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Fungal Invertase as Aid for Production of Ethanol from Sugarcane Bagasse

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Abstract: The present study is aimed at the production of bio-ethanol from two fungal strains with the addition of the enzyme invertase using sugarcane bagasse as the substrate. Optimization for production of ethanol from sugarcane bagasse was determined by enzymatic saccharification using fungal strains. Enzymatic hydrolysis of sugarcane bagasse and ethanol fermentation from lignocellulosic hydrolysate were investigated by three different methods namely separate hydrolysis and fermentation process, simultaneous saccharification and fermentation and co-culture methods. The enzyme invertase from two different fungal strains were supplemented and the ethanol yield was calculated. Result of the present study report growth optimization of two fungal strains capable of saccharifying sugarcane bagasse. The hydrolyzed material was then fermented with the help of yeast into ethanol. Result of the study reveals the ethanol production by *C. cladosporioides* was higher (48%) after 48 h of fermentation under static condition by using submerged fermentation.

Key words: Invertase, bio-ethanol, saccharification, co-culture, submerged fermentation

INTRODUCTION

Global warming alerts and threats are on the rise due to the utilization of fossil fuels. Alternative fuel sources like bioethanol and biodiesel are being produced to combat against these threats. Industrial alcohol is valuable as it is widely used as solvent, germicide, antifreezer, fuel and chemical raw material. Ethanol is safer to handle, burns cleaner and reduces pollution by significantly decreasing co-quantity in the exhaust system. With the increasing shortage of petrochemicals, ethanol is expected to play a very significant role in future (Pandey *et al*., 1999). The production of ethanol from the fermentation system of continuous culture with cell recycle was also reported (Wyman *et al*., 1992). In order to produce larger quantities of ethanol to cater the present needs, the alcohol can be produced from a number of renewable resources such as saccharine materials, starchy materials, cellulose materials and industrial wastes. Wide variety of the micro organisms like yeast and bacteria are used to ferment the above said materials to produce ethanol (Sulia and Shantharam, 1992). Bioethanol can be produced from a range of cellulose (Arthe *et al*., 2008). Lignocellulosic material is a less expensive source of carbon and includes wheat straw (Doppelbauer *et al*., 1987; Abd El-Nasser *et al*., 1997), sugar cane bagasse (Kawamori *et al*., 1986; Aiello *et al*., 1996), aspen wood (Martin *et al*., 1986), willow (Reczey *et al*., 1996) corn

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cobs and waste news-paper (Maheshwari *et al*., 1994; Chen and Waymann, 1991). Among the agricultural residues, sugarcane bagasse is a substrate of high potential for biotechnological processes. The polymeric material comprises of 44 to 40% cellulose, 24 to 28% hemicellulose and 10 to 14% lignin (Darce *et al*., 1985; Rodrigues *et al*., 2001). Inversion of sugar and fermentation activities of yeasts are quite important for efficient fermentation. The yeast strains must contain adequately balanced invertase and fermentation activities for good production of ethanol. Therefore, the present study aimed in evaluating the production of ethanol with *C. cladosporioides* and *A. fumigatus* using sugarcane bagasse as substrate.

MATERIALS AND METHODS

Organism and Inoculum Preparation

Fungal strains were isolated from soil of sugarcane field Coimbatore, India by dilution plate method. Culture was screened for invertase enzyme production and fungal strains *A. fumigatus* and *C. cladosporioides* selected for the production of invertase was prepared from 4 days old slant culture.

Substrate Pretreatment

The sugarcane bagasse was obtained from the market, Coimbatore and sliced .The sliced pieces were spread on the trays and then sieved which was used as substrate. The lignocellulosic waste sugarcane bagasses were pretreated with 1 N NaOH for 1 h at 100°C. The pretreated substrates were washed thoroughly with distilled water, dried at room temperature and stored in a dessicator.

Enzyme Preparation

The medium used for enzyme production under submerged fermentation comprised of (g L^{-1}): sucrose 20, yeast extract 10, ammonium sulphate 1.0, magnesium sulphate 0.75, potassium dihydrogen phosphate 3.5, pH 5.0. Cultivation was carried out in 250 mL Erlenmeyer flasks each containing 50 mL of sterile medium and inoculated with the fungal culture. After inoculation (10^6 spores mL^{-1}), the flasks were incubated at 30°C for 4 days at 125 rpm. At the end of fermentation, the supernatant was harvested by centrifugation at 10,000 rpm for 10 min (4°C) and was used as crude enzyme extract.

Saccharification of Substrate

The enzyme preparation was added to the pretreated substrates suspended in sodium citrate buffer 0.05 M (1% w/v), pH 5, at a concentration of 1 IU invertase activity per gram of suspension and incubated at 50°C for 24 h. After saccharification, the reducing sugars released were estimated by DNS method and the percent saccharification was calculated by the formula proposed by Tewari *et al*. (1988). The cellulose content was estimated following the method of Updegroff (1969).

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugar formed} \times 0.9 \times 100}{\text{Cellulose content of pretreated substrate}}$$

Fermentation

The hydrolysates obtained after saccharification process were further fermented to ethanol by employing the yeast (*Saccharomyces cerevisiae*) and selected fungal isolates (*A. fumigatus* and *C. cladosporioides*) were maintained on fermentation medium proposed by Barron *et al*. (1995).

The ethanol production was carried out using three steps:

- **Separate hydrolysis and fermentation process:** The hydrolysates obtained from saccharification processes were fermented by *Saccharomyces cerevisiae* (0.1% w/v) for 72 h at 30°C
- **Simultaneous saccharification and fermentation:** In this process, the saccharification and fermentation were performed in the same vessel at 30°C. The saccharifying enzymes (5 IU invertase mL^{-1}) was used for saccharification and the fermentation was carried out using organism, *Saccharomyces cerevisiae* (1 g/100 mL). Samples were analyzed for ethanol production at regular time intervals of 12 h for 72 h. The theoretical yield was calculated by assuming that all the potential invertase in the pretreated material was available for fermentation. The experiments were performed in triplicate
- **Co-culture:** The spore suspension (10^6 mL^{-1}) of the fungi and *Saccharomyces cerevisiae* cells was added simultaneously to the production medium amended with pretreated substrate sugarcane bagasse and incubated at 30°C on a rotary shaker at 125 rpm for a period of 72 h

Estimation of Ethanol

Determination of ethanol content was done by spectrophotometric method (Caputi *et al*., 1968; Patel *et al*., 2007).

Ethanol Yield

The ethanol yield was calculated by the modified formula proposed by Gunasekaran and Kamini (1991).

$$\text{Ethanol yield (\%)} = \frac{\text{Ethanol produced} \times 100}{\text{Reducing sugar utilized}}$$

Statistical Analysis

Data were subjected to analysis of variance by One Way ANOVA using AGRES software and Duncan's Multiple Range Test (DMRT) (Duncan, 1955).

RESULTS AND DISCUSSION

The crude enzyme extract of invertase prepared from two fungal strain *A. fumigatus* and *C. cladosporioides* were added in the fermentation medium for the production of ethanol. The production was carried out in three different method namely separate hydrolysis and fermentation, simultaneous saccharification and fermentation and co-culture methods.

Effect of invertase supplementation is shown in Fig. 1. Invertase supplementation enhanced the production of reducing sugar from sugarcane waste with time.

Saccharification of sugarcane bagasse and production of reducing sugar at 48 h of incubation was more incase of *C. cladosporioides* when compare to *A. fumigatus*. Results are mean of three independent determinations. Lines correspond to standard deviation.

Ethanol yield of the hydrolysates obtained from two isolates *A. fumigatus* and *C. cladosporioides* were 33.96 and 48.59%, respectively (Table 1) by separate hydrolysis and fermentation method in 48 h. Krishna *et al.* (2001) have reported ethanol yield of 2-2.5% (w/v) in 72 h SSF of lignocellulosic wastes with thermo tolerant yeast at 10% (w/v) initial substrate concentration. Microwave alkali pretreated straw yielded 25.8 g L^{-1} ethanol with a yield

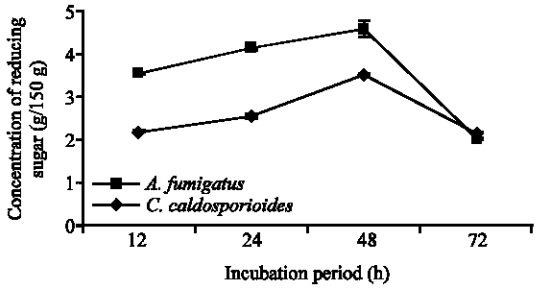

Fig. 1: The effect of invertase supplementation

Table 1: Ethanol production by *saccharomyces cerevisiae* from sugarcane bagasse hydrolysates of *A. fumigatus* and *C. cladosporioides* (separate hydrolysis and fermentation)

	A. fumigatus			*C. cladosporioides*		
Incubation period (h)	RSU	EP	EY (%)	RSU	EP	EY (%)
12	3.52±0.01^{a}	0.09±0.05^{a}	2.579	2.18±0.04^{a}	0.04±0.02^{a}	1.808
24	4.10±0.08^{b}	1.20±0.33^{ba}	29.68	2.53±0.01^{b}	0.95±0.05^{b}	37.490
48	4.57±0.02^{c}	1.55±1.18^{c}	33.968	3.50±0.18^{c}	1.64±0.03^{c}	48.592
72	2.01±0.01^{d}	0.21±0.16^{da}	10.427	2.15±0.01^{da}	0.31±0.02^{d}	14.452

EP: Ethanol production (g L^{-1}); RSU: Reducing sugar utilized; EY: Ethanol yield (%). Values are Mean±SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis

Table 2: Ethanol production from sugarcane bagasse by simultaneous saccharification and fermentation by *A. fumigatus* and *C. cladosporioides*

	A. fumigatus		*C. cladosporioides*	
Incubation period (h)	EP	EPS	EP	EPS
12	0.02±0.00^{a}	0.00±0.00^{a}	0.05±0.00^{a}	0.01±0.00^{a}
24	0.28±0.02^{b}	0.03±0.00^{b}	0.49±0.00^{b}	0.05±0.00^{b}
48	1.42±0.01^{c}	0.14±0.00^{c}	1.92±0.01^{c}	0.19±0.00^{c}
72	1.32±0.00^{d}	0.13±0.00^{d}	1.83±0.00^{d}	0.18±0.00^{d}

EP: Ethanol production (g L^{-1}); EPS: Ethanol production g g^{-1} substrate. Values are Mean±SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis

of 57.5% and alkali pretreated straw yielded 23.7 g L^{-1} with a yield of 0.35 g g^{-1} cellulose under anaerobic conditions (Panagiotou *et al*., 2005).

The pretreated sugarcane bagasse was subjected to simultaneous saccharification (with fungal enzymes) and fermentation (with *Saccharomyces cerevisiae*). It was observed that in this process, the hydrolytic enzymes produced by the *C. cladosporioides* was effective in hydrolysing the polymeric sugar substrates into simple sugars. Ethanol production was 0.1415 g g^{-1} by *A. fumigatus* and 0.1915 g g^{-1} in the case of *C. cladosporioides* (Table 2). It was very less when compared with the simultaneous Saccharification and fermentation using *Aspergillus niger* and *Saccharomyces cerevisiae* were 6.2 to 6.0 g/L/h for chicory and dahlia inulins by Ohta *et al.* (1993).

In the present study, co-culture technique was employed for ethanol production in which the selected fungal along with *Saccharomyces cerevisiae* were simultaneously inoculated into the production medium, amended with pretreated sugarcane bagasse. In this process, *A. fumigatus* yielded 0.1420 ethanol g g^{-1} substrate after 24 h of incubation; *C. cladosporioides* yielded 0.1865 g g^{-1} of substrate after 24 h of incubation (Table 3). Present result was supported by Gunasekaran and Kamini (1991), who reported that the ethanol production was 1.0 g L^{-1} h from lactose using co-immobilized yeast and *Z. mobilis* in alginate gel.

Table 3: Ethanol production from sugarcane bagasse by co-culture of *A. fumigatus* and *C. cladosporioides*

	A. fumigatus		*C. cladosporioides*	
Incubation period (h)	EP	EPS	EP	EPS
12	0.52±0.00[a]	0.05±0.00[a]	0.85±0.00[a]	0.34±0.01[a]
24	1.32±0.00[b]	0.13±0.00[b]	1.44±0.00[b]	0.14±0.00[b]
48	1.42±0.00[c]	0.14±0.00[c]	1.77±0.00[c]	0.18±0.00[c]
72	1.22±0.00[d]	0.12±0.00[d]	1.43±0.00[d]	0.14±0.00[d]

EP: Ethanol production (g L^{-1}); EPS: Ethanol production g g^{-1} substrate. Values are Mean±SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis

Results from Table 3 show that the ethanol yield was maximum in case of *C. cladosporioides* followed by *A. fumigatus*.

CONCLUSION

Fermentation of sugarcane bagasse at pH 5.0 in the presence of invertase effectively increased the ethanol yield. Batch fermentation of sugarcane bagasse by *A. fumigatus* and *C. cladosporioides* produced 33.968 and 48.592% ethanol yield in 48 h fermentation respectively. According to these result the sugarcane bagasse could be known as alternative substrate to be used for biotechnical purposes for ethanol production and among two organisms the ethanol productivity by *C. cladosporioides* is better than the *A. fumigatus* by Simultaneous saccharification.

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