RESEARCH PAPER

Asian Journal of Bio Science (April, 2010) Vol. 5 Issue 1 : 73-76

Ethanol production by *Saccharomyces cerevisiae* from wheat and rice bran hydrolysates of *Aspergillus flavus, A. niger* and *Trichoderma viride*

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(Accepted : February, 2010)

The objective of this work was to study the potential effect of three fungal xylanase enzymes for ethanol production. The selection of *Saccharomyces cerevisiae* strains to ferment sugars obtained from the wheat bran and rice bran at temperatures above 35^0C with high ethanol yield has become a necessity. In this work *S.cerevisiae* strains were screened for their ability to grow and ferment xylose in the culture filterate produced by *Aspergillus flavus,A.niger* and *Trichoderma viride*.The results obtained from this study showed that the wheat/rice bran possessed an excellent potential for agro-residue based ethanol production.

Key words : Wheat bran, Rice bran, Ethanol, Yeast, Fermentation

INTRODUCTION

Lignocellulosic biomass can be used to produce ethanol, a liquid biofuel that can replace fossil transportation fuels (such as gasoline). Bioethanol is already used in several countries for e.g. Brazil, USA and Sweden either pure or as a blend with gasoline. The bioethanol used today is mainly produced from lignocellulosic biomass. The process for ethanol production from biomass is more complicated than producing it from sugar or starch. Processes to obtain ethanol from lignocellulose based on enzymatic hydrolysis are promising methods to produce bio-fuel with low cost (Gonsalves, 2006).

Wheat bran and rice bran are major raw material used in many industries. The substrates left over from the production processes are abundant and still contain a high amount of carbon content. Mostly these two brans can be used as animal feed due to its high content of protein and other nutrients which are necessary for animal growth. Use of wheat and rice bran as raw material in ethanol production not only reduces waste material but also lowers the cost of ethanol production.

Several studies were made by researchers and shown that higher ethanol yields could be obtained in simultaneous saccharification and fermentation (SSF) processes compared with separate hydrolysis and fermentation (SHF). *Saccharomyces cerevisiae* is used widely and traditionally for industrial ethanol production because of its ability to produce high concentrations of ethanol from sugars and because of its high tolerance to ethanol and other inhibitory compounds.

Two technologies used to convert cellulose and hemicellulose to fuel ethanol are acid and enzymatic hydrolysis. The most common is acid hydrolysis.

Acidic hydrolysis is an effective method used for raw material pretreatment in ethanol production. Although acids are powerful agents used for biomass hydrolysis, concentrated acids are toxic, erosive and hazardous. Handling high concentrations of acid requires reactors that are resistant to erosion in raw material pretreatment. Diluted acid hydrolysis has been successfully developed for pretreatment of cellulose materials. Diluted sulphuric acid (H_2SO_4) can achieve significant results.

Another method of hydrolysis is enzymatic hydrolysis. Enzymes are naturally occurring plant proteins that cause certain chemical reaction to occur. However, for enzyme to work, they must obtain access to the molecules to be hydrolyzed (Gray *et al.*, 2006).

In this present paper, utilization of xylanase containing xylose produced from *Aspergillus flavus, A.niger and Trichoderma viride* was used as the substrate for the growth of *Saccharomyces cerevisiae* and production of bioethanol.

MATERIALS AND METHODS

Yeast strain and media :

The yeast used in these studies was *Saccharomyces cerevisiae* known as bakers yeast was purchased from Sakthi Sugars, Coimbatore (T.N.). The yeast culture was maintained in medium contained 20g of glucose, 20g of agar, 5g of peptone, and 5g of $MgSO_4.7H_2O$ per liter. The growth medium utilized in the liquid inoculation contained 50g glucose, 5g of yeast extract, 1g of KH_2PO_4, 0.3 g of NH_4Cl and 2g of $MgSO_4.7H_2O$ per liter.

Inoculum preparation :

Cell suspension (10ml) prepared from 2 days old slant culture was inoculated into 100ml of medium and incubated at 30^0C for 48h on a rotary shaker. The cells were then collected by centrifugation. The inoculum concentration of 0.1 per cent (dry weight/volume) was used for the fermentation process.

Production of ethanol from agro residues (Wheat / rice bran):

Ethanol production was carried out in two steps (1) Saccharification of agro residues by fungi or fungal enzymes (2). Fermentation of sugar rich hydrolysates obtained from the saccharification process by *Saccharomyces cerevisiae*.

Saccharification :

The agro-residues wheat bran and rice bran rich in lignocellulosics was used as substrate for ethanol production.

Substrate pretreatment :

The substrate was pretreated with 1N NaOH for 1h at 100^0C.The pretreated substrate was washed thoroughly with distilled water, dried at room temperature and stored in a airtight container.

Saccharifying enzyme production:

Carter and Bull(1969) medium amended with pretreated substrate (1% w/v was inoculated with fungal spore suspension (10%v/v) having a concentration of 10^6 spores/ml and incubated in orbital shaker (125 rpm) at 30^0C for 5 days. After 5 days, the culture broth was filtered and centrifuged at 10,000 rpm for 20 min at 4^0C.The clear supernatant was used as saccharifying enzyme and analyzed for xylanase activity.

Saccharification of substrate :

The enzyme preparation was added to the pretreated substrate suspended in 0.05m, pH 5.0, sodium citrate buffer (1%w/v) at a concentration of 5.26 IU xylanase activity per ml of suspension and incubated at 50^0C for 72 h at 100 rpm. After enzyme addition the yeast inoculum from the selected strain was added.

Fermentation process :

Separate hydrolysis and fermentation process :

In this process, the hydrolysates obtained from saccharification processes were amended with the components of the production medium except that wheat/ rice bran and fermented by *Saccharomyces cerevisiae* (0.1% w/v) for 72 h at 30^0C. Ethanol produced was estimated at regular time intervals of 12h by the method of Caputi *et al*. (1968).

Simultaneous saccharification and fermentation :

In this process, the saccharification and fermentation were performed in the same vessel at 30^0C. Samples were analyzed for ethanol production at regular time intervals of 12 h for 72 h.The theoretical SSF yield was calculated by assuming that all the potential xylose in the pretreated material was available for fermentation. The experiments were performed in triplicate.

Ethanol estimation :

An a liquot volume (1ml) of the sample was made up to 25 ml and distilled at 78^0C.The distillate 3ml was collected in a flask containing 25 ml of chromic acid and made up to 50 ml of chromic acid and made up to 50ml and kept in a water bath at 60^0C for 30min. After 30 min, the contents were cooled and the colour intensity was measured at 600nm in calorimeter. Calibration curve was drawn using ethanol as standard.

Ethanol yield :

In separate saccharification and fermentation process, while testing the fermentation broth for ethanol content, the initial and final residual reducing sugar content of the broth was determined (Miller, 1959).The difference between the initial and final reducing sugar content was interpreted as reducing sugars utilized (RSU).The ethanol yield was then calculated by the modified formula proposed by Gunasekaran and Kamini (1991).

$$\textbf{Etanol yield} = \frac{\textbf{Ethanol produced x 100}}{\textbf{Reducing sugar utilized}}$$

GC Chromatographic analysis :

GG analysis of fermentation broth to estimate the ethanol content was carried out using GC instrument with FID detector. This experiment was carried out under the following operating conditions.

Instrument used: Aglient technologies:GC6890N, Detector: FID

Temp. ramp:

45^0C-4 min; 10-100^0C-1minhot, 15-200^0C-10min, Total run time: 29.17sec, Injection volume: 0.2 ul, Column used: DB 5ms.30mx0.25 midx 0.2µ flim thickness.

RESULTS AND DISCUSSION

Enzymatic hydrolysis performed separately from

Table 1 : Ethanol production by *Saccharomyces cerevisiae* from wheat bran/rice bran hydrolysates of *A. flavus, A.niger* and *T.viride* (Separate hydrolysis and fermentation)

Incubation period (h)	*A. flavus*			*A. niger*			*T. viride*		
	RSU	EP	EY (%)	RSU	EP	EY (%)	RSU	EP	EY (%)
12	0.000	0.00	0.000	0.000	0.00	0.000	0.000	0.00	0.000
24	3.212	0.05	1.556	2.212	0.04	1.808	3.142	0.06	1.909
48	4.012	1.57	39.132	3.561	1.65	46.335	3.124	1.59	50.896
72	3.912	1.62	41.411	4.512	1.25	27.703	4.563	1.55	33..968
96	2.212	0.91	41.131	3.124	0.85	27.208	2.123	1.23	57.936
144	2.014	0.21	10.427	2.145	0.31	14.452	1.236	0.54	43.689

Initial reducing sugar content of the hydrolysate (g/l)
Aspergillus flavus = 4.526 *A. niger* = 4.365 *Trichoderma viride* = 3.962
The values are means of three replicates; RSU - Reducing sugar utilized (g/l);
EP - Ethanol production (g/l); EY - Ethanol yield (%)

fermentation step is known as separation and fermentation (Sreenath *et al.*, 2001; Wingren *et al.*, 2003). In the present study hydrolysates obtained by *A.flavus* enzyme was 3.212g/L, in *A.niger* hydrolysates it was 2.212 g/L and it was 3.142g/L in *T.viride.*Ethanol yield in these hydrolysates were 41.411, 46.335 and 57.936%, respectively (Table 1). These results revealed that the *T.viride* hydrolysates was good for saccharification of agro-industrial wastes. The culture filterate was analyzed in Gas chromatography (GC) to find out the percentage of ethanol by comparing it with standard ethanol. The results showed 0.417 % of ethanol in *A.flavus*, 0.280 % in *A.niger* and 0.381% in *T.viride*. In these results *A.flavus* was found to produce maximum percentage of ethanol.

Hari Krishna *et al.*(2001) have reported ethanol yield of 2-2.5 % (w/v) in 72h SSF of lignocellulosic wastes with thermo tolerant yeast at 10 % (w/v) initial substrate concentration. Microwave alkali pretreated straw yielded 25.8gl^{-1} ethanol with a yield of 57.5% and alkali pretreated straw yielded 23.7gl^{-1} with a yield of 0.35g/g cellulose under anaerobic conditions (Panagiotou *et al.*, 2005). Addition of xylanase along with cellulase resulted in synergetic effect on ethanol production in SSCF (Simultaneous saccharification and co-fermentation) using SAA-treated barley hull and recombinant *E. coli* (KO11). With 3% w/v glucan loading and 4 ml of xylanase enzyme loadings, the SSCF of the SAA treated barley hull resulted 24.1 g/l ethanol concentration at 15 FPU cellulase/g-glucan loading, which corresponds to 89.4% of the maximum theoretical yield based on glucan and xylan (Kim *et al.*, 2008).

In the present study, the pretreated wheat and rice bran were subjected to simultaneous saccharification (with fungal enzymes) and fermentation (with *S.cerevisiae*). The reducing sugars obtained from saccharification process were 3.66 mg/ml in *Aspergillus flavus*, 2.56 mg/ml in *Aspergillus niger* and 6.15 mg/ml in *Trichoderma viride*. In this SSF process 0.1789 g/g of ethanol was obtained after 48h of fermentation in *Aspergillus flavus*. Next to *A. flavus, Aspergillus niger* yielded 0.1665 g/g ethanol; *Trichoderma viride* enzyme yielded 0.1854 g/g ethanol (Table 2). The results of GC analysis showed 0.74% in *Aspergillus flavus*, 0.49% in *Aspergillus niger* and 1.61% in *Trichoderma viride*. In these results *Trichoderma viride* was found to produce maximum percentage of ethanol.

Table 2 : Ethanol production from wheat bran/rice by simultaneous saccharification and fermentation by *Aspergillus flavus, Aspergillus niger* and *Trichoderma viride* (SSF)

Incubation period(h)	*A. flavus*		*A. niger*		*T. viride*	
	EP	EPS	EP	EPS	EP	EPS
12	0.000	0.0000	0.000	0.0000	0.000	0.0000
24	0.010	0.0010	0.023	0.0023	0.020	0.0020
48	0.254	0.0254	0.356	0.0356	0.342	0.0342
72	1.789	0.1789	1.665	0.1665	1.854	0.1854
96	1.632	0.1632	1.654	0.1654	1.356	0.1356
144	1.632	0.1632	1.654	0.1654	1.356	0.1356

EPS-Ethanol production g/g substrate; Initial concentration of the substrate 1% (w/v).

From the present study, it can be concluded that the lignocellulosic waste, wheat and rice bran can be used as raw material for ethanol production. The alkali pretreatment of the substrate followed by saccharification and fermentation could yielded good amounts of ethanol.

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