Mycobiology 32(4): 170-172 (2004)

Effect of Different Pretreatment Methods on the Bioconversion of Rice Bran into Ethanol

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(Received November 26, 2003)

The efficiency of acid, enzyme and microbial pretreatment of rice bran was compared based on the content of cellulose, hemicellulose, reducing sugars and xylose in the substrate. An isolate of *Aspergillus niger* or a strain of *Trichoderma viride* (MTCC 800) was employed for microbial pretreatment of rice bran in solid state. Acid pretreatment resulted in the highest amount of reducing sugars followed by enzyme and microbial pretreatment. *A. niger* showed a higher rate of hydrolysis than *T. viride*. The rice bran hydrolysate obtained from the different methods was subsequently fermented to ethanol either by *Zymomonas mobilis* (NCIM 806) or by *Pichia stipitis* (NCIM 3497). *P. stipitis* fermentation resulted in higher ethanol (37% higher) and biomass production (76~83% higher) than those of *Z. mobilis*. Maximum ethanol production resulted at 12 h in *Zymomonas* fermentation, while in *Pichia* fermentation, it was observed at 60 h. Microbial pretreatment of rice bran by *A. niger* followed by fermentation employing *P. stipitis* was more efficient but slower than the other microbial pretreatment and fermentation.

KEYWORDS: ***Aspergillus niger*, Ethanol, Fermentation, *Pichia stipitis*, *Trichoderma viride*, Xylose, *Zymomonas mobilis***

The potential of bioconversion of cellulosic materials is frequently considered because of their high availability and low cost. The microbiological conversion of organic matter to useful products is becoming increasingly attractive as a method of waste treatment and resource recovery.

Cellulosic or lignocellulosic waste biomass are to be pretreated by acids, alkalis or enzymes to break them down to simple sugar molecules which can be fermented into ethanol by bacteria or yeasts. A number of yeasts like *Pichia stipitis*, *Candida shehatae* and *Pachysolan tannophilus* were identified as successfully fermenting pure xylose solutions (Hinman *et al.*, 1989). Apart from yeasts, an ethanologenic bacterium *Zymomonas mobilis* has been the main focus for ethanol fermentation in recent years (Rhee *et al.*, 1986; Kim *et al.*, 1988).

The present study compared the hydrolytic efficiency of different pretreatment methods on rice bran and their effect on the ability of *Zymomonas mobilis* and *Pichia stipitis* in fermenting the rice bran hydrolysate.

Materials and Methods

Cultures of *Trichoderma viride* (MTCC 800), *Zymomonas mobilis* (NCIM 806) and *Pichia stipitis* (NCIM 3497) were obtained from the Institute of Microbial Technology, Chandigarh and the National Chemical Laboratory, Pune, India, respectively. An isolate of *Aspergillus niger* was recovered from naturally contaminated rice bran. The fungi were subcultured on PDA medium as slants and stored at 4°C. Subcultures of the bacterium and yeast were made and maintained on Rich medium containing 20 g of agar, 2 g of KH_2PO_4, 20 g of glucose and 10 g of yeast extract/litre.

Rice bran containing 60% (w/w) water was taken in individual conical flasks, which were autoclaved at 121°C for 15 min. Sections (7 mm) of 4 days old mycelium of *A. niger* and *T. viride* were used for inoculating the experimental flasks containing 20 g of the rice bran slurry under sterile conditions. The flasks were incubated at room temperature.

In a set of preliminary experiments, samples of substrates undergoing fermentation were removed daily for 6 days and the content of reducing sugars and xylose (Miller, 1959) were analysed. The activities of CMCase, FPA and β-glucosidase (Ray *et al.*, 1993) were assayed on alternate days. Based on the results of the preliminary experiments, the fungi were inactivated on the third day by heating the culture for 5 min at 100°C. Single batch bioconversion technique was followed in which fermented rice bran sample was directly used as the substrate for liquid state fermentation (LSF).

For acid pretreatment, rice bran (5% w/v) taken in 250 *ml* Erlenmeyer flask was saccharified by dilute H_2SO_4 (0.5 %) at 100°C for 2 h and the pH of the hydrolysate was neutralized by using 1 N NaOH. For enzyme saccharification, rice bran slurry in water (5% w/v) taken in 250 *ml* Erlenmeyer flasks was autoclaved at 121°C for 15 min. After cooling, 0.15 *ml* cellulase enzyme was added

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to each flask. pH of the sample was changed to 5 and the flasks were kept incubated at 60°C for 1 h. The pH of enzyme pretreated rice bran hydrolysate was neutralized with 1 N NaOH.

Reducing sugar and xylose after saccharification by all the three methods were estimated by DNS method (Miller, 1959). These flasks containing acid, enzyme or fungal pretreated rice bran hydrolysate were inoculated with liquid cultures of either *Zymomonas mobilis* or *Pichia stipitis* (10% v/v) under aseptic conditions. Liquid state fermentation was carried out at room temperature (30 ± 2°C) in a rotary shaker (Remi, India) for 2~3 days. The fermented samples were drawn at an interval of 12 h and analysed for reducing sugar, xylose (Miller, 1959), biomass (Doelle and Greenfield, 1985) and ethanol (Caputi *et al.*, 1968).

Results and Discussion

The yield of reducing sugar after acid hydrolysis was 66% while enzyme hydrolysis resulted in 45% saccharification. Dilute acid pretreatment has been agreed upon by several workers (Schele *et al.*, 1991; Torget *et al.*, 1991) as the best hydrolytic method for near term commercial application. Microbial pretreatment was less efficient with *A. niger* showing 13% saccharification and *T. viride* saccharified 9.5% of the carbohydrates on the third day of fermentation (Table 1).

Table 1. Effect of the type of hydrolytic treatments on the yield of reducing sugar, xylose and saccharification of rice bran

Treatments	Reducing sugar (g/l)	Xylose (g/l)	Saccharification (%)
Acid	14.08±1.30	5.66±0.02	66.0
Enzyme	11.45±0.70	3.20±0.03	45.0
Aspergillus niger	2.94±0.08	1.33±0.07	13.1
Trichoderma viride	1.87±0.10	0.92±0.01	9.5

Values are mean±SE of 3 replicates.

The highest cellulase activity (in terms of FPase, CMCase and β-glucosidase activities) was exhibited by *A. niger* and its cellulase activities peaked earlier than in *T. viride* (Table 2). This explains the higher amount of reducing sugars in the rice bran hydrolysed by *A. niger*, when liquid state fermentation was initiated at the end of 3 days of fermentation in solid state. Similar findings have been reported by Ray *et al.* (1993) and Reddy *et al.* (1998).

The maximum utilization of reducing sugars in both acid and enzyme hydrolysate of rice bran by *Z. mobilis* occurred within 12 h of fermentation, while *Pichia* took 48 h to produce the same effect. The biomass of *Pichia* was higher than that of *Zymomonas* in all the rice bran hydrolysates by the different methods and the biomass

Table 2. Effect of enzymes viz., carboxy methyl cellulase (CMCase), filter paper degrading enzyme (FPase) and β-glucosidase of *Aspergillus niger* and *Trichoderma viride* during SSF on rice bran

Organisms	Fermentation (h)								
	CMCase (IU/ml) β-glucosidase (IU/ml)			FPA (IU/ml)			β-glucosidase (IU/ml)		
	2	4	6	2	4	6	2	4	6
A. niger	0.36±0.03	0.66±0.01	0.50±0.09	0.18±0.01	0.32±0.01	0.22±0.01	0.59±0.02	1.3±0.13	0.84±0.02
T. viride	0.27±0.01	0.47±0.02	0.62±0.01	0.10±0.01	0.22±0.01	0.36±0.01	0.37±0.02	0.7±0.05	1.10±0.02

Values are mean±SE of 3 replicates.

Table 3. Effect of the type of hydrolytic treatments on the production of reducing sugars (g/l) during liquid state fermentation (LSF) of rice bran hydrolysate by *Zymomonas mobilis* and *Pichia stipitis*

Treatments	Reducing sugars (g/l)/Fermentation (h)										
	Z. mobilis					*P. stipitis*					
	0	12	24	36	48	12	24	36	48	60	72
Acid	4.08 ±1.30	2.68 ±0.10	1.32 ±0.01	0.56 ±0.03	0.32 ±0.02	11.32 ±1.60	8.68 ±0.9	4.70 ±0.28	1.1 ±0.14	0.60 ±0.07	0.39 ±0.05
Enzyme	11.45 ±0.70	2.40 ±0.30	1.22 ±0.10	0.99 ±0.07	0.74 ±0.03	8.05 ±0.10	6.13 ±0.57	4.84 ±0.14	1.99 ±0.13	0.94 ±0.04	0.65 ±0.05
Aspergillus niger	2.94 ±0.07	1.06 ±0.01	0.53 ±0.18	0.40 ±0.03	0.36 ±0.02	1.6 ±0.13	0.97 ±0.09	0.61 ±0.04	0.52 ±0.03	0.41 ±0.04	0.28 ±0.02
Trichoderma viride	1.87 ±0.10	0.94 ±0.03	0.83 ±0.06	0.56 ±0.04	0.40 ±0.03	1.3 ±0.09	0.95 ±0.08	0.56 ±0.04	0.45 ±0.03	0.41 ±0.13	0.24 ±0.02

Values are mean±SE of 3 replicates.

Table 4. Effect of the type of hydrolytic treatments on the production of biomass (g/*l*) during liquid state fermentation (LSF) of rice bran hydrolysate by *Zymomonas mobilis* and *Pichia stipitis*

Treatment	Biomass (g/*l*)/Fermentation (h)									
	Z. mobilis					*P. stipitis*				
Acid	1.14±0.04	1.81±0.04	1.98±0.05	2.05±0.12	1.13±0.10	1.25±0.05	1.93±0.02	2.95±0.02	3.34±0.15	3.76±0.35
Enzyme	1.12±0.21	1.38±0.06	1.66±0.05	1.74±0.06	0.87±0.03	1.12±0.10	1.84±0.09	2.11±0.21	2.54±0.20	3.02±0.15
Aspergillus niger	1.08±0.10	1.39±0.03	1.60±0.02	1.62±0.04	0.77±0.22	1.11±0.10	1.72±0.06	2.04±0.24	2.51±0.38	2.57±0.20
Trichoderma viride	0.94±0.08	1.21±0.18	1.30±0.17	1.41±0.08	0.74±0.03	0.82±0.03	1.64±0.30	1.92±0.02	2.39±0.07	2.48±0.15

Values are mean±SE of 3 replicates.

Table 5. Effect of type of hydrolytic treatments on the production of ethanol (g/*l*) during liquid state fermentation (LSF) of rice bran hydrolysate by *Zymomonas mobilis* and *Pichia stipitis* in different time (h)

Treatments	Ethanol (g/*l*)/Fermentation (h)										
		Z. mobilis				P. *stipitis*					
	0 (THS)	12	24	36	48	12	24	36	48	60	72
Acid	19.74	5.03 ±0.5	3.07 ±0.2	2.25 ±0.11	1.36 ±0.10	1.16 ±0.10	2.81 ±0.12	4.44 ±0.48	7.07 ±0.60	9.48 ±0.35	7.15 ±0.30
Enzyme	14.65	3.88 ±0.40	2.60 ±0.18	1.68 ±0.10	1.08 ±0.03	1.80 ±0.10	2.70 ±0.17	3.81 ±0.30	4.83 ±0.21	6.58 ±0.50	5.46 ±0.55
Aspergillus niger	4.27	1.40 ±0.22	0.85 ±0.05	0.74 ±0.15	0.74 ±0.05	0.50 ±0.06	0.82 ±0.08	1.20 ±0.10	1.90 ±0.14	2.21 ±0.25	1.15 ±0.09
Trichoderma viride	2.79	1.18 ±0.03	0.72 ±0.50	0.61 ±0.02	0.50 ±0.04	0.43 ±0.03	0.55 ±0.01	0.74 ±0.05	1.06 ±0.12	1.90 ±0.15	0.85 ±0.05

THS : Total hydrolysable sugar (g/*l*)/ Values are mean ± SE of 3 replicates.

production was unaffected by the method of hydrolysis (Tables 3, 4). *Pichia* fermentation resulted in a higher yield of ethanol than did *Zymomonas* fermentation, but the duration of fermentation was longer. The reasons for these results may be due to the findings that in *P. stipitis*, xylose and cellulose utilization commenced only after the glucose was depleted and ethanol production from cellobiose continued even after exhaustion of xylose (Table 5) as reported by Dupreez *et al.* (1986).

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