

Partial Purification and Characterization of Two Endo- β -1, 4-glucanase from *Trichoderma* sp. (Shmosa tri)

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Abstract: Two endoglucanases (EC 3.2.1.4) from *Trichoderma* sp. (shmosaTri) FJ937359 were purified to homogeneity using ammonium sulfate precipitation and gel filtration. Purity was confirmed by SDS/PAGE. Enzymatic properties and molecular weights were determined. Molecular weights of CMCase I and II were 58 and 34 KDa, respectively. The effect of temperature on the 2 endoglucanase activity was studied and results showed that optimum activity obtained at 50°C for both CMCase I and II. The enzymes withstand 60 min at 50°C without loss of enzymatic activity. CMCase I and II retained 14.0 and 26.5 % of their original activities at 70°C after 90 min. The optimum pH for CMCase I and II was 5.0. Results also show that CMCase I was active at room temperature after 24 hrs over a broad pH range (3.0-9.0) while CMCase II was relatively stable in pH range (4.0-6.0). Among different kinds of substrates, both enzymes showed a high preference for carboxymethyl cellulose while both CMCase I and II did not show any hydrolytic activity against chitin, starch and cellobiose. On the other hand both CMCase I and II have relatively low hydrolytic activity towards β glucan and xylan. All metallic ions used as well as EDTA and SDS at a concentration of 20 μ g/ml of reaction mixture have an inhibitory effect on both CMCase I and II.

Key words Endoglucanase; *Trichoderma* sp.; Carboxymethyl cellulose; Substrate specificity.

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). It is composed of β -1, 4-glucose units linked by β -1, 4-D-glycosidic bond, Cellulolytic enzymes degrade cellulose by cleaving the glycosidic bonds (Han *et al.*, 1995).

Cellulases, responsible for the hydrolysis of cellulose, are composed of a complex mixture of enzyme. Cellulases divided into three major enzyme classes (Goyal *et al.*, 1991; Rabinovich *et al.*, 2002a, b). These are endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β -glucosidase (EC 3.2.1.21) (Krässig, 1993 and Gielkens *et al.*, 1999). Endoglucanase, often called carboxymethylcellulase (CM)-cellulase, which attack randomly at 1,4-D-glycosidic bonds in cellulose (Siddiqui *et al.*, 1998). Endoglucanases attack multiple internal sites in the amorphous regions of the cellulose fibre opening-up sites for subsequent attack by the cellobiohydrolases (Wood, 1992 and Safari Sinigani *et al.*, 2005).

Trichoderma species are common soil inhabiting fungi with a strong ability to degrade cellulose (Frisvad and Thrane 2000). The fungus produces a complete set of cellulases that are able to cleave the β 1,4-glycosidic bonds present in cellulose or cellulose derivatives (Ilme'n *et al.*, 1997). One of the most extensively studied cellulolytic organisms is the soft rot fungus *Trichoderma reesei* Teeri *et al.*, (1992) and Kubicek *et al.*, (1993) stated that *Trichoderma reesei* produces two exoglucanases, five endoglucanases. In terms of enzyme novelty for industrial applications, interest is focused on not only finding enzymes which could break down cellulose much more rapidly but also enzymes which could withstand pH and higher temperature.

This study interesting in purification of two cellulases from *Trichoderma* sp. shmosa (Tri). In addition, different substrates were used to analyze the substrate specificity of the partially purified enzymes. Other biochemical properties of these two enzymes were also studied.

MATERIALS AND METHODS

Organism, Cultivation and Growth Conditions:

Trichoderma sp. (shmosaTri) FJ937359 previously isolated from Sharkia, Egypt, was cultivated on Czapek Dox medium containing (1%) wood dust as a sole carbon source. Culture was incubated for 14 days at 30°C on orbital shaker at 150 rpm. At the end of incubation time, wood dust residues were removed and filtrate was centrifuge at 5000 rpm. The clear supernatant was considered as the source of crude enzymes (Rajoka and Malik, 1997).

Determination of Enzyme Activity:

Endoglucanase activity was routinely measured according to (Miller, 1959). The enzyme solution, 1 ml in appropriate dilution, was added to 1 ml of 1% carboxymethyl cellulose (CMC) dissolved in 50 mM sodium acetate buffer, pH 5.0. After incubation at 50 °C for 60 min, the reaction was stopped by the addition of 3 ml dinitrosalicylic acid reagent. After 10 min in a boiling water bath, the enzymatic hydrolysis of CMC was determined at 540 nm. One unit of CMCase activity was expressed as the amount of protein that liberated reducing sugar equivalent to glucose per minute under assay condition.

Determination of Protein Concentration:

The protein content of the crude enzyme preparation was assayed by Folin- phenol reagent according to the protocol adopted by Lowry *et al.* (1951) using bovine serum albumin as a standard.

Purification of Endoglucanases (CMCases):

Trichoderma sp. Shmosa Tri FJ937359 culture filtrate was centrifuged for 10 min at 5000 rpm. The clear supernatant used as source of crude enzyme were subjected to slow addition of ammonium sulphate with stirring until the desirable saturation 80%. The obtained precipitated protein was resuspended in known volume of 0.1 M citrate phosphate buffer (pH 5.0). Dialysis via cellophane bags against pure sucrose crystals (Shindia *et al.*, 2005) then was dialyzed against citrate-phosphate buffer (pH 5.0). A pharmacia column (2.5 x 90 cm) of sephadex G₁₀₀ was used for purification of the dialyzed enzyme. The sephadex G₁₀₀(10 gm) was swollen in 0.1 M citrate phosphate buffer (pH 5.0) for 24 hour at 4.0°C, sodium azide (0.02%) was added to prevent any microbial growth. Enzyme preparation was applied carefully to the column; fractions were collected and assayed for CMCase activity and protein content. Sharp peaks fractions were collected and used for gel electrophoresis.

Estimation of Enzymes Molecular Weights:

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE):

For molecular weights determination, the enzyme preparations and known molecular weight marker were subjected to electrophoresis according to Bollag and Edelstein (1991) with 10% acrylamide gel; 0.2% CMC was incorporated into the separating gel prior to the addition of ammonium persulphate. After electrophoresis, the gel was stained with Coomassie Blue R dye. For CMCase activity, the gel washed at room temperature in solution A (sodium phosphate buffer, pH 7.2, containing isopropanol 40%), solution B (sodium phosphate buffer, pH 7.2) for 1 h, respectively then solution C (sodium phosphate buffer, pH 7.2, containing 5 mM β-mercaptoethanol and 1 mM EDTA) at 4 °C overnight. The gel was then incubated at 37 °C for 4 h, stained with 1% Congo red for 30 min, destained in 1 M NaCl for 15 min Kluepfel (1988), clear bands indicated the CMCase activity.

Enzyme Characteristics:

Effect of Substrate Concentration on (CMCase I and II) Activities: The CMCase activity was measured for the two enzymes (CMCase I and II) at different concentrations of the substrate (CMC): 0.12, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 and 3.0 % at pH 5.0. Activity was determined using DNS (3, 5-dinitrosalicylic acid) reagent (Miller, 1959).

Effect of Reaction Time on (CMCase I and II) Activities:

CMCase activity was determined for the two enzymes CMCase I and II at different incubation time of reaction mixture; 15, 30, 60, 90, 120,150 and 180 min.

Effect of Reaction Temperature on (CMCase I and II) Activities:

The enzymatic activity of (CMCase) was estimated for CMCase I and II at the following reaction temperatures 20, 30, 40, 50, 60 and 70°C.

Thermal Stability of (CMCase I and II):

The thermal stability of CMCase I and II were tested by preheating of enzymes at 40, 50, 60 and 70 °C, activity was estimated every 30 min for 2 hours.

Effect of PH-value of Reaction Mixture on (CMCase I and II) Activity:

The pH optima of the CMCase enzymes were determined at a pH range from 2 to 9, using citrate–phosphate buffer (0.1 M citric acid, 0.2 M Na₂HPO₄) and Tris buffer (0.08 M Tris, 0.1 M HCl).

pH-stability of (CMCase I and II):

The pH stability of purified (CMCase) enzymes was determined by preincubation of enzymes at a pH range from 2 to 9 for 24 hours according to Hurst *et al.*, (1977) then enzymes activities were assayed as previously described at each pH value.

Effect of Some Metallic Ions on (CMCase I and II) Activities:

The effect of some ions (KCl, NaCl, CaCl₂, CoCl₂, CuSO₄.5H₂O, ZnSO₄.7H₂O, MgSO₄.7H₂O) as well as EDTA and SDS at the concentration of 20 µg/ml of reaction mixture on (CMCase I and II) was studied.

Enzyme Substrate Specificity:

The specificity of the enzymes for their substrates was investigated using different kind of substrates as: CMC, chitin, starch, β- glucan, xylan and cellobiose. Activities of CMCase I and II were assayed.

RESULTS AND DISCUSSION

Partial Purification of CMCase:

The crude enzymes obtained from culture filtrate of *Trichoderma sp.* Shmosa Tri FJ937359 grown on wood dust, incubated at 30°C for 14 days at 150 rpm was subjected to purification protocol including: salting out with ammonium sulphate, dialysis via cellophane bag and gel filtration using column chromatography. Results showed in table (1) indicated that the purification of (CMCases) increased by 1.26 fold by salting out compared with the crude enzyme. Macris, (1987) stated that 100% of CMCase precipitated in the range of 0 to 80% ammonium sulfate saturation. Many workers used ammonium sulphate for precipitation of Cellulase (Sul, *et al.*, 2004 and Bakare *et al.*, 2005). The results showed also that there are two peaks I and II for CMCase with specific activities 170.53 and 166.51 u/mg, respectively. The peaks were used for subsequent characterization and properties of enzymes. these purification techniques are comparable to those reported by Tong *et al.*, (1980) using sephadex G-100; Inglin *et al.*, (1980) using sephadex G-150; Sadia *et al.*, (2005) using (sephadex G -50 and G -200) and Dutta *et al.*, (2007) using superdex-200 HR.

Table 1: Purification profile (CMCases) produced by *Trichoderma sp.* grown on wood dust.

Purification steps	Protein (mg/ml)	Total protein (mg)	Total activity (U/ml)	Specific activity (U/ mg)	Recovery (%)	Fold
Crude enzyme	0.340	595	29505	49.58	100	1
Precipitate with (NH ₄) ₂ SO ₄	0.237	47.4	2980	62.86	10.09	1.26
Gel filtration Peak I	0.132	3.3	562.75	170.53	1.90	3.43
Sephadex G ₁₀₀ Peak II	0.152	3.8	632.75	166.51	2.14	3.35

Enzymes Molecular Weights:

The molecular weights of CMCases were estimated using the technique of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results showed that there were two clear bands appeared (I, II) when stained with 1% Congo red and destained in 1 M NaCl, indicated the presence of CMCase activity. The results showed also that the molecular weights of the 2 enzymes I, II were, 58 KDa and 34 KDa, respectively Fig (1). These findings are in agreement with other investigators that reported the presence of different isoenzymes with different molecular weights of CMCase produced by various microorganisms (Bhikhabhai *et al.*, 1984; Kim *et al.*, 1994 and Petrova *et al.*, 2009). Holt and Hartman (1994) used zymogram

method to detect endoglucanases from *Trichoderma reesei*. Sul *et al.*, (2004) determined the molecular weight of EG endoglucanase purified from the culture filtrate of *Trichoderma* sp. C-4, it was 51 kDa. The EGs of the mesophilic fungi *Trichoderma reesei* and *Phanerochaete chrysosporium* have molecular weight range from 25 to 50 kDa (Perez *et al.*, 2002). However carboxymethyl cellulase purified from *Trichoderma viride*, was examined by (SDS-PAGE) and the molecular weight was 66kDa (Liu and Xia 2006). Petrova *et al.*, (2009) purified two endoglucanases to homogeneity from the culture filtrate of a mutant strain *Trichoderma* sp. M₇ (EG-III and EG-IV) with molecular weight of 49.7 and 47.5 kDa. The difference in the molecular weights of CMCCase enzymes may due to the biological aspect of the fungal strains.

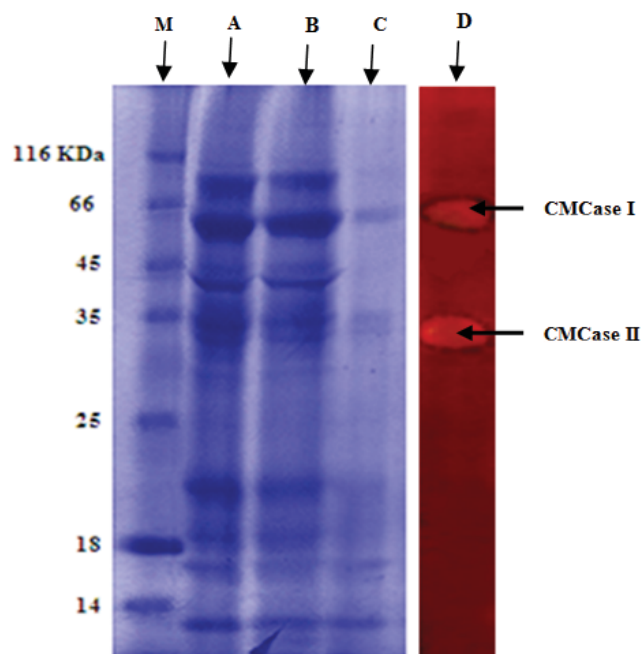


Fig. 1: SDS-PAGE profile of CMCCase (I, II) from *Trichoderma* sp. (Tri) Shmosa Tri. (M) Standard protein markers, (A) Crude enzyme preparation, (B) dialyzed enzyme preparation, (C) active fractions of column chromatograph and (D) zymogram stain with Congo red

Enzymes Characteristics:

Effect of Substrate Concentration on (CMCase I and II) Activities:

The results in Fig. (2) illustrated that, the optimum specific activities of CMCCase I and II obtained at substrate concentration of 1%. The increase of substrate concentration more than 1 % (w/v) cause decrease in specific activities in both enzymes. This was probably because at high substrate concentration, the substrate molecules were too much around the enzyme molecules, crowd the active site or they may bound to regions, which are not the active site. These results are supported by Bakare, *et al.*, (2005) they reported that the activities of cellulases were greatly influenced by the concentration of the substrate, with fixed enzyme concentration, an increase in the concentration of substrate results in increase in enzyme activity until a saturation point is reached beyond which enzyme activity decreases. Result showed also that K_m value of CMCCase I and II were 4.0 and 3.1 mg per ml, respectively. K_m value denotes the amount of substrate needed to achieve half the maximal initial reaction velocity (Tong *et al.*, 1980; Bakare, *et al.*, 2005). Petrova *et al.*, (2009) purified two endoglucanases EG-III and IV from *Trichoderma* sp. M₇, exhibited K_m of 2.9 and 3.8 mg ml⁻¹, respectively.

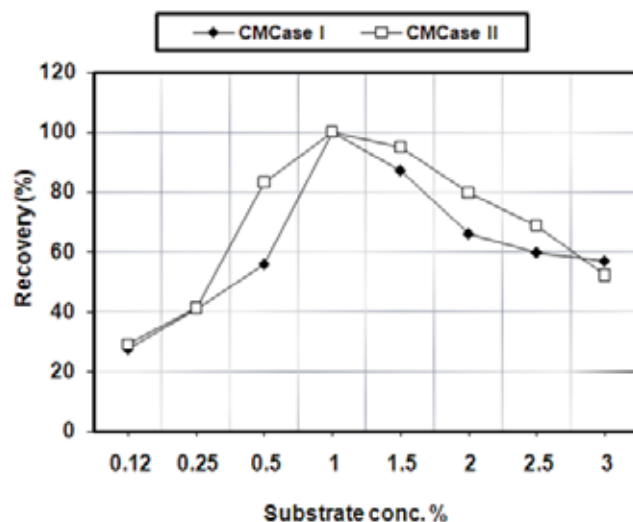


Fig. 2: Effect of substrate concentrations on the activities of the purified (CMCase I) and (CMCase II) produced by *Trichoderma sp.* grown on wood dust.

Effect of Reaction Time on (CMCase I and II) Activities:

The results presented in Fig (3) showed that the highest specific activities of CMCase I and CMCase II were recorded after one hour. Results indicated also that CMCase I and II retained 25.2 and 42.3 % of their activities respectively, after 3 hours reaction time. Our data, in coincidence with the previous studies that recorded the amount of reducing sugar produced under the action of cellulases were gradually increase with the increase of incubation time and reach the maximum at 60 min for *Trichoderma koningii* and *Aspergillus niger* (El-Zawahry and mostafa 1983; shindia, 1990).

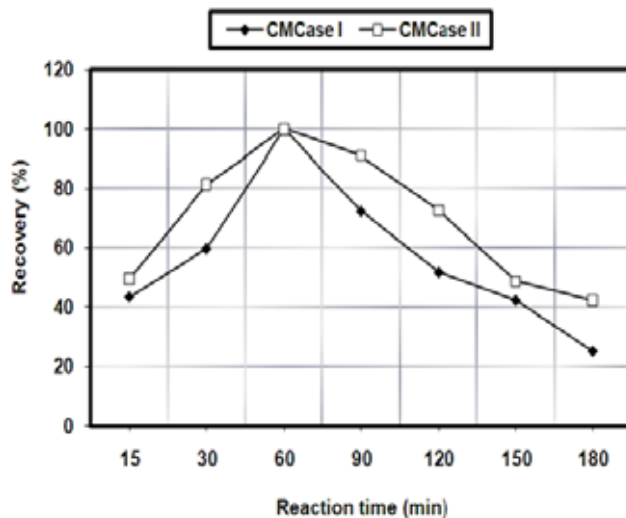


Fig. 3: Effect of reaction time on the activities of the purified (CMCase I) and (CMCase II) produced by *Trichoderma sp.* grown on wood dust wood dust.

Effect of Reaction Temperature and Thermal Stability on (CMCase I and II):

Data in Fig (4) illustrated that the specific activities of CMCase enzymes increased with the increasing of reaction temperature, reaching its optimum values at 50°C for both CMCase I and II. In general, cellulases have high temperature optima when compared with other enzyme systems (Tong *et al.*, 1980). Cellulolytic enzymes activities increased up to 60°–65°C for the three enzyme components from *Trichoderma longibrachiatum* (Kalra *et al.*, 1986). Ülker and Sprey, (1990) purified low molecular weight endoglucanase

from *Trichoderma reesei* with optimal temperature 52°C. Petrova *et al.*, (2009) stated that optimal temperature values for two purified endoglucanases EG-III and EG-IV from *Trichoderma sp.* M₇, were found to be 60°C and 50°C, respectively. Our results showed that the thermal inactivation of the purified CMCCase I and II enzymes increased with the increasing of preheating temperature as well as exposure time Fig (5). The enzymes can withstand 60 min at 50°C without loss of enzymatic activities. The results also illustrated that CMCCase I and II retained 14.0 and 26.5 % of their original activities at 70°C after 90 min. In accordance with our results CMCCase and FPase from *Trichoderma sp.* A-001 grown on various carbon sources lost 20–33% of their activities when kept at 60°C for 4 hours before assaying while beta-glucosidase lost only 37% of its activity when maintained at 70°C for 4 h (Gashe, 1992).

Endoglucanase purified from the culture filtrate of *Trichoderma sp.* C-4 was assayed at various temperatures ranging between 30°C and 70°C and the optimum temperature was 50°C. The enzyme showed stability at 50°C for 60 min but lost 50% of its maximal activity after 10 min at 60°C (Sul *et al.*, 2004). Thermostable enzymes produced by *Trichoderma spp.* were recorded by many workers (Gashe, 1992; Seyis and Aksoz 2004; Harighi *et al.*, 2007). Heat stable endoglucanases were also purified from other mesophilic fungi (Macris, 1984; Shuyan *et al.*, 2006 and Elshafei *et al.*, 2008).

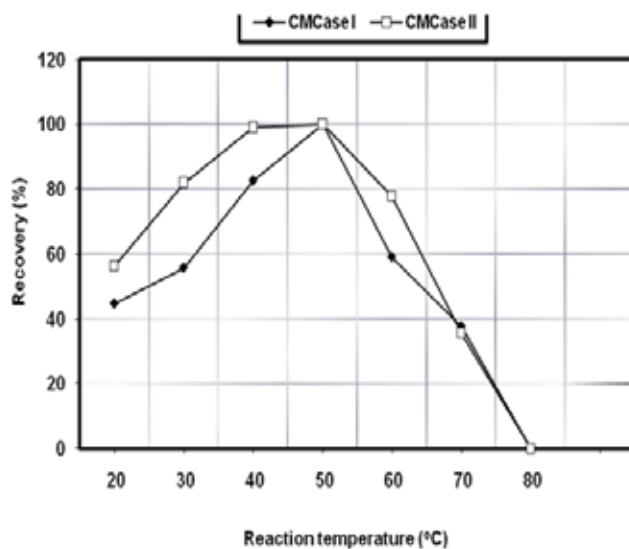


Fig. 4: Effect of reaction temperature on the activities of the purified (CMCase I) and (CMCase II) produced by *Trichoderma sp.* grown on wood dust.

Effect of PH Values of Reaction Mixture and PH Stability on (Cmcase I and II):

Results in Fig. (6) showed that the specific activities increase gradually with the increasing of the pH value of the reaction mixtures till reaching their maximal values at pH 5.0 for both CMCCase I and II. Kalra *et al.*, (1986) stated that purified enzyme preparation from *Trichoderma longibrachiatum* showed an optimal pH of 5.0 for CM cellulase. Our results were also confirmed by other workers (Sul *et al.*, 2004; Petrova *et al.*, 2009). However Carboxymethyl cellulase purified from *Trichoderma viride*, showed optimum pH 4.0 at 50 °C (Liu and Xia 2006). The results also revealed that both CMCCase I and II retain 23.2 and 22.9% from their activity at pH value of 9.0, respectively. The results in Fig (7) show that CMCCase I was active at room temperature after 24 hrs over a broad pH range (3.0-9.0). On the other hand CMCCase II was relatively stable in pH range (4.0-6.0). In general, the pH-stability curves of the enzymes are much broader than the pH-activity curves (Tong *et al.*, 1980).

Effect of Some Metallic Ions and Chemicals Substances on Cmcase I and II:

The obtained results in Fig. (8) showed inhibitory effect on the activities of CMCCase I and II recorded with all tested compounds but CMCCase I was more sensitive than CMCCase II. Co⁺² was the most inhibitory ion for CMCCase I while Hg⁺² was the most inhibitory ion for CMCCase II. Liu and Xia (2006) stated that heavy metal ions such as Hg²⁺, Ag⁺ have significantly or completely inhibitory effect on CMCCase purified from *Trichoderma viride*. Petrova *et al.*, (2009) proved that Mn²⁺, Cu²⁺ and Pd²⁺ strongly inhibited EG-III and EG-IV

endoglucanases purified from *Trichoderma* sp. These inhibitory effect may be due to these ions have been reported as heavy metals and generally toxic to some organisms. The results also showed complete inactivation in presence of EDTA on the two enzymes. EDTA is known as an ionic chelator (Ali and Sayed, 1992) and its inhibition ability indicates that specific ions might be actively involved in the catalytic reaction of the enzyme (Kotchoni *et al.*, 2006).

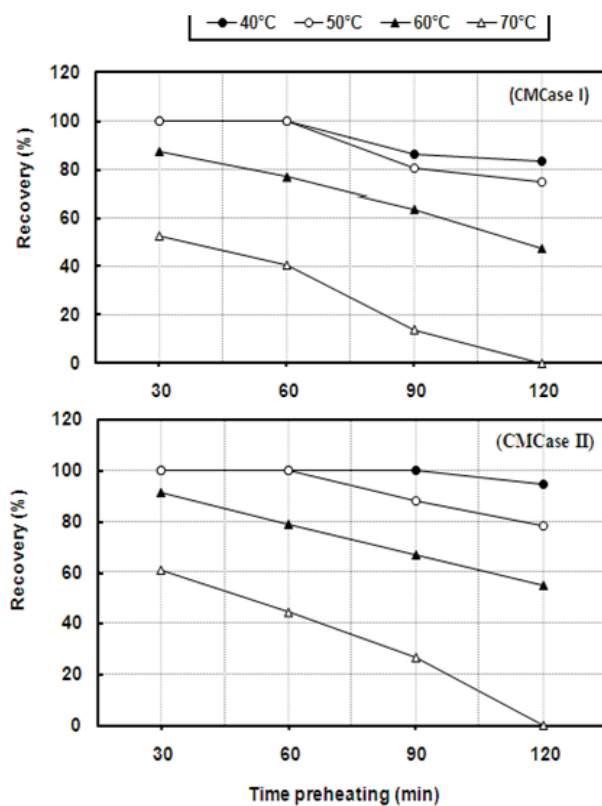


Fig. 5: Thermal stability of CMCase I and II produced by *Trichoderma* sp. grown on wood dust.

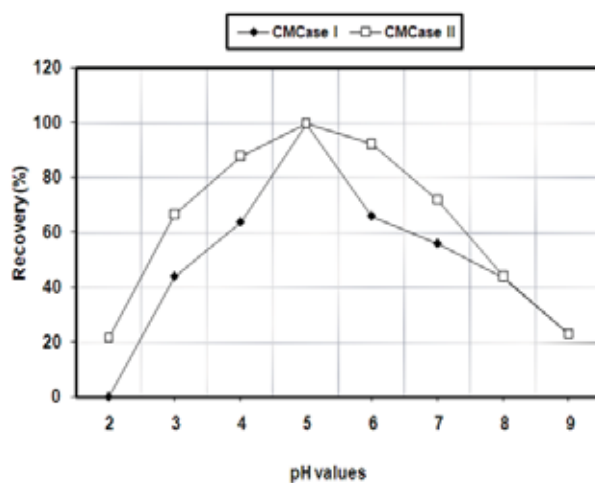


Fig. 6: Effect of pH values of reaction mixture on the activities of the purified (CMCase I) and (CMCase II) produced by *Trichoderma* sp. grown on wood dust

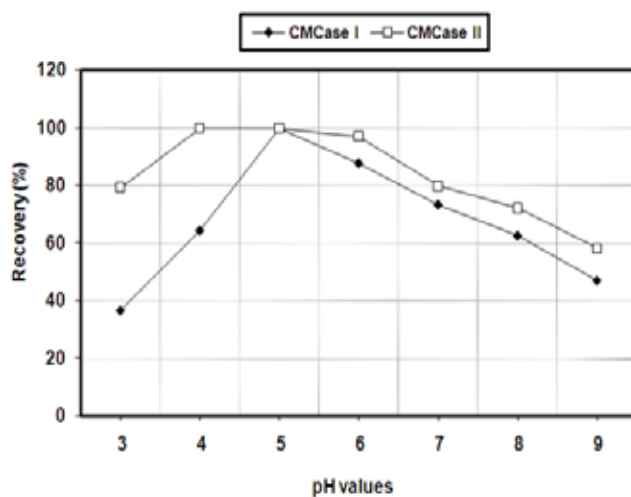


Fig. 7: Effect of pH values of reaction mixture on the activities of the purified (CMCase I) and (CMCase II) produced by *Trichoderma sp.* grown on wood dust

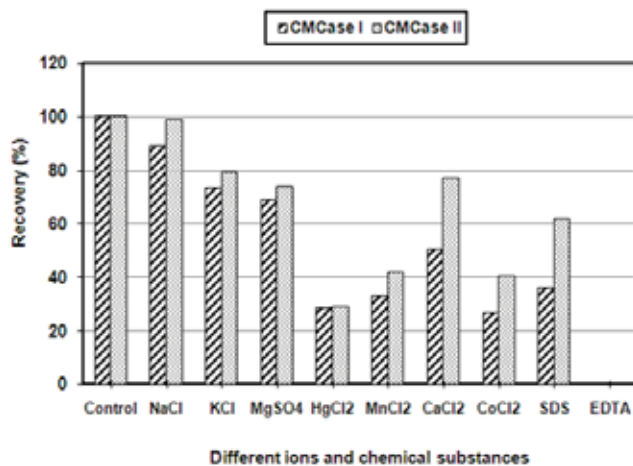


Fig. 8: Effect of some metallic ions and chemicals on CMCase I and II produced by *Trichoderma sp.* grown on wood dust.

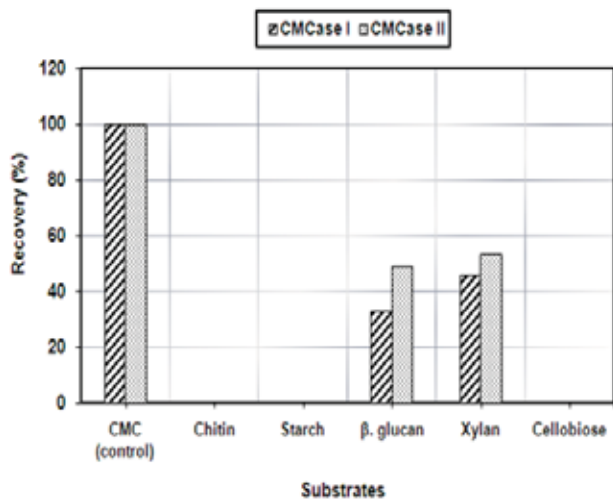


Fig. 9: Substrate specificity of CMCase I and II produced by *Trichoderma sp.* grown on wood dust.

Substrate Specificity of Cmcase I and II Enzymes:

The results in Fig. (9) illustrated that both enzymes have high hydrolytic activity towards carboxymethyl cellulose CMC while both CMCCase I and II did not show any hydrolytic activity against chitin, starch and cellobiose. On the other hand both CMCCase I and II have relatively low hydrolytic activity towards β glucan and xylan. According to Ülker and Sprey, (1990) purified endoglucanase from *Trichoderma reesei*, gave a strong increase in CMC-fluidity but the enzyme had no specificity toward crystalline cellulose (Avicel) or xylan. Petrova et al., (2009) stated also that purified endoglucanases from *Trichoderma sp.* EG-IV catalyzed the hydrolysis of Na-CMC whereas EG-III displayed high activity towards xylan.

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