Properties of Extracellular Carboxymethyl Cellulase Produced by *Aspergillus terreus* DSM 826 using some Agricultural Wastes

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ABSTRACT

Recently, cellulases gained significant commercial importance due to their potential applications in food, animal feed, detergents, paper and pulp and textile industries. A thermostable extracellular carboxymethyl cellulase (CMCase) produced by *Aspergillus terreus* DSM 826 has been isolated using rice straw or sugar cane bagasse as sole source of carbon for fungal growth. Some of the properties of the produced enzyme were studied. Optimal conditions for enzyme activity were established in relation to pH, temperature and thermal stability behaviour. Results obtained indicate that optimum degree of temperature was found to be 50 °C and the enzyme was found to be heat stable at that degree when incubated for 40 min for both agricultural wastes. The extracellular CMCase gave a broad activity with wide range of pH values with maximum activity at pH values of 4.8 and 4.5 using rice straw and sugar cane bagasse, respectively. The specific activity of dialyzed enzyme (for 18 hrs) was significantly higher than those of the non-dialyzed one and the addition of EDTA to the reaction mixture containing the crude CMCase does not affect CMCase activity. On testing different metal ions on the activity of crude CMCase it was found that Hg²⁺ exhibited inhibition at a final concentration of 5 × 10⁻³ M, whereas no remarkable effect could be detected with all the tested metal ions except with Co²⁺ that caused about 30 % increase in CMCase activity.

Key Words: Rice straw, sugar cane bagasse, carboxymethyl cellulase (CMCase), *Aspergillus terreus*.  

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INTRODUCTION

The biotechnology of cellulases and hemicellulases began in the early 1980s, first in animal feed and then in food applications. Subsequently, these enzymes were used in textile, laundry and pulp and paper industries. Today, these enzymes account for approximately 20 % of the world enzyme market, mostly from *Trichoderma* and *Aspergillus* (Coral et al., 2002).

Cellulases refer to a group of enzymes which act together to hydrolyze cellulose into soluble sugars and provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen et al., 2005 and Lee et al., 2008). Cellulases are inducible enzymes, which are synthesized by microorganisms during their growth on cellulose materials (Lee and Koo, 2001). A number of fungi and bacteria capable of utilizing cellulose as a carbon source have been identified (Kim et al., 2003). Cellulases produced by fungi such as the *Aspergillus*, *Rhizopus* and *Trichoderma* species have been extensively studied by several researchers (Murashima et al., 2002 and Saito et al., 2003). Cellulases, responsible for the hydrolysis of cellulose, are composed of a complex mixture of enzyme proteins with different specificities to hydrolyze glycosidic bonds. The complete enzymatic hydrolysis of cellulose materials needs different types of cellulase (Lee et al., 2008). Cellulases can be divided into three major enzyme activity classes (Goyal et al., 1991 and Rabinovich et al., 2002 a and b). These are endoglucanase or endo-1, 4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Endoglucanases, often called carboxymethyl cellulases (CMCase), are proposed to initiate attack randomly at multiple internal sites in the amorphous regions of the cellulose fibre opening-up sites for subsequent attack by the cellobiohydrolases (Wood, 1991). Cellobiohydrolase, often called an exoglucanase, is the major component of the fungal cellulase system accounting for 40-70% of the total cellulase proteins and can hydrolyze highly crystalline cellulose (Esterbauer et al., 1991). β-glucosidase hydrolyze glucose dimers and in some cases cello-oligosaccharides to glucose.
In the present work, a series of experiments was designed to elucidate some of the properties of the extracellular CMCase and establish the optimum conditions for the CMCase activity produced by a filamentous fungus. Such information is necessary to disclose some aspects of the biochemical nature as well as to evaluate the possible application feasibility of this enzyme.

**MATERIALS AND METHODS**

**Microorganism:**
Aspergillus terreus DSM 826 was obtained from Deutsche Sammlung Von Mikroorganismen, Göttingen, Germany, maintained on slants of modified solid Czapek-Dox's medium and was stored at 4°C at the culture collection of the Department of Microbial Chemistry, National Research Centre, Cairo, Egypt.

**Media for Carboxymethyl Cellulase Production:**
A. terreus DSM 826 used in this study was grown on modified Czapek-Dox's liquid medium containing rice straw or sugar cane bagasse pith 2 % as a carbon source for fungal growth. This medium contains (g/l): Rice straw or sugar cane bagasse pith, 20.0; NH₄Cl, 1.26; KH₂PO₄, 1.0; MgSO₄·7H₂O, 0.5; KCl, 0.5; Tween-80, 4.0 and 1.0 ml for liquid medium containing rice straw and sugar cane bagasse pith, respectively. The medium was adjusted to an initial pH value of 5.0. The medium were sterilized by autoclaving at 1.5 atmosphere and 126°C for 20 min. Rice straw and sugar cane bagasse were brought from Al-Sharkia and Al-Giza Governorates, respectively.

**Cultivation of Microorganism:**
Conidia were scrapped from mycelia, which were grown on slants for 7 days at 30°C and suspended by hand shaking in sterile cold distilled water. Five ml aliquots of this suspension were used to inoculate, under aseptic conditions, 250 ml Erlenmeyer flasks each containing 50 ml of sterile medium. The flasks were incubated at 30°C for three days under shaking condition (150 rpm) using New Brunswick scientific Co. Inc. Edison N. J. USA shaker. At the end of the incubation period, the cultures were filtrated using Whatman No.1 filter paper. The culture filtrate was used directly for enzyme activity determination.

**Pretreatment of Lignocellulosic Wastes:**
Rice straw and sugar cane bagasse were ground and soaked in NaOH (1 %) solution (w/v) for 1 hr at room temperature (100 g waste /1L NaOH (1%) solution) in 3 L conical flask. Wastes were then filtered and washed using running tap water until neutralization (pH 7.0), then washed with distilled water, dried in an oven at 60°C to be used as a carbon source in the basal salt medium.

**Analytical Methods:**
Protein content was estimated by Lowry, et al. (1951). Reducing sugars released were determined by the dinitrosalicylic acid method (Miller, 1959). One unit of enzyme activity is defined as the amount of protein that catalyzed the formation of one μmol of glucose under the experimental standard conditions.

**Enzyme Assay:**
Carboxymethyl cellulase (CMCase, Endo-1, 4-β-D-glucanase, E.C. 3.2.1.4) activity was determined according to Adsul, et al. (2004). The total reaction mixture of 1 ml contained a 0.5 ml of 1 % (w/v) CMC solution in citrate buffer (50 mM, pH 4.8) and 0.5 ml of the enzyme. The reaction mixture was incubated at 50°C for 30 min. After incubation, the enzyme activity was stopped by adding 3ml DNS reagent; tubes were then placed in a water bath at 90°C for 15 min., 1 ml of sodium potassium tartrate was added to each tube before cooling. The optical density of samples was immediately measured at 575 nm using Bousch and Lomb (Spectronic 710) spectrophotometer.

**Buffer:**
Sodium citrate buffer was prepared according to the method presented by Gomori (1955). Moreover, the final pH was accurately adjusted by using pH M62 Standard pH meter.

**RESULTS**

**Effect of Incubation Temperature:**
This factor was studied by incubation the crude enzyme with the standard reaction mixture (previously reported in Materials and Methods Section) in water baths set at different temperatures ranging between 10°C and 80°C. The data obtained are graphically illustrated in (Figure 1). The crude CMCase shows progressive increased activity with the increase in reaction temperature up to 50°C which represents the optimum temperature for enzyme activity. By increasing the temperature up to this value, a notable decrease in enzyme activity was observed.

**Thermal Stability Behaviour:**
Aliquots of the crude enzyme were heated inside thin walled glass tubes (in the absence of substrate) in water baths set at different temperatures (50, 60, 70 and 80°C) for different incubation periods up to 40 minutes. Identical aliquots of enzyme were removed at different time intervals, cooled and assayed for CMCase activity using the standard
procedure. Results obtained in (Figure 2) Indicate that the crude CMCase from *A. terreus* DSM 826 was heat stable upon heating. The enzyme was found to be stable at 50°C (the optimum temperature for enzyme activity) for 40 min. By increasing the incubation temperature of cell free filtrates (crude enzyme) up to this value and prolonging the storing time, the rate of enzyme activity began to decrease. Complete loss of enzyme activity was recorded at 80°C for 30 min in case of cell free filtrates prepared from medium containing rice straw. The previously mentioned results revealed high thermal stability of the crude enzyme obtained in case of using rice straw and bagasse as agricultural wastes in the medium by *A. terreus* DSM 826.

**Effect of pH Values:**
The effect of pH on crude CMCase was examined using sodium citrate buffer, pH 3.0 - 6.0. This experiment has been constructed by incubating the crude enzyme with different pH values buffered system (0.1 M sodium citrate buffer; pH 3.0-6.0) and CMC dissolved in 0.05M sodium citrate buffer (pH 4.8) as substrate for 30 minutes at 50°C. Results obtained in (Figure 3) indicate that, the activity increases gradually from pH 3.0 to 4.5 and showed its maximum activity at pH values of 4.8 and 4.5 with crude CMCase of *A. terreus* DSM 826 using rice straw and sugar cane bagasse as carbon sources, respectively, activity was then decreased by increasing the pH value above 5.0. Results also indicate that the crude CMCase of *A. terreus* DSM 826 give a broad range of activity with the tested pH values.

**Reaction Progress with time (time curve):**
The crude CMCase was used in standard reaction mixture that incubated at 50°C. Samples were withdrawn periodically for a period of 30 minutes (0, 2, 4, 6, 8, 10, 15, 20 and 30 min.) and tested for CMCase activity. The results are graphically illustrated in (Figure 4). It is obvious that the enzyme activity was more or less linear with reaction time only up to 10 min., after which the reaction linearity gradually deviated and disappeared upon extended incubation time. According to these results, the use of 10 min. at 50°C was found to be the suitable conditions for enzyme assay.

**Effect of Protein Concentration:**
The crude CMCase enzyme was diluted in an orderly manner and then used as an enzyme source with standard reaction mixtures. The final protein concentration in the reaction mixtures ranged between 0.095 to 0.885 and between 0.035 to 0.315 mg/ml for rice straw and sugar cane bagasse, respectively. The enzyme activity response as related to protein concentration is graphically illustrated in (Figures 5 and 6) for rice straw and sugar cane bagasse, respectively. Results show that enzyme activity exhibited a linear response to protein concentration up to 0.760 and 0.245 mg/ml in the corresponding reaction mixture for rice straw and sugar cane bagasse, respectively.

**Effect of Dialysis:**
This experiment aimed to find out whether or not the crude CMCase requires a metal ion(s) in the process of catalysis. This was accomplished by dialyzing cell free filtrate (crude enzyme) and comparing the specific activity of dialyzed and non-dialyzed enzyme. A known volume of crude enzyme was dialyzed against 200 volumes of 0.05 M sodium citrate buffer pH 4.8 for 1 hr and 18 hrs under agitation. Data presented in (Table 1) show that the specific activity of dialyzed enzyme (dialyzed for 18 hrs) was significantly higher than those of the non-dialyzed enzyme as a notable decrease in protein concentration resulting from dialysis process. The results obtained may indicate the non-requirement of certain metal or cofactor for enzyme activity or the cofactor might be bound to the enzyme i.e. non-dialyzable.

<table>
<thead>
<tr>
<th>Type of cell free filtrate</th>
<th>Rice straw</th>
<th>Sugar cane bagasse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-dialyzed</td>
<td>0.57</td>
<td>1.86</td>
</tr>
<tr>
<td>Dialyzed (1hr)</td>
<td>0.51</td>
<td>1.86</td>
</tr>
<tr>
<td>Dialyzed (18hr)</td>
<td>0.41</td>
<td>2.17</td>
</tr>
</tbody>
</table>

**Effect of Some Metal Salts:**
From the preceding experiment, it was shown that CMCase does not affected by addition of EDTA indicating its no requirement for a metal ion(s), so the present experiment was carried out to emphasis the previous results and the effect of metal ions on crude CMCase activity. Different mineral salts of various cations Na⁺, K⁺, Hg²⁺, Mg²⁺,Co²⁺,Ca²⁺,Cu²⁺, Mn²⁺ and Zn²⁺ were added at a final concentration of **Table 1: Effect of dialysis on CMCase activity of *A. terreus* DSM 826.**

<table>
<thead>
<tr>
<th>Protein (mg)</th>
<th>Activity (U/ml)</th>
<th>Specific activity (U/mg protein)</th>
<th>Protein (mg)</th>
<th>Activity (U/ml)</th>
<th>Specific activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-dialyzed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialyzed (1hr)</td>
<td>0.51</td>
<td>1.86</td>
<td>0.54</td>
<td>1.78</td>
<td>3.27</td>
</tr>
<tr>
<td>Dialyzed (18hr)</td>
<td>0.41</td>
<td>2.17</td>
<td>0.41</td>
<td>2.08</td>
<td>5.03</td>
</tr>
</tbody>
</table>

Reaction mixture contained: CMC, 5 mg dissolved in 0.5 ml sodium citrate buffer 0.05M (pH 4.8); crude protein, as indicated; total volume, 1ml; reaction time, 10 min and reaction temperature and 50°C.

**Effect of Ethylene Diamine Tetra Acetate (EDTA):**
The present experiment was carried out to test the effect of EDTA as a metal chelating agent. As shown in (Table 2) it is clear that the addition of EDTA at three different concentrations namely 5 × 10⁻³ M, 1 × 10⁻² M and 2 × 10⁻² M to the reaction mixture of the crude CMCase of *A. terreus* DSM 826 using rice straw or sugar cane bagasse as a carbon source doesn’t affect CMCase activity.
5 × 10⁻³ M and 1 × 10⁻² M to the reaction mixtures containing (18 hrs) dialyzed cell free filtrate (CFF) from both rice straw and sugar cane bagasse. After incubation the relative enzyme activities were recorded in (Table 3). A control reaction mixture that did not contain any of these metal salts was made. The activities in the presence and absence of these metal salts were then compared. Results show that in case of rice straw CFF and sugar cane bagasse CFF, the addition of HgCl₂ at lower concentration (5 × 10⁻³ M) caused great inhibition but not complete inhibition, while all other tested metal ions have not any remarkable effect on crude CMCase activity except Co²⁺, which caused CMCase activation by about 30%. At higher concentration (1 × 10⁻² M) in case of rice straw CFF, CMCase inhibition was occurred almost with all tested metal ions except in case of Na⁺, K⁺ and Ca²⁺, while in case of sugar cane bagasse CFF, CMCase activity was not affected by metal ions except the appearance of an obvious inhibition with the two concentrations used of Hg²⁺ (5 × 10⁻³ M, 1 × 10⁻² M), Cu²⁺ (1 × 10⁻² M) and Zn²⁺ (1 × 10⁻² M).

**Table 3: Effect of some metal salts on CMCase activity of A. terreus DSM 826.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rice straw</th>
<th>Sugar cane bagasse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NaCl</td>
<td>116</td>
<td>107</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>104</td>
<td>104</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>96</td>
<td>107</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>116</td>
<td>107</td>
</tr>
<tr>
<td>KCl</td>
<td>116</td>
<td>96</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>100</td>
<td>112</td>
</tr>
<tr>
<td>CoSO₄·7H₂O</td>
<td>133</td>
<td>85</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>108</td>
<td>107</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>104</td>
<td>100</td>
</tr>
</tbody>
</table>

N.D: not determined.

**Figure 1: Effect of incubation temperature on the activity of crude CMCase of A. terreus DSM 826.**

Reaction mixture contained: CMC, 5 mg dissolved in 0.5 ml sodium citrate buffer 0.05M (pH 4.8); crude protein for rice straw, 0.960 mg protein; crude protein for sugar cane bagasse, 0.480 mg protein; total volume, 1 ml; reaction time, 30 min. and reaction temperature, as indicated.

**Figure 2: Thermal stability behaviour of the crude CMCase produced by A. terreus DSM 826.**

Reaction mixture contained: CMC, 5 mg dissolved in 0.5 ml sodium citrate buffer 0.05M (pH 4.8); crude protein for rice straw, 0.960 mg protein; crude protein for sugar cane bagasse, 0.480 mg protein; total volume, 1 ml; reaction time, 30 min. and reaction temperature, 50°C.
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Figure 3: Effect of reaction pH values on the activity of crude CMCase from *A. terreus* DSM 826.
Reaction mixture contained: CMC, 5 mg dissolved in 0.5 ml sodium citrate buffer 0.05M (pH 4.8); crude protein for rice straw, 0.480 mg protein; crude protein for sugar cane bagasse, 0.300 mg protein; total volume, 1 ml; reaction time, 30 min. and reaction temperature and 50°C.

Figure 4: Reaction progress with time (time curve) of crude CMCase of *A. terreus* DSM 826.
Reaction mixture contained: CMC, 5 mg dissolved in 0.5 ml sodium citrate buffer 0.05M (pH 4.8); crude protein, crude protein for rice straw, 1.020 mg protein; crude protein for sugar cane bagasse, 0.702 mg protein; total volume, 1 ml; reaction time, as indicated and reaction temperature and 50°C.

Figure 5: Effect of protein concentration on CMCase activity of *A. terreus* DSM 826 using rice straw as a carbon source.
Reaction mixture contained: CMC, 5 mg dissolved in 0.5 ml sodium citrate buffer 0.05M (pH 4.8); crude protein, as indicated; total volume, 1 ml; reaction time, 10 min. and reaction temperature and 50°C.

Figure 6: Effect of protein concentration on CMCase activity of *A. terreus* DSM 826 using sugar cane bagasse as a carbon source.
Reaction mixture contained: CMC, 5 mg dissolved in 0.5 ml sodium citrate buffer 0.05M (pH 4.8); crude protein, as indicated; total volume, 1 ml; reaction time, 10 min. and reaction temperature and 50°C.
DISCUSSION

The results obtained in this study indicate that the crude CMCase from A. terreus DSM 826 belongs to the enzymes of thermophilic nature. Compared with other cellulases, it was found that optimal temperature is similar to that of the CMCase from Aspergillus glaucus XC9 (Chang et al., 2006), Cellulomonas sp. (Emtiazi and Nahvi, 2000), Cellulomonas flavigena (Sami et al., 1988), Trichoderma reesei (Wang, 1999) which had an optimal activity at 50°C. While the crude CMCase from other microorganisms had optimal activities at slightly higher temperatures such as 55°C for CMCase from Arachniotus citrinus (Saleem et al., 2005), Agaricus blazei (Jiang et al., 2001), Streptomyces sp. EC22 (Okeke and Paterson, 1992) and Streptomyces sp. S36-2 (Silva et al., 1993). Extracellular alkaline crude CMCase from Bacillus sp. B38-2 had optimal activity at 60°C (Silva et al., 1993), while Silva, et al. (2005) reported that the crude CMCase from thermophilic fungus Thermoasus aurantiacus had optimum temperature for activity at 75°C. On the other side, optimum CMCase activity was observed at mesophilic range of temperature. These studies recorded a degree of 40°C for Aspergillus niger Z10 (Coral et al., 2002) and of Cellulomonas, Bacillus and Micrococcus spp. (Immanuel et al., 2006). CMCase from both Streptomyces omiyaensis (Alam et al., 2004) and Clostridium papyrosolvens (Garcia et al., 1989) had a maximum activity at 45°C.

The results also demonstrate high thermal stability behaviour of the crude enzyme obtained in case of using rice straw and sugar cane bagasse as agricultural wastes in the medium by A. terreus DSM 826. Sun, et al. (2006) reported that the CMCase activity of Acidothermus cellulolyticus after 6 hrs of heating at 60°C was found to be almost the same as the original activity when heated at 80°C. They also found that CMCase activity declined gradually to 38 % of its maximum activity within 1hr and almost lost all of its activity after 3 hrs. Silva et al. (2005) reported that the crude CMCase from Thermoasus aurantiacus shows 100 % stability to the treatment at 60°C and started to undergo denaturation above this temperature. At 70°C, 68 % of the initial activity was still recovered after heat treatment. Similarly, the CMCase of Trichoderma reesei was found to be still stable to some extent up to 60°C (Wang, 1999). In the present study, the crude CMCase obtained from A. terreus DSM 826 showed cellulolytic activity in a broad range of pH between 3.0 and 6.0 and the maximum CMCase activity was obtained at pH values of 4.8 and 4.5 with the crude CMCase using rice straw and sugar cane bagasse as carbon sources, respectively. The increase or decrease of pH values above or below these optimal values result in a gradual decrease in CMCase activity.

The results obtained are in agreement with those obtained from Acidothermus cellulolyticus, Trichoderma reesei, Streptomyces sp. EC22 where CMCase activity has optimum pH of 5.0 (Sun et al., 2006, Wang, 1999 and Okeke and Paterson, 1992). On the other hand, many authors reported less acidic condition for CMCase activity such as the crude CMCase from Streptomyces omiyaensis, pH 6.5 (Alam et al., 2004), Cellulomonas flavigena, pH 6.5 (Sami et al., 1988), Cellulomonas sp., pH 6.0 (Emtiazi and Nahvi, 2000) and Thermoasus aurantiacus, pH 5.5 (Silva et al., 1993) reported that optimal pH of the crude CMCase activity ranged from 6.0 to 7.0 for Streptomyces sp. S36-2 and 7.0 to 8.0 for Bacillus sp. B38-2. Immanuel, et al. (2006) found that the crude CMCase produced from Cellulomonas, Bacillus and Micrococcus spps. hydrolyzes substrate with maximum activity at pH 7.0. CMCase activity of Aspergillus niger Z10 had a broad pH range between 3.0 and 9.0. The enzyme shows two major activity peaks at pH 4.5 and 7.5. This result is probably due to the presence of two isoenzymes or subunits in the enzyme preparation (Coral et al., 2002).

The results also indicate that crude CMCase activity of A. terreus DSM 826 was not dependent on a metallic cofactor at its active site, as EDTA, a chelating agent, had no effect on the activity which is in agreement with the results obtained by Ng and Zeikus (1981). In addition, β-glucosidase from Aspergillus oryzae and Candida peltata were not affected by 10 mM EDTA (Riou et al., 1998 and Saha and Bothast, 1996). In this study and in case of rice straw and sugar cane bagasse cell-free filtrate (CFF), an inhibition was exhibited by Hg²⁺ at the lower concentration (5×10⁻³M), whiles no remarkable changes could be detected with all the tested metal ions except with Co²⁺ that caused about 33% increase in CMCase activity. In case of rice straw CFF and at higher concentration of metal ions (1×10⁻²M), CMCase inhibition with variant degrees was occurred almost with all tested metal ions except with Na⁺, K⁺ and Ca²⁺. While in case of sugar cane bagasse CFF, CMCase activity was not affected by metal ions except inhibition effect that appeared with Hg²⁺, Cu²⁺ and Zn²⁺.

REFERENCES


