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Ethanol Production from Rice (*Oryza sativa*) Straw Biomass by Separate Hydrolysis and Fermentation

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This paper presents a study on the use of rice (*Oryza sativa*) straw biomass for ethanol production. Alkali treated rice straw was successfully converted to ethanol using commercial cellulase and *Saccharomyces cerevisiae* HAU-1 strain. Treatment of 0.5 mm straw with 2% sodium hydroxide at 15 psi for 1 h resulted in 70% lignin removal along with 88% cellulose recovery. The optimum concentration of cellulase enzyme and hydrolysis temperature was found to be 7.5 FPU/ g substrate at 50°C that resulted in about 75% sugar release from polysaccharides. Fermentation of enzymatic hydrolysate by *Saccharomyces cerevisiae* (HAU-1) resulted in production of 20.46 g/l ethanol after 72 h incubation at 30°C.

Key words: Bioethanol, Rice straw, *Saccharomyces cerevisiae*, Fermentation.

Contemporary industrial developments and rapid pace of urbanization have called for environmentally sustainable energy sources. Ethanol made from biomass provides unique environmental, economic strategic benefits and can be considered as a safe and cleanest liquid fuel alternative to fossil fuels (Chandel *et al.,* 2007). Ethanol is economically produced from sugarcane and corn in Brazil and America, respectively, but because of food security, feed based resources can not be used for long and eventually reliance moved to non-feed resources. The priority in global future ethanol production, therefore, lies on lignocellulosic processing (Kuhad, 2012).

The straw residue available from rice (*Oryza sativa*) crop could serve as a potential substrate for ethanol production as rice is the staple food for most of the world's human population and is the third most important grain crop behind wheat and corn in the world. Global rice production, in 2012, was 730.2 million tonnes (486.9 million tonnes on milled basis) (FAO rice market monitor, 2013; updated as of 24 Jan, 2013) resulting copious production of rice straw. Rice straw puts a burden on farmers and is generally disposed off by field burning which not only raises environmental constraints but also challenge the public health, consequently, if the locally available rice straw could be used for ethanol production it would be beneficial both in terms of environmental concern as well as to the farmers.

Lignification, silcification and crystallinity of cellulose are major barriers in the process of conversion of lignocellulosic biomass into ethanol. It is essential to alter or remove structural and compositional impediments to hydrolysis by pretreatment to improve the rate of hydrolysis. Hydrolysis of cellulose by acids or enzymes (cellulase) result into monomers. Enzymatic methods have the advantage of being highly specific, ecofriendly and no inhibitory products are formed (Wati *et al.,* 2007). Sugars generated as

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a result of hydrolysis are then fermented to ethanol using suitable yeast strains.

The present study aims to compare the effect of different mesh size of rice straw on improving the delignification efficiency as well as the effect of temperature, incubation time and cellulase concentration on rate of hydrolysis and ethanol production from the hydrolysate.

MATERIALS AND METHODS

Pretreatment of the straw

Rice straw of variety "Pusa-1" was procured from farmers' fields and dried at 50°C. It was analyzed for various components using standard procedures (Anonymous, 1970). For pretreatment, straw of different mesh size (0.5-2.0 mm) was immersed in sodium hydroxide solution (2%) at 1:10 (solid: liquid) followed by autoclaving at 15 psi for 1 h. Residue was washed with water to make it alkali free and dried at 50°C for further use.

Enzyme and yeast strain

A commercial preparation of cellulase enzyme (Palkosoft super 720) was kindly supplied by Maps India Ltd. Ahmedabad, Gujarat and enzyme activity was measured according to the standard procedure (Ghosh, 1987). A fast fermenting yeast strain of *Saccharomyces cerevisiae* HAU-1 was obtained from culture collection, Department of Microbiology, CCS HAU, Hisar and maintained on medium containing 20.0 g glucose, 20.0 g peptone and 10.0 g yeast extract L^{-1} at pH 5.0 by regular sub culturing and stored at 4°C until use.

Saccharification

Dry alkali treated rice straw was suspended in citrate buffer (pH 5.0) at 1:10 (solid: liquid) and the enzyme was added at different concentrations (5.0-10.0 FPU/ g substrate). The hydrolysis was carried out in a shaking water bath at varying temperature (40-50°C) for different time intervals (0.5–2.5 h). The hydrolysate was centrifuged at 5000 rpm for 15 min and total reducing sugars were estimated in the supernatant by di-nitrosalicylic acid method (Miller, 1959).

The saccharification value was calculated as:

$$\frac{\text{Reducing sugars produced}}{\text{Cellulose content in substrate}} \times 0.9 \times \text{Dilution}$$

Fermentation

The biomass of yeast after overnight growth at 30°C in yeast extract peptone sucrose (YEPS) medium containing: yeast extract 5.0, peptone 5.0 and sucrose 60.0 (g L^{-1}) was centrifuged at 5000 rpm for 15 min and inoculated into the hydrolysate @ 1.0% (w/v). The hydrolysate was supplemented with 0.3% urea and fermented at different temperatures (30-40°C). The ethanol content in fermented samples was analyzed colorimetrically (Caputi *et al.,* 1968).

Statistical analysis

Data were analyzed for statistical significance by the application of complete randomized design (CRD). A 5% probability level ($p = 0.05$) was used to accept or reject the null hypothesis.

RESULTS AND DISCUSSION

Analysis of rice straw revealed 35.07% cellulose, 24.85% hemicellulose, 6.29% lignin, 49.82% organic carbon and 0.85% nitrogen on dry weight basis (Table 1).

Pretreatment

To make the cellulose and hemicellulose amenable to hydrolysis, lignin fraction needs to be removed and therefore, straw biomass is pretreated with alkali (Chandel *et al.,* 2007). Efficacy of delignification depends directly on the available surface area. Alkali treatment of different mesh size (0.5-2.0 mm) rice straw revealed that maximum lignin removal occurred was not occurred with 0.5 mm mesh size where only 1.82% lignin was left whereas lignin content in 1.0 and 2.0 mm mesh size paddy straw after pretreatment was 3.96 and 5.74%, respectively (Table 2). Delignification was accompanied with increase in cellulose content and decrease in hemicellulose content compared to untreated rice straw. Whereas cellulose content

Table 1. Analysis of rice straw

Parameters	% (w/w)*
Cellulose	35.07±0.42
Hemicellulose	24.85±0.37
Lignin	06.29±0.19
Organic carbon	49.82±0.08
Total nitrogen	00.85±0.03

*Mean ± Standard error (S.E.)

increased from 35.07 to 73.43% in alkali treated straw of 0.5 mm mesh size, the hemicellulose content decreased from 24.85 to 16.16% (Table 2). Similar kind of increase in cellulose and decrease in hemicellulose content was reported by Kim *et al*. (2011) for two stage pretreatment of rice straw using aqueous ammonia and dilute sulphuric acid.

The recovery of total solids after pretreatment was applied to evaluate the effect of alkali treatment on different mesh size straw. Larger the mesh size of rice straw, higher was the recovery of total solids and maximum 49.04% total solids were recovered for 2.0 mm mesh size. Decrease in mesh size decreased the retrieval of total solids as the corresponding values for 1.0 and 0.5 mm mesh size were found to be 46.06 and 43.27% (Table 2).

Observing the effect of alkali treatment on lignin removed and total solids recovered with different mesh size of rice straw, polysaccharide (cellulose and hemicellulose) recovery was expressed in terms of mass balance. It was observed that a maximum of 95.69% of cellulose was recovered from 2.0 mm mesh size paddy straw while this value was found to be 88.26% with 0.5 mm mesh size paddy straw. Hemicellulose recovery after pretreatment, however, ranged between 27.24-29.59% for 0.5- 2.0 mm mesh size paddy straw (Fig.1). Cellulose recovery was found to be about 3-fold higher than hemicellulose recovery. The most obvious reason for lesser recovery of hemicellulose is its solubility in alkali.

Evaluating the effect of alkali treatment on cellulose, hemicellulose and lignin content of different mesh sized rice straw, 0.5 mm mesh size was found to be best suited for delignification as ascertained by minimum residual lignin and comparable recovery of cellulose and hemicellulose than larger size paddy straw. It was processed for fuel ethanol production by separate hydrolysis and fermentation (SHF).

Saccharification

Prior to ethanolic fermentation by yeast, cellulosic and hemicellulosic components of rice straw need to be processed by saccharification

Table 2. Effect of alkali pretreatment on various components of different mesh size rice straw

Component	Mesh size (mm)*			
	0.5[a]	1.0[b]	2.0[c]	CD (p=0.05)
Cellulose (%)	73.43±0.76	70.84±0.18	68.98±0.49	1.68
Hemicellulose (%)	16.16±0.24	15.98±0.10	13.72±0.28	0.88
Lignin (%)	01.82±0.11	03.96±0.27	05.74±0.09	0.60

a. 43.27 % solids recovered
b. 46.06 % solids recovered
c. 49.04 % solids recovered
*Mean±Standard error (S.E.)

Table 3. Ethanol production from pretreated rice straw by separate hydrolysis and fermentation (SHF) at different temperatures

Time (h)	Ethanol production (g/l)			
	30°C	35°C	40°C	Mean
24	14.07	13.36	06.97	11.47
48	19.57	16.89	08.81	15.09
72	20.46	19.02	08.81	16.09
Mean	18.03	16.42	8.19	

CD (p=0.05)
Temperature (A): 0.594
Time interval (B): 0.594
Interaction (A×B): 1.029

Table 4. Ethanol yield per kg rice straw at different temperatures

Time (h)	Ethanol production (g/l)			
	30°C	35°C	40°C	Mean
24	178.33	170.00	88.33	145.56
48	248.33	215.00	111.66	191.67
72	260.00	241.66	111.66	204.44
Mean	228.89	208.89	103.89	

CD (p=0.05)
Temperature (A): 7.623
Time interval (B): 7.623
Interaction (A×B): 13.203

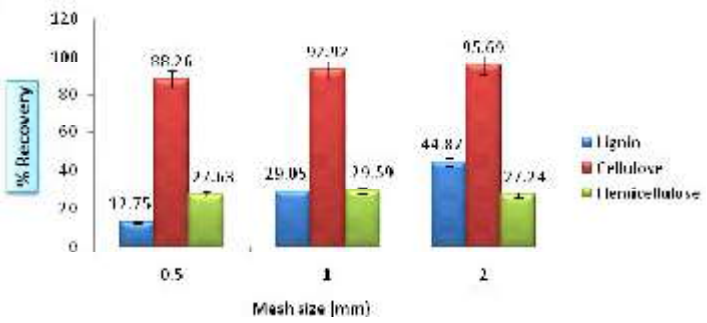

Fig. 1. Recovery of cellulose, hemicellulose and lignin from rice straw after alkali treatment

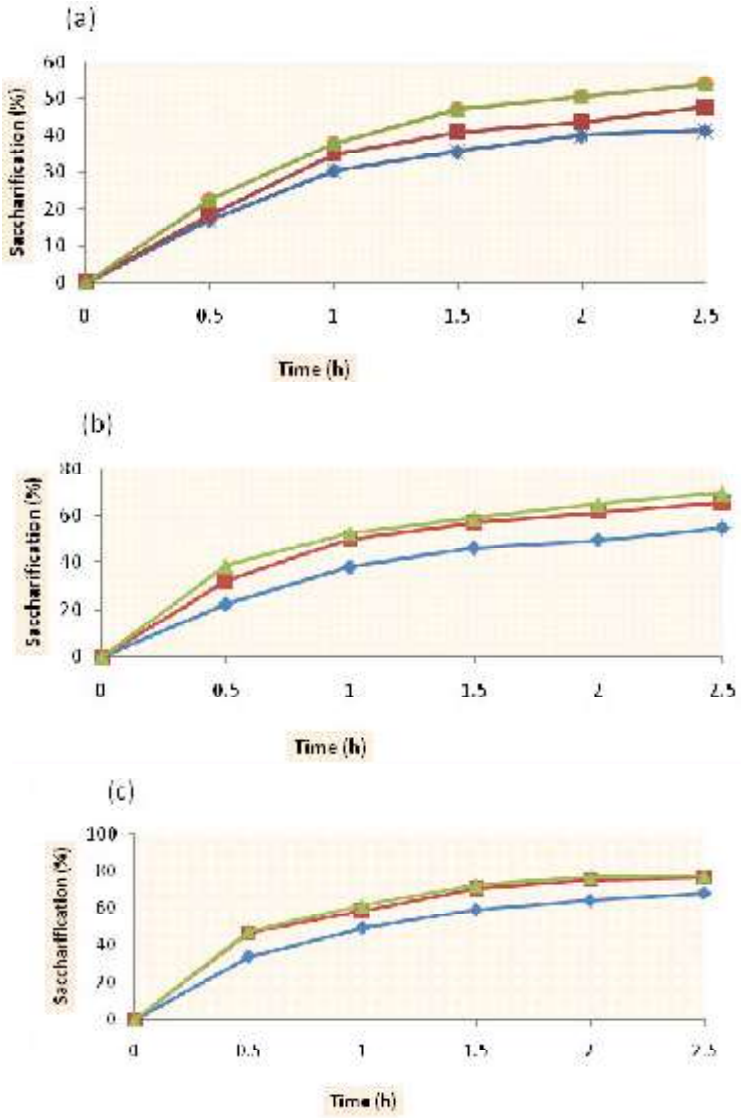

Fig. 2. Effect of cellulase concentration (¨) 5.0 ( ) 7.5 (D) 10.0 FPU/g substrate on saccharification of delignified rice straw at different temperatures (a) 40°C (b) 45°C (c) 50°C

technology in order to release fermentable sugars. Hydrolysis of delignified rice straw was carried out using commercial cellulase and the effect of enzyme concentration, temperature and incubation time on the amount of total reducing sugars released and consequently on saccharification was investigated. It was observed that saccharification increased with increase in enzyme concentration. Nevertheless, hydrolysis studies at different temperatures (40, 45 and 50°C) revealed that at 50°C, 50% saccharification occurred within first hour of hydrolysis with cellulase concentration 5.0 FPU/g while at 45°C same level of saccharification was achieved with 7.5 FPU/g cellulase concentration and at 40°C, about 2-fold higher cellulase concentration and longer incubation time was required for 50% saccharification. In all experiments, saccharification was fastest at 50°C; however, optimum cellulase concentration was connected to residence time. Increase in cellulase concentration from 7.5 to 10.0 FPU/g and incubation time from 2.0 to 2.5 h increased saccharification only slightly (from 76 to 77%). Therefore, examining the effect of temperature and enzyme concentration over time, biomass was hydrolyzed optimally at 50°C loaded at 7.5 FPU/ g enzyme concentration for 2 h resulting in maximum saccharification of 75% (Fig. 2).

Abedinifer *et al*. (2009) reported 60-71% sugar yield from rice straw at varied substrate concentration (20-100 g/L) using commercial cellulase loaded at 15 FPU/g at 45°C and pH 5.0 after 48 h incubation by SHF. Kim *et al* (2011) developed a two-stage pretreatment process using aqueous ammonia and dilute sulfuric acid in a percolation mode to improve production of fermentable sugars from rice straw and reported 89.0 and 71.7% sugar conversions of cellulose and hemicellulose into glucose and xylose by enzymatic and acid hydrolysis, respectively.

Fermentation

The fermentation of enzymatic hydrolysate obtained as a result of hydrolysis under optimum conditions revealed that ethanol fermentation was rapid during first 24 h of incubation as more than 50% ethanol (11.47 g/l) was produced during this period, thereafter, fermentation progressed slower. Statistical analysis of fermentation showed a significant effect of incubation time (CD= 0.594; p=0.05) as depicted by production of 16.09 g/l ethanol after 72 h incubation. Fermentation study with respect to temperature revealed 18.03 g/l ethanol production and statistically significant (CD= 0.594; p=0.05) (Table 3). The ANOVA revealed a significant interaction between fermentation time and temperature (CD=1.029; p=0.05). A comparison of ethanol production at all three temperatures showed that in an endeavor of ethanol production from rice straw where saccharification and fermentation were carried out separately, the most suitable temperature for efficient fermentation was 30°C resulting in ethanol production of 20.46 g/l after 72 h incubation (Table 3). Statistically significant effect of fermentation time (CD=7.623; p=0.05) as well as temperature (CD=7.623; p=0.05) on ethanol yield was observed. Combined effect of temperature and time resulted in the production of maximum 260.00 ml ethanol/ kg delignified rice straw at 30°C after 72 h incubation (CD=13.203; p=0.05) (Table 4).

In a study conducted by Zhong *et al*., (2009) on ethanol production from rice straw combining AFEX pretreatment with *S. cerevisiae* 424A(LNH-ST) in separate hydrolysis and fermentation, final ethanol yield of 175.6 g EtOH/kg untreated rice straw (37.0 g/l) was observed using commercial cellulase (Spezyme® CP) at 15 filter paper unit/g of glucan, with simultaneous addition of Multifect® Xylanase at 2.67 mg protein/g glucan and Multifect® Pectinase at 3.65 mg protein/g glucan. Pasha *et al*., (2012) studied sequential cellulase production, saccharification and fermentation of rice straw and reported 15.6 g/l final ethanol concentration.

CONCLUSION

The current work shows the successful ethanol production from rice straw biomass by separate hydrolysis and fermentation. Mesh size of rice straw has significant impact on delignification where smaller mesh size allows better penetration of alkali resulting in effective removal of lignin coupled with good polysaccharides recovery. However, utilization of pentose sugars from hemicellulose fraction using pentose fermenting yeast strain in combination with *S. cerevisiae* may result in further improvement in ethanol yield.

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