

Original Research Article

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## A Novel Alkaline, Highly Thermostable and Oxidant Resistant Carboxymethyl Cellulase (Cmcase) Produced by Thermophilic *Bacillus sonorensis* CY-3

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### ABSTRACT

#### Keywords

*Bacillus sonorensis*, Thermostable enzyme, Cellulase activity, Oxidant resistance, Carboxymethylcellulase.

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The bacterial strain a thermophilic carboxymethyl cellulase-producing was screened from biocompost. The strain was identified as *Bacillus sonorensis* CY-3 according to morphological, biochemical and molecular analysis, then it was optimized for the production of carboxymethyl cellulase (CMCase). Enzyme was optimally produced in a Luria Broth (LB) medium containing carboxymethyl cellulose (CMC) at pH 9.0 and 55°C. Two bands were found with molecular mass 80 kDa and 72 kDa by use SDS-PAGE and the Vmax and Km were measured 380.19 U/ml and 5.82 mg/ml, respectively. The partially purified enzyme has showed optimal activity at pH 9.0 and 100°C while it was stabled from pH 6.0 to 13.0 with more than 65% activity. It was found to have the properties of enzyme highly thermostability, pH stability, and stability in the presence of some additives that made potentially useful in textile, laundry, and other industrial applications.

### Introduction

Cellulosic materials, which is the product of plant biomass that compose the cell of all higher plants, is the most renewable and abundant source of fermentable carbohydrates in the world (Christakopoulos *et al.*, 1999). They hydrolyzed into soluble sugars by cellulases. Cellulases are hydrolysing the  $\beta$ -1,4 linkages in cellulose. This cellulolytic activity occurs by the synergistic effect of three major components; endo- $\beta$ -glucanase (EC 3.2.1.4), exo- $\beta$ -glucanase (EC 3.2.1.91) and  $\beta$ -glucosidase (EC 3.2.1.21) and they are

classified into two groups: endoglucanases (EC 3.2.1.4) and cellobiohydrolases (EC 3.2.1.91) (Cavaco-Paulo, 1998; Christakopoulos *et al.*, 1999; Xu *et al.*, 2007; Liu *et al.*, 2008; Das *et al.*, 2010). Alkaliphilic *Bacillus* sp. produce a massive amount of extracellular alkaline adapted enzymes such as amylases, cellulases, pectinases and proteases, that they are good and advantageous in industry (Singh *et al.*, 2004; Fujinama and Fujisawa, 2010). The potential of cellulases has been revealed in a broad

range of processes in the textile such as biopolishing, biostoning and stonewashing; furthermore they are used for the production of food, energy, laundry detergent additives and with xylanases for deinking of waste paper (Nielsen *et al.*, 2007; Fujinama and Fujisawa, 2010).

In this study we have purified and characterised an alkaline, thermophilic, oxidant resistance CMCase from thermophilic *Bacillus sp.* CY-3.

## Materials and Methods

### Bacterial strain and culture conditions

Alkaline CMCase-producing *Bacillus sp.* CY-3 were screened from alkaline soil samples near agricultural waste in Adana, Turkey. For selection of gram-positive spore forming bacteria the samples were incubated at 80°C for 10 min (Chang *et al.*, 2012). Cultures were subjected to single-colony isolation on the solid medium at 55°C for 26h. Following, the single-colonies were grown on CMC containing solid medium containing: (g/l<sup>-1</sup>): Pepton 10, Yeast extract 5, NaCl 5, CMC 6, Agar agar 15; the pH was adjusted to 9.0 with NaOH (set prior to sterilization) at 55°C for 26h. Afterward, CMCase positive isolates were determined staining with Congo Red solution (1%) (Chang *et al.*, 2012, Wang *et al.*, 2010).

The strain was identified by studying its morphological and biochemical characteristics (Xu *et al.*, 2007; Vos *et al.*, 2009; Caf *et al.*, 2012) Molecular identification of the strain was carried out by analyzing of its 16S rDNA gene sequences. The extraction of genomic DNA and 16S rDNA amplification was realized by the polymerase chain reaction (PCR) with two universal primers. Subsequently, the PCR product was purified by Wizard® SV Gel and PCR Clean-Up System-Promega.

The sequence of the isolate was aligned with those in the NCBI GenBank database for similarity search and were performed by using ClustalW software and MEGA6.06 program. The phylogenetic trees were created using the neighbour-joining method (Balasubramanian and Simoes, 2014; Caf *et al.*, 2014).

### Optimization of medium composition

Culture conditions such as different temperatures, pHs, carbon and nitrogen sources, salt concentrations, and substrate (CMC) concentrations were optimized for enzyme production.

For this purpose production medium containing different carbon sources (fructose, glucose, maltose and sucrose) and different nitrogen sources (beef extract, yeast extract, tryptone, casein and peptone), different NaCl concentrations (0.1–1%, with increments of 0.1%), different CMC concentration (0.1–1%, with increments of 0.1%) and incubated under different temperature (0–50°C, with increments of 5°C) and pH (3.0–9.0 with increments of one unit) were analyzed for enzyme production by the selected *Bacillus* strain (Shanmughapriya *et al.*, 2010; Caf *et al.*, 2012; Caf *et al.*, 2014).

### Enzyme production

Strain *Bacillus* CY-3 was grown under optimized culture condition in the 0.6% CMC medium at 55°C for 26 h at 190 rev/min. The culture was centrifuged at 8000 x g at 4°C for 15 min and the supernatant was used for partial purification and biochemical characterization (Caf *et al.*, 2012; Caf *et al.*, 2014).

### Partial purification of crude enzyme

The supernatant of culture was precipitated with chilled acetone and was left at -30°C for

4 h. The precipitate was recovered by centrifugation at 12 000 x g for 20 min at 4°C. After centrifugation the sediment, dissolved in phosphate buffer (1mM) at pH 7.0 (Chang *et al.*, 2012).

### **Estimation of protein content**

The protein content in the partial purified Enzyme solution was estimated by the method of Lowry (Hafiz, 2005; Lowry, 1951).

### **Enzyme assay**

Diluted enzyme solution, 0.5 ml was mixed with 0.5 ml 1% (w/v) CMC in 0.1M glycine /NaOH buffer, pH 9.0, and incubated at optimum temperature for 60 min. The reaction was stopped by the addition of 3,5-dinitrosalicylic acid (DNS) solution, boiled for 5 min, and then cooled in water. The absorbance was measured at 540 nm in a 5500 spectrophotometer (Christakopoulos *et al.*, 1999; Arian *et al.*, 2003; Rastogi *et al.*, 2010).

### **Influence of pH, temperature on the enzyme activity and stability**

The optimum pH activity of enzyme was determined using different pH buffers: 0.01M sodium phosphate buffer (pH 6.0-8.0), 0.01M glycine buffer (pH 8.0-10.0), 0.01M borax buffer (pH 11.0-13.0). And the optimum temperature was tested at different temperatures (20-110°C) for 1 h. The pH stability study of the partial purified enzyme was measured after 1 h of preincubation in different pH buffers. Afterward residual activity was determined under optimized assay conditions considering control as 100%. The temperature stability was measured by preincubating the enzyme at different temperatures for 1 h. The residual activity was determined at optimum temperature for at optimum temperature for 60 min considering

control as 100% (Das *et al.*, 2010; Chang *et al.*, 2012).

### **Influence of different NaCl concentrations on enzyme stability**

The stability of enzyme was measured under optimized assay conditions considering control as 100% at different NaCl concentrations (0.5-5 M) after pre- incubation at 55°C for 60 min (Caf *et al.*, 2012).

### **Influence of effectors on enzyme activity**

For measurement the effect of various additives the enzyme was pre-incubated at 55°C for 60 min in different effectors. Afterward residual activity was determined under optimized assay conditions considering control as 100% (Chang *et al.*, 2012, Caf *et al.*, 2014).

### **Determination of Molecular Weight and Zymogram Analyses**

The molecular weight of the partially purified enzyme was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 5% stacking gel and 10% separating gel including CMC (0.1%). Molecular weight was determined by comparing of Standard protein molecular weight markers (Sigma SDS6H2, 29.000, 45.000, 66.000, 97.000, 116.000, 200.000 Da). After electrophoresis the gel was cut into two pieces, markers was stained with Coomassie Brilliant Blue R-260 and destained with methanol-acetic acid-water solution (1:1:8), other piece was subjected to renaturation solutions containing (I,II and III) containing: Renaturation solution I: 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.2), isopropanol 40% for 1 h. Renaturation solution II: 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 50mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.2) for 1 h and at last in renaturation solution III: 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.2), 5 mM β-

mercaptoethanol and 1 mM EDTA at 4°C overnight, respectively. After that, the gel was incubated at 45°C for 5 h and stained with Congo red (Chang *et al.*, 2012, Shanmughapriya *et al.*, 2010).

### **Chromatography of hydrolysed products**

Enzyme solution 2 ml was incubated with 0.5 ml 2% (w/v) CMC in 0.1 M glycine/NaOH buffer, pH 9.0, and incubated at optimum temperature for 60 min. And the hydrolysis products (0.5 µl) were assayed on silica gel plates using a chloroform: acetic acid: water (6:7:1) solvent system. Afterward, the spots were visualized by spraying with acetone solution including: aniline (1.0%, v/v), diphenylamine (1.0%, w/v), orthophosphoric acid (10%, v/v) and baking in oven at 120°C for 45 min (Singh *et al.*, 2001; Voget *et al.*, 2006).

### **Kinetic determination**

Kinetic studies were performed with different CMC concentrations (0.05–0.5%) and times (0-30 min) in 50mM glycine–NaOH buffer (pH 9.0) at 80°C. The kinetic constant  $K_m$  and  $V_{max}$  were determined according to Lineweaver–Burk double reciprocal plot (Trivedi *et al.*, 2011; Caf *et al.*, 2012).

## **Results and Discussion**

### **Isolation of alkaline thermophilic *Bacillus* sp.**

A total of 8 *Bacillus* sp. isolates secreting protease negative alkaline cellulase were screened from biocompost-waste, were selected from 84 colonies secreting alkaline cellulase. Of these, isolate CY-3 showed a large zone of hydrolysis and exhibit significant enzyme activity on was selected for cellulase production. The isolate was Gram positive, rod shaped, spore forming

bacterium and aerobic. With the respect to this results of various morphological and biochemical characteristic, it was identified as belonging to the genus *Bacillus*. The strain grew well between 30–60°C and at a wide pH range of 8.0 to 11.0 and the optimum enzyme synthesis occurred at 55°C and pH 9.0 on CMC plate.

### **Determination of molecular mass**

Partial purified cellulase appeared as two different polypeptide band on SDS-PAGE and had the molecular masses of 80 kDa and 72 kDa, respectively (As shown in Fig.1.). Both protein also showed clear bands on the zymogram gel. Although, the cellulase activity band for 72 kDa protein was faintly.

### **pH and temperature optima and stability of carboxymethylcellulase (CMCase)**

A pH range from 6.0 to 12.0 was used to study the effect of pH on enzyme activity. The optimum activity was observed at pH 9.0 and there was another peak at pH 11.0 (about 83%) (As shown in Fig.2.). And it was almost completely stable from pH 6.0 to 12.0 with about 70% residual activity (As shown in Fig.3.). The optimum temperature of endoglucanase was 100°C (As shown in Fig.4.) and the enzyme was stable with more than 85% residual activity in different temperature (20–110°C) (Fig. 5).

### **Effect of various effectors**

The residual enzyme activity result have given in Table I. The Enzyme was slightly inhibited in the presence of 5 mM EDTA,  $MnCl_2$ ,  $ZnCl_2$ ,  $MgCl_2$ , 1% SDS, Triton-X-100, 0.1% Tween 20, Tween 80, mercaptoethanol, 5 mM phenantroline, 3 mM PMSF, iodoacetamide and 8 mM urea up to 34, 53, 29, 36, 17, 32, 31, 29, 26, 33, 13, 34, and 30 respectively. On the other hand, it was

increased in the presence of CaCl<sub>2</sub>, CoCl<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> (31, 21 and 16%, respectively).

**Effect of different NaCl concentration on carboxymethylcellulase (CMCase) activity and stability**

The activity was stable in different NaCl concentration from 3 to 30% with more than 66°C activity (As shown in Fig.6.).

**Chromatography of hydrolysed products**

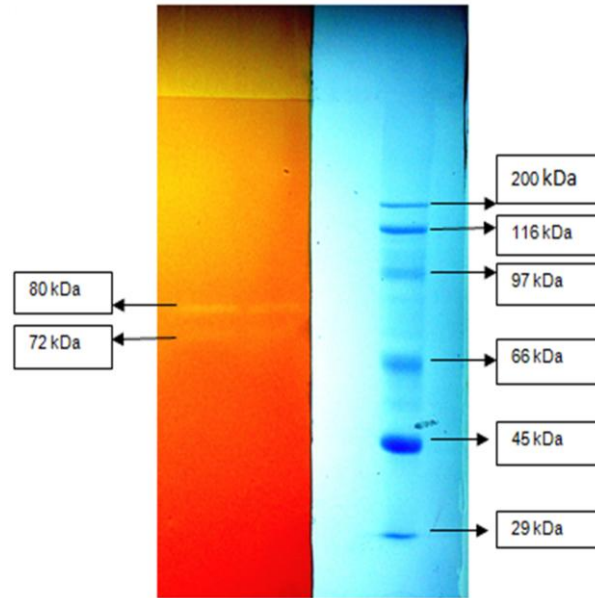
After 2 h incubation of enzyme-substrate mixture, the thin layer chromatography of the CMC hydrolysate revealed the presence of maltoz, maltotrioz, etc. This result suggested that the CMCase called CY-3 is a very good producer of maltose (Fig. 7).

**Table.1** Effect of different effectors with various concentration on the activity of carboxymethyl cellulase (CMCase) from *Bacillus sp. CY-3*

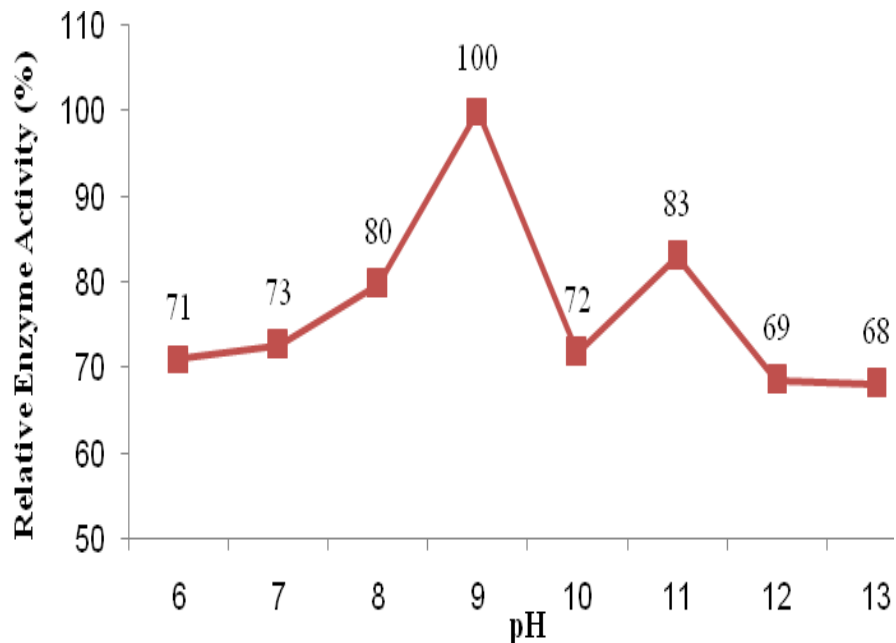
Effectors	Concentration	Relative enzyme activity (%)
Control	None	100
EDTA	5 mM	65
CaCl <sub>2</sub>	5 mM	131
CoCl <sub>2</sub>	5 mM	121
MnCl <sub>2</sub>	5 mM	47
ZnCl <sub>2</sub>	5 mM	71
MgCl <sub>2</sub>	5 mM	64
SDS	1 %	83
Triton X-100	1 %	68
Tween 20	0,1 %	69
Tween 80	0,1 %	71
β-mercaptoethanol	1 %	75
1,10-phenantroline	5 mM	67
Idoasetamide	3 mM	66
PMSF	3 mM	87
Urea	8 M	69
H <sub>2</sub> O	0,1 %	11

**Fig.1** SDS-PAGE zymogram analyse of CY-3 carboxymethyl cellulase (CMCase).

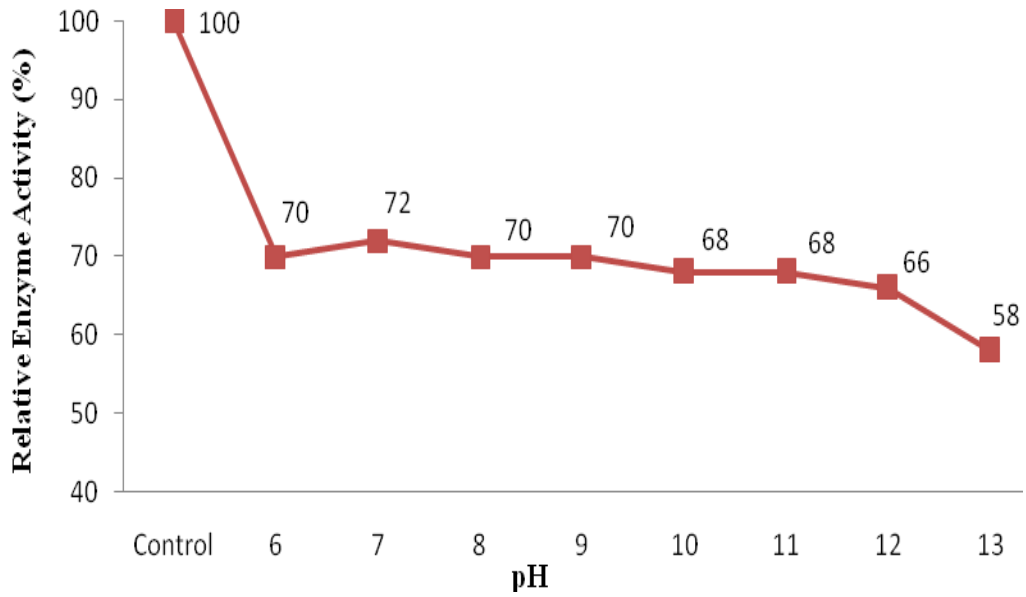
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 5% stacking gel and 10% separating gel including 0.1% CMC. Lane1 and 2) Fragments resulting by CY-3 carboxymethyl cellulase activity produced by *B. sonorensis* CY-3 stained with %0.1 Congo red; Lane 3) Molecular weight marker (29-200 kDa) stained with Coomassie Brilliant Blue R-260



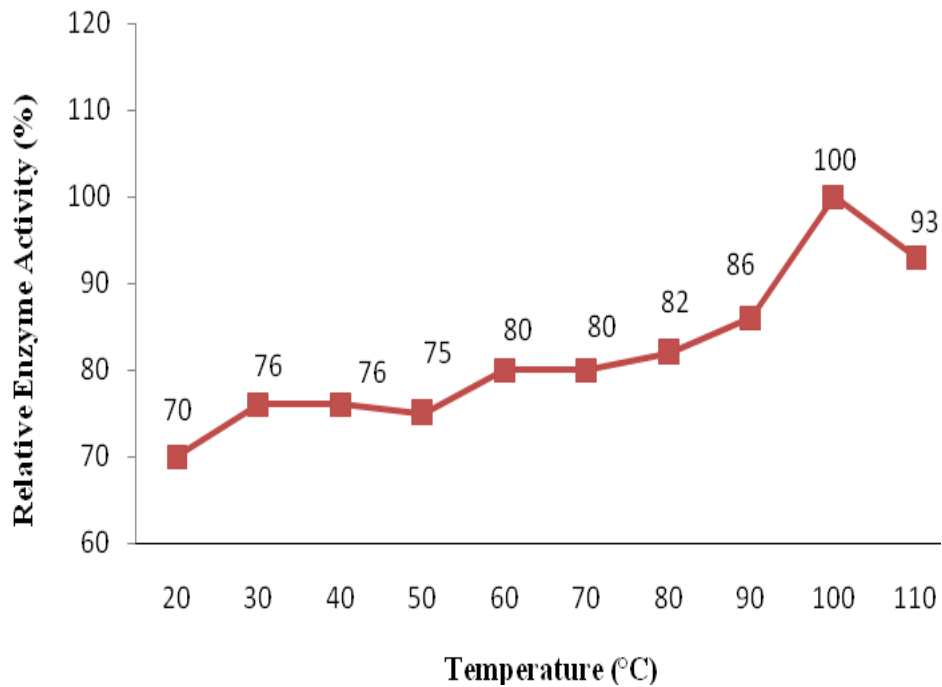
**Fig.2** Effect of pH on the activity of CY-3 carboxymethyl cellulase (CMCase)



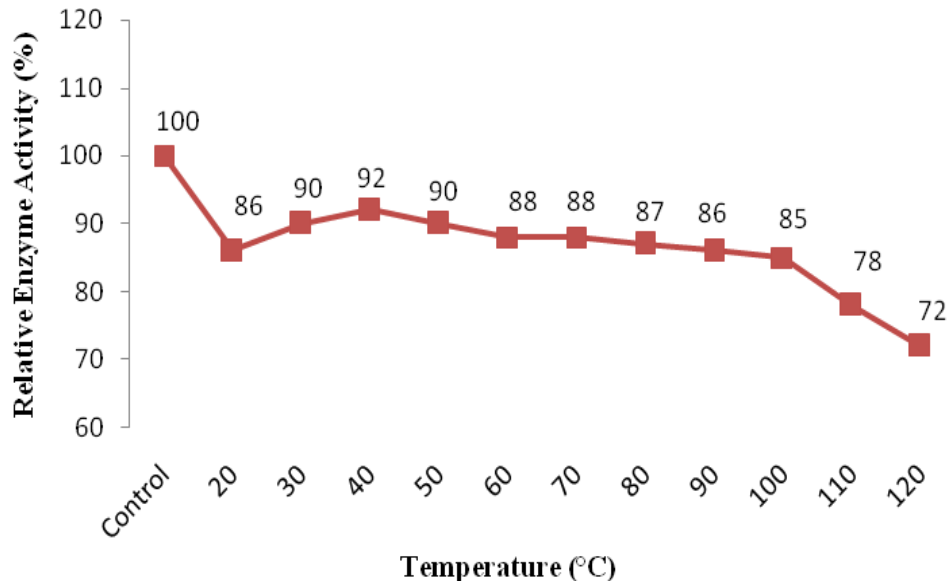
**Fig.3** Effect of pH on the stability of CY-3 carboxymethyl cellulase (CMCase)



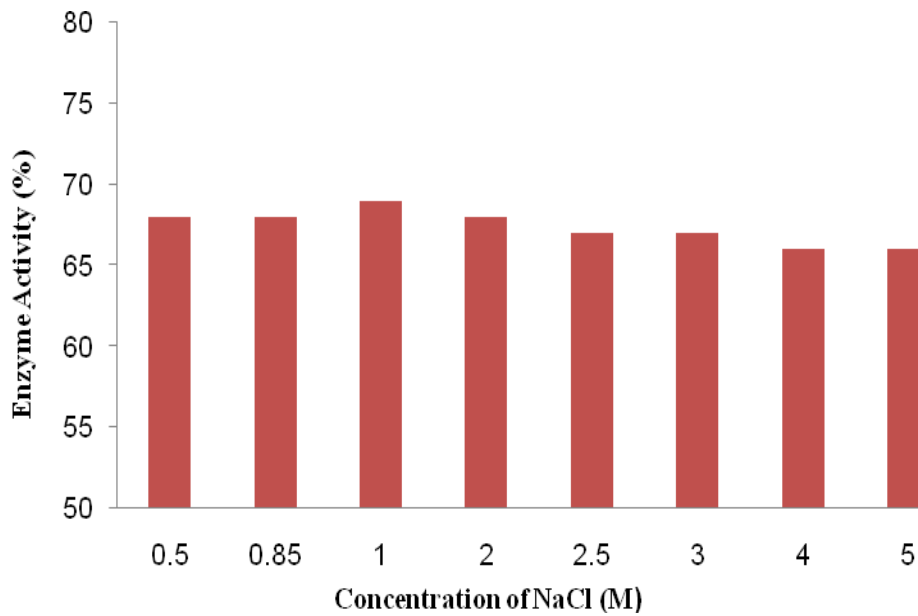
**Fig.4** Effect of temperature on the activity of CY-3 carboxymethyl cellulase



**Fig. 5.** Effect of thermal stability of CY-3 carboxymethyl cellulase (CMCase)

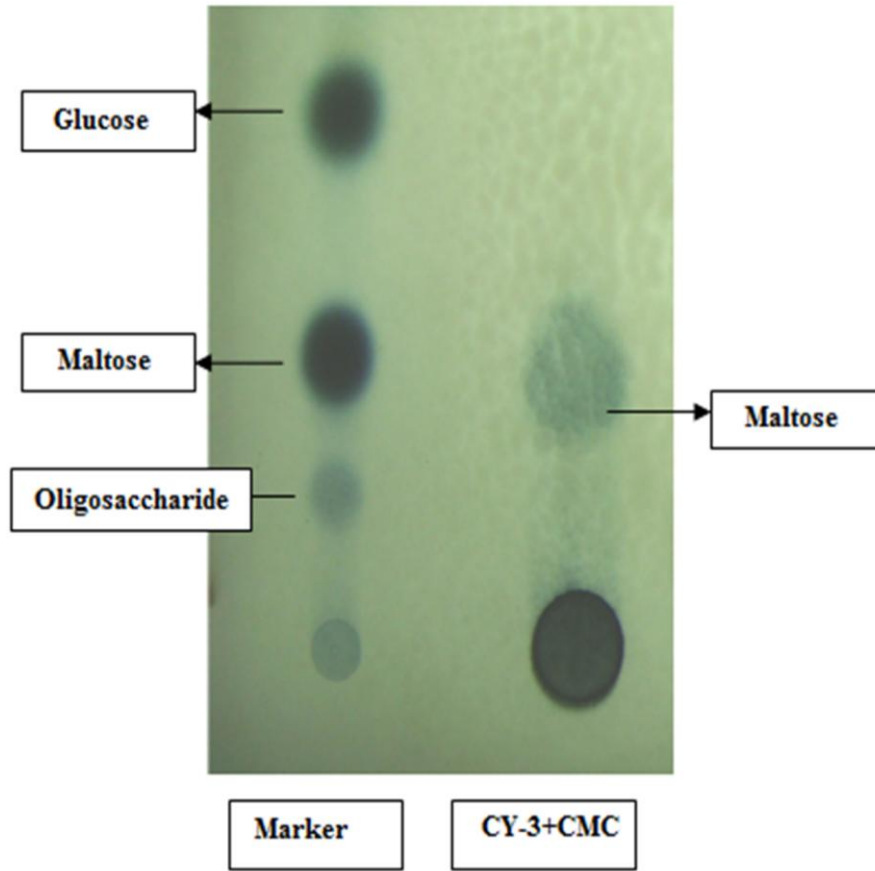


**Fig.6** Effect of different NaCl concentration on CY-3 carboxymethyl cellulase (CMCase) activity

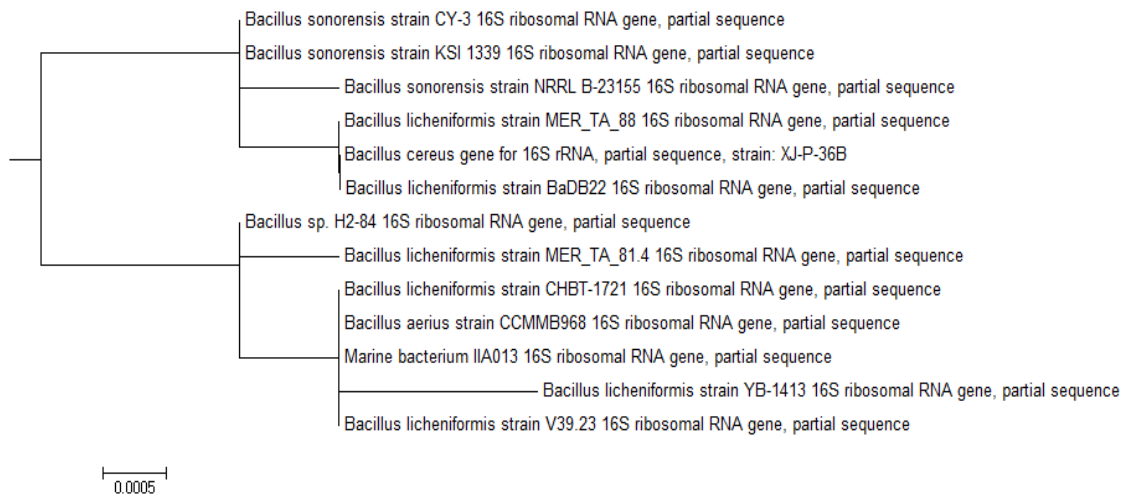




**Fig.7** Thin layer chromatography showing the hydrolysed end products of carboxymethyl cellulase (CMCase) from *Bacillus sonorensis* CY-3



**Fig.8** Phylogenetic tree of isolate *Bacillus* sp. CY-3 showing the relationship with other members of the genus *Bacillus* sp. using 16S rDNA sequence



Isolate CY-3 was showed the largest zone of hydrolysis and was selected for cellulase production. The diameter of halo directly reflected the ability to produce cellulases and the bigger the diameter of halo, the higher the enzyme concentration for liquid culture have been reported by Liu *et al.*, (2008), Theather and Wood (1982). The isolate was Gram positive, rod shaped, spore forming bacterium and aerobic. The 16S rRNA gene sequence (accession no. KJ792668) of isolate CY-3 showed 99% similarity with *Bacillus sonorensis* and with the respect to this results of various morphological, biochemical molecular characteristic, it was classified as *Bacillus* species, belongs to phylum Firmicutes, class Bacilli, order Bacillales and family Bacillaceae (Fig. 8). The strain grew well between 30-60°C and at a wide pH range of 8.0 to 11.0 and the optimum enzyme synthesis occurred at 55°C and pH 9.0 in the culture medium containing 0.6% CMC. The optimum incubation period for enzyme production was 48 hours. Alkaliphiles are defined as microbes growing optimally within pH 9.0-12.0, although the optimal pH varies depending on the growths conditions (Fujinama and Fujisawa, 2010; Arabaci *et al.*, 2013). The bacterium is called typically alkaliphilic, as it grows optimally at pH values above 8.0, but cannot grow or grows poorly at the near neutral pH value of 6.5 (Chang *et al.*, 2012). Thermophilic bacteria are the organisms which can grow and produce such compounds optimally high temperature. Thermophiles are further subcategorized on the basis of their temperature tolerance: for instance, facultative thermophiles, can grow at temperatures between 50°C-65°C, but also grow also at 37°C; obligate thermophiles have maximum growth temperatures of 65°C-70°C, and will not grow below 40°C; extremely thermophiles can grow between 40°C-70°C with an optimal growth temperature of about 65°C and hyperthermophiles, mainly

comprising of archae, can grow over 90°C with a range of optimal temperatures between 80°C-115°C (Horikoshi, 1999; Kikani *et al.*, 2010). Specialized proteins called 'chaperonins' are produced by these organisms, which help, after their denaturation to returned the proteins to their native form and restore their functions and their cell membrane is made up of saturated fatty acids (Haki and Rakshit, 2003). According to these results the isolate *Bacillus sp.* CY-3 is called thermophilic and alkaliphilic bacterium.

Partial purified cellulase appeared as two different polypeptide band on SDS-PAGE and had the molecular masses of 80 kDa and 72 kDa, respectively (As shown in Figure 1). The similar result of this zymogram analyses was reported for the cellulases by Gelhaye *et al* (1993), Hoshino (2000), Coral *et al* (2002) and Odeniyi *et al* (2009). These results suggested that the enzyme have two subunits or dimeric structure (Arabaci *et al.*, 2013).

On optimum pH analyses CY-3 showed two peaks at pH 9.0 and 11.0. But at pH 9.0 was the activity value higher than pH 11.0. These results supported this zymogram analysis (Caf *et al.*, 2012). And it was almost completely stable from pH 6.0 to 12.0 for 60 min (about %70 residual activity, as shown in Fig.4.). The enzyme was extremely stable at 20 to 110°C after more than 60 min incubation with CMC substrate with more than 92% macroactivity. These values are in accordance with these reports by Jang and Chen (2003), Wang *et al.*, (2010) and Rostagi *et al.*, (2010) for alkaline cellulase. This result are thought that the thermostable cellulase may provide spacious application in biopolishing process of cotton in the textile industry where requires cellulase stable at high temperature about 100°C and in the food and sugar industry, where high- temperature processes such as pasteurization are used (Haki and Rakshit,

2003). In this study CMCCase activity was stimulated by  $\text{Co}^{2+}$  to 121%, while the presence  $\text{Mg}^{2+}$  reduced to 64%. The results are quite similar to that reported for this enzyme from other source (Chang *et al.*, 2011).  $\text{Co}^{2+}$  ions caused a significant increase (Mansfield *et al.*, 1998; Christakopoulos *et al.*, 1999; Chang *et al.*, 2011) while  $\text{Mn}^{2+}$  caused a decrease in the activity of enzymes (Mawadza *et al.*, 2000; Liu *et al.*, 2008). In this study enzyme activity was stimulated by  $\text{Ca}^{2+}$  ions to 131%. The activity was stimulated by  $\text{Ca}^{2+}$  ions rather than  $\text{Co}^{2+}$  ions. These results suggest that the enzymes required  $\text{Ca}^{2+}$  and  $\text{Co}^{2+}$  ions for thermal stability (Ito, 1997).  $\text{Ca}^{2+}$  ions had a considerable enhancement on the thermal stability. The stimulation by  $\text{Ca}^{2+}$  and  $\text{Co}^{2+}$  ions has been reported very unusual for alkaline endoglucanases (Christakopoulos *et al.*, 1999; Liu *et al.*, 2008; Lee *et al.*, 2008). However, Mansfield *et al.* (1998) reported that  $\text{Ca}^{2+}$  ions required by enzymes with the former increasing the substrate binding affinity of the enzyme, protecting them from conformational changes and stabilizing the conformation of the catalytic site. The  $\text{Ca}^{2+}$  binding sites determined for some bacterial enzymes contain a number of co-coordinating aspartic acid (Asp), glutamic acid (Glu) residues (Theather and Wood, 1982; Chang *et al.*, 2012).

The inhibition by iodoacetamide (34%) suggesting tryptophan and free thiol groups to be necessary for the enzyme activity. Tryptophan residues are not directly associated with active site but are essential for substrate binding in the cellulose binding domain of the cellulases (Bray *et al.*, 2006; Lee *et al.*, 2008). The non-ionic detergents Triton X-100, Tween 20, and Tween 80 and SDS showed slightly inhibitory effect, 31, 29, 30% respectively (Table 1). Similar findings have been made by other workers (Christakopoulos *et al.*, 1999; Singh *et al.*,

2004; Chang *et al.*, 2012). Cellulases for detergent use should maintain activity in their presence. These results showed that our enzyme is a good source for detergent additive (Arabaci *et al.*, 2013).

In this study  $\text{Zn}^{2+}$  ions decreased activity of CMCCase. The inhibition by  $\text{Zn}^{2+}$  (29%) can be a result of inhibitory effects of heavy metals on enzymes. This result suggested that these groups are present at the active site of the enzyme. Because of thiol groups are targets for the heavy metals. In another study was reported that the inhibition of enzymes by Zn is an indication of thermostability for an enzyme. In addition, the 1,10-phenantroline and EDTA decreased the activity of CMCCase about 35%. The massive inhibition observed against these inhibitors suggested that the partial purified cellulase is a metalloprotein (Theather and Wood, 1982; Chang *et al.*, 2012). However, the CY-3 CMCCase activity was slight inhibited (13%) by PMSF (3mM). Thus, this result is thought that the enzyme do not possesses modification of a serine (ser) residue at the active site, because PMSF is known as serine protein inhibitors (Theather and Wood, 1982; Chang *et al.*, 2012; Arabaci *et al.*, 2013). On the other hand, it was found that the enzyme activity of CY-3 CMCCase was not influenced by  $\text{H}_2\text{O}_2$ . Similar results have been reported by Joo and Chang (2005).

In conclusion the results of this study have shown that the enzyme CY-3 is a thermophilic, highly thermostable, alkaline, pH stable, highly resistant to  $\text{H}_2\text{O}_2$  and other effectors (non-ionic detergents, SDS etc.) Cellulases for detergent use should maintain activity in their presence. These results showed that our enzyme is a good source for detergent additive. On the other hand, our enzyme showed optimum activity at 100°C with highly stability at 20-110°C for 60 min over than 92%. This result are thought that the thermostable cellulase may provide spacious

application in biopolishing process of cotton in the textile industry where requires cellulase stable at high temperature about 100°C (Haki and Rakshit, 2003) and in the food and sugar industry, where high- temperature processes such as pasteurization are used.

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