African Journal of Biotechnology Vol. 4 (1), pp. 26-30, January 2005
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2004 Academic Journals

Full Length Research Paper

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

Habib Onsori[1,3]*, Mohammad Raza Zamani[2], Mostafa Motallebi[2], Nosratollah Zarghami[3]

[1]Department of Biology, Islamic Azad university, Marand, Iran.
[2]National Research Center for Genetic Engineering and Biotechnology of Iran.
[3]Department of Molecular Biochemistry and RIA, Applied Drug Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Accepted 13 September, 2004

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-β-1,4-glucanase. Properties of endo-β-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-β-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-β-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 HindII and PstI enzymes.

Key words: *Aspergillus sp.*, Endo-β-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 β-1,4 glucose units linked by a β-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 β-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-β-1,4glucanase, EC 3.2.1.4), exoglucanases or cellobiohydrolases (exo- β-1,4-glucanase, EC 3.2.1.91), and β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) (Gielkens et al., 1999; Kang et al., 1999; Parry et al., 1983). Endoglucanases randomly hydrolyze internal β-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).

*Correspondingauthor. E-Mail: onsori1356@yahoo.com, nzarghami@hotmail.com Tel: +98-411-3363234 Ext 241. Fax: +98-411-3363231

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

Isolates	Code	ATCC number	Diameter of Clearing zone	CMCase specific activity(U/mg)
A. niger	5010	9172	35mm	8.50
A. niger	5011	16404	38mm	2.56
A. niger	5012	9029	40mm	4.191
A. terreous	5021	10029	28mm	3.53
A. awamori	5097	16877	37mm	2.90
A. niger	5154	1004	20mm	2.43
Aspergillus sp.	R1	Rijab	un clear	3.53
Aspergillus sp.	R2	Rijab	35mm	11.19
Aspergillus sp.	R3	Rijab	33mm	11.69
Aspergillus sp.	R4	Rijab	50mm	11.80
Aspergillus sp.	M2	Marand	21mm	11.38
Aspergillus sp.	M3a	Marand	25mm	5.82
Aspergillus 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 β-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 Cullection (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 28ºC. Cultures were stored at 4ºC.

Plate Screening

For plate screening, Caboxymethylcellulose-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 (~10^5) was inoculated into the wells. Plates were incubated at 28ºC for three days fallowed by 18 h at 50ºC. 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 β-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 Erlenmayer flask containing 200 ml of Mandels medium, CMC as carbon source and Lactose as inducer at pH 8, was inoculated with 10^6 conidia. Culture was incubated in an orbital shaker incubator at 30ºC for 10 days at 150 rpm. At 48 h intervals samples of 8 ml were collected and centrifuged at 12000 rpm (4ºC) 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 µmol 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 Erlenmayer flasks which contained 500 ml of growth medium with optimum conditions were incubated at 30ºC 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 4ºC. After centrifugation at 14000 rpm for 15 min at 4ºC, 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 H_2O and was dialyzed against double

distilled H_2O for 48 h at 4ºC. 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 Edlesein (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 shaked 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 94ºC for 90 s, annealing at 62ºC for 30 s and extension at 72ºC for 45 s, with final extension at 72ºC for 180 s.

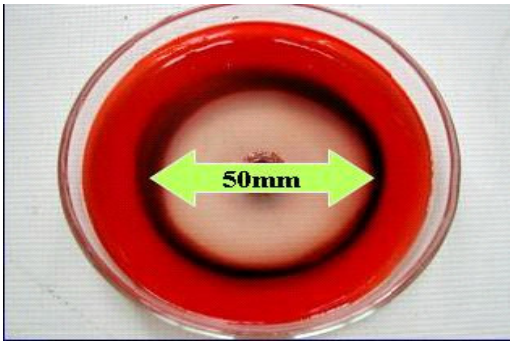


Figure 1. Plate screening of CMCase in the surrounding of colonies and Congo Red dye staining for displaying clearing zone.

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.

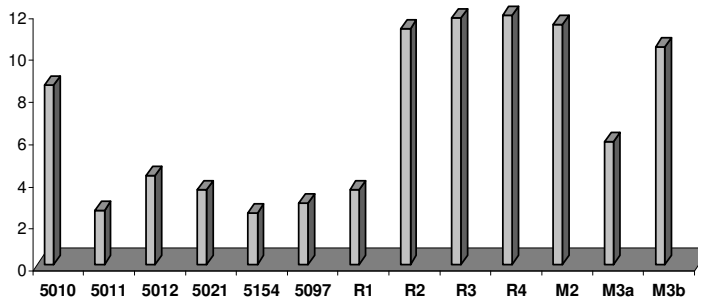


Figure 2. CMCase specific activity for 13 isolates in optimum conditions

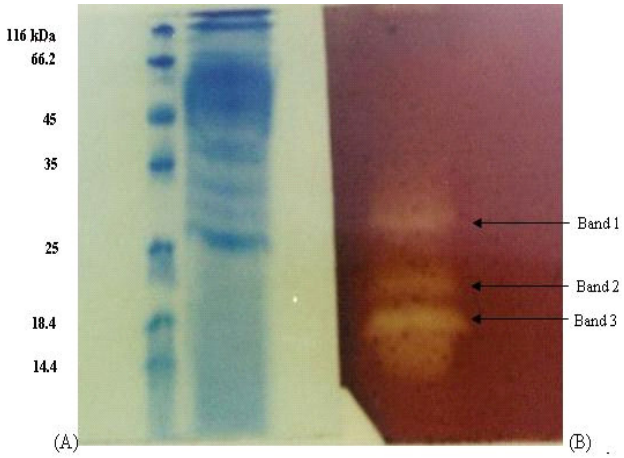


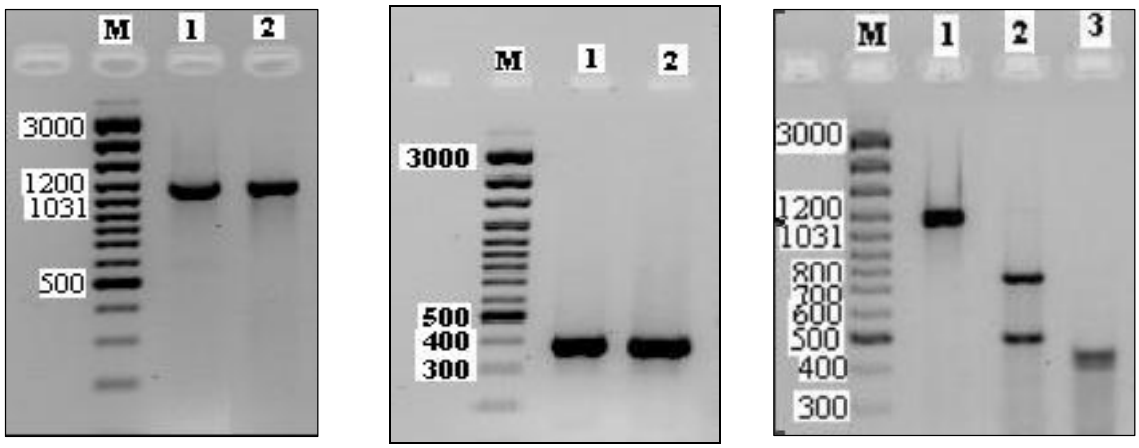
Figure 3. 12% SDS-PAGE of crude CMCase stained for the protein pattern (A) and activity pattern (B) for determination of molecular weight. From left to right, lane 1: contains the molecular weight markers [β-galactosidase (116.0), bovin serum albumin (66.2), ovalbumin (45.0), lactate dehydrogenase(35.0), RE *Bsp*981 (25.0), β-lactoglobulin (18.4) and lysozyme (14.4kD)]. Lane 2: Coomassie blue G250 dye staining of crude protein. Lane 3: CMCase activity with Congo Red dye staining (band 1: 28 kD, band 2: 23 kD and band 3:18.5 kD).

CMCase was obtained from *Aspergills* 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-β-1,4-glucanase. We identified *Aspergillus* sp. (R4) as an over producer of endo-β-1,4-glucanase among 13



4A 4 B. 4C.

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

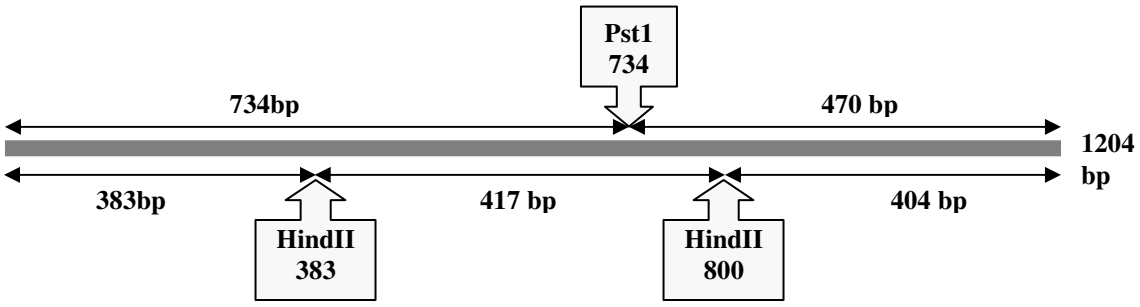


Figure 5. Restriction map for HindII and PstI on the amplified fragment of CMCase gene.

different *Aspergillus* species. Enzyme specific activity for *Aspergillus* sp. (R4) of 11.80 (U/mg) indicate that this isolate is over producer of CMCase. 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-β-1 ,4-glucanase and amplified its coding region.

ACKNOWLEDGEMENT

We would like to acknowledge the kind assistance of B. Alani and H. Habibollahi, M. Badri and B. Alani of the Applied Drug Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

REFERENCES

Akiba S, Kimura Y, Yamamoto K, Kumagai H (1995). Purification and characterizationof a protease-resistant cellulase from *Aspergillus niger*. J. Ferment. Bioeng. 79(2):125-130.

Beguin P (1990). Molecular biology of cellulose degradation. Annu. Rev. Microbiol. 44:219-248.

Coral G, Arikan B, Unaldi MN, Guvenmes H (2002). Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 wild-type Strain. Turk J. Biol. *26*:209-213.

Dan S, Marton I, Dekel M, Bravdo BA, He S, Withers SG, Shoseyov O (2000). Cloning, expression, characterization, and nucleophile identification of family 3, *Aspergillus niger* b-Glucosidase. J. Biol. Chem. 275 (7): 4973-4980.

Gielkens MMC, Dekkers E, Visser J, Graaff L H (1999). Two cellubiohydrolase-encoding genes from *Aspergillus niger* require D-Xylose and the xylanolytic transcriptional activator XlnR for their expression. Appl. Environ. Microbiol. 65(10):4340-4345.

Han S J, Yoo Y J, Kang H S (1995). Characterization of a bifunctional cellulase and Its structural gene. J. Biol. Chem. 270(43):26012-26019.

Hong J, Tamaki H, Akiba S, Yamamoto K, Kumaga H (2001). Cloning of a gene encoding a highly stable endo-β-1,4-glucanase from *Aspergillus niger* and Its expression in Yeast. J. Biosci. Bioeng. 92(5):434-441.

Kader AJ, Omar O (1998). Isolation of cellulolytic fungi from Sayap-Kinabalu Park, SABAH. ASEAN Review of Biodiversity and Environmental Conservation (ARBEC).

Kader AJ, Omar O, Feng LS (1999). Isolation of cellulolytic fungi from the Bario Highlands, Sarawak. ASEAN Review of Biodiversity and Environmental Conservation (ARBEC).

Kang S W, Ko EH, Lee JS, Kim SW (1999). Over production of β-glucosidase by *Aspergillus niger* mutant from lignocellulosic biomass. Biotechnol. Lett. 21:647-650.

Mandels M, Andreotti R, Roche C (1976). Measurment of saccharifying cellulose. Biotechnol. Bioeng. Symp. 6:21-34.

Murai T, Ueda M, Kavaguchi T, Arai M, Tanaka M (1998). Assimilation of cellooligosaccharides by a cell surface-engineered Yeast expressing b-glucosidase and carboxymethylcellulase from *Aspergillus aculeatus*. Appl. Environ. Microbiol. 64(12):4857-4861.

Parry JB, Stewart JC, Heptinstall J (1983). Purification of the major endoglucanase from *Aspergillus fumigatus* Freseius. Biochem. J. 213: 437-444.

Peij N, Gielkens MMC, Veries RP, Visser J, Graaff LH (1998). The transcriptional activator XlnR Regulates both xylanolytic and endoglucanase gene expression in *Aspergillus niger*. Appl. Environ. Microbiol. 64(10):3615–3619.

Siddiqui KS, Shemsi AM, Anwar MA, Rashid MH, Rajoka MI (1998). Partial and complete alteration of surface charges of carboxymethylcellulase by chemical modification: thermostabilization in water-miscible organic solvent. Enzyme Microb. Technol. 24:599-608.

Suto M, Tomita F (2001). Induction and Catabolite repression mechanisms of cellulase in fungi. J. Biosci. Bioeng. 92(4):305-311.

Zaldivar M, Velasquez JC, Contreras I, Perez LM (2001). Trichoderma auroviride 7-121, a mutant with enhanced production of lytic enzymes: its potentioal use in wast cellulose degradation and/or biocontrol. EJB. 4(3):1-9.