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Full Length Research Paper

Production and characterization of endo-β-1,4-glucanase from *thermophilic fungus*

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Chaetomium thermophile **produces substantial extra-cellular cellulase on several cellulosic substrates (wheat straw, wheat bran and corn straw etc.) including simple sugars. 2% (w/v) wheat straw, wheat bran and corn straw were used as carbon source and urea was used as a nitrogen source. Experiment was conducted in a 20-l stirred tank reactor under the following conditions: pH 5.0, stirring speed 300 rpm, temperature 45°C and aeration 50% (DO). The samples were withdrawn after every 12 h regularly and enzyme activity was assayed. Endo-β-1,4-glucanase activities on wheat straw, wheat bran and corn straw were 2.51, 1.62 and 5.10 U/ml, respectively and extra-cellular protein contents were 1.48, 1.17 and 2.88 mg/ml, respectively. Specific activity of enzyme after ammonium sulphate precipitation and Fast Protein Liquid Chromatography (FPLC) was 2.1 and 3.3 U/mg of protein, respectively. SDS-PAGE showed only a single band at 53 kDa. The optimum temperature and pH of the CMCase were 60°C and from 6.0 - 7.5, respectively.**

Key words: *Chaetomium thermophile,* cellulase, fermentation, extra-cellular enzymes, agro-wastes.

INTRODUCTION

Cellulases are carbohydrases that cleave the β-1,4-linkage of cellulose in addition to degrading cello-dextrin or cellobiose. Biological degradation of cellulose involves the synergistic action of three enzymes, namely endo-β-1,4-glucanase (EC 3.2.1.4), exo-β-1, 4-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Endo-β-1,4-glucanase or carboxymethyl cellulase (CMCase), hydrolyzed cellulose in random fashion producing oligos and reducing polymer length, while exo-β-1,4-glucanase (cellobiohydrolase) cleave cellobiosyl residues from the non-reducing end of cellulose chain. Then, cellobiose is hydrolyzed by β- glucosidase to yield two glucose units (Coughlan et al., 1985). Many cellulolytic organisms produce cellulases under suitable conditions that perform cellulolysis necessary for cell growth.

Fungi constitute a most fascinating group of organisms and are most common industrial source for hemicellulases such as glucanases, xylanases, galactanaseses, etc. Their extra-cellular enzymes have almost same optimum temperature as for the growth of that micro-organism. In general they are more heat stable than those of mesophilic fungi. Thermophilic fungi such as *Chaetomium thermophiles, Mucar meihei, Sporotrichum thermophile* and *Asperigillus fumigatus* thrive at temperatures of 40 - 60°C. They have immense commercial importance because of producing extra-cellular thermostable enzymes (Lin et al., 1999).

The objective of this study was to produce CMCase from cheap and easily available carbon sources by using *C. thermophiles.* Furthermore, an attempt was made for characterization and partial purification of the enzyme.

MATERIALS AND METHODS

Organism and growth conditions

C. thermophile collected from NIBGE (National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan) was maintained on yeast extract (YE) and malt agar (MA). Shaken culture (200 rpm) was conducted at 45°C in Eggin and Pugh medium (Eggin and Pugh, 1962) of the following composition (g/l): KH_2PO_4 1.0, $(NH_4)_2SO_4$ 0.5, KCl 0.5, $MgSO_4$ 0.2, $CaCl_2$ 0.1, YE 0.5, urea 1.5.

Inoculum was prepared as following: 100 ml E and P medium was dispend into a 500-ml conical flask. The medium was autoclaved for 15 min (121°C) and was seeded with spores of *C. thermophiles*. A filter-sterilized (50% w/v) stock solution of glucose (4 ml) was added

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as carbon source. The inoculum was cultivated on a rotary shaker at 45°C and 150 rpm for 48 h.

Enzyme production on different carbon sources

Enzyme production was carried out in 20-l fermentor (B-Braun, Biostate) using different carbon sources i.e. wheat straw, wheat bran and corn straw (2% w/v) at 45°C, air 6.0 l/m, agitation 300 rpm, pH was allowed to fluctuate naturally and dissolved oxygen (DO) was kept at 50%, using cascade mechanism. The inoculum was prepared in glucose medium at 45°C on a rotary shaker (200 rpm), 10% (v/v) of inoculum was transferred to bioreactor ascetically and allowed to continue for 5 days. Samples were taken out at regular intervals and enzyme activity was checked using standard assay. Culture was centrifuged at 48,000 x g for 10 min at 25°C. The biomass pellets were discarded and the supernatant containing crude enzyme was stored at 4°C. Sodium azide (0.02%) was added to avoid contamination.

Ammonium sulphate precipitation

The supernatant from liquid culture conducted on the medium, added with wheat straw was treated with ammonium sulphate (20 – 80% saturation) and incubated at 4°C for 10 h. The precipitate was collected by centrifugation at 48,000 x g for 30 min, dissolved in 0.1 M Tris-HCl buffer of pH 8.0 and dialyzed overnight against the same buffer.

Fast protein liquid chromatography (FPLC)

The sample obtained after 70% ammonium sulphate precipitation was subjected to sequential fractionation by anion exchange chromatography on a Hi-Load Q Sepharose column, anion-exchange chromatography on a Mono-Q column (Amersham Bio-sciences, UK) and gel filtration chromatography (Deutscher, 1990; Grabski et al., 1991). The sample was loaded on a Hi-load Q-Sepharose column (30 x 120 mm) at a flow rate of 2 ml/min. A linear gradient of NaCl from 0–0.6 M Tris–HC1 buffers at pH 5, 8 and 9.5 were used as elution buffers. The 10 ml dialyzed fractions were loaded onto a Mono-Q column (15 x 100 mm) at a flow rate of 1 ml/min and eluted with a linear gradient of 0 – 0.5 M NaCl in 20 mM ethanolamine at pH 9.5. Pooled active fractions were loaded onto a 15 x 300 mm Superose 12 column equilibrated in 0.1 M Tris–HCl buffer pH 7.0 containing 0.1 M NaCl at a flow rate of 0.5 ml/min.

Assessment of homogeneity and molecular weight determination

SDS-PAGE (12%) was carried out according to method of Laemmli (1970) in order to assess homogeneity of the purified preparation as well as to determine the molecular weight of the enzyme. The molecular weight of the enzyme under non-denaturing conditions was determined by gel filtration on Bio-GelA- 0.5 (Bio-Red Laboratories) as described by Andrews (1965). The standard protein markers used were: phosphorylase b (94 kDa), glutamate dehydrogenase (53 KDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), lysozyme (14.4 kDa) and cytochrome c (12.4 kDa).

Enzyme assay

Endo-β-1,4-glucanase activity was assayed by using carboxymethyl cellulose (CMC) as a substrate according to the method of Prasertsan (1997). In particular, 1.0 ml of properly diluted enzyme sample was added to 1% CMC solution in 50 mM citrate phosphate buffer at pH 5.0 and subsequently incubated at 50°C in a shaken water-bath. The reaction was stopped by adding 3 ml DNS (3,5-dinitro salicylic acid) reagent (Miller, 1995) and reaction mixture was incubated at 100°C for 10 min. Enzyme activity was calculated by taking absorbance at 550 nm.

Determination of extra-cellular protein

The protein of the samples was estimated using the Bradford method with bovine serum albumin (A-4503, Sigma) as the protein standard (Bradford, 1976).

Temperature, pH optima and heat stability

In all the determinations, the CMCase activity was measured using carboxymethyl cellulose a substrate. To estimate optimum temperature, pH and heat stability, the activity was calculated by carrying out the above standard assays at several temperatures (40 - 90°C) and pH values (4.0 - 8.5) using 50 mM citrate phosphate buffer (Chakrabarty et al., 2000). The thermo-tolerance of the enzyme was studied by heating the enzyme at different temperatures (40 - 90°C) for 15 min.

RESULTS AND DISCUSSION

Time course study of enzyme production

Fermentation was carried out from 12 - 120 h to determine the optimum fermentation period. Enzyme production was started after 72 h, the production rate was increased up-to 96 h, retained steady up-to 105 h and eventually decreased with passage of time (Figure 1). This means that the optimum time period for production of enzyme is up to 96 h. These results are also supported by earlier studies on *Aspergillus candidus* and *Thermoascus aurantiacus* (Warsywoda et al., 1992; Kalogoris et al., 1999).

Effect of carbon sources

In order to check the effect of carbon sources on the production of CMCase, enzyme production was done on different carbon sources. Table 1 reveals that CMCase activity was high for corn straw (5.10 U/ml) as a carbon source instead of wheat straw (2.51 U/ml) and wheat bran (1.62 U/ml). The similar results were obtained in bubble column reactor from *Asperigillus niger* in external-loop air-lift reactor (Kang et al., 1994).

Optimum temperature

The activity of the enzyme was measured at different temperatures (40 - 90°C). The CMCase retained its 100 and 71% activity at 60 and 80°C, respectively, for 15 min (Figure 2). Thus extra-cellular enzyme from *C. thermo-*

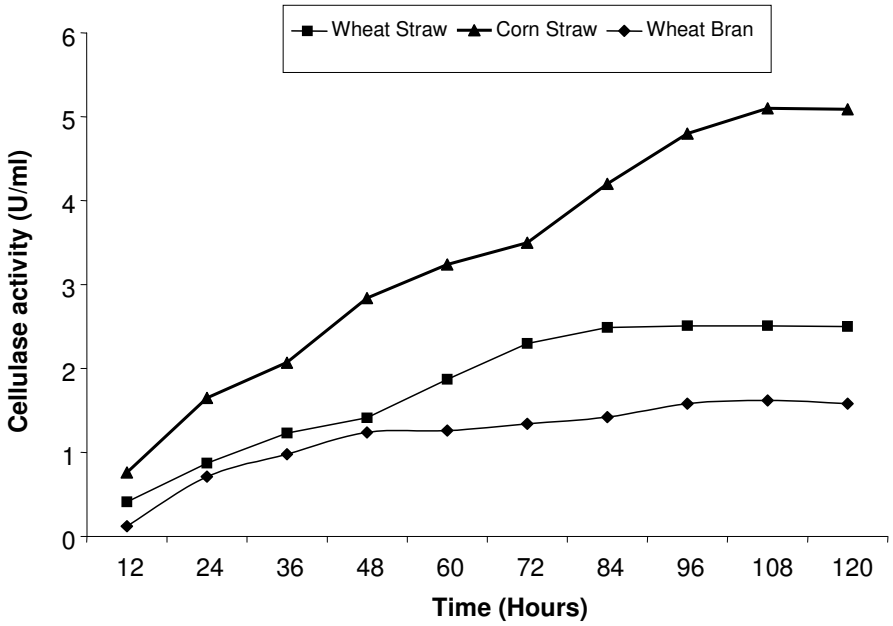

Figure 1. Time course study of production of cellulase on different carbon sources by *Chaetomium thermophile*.

Table 1. Activity of cellulase produced by *Chaetomium thermophile* on different carbon sources.

Carbon source	Cellulase activity (U/ml)	Extra cellular protein (mg/ml)	Specific activity (U/mg)
Wheat straw	2.51	1.48	1.70
Wheat bran	1.62	1.17	1.38
Corn straw	5.10	2.88	1.77

phile was found stable at high temperature and it is in good agreement with the work of Gomes et al. (2000) and Kawamori et al. (1987).

Optimum pH

To determine the optimum pH, enzyme activity was checked at different pH values (4.0 - 8.5). The enzyme retained its 100% activity at pH values from 6.0 - 7.5 and 86% activity at pH 8.0 (Figure 3).

Heat stability

CMCase activity was measured by incubating the enzyme at various temperatures from 30 - 70^{o}C at pH 7.0 for 4.0 h. The enzyme retained its 80% activity at 45^{o}C for 4 h (Figure 4). The use of thermostable enzymes to carry out hydrolysis at high temperature is advantageous because they speed up the reaction rate and prevent microbial contamination. It also increases the economical and technical vialability of the process (Durand et al., 1984; Gomes et al., 1994).

Purification of CMCase

All the purification steps are summarized in Table 2. The crude enzyme was first precipitated with 20 to 80% ammonium sulfate saturation. It was found that 70% ammonium sulphate saturation was optimal for CMCase precipitation. The specific activity was increased from 1.7 to 2.1 U/mg and 54% yield was obtained. Then the crude enzyme was subjected to Fast Protein Liquid Chromatography (FPLC) that came out with 40% yield and 3.3 U/mg specific activities in accordance with already reported results for *Thermomyces lanuginosus* (Lin et al., 1999).

Molecular weight

The molecular weight of cellulase was determined by SDS-PAGE, as described in materials and methods section. On SDS-PAGE, CMCase showed only a single band indicating cellulolytic activity in the gel. The molecular weight of this band was estimated to be around 53 kDa (Figure 5).

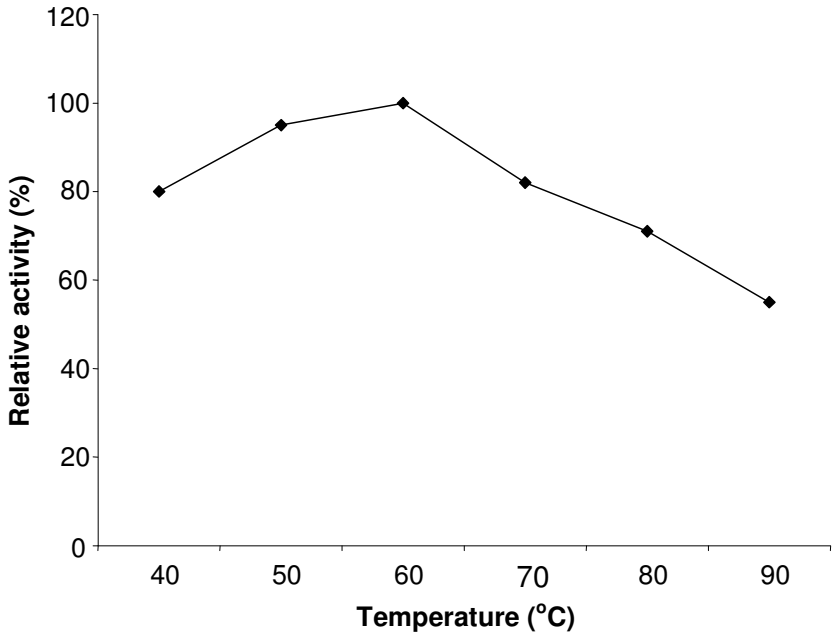

Figure 2. Optimum temperature range of CMCase from *Chaetomium thermophile.*

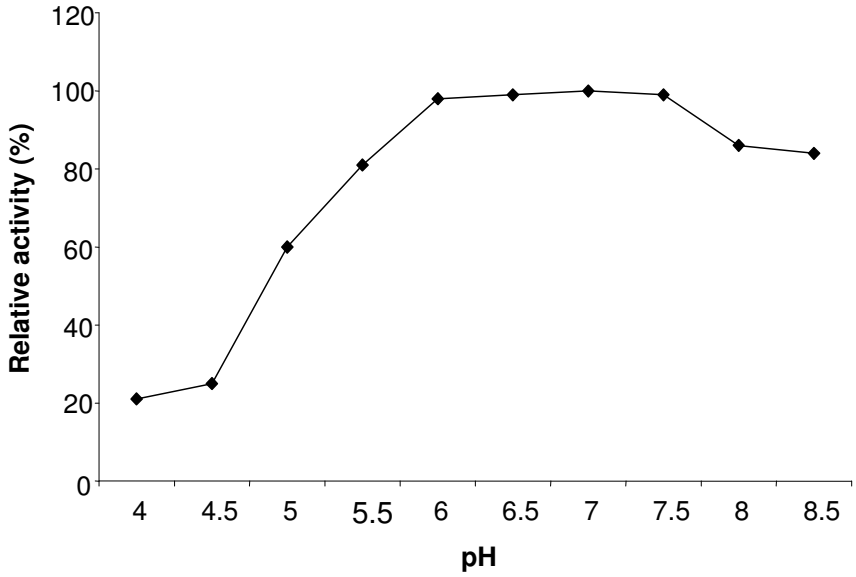

Figure 3. Optimum pH range of CMCase from *Chaetomium thermophile.*

Conclusion

It was concluded that corn straw is best carbon source than wheat bran and wheat straw. The enzyme activity was best from 84-96 h, after this activity of enzyme start to come down. The optimum temperature and pH of the CMCase are 60°C and pH 6.0 - 7.5, respectively. As SDS-PAGE showed only a single band at 53 kDa it means that endo-β-1, 4-glucanase from *C.thermophile* possesses a monomeric structure.

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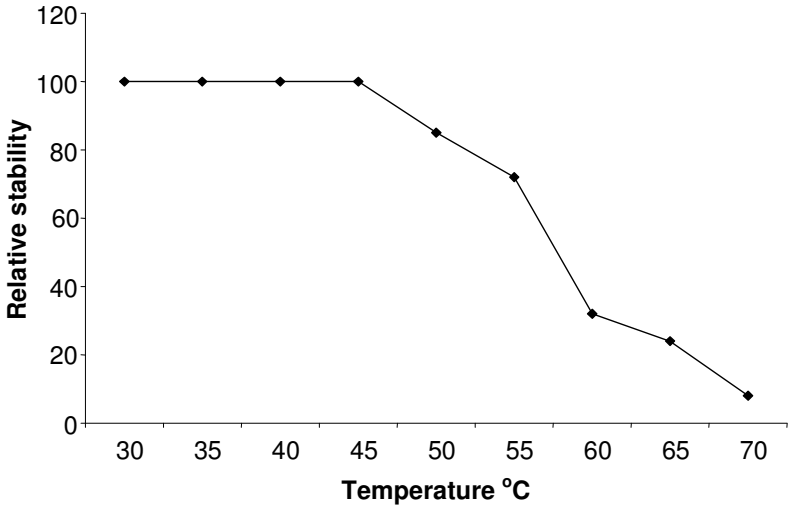

Figure 4. Heat stability of CMCase from *Chaetomium thermophile.*

Table 2. Extraction profile of cellulase from *Chaetomium thermophile.*

Purification step	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	251	148	1.7	100	1.0
$(NH_4)_2SO_4$ precipitation	165	80	2.06	54	1.04
Hi-load anion Exchange	152	71	2.14	50.4	1.02
Mono-Q- anion Exchange	142	65	2.18	46.2	1.51
FPLC	105	32	3.3	40.1	1.57

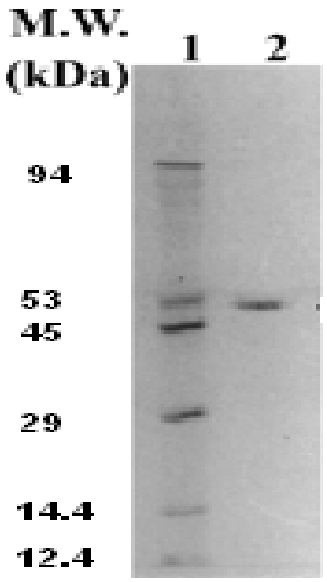

Figure 5. SDS-PAGE of the purified CMCase from *Chaetomium thermophile* on 12% polyacrylamide gel. Lane 1: molecular weight marker, Lane 2: purified enzyme.

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