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Optimization of Culture Conditions Affecting Fungal Cellulase Production

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ABSTRACT

Local isolated Fungal cultures including *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium avenaceum* and *Cephalosporium acremonium* were employed for cellulase production. The current study aimed at optimization conditions of cellulase production from our local fungal strains. These fungi were grown as stand cultures in 1000 mL conical flasks containing cellulose powder medium for screening their ability for utilizing cellulose as main carbon source for cellulase production. *A. niger* was chosen on the basis of the best mean cellulase activity reached 0.076 U mL^{-1}, for optimizing culture condition for cellulase production. Wheat straw was used as a sole carbon source for the enzyme production at a concentration of 20% (w/v). The highest activity reached 0.097 U mL^{-1} was obtained under the optimum conditions of cellulase production including 4% inoculum size, 72 h incubation period, initial pH 6 of the growth medium with using meat extract as a sole nitrogen source at a concentration of 0.33 g L^{-1} . The result obtained indicated the possibility of cellulase production from *A. niger* local strain using wheat straw as a sole carbon source.

Key words: Cellulase production, *A. niger*, wheat straw, banana wastes, optimum conditions, nitrogen source

INTRODUCTION

Cellulases are among the industrially important hydrolytic enzymes and are of great significance in present day biotechnology, these enzymes produced by numerous microorganisms such as *Aspergillus*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Myrothecium*, *Paecilomyces*, *Penicillium* and *Trichoderma* species (Haight, 2005; Azzaz, 2009).

Cellulases are a group of fibrolytic enzymes which cooperatively hydrolyze plant cell wall fibers into glucose, cellobiose or oligosaccharides (Murad and Azzaz, 2010; Chinedu *et al*., 2010). Three types of cellulase enzymes are involved in the cellulase hydrolysis process including cellobiohydrolase, endoglucanase or carboxy methylcellulase (CMCase) and **β**-glucosidases (Bhat, 2000; Saber *et al*., 2010).

Many research being achieved in cellulases production and their characterization during recent years (Rajoka and Malik, 1997; Murad and Azzaz, 2010; Khan and Husaini, 2006; Milala *et al*., 2009; Ong *et al*., 2010; Roslan *et al*., 2011). Since the production of cellulase enzyme is a major process and economically viable, major attention has been given to use lignocellulosics as substrate for cellulase production. The level of cellulase activity and its application depend on

the microbial producing strain, the media composition and process control (Ghose, 1987; Kheng *et al*., 2006).

Fungi are the main cellulase producing microorganisms though a few bacteria and actinomycetes have also been reported to yield cellulases (Milala *et al*., 2009). Arunachalam *et al*. (2010) reported that the biotechnology application of cellulases began in the early 1980s in the animal feed followed by food applications and account for approximately 20% of the world enzyme market. There are huge amounts of agricultural wastes can be used for cellulases production including rice straw, wheat straw and banana wastes (Roslan *et al*., 2009; Murad and Azzaz, 2010). Shahriarinour *et al*. (2011) has mentioned the great interest in utilizing cellulose wastes as feedstock through fermentation processes thereby converting low cost starting materials into products of great value. There are few studies on cellulase production from raw biomass such as rice straw (Roslan *et al*., 2011).

Cellulase with its immense importance is being imported for use in Egypt at a high cost. The local production of such enzymes may reduce the cost of importation and encourage self-reliance. This study was carried out to produce fungal cellulase under the optimum fermentation conditions.

MATERIALS AND METHODS

This study was carried out from January, 2010 to December, 2010. In the Dairy Department, National Research Centre, Dokki, Giza, Egypt.

Waste materials used as substrates: Banana wastes, rice straw, wheat straw and corn stalks were collected after harvesting. The air-dried wastes were cut into 0.5-1 cm then dried at 70°C for 24 h in air-circulation oven and ground to powder from 5-10 mm (Hattaka, 1983) in an electric grinder then packed and stored in dry place at room temperature till use.

Fungal cultures, media and inoculum preparation: Four fungal cultures were used for screening their ability of utilizing cellulose as main carbon source for cellulase production; *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium avenaceum* and *Cephalosporium acremonium* were obtained from laboratory of plant pathology of National Research Center, Cairo, Egypt. These fungal cultures were cultivated and maintained on Potato Dextrose Agar medium (PDA). Malt medium containing malt extract (30 g L^{-1}); yeast extract (5 g L^{-1}) was used for preparing the activated fungal inocula; Cellulose Powder Medium (CPM) recommended by Fadel and Foda (1993) was used for growth and cellulase production. The medium has the following composition (g L^{-1}) NaCl, 6.0; $(NH_4)_2SO_4$, 1.0; K_2HPO_4, 1.0; $MgSO_4$, $7H_2O$, 0.05; $CaCl_2$, 0.1; Yeast extract, 0.5; Peptone, 0.5; Glucose, 4.0; Cellulose powder, 2.0 and medium pH was adjusted to pH 6.0. Spores of fungi were transferred from surface of the actively growing slants of (PDA) medium to 250 mL conical flasks each containing 50 mL of malt medium. After incubation on rotary shaker (120 rpm) at 29±1°C for 48 h, the grown cultures were employed as inocula for experimental 1000 mL conical flasks containing 100 mL (CPM) medium at rate of 5% (v/v) inoculum size.

Culture conditions for cellulase production: Static cultures were used for studying fungal cellulase production under variable condition including fungal cultures effect, substrate source, inoculum size, incubation period, initial pH and nitrogen source. The general procedure included use of triplicate of 1000 mL conical flasks each containing 100 mL of CPM. The effect of fungal cultures was studied through inoculation of 3 flasks each with one of the four mentioned fungal

cultures incubated for three days at 29±1°C and the levels of cellulase activities were determined in the cultures filtrate. Effect of substrate source was investigated through replacing of cellulose powder in CPM by 20% (w/v) of different cellulolytic waste materials including banana wastes, rice straw, wheat straw and corn stalks. The fermented substrate for each flask was mixed with 25 mL of 0.02 M acetate buffer (pH 5.0) by shaking in a rotary shaker (120 rpm) for one hour at room temperature to extract the enzyme and the extracted mixture was filtered and collected for cellulase activity assay. Effect of inoculum size ranged from 1 to 10% (v/v) on cellulase activity by tested fungal cultures was studied. The influence of incubation period was studied through determination of cellulase activities after 24, 48, 72, 96 and 120 h. Effect of the initial pH of growth medium was studied through adjusting the initial pH values in a range between 3 and 8 using either NaOH or HCl 0.1 N. Effect of nitrogen source included the use of three inorganic salts (ammonium sulphate, ammonium chloride and sodium nitrate; and three organic sources (meat extract, yeast extract and peptone) were studied. Various nitrogen sources were used separately at an equivalent concentration of 0.33 g N L^{-1} media as recommended by (Murad and Azzaz, 2010) these nitrogen sources replaced the original nitrogen present in the CPM. The level of a parameter optimized in an experiment was maintained in the subsequent studies.

Enzyme assay: The carboxymethyl-cellulase activity (CMC) for resultant enzyme was determined according to Mandels *et al.* (1974). The reducing sugar liberated was determined by modified Dinitrosalicylic acid method (DNS) of Miller (1959). One cellulase unit is defined as the amount of enzyme that liberates reducing sugar at the rate of one μmol mL^{-1} min^{-1} under assay condition.

Statistical analysis: The significance of the results was determined by the Analysis of Variance (ANOVA) evaluated by Duncan's multiple range tests (at 0.05), using COSTAT software, product of Cohort software Inc., Berkley, California (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of fungal cultures on cellulase activity: The capability of four fungal cultures on cellulase production on CPM was shown in Fig. 1. *A. niger* gave the highest ($p<0.05$) cellulase activity (0.076 U mL^{-1}) followed by *C. acremonium* (0.039 U mL^{-1}) while the other fungal cultures gave low activity especially *F. oxysporum* gave the lowest cellulase activity. A capacity to degrade cellulose is a character distributed among a wide variety of aerobic, facultative aerobic, anaerobic bacteria and fungi. These characteristics are involved in few species of fungi and bacteria (Gooday, 1979). Fungi, *Trichoderma* spp., *A. niger*, *A. flavus* and *Penicillium* sp. have been reported to be main sources of cellulase, hemicellulase, pectinase and xylanase (Chandra *et al.*, 2007). It has been reported in the recent studies that higher levels of cellulases were obtained with *A. niger* (Hanif *et al.*, 2004). This is in line with we found in the present study, so *A. niger* was chosen for further studies on CPM.

Effect of substrate source: The use of available lignocellulosic wastes as carbon source in the growth medium would reduce the costs of enzyme production. As shown in Fig. 2, wheat straw as lignocellulosic substrates gave the highest cellulase production ($p<0.05$) with *A. niger* (0.18 U mL^{-1}), while the pure cellulose in CPM gave the lowest cellulase production (0.09 U mL^{-1}). The variability in amount of cellulase production it may be due to the influence of substrate (carbon

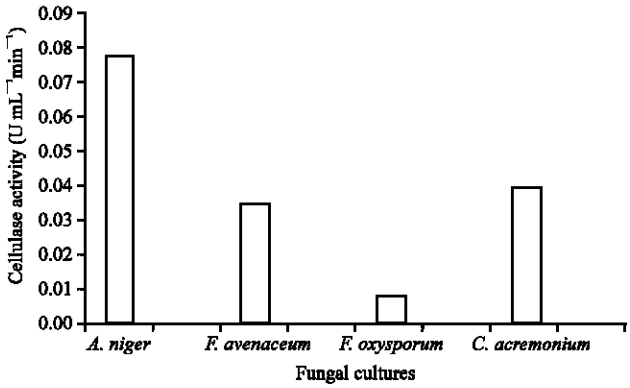

Fig. 1: Fungal cultures tested for cellulase production

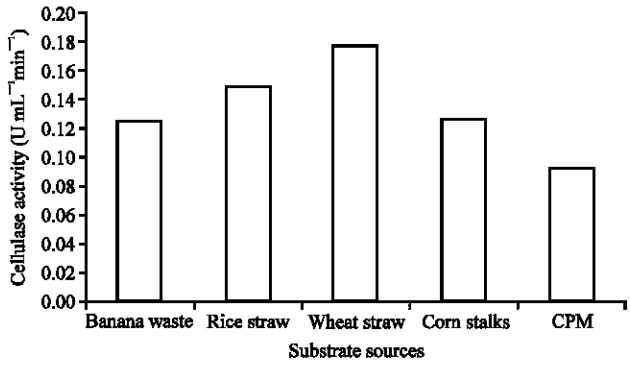

Fig. 2: Effect of substrate source on cellulase production by *A. niger*

source) on the growth of cellulolytic organisms (Lakshmikant and Mathur, 1990). Some environmental factors are also influenced the growth of organisms as well as maximum enzyme production including optimum temperature, pH, salt concentration etc. (Immanuel *et al*., 2006). Several studies of cellulase enzyme production by using different substrates and microorganisms have been found with different yields. This as mentioned by Alam *et al*. (2008) may be attributed to the ability of some substrates to induce enzyme production to degrade specific combinations of polysaccharides linked to other components found in the carbon source. The superiority of tested cellulolytic waste materials over cellulose as a carbon source in CPM for cellulase production may be due to that cellulolytic waste materials can act as a source of carbon, nitrogen and minerals as well as growth factors. Ojumu *et al*. (2003) tested bagasse, corncob and sawdust as lignocellulosic substrates for production of cellulase enzyme using *Aspergillus flavus*, sawdust gave the best enzyme activity while mixture of rice straw and wheat bran showed better results in submerged fermentation for the production of cellulase and hemicellulase by *A. niger* KKS (Kang *et al*., 2004). From the previous data, wheat straw was selected as a sole carbon source for conducting further studies on cellulase production by *A. niger*.

Effect of inoculum size: *A. niger* has exhibited different responses to variations in inoculum size from 1 to 10% (v/v) The data shown in Fig. 3. Indicated that the production of cellulase by *A. niger* was increased significantly ($p<0.05$) by increasing inoculum size up to 4% (0.077 U mL^{-1}). Further

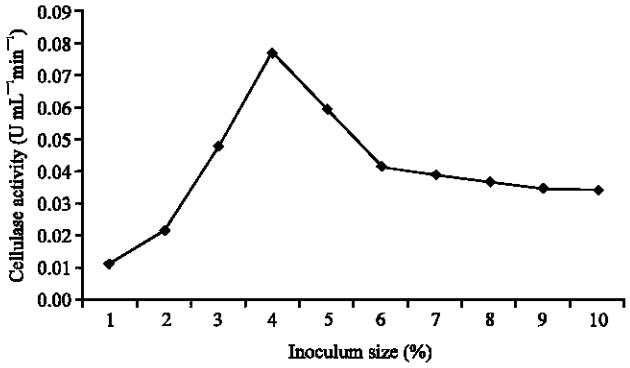

Fig. 3: Effect of inoculum size on cellulase production by *A. niger*

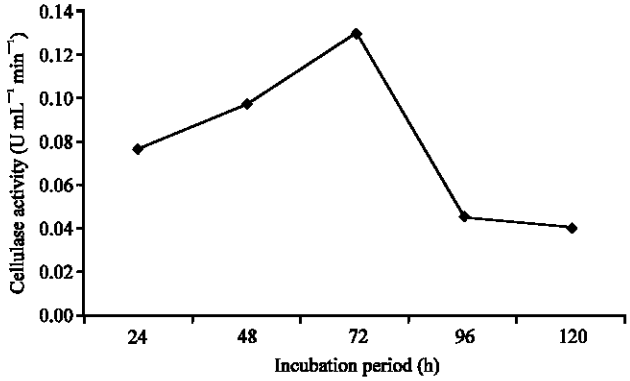

Fig. 4: Effect of incubation period on cellulase production by *A. niger*

increasing in inoculum size up to 10% led to decrease in cellulase production by *A. niger*. Zhang *et al.* (2001) investigated the effect of inoculum size on cellulase synthesis by *Trichoderma viride* they reported that the impact of the amount of inoculant on cellulase production was small and 5% inoculum was the most suitable, also Alam *et al.* (2005) revealed that the higher cellulase activity of 0.0413 unit was achieved with 5% (v/w) of inoculum size when fermented oil palm biomass by *Trichoderma harzianum*. Omojasola *et al.* (2008) found that amount of cellulase activity was decreased at inoculum sizes above 6 and 8% for pineapple peel and pineapple pulp fermentation by *A. niger*. The decrease in cellulase production with further increase in inoculum might be due to clumping of cells which could have reduced sugar and oxygen uptake rate and enzyme release (Omojasola *et al.*, 2008). From the previous data, 4% inoculum size was selected for conducting further studies on modified CPM by *A. niger*.

Effect of incubation period: The production of cellulase on modified CPM was monitored for a period of 120 h (Fig. 4). The highest cellulase activity ($p<0.05$) was recorded after 72 h of incubation with *A. niger* grow on wheat straw. This result is in line with this obtained by Chandra *et al.* (2007) who found that maximum cellulase activity was recorded on 72 h of incubation with groundnut fodder, wheat bran and rice bran fermented by *A. niger* also, Milala *et al.* (2005) reported that cellulase show maximum activity after 72 h of fermentation by

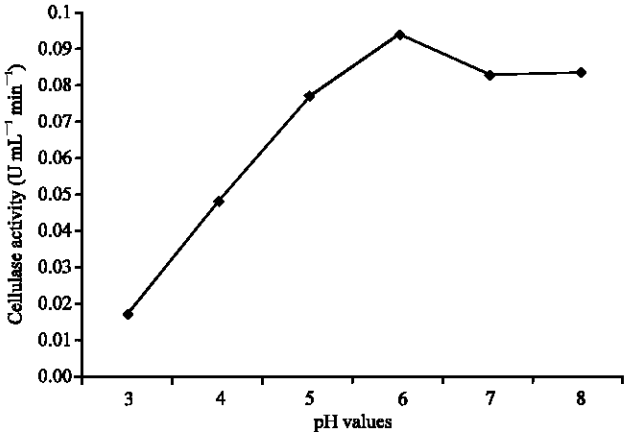

Fig. 5: Effect of initial pH of growth medium on cellulase production by *A. niger*

A. niger grow on maize straw and rice husk. In addition the maximum cellulase activity was obtained by *Bacillus subtilis* after 72 h of fermentation with banana waste (Krishna, 1999). On the other hand, Kang *et al.* (2004) found that the highest cellulase activity was obtained after 5-6 days of fermentation by *A. niger* grow on rice straw, while Ojumu *et al.* (2003) stated that *A. flavus* grown on sawdust, bagasse and corncob gave the highest cellulase activity at 12 h of fermentation. In our study the decrease in cellulase activity after more than 72 h might be due to denaturation of the enzyme, resulting from variation in pH during fermentation as reported by Krishna (1999), or may be due to cumulative effect of cellobiose, a dimer of glucose which is known to inhibit both endoglucanase and β-glucosidase (Howell and Mangat, 1978). From the previous data, 72 h incubation period was selected for conducting further studies on modified CPM by *A. niger*.

The effect of initial pH: As shown in Fig. 5 initial pH of the medium has profound effect on cellulase production. The cellulase production by *A. niger* in varying pH of CPM showed highest values of cellulase activity ($p<0.05$) at pH 6.0 (0.094 U mL^{-1}), more over when the pH level increased, the enzyme production was decreased. The initial pH of the medium has a great effect on the growth of the organism, permeability membrane, as well as on the biosynthesis and stability of the enzymes (Shoichi *et al.*, 1985; Poorna and Prema, 2007). It was reported that optimal pH for CMCase from *A. niger* was found to be 6 to 7 (Parry *et al.*, 1983). But Akiba *et al.* (1995) reported that the production was high at pH 4.0 and 4.5 by *A. niger*. Coral *et al.* (2002) observed that the enzyme activity has a broad pH range between 3 and 9. These data suggest that the enzyme systems within the same species may vary, depending on the strain under study. Based on the results obtained, the initial pH of the medium was adjusted to pH 6 for *A. niger* in subsequent studies on modified CPM.

Effect of nitrogen source: The data shown in Fig. 6 revealed that among the 6 nitrogen sources tested, the meat extract was found to be the best nitrogen source producing the highest level of cellulase activity ($p<0.05$) by *A. niger* (0.097 U mL^{-1}). This data indicating that the source of nitrogen should be organic for better results. Our results are in line with the work of Abou-Taleb *et al.* (2009) who reported that organic nitrogen sources were found to be more suitable

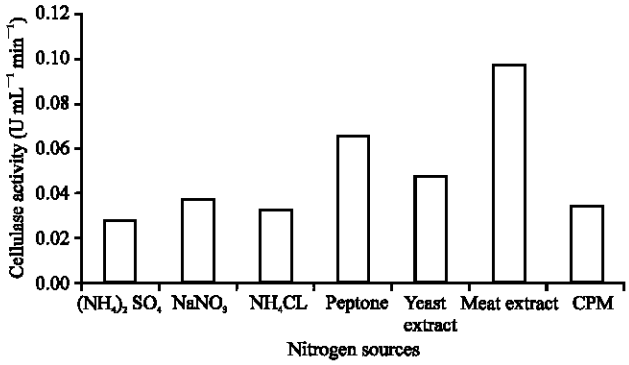

Fig. 6: Effect of nitrogen source on cellulase production by *A. niger*

for optimizing cellulase production by *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* C23 than inorganic sources while Xavier and Lonsane (1994) indicated that the source of nitrogen should be inorganic for better results. Differences in titres of enzyme yields in different studies can be attributed to use of different materials as solid matrix, different cultural practices and different organisms (Chandra *et al*., 2007).

CONCLUSION

The results obtained from this investigation have designated the superiority of *A .niger* over the other tested fungal cultures for production of cellulase. On the other hand wheat straw induced the highest level of cellulase activity compared with the other agriculture wastes. More investigations are needed for production of cellulases which imported for use in Egypt at a high cost. The utilization of agricultural wastes as substrates may be promising for production of cellulases.

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