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Amylase Production by ASPERGILLUS NIGER for Bioethanol Production under Solid State Fermentation

KEYWORDS: Bioethanol ,Amylase, Aspergillus niger

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ABSTRACT *The delignified agro residues using 2% NaOH (sugarcane bagasse, potato peel and rice bran) were used for amylase production by Aspergillus niger under solid state fermentation. The highest amylase activity was recorded with rice bran at pH 5.5, temperature 400C and incubation time 120h. Resultant crude enzyme of 5.0 U/ml was applied for hydrolysis of all pre-treated substrates in 0.1M sodium acetate buffer (pH 4.8). Hydrolysates thus obtained were used for ethanol production by inoculating Saccharomyces cerevisiae, and maximum bioethanol was recovered after 48 hr of incubation.*

INTRODUCTION

India consumes more than 250 million tones fuels every year and ranked fifth in the world in term of fossil fuel consumption (Shukla, 2005). In India, ethanol is primarily produced from molasses which is a byproduct from sugar mills (Peterson, 2006). The first phase of the ethanol-blended petrol was launched in January 2003. Even at the 5 percent blend level there is a shortfall of 225 million litres of ethanol for the oil companies whose current demand is 435 million litres (Anonymous, 2006). Most targeted alternate source for ethanol production are the agro residues e.g., wheat which contains 35-40 per cent of cellulose shows ethanol yield of 5.3 g/l of hydrolysate (Ahring et al., 1999). Likewise about 95 per cent of cellulose in the pretreated bagasse pulp residue was converted to glucose enzymetically. Fermentation of this hydrolysate with Saccharomyces cerevisiae gave 90 per cent production (Azzam, 1989). Potato represents an attractive feedstock for bioethanol. Ethanol yields from potato tubers are approximately 1400-1800 L/ha (Babu et al. 2010). Since lignocelluloses biomass is naturally recalcitrant to enzymatic hydrolysis, pretreatment is essential to improve its enzyme digestibility and also to obtain solubilized sugar (Tassinari et al., (1980). Amylases are able to hydrolyse the polymer to monomer sugar glucose, which is naturally fermented to ethanol by the yeast Saccharomyces cervisiae. Solid-state fermentation is a technology that allows transforming agro industrial waste into many valuable bioproducts, like ethanol Rodriguez et al., (2010) utilizing different fungal cultures Aspergillus foetidus, Aspergillus niger, Phanerochaete chrysosporium, Trichoderma viridae (Geeta et al., 2002).

The present study focused on production and optimisation of amylase by A. niger on pre-treated substrates which could be employed for bioethanol production.

MATERIALS AND METHODS

Organisms

Pure cultures of Aspergillus niger and Saccharomyces cerevisiae were maintained on potato dextrose agar 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°C. The pretreated substrates were allowed to cool, filtered and neutralized by acetic acid and NaOH and then dried at 60 °C in an oven for 12h.

Inoculums preparation

Vegetative inoculum used was prepared according to the method of Haq et al. (1998).

Solid state fermentation

Amylase was produced by pretreated substrates (5g) with 20 ml of basal salt solution (g/l :KH_2PO_4: 10, $MgSO_4$:2, NaCl :2and $MnSO_4$:0.5) autoclaved at 121°C for 30 min. in 250 ml Erlenmeyer flasks. After cooling, the flasks were inoculated with 1 ml of fungal spore suspension and incubated at 30°C for 4days.

Optimization of process parameter

Enzyme production was carried out with different pH (3.5, 4.5, 5.0, 5.5, 6.5, and 7.0), temperature (30, 40, 50 and 60°C) and incubation period (24, 48, 72, 96 and 120).

Enzyme Extraction

Fermented matter was dissolved in 0.1 M sodium acetate buffer, pH 4.8. The mixture was homogenized for 30 minutes at 200 rpm and filtered through Whatman filter paper No. 1. The filtrate was used as crude enzyme.

Amylase Assay

In a test tube, 1 ml of starch solution and 1 ml of properly diluted crude enzyme was taken. The mixture was incubated at 27°C for 15 min in water bath. The reaction was stopped by 3 ml of 3, 5- Dinitrosalicylic acid. 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 560 nm against blank (Sadasivam and Manickam, 1996). A unit of amylase was expressed as mg of maltose produced during 15 min incubation with 1% starch.

Estimation of reducing sugar

Reducing sugar was estimated by dinitrosalicylic acid (DNSA) (Miller, 1959). :0.5 ml of sample was drawn from every treatment into test tube. The volume was made up to 3 ml with

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distilled water, 3 ml DNSA was added to each 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 optical density 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

Reaction mixture containing 5g SB in 100 ml 0.1 M citrate buffer pH 4.8 was mixed with 5 U/ml of crude enzyme,the pH was adjusted to 4.5. It was incubated on rotary shaker at 50°C, 75 rpm for 24 hrs. After that, samples were boiled for 2 minutes to denature enzyme and then centrifuged at 5000 rpm for 15 minutes. The supernatant was collected.

The starchy substrates, RB and PP were mixed with water and amylase and heated at very high temperature (120-140°C) and then cooled down (90°C). Further glucoamylase was added to convert the liquefied starch to fermentable sugar.

Solid state fermentation of Bioethanol

The hydrolyzed samples in 250ml Erlenmeyer flasks were sterilized at 121°C for15 min, allowed to cool, aseptically inoculated with 2 ml of 24 hr old culture of S. cerevisiae and incubated under anaerobic conditions for 7 days. Harvesting was done by centrifuge at 5000 rpm for 20 minute.

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°C for 6-7 hrs was used to heat the round-bottomed flask containing the fermented broth.

RESULTS

Effect of process parameters on amylase production

pH

RB (13.49 U/ml/min) at pH 5.5 and PP (11.91U/ml/min) at pH 6.5 had shown maximum enzyme activities which were nearly six times maximum activity of SB (2.99 U/ml/min) at ph5.0 (Table 1). The role of pH factor attributed to affect the permeability of cells as well as stability of produced enzyme (Mase et al. 1996). The obtained results are close to the findings of Gupta et al. (2010).

Incubation period

Both RB (14.07 U/ml/min) and PP (13.50 U/ml/min) displayed their maximum production of amylase at 120 h of incubation period. maximum activity of SB (3.74 U/ml/min) at 96 h was even lesser than the minimum activities recorded for PP (8.61 U/ml/min) and RB (9.34 U/ml/min) (Table 1). Monga et al. (2011) also obtained the similar results.

Temperature

There was no sharp decline or increase activity recorded for varied temperature 30-60°C for any of the substrate. RB (13.37 U/ml/min) at 40°C, potato peel (12.77 U/ml/min) at 50°C and SB (3.87 U/ml/min) at 60°C produced maximum enzyme. Here also, minimum activities from RB (11.54 U/ml/min) and PP (10.81 U/ml/min) were about double activity than SB (Table 1). The result obtained were in accordance with Nimbkar et al. (2010); Suganthi et al. (2010).

Table 1. Effect of different parameters on amylase production

pH / Substrate	AMYLASE ACTIVITY (U/ml/min)					
	3.5	4.5	5.0	5.5	6.5	7.0
RB	10.27	10.82	11.55	13.49	11.64	9.23
PP	8.36	8.93	10.48	10.94	11.91	9.76
SB	1.28	1.75	2.99	2.54	1.80	1.13
Incubation period (h) / Substrate	24	48	72	96	120	
RB	9.34	11.26	12.15	12.73	14.07	
PP	8.61	9.98	10.78	11.93	13.50	
SB	2.15	2.69	3.26	3.74	3.27	
Temperature (°C) / Substrate	30	40	50	60		
RB	12.59	13.37	11.54	12.60		
PP	10.85	12.54	12.77	10.81		
SB	1.55	2.91	3.49	3.87		

Ethanol Production

SB [6.11% (v/v)] with concentration of reducing sugars (0.282 mg/ml) resulted in maximum ethanol production after 48 h of incubation whereas PP [5.10% (v/v)] with (0.197 mg/ml) reducing sugars yielded next best production at 72 hr. Minimum ethanol was recovered after 96 h of fermentation in case of RB [3.59% (v/v)] the amount of reducing sugars was (0.273 mg/ml). Current results showed the concentration of reducing sugar goes down consistently during the first 72h of fermentation. This was due to rapid increase in ethanol at the same time. (Ado et al., 2009; Behra et al., 2010).

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