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Short Communication

Induction of Cellulase Biosynthesis by Cellobiose Octaacetate in *Aspergillus humicola*

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***Aspergillus humicola*, one of the major cellulase-producing fungi, was used in this study for carboxymethylcellulase (CMCase) production using Winstead's basal broth supplanted with cellobiose octaacetate (COA), a synthetic carbon source. Under all conditions, the enzyme biosynthesis was remarkably increased when the inducer COA was added to the production medium containing carboxymethylcellulose (CMC). Maximum enzyme production (1.62 U/ml) was achieved in COA-containing at 37°C. The enzyme production was highest at initial pH 5.5 and after 7 days incubation. The enzyme exhibited maximum activity at 40°C with a reaction pH 5.5. CMCase activity was inhibited by its own substrate CMC at concentration higher than 1.0%. The study clearly demonstrated that COA is a good inducer for extracellular CMCase production by the fungus.**

Keywords: *Aspergillus humicola*, Carboxymethylcellulase (CMCase), Carboxymethylcellulose (CMC), Cellobiose octaacetate (COA)

Microbial sources of cellulase enzymes are of great importance in current economic value because of their versatile industrial and commercial applications. As the production of microbial enzymes has a great impact on the whole process economy, it is enviable to select effective but inexpensive and readily accessible substrates in enzyme production and to optimise the culture medium and culture conditions influencing the enzyme productions[1]. There are numerous reports on cellulase-producing fungi[2], however, only a few provide high activities of enzymes with commercial success[3-4]. In our search for cellulolytic fungi, *Aspergillus humicola* was found to be a potent producer of cellulase enzyme under suitable nutritional and environmental conditions. The present study was attempted to evaluate the influence of cellobiose octaacetate (COA) on the inductive formation of cellulase production by the fungus under different cultural conditions, and also to determine the optimum conditions for the enzyme activity.

A potential cellulolytic fungal isolate, *Aspergillus humicola*, was isolated from the wheat straw and was maintained on Czapek's and potato dextrose agar media. The fungus was identified according to taxonomical criteria described by Gilman[5]. Primarily, the isolate was screened for the cellulolytic activity using plate clearing assay method on Winstead's agar medium[6] containing 1.2% CMC according to Anwar and Zaman[7]. Induction of cellulase by cellobiose octaacetate (COA) was performed in 100-ml conical flask containing 50 ml Winstead's broth medium[6] and 1.2% CMC supplemented with or without the inducer COA (0.6%)[8]. Each flask was inoculated with 3-days-old fungal culture, and was incubated for 72 h at 27°C for cellulase production. The fungal culture was filtered through Whatman No.1 filter paper and the filtrate was then centrifuged at 8,000 rpm for 15 min. The clear supernatant was recovered and stored at 4°C with few drops of sodium azide to avoid contamination. The optimum cultural conditions, such as incubation period, initial pH and incubation temperature, for production of cellulase in shake-flask culture were determined as described by Shibli *et al*.[9].

Cellulase (CMCase) activity was assayed using 1.0% (w/v) solution of carboxymethylcellulose (CMC) in 0.1 *M* citrate buffer (pH 4.6) as substrate. The reaction mixture (5 ml), containing 2.0 ml substrate, 2.0 ml enzyme solution and 1.0 ml citrate buffer (0.1 *M*), was incubated at 35ºC for 120 min. The reducing sugars released were measured as glucose equivalents by Nelson's (1944) modification of Somogyi method[10]. One unit (U) of enzyme activity was defined as 1 µmol of reducing sugar released, as glucose equivalent, in 1 min under the assay conditions. Effect of substrate (CMC) concentration (0.1-2.0%), incubation temperature (35°-60°C) and assay pH (3.5-6.0) on cellulase activity was determined according to Saxena *et al*.[11].

The fungus, identified as *Aspergillus humicola* according to Gilman[5], was used for extracellular cellulase (CMCase) production in shake-flask cultures. It produced appreciable level of CMCase when grown in Winstead's broth medium supplemented with CMC (1.2%). The induction of CMCase was increased considerably when the production medium was supplemented with 0.6% cellobiose

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octaacetate (COA). Table 1 shows the influence of incubation temperature, initial pH and incubation period on CMCase production from the fungus when grown in CMC-containing medium supplemented with or without COA. Production of the enzyme was very dependant on the environmental conditions. However, under all conditions, the enzyme production was remarkably enhanced by the addition of COA in the medium. Different microorganisms vary in their incubation temperature, medium pH and incubation period for production of hydrolytic enzymes[12]. CMCase production by the fungus was highest in CMC plus COA-containing medium at 37°C, but in CMC-containing medium it was highest at 27°C. Therefore, the optimum temperature for CMCase production by the fungus might lie between 27° and 37°C. Production of CMCase by some mesophilic organisms at 37°C was reported by several workers[13-14].

Table 1. *Effect of culture temperature, initial pH and incubation period on extracellular CMCase synthesis from Aspergillus humicola on inducing medium containing CMC (1.2%) with or without COA (0.6%) supplementation*

Incubation condition	Enzyme activity in presence of CMC		Enzyme activity in presence of CMC + COA	
	CMCase (U/ml)	Relative activity (%)	CMCase (U/ml)	Relative activity (%)
Temperature[a]				
27°C	1.07	100.0	1.21	74.7
37°C	0.91	85.0	1.62	100.0
45°C	0.83	77.6	1.11	68.5
Initial pH[b]				
4.5	0.89	100.0	1.17	100.0
6.5	0.20	22.5	0.38	32.5
8.5	0.08	9.0	0.18	15.4
Time course[c]				
3 days	0.47	51.6	0.67	41.4
5 days	0.60	65.9	1.10	67.9
7 days	0.91	100.0	1.62	100.0
10 days	0.83	91.2	0.93	57.4

COA = Cellobiose octaacetate; [a]Shake-flask culture with an initial pH 4.6 for 7 days; [b]Shake-flask cultures at 37°C for 7 days; [c]Shake-flask culture with an initial pH 4.6 at 37°C

Maximum enzyme production by *A. humicola* was obtained at pH 4.5 regardless of the carbon source or inducer (Table 1). The enzyme production drastically dropped down at pH 6.5, which corresponded to only one-fifth of the activity achieved at pH 4.5. There are many reports on the requirement of pH of culture medium for extracellular enzyme production by fungi and bacteria, and in most case the maximum lies between pH 4.5 and 5.5[4] and between pH 4.5 and 8.5[13,15-17], respectively.

Highest CMCase activity was observed after 7 days of incubation, and thereafter the enzyme activity was decreased (Table 1). The plausible reason for decreased enzyme activity after prolonged incubation might be due to autolysis of the mycelia and/or loss of enzyme stability. Under optimum temperature (37°C) and pH (4.5) the enzyme production was about 1.8-fold higher in the medium containing COA (1.62 U/ml) as compared to the medium devoid of COA (0.91 U/ml). The role of a compound to act as inducer of cellulase biosynthesis varies from organism to organism. Sophorose is a strong inducer of cellulase in *Trichoderma* species[18], whereas it represses cellulase production in *Acetivibrio cellulolyticum*[19]. Cellobiose is an excellent inducer in *Neurospora crassa*[20] and *Sporotrichum pulverulentum*[21], but it is a relatively poor inducer in *Trichoderma reesei*[22]. In this study, the synthetic inducer cellobiose octaacetate (COA) exhibited good inducing property for cellulase biosynthesis by the fungus. For many other organisms cellobiose and cellulose are potential inducers of cellulase[23-25].

Activity of CMCase was assayed over the temperature range from 35-60°C. The temperature-activity of CMCase is shown in Table 2. Maximum activity of the enzyme was found at 40°C showing about 39% and 83% of its highest activity at 30°C and 50°C, respectively. The temperature-activity profile is similar to those observed for some other mesophilic fungi[4,11]. Fungal cellulases generally have lower pH optima for activity than bacteria[4]. The optimum pH observed for the CMCase of *A. humicola* seems consistent with these. The enzyme showed a sharp optimum on the pH-activity profile at pH 5.5 (Table 2). The pH-activity profile is similar to those observed with cellulase from other fungi[4,26].

Table 2. *Effect of temperature, pH and substrate concentration on CMCase activity from A. humicola*

Reaction condition	CMCase activity (U/ml)	Relative activity (%)
Reaction temperature[a]		
30°C	0.85	38.5
35°C	1.04	47.6
40°C	2.21	100.0
45°C	1.88	85.1
50°C	1.83	82.8
55°C	1.22	55.2
60°C	0.43	19.5
Reaction pH[b]		
3.5	0.51	27.4
4.0	0.61	32.8
4.5	1.06	57.0
5.0	1.26	67.7
5.5	1.86	100.0
6.0	0.83	98.4
Substrate concentration[c]		
0.1%	0.65	28.6
0.5%	1.07	47.1
1.0%	2.27	100.0
1.5%	1.32	58.1
2.0%	0.91	40.1

[a]Assay was performed using CMC (1.0%) as substrate with reaction pH 4.5 at various temperatures for 60 min. [b]Assay was performed using CMC (1.0%) as substrate with various reaction pH at 37°C for 60 min. [c]Assay was performed using various concentration of CMC as substrate with reaction pH 5.5 at 40°C for 60 min.

The activity of cellulase (CMCase) was determined with different concentrations (0.1-2.0%) of substrate (CMC). The effect of substrate concentration on the hydrolysis rate of CMC is shown in Table 2. It was observed that the enzyme was inhibited by its own substrate at concentrations above 1.0%. Bedino *et al*.[27] also reported similar result for an intracellular β-glucosidae of a thermophilic fungus *Thermoascus aurantiacus*. The enzyme activity increased with the increase in substrate concentration up to 1.0%, and the activity decreased gradually with further increase in substrate concentration. The rate of product formation reduced to half the maximum value at substrate concentration somewhere between 1.5% and 2.0%.

The present study clearly shows that the synthetic substrate, cellobiose octaacetate (COA) has the ability to induce cellulase production by *A. humicola*. Therefore, it could be incorporated in production medium for increased production of cellulase by the fungus. The fungus merits further attention as a potential source of extracellular cellulolytic enzymes.

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