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Bioethanol Production by *A. Niger* Produced Cellulase by Solid State Fermentation

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Abstract: ***Bioethanol is the most suitable renewable alternative to fossil fuel as its quality constituent match with diesel and petrol. the agro residues (sugarcane bagasse, potato peel and rice bran) were delignified by 2% NaOH and subjected for production of cellulase by Aspergillus niger under solid state fermentation. The highest cellulase activity was attained with sugarcane bagasse at pH 4.5, temperature-30^{0}C and incubation time 96h. The resultant crude enzyme of 5.0 U/ml were applied for saccharification of all pre-treated substrates in 0.1M citrate buffer (pH 4.8) and obtained maximum reducing sugars from sugarcane bagasse at pH 7.0, 40^{0}C temperature and incubation time 120h.Hydrolysates thus obtained were used for ethanol production by inoculating Saccharomyces cerevisiae under different fermentation conditions and observed maximum production with sugarcane bagasse at pH 5.5 after 48 h at 35^{0}C.***

Keywords*:* Bioethanol, Aspergillus niger, Cellulase

1. Introduction

As concern about global warming and dependence on fossil fuels grows, an eco-friendly bioethanol has emerged as the most suitable renewable alternatives to fossil fuel especially when the oil peak is estimated to reach sometime between 1996 and 2035 **(Demirbas, 2008).** It has widespread uses in industries, antifreeze compounds, pharmaceutical rocket and motor fuels etc. **(Zaldivar *et al.* (2001).**With the provision of addition of 5-10 percent of ethanol in petrol and diesel there has been a substantial rise in ethanol production in last few years. Brazil produces the maximum amount of ethanol (15,099 million liters year^{-1}) followed by the U.S. (13,381 million liters year^{-1}), China (3,649 million liters year^{-1}) and India (1,749 million litresyear^{-1}). In India, ethanol is primarily produced from molasses which is a byproduct from sugar mills **(Peterson, 2006).** Ethanol, which is generally expected to be the first major commercial product of emerging cellulosic biofuel technology, has great potential to lessen a country's dependency on fossil fuel **(Lynd *et al.*, 1996).** Cellulosomes are naturally occurring elaborate enzyme complexes found in many microorganisms that can efficiently hydrolyze cellulose based on the high level of enzyme-substrate synergy. An ideal microorganism should possess the capability of efficient enzyme production and simultaneous cellulose saccharification and ethanol fermentation (SSF). *Saccharomyces cerevisiae* is an attractive candidate because of its high ethanol productivity and inherent ethanol tolerance but it lacks amylolytic enzymes and unable to directly convert the starch into ethanol. Therefore, it is necessary to use starch enzymes producing strains in order to get free carbohydrates**.** Among mold species, *Aspergillus niger* is well known for its cellulolytic and amylolytic activities and its ability to produce fermentable sugars from cellulosic waste and achieve a good reducing sugar extraction level **(Zayed and Meyer, 1996).**

The present study focused on a preliminary evaluation of cellulase enzyme production by *Aspergillus niger* on biomass solids after pre-treatment to assess whether this route could enhance performance on inexpensive biomass and reduce enzyme production costs which could be finally utilised for the production of bioethanol.

2. Materials and Methods

Organisms

Pure cultures of *Aspergillus niger* and *Saccharomyces cerevisiae* were maintained on potato dextrose agar (PDA) slants, grown at 30c for 5 days and stored.

Pre-treatment of substrates

Agro residues sugarcane bagasse (SB), rice bran (RB) and potato peel (PP) were finely grinded and treated with 2% alkali (NaOH) to delignify and autoclaved for 1 h at 121^{0}C. The pretreated substrates were allowed to cool, filtered and neutralized by acetic acid and NaOH and then dried at 60 ^{0}C in an oven for 12h.

Inoculum Preparation

The young colonies of *Aspergillus niger* were aseptically picked up and transferred to PDA slants and incubated at 30^{0}C for 72 h for maximum growth. The spores were harvested using sterilized water with 0.1% Tween 80 **(Smith *et al.*, 1996).**

Solid state fermentation

Cellulase was produced using pretreated agro residues (5g) kept separately in a 250 mL Erlenmeyer flasks moistened with 20ml Mandel's medium pH 4.8 [(%w/v): $(NH_4)_2SO_4$, (0.14%); KH_2PO_4 (0.2%); $CaCl_2{\cdot}2H_2O$(0.03%); $MgSO_4{\cdot}7H_2O$ (0.03%); urea (0.03%); peptone (0.075%) and yeast extract (0.025%). Trace mineral stock solution (100X) contained 0.5g/l $FeSO_4$; 0.016 g/ l $MnSO_4$; 0.014 g/ l $ZnSO_4$; 0.37g/ l $CoCl_2$], medium was autoclaved and cooled later 1mL of fungal spore suspension was added. The flasks were then incubated at 30^{0}C for 5 days.

Optimization of process parameters for Cellulase production

Cellulase production was optimized with respect to various environmental parameters:pH (3.5, 4.5, 5.0, 5.5, 6.5, and 7.0); temperature (30, 40, 50 and 60^{o}C) and incubation period (24, 48, 72, 96 and 120 hrs).

Extraction of crude enzyme

The fermented material was mixed with 50 ml of 0.1 M citrate buffer, pH 4.8, the substrates were homogenized on a rotary shaker for 30 minutes at 200 rpm. The solids were removed from the homogenate by filtration. The filtrate was used for analytical studies.

Cellulase Assay

Bioassay (FPase) was carried out according to **Mandel *et al.* (1976)**:0.5 ml crude enzyme and 1.5 ml (0.05 M) citrate buffer pH 4.8 were added to test tubes containing 50 mg of Whatman filter paper No.1.The test tubes were incubated in water bath adjusted at 50^{0}C for 1 h cooled and 3 ml of 3, 5-Dinitrosalicylic acid (DNSA) was added. The tubes were kept on a vigorously boiling water bath for 5 min, cooled, volume of test tubes was increased up to 10 ml by adding distilled water, and then absorbance was read at 540 nm against blank of sugar free sample. One unit of enzyme activity is the one mole of reducing sugar in terms of glucose released per minute.

Estimation of reducing sugars

Reducing sugar was estimated by DNSA (**Miller, 1959):** 0.5 ml of sample was taken from every treatment into test tubes. The volume was made up to 3 ml using distilled water. DNSA, 3 ml, was added to each sample and mixed. The reagent blank contained 3 ml of each distilled water and DNS reagent. All tubes were kept on boiling water bath for 5 minutes, cooled and absorbance was read at 510 nm. The amount of glucose produced was calculated by referring to the standard plot using glucose as the reducing sugar.

Enzymatic hydrolysis of substrates

The reaction mixture contained 5g of treated substrate in 100 ml 0.1 M citrate buffer (pH 4.8) with 5 U/ml of crude enzyme. The pH was adjusted to 4.5 and flasks were incubated on rotary shaker at 50^{0}C, 75 rpm for 24 h. The samples were boiled for two minutes to denature enzyme, centrifuged at 5000 rpm for 15 minute and the supernatants was collected for fermentation.

Solid state fermentation of Bioethanol

The flasks containing the hydrolyzed samples were sterilized at 121^{o}C for15 min, allowed to cool, were aseptically inoculated with 2 ml of 24 h old yeast culture of *S. cerevisiae* and incubated under anaerobic conditions for 7 days. Harvesting was done by centrifuge at 5000 rpm for 20 minute**.**

Process Optimization for Bioethanol production

Fermentation process was optimized at different parameters:pH (3.5, 4.5, 5.0, 5.5, 6.5, and 7.0); temperature (30, 40, 50 and 60^{o}C) and incubation period (24, 48, 72, 96 and 120 hrs).

Bioethanol Purification by fractional distillation

The fermented substrates were filtered and dispensed into round bottom flask fixed to a distillation column enclosed in running tap water. A conical flask was fixed to the other end of the distillation column to collect the distillate. A heating mantle with the temperature adjusted to 78^{o}C for 6-7 hrs was used to heat the round-bottomed flask containing the fermented broth.

3. Results

3.1 Effect of process parameter on cellulase production

a) pH

sugarcane bagasse achieved maximum activity (13.14 U/ml/min) at pH 3.5.Maximum activity (6.23 U/ml/min) was observed with rice bran at pH 5.5 while potato peels released enzyme towards neutral concentration at pH 6.5 and displayed maximum only 3.63 U/ml/min. The variation of pH from the optimum levels causes denaturation of the enzyme and reduces enzyme synthesis ability. **Muhammad *et al.* 2007; Ilyas *et al.* 2011** also recorded the same.

b) Temperature

The maximum activity 8.34 U/ml/min was recorded at 30^{0}C with the use of SB. The optimum temperature for rice bran and potato peel was observed to be 40^{0}C with their respective enzyme activities 5.18 U/ml/min and 3.22 U/ml/min. The activity falls quickly above the optimal temperature which indicates that cellulase is highly sensitive towards temperature. Similar results were obtained by **Sherief *et al.*, 2010; Muhammad *et al.*, 2010.**

c) Incubation period

Cellulase activity was found gradually increased and reached its maximum 14.15 U/ml/min and 7.29 U/ml/min respectively with substrates SB and RB at 96hr of incubation time after that enzyme activity slowly decreased whereas 72h of incubation yielded more activity with PP i.e. 3.62 U/ml/min. Moreover, at 120h of fermentation, the yield of enzyme from SB remained higher than other substrates. **Eyini *et al.* (2004); Singhania *et al.* (2006))** observed the same time period

3.2 Effect of enzymatic hydrolysis on the yield of reducing sugar

The data obtained reveals the positive effect of hydrolysis over the release of reducing sugar.The variations in pH and different sources of nitrogen generated maximum reducing sugar in comparison to the variations in other studied parameters. **(Table 1).**

Table 1: Effect of enzymatic hydrolysis on the yield of reducing sugar

Substrates→ / *Parameters↓*	*Reducing Sugar (mg/ml)*		
pH	*Rice bran*	*Potato peel*	*Sugarcane bagasse*
3.5	0.94	1.12	1.13
4.5	1.04	1.17	1.18
5	1.1	1.02	1.21
5.5	1.12	1.23	1.31
6.5	1.14	1.27	1.35
7	1.16	1.32	1.4
Incubation Period (h)			
24	0.78	0.93	1.1
48	0.83	0.98	1.15
72	0.86	1.05	1.21
96	0.89	1.14	1.24
120	0.9	1.18	1.27
Temperature (^oC)			
25	0.72	0.96	0.99
30	0.94	1	1.02
35	1.04	1.07	1.11
40	1.11	1.12	1.14

3.3 Effect of process parameter on bioethanol production

a) pH

The production gradually increased from pH 3.5 and yielded maximum at pH 5.5 with SB at pH 5.0 with rice bran, at pH 4.5 with potato peel and ensured respective ethanol yield 6%, 4.77% and 5.06% (v/v) which was seen with much consumption of sugars and accorded only 0.349, 0.373 and 0.262 mg/ml reducing sugar respectively at the their pH optima. The result collaborate with **Adarsh *et al.* 2010; Suresh *et al.*, 1999).**

b) Temperature

Data shows a clear alteration in ethanol production with changing temperature. Maximum ethanol production from SB[4.41% (v/v)] and RB[3.58% (v/v)] was obtained at slightly higher temperature i.e. 35^oC whereas PP yielded maximum [3.92% (v/v)] at 30^oC. The best ethanol concentrations were obtained at 30^oC and 35^oC **by Roukas,1994**.

c) Incubation period

After 48 h of incubation, maximum ethanol [4.64% (v/v)] was obtained from SB with reducing sugars consumption (0.219 mg/ml) next best yield [4.42% (v/v)], was recovered after 72 h of fermentation in case of PP with 0.274 mg/ml reducing sugars. RB yielded least [4.02% (v/v)] ethanol, after 96 h of incubation. The ethanol production by sugarcane bagasse was less than that of potato peel and rice bran at 120h.The results were in accordance with **Venkatachalam *et al.*, 2010.**

4. Future Prospect

Sugarcane bagasse is economically best substrate for bioethanol production amongst all the agro-residues used in study. *Aspergillus niger* has the potential to utilize agricultural waste for production of cellulase enzyme in SSF.Production of bioethanol can be obtained on inexpensive and easily available substrate, sugarcane bagasse. This can have important implications for the establishments of a robust and cost effective process in biofuel production at larger scale.

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