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Full Length Research Paper

Nutrient effects on production of cellulolytic enzymes by *Aspergillus niger*

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The production of cellulase (filter paper activity, endoglucanase and β-glucosidase) by *Aspergillus niger* on three media in liquid shake culture was compared. The culture filtrate of this organism exhibited relatively highest activity of all three enzymes and extracellular protein content at 7-day interval during the course of its growth on Czapek-Dox medium supplemented with 1.0% (w/v) cellulose. Urea as a nitrogen source and pH 5.0 were found to be optimal for growth and cellulase production by *A.niger*. Among various soluble organic carbon sources and lignocelluloses tested in this study, carboxymethylcellulose and sawdust at 1% supported maximum production of all three enzymes by *A.niger.*

Key words: *Aspergillus niger*, cellulase activity, nutrients, β-glucosidase.

INTRODUCTION

Cellulose is the most abundant polymer in the biosphere with its estimated synthesis rate of 10^{10} tonnes per year (Schlesinger, 1991; Singh and Hayashi, 1995; Lynd et al., 2002). Cellulose-rich plant biomass is one of the foreseeable and sustainable source of fuel, animal feed and feed stock for chemical synthesis (Bhat, 2000). The utilization of cellulosic biomass continues to be a subject of worldwide interest in view of fast depletion of our oil reserves and food shortages (Kuhad et al., 1997; Gong et al., 1999). The conversion of cellulosic mass to fermentable sugars through biocatalyst cellulase derived from cellulolytic organisms has been suggested as a feasible process and offers potential to reduce use of fossil fuels and reduce environmental pollution (Dale, 1999; Lynd et al., 1999). Complete enzymatic hydrolysis of enzymes requires synergistic action of 3 types of enzymes, namely cellobiohydrolase, endoglucanase or carboxymethylcellulase (CMCase) and β-glucosidases (Bhat, 2000). However, the high cost of production of these enzymes has hindered the industrial application of cellulose bioconversion. One of the different approaches to overcome this hindrance is to make continuous search for organisms with secretion of cellulase enzymes in copious amounts and to optimize enzyme production with them. In this paper, effects of nutrient on cellulase production by *Aspergillus niger*, a local isolate, in submerged fermentation in a laboratory study are presented.

MATERIALS AND METHODS

Culture conditions and enzyme production

A. niger isolated from soil contaminated with effluents of cotton ginning industry (Reddy et al., 1998) was used in this study. Sterile 50 ml of three different media (minimal, basal and Czapek-Dox) each amended with 1% cellulose as carbon source was distributed in sterile 250 ml Erlenmeyer flask. Meanwhile, the spore suspension was prepared in sterile distilled water from 6-day old cultures of *A. niger* grown on Potato-Dextrose Agar (PDA) slants. The flasks were inoculated with a density of 2 x 10^6 spores and incubated at 28°C on a rotary shaker (180 rpm). Flasks were withdrawn at 7-day intervals over a period of 3 weeks and filtered through Whatman No.1 filter paper to separate mycelial mat and culture filtrate. Biomass of the culture was dried at 70°C in an oven until constant weight and then measured. Fungal growth was expressed in terms dry weight of mycelial mat (mg/flask). The content of soluble protein in the culture filtrate was estimated according to method of Lowry et al. (1951) with bovine serum albumin as a standard. Total activity of cellulase complex and/or individual component enzyme activities in the culture filtrate were determined as per procedures described below. In view of maximum growth and cellulase activity on the Czapek-Dox medium

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Table 1. Growth, total cellulolytic activity (Filter paper activity*) and protein secretion by *A. niger* on different media.

Incubation Time (week)	Minimal Media			Basal Media			Czapeck Dox		
	Dry weight of mycelial mat (mg/flask)	Total cellulolytic activity (FPU/ml)	Total Protein content (µg/ml)	Dry weight of mycelial mat (mg/flask)	Total cellulolytic activity (FPU/ml)	Total Protein content (µg/ml)	Dry weight of mycelial mat (mg/flask)	Total cellulolytic activity (FPU/ml)	Total Protein content (µg/ml)
1	165	1.01	64	245	1.59	120	382	1.72	150
2	286	0.89	116	328	0.98	176	410	1.45	200
3	526	0.59	110	567	0.67	65	650	1.10	120

Values represented in the table are average of results of two separately conducted experiments.
* Filter paperase is expressed in terms of filter paper units (FPU). One unit is the amount of enzyme in the culture filtrate that releasing 1 µmole of reducing sugar from filter paper per min.

at 7th day interval, subsequent experiments were carried out on this medium to find out the influence of dose response of cellulose supplementation (with a range of 0.5 to 2.0%), supplementation of carbon sources, nitrogen and ligninocellulose on growth, secretion of extracellular protein and cellulase production by *A. niger* at only 7th day interval.

Enzyme assay

Filter paper activity (FPA) for total cellulase activity in the cultural filtrate was determined according to the method of Mandels et al. (1976). Aliquots of appropriately diluted culture filtrate as enzyme source was added to Whatman No.1 filter paper strip (1 X 6 cm; 50 mg) immersed in one millilitre of 0.05 M sodium citrate buffer of pH 5.0. After incubation at 50°C for 1 h, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per ml per min. Endoglucanase activity (carboxymethylcellulase; CMCase) was measured as described previously (Ghosh, 1987) using a reaction mixture containing 1 ml of 1% carboxymethyl cellulose (CMC) in 0.05 M citrate acetate buffer (pH 5.0) and aliquots of suitably diluted filtrate. The reaction mixture was incubated at 50°C for 1 h and the reducing sugar produced was determined by DNS method. One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per min. β-glucosidase activity was assayed by the method of Herr (1979). β-glucosidase activity was measured in 1ml of 5 mM p–nitrophenyl-β-D-glucopyranoside (PNPG) in 0.05 M citrate buffer (pH 5.0) and aliquots of appropriately diluted culture filtrate and incubated at 50°C for 30 min. The reaction was terminated by addition of 4 ml of 0.1 M NaOH-glycine buffer solution and the released p-nitrophenol was read at 410 nm. The activity was expressed in terms of liberation of p-nitrophenol from p–nitrophenyl-β-D-glucopyranoside (PNPG). One unit of the enzyme activity was defined as the amount of enzyme producing 1 µmole of p-nitrophenol per min.

RESULTS AND DISCUSSION

A. niger was cultured on three liquid media (minimal, basal and Czapek-Dox) amended with 1.0% cellulose at 28°C under shaking conditions. During cultivation, growth and total cellulolytic activity (filter paper activity) and protein contents in the culture filtrate were monitored at 7-day interval over a period of 3 weeks and are presented in the Table 1. Growth of *A.niger* in terms of dry weight of mycelial mat on all 3 media increased during the course of incubation in this study. Of 3 media tested in the study, Czapek-Dox medium supported the maximum growth of *A. niger*. *A. niger* grown on 3 media exhibited highest cellulolytic activity at 7th day interval followed by decline activity at lateral intervals. Maximum cellulolytic activity of about 1.7 FPU per milliliter of culture filtrate on Czapek-Dox medium at 7-day interval was recorded. The extracellular protein content in the culture filtrate of 3 media followed the same pattern of growth with maximal values in the respect of Czapek-Dox medium. Thus, it is clearly evident from the results of the present study that Czapek-Dox medium appeared to be superior for growth and cellulase production by *A. niger*. Similar observations with another fungus, *Humicola fuscoatra* was made by Rajendran et al. (1994). The cellulolytic activity of *A. niger* used in the present study is comparable to that of the most well studied fungus, *Trichoderma reesei* whose wild type or mutant cells in free status produced cellulase within a range of 1-2 FPU/ml on various media (Tamada et al., 1989; Domingues et al., 2000). The production of cellulase by wild type cells of *Bacillus pumilus* (Kotchoni and Shonukan, 2002) and *Cellulomonas biazotea* (Rajoka et al., 1998) and *Trichoderma aureoviride* (Zaldivar et al., 2001) in liquid did not exceed 1.5 U/ml.

Dose response of cellulose supplementation within a range of 0.5-2.0% on cellulase production of *A. niger* was examined. Yields of fungal biomass, protein content and cellulase production derived from the growth of *A. niger* on Czapek-Dox medium with different cellulose concentrations at 7th day interval were presented in the Table 2. The growth increased with increase in the concentration of cellulose up to 1% (W/V). Further increase in cellulose concentration beyond 1% level did not result in proportionate increase in yields of fungal biomass and protein content. Production of individual components of cellulase by *A. niger* in response to cellulose dose followed the same trend as noticed for growth. The results clearly showed that supplementation of cellulose at 1.0% was optimal for cellulase production. Similarly, maximum endoglucanase activity (690 ηkat/ml)

Table 2. Effect of cellulose concentration on cellulase production by *A. niger*.

Cellulose Concentration g (%)	Dry weight of mycelial mat (mg/flask)	Protein (µg/ml)	Cellulase		
			FP (U/ml[a])	CMCase[b] (U/ml)	β-glucosidase[c] (U/ml)
0.5	587	590	1.06	0.325	0.60
1.0	917	920	1.73	0.642	1.02
1.5	900	900	1.75	0.650	1.12
2.0	905	910	1.74	0.641	1.07

Values represented in the table are averages of results of two experiments.
[a]Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 µmole of reducing sugar from filter paper per min.
[b]Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxymethyl cellulose per min.
[c]One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1µmole of *p*-nitrophenol per min.

Table 3. Effect of supplementation of carbon source on cellulase production by *A. niger*.

Carbon Source	Dry weight of mycelial mat (mg/flask)	Protein (µg/ml)	Cellulase		
			FPU/ml[a]	CMCase[b] (U/ml)	β-glucosidase[c] (U/ml)
Glucose	1312	590	0.154	0.38	0.46
Cellulose	557	920	1.632	0.542	1.02
Carboxymethyl cellulose	735	900	1.640	0.521	1.12
Cellobiose	765	910	1.021	0.48	0.66

Values represented in the table are averages of results of two separately conducted experiments.
[a]Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 µmole of reducing sugar from filter paper per min.
[b]Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxymethyl cellulose per min.
[c]One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1µmole of *p*-nitrophenol per min.

was recovered on the medium with cellulose at 10 g/L (Haapela et al., 1995). Cultivation of *Trichoderma harzianum* on microcrystalline cellulose at 10 g/L yielded 3000 U/L of CMcase and 400 U/L of filter paper activity (Rousses and Raimbault, 1982).

Influence of supplementation of different carbon sources to Czapek-Dox medium on cellulase production by *A. niger* was examined. Among carbon sources tested in this study, carboxymethyl cellulose served the best source followed by cellulose for cellulase production (filter paper activity) and secretion of extracellular protein by *A. niger* (Table 3). Activities of even other individual components of cellulase, such as carboxymethyl cellulase (CMCase) and β-glucosidase were also highest in culture filtrate of *A. niger* grown in the presence of carboxymethyl cellulose. Inclusion of glucose in the medium supported the maximal growth of *A. niger* but resulted in minimal production of cellulase and secretion of extracellular proteins. Similarly, induction of cellulase production by *Humicola fuscoatra* in presence of cellulosic substrates has been reported by Rajendran et al. (1994). *Volvariella diplasia* produced cellulolytic enzymes (550 U of CM-cellulase and 69 U of filter paper cellulase) when grown in shake culture at pH 5.4 and 28°C with 0.5% cellulose powder (Puntambekar, 1995). Cellulase production was higher upon growth of *Trichoderma harzianum* (Mes-Hartree et al., 1988) and *A. niger* (Hanif et al., 2004) on cellulosic substrates. This observation is well in agreement with the result of the present study. Low level of cellulolytic enzymes in the presence of glucose in this study could be attributed to repression of synthesis of cellulolytic enzymes involved in the utilization of cellulose by easily metabolisable carbon, glucose that was demonstrated in many organisms (Ruijter and Visser, 1997; Suto and Tomita, 2001). However, insensitization of this repression by mutations resulted in higher production of cellulase even in the presence of glucose (Kotchoni and Shonukan, 2002).

Cellulose production by *A.niger* grown on different nitrogen sources at concentration of 0.03% nitrogen-N was compared (Table 4). The effectiveness of nitrogen source in supporting cellulase production along with growth, secretion of extracellular protein by *A. niger* decreased in the following order: Urea>peptone>$NaNO_3$> yeast extract. Activities of CMCase and β-glucosidase in culture filtrate of *A. niger* grown on urea were also very

Table 4. Effect of supplementation of nitrogen source on cellulase production by *A. niger*.

Nitrogen Source	Dry weight of mycelial mat (mg/flask)	Protein (μg/ml)	Cellulase		
			FP (U/ml[a])	CMCase[b] (U/ml)	β-glucosidase[c] (U/ml)
Control[d]	520	150	1.602	0.542	1.02
Urea	913	290	1.682	0.824	1.122
Peptone	834	280	1.214	0.421	1.060
$NaNO_3$	749	170	1.122	0.401	0.940
Yeast extract	712	90	0.526	0.348	0.895

Values represented in Table are averages of results of two separately conducted experiments.
[a]Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 μmole of reducing sugar from filter paper per min.
[b]Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 μmole of reducing sugar from carboxymethyl cellulose per min.
[c]One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1μmole of *p*-nitrophenol per min.
[d]Contained 1% cellulose (w/v) without supplementation of nitrogen.

Table 5. Cellulase production on lignocelluloses by *A. niger*.

Lignocellulose	Dry weight of mycelial mat (mg/flask)	Protein (μg/ml)	Cellulase		
			FPU/ml[a]	CMCase[b] (U/ml)	β-glucosidase[c] (U/ml)
Control[d]	520	720	1.632	0.542	1.020
Saw-dust	913	1310	2.412	0.775	1.322
Jowar-straw	834	530	1.52	0.627	0.998
Dry leaves of tobacco	749	470	1.41	0.513	0.962
Rice-straw	712	430	0.96	0.660	1.025

Values represented in Table are averages of results of two separately conducted experiments.
[a]Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 μmole of reducing sugar from filter paper per min.
[b]Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 μmole of reducing sugar from carboxymethyl cellulose per min.
[c]One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1μmole of *p*-nitrophenol per min.
[d]Contained cellulose at 1% (w/v) instead of lignocellulose.

high to the tune of 1.7 and 1.12 units/ml, respectively. Similarly, increase of urea concentration from 2 to 6 g/L and reduction of yeast extract from 6 to 4 g/L in the medium improved endocellulase production of bacterium, *Clostridium thermocopriae* (Jin and Toda, 1989). Casamino acids, irrespective of carbon sources used, highly stimulated extracellular production of β-glucosidase by *Termitimyces clypeatus* (Khowala and Sengupta, 1992). In contrast, the growth of *Trichoderma reesei* on production medium without nitrogen source increased cellulase production (Turker and Mavituna, 1987).

The use of purified cellulosics as substrates is uneconomical for large scale production of cellulase. Therefore cheaply available agricultural lignocellulose wastes were tested to find out whether they could support the production of cellulase by *A. niger* at 1% (w/v) level. Comparison of yields of cellulase (FP activity), obtained by growth of *A. niger* on untreated lignocelluloses, indicated most effectiveness of saw dust (Table 5). High activities of other enzyme components (CMCase and β-glucosidase) in culture filtrate of *A. niger* grown on saw dust could be related to its high growth of fungus and secretion of extracellular protein. The rate of bioconversion of cellulosic substrates, stream pretreated spruce and solka-floc cellulose by cellulolytic enzymes derived from the growth of *Trichoderma reesei* Rut C-30 in fermentor was higher than that achieved with commercially available celluloclast and logen cellulase (Szengyel et al., 2000). Similarly, *Humicola grisea* yielded maximum production of endoglucanase (CMCase) and filter paper activity upon its growth on lignocellulose of banana stalk (Soundar and Chandra, 1987). But, the culture filtrate of this fungus contained low activity of β-glucosidase. The inclusion of sludge from straw product factory in the medium at 3% (w/v) level supported the production of cellulases by *Trichoderma pseudokoningi* (Maheswari et al., 1990). According to studies of Gonzalez et al. (1986), higher enzymatic activities (FPA and β-glucosidase) were obtained with growth of *Trichoderma reesei* GM 9414 on wheat straw rather than on solka-floc as carbon source. A lignocellulolytic fungus, *Gliocladium* sp. TUBF-498, produced 77 filter paper units of cellulase activity and 246 U of β-glucosidase activity

per g of dry weight of processed popular wood as compared to 100 filter paper units and 92 U of β-glucosidase by the mutant reference strain, *Trichoderma reesei* Rut C- 30, on the same substrate in a stirred tank fermentation (Szakacs and Tengerdy, 1997). In a comparative study of cellulase production on different natural cellulosic substrates by *T. reesei* Rut C-30 highest titers of 4 IU/ml on popular wood at 2% level in both shake flask and fermentor was obtained (Shin et al., 2000). *Pleurotus sajor-caju* produced higher activities of 3 components of cellulase complex on alkali-treated cotton waste than on untreated cotton wastes (Tan and Wahab, 1997). Similarly, *A. niger* exhibited higher overall cellulolytic activity on untreated saw wood in the present study. The difference in the production of cellulolytic enzymes on a variety of lignocelluloses by different organisms could be assessed to various factors such as variable cellulose content in lignocellulose derived from different plant sources, heterogeneity of structure and cellulolytic abilities of the organisms at different degree. However, the production of cellulase on the treated lignocellulose further needs to be assessed.

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