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Cellulase Production and Enzymatic Hydrolysis of Some Selected Local Lignocellulosic Substrates by a Strain of *Aspergillus niger*

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Abstract: The ability of selected cellulosic substrates to induce cellulase enzyme production by *Aspergillus niger* and for the ability of the induced enzyme to saccharify the substrates was assessed. The cellulosic substrates tested were filter paper, absorbent cotton wool, newspaper, corn cobs, groundnut shell and saw dust. Treated and untreated corn cob and groundnut shell were the best carbon sources for the induction of cellulolytic enzymes while filter paper was a poor carbon source. Enzyme activity was highest at 2% substrate concentration. Hydrolysis of the cellulosic substrates showed that the most resistant substrates were cotton wool and filter paper which produced 1.90 and 2.25% saccharification respectively. Treated corn cob gave the highest saccharification rate of 5.0% after 48 h. Reducing sugar yield ranged from 0.75 mg mL^{-1} for filter paper at 1 h to 5.55 mg mL^{-1} for treated corn cob at 48 h.

Key words: *Aspergillus niger*, cellulosic substrates, cellulolytic enzymes, saccharification

INTRODUCTION

Cellulose represents the major constituent of plant cell wall polysaccharide and consists of linear β-1,4-linked D-glucopyranoside chains that are condensed into crystalline structures called microfibrils. In addition to the crystalline structure, cellulose contains non-crystalline (amorphous) regions within the microfibrils (de Vries and Visser, 2001). In a few cases such as cotton balls, cellulose is present in a nearly pure state. In most cases, however, the cellulose fibres are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin (Lynd *et al.*, 1999). The three classes of enzymes involved in the biodegradation of cellulose are endoglucanases (EC 3.2.1.4) which hydrolyze cellulose to glucooligosaccharides; exoglucanases including cellobiohydrolases (EC 3.2.1.91) release cellobiose from crystalline cellulose and β-glucosidases (EC 3.2.1.21) which degrade oligosaccharides to glucose (Lynd *et al.*, 2002).

Microbial utilization of the inexhaustible cellulosic biomass for the production of industrial chemicals such as ethylene, butadiene, hydroxymethyl furfural and levulinic acid, protein-rich food and feeds and preparation of cellulose polymers will help to meet energy and food demands (Cowling and Kirk, 1976; Ghosh and Singh, 1993).

Native cellulose is very resistant to microbial attack due to its high crystalline structure and the presence of lignin which reduces its susceptibility to attack by microorganisms (Garg and Neelakantan, 1981). Degree of polymerization, admixture with impurities and specific surface area also interfere with the mode of interaction between enzymes and cellulose molecules (Wood and Saddler, 1988). Pretreatment of cellulose can enhance its physical configuration or decrease the degree of polymerization (Maheshwari *et al.*, 1993).

The genus *Aspergillus* is a group of filamentous fungi with a large number of species. The black aspergilli have a number of characteristics which make them ideal organisms for industrial applications, such as good fermentation capabilities and high levels of protein secretion. In particular, the wide range of enzymes produced by *Aspergillus* for the degradation of plant cell wall polysaccharides is of major importance to the food and feed industry (de Vries and Visser, 2001).

This study reports the cellulase induction by a strain of *Aspergillus niger* and its saccharification of some local cellulosic substrates for sugar production.

MATERIALS AND METHODS

Microorganism: Aspergillus niger was selected from strains obtained from composite soil samples in domestic refuse dumps in Yola, Northern Nigeria. Identification of the isolate was by reference to Barnett (1960) and Domsch *et al.* (1980) Pure cultures were maintained on Potato Dextrose Agar (Oxoid) slants by subculturing every four weeks.

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Substrates: Whatman No. 1 filter paper and absorbent cotton wool served as pure cellulosic substrates, while newspaper, corn cob, groundnut shell and saw dust served as complex cellulosic substrates. The saw dust, groundnut shell, corn cob and newspaper were obtained locally.

Pretreatment of substrates: Corn cobs, groundnut shell and saw dust were chopped into small pieces of 2 cm length with the help of a cutter, washed thoroughly to remove surface dust and then dried at 65°C in an incubator. Twenty grams of the dried substrates was autoclaved for 1 h at 121°C with 100 mL 0.25 M NaOH and then neutralized with 0.25 M HCl. The residue were washed with distilled water and then dried at 65°C (Singh *et al*., 1988).

Cellulase induction: The basal medium used for cellulase production was modified medium of (Deacon, 1995) containing (in g L^{-1}) Yeast extract, 2.0; $NaNO_3$, 5.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5 and $FeCl_3$, 0.001. The substrates were added at 0.5, 1.0 or 2.0% concentration. Twenty five millilitres of the basal medium and substrate were placed in a flask and sterilized by autoclaving. The flasks were inoculated with three 4 mm discs of 5 day-old culture of *Aspergillus niger* and incubated at 30°C for 7 days. Thereafter the contents were filtered through Whatman No. 1 filter paper and the filtrate used for endoglucanase, exoglucanase and filter paper activity.

Enzyme assay: Endoglucanase activity was determined according to the method of Mandels and Weber, (1969). Half millilitre of 1% Carboxy Methyl Cellulose (CMC) in 0.1 M citrate buffer (pH 5.5) was placed in a test tube and 0.5 mL of culture filtrate added. The reaction mixture was incubated at 30°C for 1 h and the reaction terminated by adding 1 mL 3,5-Dinitrosalicylic Acid (DNSA) reagent. The tubes were heated at 100°C in boiling water bath for 15 min and then cooled at room temperature. The final volume was brought to 12 mL by addition of 10 mL-distilled water. The absorbance was read at 540 nm.

Filter paper Activity (FPA) was estimated by the method of (Mandels *et al*. (1976). This was done by placing 0.5 mL of culture filtrate and 1.0 mL citrate buffer pH5.0 in a test tube. A 1×6cm filter paper strip (50 mg) was added and the tube incubated at 50°C for 1 h. Then 3 mL DNSA reagent was added and the mixture boiled for 15 min to terminate the reaction. Ten milliliters of distilled water was added and absorbance read at 540 nm.

β-glucosidase activity was determined by mixing 1 mL of the culture filtrate with 1 mL of 0.1 M citrate buffer (pH 5.0) containing 5 mg cellobiose (Fisher Scientific Co.). The mixture was incubated at 50°C for 30 min. The reducing sugar released was estimated by the DNSA method (Saddler *et al*. 1985).

Enzyme activity is expressed as μmol glucose released min^{-1} mL^{-1} of culture filtrate as enzyme solution.

Table 1: Effect of different cellulosic substrates on the production of cellulolytic enzymes by *Aspergillus niger*

Concentration of cellulosic substrates	Cellulolytic enzyme (U mL^{-1})			
	%(w/v)	FPA	Endolucanase	β-glucosidase
Filter paper	0.5	0.65	0.65	4.88
	1.0	1.30	1.30	8.45
	2.0	0.65	1.38	9.43
Cotton wool	0.5	0.65	6.50	5.53
	1.0	1.38	1.45	6.50
	2.0	2.60	3.58	10.40
Newspaper	0.5	0.65	3.90	9.80
	1.0	1.30	5.85	10.40
	2.0	1.38	4.88	1.37
Untreated corn cob	0.5	4.88	7.48	14.63
	1.0	5.53	9.10	18.20
	2.0	6.50	10.40	21.13
Treated corn cob	0.5	3.58	8.13	14.95
	1.0	5.53	13.65	20.15
	2.0	8.13	16.25	23.73
Untreated groundnut shell	0.5	1.30	4.88	7.15
	1.0	2.60	5.85	8.45
	1.0	1.45	3.58	16.90
Treated groundnut shell	0.5	0.65	1.30	5.85
	1.0	1.38	3.58	7.48
	2.0	1.45	4.88	9.43
Untreated sawdust	0.5	1.30	2.60	7.48
	1.0	1.38	5.53	10.08
	2.0	2.60	7.15	11.70
Treated sawdust	0.5	1.45	3.58	7.80
	1.0	2.93	7.15	11.70

Values represents mean of 2 determinations

Hydrolysis of substrates: Modified method of Mandels *et al*. (1974) and Bastawde (1992) was used for hydrolyzing the substrates. One hundred milligram dry weight equivalent of cellulosic material in 1 mL of 0.1 M citrate buffer (pH 5.5) was incubated with 1 mL crude culture filtrate at 50°C for 48 h. The amount of reducing sugar released was determined by the dinitrosalicylic acid method of Miller. The saccharification was calculated using the relationship: (Shewale and Sadana, 1979).

$$\%\,\text{saccharification} = \frac{\text{amount of reducing sugar}}{\text{amount of substrates}} \times \frac{162}{100} \times 100$$

RESULTS

Treated and untreated corn cob and untreated groundnut shell were the best carbon sources for production of cellulolytic enzymes (Table 1). Treated corn

Table 2: Production of reducing sugar by *Aspergillus niger* culture filtrate acting on different cellulosic substrates

	Reducing sugar (mg mL^{-1})			
Substrate	1 h	4 h	24 h	48 h
Filter paper	0.75	0.75	1.90	2.50
Newspaper	1.16	1.16	2.80	3.65
Cotton wool	1.10	0.30	1.40	2.10
Untreated corn cob	3.00	3.00	3.35	4.25
Treated corn cob	1.40	2.00	4.25	5.55
Untreated sawdust	1.80	1.85	2.40	4.25
Treated sawdust	2.25	2.15	3.15	4.35
Untreated groundnut shell	3.50	3.50	4.35	5.10
Treated groundnut shell	1.10	1.10	1.90	5.00

Values represents mean of 2 determinations

Table 3: Saccharifiction of cellulosic materials by culture filtrate of *Aspergillus niger*

	% Saccharification			
Substrate	1 h	4 h	24 h	48 h
Filter paper	0.68	0.68	1.71	2.25
Newspaper	1.44	1.44	2.52	3.30
Cotton wool	0.01	0.27	1.26	1.90
Untreated corn cob	2.70	2.70	3.00	3.80
Treated corn cob	1.36	1.80	3.80	5.00
Untreated sawdust	1.62	1.66	2.16	3.80
Treated sawdust	2.00	1.90	2.80	3.90
Untreated groundnut shell	3.20	3.20	3.90	4.60
Treated groundnut shell	1.00	1.00	1.70	4.50

cobs gave the highest activity for the cellulolytic enzymes. Filter paper used as carbon source gave the least activity for the cellulolytic enzymes assayed. Enzyme activity was highest at 2% substrate concentration. Lower enzyme activity was obtained when *A. niger* was grown on untreated substrate except for groundnut shell where the untreated substrate produced higher enzyme activities than the treated groundnut shell samples. Statistical analysis of the results using Duncan's Multiple Range Test (DMRT) showed that there was no significant difference in the β-glucosidase activities of the substrates using treated saw dust and newspaper. Also endoglucanase activities showed no significant difference when newspaper, untreated groundnut shell and untreated saw dust were used as substrates.

The result of hydrolysis of cellulosic substrates with *A. niger* culture filtrate is presented in Table 2 and 3. The most resistant substrates were cotton wool and filter paper which produced 1.90 and 2.25% saccharification, respectively after 48 h. Reducing sugar yield ranged from 0.75 mg mL^{-1} for filter paper at 1 h to 5.55 mg mL^{-1} for treated corn cob at 48 h. For all the substrates hydrolyzed, the maximum reducing sugar yield was obtained at 48 h.

There was little or no difference in reducing sugar yield and saccharification after 1 and 4 h incubation. Treated corn cobs gave the highest saccharification rate of 5.0% after 48 h. Except for groundnut shell, the treated substrates gave a higher production of reducing sugar and saccharification after 48 h.

DISCUSSION

The result of using different cellulosic materials as substrates for production of cellulase enzyme showed that treated corn cob at 2% concentration were the most susceptible of the substrates producing the highest activity for FPA (8.13U mL^{-1}) and beta-glucosidase (23.73U mL^{-1}). El-Naghy *et al.* (1991) reported 2% substrate concentration as being optimum for cellulase production for S*porotrichum thermophile* in culture media containing different cellulosic materials as sole carbon source. Mandels (1995) reported that cotton was a poor substrate because it has little available surface and high crystallinity. This may be responsible for the low reducing sugar values obtained in this study. The complex substrates (newspaper, groundnut shell and saw dust) were poor inducers of the enzymes probably due to the lignin in these substrates. In nature, it is known that lignin physically encrusts cellulose, making it resistant to enzymatic degradation (Kirk and Farrel, 1987). This leads to shortage of utilizable carbohydrates in lignin-rich substrates and consequently poor growth of organism and low enzyme yield. These lignified cellulosics however produced more quantities of cellulase enzyme after pretreatment. This agreed with the work of Singh *et al.* (1990) who reported that higher values of reducing sugar were obtained from pretreated substrates compared to untreated substrates. In this study, it was observed that only treated groundnut shell showed less activity when compared to the untreated groundnut shell. There was good elaboration of beta-glucosidase by *A. niger* using the substrates. This may be indicative of the important role of β-glucosidases in the enzymatic hydrolysis of cellulosic materials as stated by Shewale (1982).

The culture filtrate of the *A. niger* strain showed low saccharifying rates on cellulosic materials (0.68% - 5.0%). The highest saccharification rate (5.0%) was obtained for treated corn cob at 48 h. This value is lower than the values reported by Mandels *et al.* (1974) of 9.9% saccharification for cotton, 59.4% for filter paper and 41.8% for newspaper after 48h incubation with *Trichoderma viride* culture filtrate, but agreed with the result of El-Naghy *et al.* (1991) who reported 3.5% saccharification for saw dust, 1.5% for cotton and 3.0% for newspaper using *Sporotrichum thermophile* culture filtrate. The low level of saccharification may be indicative of the possible inhibition of enzyme activity due to the lignin content in the substrates (Bastawde, 1992). Saccharification level of treated substrates was higher than that of untreated substrates. Treatment of cellulosic substrates was reported to increase susceptibility to enzymatic hydrolysis by culture filtrates of *Sclerotium rolfsii* (Shewale and Sadana, 1979) and *Sporotrichum thermophile* (El-Naghy *et al.*, 1991).

CONCLUSION

The cellulosic substrates used in this study were saccharified by the culture filtrate of *A. niger* to different degrees. Pretreatment of the substrates increased their ability to produce cellulolytic enzymes and reducing sugars and the rate of saccharification. Corn cob was the most active for the production of reducing sugars and for saccharification. Filter paper was the least active of the substrates. Corn cob which is a readily available agrowaste in Nigeria may be an easy source for production of reducing sugar.

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