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ORIGINAL ARTICLE

Shake-flask production of bioethanol from corn cobs by a two step process using moulds and yeasts

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ABSTRACT
Production of bioethanol from agricultural wastes is a significant initiative to meet the increasing demand for alternative fuel. The cellulose, hemicellulose and lignin residues of agronomic wastes can be utilized to yield bioethanol. The present research examines the suitability of corn (Zea mays L.) cob for bioethanol production. Bioethanol production involved two steps- hydrolysis of the lignocellulosic content of corn cob into fermentable sugars, followed by ethanolic fermentation by Saccharomyces cerevisiae and Pichia stipitis. Corn cobs were initially pre-treated using heat, acid, alkali and acid-alkali combination, respectively. The pre-treated corn cobs were subjected to solid state fermentation employing selected fungi with cellulolytic traits. The reducing sugars obtained were estimated by dinitro salicylic acid method and subjected to ethanolic fermentation by yeasts. The amount of ethanol produced was estimated. The cellulolytic fungi were identified as Trichoderma reesei and Aspergillus niger. Fermentation of the available reducing sugars by yeast co-culture resulted in ethanol production ranging between 10.10%-14.12% (v/v). Thus pre-treatment of corn cob may prove to be an effective means of obtaining fermentable sugars and can be utilized industrially to produce bioethanol by using different yeasts.
Keywords: *Solid state fermentation, cellulases, cellulolytic fungi, bioethanol, yeasts*

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INTRODUCTION

Due to depletion of fossil fuels such as petroleum, coal and natural gas, there is growing concern regarding the availability of these natural resources in future [1]. Ethanol derived from a renewable biological source is an attractive alternative, as it can be blended with gasoline/petrol and used with no or very little alteration to existing engines [2]. Most of the agricultural residues available all over the world consist of lignocellulosic residues. The lignocellulosic biomass (LB) is suited for bioethanol production and can be pre-treated physically and chemically followed by enzyme treatment to obtain fermentable sugars to be used in bioethanol production [3, 4].

Cellulose is the principal component of LB and the degree of polymerization and crystallinity of cellulose varies from species to species. This is known to have a significant impact on hydrolytic process [3]. Lignin (20 to 35% of LB) acts as the cementing agent and is an impermeable barrier against enzymatic attack on cellulose [5].

In this research, an attempt was made to use efficient cellulolytic fungi for hydrolyzing LB under solid state fermentation (SSF), a cost effective approach adopted for obtaining enzymes**,** followed by ethanol fermentation using *Saccharomyces cerevisiae* and *Pichia stipitis*.

MATERIALS AND METHODS

Processing of the substrate

Corn cobs were collected from corn vendors and corn sifting units around Bangalore. The samples were air-dried, cut into small pieces and ground with a blender into a fine powder. The powder was weighed and autoclaved for 20 min at 15 psi.

Isolation and screening of microorganisms from wood chips

Fungi were isolated from wood chips by spreading on potato dextrose agar (PDA) plates and identified based on their macroscopic and microscopic characteristics. Fungal forms isolated were screened for cellulolytic activity. Cellulase activity was determined by filter paper activity (FPA) and expressed as filter paper unit (FPU) [6]. The selected cellulolytic fungi were identified as *Trichoderma reesei* and *Aspergillus niger* and designated as *T. reesei* JUBT0014 and *A. niger* JUBT0015.

Pre-treatment of substrate

Heat treatment

The samples were heat hydrolyzed by maintaining the temperature at 110 -120°C in an incubator for a period of 15 min [7].

Acid hydrolysis

The substrate was treated with 2.5% H_2SO_4 (v/v) which gives best result according to Suhardi et al [8] and incubated for 1 h at 120°C-130°C. The contents were neutralized with 5% (w/v) calcium hydroxide and dried in a hot air oven at 50°C.

Alkali hydrolysis

The substrate was autoclaved and treated with 0.2 M NaOH for 4 hours at room temperature (~ 30 °C). The contents were neutralized with 3% HCl till the pH became 6.0-6.5.

Acid and alkali hydrolysis

The substrate was first treated with 2.5% H_2SO_4 for 1 h at 120-130 °C and the contents were subjected to 0.2 M NaOH for 4 h at room temperature (~ 30 °C). The contents were neutralized with 3% HCl.

Inoculation and incubation of substrate

Spore suspension was prepared by taking loopful fungal spores in 10 ml of physiological saline. A uniform spore suspension was obtained by mixing vigorously and absorbance measured under white light. Fungal spore suspension of *T. reesei* JUBT0014 and *A. niger* JUBT0015 (1 ml each) were inoculated to the pre-treated substrate, followed by incubation at room temperature for four days (incubating longer than 4 days resulted in loss of reducing sugars due to fungus utilization).

Estimation of reducing sugars formed

The amount of reducing sugars and cellulase activity were measured by 3, 5- dinitrosalicylic acid (DNS) method using glucose as standard [9]. One unit of cellulase activity was defined as the amount of enzyme that released one µg of reducing sugar per minute under standard assay conditions.

Ethanolic fermentation

Following inoculation with 5% v/v of *S. cerevisiae* culture (10^6 cells/ml), *Pichia stipitis* (5% v/v) and a mixture of equal amounts of *S. cerevisiae* and *P. stipitis* (10^6 cells/ml), fermentation of the pre-treated and saccharified LB was carried out for a period of 6 days. After the 6^{th} day, the contents were filtered till the solution became clear. The amount of ethanol produced was estimated by using ceric ammonium nitrate method [10].

Statistical Analysis

Each experiment was carried out in triplicate and the data have been graphically presented as mean ± standard deviation (n=3). t-test was performed using graph pad prism software (version 5.03) with heat treated sample as control. The difference between control and experimental samples was found to be significant at p=0.05.

RESULTS AND DISCUSSION

When *T .reesei* JUBT0014 was inoculated, cellulase activity was found to be 0.161 µg/ml/min and reducing sugars produced was 9.215 mg/ml. In case of *A. niger* JUBT0015 inoculation, cellulase activity was found to be 0.153 µg/ml/min and reducing sugars produced was 9.18 mg/ml, whereas in dual culture, the cellulase activity was found to be 0.296 µg/ml/min and reducing sugars produced was 9.277 mg/ml (figures 1 and 2). 6 days of *S. cerevisiae* and *P. stipitis* assisted fermentation of acid pre-treated substrate, resulted in highest alcohol production (14.12%). Data shown in figures 3-5. It was found that there was increase in the

percentage of alcohol when LB was fermented with combination of yeasts i.e., *S. cerevisiae* and *P. stipitis* (figure 4).

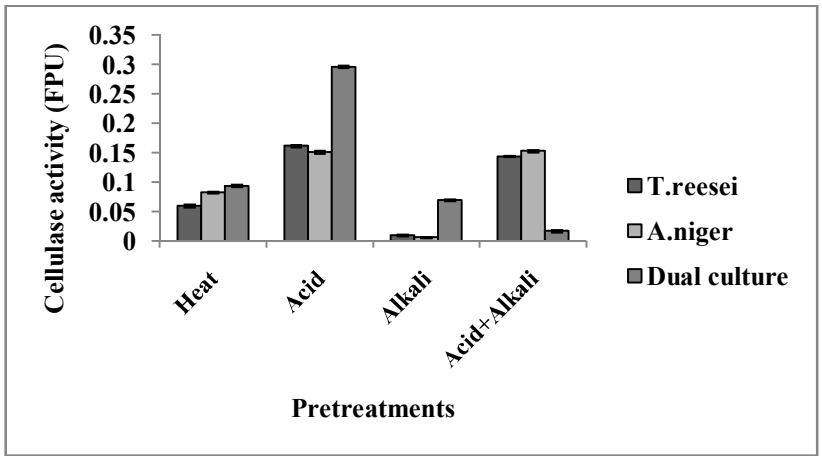

Figure 1: Cellulase production by inoculation of pre-treated substrate with *T.reesei* JUBT0014, *A. niger* JUBT0015 and dual culture, Data represent mean ± (n=3); P<0.05

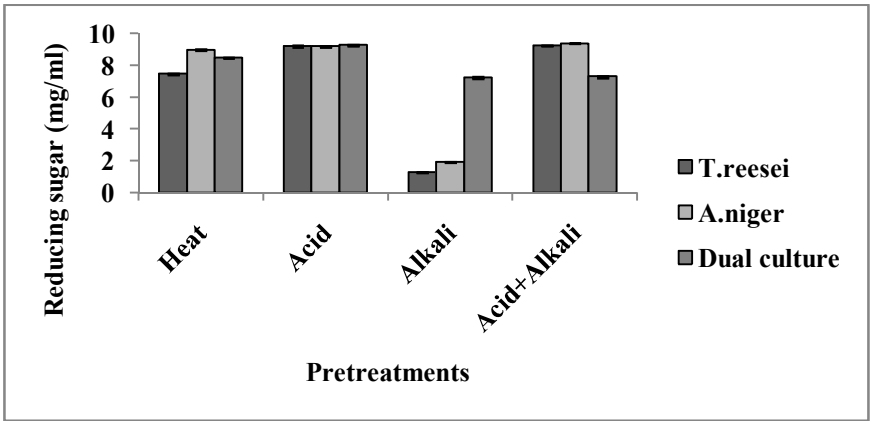

Figure 2: Reducing sugars produced by inoculation of pre-treated substrate with *T. reesei* JUBT0014, *A. niger* JUBT0015 and dual culture, Data represent mean ± (n=3); P<0.05

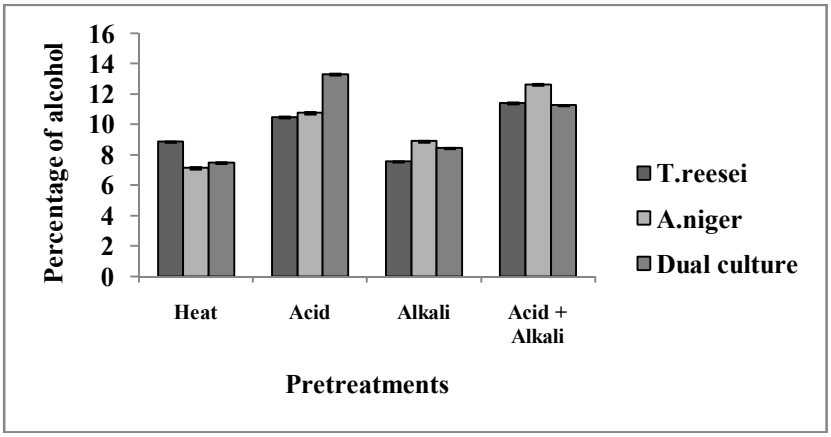

Figure 3: Ethanol production after the substrates were saccharified with *T. reesei* JUBT0014, *A. niger* JUBT0015 and dual culture and fermented with *S. cerevisiae*. Data represent mean ± (n=3); P<0.05

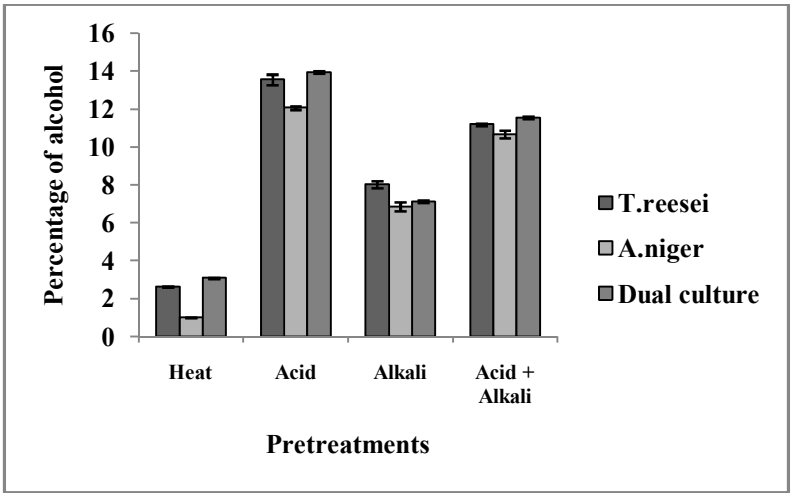

Figure 4: Ethanol production after the substrates were saccharified with *T. reesei* JUBT0014, *A. niger* JUBT0015 and dual culture and fermented with*, P.stipitis*. Data represent mean ± (n=3); P<0.05

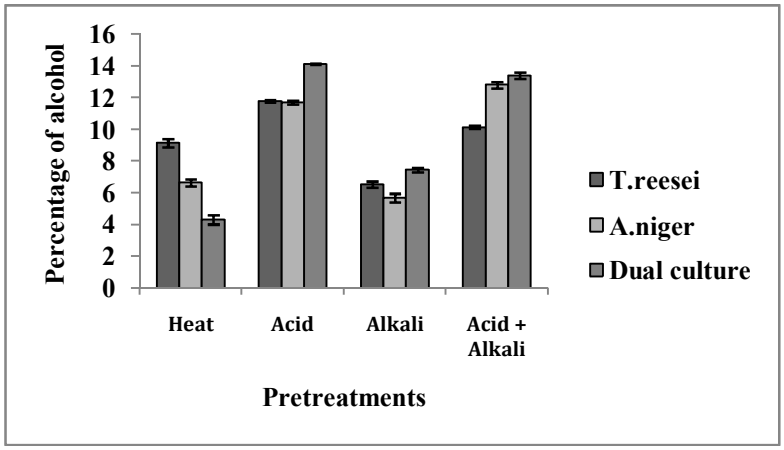

Figure 5: Ethanol production after the substrates were saccharified with *T. reesei* JUBT0014, *A. niger* JUBT0015 and dual culture and fermented also by dual culture of two yeast cultures. Data represent mean ± (n=3); P<0.05

Similar result was reported by Itelima *et al*. [11] where saccharification and fermentation of corn cob to bioethanol involved a co-culture of filamentous fungus *A. niger* and the yeast *S. cerevisiae* to yield 10.08% (v/v) of alcohol. Firoz *et al*. [12] worked on thermotolerant *T. viride* for enzymatic hydrolysis of pre-treated lignocellulosic waste followed by fermentation with *S. cerevisiae* and the percentage of ethanol production was considerably low (1.15%) as compared to present work**.** The probable reason behind this observation could be that LB contains C5 sugars as well as C6 sugars and all the sugars have to convert to reducing sugars so that the amount of bioethanol can be increased [13]. In the present work efforts have been made to increase the percentage of ethanol by using yeasts with the capability to ferment both C5 as well as C6 sugars. Comparing the data with the previous research, it can be understood that better pre-treatment [14] and use of mixed culture of yeasts [15] can improve the ethanol production.

Saliu and Sani [16] used corn cob for ethanol production involving *A. niger* and *Penicillium decumbens* for cellulolytic hydrolysis of alkali pre-treated substrate. When the fermentation was carried out with *S. cerevisiae* along with *A. niger* the percentage of ethanol produced was found to be 2.67% (v/v) and when fermented simultaneously with *S. cerevisiae* and *P. decumbens* the percentage was 0.56% (v/v) which was still less when compared to the present observation.

Ado *et al*. [17] studied the effect of *A. niger* and *S. cerevisiae* co-culture for improvement of ethanol yield from corn cob by fermentation and examined the efficiency of the process to yield ethanol and reduce a

saccharification step. Results indicated that the final yield was significantly lower (4.17%) than that reported in the present study.

The present study resulted in the production of bioethanol to the extent of 14.13% v/v of the final filtrate, which was significantly higher than the ethanol yield reported earlier. Therefore, the materials and methodology used in the present study has better potential for commercial application. Of the total cost of bioethanol production, 20% is the cost of production of fungal enzymes [2]. In the present work, the use of SSF with *T. reesei* JUBT0014 and *A. niger* JUBT0015 has been effective in breakdown of lignocelluloses and further saccharification, thus eliminating the cost of enzyme production.

CONCLUSION

Bioethanol from lignocellulosic biomass may act as alternative fuel. Production of ethanol from corn cob provides dual advantage: reduction in the accumulation of cellulosic waste and effective utilization of agronomic wastes for energy production. Thus C5 and C6 sugars in corn cob can be fermented to ethanol using yeast strains capable of fermenting both hexose and pentose sugars simultaneously which in turn economize ethanol production from lignocellulosic biomass.

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