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Optimization of Pretreatment, Enzymatic Saccharification and Fermentation Conditions for Bioethanol Production from Rice Straw

KEYWORDS: Rice straw, Pretreatment, Saccharification, Fermentation

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ABSTRACT *Pretreatment of rice straw was optimized by soaking it in 2% H2SO4 (1:8) followed by autoclaving at 15 psi for 90 min resulting in 25% solubility of cellulose along with decrease in hemicelluloses and lignin contents. Pretreatment also released 70.5 mg xyloses and 296.2 mg hexoses per gram of straw in the acidic solution that upon fermentation with a co culture (5% v/v) of S.cerevisiae and P.tannophilus (1:1) produced 0.98%, v/v of ethanol. Saccharification of pretreated rice straw, optimized [S] and [E] revealed 5% substrate and 15 FPU of partially purified enzyme, yielding 41.8% saccharification efficiency. The fermentation of saccharified rice straw produced 0.67%, v/v ethanol at a fermentation efficiency of 78.6%. The validation of fermentation of saccharified rice straw to 3 L (@ 200g straw) revealed 6.1g of absolute alcohol at a fermentation efficiency of 55%.*

INTRODUCTION:

Rice straw is one of the abundant lignocellulosic wastes of the world with an annual production of 731 MT of which Asia contributes about 90%. This amount of straw can produce 205 billion litres of ethanol /year (Kim and Dale, 2004). Inspite of this huge potential of rice straw, its practical application is marked with recalcitrance that requires stringent physicochemical techniques to open up its bound sugars. This so called pretreatment has been studied and literature reports use of acid, alkali or even different solvents either alone or coupled with steaming or other physico-chemical procedures (Kang and Kim, 2012). Pretreatment while softening the crystalline meshwork of rice straw also releases bulk of hemicelluloses sugars in the reaction mixture as xylans. While the latter can be fermented to ethanol by a number of xylose fermenting yeasts like Pachysolan tannophilus, Candida etc, the cellulose of loosened solid straw is first saccharified by cellulases and then fermented to ethanol. A number of fungi specially Trichoderma harzianum, T.reesei and Aspergillus sp. produce cellulase and convert cellulose to glucose (Kocher et al., 2008). The latter is then fermented to ethanol by the traditional S.cerevisiae and other hexose fermenting yeasts.

In the present study, rice straw was optimized for pretreatment, saccharified by a crude cellulase from our local isolate Trichoderma harzianum Rut C-8230 and then fermented with P.tannophilus NRRL Y-2460 and S.cerevisae strain 35.

MATERIALS AND METHODS:

Procurement and Analysis of rice straw

The paddy straw obtained from local market was chopped into 5-7 cm pieces and dried in an oven at 70°C for 24 h. It was then ground (40 mesh) and packed in poly bags. Physical analysis of the sample revealed that the Bulk densities of raw and gound straw were 87 and 297 kg m^{-3}, respectively and moisture content was 8.0%. The composition of paddy straw was 55.4, 22 and 10.3% of cellulose, hemicelluloses and lignin, respectively (Crampton and Maynard, 1938) and Goering and Vansoest, 1970).

Pretreatment of rice straw

The pretreatment of water washed paddy straw was carried out by taking 5 gm of rice straw that was soaked overnight in 75 ml of 2% H_2SO_4, 2.5% NaOH or a solution (1:1) of 96% C_2H_5OH + 8% NaOH (as three separate treatments). The soaked straw was autoclaved at 15 psi for 90 minutes followed by sudden release of pressure by opening of steam release and safety valves.

Fermentation of pretreatment hydrolysate

The acid hydrolysate obtained from the pretreated rice straw was decanted into sterilized flasks, their pH adjusted to 5.0 and this hydrolysate was divided into 3 sets of triplicate flasks each containing 50 ml of hydrolysate. The 3 sets were inoculated @ 5% (v/v) with active cultures of S. cerevisiae 35, P. tannophilus and a combination of the two (1:1), respectively and incubated in a BOD incubator (28°C) upto 48h. The periodic samples were analyzed for ethanol production by the method of Caputi and Wright (1969).

Saccharification of pretreated rice straw

The pretreated rice straw was dried in an oven at 60°C for 3 h and used for saccharification by partially purified cellulase of Trichoderma harzianum MTCC 8230 (Kocher et al., 2010). The saccharification was carried out by incubating a mixture (100 ml) of pretreated rice straw, 0.1 M citrate buffer (pH 5.0) and cellulase in a water bath cum shaker at 45°C (100 rpm) for 72h. The saccharification was estimated by reducing sugars produced (Miller, 1959) and calculated as:

$$\text{Saccharification efficiency (\%)} = \frac{\text{Released sugars (g)}}{\text{Pretreated substrate(g)}} \times 0.9 \times 100$$

The saccharification was optimized with respect to concentration of pretreated rice straw (5- 15%) and partially purified cellulose (5-25 FPU/30 ml).

Ethanol production from saccharified rice straw

Ethanol production from rice straw was studied by initially taking a 300 ml reaction mixture containing 10 and 20 g pretreated rice straw, saccharified with 7.5 FPU of partially purified cellulase/g of rice straw and fermented with a 5% (v/v) inoculum of S.cerevisiae. The periodic samples were estimated for reducing sugars and ethanol as described above. The ethanol production process was then validated in a steel fermenter (7L capacity, Bioage, Mohali, India) at 3.0 L scale.

RESULTS AND DISCUSSION:

The results presented in table 1 revealed that the combined acid and steam (15 psi for 90 minutes) treatment was best with hexose and xylose levels of 287.6 and 82.5 mg/g of rice straw, respectively. On the other hand, the alkali and C_2H_5OH + NaOH treatments were found to produce lesser amounts of hexose sugars (7.6 mg/g and 9.8 mg/g respectively) and xylose (67.8 mg/g and 72.1 mg/g respectively). Earlier, Yoswathana and Phuriphipat (2010) standardized pretreatment of rice straw using acid and ultrasonic treatment with a

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37% recovery of sugars. Although alkali treatment led to more delignification of rice straw, the acid treatment produced low levels of furfurals (inhibit fermentation) and high levels of reducing sugars and xylose (Table 1). At increased pressure of 20 psi and 25 psi, there was more solubilization of cellulose and hemicellulose but it was accompanied with high levels of furfurals and low levels of xyloses (Gupta and Sharma, 2008). Both cellulose and hemicelluloses were solubilized with cellulose solubility of 16-25%. Also, sufficient appearance of free sugars in hydrolysate suggested that hydrolysis took place. The values of cellulose decreased in all the treatments, which has been reported elsewhere and there was 16-25% more cellulose available for saccharification. Hence, the optimized parameters of overnight soaking of paddy straw in 2% H_2SO_4 in substrate: acid ratio of 1:8 followed by steaming at 15 psi for 90 minutes were selected for pretreatment of paddy straw in further experiments. The dilute acid pretreatment is also considered a preferred method for its easy operation, better sugar recovery and comparatively low cost (Yoswathana and Phuriphipat., 2010; Chandel et al., 2011).

The acid hydrolysate obtained from the pretreated rice straw after neutralization with NaOH and fermented with 5% (v/v) of either S. cerevisiae, P. tannophilus alone or in a combination of the two (1:1) revealed that the combination was superior with 83% fermentation efficiency and a yield (on the basis of available hexoses and pentoses) of 0.16 g/g (Table 1). In literature, ethanol yields ranging from 0.24 to 0.51 g/g are reported from pretreated hydrolysate of different lignocellulosics (Chandel et al., 2011). Yoswathana and Phuriphipat (2010) reported 1.69% (v/v) ethanol from hydrolysate of pretreated rice straw. This may be attributed to the stringent conditions applied for its pretreatment due to the presence of high silica content (Binod et al.,2010).

The effect of pretreated rice straw concentration (1, 2 and 3 g/30 ml) on saccharification (Table 2) revealed a maximum saccharification efficiency of 35.6% with 2g/ 30 ml rice straw. which was selected for the subsequent experiments.

The effect of partially purified cellulase concentration was studied by varying its concentration from 5- 25 Filter Paper Units (FPU) in a reaction mixture of citrate buffer containing 2 g pretreated rice straw. The results presented in table 2 revealed that a maximum of 116 mg/g reducing sugars were obtained after 72 h of incubation in a water bath cum shaker with 15 FPUs of cellulase. The corresponding saccharification efficiency was also improved to 41.8 %. The same was also comparable with the control (Carboxymethy cellulase, a soluble form of cellulose) where 51% saccharification efficiency was realized. Therefore, 15 FPU of partially purified cellulase / 2g pretreated rice straw (i.e. 7.5 FPU/g of rice straw) of cellulase for a incubation period of 72 h were optimized conditions for saccharification. Earlier, Karimi et al (2006) standardized 15 FPU/ g DM of commercial cellulase for saccharification of dilute acid pretreated rice straw. Singh and Bishnoi (2012) used 10 FPU and 100 IU b-glucosidase/g dry solids alongwith 0.15% Tween 80 for obtaining 84% saccharification.

Ethanol production from rice straw in 300 ml reaction mixture containing 10 and 20 g pretreated rice straw revealed that the reducing sugars (mg/g) obtained were at par and hence the saccharification efficiencies. At 33% saccharification, 2.8 and 5.6g reducing sugars were available from 10 and 20 g pretreated rice straw, respectively (Table 3). The straw free reducing sugar milieu was then used for ethanol fermentation.

The results presented in table 3 revealed that like sugars produced, ethanol production was double with sugars obtained from 20 than 10 g rice straw. The total ethanol production was 0.80 and 1.53g/300 ml with fermentation efficiencies of 56.4 and 53.5% respectively from 10 and 20 g pretreated straw. Therefore, 20 g rice straw/300 ml was used for validation. of ethanol production whereby, 200 g of pretreated rice straw was saccharified with partially purified cellulose (7.5 FPU/g) in a total volume of 3 L for 72 h (28°C) at 100 rpm with an air supply of 2 vvm. The results presented in table 3 revealed a total production of 21.7g of reducing sugars with a saccharification efficiency of 34.9%. Earlier Karimi et al (2006) used a rice straw loading of 50 and 100g pretreated rice straw/ liter of saccharified wort. The saccharified wort (milieu) was freed of the spent rice straw by decantation with a recovery of 2.85 L. The latter was inoculated with an active culture of S.cerevisae strain 35 (grown for 24h in Glucose yeast extract broth) @ 5% (v/v) and the air supply and shaking were shut off. Results presented in table 4 revealed an ethanol production of 6.1 g/3L in 72h that correspond to 55% fermentation efficiency. This corresponds to an ethanol yield of 30.5 g/Kg of pretreated rice straw. Theoretically, @ 50.4 % cellulose of pretreated rice straw, 257.5 g ethanol/Kg rice straw is expected. Our low ethanol could be due to the fact that we obtained 287.5 mg/g glucose at the level of acid pretreatment and we incorporate these values i.e. from fermentation of pretreated hydrolysate (Table2), 160 g ethanol (out of the total theoretical amount of 220 g, based on 22% hemicelluloses), a total of 190.5 g ethanol/ Kg pretreated rice straw was obtained (from a maximum possible of 332.2 g ethanol/Kg pretreated rice straw) at an efficiency of 57.34%. Earlier, Karimi et al (2006) reported 208 ml ethanol/ Kg of rice straw and Singh and Bishnoi (2012) reported an ethanol yield of 0.50, 0.47, and 0.48 (g/g) by S. cerevisiae, S. stipitis, and by co-culture, respectively, using pretreated rice straw hydrolyzate. The co-culture of S. cerevisiae and S. stipitis produced 25% more ethanol than S. cerevisiae alone and 31% more ethanol than S. stipitis alone.

CONCLUSION:

The present work revealed that acid cum steam treatment is the best pretreatment for rice straw. An indigenous isolate of T.harzainum was standardized for saccharication of pretreated rice straw as 7.5 U/g which on fermentation revealed an ethanol yield of 0.31 g/g of rice straw.

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Table 1. Physico-chemical pretreatment of rice straw and frmentation of acid hydrolysate by S.cerevisiae and P.tannophilus..

PRETREATMENT					
S. No	Measurable parameters	Pretreatment (with post steaming at 15 p.s.i. for 90 min)			Control
		2% H_2SO_4 (1:8)*	2.5% NaOH (1:15)*	96% C_2H_5OH + 8% NaOH 1:1 (1:8)*	
1.	[1]Cellulose	25.4	34.8	30.1	50.4
2.	[2]Hemicellulose	9.8	7.9	14.7	22.0
3.	[3]Lignin	7.2	2.0	2.0	10.3
4.	Reducing sugars (mg/g)	287.6	7.6	9.8	--
5.	[4]Xyloses (mg/g)	82.5	67.8	72.1	--
6.	Furfurals (mg/g)	0.1	--	--	--
FERMENTATION					

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Time (h)	Ethanol, % w/v		
	Fermenting Yeasts		
	S.cerevisiae	P.tannophilus	S.cerevisiae + P.tannophilus (1:1)
24	0.22	0.10	0.29
48	0.69	0.34	0.78
[a]Fermentation efficiency (%)	69.0	36.3	83.0
[b]Yield, g/g	0.14	0.07	0.16

C.D. 5% = [1]2.46, [2]1.81, [3]1.32 and [4]4.93

[1]Sugars available were hexose (296 mg/g) and xylose (70.5 mg/g)= 1.83 g, 0.94 g of theoretical ethanol.

[2]Calculated on the basis of straw (5 g in 75 ml of pretreatment solution)

Table 2. Effect of concentration of pretreated (PT) rice straw and cellulase on saccharification of PT rice straw.

Parameters	Reducing sugars (mg/g) at Time (h)			Saccharification efficiency (%)
	24	48	72	
[1]PT rice straw concentration (g/30 ml)				
1.0	21.2	68.9	89.6	32.0
2.0	21.6	81.1	98.8	35.6
3.0	23.0	67.0	87.4	31.5
[2]Cellulase (FPU/30 ml), PT rice straw, 2g/30 ml				
5.0	16.5	17.4	24.7	8.9
10.0	22.1	58.2	103.9	37.4
15.0	23.2	93.2	116.0	41.8
20.0	24.4	59.3	92.8	33.4
25.0	25.3	86.1	103.9	37.4
Control (0.5 g CMC /100 ml)	39.0	52.6	56.8	51.1

C.D. 5% = [1]2.65, [2]3.05

The reaction flasks were incubated in a water bath cum shaker at 45°C

Table 3. Fermentation of PT rice straw by S.cerevisiae under optimized saccharification conditions.

PT straw (g)	Reaction volume (L)	[1]Reducing sugars (mg/g)	Saccharification efficiency (%)	[2]Total ethanol (g)	Ethanol (g/g)	[3]Fermentation efficiency (%)
10	0.3	114.5	36.8	0.80	0.08	56.4
20	0.3	115.8	37.2	1.53	0.77	53.5
200	3.0	108.9	34.9	6.1	0.31	55.0

[1]Total reducing sugars available per 10, 20 and 200g were 2.8, 5.6 and 56g, respectively

[2]Theoretical ethanol production was 1.43, 2.86 and 11.08g per 2.8, 5.6 and 56g sugars, respectively.

[3]Fermentation efficiency was calculated on the basis of actual sugars available.

REFERENCE 1. Kim S and Dale BE 2004 Global potential bioethanol production from wasted crops and crop residues, Biomass Bioenergy. 26: 361-375. | 2. Kang HK and Kim D 2012 Efficient bioconversion of rice straw to ethanol with TiO2/UV pretreatment, Bioprocess Biosyst Eng. 35: 43-48. | 3. Kocher GS Kalra KL and Banta G Optimization of cellulase production by submerged fermentation of rice straw by Trichoderma harzianum Rut-C 8230, The Internet J Microbiology. 5 DOI: 10.5580/1658. | 4. Crampton EW and Maynard LA The relation of cellulose and lignin content to the nutritive value of animal feeds, J. Nutrition 15: 32. | 5. Goering HK and Van Soest PJ 1970 Forage fiber analysis (Apparatus, reagent, procedures and some applications), USDA Handbook No. 379. | 6. Caputi Jr A and Wright D (1969) Collaborative study of the determination of ethanol in wine by chemical oxidation, J Assoc Off Anal Chem, 52: 85. | 7. Miller GL (1959) Use of Dinitrosalicylic Acid Reagent for determination of. Reducing Sugar, Anal. Chem. 31: 426-428. | 8. Yoswathana N and Phuriphipat P 2010 Bioethanol Production from Rice Straw, Energy Research Journal. 1: 26-31. | 9. Gupta PK and Sharma R 2008 Integrated use of paddy straw, (Component C) Annual Report of Adhoc research project, Punjab Agricultural University, Ludhiana. p.73-92. | 10. Chandel AK Chandrasekhar G Radhika K Ravinder R and Ravindra P 2011 Bioconversion of pentose sugars into ethanol: A review and future directions, Biotechnology and Molecular Biology Review. 6: 8-20. | 11. Karimi K Emitiazi G and Taherzadeh MJ 2006 Ethanol production from dilute acid pretreated rice straw by simultaneous saccharification and fermentation with Mucor indicus, Rhizopus oryzae and Sacharomyces cerevisiae. Enz Microb Tech. 40: 138-144. | 12. Binod P Sindhu R Singhania RR Vikram S Devi L Nagalakshmi S Kurien N Sukumaran RS and Pandey A 2010 Bioethanol production from rice straw: An overview, Biores. Technol 101: 4767-4774. | 13. Singh A and Bishnoi NR 2012 Optimization of enzymatic hydrolysis of pretreated rice straw and ethanol production, Appl Microbiol Biotechnol. 93: 1785-1793. | 14. Zhong C Lau MW Balan V Dale BE and Yuan YJ 2009 Optimization of enzymatic hydrolysis and ethanol fermentation from AFEX-treated rice straw, Appl Microbiol Biotechnol. 84: 667-676. |