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Production, Partial Purification and Proteolysis of Carboxymethylcellulases from *Arachniotus citrinus*

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ABSTRACT

Carboxymethylcellulases (CMCases) of *Arachniotus citrinus* were produced under solid state fermentation on different carbon sources including wheat bran, corn cob, alkali treated corn cob and mixture of wheat bran and alkali treated corn cob at 30°C for 15 days. In this case, mixture of wheat bran and corn cob was observed to be the best carbon source as maximum specific activity (4.2 U mg^{-1} proteins) for crude CMCases was obtained on it. CMCases were partially purified by ammonium sulphate precipitation. The onset of CMCases precipitation occurred at 35% and completed at 75% saturation of ammonium sulphate at 0°C. The purification after ammonium sulphate precipitation was 1.72-fold while 86% CMCases were recovered. After dialysis, specific activity of CMCases was 7.29 U mg^{-1}. CMCases were found resistant against chymotrypsin and subtilisin as residual activity of CMCases against these proteases was 85 and 70% after 70 min, respectively.

Key Words: *Arachniotus citrinus;* Wheat bran; Corn cob; Fermentation; Cellulases

INTRODUCTION

Cellulases are consortium of free enzymes which comprise of endoglucanases (ß-1, 4-D-glucan-4-glucanohydrolase, EC 3.2.1.4, Carboxymethylcellulase, EG), exoglucanases (ß-1, 4-D-glucan-4-glucohydrolase, EC. 3.2.1.91, Cellobiohydrolase, CBH) and cellobiases (ß-D-glucoside glucohydrolase, EC. 3.2.1.21, ß-1, 4-D-glucosidase) are found in many of the 57 glycosyl hydrolase families. (Siddiqui *et al.*, 2000) The right proportion of these enzymes acts synergistically for maximum saccharification. Endoglucanase cleaves internal ß -1, 4-glucan chain links in cellulose at randomly (Schulein, 2000) and opens the molecules for cellobiohydrolases which hydrolyse the bonds at non-reducing end of crystalline cellulosic chain producing cellobiose. Cellobiases split the disaccharide units and convert cellobiose into glucose and thus complete the cellulolysis (Duenas *et al*., 1995). Cellulases have been widely used in detergents and textile industry for desizing, stain removing, fabric softening, depilling, pilling prevention as anti-redepositors, colour care agents, stone washing, biopolishing, biofinishing and smooth surfacing of cotton fabric. Proteases resistant cellulases are preferably used in soap and detergent industries as its activities remain intact against proteolytic agents (Godfrey & West, 1996). Other functions of cellulases which are of great ecological and commercial importance are amelioration and redunting of municipal, forestry and agricultural waste from paper, lumber and textile industries to control environmental pollution, bio-composting to produce natural organic fertilizers, production of food and feed supplements for cattle and poultry feed stocks, production of plant protoplast for genetic manipulation, preparations of pharmaceuticals, baking, malting and brewing, extraction of fruit juices and processing of vegetables, botanical extraction for maximum oil yield, processing of starch and fermenting tea and coffee (Petre *et al*., 1999).

The properties of pulp and quality of paper can be markedly improved by cellulase treatment. These enhance stretch and tensile index of paper. Removal of ink is an important part of secondary fiber processing in paper manufacturing. Cellulases can increase the efficiency of deinking process. Mixture of cellulases and hemicellulases can improve the drainage rate of mechanical pulp.

Cellulases are produced by a number of microbes including bacteria, yeast and fungi (Kubicek *et al*., 1991). Techniques of solid state fermentation are recently gaining great interest from researchers as these offers several economical and practical advantages: higher product concentration, improved product recovery, very simple cultivation equipment, reduced waste water output, lower capital investment and plant operation costs (Muniswaran *et al*., 1994). Endoglucanases have been purified and characterized from a variety of fungi and bacteria. Carboxymethyl cellulases (CMCases) from *Aspergillus niger* and *Cellulomonas biazotea* have been also purified (Siddiqui *et al*., 1997).

The search for a novel and improved microbial strain, having hyper cellulase productivity with more activity and high stability against temperature pH and proteases under

non-aseptic conditions might make the process more economical. Therefore, a novel fungal strain, *Arachniotus citrinus* has been used for cellulases production and characterization.

MATERIALS AND METHODS

Arachniotus citrinus was obtained from the Department of Plant Pathology, University of Agriculture, and Faisalabad, Pakistan. The culture was maintained on potato dextrose agar slants according to Rashid (1997).

Preparation of carbon sources. Corn Cob 40 mesh size was obtained from CPC Rafhan, Faisalabad, Pakistan. It was soaked in 2% (w/v) NaOH aqueous solution in 1:5 (w/v) ratio of corn cob powder for 24 h. It was filtered through muslin cloth. The residue was washed and rinsed thoroughly with tap water. Finally it was soaked, washed and rinsed with distilled water till neutrality (pH 7). It was kept in oven at 70°C for 48 h to dry and store in polythene bags to protect it from moisture and environmental effects. Wheat bran (40 mesh size) was obtained from flour mill. Delignified corn cob powder and wheat bran were mixed uniformly in 1:1 (w/w) ratio.

Inoculum preparation. Inoculum was prepared as described by Iqbal *et al*. (1991). 150 mL of Vogel medium was added per 500 mL conical flask. About 20, chromic acid washed marble gravels were added to each flask after thoroughly washing with water to break the mycelia of the fungus. Flasks were autoclaved for 20 min at 121°C (1.1 kg cm^{-2}). 50% (w/v) stock solution of glucose was prepared and autoclaved at 121°C (1.1 kg cm^{-2}) for 5 min. The glucose solution was aseptically transferred to each flask to get a final concentration of 2% (v/v). A loopful of spores of *Arachniotus citrinus* was transferred aseptically to each flask in laminar air flow. Flasks were shaken on orbital shaker at a speed of 110 rpm at 30°C for one day.

Production of carboxymethylcellulases. *Arachniotus citrinus* was grown on solid state as fungal cultures adapted to grow in nature. First *A. citrinus* was grown on various carbon sources for maximum CMCases production. After optimization of carbon source, 100 conical flasks of 250 mL capacity were taken. Each flask contained 15 g mixture of wheat bran and alkali treated corn cob (the best carbon source observed) in 1:1(w/w) ratio and soaked with 30 mL distilled water i.e. 1:2 (w/v) ratio. Finally flasks were plugged with cotton and covered with aluminum foil then autoclaved at 121°C (1.1 kg cm^{-2}) for 20 min. 4 mL of inoculum was sprinkled aseptically on the carbon source containing flasks and incubated at 30°C for two weeks to produce cellulases. The cellulases were harvested by adding 15 mL of distilled H_2O in each flask and shake vigorously. Crude enzyme extract was filtered through muslin cloth and centrifuged at 15600 X *g* at 4°C for 30 min and finally total proteins were estimated and carboxymethylcellulases activity was determined in crude extract.

Protein estimation. Proteins were estimated using Bradford Method (Bradford, 1976). Bovine serum albumin (BSA) was used as standard. Stock solution for standard curve was prepared by dissolving 1 mg of BSA in 1 mL of distilled water. The standard solution was prepared by adding 100 µL of BSA stock solution in 900 µL distilled water. 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µL of this standard solution were added in tests tubes containing 1 mL of Bradford reagent. The volume of all tubes was made equal by adding distilled water. 100 µL of distilled water was added to another test tube containing 1 mL Bradford reagent. It was used as reagent blank. All tubes were stirred and kept at least for 15 min. The absorbance was taken within 15-30 min at 595 nm (Fig. 1).

Carboxymethylcellulase (CMCase) assay. Endoglucanase activity was determined using 1.5% w/v sodium salt of carboxymethylcellulose (CMC) as substrate in 50mM acetate buffer having pH 5 at 40°C (Nakamura & Kitamura, 1988). Reducing sugars were determined colorimetrically with DNS reagent (Miller, 1959). CMC was hydrolysed by endoglucanases to yield cellobiose (Fig. 2, 3).

Standard curve for CMCase activity. Stock solution for glucose (20 mM) and cellobiose (10 mM) were prepared. Glucose and cellobiose standard solution were separately pipetted out into a series of numbered test tubes 10-100 µL with an increment of 10. The volume was made up 1 mL by adding acetate buffer pH 5. In each test tube, 3 mL of DNS reagent was added and reaction mixture was boiled for 10 min in water bath and cooled in ice. O.D. was taken at 550 nm (Ng & Zeikus, 1988).

The endoglucanase activity was measured in terms of micro moles of glucose equivalent from standard curve. Therefore, one unit of endoglucanase (CMCase) activity in micro moles of glucose liberated at 40°C pH 5.0 per min per mL was calculated from the following:

$$\mathrm{U\,ml^{-1}min^{-1}} = \frac{\Delta A_{550}}{\Sigma_m} \times \frac{V_t \times D_f}{V_e \times t}$$

Where,

ΔA= absorbance; Σ_m = extinction coefficient; V_t = total volume of reaction mixture; V_e = volume of enzyme; D_f = dilution factor; T = time in min; U = enzyme activity

Ammonium sulphate precipitation. Endoglucanases obtained from *Arachniotus citrinus* were centrifuged at 18,000 rpm (39200 X *g*), concentrated by freeze drying and dialyzed to remove ammonium sulphate.

Optimization of ammonium sulphate. Different amounts of solid ammonium sulphate were added separately to 1 mL of crude dialyzed CMCase concentrate in eppendorf tubes to get 10-90% saturation at 0°C and stirred. These tubes were left for over night at 4°C and centrifuged at 12000 rpm for 15 min. The supernatant were assayed for endoglucanase activity. After optimization of ammonium sulphate precipitation, the crude enzyme concentrate was placed in ice bath and solid ammonium sulphate was dissolved bit by bit to attain initially 35% saturation at 0°C. Then it was

centrifuged at 18,000 rpm (39200 X *g*) for 30 min at 4°C. The pellet of precipitated protein was discarded. In supernatant more solid ammonium sulphate was added to attain 55% saturation at 0°C. It was again kept for a night at 4°C and centrifuged like previously. This time pellet was collected and more ammonium sulphate was added in the supernatant to attain 80% saturation at 0°C. Pellet of precipitated protein was obtained in the same manner as mentioned above and supernatant was discarded. The pellets were dissolved in distilled water and dialyzed against distilled water for 24 h with four changes of equal intervals to remove ammonium sulphate because it inhibits the catalytic function of enzyme. Total proteins and CMCase activity was determined before and after dialysis of ammonium sulphate precipitated CMCases and finally lypholyzed to powder.

Effect of proteases. Effect of proteases on endoglucanase was determined according to Siddiqui *et al.* (1997). 0.2 mg mL^{-1} Chymotrypsin in 10 mM Tris/HCl pH 7 containing 2 mg of endoglucanase and 0.5 mg mL^{-1} subtilisin in 10 mM Tris/HCl pH 7 containing 1 mg endoglucanase were separately incubated at 30°C. Different time course aliquots were withdrawn and immediately assayed for enzyme activity.

RESULTS AND DISCUSSION

Production of cellulases. Solid state fermentation systems offer several economical and practical advantages such as: higher product concentration, improved product recovery, very simple cultivation equipment, reduced waste water output, lower capital investment and lower plant operation costs (Muniswaran *et al*., 1994). Therefore, *Arachniotus citrinus*, a mesophillic fungal strain was grown under solid state fermentation conditions. The specific activities of Carboxymethylcellulase (CMCases) produced after fifteen days at 30°C were 3.0 Umg^{-1} on wheat bran, 4.75 Umg^{-1} on corn cob and 4.25 Umg^{-1} when mixture of wheat bran and corn cob was used (Table I). Though specific activity in case of corn cob alone was higher but the total protein contents were very low. So, it seemed difficult to proceed for purification due to enzymes loss during purification process. Therefore, mixture of wheat bran and alkali treated corn cob was observed to be most suitable substrate for CMCases production and selected for hyper production of CMCases. The crude enzyme extract was purified after dialyzing it against distilled water.

Purification of CMCases. Crude enzyme was partially purified by subjecting it to ammonium sulphate precipitation. The precipitations of endoglucanases by ammonium sulphate were optimized. The onsets of precipitation of CMCases occurred at 35% and were completely precipitated at 75% saturation of ammonium sulphate at 0°C (Fig. 4). The CMCases from *Aspergillus niger* precipitated between 45-65% ammonium sulphate saturation at 0°C (Siddiqui *et al*., 2000). The cellulases were 1.72-fold purified after precipitation. The recovery of enzyme was about 86% (Table II). Moreover, ammonium sulphate inhibited CMCases activity and the enzyme was reactivated after the removal of salt through dialysis (Fig. 5).

Table I. Production of CMCases from *A. citrinus* on various carbon sources

Carbon sources	Units*	Protein(mg)	specific activity(U/mg)
Wheat bran	213	70	3.0
Corn cob	78	64	1.2
Alkali treated corn cob	190	40	4.75
Mixture of Alkali treated corn cob & wheat bran	316	75	4.20

*μmoles of glucose/ml/min

Table II. Purification of CMCases from *Arachniotus citrinus*

	Crude extract	$(NH_4)_2SO_4$ ppt. (before dialysis)	$(NH_4)_2SO_4$ ppt. (after dialysis)
Units	4424	2487	3801
Protein	1040	530	521
Specific activity	4.25	4.69	7.29
Purification factor	1.0	1.10	1.71
% recovery	100	56	86

Effect of proteases on stability. The stability of CMCases from *A. citrinus* was checked against chymotrypsin and subtilisin. The CMCase was found to be very resistant against chymotrypsin as 90% of activity was retained after 70 min of protease attack. Subtilisin based nicking of CMCases showed a gradual decreasing trend of CMCase activity. The residual activity after 70 min of subtilisin treatment was about 70% (Fig. 6). The stability refers to the catalytic function of enzyme. There are hardly few reports on proteolysis of CMCases. Ashfaq *et al.* (1991) had reported the partial proteolysis by trypsin on endoglucanase and β-glucosidase components of *Cellulomonas biazotea* NIAB 422 but results were not clearly mentioned. Siddiqui *et al.* (1996) reported that 25% of endoglucanase activity of *A. niger* was lost after 32 h treatment of thermolysin. However, in present study, strong resistance profile of CMCases from *A. citrinus* against proteases was observed. This indicates better storage capacity of this enzyme because its catalytic sites were not destroyed during proteolysis. These results favour its application in food and feed for livestock, poultry, detergent, and soap industries.

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Fig. 1. Standard curve for proteins estimation

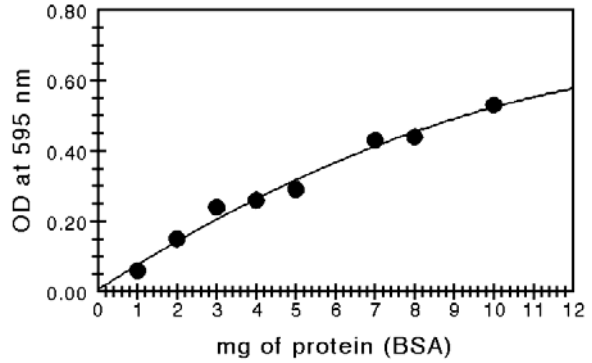


Fig. 2. Standard curve of glucose estimation and determination of molar extinction coefficient

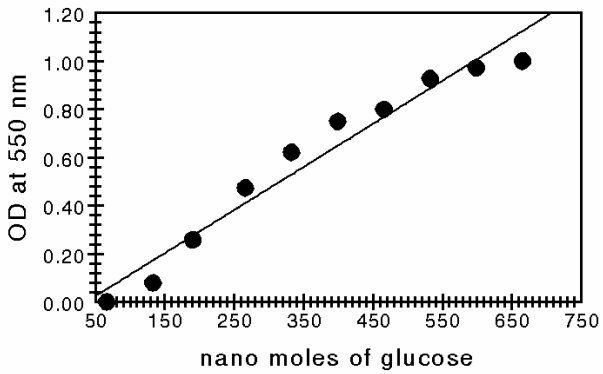


Fig. 3. Standard curve of cellubiose estimation and determination of molar extinction coefficient

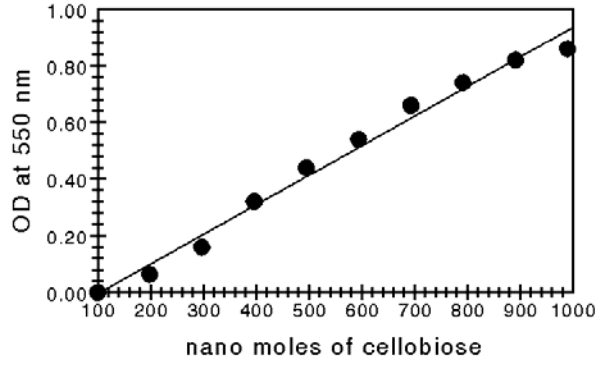


Fig. 4. Optimization of Ammonium sulfate concentration for precipitation of Carboxymethylcellulases from *Arachniotus citrinus*

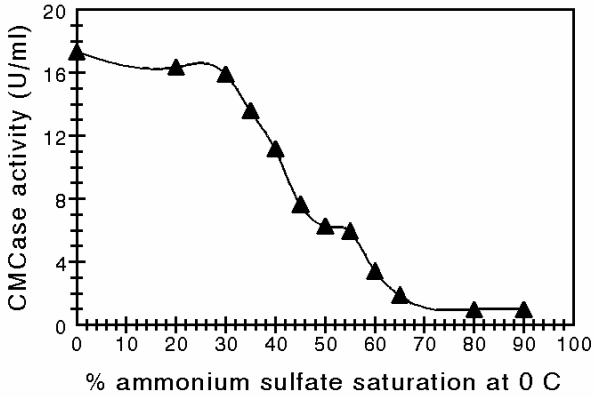


Fig. 5. Effect of ammonium sulfate on activity of CMCases

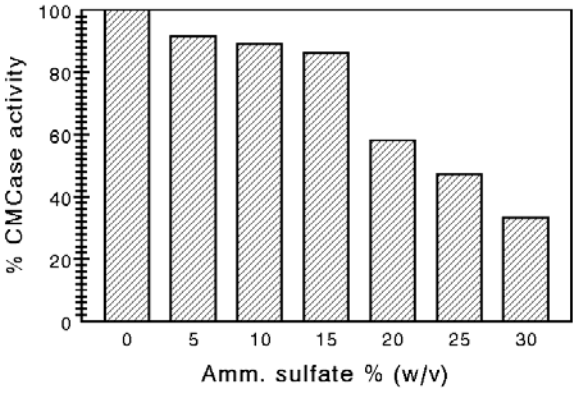
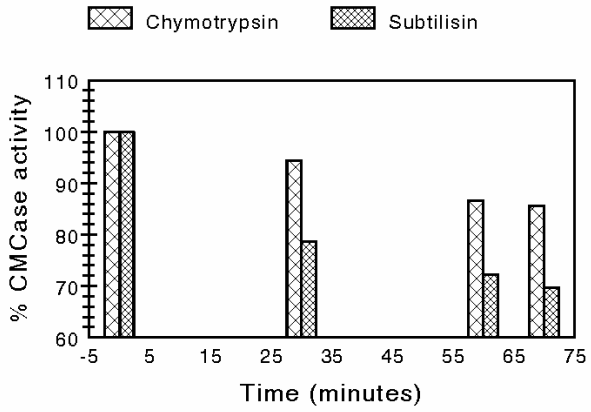


Fig. 6. Effect of proteases on stability and catalytic function of CMCases



CONCLUSION

The results indicate that wheat bran is a good carbon source for production of enzymes. Proteolytic decay occurs and enzymes should not be kept for longer time. These must be used as earlier as possible. Residual activity against chymotrypsin is greater than bacterial proteolytic enzyme subtislin.

REFERENCES

Ashfaq, R., A. Bashir, M.I. Rajoka, K.A. Malik, C.A. Batt, S.H.M. Naqvi and M.I.H. Aleem, 1991. Characterization of cellulases from *Cellulomonas biozotia* NIAB442 using zymographic techniques. In: *Proc. Int. Symp. Biotech. Energy*, 19: 77–83

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analyt. Biochem.,* 72: 248–54

Duenas, R., R.P. Tengerdy and M. Gutierrez–Corea, 1995. Cellulase production by mixed fungi in solid substrate fermentation of baggasse. *World J. Microbiol. Biotechnol.,* 11: 333–7

Godfrey, T. and S. West, 1996. *Industrial Enzymology,* 2nd Ed. MacMillan Press Ltd., New York, USA

Iqbal, J., M.I. Rajoka and K.A Malik, 1991. Production of thermostable β–glucosidase by a mesophilic fungus *Aspergis niger* NIAB–280. *In:* Malik, K.A., S.H.M. Naqvi and M.I.H. Aleem (eds.). *Biotechnology for Energy*. Pp: 157–65. NIAB/NIBGE, Faisalabad, Pakistan

Kubicek–Pranz E.M., F. Gruber and C. P. Kubicek, 1991. The *Trichoderma* cellulase regulatory puzzle from the interior life of a secretory fungus *J. Biotechnol*., 20: 83–94

Miller, G.I., 1959. Use of Dinitrosalicylic acid reagent for determination of reducing sugars. *Analyt. Chem.,* 31: 426–8

Muniswaran, P.K.A. and N.C.L.S. Charyulu, 1994. Solid substrate fermentation of coconut coir pith for cellulase production. *Enzyme Microb. Technol*., 27: 33–6

Nakamura, K. and K. Kitamura, 1988. Cellulases of *Cellulomonas uda*. *Methods in Enzymology,* 160: 211–6

Ng, T.K. and J.G. Zeikus, 988. Endoglucanase from *Clostridium thermocellum*. *Methods in Enzymology,* 160: 351–5

Petre, M., G. Zarnea, P. Adrian and E. Gheorghiu, 1999. Biodegradation and bioconversion of cellulose waste using bacterial and fungal cells immobilized in radiopolymerized hydrogels. *Resour. Conserv. Recycling.,* 27: 309–32

Rashid, M.H., 1997. Purification and characterization of native proteolytically nicked and chemically modified ß–glucosidase from *Aspergillus niger*. *Ph.D. Thesis,* University of the Punjab, Lahore, Pakistan

Schulein, M., 2000. Protein engineering of cellulases. *Biochimica et Biophysica Acta.,* 1543: 239–52

Siddiqui, K.S., A.A.N. Saqib, M.H. Rashid and M.I. Rajoka, 2000. Carboxyl group modificaiton significantly alterd the Kinetic properties of purified Carboxymethylcellulase from *Aspergillus niger*. *Enzyme Microb. Technol.,* 27: 467–74

Siddiqui, K.S., M.J.Azhar, M.H. Rashid and M.I. Rajoka, 1997. Stability and identification of active site residue of Carboxymethylcellulases from *Aspergillus niger* and *Cellulomonas biazotea. Folia Microbiol.* 42: 312–8

Siddiqui, K.S., M.J.Azhar, M.H. Rashid and M.I. Rajoka, 1996. Activity and thermostability of Carboxymethylcellulase from *Aspergillus niger* is strongly influnced by non covalently attached polysaccharides. *World J. Microbiol. Biotechnol.,* 12: 213–6

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