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Ethanol Production from Pearlmillet Flour by *Saccharomyces cerevisiae*

Bijender Poonia, Leela Wati* and Kushal Raj

Department of Microbiology, CCS Haryana Agricultural University, Hisar - 125 004, India.

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Ethanol production from pearlmillet flour was studied by *Saccharomyces cerevisiae*. Homogenous slurry of pearl millet flour was prepared in water at 30%(w/v) concentration. Liquefaction of pearl millet flour slurry carried out with a-amylase (1.75units/g starch) at 90^{0}C for 30 min followed by saccharification with glucoamylase (16.5GA/unit starch) at 60^{0}C for 2h generated about 15.9% total reducing sugars in the hydrolysate. The fermentation of hydrolysate with *Saccharomyces cerevisiae* HAU-1 at 30^{0}C for 48h resulted in production of 11.4%(v/v) ethanol. Simultaneous saccharification also accomplished during fermentation of pearl millet flour.

Key words: Ethanol, Fermentation, liquefaction, pearl millet flour, saccharification, *Saccharomyces cerevisiae*.

Ethanol is one of the most important feed stocks for industrial chemicals and potable purposes. The demand of ethanol is increasing steadily as a promising fuel since the time of early automotive development to stabilize petroleum supplies and to save fossil reserves (Anupma and Sudarsan, 2006; Anon, 2008).

Ethanol is produced by fermentation of molasses by *Saccharomyces cerevisiae* in Indian distilleries. However, short supply and high cost of cane molasses are some of the hindrances in large scale production of ethanol. More recently, emphasis has been on the use of other substrates for ethanol production to meet the increasing demand of ethanol (Pimental and Patzek, 2005)

Sugary substrates could easily be employed for ethanol production but are comparatively expensive. Cellulosic materials are cheaper, available in plenty but their conversion to ethanol is very expensive. More recently, starchy substrates have become economically practicable for ethanol production (Reddy *et al*., 2005). Cereals (corn, barley, wheat, rice) and tubers (potato, sweat potato) have been used for the production of bioethanol in many parts of the world (Montesinos and Navarro, 2000; Shigechi *et al*., 2004; Jamai *et al*., 2007). On a dry basis, these grains contain around 60-75 per cent (w/w) of starch.

* To whom all correspondence should be addressed.

Pearl millet, a starch-cereal crop is well adapted to production systems characterized by low rainfall, low soil fertility, and high temperature. It performs well in soils with high salinity and it can be grown in area where other cereals crop, such as wheat as maize, would not survive. Pearl millet has ability to yield higher than all other cereals with low fertilizer inputs. Further this is nutritionally rich crop with high content of protein, starch, fat, carbohydrate and minerals. The calorific value of pearl millet is equivalent 360Kcal/100g (http://en.wikipedia.org/wiki/ Millet). The utilization of pearl millet is limited that too to smaller segment of population because of its short storage life and processed pearl millet products are not available. Efforts are needed to improve its shelf life and to develop suitable commercially viable technologies for development of fermented beverages and ethanol from pearl millet. Therefore the studies were planned to develop suitable technology for production of ethanol from pearl millet flour.

MATERIAL AND METHODS

Pearl millet seeds

Substrate

The pearl millet hybrids HHB-60, HHB-67, HHB-94, HHB-146 developed in the Department of Plant Breeding, CCS HAU, Hisar were selected randomly for the present investigation. These grains were dried at 50^{0}C and flour was prepared by grinding to fine power (210 µm). The flour of different hybrids was analysed for various components by standard methods (AOAC, 1990).

Enzyme for liquefaction and saccharification

Commercial α-amylase (Termamyl-100 , Specific activity 30 DUN units/ml) and amyloglucosidase (Amylo 300, Specific activity 400 GA units/ml) were received from Ms. Jagatjeet Industry Ltd., Jalandhar (Punjab).

Yeast culture for fermentation

A fast fermenting strain of *Saccharomyces cerevisiae* HAU-1 was obtained from culture collection Department of Microbiology, CCS Haryana Agricultural University, Hisar (Haryana) and maintained on yeast extract peptone dextrose (YEPD) agar medium containing yeast extract (1%), peptone (2%), dextrose (2%) and agar (2%).

Hydrolysis of pearl millet flour

Preparation of pearl millet flour slurry

Slurries of pearl millet flour were prepared in water at different solid liquid ratio (1:1 to 1:10) and treated with liquefying enzyme (1000 µl /100ml) at 90^{0}C for 30 min under shaking conditions. The slurry prepared by mixing 30 g flour in 70 ml water being homogenous, loose, easy to handle was used for further experiments.

Liquefaction of pearl millet flour slurry

Liquefaction of pearl millet flour slurry (30%) was carried out in a shaking water bath using varying concentration of liquefying enzyme (100-2000 µl/100ml) at different incubation temperatures (60-90^{0}C) for different time intervals (10-40 min). The progress of liquefaction was monitored by employing starch-iodine (1 g of Iodine and 2 g potassium iodide in 100 ml water) reaction.

Saccharification of liquefied starch

Saccharification of liquefied starch was carried out at 60^{0}C for different time intervals using varying concentration (100 - 1000 µl/ 100ml) of Amylo-300 containing amyloglucosidase. The reaction was monitored by the yield of total reducing sugars estimated by dinitrosalicyclic acid method (Miller *et al*., 1959).

Ethanol fermentation

Ethanol production from hydrolysate of pearlmillet flour was studied with *S. cerevisiae.* Inoculum medium for ethanol production contained dextrose (6%), peptone (0.5%), yeast extract (0.5%). Yeast cells pre grown in inoculum medium for 18 h under shaking condition (100 rpm) were centrifuged at 8000 rpm for 15 min and inoculated into hydrolysate at a concentration of 0.5% (w/v) wet weight basis. Flasks were incubated at 30±2^{0}C under stationary conditions and ethanol content was measured at an interval of 24 hr by gas liquid chromatography.

RESULTS AND DISCUSSION

Composition of various pearlmillet hybrids

In the search for alternate substrate for ethanol production, four pearl millet hybrids were evaluated. Composition of raw material plays crucial role in determining its suitability to act as

substrate for ethanol production. The pearl millet hybrids HHB-60, HHB-67, HHB-94 and HHB-146 were found to contain 52.6-56.9% starch, 2.06-2.38% nitrogen and 8.0-8.4% ether extractable matter. Highest starch content was observed in hybrid HHB-67 (Table 1). Similar composition of pearl millet seeds has been reported by various workers (Tou *et al*., 2006) suggesting that starch content in pearl millet is sufficient for its use as substrate for ethanol production.

Standardization of judicial combination of concentration of raw material slurry and hydrolytic enzymes is one of the important aspects of optimization of hydrolysis. Slurries of 45-50%(w/v) pearl millet flour were very thick while loose slurries were obtained at 25-40% concentration and 30% slurry was found optimum for hydrolysis. Aggarwal *et al*. (2001) found 25% pearl millet slurry to be judicious and optimal for liquefaction.

The hydrolysis conditions as prescribed by Jagat Jeet Industry Ltd. (Jalandhar) were applied to pearl millet flour. The Termamyl-300 conc. of 1000 ml/100ml (1.75units/g starch) emerged from optimization experiments conducted in this investigation. It was demonstrated that heat treatment of 90ºC for 30 min. under shaking condition was enough for liquefaction of pearl millet starch.

The optimized concentration of Spirizyme (amyloglucosidase) for pearl millet flour was 750 ml (16.5GA/unit starch) which produced maximum reducing sugars amounting 15.9% (w/v) after 2 hrs (Table 2) however further increase in reaction time and temperature and enzyme concentration did not have appreciable effect on saccharification (Table 3). Dabas *et al*. (1997) obtained maximum saccharification of gelatinized slurry with a combination of α-amylase and glucoamylase at a conc. of 3.5 mg/g starch and 12% reducing sugars were generated in 12 h at 40ºC. Aggarwal *et al*. (2001) used Biotempase and crude glucoamylase from *Aspergillus* sp. NA21 to hydrolyze pearl millet (25% slurry) and attained 90% saccharification under optimum condition.

Table 1. Characterization of pearl millet hybrids for different components

Pearl millet hybrids	% dry weight basis			
	Starch	Nitrogen	Ether extract	Phosphorus
HHB-60	52.6	2.35	8.4	0.68
HHB-67	56.9	2.06	7.8	0.72
HHB-94	53.6	2.16	8.2	0.64
HHB-146	55.4	2.38	8.0	0.52
CD at 0.05%	0.13	0.06	0.19	0.08

Table 2. Optimized conditions for hydrolysis of pearl millet flour

Treatment	Optimum value
Slurry conc.	30%
Liquefaction	
Conc. of enzyme (Termamyl- 300)	1000ml
Temp.	90ºC
Time	30 min
Saccharification	
Conc. of enzyme (Spirizyme)	750ml
Temp.	60ºC
Time	120 min

Ethanol production from hydrolysate of pearl millet flour

Fermentation of hydrolysate of various pearl millet hybrids at 30°C by *S. cerevisiae* at pitching rate of 0.5% (w/v) generated 10.6-11.4%(v/v) ethanol within 36 hrs (Table 4). Maximum 11.4% ethanol (was produced from hydrolysate of HHB-67 containing 15.9% initial sugars suggesting that additional sugars are generated during fermentation also. The phenomenon simultaneous saccharification and fermentation has been reported by various workers [Singh *et al*. (1995), Montesinos and Navarro

Table 3. Saccharification of liquefied starch of different pearl millet hybrid varieties at different time intervals at 60°C

Slurry conc. (%)	Conc. of Spirizyme (µl)	Treatment time (h)	Total reducing sugar (% w/v) HHB-60	HHB-67	HHB-94	HHB-146	Mean (A)
30	750	1	10.58	11.99	11.32	11.84	11.43
		2	14.95	15.89	15.21	15.36	15.35
		3	14.98	15.99	15.21	15.68	15.41
		4	15.87	16.05	15.26	15.76	15.73
Mean (B)			14.09	14.98	14.25	14.61	
Factor		CD	SE(d)	SE(m)			
A (Time)	=	0.08	0.04	0.03			
B (Sugar)	=	0.08	0.04	0.03			
AxB	=	0.16	0.08	0.05			

Table 4. Ethanol production from different pearl millet hybrids by *S. cerevisiae* at 0.5 % inoculum concentration

Pearl millet hybrids	Ethanol (% v/v) 12 h	24 h	36 h	48 h	Mean (A)
HHB-60	6.7	10.0	10.6	10.7	9.5
HHB-67	7.3	10.7	11.4	11.4	10.2
HHB-94	6.9	10.1	10.8	10.8	9.6
HHB-146	7.2	10.5	11.2	11.2	10.0
Mean (B)	7.0	10.3	11.0	11.0	
Factor		CD	SE(d)	SE(m)	
A (Hybrid)	=	0.13	0.06	0.04	
B (Time)	=	0.13	0.06	0.04	
AxB	=	NS	0.13	0.09	

Table 5. Ethanol production from different pearl millet hybrids by *S. cerevisiae* at different temperatures

Pearl millet hybrids	Ethanol (% v/v) 24 h 30ºC	35ºC	40ºC	Mean	48 h 30ºC	35ºC	40ºC	Mean
HHB-60	10.0	10.1	7.9	9.33	10.7	10.7	8.9	10.1
HHB-67	10.7	10.6	8.6	9.97	11.4	11.5	9.5	10.8
HHB-94	10.1	10.2	8.2	9.50	10.8	10.8	9.1	10.2
HHB-146	10.5	10.5	8.4	9.80	11.2	11.3	9.1	10.5
Mean	10.3	10.35	8.3		11.0	11.1	9.1	
Factor		CD	SE(d)	SE(m)	CD	SE(d)	SE(m)	
A (Hybrid	=	0.12	0.06	0.04	0.16	0.08	0.06	
B (Temp.)	=	0.10	0.05	0.03	0.14	0.07	0.05	
AxB	=	NS	0.10	0.07	NS	0.14	0.09	

Table 6. Effect of supplementation of urea, peptone and yeast extract on ethanol production from hydrolysate of different pearl millet hybrids

Supplement	Concentration	Ethanol % (v/v)			
	(% w/v)	HHB-60	HHB-67	HHB-94	HHB-146
Urea	Control	10.7	11.4	10.8	11.2
	0.10	10.6	11.4	10.6	11.4
	0.20	10.8	11.6	10.8	11.6
	0.30	10.6	11.5	10.6	11.5
Peptone	0.00	10.7	11.4	10.7	11.4
	0.10	10.8	11.6	10.8	11.5
	0.20	10.9	11.8	10.9	11.7
	0.30	10.8	11.7	10.8	11.6
Yeast extract	0.00	10.7	11.4	10.7	11.4
	0.10	10.7	11.5	10.7	11.4
	0.20	10.9	11.7	10.9	11.8
	0.30	10.9	11.5	10.9	11.6

(2000), Alfani *et al*. (2000)]. Wang *et al*. (2006) obtained ethanol yield from pearl millet ranging between 8.7-16.8% (v/v) at dry mass conc. of 20-35% with 90-95.6% fermentation efficiency.

For maximum ethanol production, a temperature of 30-35ºC is reported to be optimum for *S. cerevisiae* and *Zymomonas mobilis* (Bansal and Singh, 2003; Wang *et al*., 2006). In the present study, maximum value of ethanol was obtained at 30 and 35ºC after 36 h and further increase in temperature to 40^0C decreased ethanol production (Table 5).

Addition of nutrient such as urea, yeast extract and peptone have been known to play a vital role in boosting ethanol production and its rate (Fundora *et al*., 2000). Interestingly in the present investigation, the response to addition of nutrient into pearl millet was poor (Table 6). This suggests that pearl millet as such may be a good substrate for growth and ethanol production by *S. cerevisiae*. Pearl millet grains are rich not only in starch but also, protein, vitamins, lipids and minerals and thus is capable of supporting growth of yeast.

CONCLUSION

The results of present study indicate that significant amount of ethanol can be produced by fermentation of pearl millet starch after liquefaction and saccharification. Although this study was conducted only on laboratory scale but the information available from this work can be scaled up. An experiment on pilot scale shall help in using the technology at industrial scale.

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