Polish Journal of Microbiology
2012, Vol. 61, No 1, 51–55

ORIGINAL PAPER

Purification and Characterization of Carboxymethyl Cellulase from *Bacillus* sp. Isolated from a Paddy Field

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Received 20 February 2011, revised 14 December 2011, accepted 20 December 2011

Abstract

A microorganism hydrolyzing carboxymethyl cellulose was isolated from a paddy field and identified as *Bacillus* sp. Production of cellulase by this bacterium was found to be optimal at pH 6.5, 37°C and 150 rpm of shaking. This cellulase was purified to homogeneity by the combination of ammonium sulphate precipitation, DEAE cellulose, and sephadex G-75 gel filtration chromatography. The cellulase was purified up to 14.5 fold and had a specific activity of 246 U/mg protein. The enzyme was a monomeric cellulase with a relative molecular mass of 58 kDa, as determined by SDS-PAGE. The enzyme exhibited its optimal activity at 50°C and pH 6.0. The enzyme was stable in the pH range of 5.0 to 7.0 and its stability was maintained for 30 min at 50°C and its activity got inhibited by Hg^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Na^{2+}, and Ca^{2+}.

Key words: *Bacillus* sp., biological degradation, carboxymethyl cellulase, paddy field, cellulase purification

Introduction

Cellulase is an enzyme that hydrolyzes the β-1,4-glycosidic bonds in cellulose to release glucose units (Nishida *et al.,* 2007). It has been generally accepted that effective biological hydrolysis of cellulose into glucose requires the synergistic actions of three enzymes including endo-β-1,4-glucanase (EG; EC 3.2.1.4, randomly cleaving internal linkages), cellobiohydrolase (CBH; EC 3.2.1.91, specifically hydrolyzing cellobiosyl units from non-reducing ends), and β-*D*-glucosidase (EC 3.2.1.21, hydrolyzing glucosyl units from cello-oligosaccharides) (Perez *et al.,* 2002). As for carboxymethyl cellulose (CMC), only endoglucanases are needed to degrade it (Robson and Chambliss, 1989). Cellulases have many industrial applications from the generation of bioethanol, a realistic long-term energy source, to the finishing of textiles (Ando *et al.*, 2002; Lynd *et al.,* 2002), formulation of washing powders, extraction of fruit and vegetable juices, and starch processing (Camassola and Dillon, 2007). Many *Bacillus* sp. including *B. brevis* (Singh and Kumar, 1998), *B. pumilus* (Kotchoni *et al.,* 2006), *B. amyoliquefaciens* DL-3 (Lee *et al.*, 2008), and *Bacillus subtilis* YJ1 (Li-Jing Yin *et al.,* 2010) have been exploited for cellulase production. In this study an attempt has been made to screen carboxymethyl cellulase (CMCase)-secreting *Bacillus* sp. from a paddy field located in the southwestern region of India. The main objective of the present study was to isolate potential CMCase-secreting organisms for purification of cellulase by convenient methods. A study of this kind will improve our knowledge on the biotechnological application of bacteria found in the natural environment.

Experimental

Materials and Methods

Isolation of bacteria from a paddy field for CMCellulase secretion. A small amount of soil from paddy field was collected at Nagercoil, Kanyakumari (India). Suitable dilutions were made and the bacteria were cultured in the nutrient agar medium composed of in (g/l): peptic digest of animal tissue, 5.0; beef extract, 1.5; yeast extract, 1.5; sodium chloride, 5 and agar, 15. The pH was adjusted to 7.0 with the addition of 1N NaOH. The organisms were incubated at 37°C for 24 h.

Screening and identification of CMCase-secreting organism. For the screening of CMCelluase production, the bacterial isolates were streaked on CMC agar medium (g/l) (peptic digest of animal tissue, 5.0; beef extract, 1.5; yeast extract, 1.5; sodium chloride, 5.0,

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agar, 15, CMCellulose, 10) and incubated at 37°C for 48 h. After incubation, bacterial growth was seen as a single line on the plate. To visualize the hydrolysis zone, the agar medium was flooded with an aqueous solution of Gram's iodine. Gram's iodine forms a bluish-black complex with cellulose but not with hydrolyzed cellulose, giving a sharp and distinct zone within 3–5 min (Kasana *et al.,* 2008). Bacterial identification was conducted based on 'Bergey's Manual of Systematic Bacteriology' (Jones and Collins, 1984).

Microorganism and submerged fermentation. The *Bacillus* sp. was cultured in the nutrient medium composed of in (g/l): peptic digest of animal tissue, 5.0; beef extract, 1.5; yeast extract, 1.5; sodium chloride, 5.0 and CMCellulose, 10 (pH 7.0), and sterilized at 121°C for 20 min. A loopful culture of 18 h *Bacillus* sp. was inoculated into the nutrient medium with rotary shaking at 150 rpm at 37°C for 36 h. The culture medium was centrifuged at 6000×*g* for 20 min and passed through a 0.22-μm membrane (Rankam, NY0213SF) to remove the cells. The resulting supernatant was used for further CMCase purification.

Enzyme activity assays:

a) **Endo-β-1,4-glucanase**. Endo-β-1,4-glucanase (EG) activity was determined by incubation of 1.0 ml of 0.2% (w/v) CMC in 0.025 M phosphate buffer (pH 7.0) with 1.0 ml of appropriate concentration of enzyme at 37°C. After 30 min of reaction, 1 ml of dinitrosalicylic acid (DNS) was added and boiled in a water bath for 5 min to stop the reaction. The resulting samples were then cooled to room temperature and the absorbance measured at 540 nm (A_{540}). One unit of EG activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 μg of glucose within 1 min at 37°C (Nelson, 1944).
b) **Avicelase.** Avicelase activity was determined by incubation of 1.0 ml of 0.2% avicel (w/v) in 0.025 M phosphate buffer (pH 7.0) with 1.0 ml of appropriate concentration of enzyme at 37°C. After 30 min of reaction, the activity was measured. One unit of avicelase activity was defined as the amount of enzyme that could hydrolyze avicel and release 1 μg of glucose within 1 min at 37°C (Nelson, 1944).
c) **Filter paperase.** In this assay Whatman No. 1 filter paper was used as the substrate. Fifty milligrams of filter paper was dissolved in 0.025 M phosphate buffer (pH 7.0) and 1.0 ml of appropriate concentration of enzyme. The mixture was incubated at 37°C for 30 min and the reaction was terminated by adding 2 ml of DNS. One unit of filter paperase activity was defined as the amount of enzyme that could hydrolyze filter paper and release 1 μg of glucose within 1 min at 37°C (Nelson, 1944).

Growth and fermentation. A loopful culture of the bacterial isolate was inoculated into the minimal medium (g/l) (peptic digest of animal tissue, 5.0; beef extract, 1.5; yeast extract, 1.5; and sodium chloride, 5.0 (pH 6.5)), and incubated at 37°C in an orbital shaker at 150 rpm. Five millilitres of the culture medium was withdrawn at regular 12 h intervals and the cell density determined at 600 nm (Henriette *et al*., 1993) up to 72 h. To determine the optimum fermentation period for enzyme production the culture medium was centrifuged at 6000×*g* for 10 min. The culture supernatant was assayed for determining enzyme activity (Nelson, 1944).

Determination of protein concentration. The protein concentration of the crude enzyme as well as that of the purified enzyme was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as the standard.

Substrate specificity. The hydrolytic ability of the crude enzyme sample against 0.2% CMC, 0.2% avicel, 0.2% cellobiose, and filter paper (20 mg) in 0.025 M phosphate buffer (pH 7.0) was determined to evaluate its substrate specificity.

CMCase purification

Ammonium sulphate precipitation. The proteins in the crude preparation were precipitated by the addition of solid ammonium sulphate at 40% to 80% saturation. The precipitate was allowed to form at 4°C for 24 h, and was collected by centrifugation at 5000 ×*g* in a cold centrifuge for 20 min. The precipitate was redissolved in 10 ml of buffer A (0.025 M sodium phosphate buffer, pH 7.0 containing 0.001 M ethylene diaminetetraacetic acid (EDTA) and 0.001 M 2-mercaptoethanol).

Purification by ion exchange chromatography on diethylaminoethyl (DEAE) cellulose. DEAE cellulose was packed into a vertically mounted column (1.5×15 cm) and the flow rate of the column was set at 0.5 ml/min. The column was equilibrated with 4 bed volumes of buffer A. The enzyme concentrate obtained from ammonium sulphate precipitation was redissolved in a minimal amount of buffer A and dialysed overnight against the same buffer. The column was washed to remove all unbound proteins and a linear gradient of 0 to 0.5 M NaCl-added buffer A was used to elute the bound proteins. Fractions (2 ml) were collected at a flow rate of 0.5 ml/min. Fractions exhibiting cellulase activity were pooled and concentrated with ammonium sulphate. The precipitate was collected by centrifugation at 5000 × *g* in a refrigerated centrifuge at 4°C for 20 min, redissolved in buffer A and dialysed against the same buffer for 4 h.

Gel filtration on Sephadex G-75. Sephadex G-75 slurry was packed into a column (0.6 × 50 cm) and equilibrated with buffer A. The fractions with the highest cellulase activity from the ion exchange chromatography step were applied to the column. Fractions (2 ml) were collected at a flow rate of 0.25 ml/min. The eluate was monitored for protein concentration at 280 nm and

was also assayed for enzyme activity. Fractions with high enzyme activity were pooled and precipitated with solid ammonium sulphate. Again, the precipitate was collected by centrifugation at 5000 × *g* at 4°C for 30 min, redissolved in a minimal amount of buffer A and then dialysed against the same buffer overnight. The purified enzyme thus obtained was stored at –20°C.

SDS-PAGE and molecular mass determination. Denaturing sodium dodecyl sulphate/polyacrylamide gel electrophoresis SDS/PAGE (10%) was performed to determine the molecular mass of the cellulase following the methods of Laemmli (1970). The molecular weight of the subunit of the enzyme was estimated with phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa) markers. The sample obtained from the gel filtration column was loaded into the gel. Following electrophoresis, the gel was documented using a gel documentation system (Syngene, UK) and the molecular weight of the purified cellulase determined.

Effect of pH on enzyme activity and stability. The optimum pH for the activity of the purified enzyme was determined by using the following buffers (0.1 M): citrate buffer (pH 4.0); succinate buffer (pH 5.0–6.0); sodium phosphate buffer (pH 7.0); tris buffer (pH 8.0); glycine-NaOH buffer (pH 9.0). The stability of the enzyme at various pH was examined by incubating the enzyme solution with the above-mentioned buffers at 37°C for 30 min prior to incubation with substrate. Enzyme activity was assayed as described by Nelson (1944).

Effect of temperature on enzyme activity and stability. The temperature profile of the purified cellulase enzyme was determined by performing the routine enzyme assay at different temperatures: 30, 40, 50, 60, 70 and 80°C. To determine thermal stability, the crude enzyme was incubated (without substrate at increasing temperatures, from 30 to 80°C) for 30 min and cooled. Enzyme activity was assayed as described by Nelson (1944).

Effect of divalent ions on enzyme activity. To study the effect of divalent ions on enzyme activity, the enzyme sample was incubated with various divalent ions namely, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Na^{2+}, Hg^{2+} at 0.005 M concentration for 30 min. Enzyme activity was assayed as described by Nelson (1944).

Results

Screening and identification of cellulolytic bacteria from a paddy field. Five cellulase-secreting bacterial strains were isolated from a paddy field using spread plate technique on basal medium. Among the five bacterial strains, *Bacillus* sp. showed the highest cellulolytic activity on the CMC agar plate. The identified organism was Gram-positive, motile, rod-shaped, starch-hydrolyzing, and nitrate-, catalase- and urease-positive. The organism was seen to produce acid when fructose, galactose, mannitol, sucrose, trehalose, and cellobiose were added to it. Based on morphological and biochemical characteristics, the organism was tentatively identified as *Bacillus* sp. according to Bergey's Manual of Systematic Bacteriology.

Growth curve and fermentation. The *Bacillus* sp. attained maximum growth after 36 h of fermentation and the absorbance was 1.69 at 600 nm. The absorbance declined to 1.38, 1.14, and 0.836 at 48, 60, and 72 h of incubation, respectively. The enzyme secretion increased to its maximum of 13 U/mg protein at 36 h of fermentation. Enzyme production was found to be 3.8, 7.1, 7, 3.1 and 0.7 U/mg protein at 12, 24, 48, 60 and 72 h of fermentation, respectively (Fig. 1).

Effect of pH on enzyme production. Effect of pH on enzyme production was studied by culturing the organism at various pH levels (5.5–8.0) using 1 N HCl/NaOH. Enzyme production was 3.6, 5.1, 9.6, 11.1, and 1.4 U/mg protein at pH 5.5, 6.0, 7.0, 7.5, and 8.0, respectively. Enzyme activity was found to be high at pH 6.5 (14.4 U/mg protein) (Fig. 2).

Purification of cellulase from the *Bacillus* sp. The enzyme was purified through 3 steps including ammonium sulphate fractionation, DEAE cellulose and gel filtration column chromatography. The recovery and purification were 24% and 14.5-fold, respectively, after

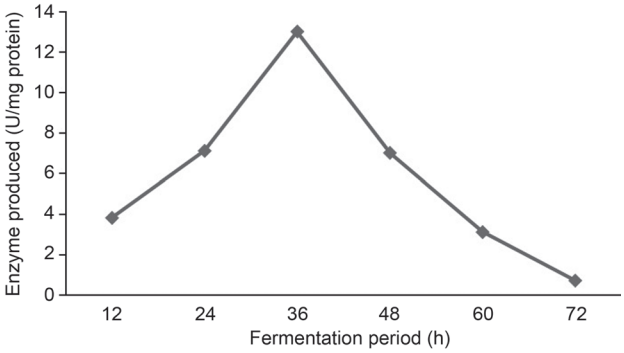

Fig. 1. Effect of fermentation period on CMCellulase by *Bacillus* sp. in submerged fermentation

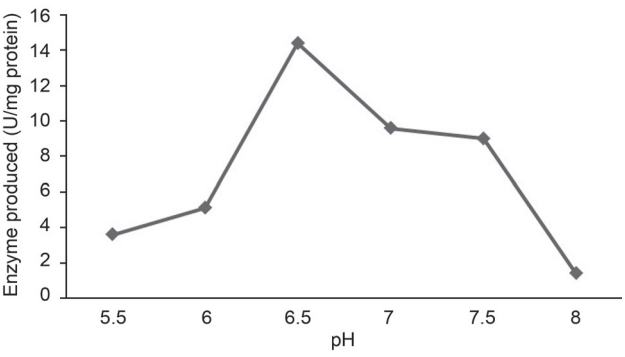

Fig. 2. Effect of pH on CMCellulase from *Bacillus* sp.

Table I
Summary of purification of CMCase from *Bacillus* sp.

Purification step	Vol. (ml)	Protein content (mg/ml)	Total amt of protein (mg)	S. activity (U/mg)	Purification (fold)
Crude extract	95	3	285	17	1
Amm. sulphate	15	9	136	31	1.8
DEAE-cellulose	12	0.68	8.2	144	8.5
Sephadex G-75	8	0.43	3.5	246	14.5

Sephadex G-75 chromatography. The purification procedure of cellulase is summarized in Table I.

Molecular mass of *Bacillus* sp. cellulase. SDS-PAGE analysis of the purified enzyme revealed a single band with a molecular mass of 58 kDa (Fig. 3). The enzyme was electrophoretically run at pH 8.3 on a 11% acrylamide gel and stained with Coomassie Brilliant Blue R-250. This was associated with cellulase activity.

Effect of pH on enzyme activity and stability. In *Bacillus* sp. cellulase activity was found to be high (19 U/mg protein) at pH 6.0. Enzyme activity was 4.1, 7.5, 15, 5.5, and 2.0 U/mg protein at pH 4.0, 5.0, 7.0, 8.0, and 9.0, respectively (Fig. 4). This enzyme was highly stable at pH 6.0 at which the relative enzyme activity was 100%. The relative enzyme activity was 27%, 73%, 67%, 42%, and 13% at the pH 4.0, 5.0, 7.0, 8.0, and 9.0, respectively.

Effect of temperature on cellulase activity and stability. The effect of cellulase activity was studied by incubating the reaction mixture at various temperatures

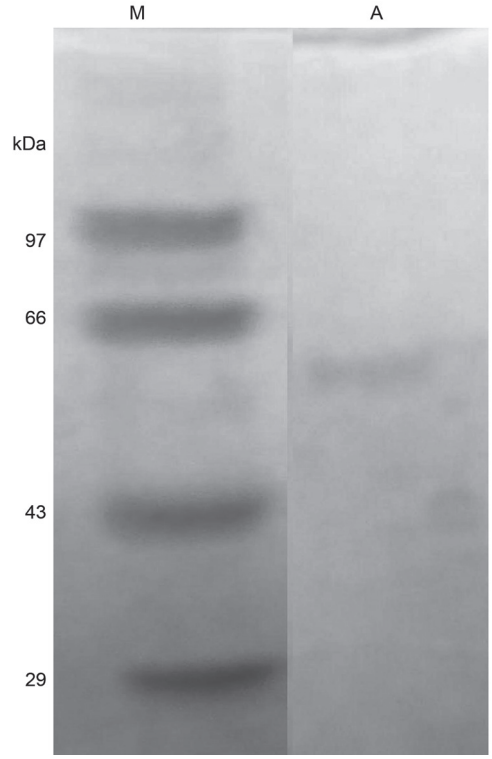

Fig. 3. SDS/PAGE of purified CMCase from *Bacillus* sp. M: Protein markers; A: Purified cellulase

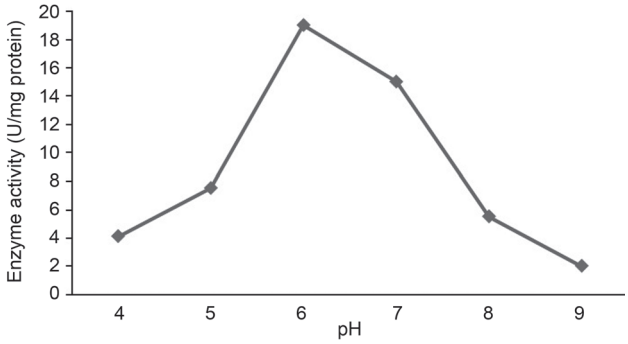

Fig.4. Effect of pH on purified CMCase from *Bacillus* sp.

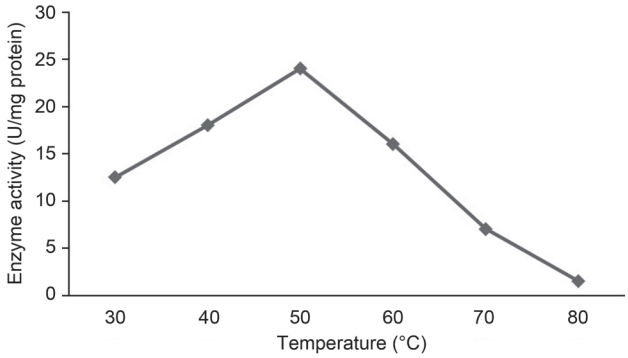

Fig. 5. Effect of temperature on purified CMCase from *Bacillus* sp.

(30–80°C). Enzyme activity was found to be high (24 U/mg protein) at 50°C. Enzyme activity was 12.5, 18, 16, 7, and 1.5 U/mg protein at 30, 40, 60, 70, and 80°C, respectively (Fig. 5). The enzyme activity was also high (100%) at the denaturizing temperature of 50°C and this indicated that it retained its activity after sample denaturation for 30 min. The relative enzyme activity was 67%, 83%, 80%, 35%, and 4% at 30, 40, 60, 70, and 80°C respectively.

Effect of divalent ions on cellulase activity. The effect of divalent ions on enzyme activity was investigated by allowing the divalent ions (0.001 M) to react with the cellulase sample**.** Among divalents, Mn^{2+} enhanced the relative enzyme activity (124%) when compared with the control (100%). Ca^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+} Na^{2+}, and Zn^{2+} inhibited the enzyme activity and the relative activity was 88%, 47%, 13%, 73%, 56%, and 81%, respectively.

Discussion

Screening of cellulase-secreting organism. In the present study five cellulase-secreting bacterial strains were isolated from a paddy field. Based on cellulolytic activity, the organism which showed more zone (3 cm) was used for CMCase production and the organism was tentatively identified as *Bacillus* sp. Growth curve was plotted for this species. CMCase activity was measured during growth in the basal medium, in which the

enzymatic activity increased up to 36 h of fermentation, before declining gradually (Fig. 1). Similar results were obtained with *Bacillus subtilis* YJ1 (Yin *et al*., 2010).

Effect of pH on enzyme production. Cellulase production was found to be high at pH 6.5 when the enzyme activity was 14.4 U/mg protein (Fig. 2). Similar results were obtained with *Bacillus licheniformis*-1 (Dhillon *et al.*, 1985) and with *Bacillus coagulans* (Odeniyi *et al.*, 2009).

Substrate specificity analysis. The crude enzyme was found to have the highest activity on CMC (16.5 U/mg protein). Significant activity was also observed with the crystalline forms of cellulose such as avicel, filter paper and cellobiose. Similar results were obtained with two other bacterial strains, *Bacillus* CH43 and HR68 (Mawadza, 2000).

Purification of CMCase. The crude protein was first precipitated with 40% to 80% ammonium sulphate saturation. Following concentration, DEAE cellulose ion exchange chromatography led to the partial purification of the enzyme. The fractions having cellulase activity were pooled and concentrated and loaded on a sephadex G-75 gel filtration column. The final sephadex G-75 gel filtration chromatography step yielded 246 U/mg protein of electrophoretically pure CMCase from the culture supernatant (Fig. 3). Similar chromatographic methods were employed for the purification of cellulase from *Bacillus subtilis* YJ1 (Yin *et al.*, 2010). SDS/PAGE revealed that the molecular mass of the purified CMCase was 58 kDa. Similar result was obtained with *Bacillus amyoliquefaciens* DL-3 (Lee *et al.*, 2008).

Effect of pH on enzyme activity and stability. The purified cellulase had optimum activity at pH 6.0. Similar results were obtained with *Bacillus subtilis* YJ1 (Yin *et al.*, 2010). This enzyme was highly stable at pH 6.0, which was similar to *Bacillus* CH43 and HR68 (Mawadza *et al*., 2000).

Effect of temperature on cellulase activity and stability. The cellulase isolated from the *Bacillus* sp. was highly active at 50°C (24 U/mg protein) and highly stable at this temperature for at least 30 min of enzyme denaturation. Similar results were found with *Bacillus* CH43 and HR68 (Mawadza *et al.*, 2000).

Effect of divalent ions on cellulase activity. Cellulase was highly active in the presence of 0.005 M Mn^{2+}, and Hg^{2+} strongly inhibited enzyme activity. Similar results were reported for *Bacillus subtilis* YJ1 (Yin *et al.*, 2010) and *Bacillus amyoliquefaciens* DL-3 (Lee *et al.*, 2008).

The progress in biotechnology of CMCellulase is remarkable and attracting worldwide attention. Overproduction of CMCase from *Bacillus* sp. can be achieved by physical, chemical methods of mutagenesis and through recombinant DNA technology. This kind of study also helps to produce CMCase in commercial scale utilizing local agro-wastes in solid state culture.

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