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RESEARCH ARTICLE

Pretreatment and production of bioethanol from different Lignocellulosic biomass

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Abstract

Overcoming the recalcitrance (resistance of plant cell walls to deconstruction) of lignocellulosic biomass is a key step in the production of fuels and chemicals. The recalcitrance is due to the highly crystalline structure of cellulose which is embedded in a matrix of polymers-lignin and hemicellulose. The main goal of pretreatment is to overcome this recalcitrance, to separate the cellulose from the matrix polymers, and to make it more accessible for enzymatic hydrolysis. Thus, advances in research have enabled the development and integration of chemical-based pretreatment into proprietary ethanol production technologies in several pilot and demonstration plants globally, with potential to scale-up to commercial levels. In this present studies report on emerging chemical pretreatment methods, highlighting recent findings and process innovations developed to offset inherent challenges via a range of interventions, notably, the combination of chemical and biological pretreatment to achieve high sugar yields at mild reaction conditions, reduce solvent loads and enzyme dose, reduce waste generation, and improve recovery of biomass components in pure forms. Various pretreatment processes for lignocellulosic biomass (Rice straw and Wheat straw) were evaluated including different concentrations (1% - 5%) of sodium hydroxide, sodium chlorite and Hydrogen peroxide. Pretreated biomass was enzymatically saccharified with fungi (*Trichoderma reesei*) and fermented using fungi (*Saccharomyces cerevisiae*) and bacteria (*Zymomonas mobilis*) respectively . The results revealed that all pretreatment processes that were subjected to enzymatic saccharification and fermentation produced ethanol. However, the maximum ethanol obtained from rice straw with *Zymomonas mobilis* (10.02 ± 1.18 g/l).

1 INTRODUCTION

Pretreatment of the biomass is followed by enzymatic hydrolysis to produce simple sugars, fermentation of sugars to produce biofuels, and then product separation (Patel et al., 2005). The pretreatment step is key for subsequent enzymatic hydrolysis and fermentation steps in order to maximize the volumetric productivity of the desired product. Cellulose and hemicellulose can be broken down into simple sugars either enzymatically or by acid hydrolysis. The hydrolysis product, six carbon sugars (hexoses), can easily be fermented to ethanol, while only a few microorganism strains can ferment the five carbon sugars (Wyman et al., 2005). There has been considerable research done in genetically modifying organisms to produce strains that are capable of fermenting both glucose and xylose to useful chemicals (Patel et al., 2006). Numerous pretreatment strategies have been developed to enhance the reactivity of

cellulose and to increase the yield of fermentable sugars. Typical goals of pretreatment include (1) production of highly digestible solids that enhances sugar yields during enzyme hydrolysis, (2) avoiding the degradation of sugars (mainly pentoses) including those derived from hemicellulose, (3) minimizing the formation of inhibitors for subsequent fermentation steps, (4) recovery of lignin for conversion into valuable coproducts, and (5) to be cost effective by operating in reactors of moderate size and by minimizing heat and power requirements.

The use of an alkali causes the degradation of ester and glycosidic side chains resulting in structural alteration of lignin, cellulose swelling, partial decrystallization of cellulose (Ibrahim et al., 2011), and partial solvation of hemicellulose (Sills et al., 2011). Sodium hydroxide has been extensively studied for many years, and it has been shown to disrupt the lignin structure of the biomass, increasing the accessibility of enzymes to cellulose and hemicellulose (Zhu et al., 2010). The alkaline process involves soaking the biomass in alkaline solutions and mixing it at a target temperature for a certain amount of time. A neutralizing step to remove lignin and inhibitors (salts, phenolic acids, furfural, and aldehydes) is required before enzymatic hydrolysis. A recent approach to lime pretreatment eliminates the solid-liquid separation step after neutralization by neutralizing the lime with carbon dioxide before hydrolysis resulting in 89% glucose recovery from leafstar rice straw (Park et al., 2010). Park and co-workers also used this method to test SSF which resulted in an ethanol yield that was 74% of the theoretical value using a mixture of Saccharomyces cerevisae after 79 hours of fermentation at 30°C.

The chlorite oxidation and wet oxidation are also used as promising oxidative delignifying pretreatments. (Bjerre et al., 1996) used wet oxidation and alkaline hydrolysis of wheat straw achieved 85% conversion yield of cellulose to glucose. Whereas, the sodium chlorite treatment yielded approximately 90 % delignification in woody material (Prosopis juliflora; Lantana camara) (Kuhad et al., 2010b). Lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of H_2O_2. The pretreatment of cane bagasse with hydrogen peroxide greatly enhanced its susceptibility to enzymatic hy- drolysis. About 50% of the lignin and most of the hemicellulose were solubilized by 2% H_2O_2 at 30^O C within 8 h, and 95% efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45 ^{O}C for 24 h. Currently most commercial cellulases are produced from *Trichoderma reesei* (Morris et al., 1993) usually used to describe a mixture of cellulolytic enzymes action is required for effective breakdown of substrate to its monomeric units. The action of cellulases involves the concerted action of (i) endoglucanases (endo-1, 4- β -glucanases, EGs) can hydrolyze internal bonds preferably in cellulose amorphous regions releasing new terminal ends, which randomly attacks the internal, β1,4-linkages (ii) cellobiohydrolase, (exo-1, 4 β --glucanases, CBHs) act on the existing or endoglucanase generated chain ends (iii) β 3-glucosidase, which hydrolyzes cellobiose to glucose.

Zymomonas mobilis is a Gram-negative, facultative anaerobic bacterium that ferments glucose, fructose, and sucrose as carbon sources (Viikari et al., 1988). These carbohydrates are metabolized via the same biochemical route, the Entner-Doudoroff pathway. *Zymomonas mobilis* are rods 2-6 µm in length and 1-1.5 µm in width, flagellated but lack spores or capsules and growing in a pH range of 3.4-7.5 (Paula. De et al., 2008). The bioconversion process is mainly consists of 3 major steps i.e, pretreatment, hydrolysis, and fermentation. The purpose of the pretreatment is to remove lignin and hemicellulose fraction, reduce cellulose crystallinity and increase the porosity of the materials. The pretreated material is subjected to the enzymatic hydrolysis and the resultant hydrolysates are fermented to ethanol using fermenting microbes (Kuhad et al., 2010b).

2 OBJECTIVES

i. To find out alternative sources of bioenergy as the prime source of biofuels
ii. To use Potential availability of some agricultural residues like rice straw and wheat straw as raw materials.
iii. To achieve high yield of sugar by different pretreatment methods like alkali, chlorite and hydrogen peroxide treatments.
iv. To achieve high yields of fermentable sugar from lignocellulosic components using microorganisms like *Trichoderma reesei*
v. To achieve high yields of ethanol at economic level using fungi S. cerevisiae and bacteria *Zymomonas mobilis*.

3 MATERIAL AND METHODS

Raw materials

Rice straw and wheat straw were obtained from local mill powdered and sieved into a 1 mm seiver. Powder of the raw material was used as carbon source.

Microorganisms

Trichoderma reesei (MTCC-4876) were obtained from, MTCC Chandigarh. The fungi produces cellulolytic

enzymes that converted carbohydrate polymers into fermentable sugars Later *Zymomonas mobilis* and *Saccharomyces cerevisiae*s were inoculated to utilize reducing sugars to ethanol.

Inoculum-preparation

Fungal cultures were inoculated onto PDA medium in the Petri plate. Spores scraped out from 7 days old slants were dispersed in desired quantity of sterile distilled water containing 0.1% Tween 80 and vortexed. Spore count was measured with haemocytometer and adjusted to $2x10^6$ spores/ml by adjustment of optical density.

Culture-medium

1000 ml of Mandels medium was prepared by adding (in gms) Urea 0.3, $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2.0, $CaCl_2.2H_2O$ 0.4, $MgSO_4.7H_2O$ 0.15, bactopeptone 1.0, and yeast extract 0.25. Trace elements were also added (in mg), $FeSO_4.7H_2O$ 0.15, $MnSO_4.H_2O$ 1.6, $ZnSO_4.7H_2O$ 1.4, $CoCl_2$ 2.0. pH was adjusted to 5.5-6.0 before sterilization (Bollok et al., 2000).

Culture-conditions

5g / 100 ml (Mandel's medium) of each substrate was taken in conical flask (250 ml).The conical flasks were plugged with cotton and sterilized at 15lbs per sq.inch for 30 minutes. The flasks were inoculated with the fungal strains in their different concentrations (ml). These flasks were incubated at room temperature for 6 days on an orbital shaker. After six days mycelium was separated by filtration through Whatman filter paper. The filtrate was used for further studies (Thimmaiah S K, 1991).

Pretreatment-Strategies

Chemical-pretreatments

The 5 grams powdered rice straw and wheat straw were taken for further process. The dried substrate was chemically treated with sodium hydroxide solution concentration (1-5%) sodium chlorite (1-5%) and H_2O_2 (1-5%). Thereafter washed several times with deionised water and then dried with hot air oven for 24 hrs at 70˚C. Later on dry fibrous cake was weighed which further processed for microbial saccharification

Biological-pretreatments

The biological pretreatment was carried out with fungal treatment, *Trichoderma reesei*.

Fermentation of Hydrolysates to Ethanol

After saccharification, *Zymomonas mobilis* and *Saccharomyces cerevisiae* was inoculated for utilization of reducing sugars to ethanol. The microorganisms strain was grown in SDDL broth (glucose 20.0, yeast extract, 5.0 g L^{-1}) at 30ºC for 48 hrs. The number of viable cells 10^9 (cfu/ml) was determined by the agar plate method using Schreder agar ($MgSO_4.7H_2O$ 0.5, $(NH_4)_2SO_4$ 1.0, KH_2PO_4 1.0, yeast extract 1.0, sucrose 20, agar 15 g L^{-1}) incubated for 24 hrs at 30ºC.

Analytical Methods

The carbohydrate content of pretreated raw materials in the culture broth was measured by Anthrone method (Sadasivam et al., 2006). The amount of reducing sugars in pretreated raw materials in the culture broth were determined by dinitrosalicylic acid (DNS) method (Miller G. L, 1959) with glucose as standard. The amount of Non-reducing sugar present in the sample was calculated by subtracting Reducing sugars from Total soluble sugars. Soluble protein was analyzed by the method of (Lowry et al., 1951) The estimation of pentose sugars was carried out using the fuming Ferric chloride (Iron solution) and orcinol reagent, as described by (Standing et al., 1972). Determination of ethanol content was done by spectrophotometric method (Caputi et al., 1968). Cellulase enzyme production was studied by FPU assay (Ghose et al., 1987).

4 RESULTS AND DISCUSSION

Pretreatment

Compositional analysis of lignocellulosic substrate

Cellulose, the dominant structural polysaccharide of plant cell walls, was ranging from 35 to 50%, followed by hemicelluloses (20-35%) and lignin (10-25%).

Pretreatment strategies (Chemical and biological pretreatments)

The effect of alkali (NaOH), Chlorite (Na-Chlorite) and Oxidative (H_2O_2) treatments on the lignocellulosic substrates (rice straw and wheat straw) were also studied for varied concentrations and pretreatment time. The maximum results for lignin loss and holocellulose gain were obtained with 5% NaOH , 5% with Na-Chlorite and 2% H_2O_2 as shown in figures 1 to 6.

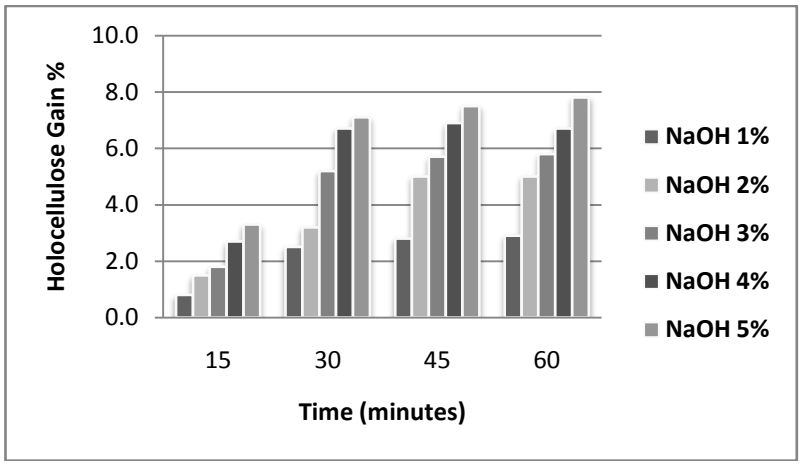

Figure 1: Effect of different concentration of alkali on holocellulose enrichment in Rice Straw at different time intervals

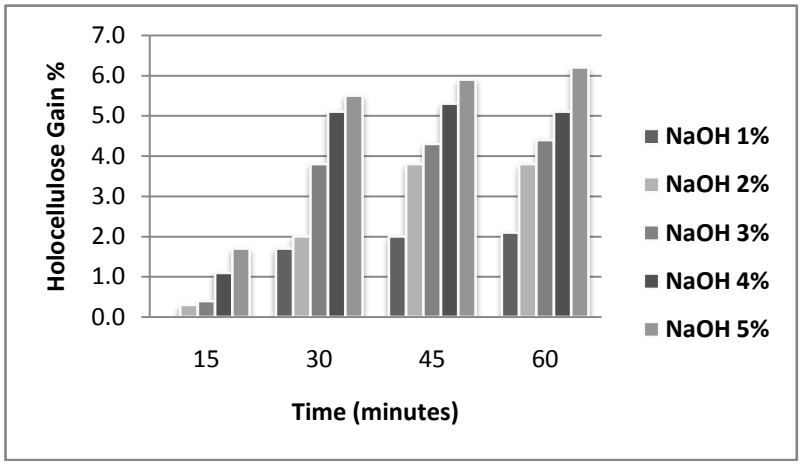

Figure 2: Effect of different concentration of alkali on holocellulose enrichment in Wheat Straw at different time intervals

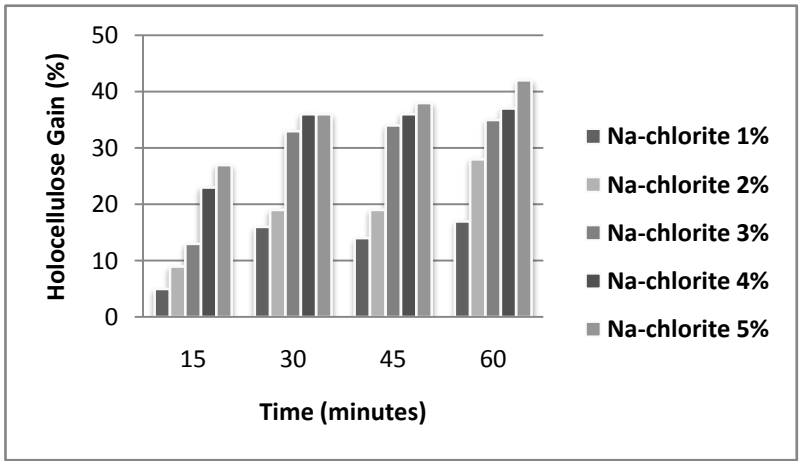

Figure 3: Effect of different concentration of Na-chlorite on holocellulose enrichment in Rice Straw at different time intervals

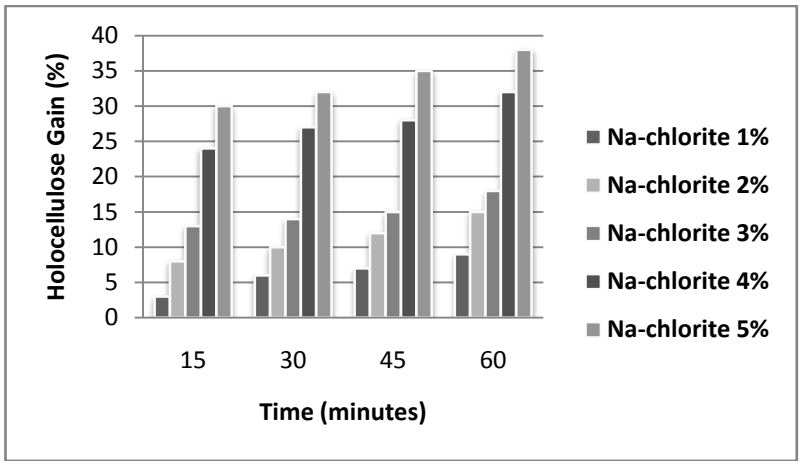

Figure 4: Effect of different concentration of Na-chlorite on holocellulose enrichment in Wheat Straw at different time intervals

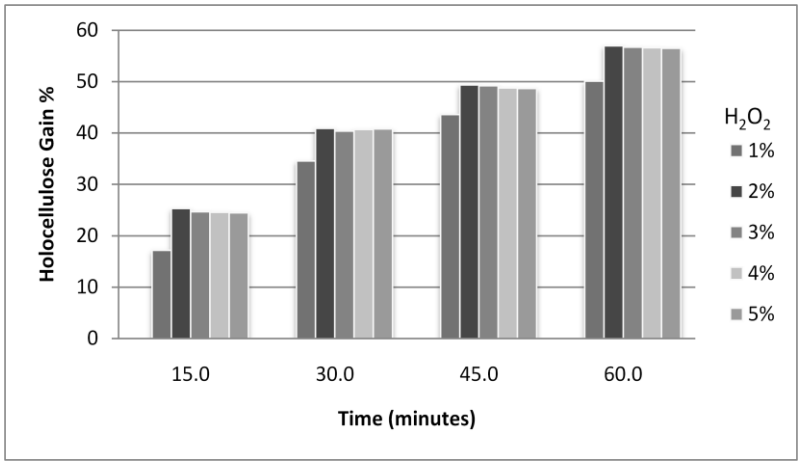

Figure 5: Effect of different concentration of H_2O_2 on holocellulose enrichment in Rice Straw at different time intervals

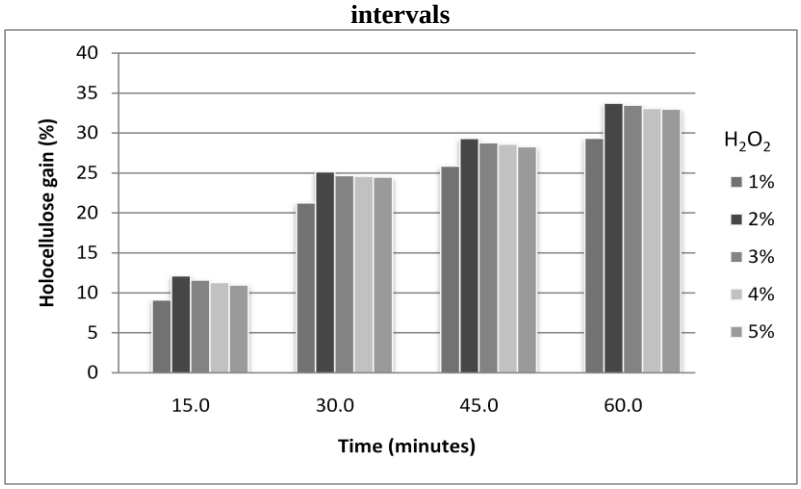

Figure 6: Effect of different concentration of H_2O_2 on holocellulose enrichment in Wheat Straw at different time intervals

Saccharification

Effect of Commercial Cellulase Enzyme on the Hydrolysis of Delignified Substrate

The substrates were treated with FPase dosages for saccharification. The optimum sugar release (810.15 ± 6.02 mg/gds) was with rice straw substrate and (690.13±5.03 mg/gds) with wheat straw.

Microbial Saccharification of Delignified Substrate

One Factor at a Time approach (figure 7and 8) of microbial saccharification (using fungi) of delignified substrate was studied. It was revealed that the optimum sugar released (175.27 ± 3 .34 mg/g and 98.68 ± 2 .43 mg/g with rice straw and wheat straw respectively) was obtained on the seventh day of saccharification.

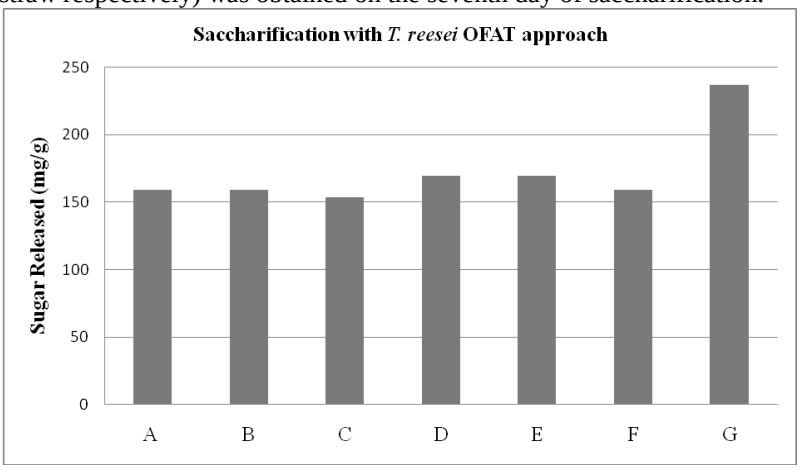

Figure 7: Rice straw saccharification where A is Time (7 days), B is Substrate (5 %), C is Microorganism Concentration (5 ml), D is Temperature (30°C), E is Agitation Rate (200 rpm), F is pH (5.5) and G Tween 80 (1% v/v)

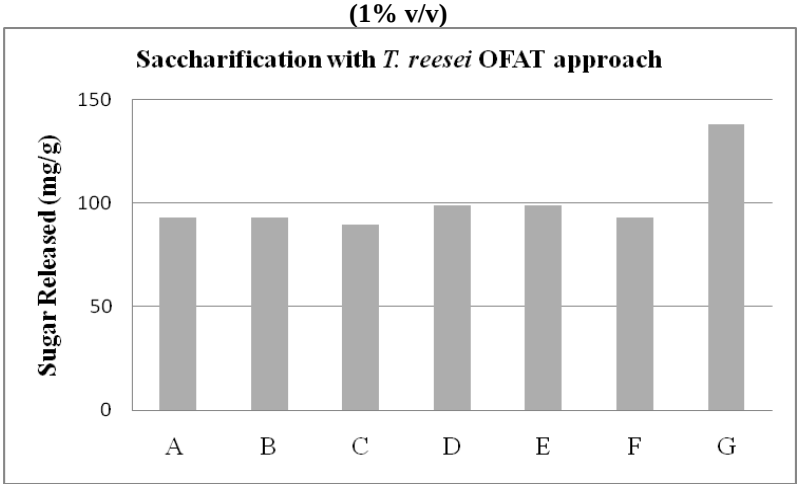

Figure 8: Wheat straw saccharification where A is Time (7 days), B is Substrate (5 %), C is Microorganism Concentration (5 ml), D is Temperature (30°C), E is Agitation Rate (200 rpm), F is pH (5.5) and G Tween 80 (1% v/v)

Fermentation of Detoxified Acid Hydrolysate

Optimization of ethanol production from detoxified acid hydrolysate using S. cerevisiae

Ethanol production increased with increase in the incubation time till 7 days of incubation. The maximum ethanol obtained from rice straw was (4.20 ± 0.12g/l) (figure 9) using S.cerevisiae and ethanol yield (0.42 g/g total sugar) whereas, the maximum ethanol obtained from wheat straw was 2.10 ± 0.11g/l using S. cerevisiae and ethanol yield was 0.16g/g total sugar (figure 10). However, the results from *Zymomonas mobilis* with rice straw was 10.02 ± 1.18 g/l ethanol (figure 11) and ethanol yield 0.43g/g. Similarly, the ethanol obtained from wheat straw with *Zymomonas mobilis* was 9.70 ± 2.32g/l ethanol (figure 12) and ethanol yield was 0.19g/l total sugar.

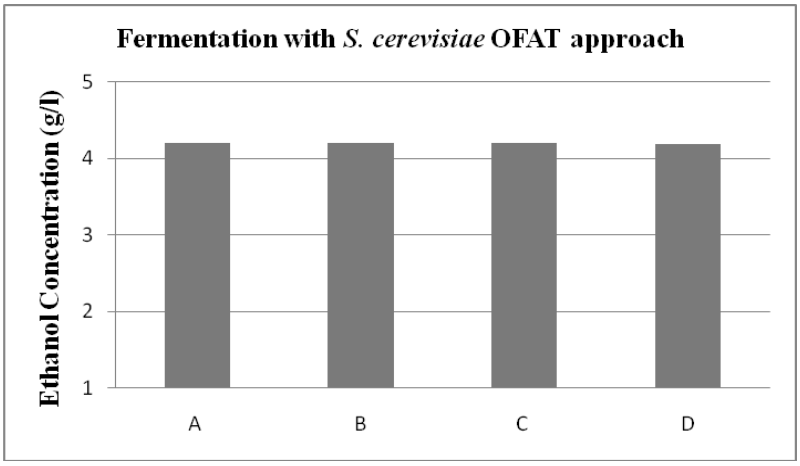

Figure 9: Rice Straw Fermentation with S. cerevisiae where A is Time (6 days), B is pH (5.5), C is Temperature (30^{O}C), D is Concentration of Soybean meal (1.20 % (v/v)

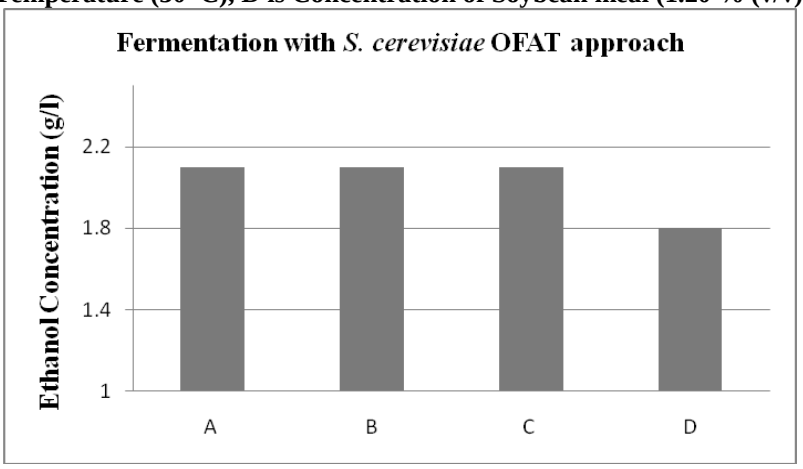

Figure 10: Wheat Straw Fermentation with S. cerevisiae where A is Time (6 days), B is pH (5.5), C is Temperature (30^{O}C), D is Concentration of Soybean meal (1.20 % (v/v)

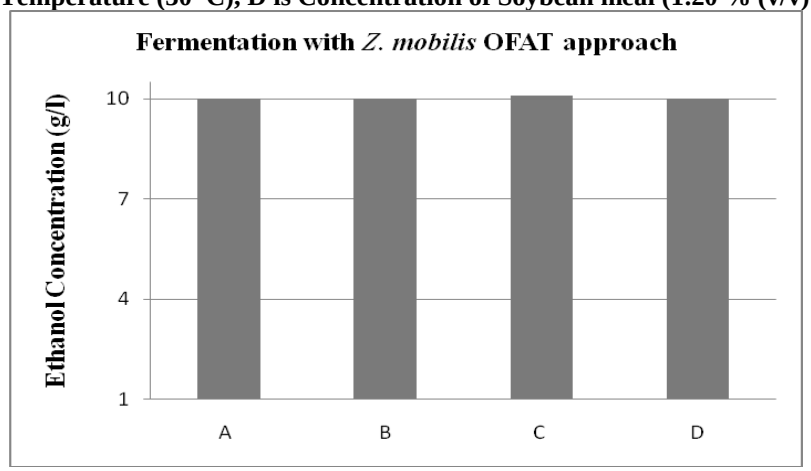

Figure 11: Rice Straw Fermentation with *Zymomonas mobilis* where A is Time (6 days), B is pH (6.0), C is Temperature (30^{O}C), D is Concentration of Soybean meal (1.20 % (v/v)

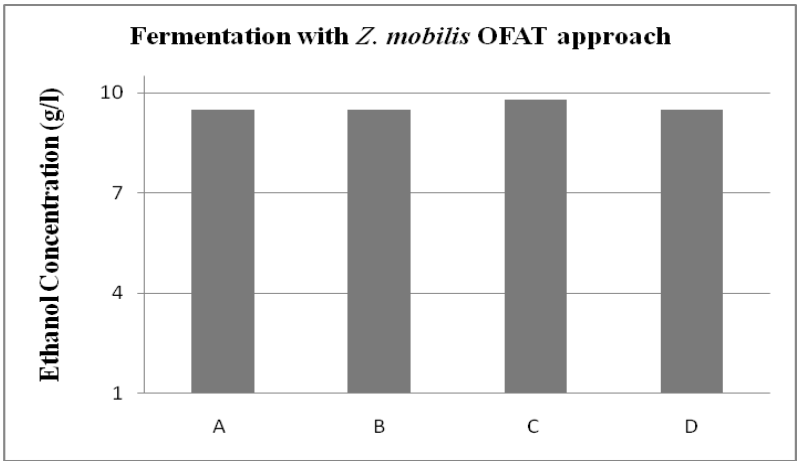

Figure 12: Wheat Straw Fermentation with *Zymomonas mobilis* where A is Time (6 days), B is pH (6.0), C is Temperature (30^{O}C), D is Concentration of Soybean meal (1.20 % (v/v)

The fermentation process would be economically viable only if both hexose and pentose sugars present in the hydrolysates are converted to ethanol. S. cerevisiae and *Zymomonas mobilis* have already been accepted as the most promising microorganisms for fermentation, as they produced considerably more ethanol from pentose and hexose sugar mixture, showed higher volumetric rate and yield of ethanol production (Nigam J.N, 2001a)

Production of ethanol by S. cerevisiaei and *Zymomonas mobilis* was found to be dependent on pH, with maximum ethanol production at pH 5.5 and 6 respectively, and lowering or elevating the pH of medium adversely affect the ethanol production.

STATISTICAL ANALYSIS

Statistical tools were applied at various stages in this study for validation of the results obtained. The correlation coefficient of various factors studied at the saccharification stage were taken. Following are the corresponding results.

Rice Straw	Microorganism Concentration (5ml)	Substrate Consistency (5%)	Temperature (30^{o}C)	Agitation Rate (200rpm)	pH (5.5)	Surfactant Concentration (1.0v/v)
Correlation coefficient (r)	0.987	0.955	0.979	0.987	0.961	0.957

Wheat Straw	Microorganism Concentration (5ml)	Substrate Consistency (5%)	Temperature (30^{o}C)	Agitation Rate (200rpm)	pH (5.5)	Surfactant Concentration (1.0v/v)
Correlation coefficient (r)	0.954	0.934	0.965	0.935	0.945	0.944

A positive correlation can be seen between the factors and time, where the value of r is higher with Rice Straw. The one way ANOVA for fermentation stage showed *Zymomonas mobilis* has taken the lesser time than S. cerevisiae for ethanol yield and the Sig. of the F value less than .05 (0.039).

ANOVA

Time

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	12.100	1	12.100	6.050	0.039
Within Groups	16.000	8	2.000		
Total	28.100	9			

5 CONCLUSION

Chemical treatment is considered to remove lignin selectively from lignocelluloses, treating rice straw and wheat straw for one hour resulted in partial solubilisation of polysaccharides. Rice straw and wheat straw with lower lignin content were more easily hydrolyzed, demonstrating that the presence of lignin in the cell

wall limits enzymatic hydrolysis. cellulosic materials holds great potential due to the widespread availability, abundance and relatively low cost. In this article *Trichoderma reesei* used for increasing saccharification value. After saccharification *Saccharomyces cerevisiae* and *Zymomonas mobilis* used for increasing ethanol production.

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