

Full Length Research Paper

Identification of over producer strain of endo- -1,4-glucanase in *Aspergillus Species*: Characterization of crude carboxymethyl cellulase

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Cellulases are a group of hydrolytic enzymes capable of degrading cellulose to smaller sugar components like glucose units. These enzymes are produced by fungi and bacteria. The aim of this research was to identify a *Aspergillus species* with over production of endo- -1,4-glucanase. Properties of endo- -1,4-glucanase/carboxymethylcellulase (CMCase) from a culture filtrate of the *Aspergillus sp.* was also studied. *Aspergillus sp.* (R4) was selected as over producer of endo- -1,4-glucanase among 13 different species. SDS-PAGE activity staining with 1% Congo Red solution revealed three protein bands showing cellulolytic activity. The molecular weights of these proteins were estimated to be approximately 18.5, 23 and 28 kD. Also, conservative region of endo- -1,4-glucanase coding gene was studied by polymerase chain reaction (PCR). Amplified fragments with 1204 bp and 399 bp were confirmed by restriction pattern with HindIII and PstI enzymes.

Key words: *Aspergillus sp.*, Endo- -1, 4-glucanase, CMCase, SDS-PAGE, PCR.

INTRODUCTION

Cellulose is a major polysaccharide constituent of plant cell walls and one of the most abundant organic compounds in the biosphere (Murai et al., 1998; Hong et al., 2001). Cellulose has enormous potential as a renewable source of energy (Coral et al., 2002) and several microorganisms use it as a carbon source. It has also attracted the interest of biotechnologists, who wish to use it as a renewable source of fuels and chemicals (Beguin, 1990). Cellulose is an unbranched glucose polymer composed of an -1,4 glucose units linked by a -1,4-D-glycosidic bond (Gielkens et al., 1999; Han et al., 1995). Cellulolytic enzymes degrade cellulose by cleaving the glycosidic bonds (Han et al., 1995). A

number of fungi and bacteria are capable of producing multiple groups of enzymes, which are collectively known as cellulases, that act in a synergistic manner to hydrolyze the -1,4-D-glycosidic bonds within the cellulose molecules (Akiba et al.1995). Cellulases can be classified into three types: endoglucanases or carboxymethyl cellulases (CMCases) (endo- -1,4glucanase, EC 3.2.1.4), exoglucanases or cellobiohydrolases (exo- -1,4-glucanase, EC 3.2.1.91), and -glucosidases (-D -glucoside glucohydrolase, EC 3.2.1.21) (Gielkens et al., 1999; Kang et al., 1999; Parry et al., 1983). Endoglucanases randomly hydrolyze internal -1,4- D-glycosidic bonds in cellulose (Siddiqui et al., 2000). As a result, the polymer rapidly decreases in length, and the concentration of the reducing sugar increases slowly. Exoglucanases hydrolyze cellulose by removing the cellobiose units from the nonreducing end of cellulose (Han et al., 1995; Akiba et al., 1995).

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Table 1. The characteristics of fungi isolates used in this study.

Isolates	Code	ATCC number	Diameter of Clearing zone	CMCase specific activity(U/mg)
<i>A. niger</i>	5010	9172	35mm	8.50
<i>A. niger</i>	5011	16404	38mm	2.56
<i>A. niger</i>	5012	9029	40mm	4.191
<i>A. terreus</i>	5021	10029	28mm	3.53
<i>A. awamori</i>	5097	16877	37mm	2.90
<i>A. niger</i>	5154	1004	20mm	2.43
<i>Aspergillus</i> sp.	R1	Rijab	un clear	3.53
<i>Aspergillus</i> sp.	R2	Rijab	35mm	11.19
<i>Aspergillus</i> sp.	R3	Rijab	33mm	11.69
<i>Aspergillus</i> sp.	R4	Rijab	50mm	11.80
<i>Aspergillus</i> sp.	M2	Marand	21mm	11.38
<i>Aspergillus</i> sp.	M3a	Marand	25mm	5.82
<i>Aspergillus</i> sp.	M3b	Marand	23mm	10.31

Cellulase enzymes are produced by several organisms including fungi and bacteria. In general, bacterial cellulases are constitutively produced, whereas fungal cellulases are produced only in the presence of cellulose (Suto and Tomita, 2001). Filamentous fungi, particularly *Aspergillus* and *Trichoderma* species, are well known as efficient producers of these cellulases (Peig et al., 1998). In the current study an over producer of -1,4-endoglucanase (CMCase) was selected among 13 isolates of *Aspergillus* sp. Protein pattern and cellulolytic activity of this isolate was studied by SDS- PAGE. Also, conservative region of CMCase coding gene was amplified by PCR using specific primers.

MATERIALS AND METHODS

Fungal strains and culture conditions

Of the 13 isolates of *Aspergillus* sp., 6 were obtained from Persian Type Culture Collection (PTCC) and other strains were isolated from soil samples and sooty molds on tree from various locations within Iran such as Rijab (R) and Marand (M) cities (Table 1). All strains were selected for their ability to grown on cellulose as sole carbon source. They were grown on Potato Dextrose Agar (PDA) plates for 5-7 days at 28°C. Cultures were stored at 4°C.

Plate Screening

For plate screening, Carboxymethylcellulose-Agar (CMC-Agar) medium was used. This medium consist of: 1.00% (w/v) CMC, 0.65% (w/v) NaNO₃, 0.65% (w/v) K₂HPO₄, 0.03% (w/v) Yeast extract, 0.65% (w/v) KCl, 0.3% (w/v) MgSO₄, 0.065% (w/v) glucose, 1.7% (w/v) agar and 0.1% (v/v) triton X-100. Also, conidia from one-week-old PDA plates were suspended in sterile H₂O. A small well created in the middle of the screening plates and same number of conidia of each strain (~10⁵) was inoculated into the wells. Plates were incubated at 28°C for three days followed by 18 h at 50°C. For cellulolytic activity observations, plates were stained with 1% Congo

Red dye for 0.5-1 h followed by destaining with 1 M NaCl solution for 15-20 min.

Production of extracellular -1,4 endoglucanase (CMCase) in shake flasks

Fungi determined to have cellulolytic activity were then cultured in Mandels salt medium with optimum conditions for enzyme assay. 0.5 L Erlenmeyer flask containing 200 ml of Mandels medium, CMC as carbon source and Lactose as inducer at pH 8, was inoculated with 10⁶ conidia. Culture was incubated in an orbital shaker incubator at 30°C for 10 days at 150 rpm. At 48 h intervals samples of 8 ml were collected and centrifuged at 12000 rpm (4°C) to remove solids. The supernatant was measured for enzymatic activity. CMCase activity was determined by the dinitrosalicylic acid (DNS) method (Mandels et al., 1976), where one IU (International Unit) is defined as the amount of enzyme releasing 1 mol glucose per minute (units/ml) (Zaldivar et al., 2001; Kader et al., 1998, 1999).

Study of some properties of crude CMCase

Aspergillus sp. (R4) wild type strain was used as the enzyme source. Cultivation was carried out in modified Mandels liquid medium containing CMC as sole carbon source. For growth of the organism and enzyme production, 1 L Erlenmeyer flasks which contained 500 ml of growth medium with optimum conditions were incubated at 30°C on an orbital shaker (150 rpm) for 6 days. Mycelia was removed by filtration and the filtrate was used for crude enzyme preparation.

Ammonium sulfate precipitation and concentration

For protein precipitation, solid ammonium sulfate was added slowly to filtrate to give a final (NH₄)₂SO₄ concentration of 48.3% (w/v) and the solution was left overnight at 4°C. After centrifugation at 14000 rpm for 15 min at 4°C, the supernatant was removed and pellet of precipitated proteins was kept and dried at laboratory temperature. Then pellet which contained CMCase was dissolved in 5 ml of double distilled H₂O and was dialyzed against double

distilled H₂O for 48 h at 4°C. This was further dialyzed against 50% (w/v) PEG in order to concentrate the protein sample.

Sodium dodecyl sulfate-poly acryl amid gel electrophoresis (SDS-PAGE)

The crude enzyme preparations and known molecular markers were subjected to electrophoresis by the modified method of Bollag and Edlsein (Coral et al., 2002) with the use of 12% acrylamide gel; 0.2% CMC was added into the separating gel prior to the addition of ammonium persulfate and TEMED for polymerization. For molecular weight determination, the gel was stained with Coomassie blue G250. For CMCase activity, the gel was soaked and gently was shaken in solution A (50 mM Sodium phosphate buffer, pH 7.2 containing 25% isopropanol) for 1 h and solution B (50 mM Sodium phosphate buffer, pH 7.2) for 30 min to renature the enzyme proteins. The gel was then stained with 1% Congo Red solution for 30 min and destained with 1 M of NaCl solution.

Polymerase chain reaction (PCR)

Genomic DNA was prepared using cetyltrimethylammonium bromide (CTAB method) as described by Dan et al. (2000). PCR was done with three specific primers: EngF1 (5'-GCT GGT TCC GCG TTG GCT GTG C-3') as forward primer, and EngR1 (5'-GCA TCT GCG ACC CCA CCC AGT T-3') and EngR2 (5'-TCG TCA GCA TTA AGT CAA CAC CAA GTG G-3') as reverse primers based on the DNA sequence of eglB gene (Hong et al., 2001). Two PCR amplifications were conducted with primer pairs (EngF1 and EngR1) and (EngF1 and EngR2) with the denaturing at 94°C for 90 s, annealing at 62°C for 30 s and extension at 72°C for 45 s, with final extension at 72°C for 180 s.

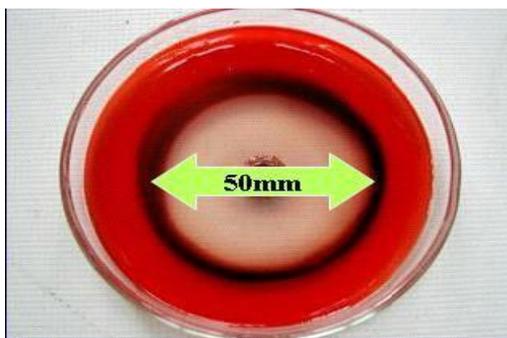


Figure 1. Plate screening of CMCase in the surrounding of colonies and Congo Red dye staining for displaying clearing zone.

RESULTS

The selection of over producing cellulase isolates was based on the diameter of clearing zone surrounding the small well on the plate screening medium (Figure 1). The size of clearing zone diameter for each isolate are shown in Table 1.

Enzyme specific activity for the 13 isolates is shown in Table 1 and Figure 2. Results showed that in optimized conditions, *Aspergillus* sp. (R4) has highest enzyme specific activity among total isolates.

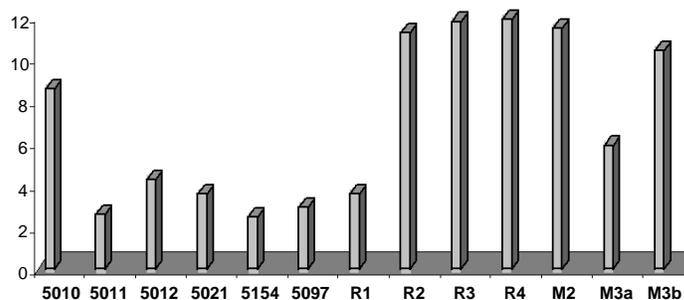


Figure 2. CMCase specific activity for 13 isolates in optimum conditions

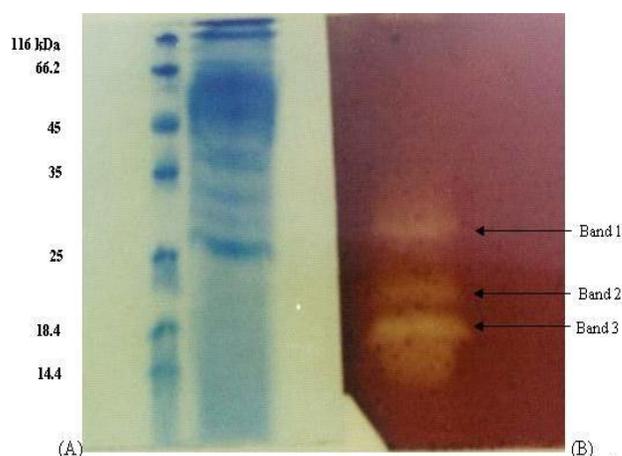


Figure 3. 12% SDS-PAGE of crude CMCase stained for the protein pattern (A) and activity pattern (B) for determination of molecular weight. From left to right, lane 1: contains the molecular weight markers [β -galactosidase (116.0), bovin serum albumin (66.2), ovalbumin (45.0), lactate dehydrogenase(35.0), RE *Bsp981* (25.0), β -lactoglobulin (18.4) and lysozyme (14.4kD)]. Lane 2: Coomassie blue G250 dye staining of crude protein. Lane 3: CMCCase activity with Congo Red dye staining (band 1: 28 kD, band 2: 23 kD and band 3:18.5 kD).

CMCase was obtained from *Aspergillus* sp. (R4) and three bands showing cellulolytic activity were detected on the electrophoresis gel (Figure 3).

The expected PCR products with 1204 bp (Eng F1 and EngR1 primers) and 399 bp (EngF1 and EngR2 primers) were amplified (Figure 4). The 1204 bp was confirmed by digestion with HindII and PstI restriction enzymes (Figures 4C, 5).

DISCUSSION

Aspergillus sp. is known to produce a variety of cellulolytic enzymes including carboxymethyl cellulase or endo- -1,4-glucanase. We identified *Aspergillus* sp. (R4) as an over producer of endo- -1,4-glucanase among 13

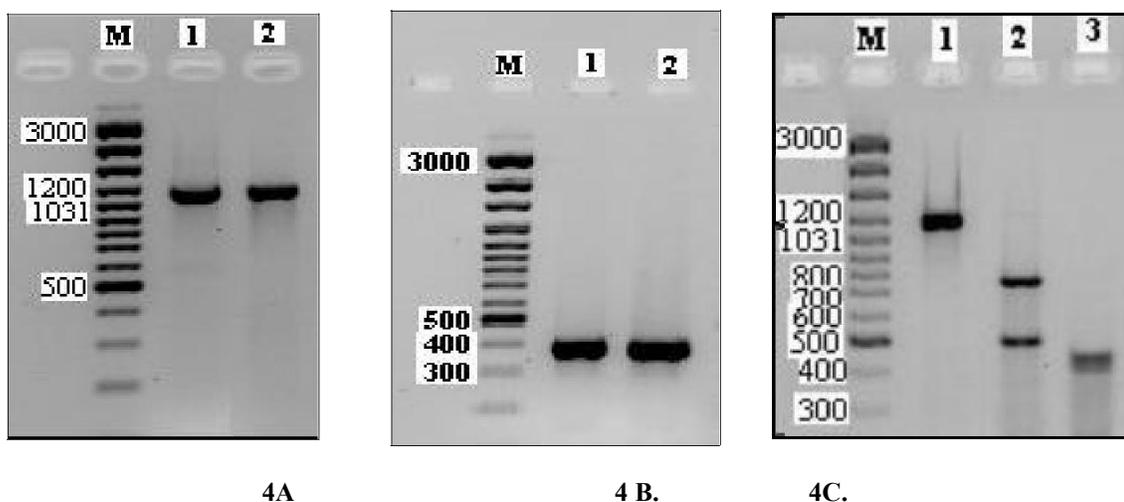


Figure 4 (A). PCR product of CMCCase gene fragment using EngF1 and Eng R1 primers. **(B)** PCR product of CMCCase gene fragment using EngF1 and Eng R2 primers. **(C)** HindII and PstI restriction enzymes digestion of the fragment amplified with Eng F1 and Eng R1 primers.

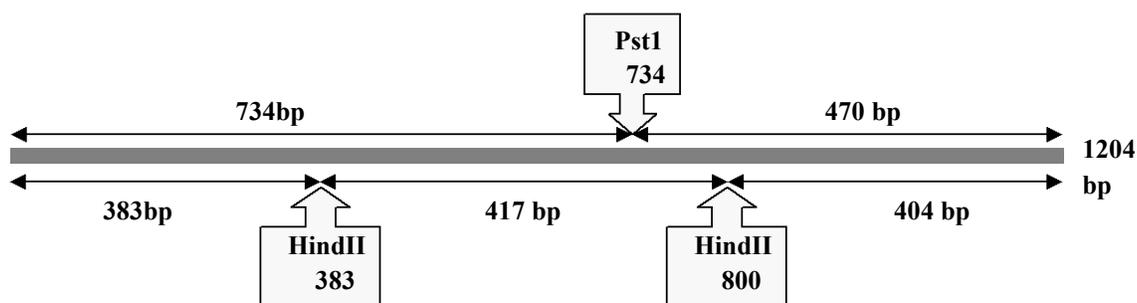


Figure 5. Restriction map for HindII and PstI on the amplified fragment of CMCCase gene.

different *Aspergillus* species. Enzyme specific activity for *Aspergillus* sp. (R4) of 11.80 (U/mg) indicate that this isolate is over producer of CMCCase. Three bands showing cellulolytic activity were detected on gel during electrophoresis of the crude enzyme. The molecular weights of these bands were estimated to be 18.5, 23 and 28 kD. These bands (proteins) may be isoenzymes or the different subunits of the same enzyme protein on electrophoresis gel (Coral et al., 2002).

Polymerase chain reaction with specific primers amplified 399 and 1204 bp fragments. These primers previously designed based on the DNA sequence of *eglB* (endoglucanase B) gene (Hong et al. 2001).

Identification of over producers of cellulase enzymes and cloning of their genes in prokaryotes can help industries exploit these enzymes. Here, we have identified an over producer of endo- α -1,4-glucanase and

amplified its coding region.

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