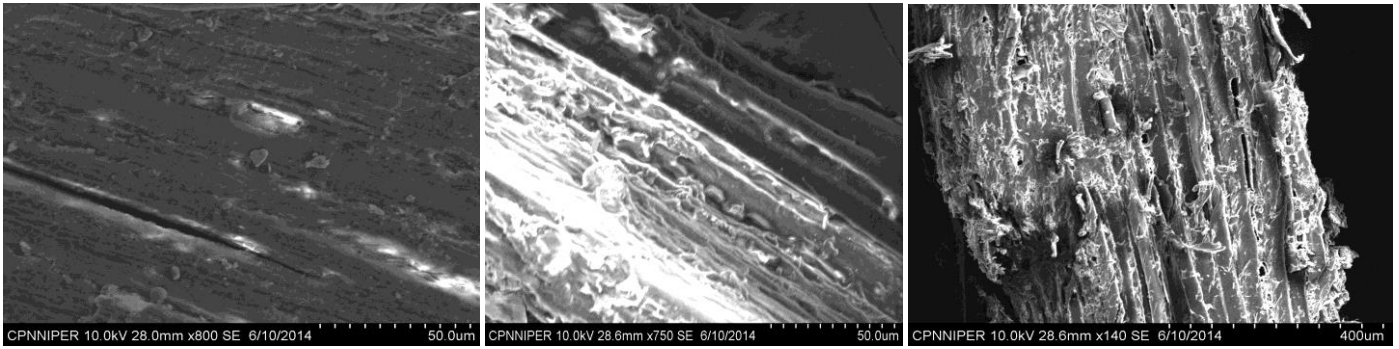**Fig 2:** Quantification of inhibitors using HPLC

Fig 3a) SEM micrographs of **a).** Untreated pine wood **b).** Pretreated pine wood **c).** Enzymatically saccharified pine wood
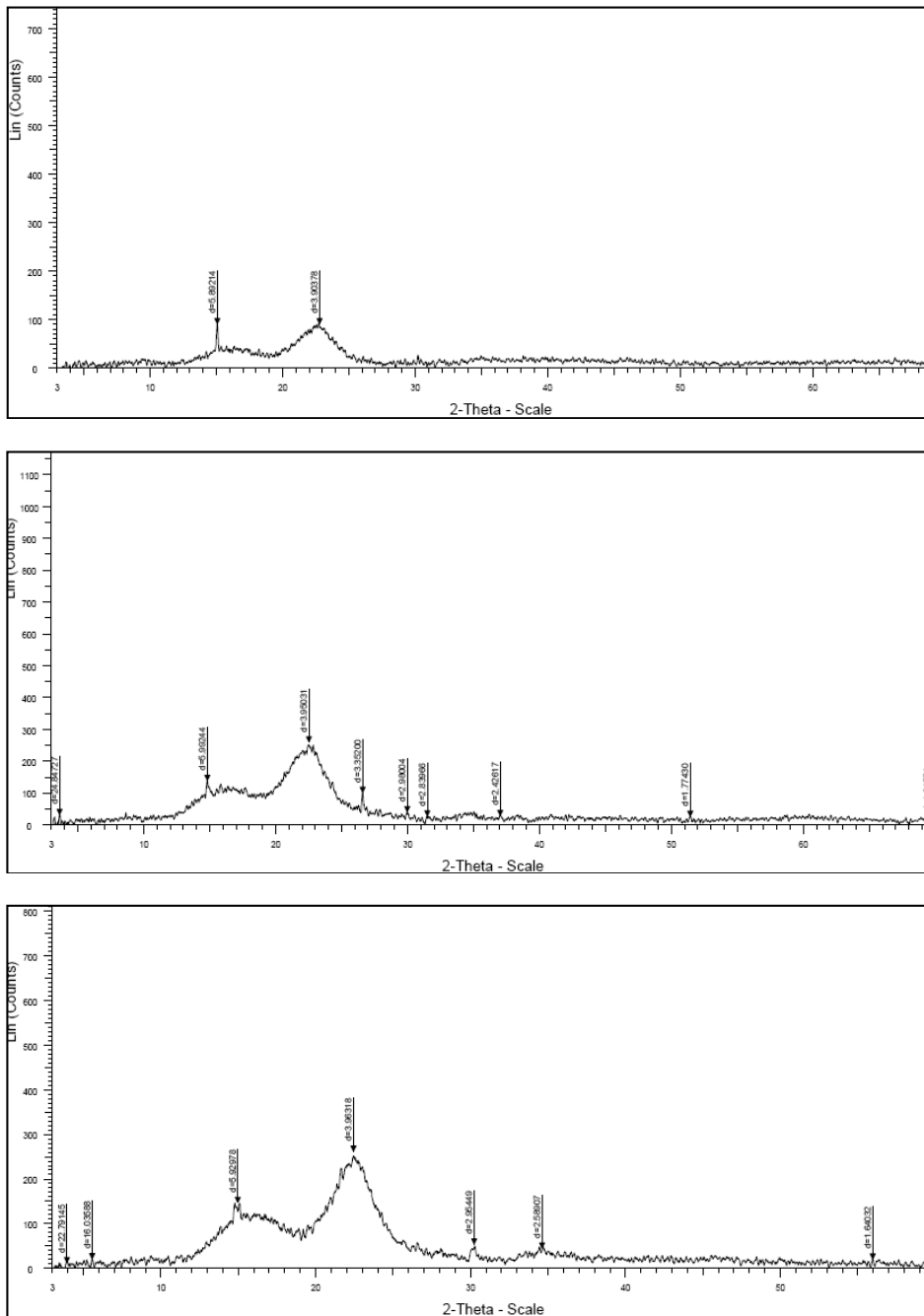

Fig 3b) X-ray diffraction pattern of **a).** Untreated pine wood **b).** Pretreated pine wood **c).** Enzymatically saccharified pine wood
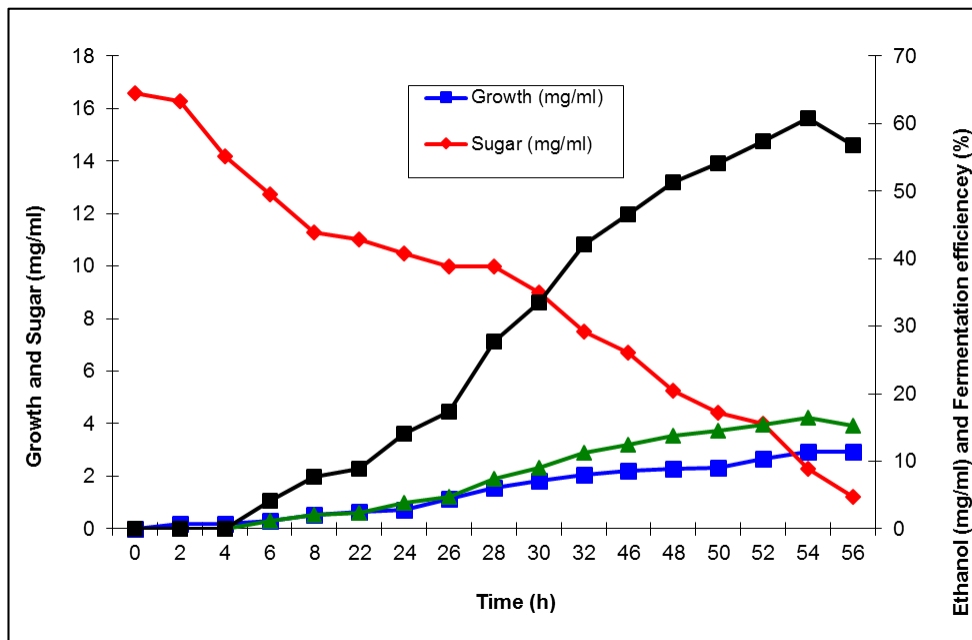
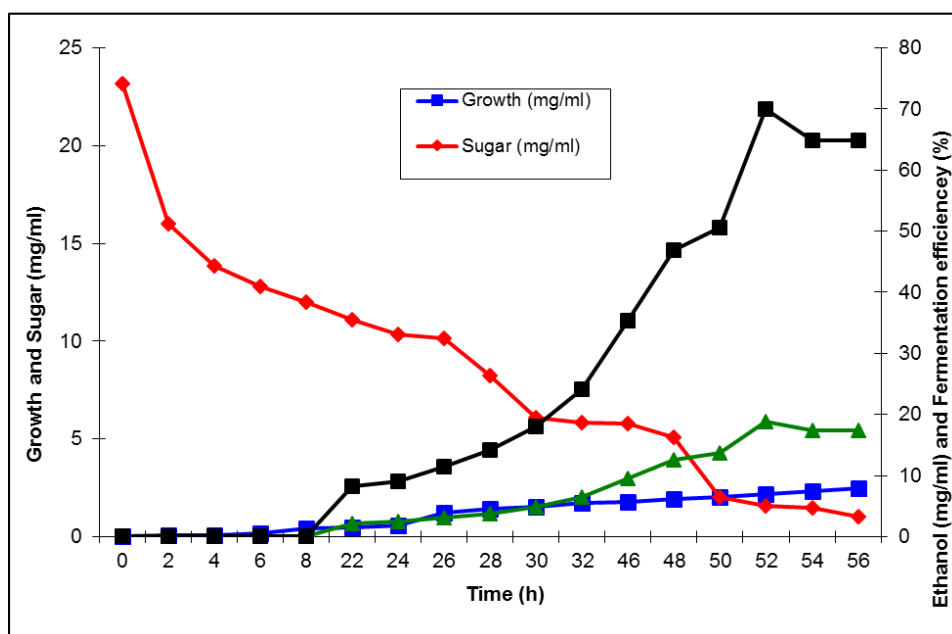**Fig 4:** Time course of fermentation during separate hydrolysis and fermentation

Fig 5: Time course of fermentation during simultaneous saccaharification and fermenation

Conflict of interest
Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human and/or animal subjects (if exists) respect the specific regulations and standards.

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