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A New Halo-alkaliphilic, Thermostable Endoglucanase from Moderately Halophilic *Bacillus* sp. C14 Isolated from Van Soda Lake

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ABSTRACT

Bacillus sp. C14 isolated from Van Soda Lake, Turkey, produced halo-alkaliphilic endoglucanase at 37ºC at pH 9. Analyses of the enzyme for molecular mass and cellulolytic activity carried out by CMC SDS-PAGE electrophoresis revealed a single band with molecular mass 61 kDa. The partially purified enzyme showed optimum activity at pH 11 and 50ºC. Thermal stability of the enzyme was approximately 89% at 20-40ºC for 30 min. The pH stability of enzyme was observed between pH 6 and 12 with an average of 65% of retaining activity for 24 h. Enzyme activity was increased in the presence of 5 mm $CaCl_2$ (132%) and inhibited in the presence of 5 mm $ZnCl_2$, EDTA, KCl, PMSF, Urea (8 M), Triton X-100 (1%), β-mercaptoethanol (1%) and SDS (1%) up to 23, 7, 60, 57, 58, 48, 31 and 19%, respectively. The maximal enzyme activity was observed at 20% NaCl concentration by 132% and enzyme retained 75 and 70% of its original activity for 1 and 6 h, respectively. The enzyme was thermostable- highly alkaline, halophilic, suitable for application in industries like beverage, textile and ethanol production from cellulosic material.

Key Words: Alkaliphilic; *Bacillus* sp.; CMCase; Endoglucanase; Halophilic; Thermostable

INTRODUCTION

Cellulose is the most abundant and renewable biomass on earth and cellulases are the enzymes, which hydrolyze it into soluble sugars. Surely, microbial cellulases are the most economic and available sources. Cellulolytic activity is a multicomponent enzyme system and consists of three major components; endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Lynd *et al*., 2002; Bhat, 2000).

Major industrial applications of cellulases are in textile industry for 'bio-polishing' of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness (Cavaco-Paulo, 1998). They are also used in animal feed for improving the nutritional quality and digestibility, in processing of fruit juices and in baking, while de-inking of paper is yet another emerging application (Tolan & Foody, 1999). Another promising area of the cellulases is the bioconversion of renewable cellulosic biomass to sugars for fermentation of bioethanol and biobased products on large scale (Cherry & Fidantsef, 2003).

Among microorganisms *Bacillus* sp. produce various extracellular enzymes of industrial importance (Priest, 1977) including cellulases. Though alkaline cellulase from alkalophilic bacilli has been studied extensively (Hakamada *et al*., 2002; Singh *et al*., 2004), limited reports are available for alkaline cellulases from moderately halophilic (or halotolerant)-alkaliphilic *Bacillus*.

In this work, we isolated alkaliphilic halotolerant microorganism, *Bacillus* sp. C14, from the soda lake in Turkey, producing carboxymethylcellulase (Endoglucanase) and described partial purification and characterization of the enzyme.

MATERIALS AND METHODS

Organisms and cultivation conditions. *Bacillus* sp. C14 was isolated from coast sediment samples collected from the shoreline of the Soda lake, Van, Turkey. The samples were incubated at 80ºC for 10 min for selection of gram-positive spore forming bacteria, *Bacillus* sp. The isolates were screened for endoglucanase production on CMC-agar plates containing (g L^{-1}) Pepton 5, KH_2PO_4 1, NaCl 10% (w/v), Yeast extract 5, CMC 10, Agar agar 20. The pH was adjusted to 10 with 10% Na_2CO_3 after autoclaving (Singh *et al.*, 2001). Endoglucanase positive isolates were selected with Congo Red solution (0.1%) (Voget *et al.*, 2006).

Enzyme production. Strain C14 was fermented in CEPM (Cellulase Enzyme Production Medium) (Krishna, 1999)

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containing (g L^{-1}) Na_2HPO_4 1.18, KH_2PO_4 0.3, $NaNO_3$ 1, KCl 0.5, $MgSO_4.7H_2O$ 0.5 g, Yeast extract 0.5, Casein Hydrolysate 0.5 g, CMC 1%; pH was adjusted to 10 with 10% Na_2CO_3 after autoclaving. Cultures were grown up at 37ºC with shaking at 200 rpm until the beginning of stationary phase, according to OD measurement at 600 nm. After removal of cells by centrifugation (Hettich Universal 30 RF) (10,000 rpm, 20 min.) at 4ºC, the supernatant was used for enzyme assay and purification.

Partial purification of endoglucanase. The clear supernatant was concentrated with ethanol previously chilled at 4°C by adding drop wise with continuous stirring. The solution was left at -30°C for 24 h, the precipitate recovered by centrifugation at 10,000 rpm for 20 min at 4°C. It was then resuspended in phosphate buffer (100 mm) at pH 7.6 (Burhan *et al*., 2003).

Enzyme assay. Endoglucanase activity was assayed by adding 0.5 mL of enzyme to 0.5 mL CMC (1% v/v) in 100 mm Borax-NaOH buffer, pH 11 and incubating at 50ºC for 30 min. The reaction was stopped by the addition of 2 mL of 3, 5-Dinitrosalicylic acid reagent and $A_{550\ nm}$ was measured in a Cecil 5500 spectrophotometer (Bernfeld, 1955). Experiments were repeated three times and mean values used.

Effect of pH, temperature and NaCl concentration on activity and stability. Temperature and pH effects on enzyme activity were assayed at different temperatures ranging from 20 to 100ºC and at pH values ranging from 6 to 12 for 30 min. Following buffers were used in the reactions: 100 mm Na-phosphate (pH 6-8), Glycine-NaOH (pH 8.5-10.5) and Borax-NaOH (pH 11-12) (Burhan *et al*., 2003).

For the measurement of thermal stability, the enzyme was pre-incubated at temperatures between 20 to 100ºC for 30 min at optimum pH. The enzyme activity was determined under standard enzyme assay condition. To determine pH stability, the enzyme was pre-incubated at 50ºC for 24 h at pH 6 to 12. The effect of salt concentration on enzyme activity was tested by adding the enzyme into substrate containing NaCl, ranging from 1.5-30% at optimum temperature (50ºC) in Borax-NaOH buffer pH 11 (Pomares *et al*., 2003). Enzyme stability against NaCl was determined by pre-incubating the enzyme at 50ºC in buffer having 3, 5, 7, 10, 15, 20 and 25% of NaCl and residual activity was determined under standard assay conditions.

Effect of various metals ions, surfactants, chelating agents and inhibitors on activity. The effect of metal ions, chelating agents, surfactants and inhibitors on the activity of endoglucanase were determined by pre-incubating the enzyme in the presence of EDTA (5 mm), SDS (1%), $CaCl_2$ (5 mm), Na_2SO_3 (5 mm), $ZnCl_2$ (5 mm), PMSF (5 mm), KCl (5 mm), β-Mercaptoethanol (5 mm), TritonX-100 (1%) and Urea (8 M) for 30 min at 50ºC before adding the substrate. Subsequently relative endoglucanase activities were measured at optimum temperature (Egas *et al*., 1998; Lo *et al*., 2001). The control (without any additive) was taken as having 100% activity.

SDS-PAGE and zymogram analysis. SDS-PAGE (10%) containing CMC 1% were carried out for the determination of molecular mass and zymogram analysis (Laemmli, 1970). After electrophoresis the gel was cut into two pieces, one was used for staining and the other was subjected to renaturation solutions (Saul *et al.*, 1990) before incubation at 50ºC overnight. The bands were detected by destaining the gel in methanol-acetic acid-water solution (1:1:8 by volume) after staining process with 0.1% Coomassie Blue R 250 (Bollag *et al*., 1996). Activity staining was performed after incubating the gel in 0.1% Congo Red solution for 1 h. The activity bands were visualized after soaking the gel in 1 M NaCl solution for overnight.

Chromatography of the end products of CMC hydrolysis. CMC (1%) was digested with partially purified endoglucanase at 50ºC for overnight incubation. Previously chilled ethanol was added to enzyme-substrate mixture to stop the reaction after incubation. The end products were analyzed (5 µL) by silica gel 60 (GF_{254}) (Merck) thin layer chromatography. After developing the products with Chloroform-Acetic acid-distilled water (6:7:1, v/v/v), the spots were visualized by spraying Aniline (1% v/v), Diphenilamine(1% w/v), Orthophosphoric acid (10% v/v) in Aceton and baking in oven at 160ºC for 30 min (Singh *et al*., 2004).

RESULTS

Bacillus sp. C14 was gram positive, rod shaped, motile, catalase positive, spore forming and aerobic. On the basis of various morphological and biochemical characteristic, it was identified as *Bacillus* sp. The growth observed between pH 6 and 12 in the presence of NaCl ranged from 3 to 15%, 45ºC temperatures. The optimum enzyme synthesis occurred at 37ºC and pH 9 on CMC agar.

Determination of molecular mass. Molecular mass determined by SDS-PAGE electrophoresis revealed single band showing endoglucanase activity in gel (Laemmli, 1970). The molecular mass of this band was 61 kDa (Fig. 1).

Properties of the enzyme. The enzyme had a broad temperature range between 20 to 100ºC and the optimum activity was observed at 50°C. The mean enzyme activity was 88% between the 20 and 60ºC and 85% between 20 to 80ºC, whereas only 65% activity was retained between 90 to 100ºC (Fig. 3). The enzyme also showed a significant relative activity (92%) between pH 6 and 12 with an optimum pH 11 (Fig. 2). The pH stability of enzyme was measured by the standard assay method. Average 65% of retaining activity was observed between pH 6 and 12 (Fig. 4).

For thermal stability estimation, the retaining activity was determined at optimum pH and temperature (Fig. 5). The retained original enzyme activity was obtained from 20 to 90ºC approximately 71% for 15 min, 60% for 30 min and 36% for 60 min. On the other hand, the enzyme activity was completely lost at 90ºC for 60 min. The enzyme was highly

Fig. 1. Zymogram analysis of Endoglucanase on SDS-PAGE. The gel was cut into two pieces, the marker protein was visualized with Coomasie Brillant Blue staining and the activity of enzyme revealed by Congo Red (0.1%,w/v) Lane 1: 30 µL Enzyme, Lane 2: 10 µL of Bovine albumin as marker (66 kDa)

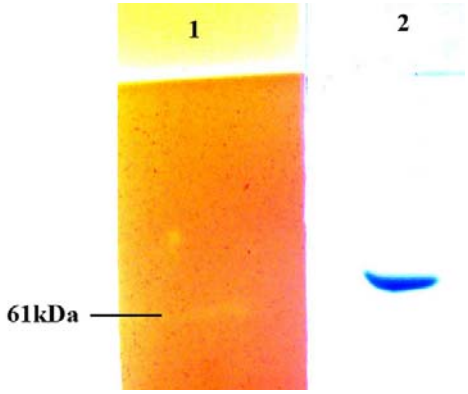


Fig. 2. Effect of pH on the activity of *Bacillus* sp. C14 endoglucanase

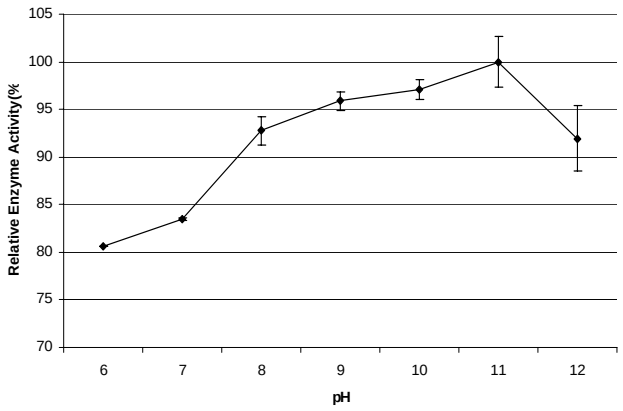


Fig. 3. Effect of temperature on the activity of *Bacillus* sp. C14 endoglucanase

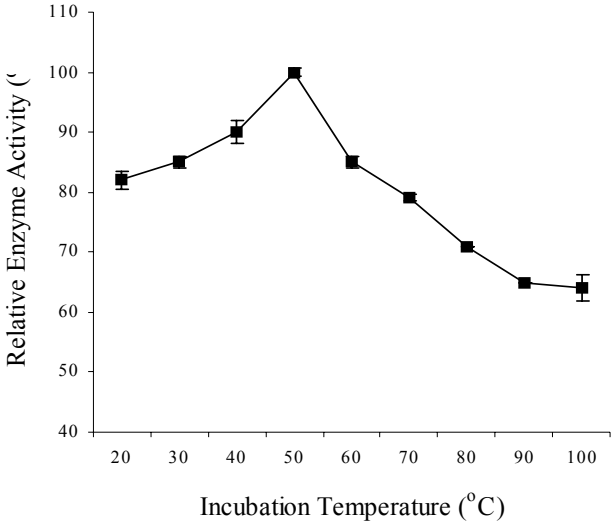
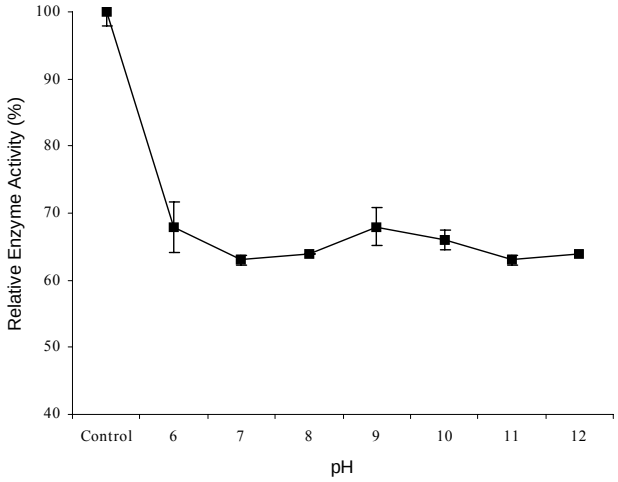


Fig. 4. Effect of pH on the stability of *Bacillus* sp.C14 endoglucanase incubated in selected buffers for 24 h



stable at 20, 30 and 40ºC with an average 94, 89 and 64% for 15, 30 and 60 min, respectively.

Endoglucanase activity was increased in the presence of NaCl ranged from 1.5% to 25% and the maximal activity was obtained with 20% NaCl (132%). The remaining enzyme activity between 3 to 15% of NaCl concentration was found approximately 72.2 and 71% for 1 and 6 h, respectively. But maximal activity was retained at 20% of NaCl concentration around 88 and 75%, endoglucanase activity between 15 and 25% NaCl was detected average 80 and 69% for 1 and 6 h, respectively (Fig. 6).

Effect of some metals ions, surfactants, chelating agents and inhibitor on enzyme activity. The enzyme was pre-incubated at 50ºC for 30 min at different concentration of the metal ions and various chemicals prior to standard enzyme activity assay. The residual enzyme activity results have given in Table I. The relative activity of enzyme was slightly reduced to 93% by EDTA (5 mm). But the enzyme was not affected by Na_2SO_3. The stimulatory effect was obtained by 5 mm $CaCl_2$ up to 132%. On the other hand, partial inhibition of original enzyme activity was obtained with SDS (1%), $ZnCl_2$ (5 mm) and β-mercaptoethanol (1%) as 19, 22 and 31%, respectively. C14 endoglucanase enzyme was also inhibited with PMSF (5 mm), KCl (5 mm), Triton X-100 (1%) and Urea (8 M) up to 57, 60, 48 and 58%, respectively (Table I).

Analysis of the end products of CMC. After overnight incubation of enzyme-substrate mixture, cellobiose was the hardly visible shortest unit of detectable sugar on thin layer chromatography. The longer oligosaccharides produced indicate that the enzyme is pretty good for oligosaccharide production (Fig. 7).

DISCUSSION

Bacillus sp. C14 showed growth at a wide range of pH from 6 to 12. *Bacillus* sp. JB-99 was also able to grow over a wide range of pH (6–12) and it required an alkaline pH (8–10) for growth and enzyme secretion (Johnvesly & Naik, 2001). The bacterium is called typically alkaliphilic, as it grows optimally at pH values above 8, but cannot grow or grows poorly at the near neutral pH value of 7 (Horikoshi, 1999). Although the optimum temperatures for enzyme production and growth were at 37ºC and the NaCl concentration was 5%, growth was not observed in the medium containing 20% NaCl. This suggested that strain *Bacillus* sp. C14 could only be called moderately halophilic. Ventosa *et al*. (1998) reported that moderately halophilic bacteria are extremophilic microorganisms that grow optimally in media containing 3-15% NaCl concentrations.

Many cellulases are produced by *Bacillus* species. Most of them belong to alkaline hydrolytic extracellular enzymes. Several bacterial strains were isolated producing alkaline cellulase. One of the strains, *Bacillus* sp. 1139, produced one cellulase that was purified and shown to have optimum activity at pH 9. The enzyme was stable over the pH range from 6 to 11 for 24 h at 4ºC (Horikoshi, 1999).

Bacillus sp. C14 strain presented a good growth between pH 6 and 12 in the presence of NaCl ranged 3 to 15% and up to 45ºC temperatures. But in 20% of NaCl medium, strain C14 did not show any growth sign. Therefore *Bacillus* sp. C14 strain could be called moderately halophilic. Ventosa *et al*. (1998) have already reported that moderately halophilic bacteria are extremophilic microorganisms that grow optimally in media containing 3-15% NaCl concentrations.

The alkaline endoglucanase from *Bacillus* sp. C14 was estimated to be 61 kDa according to the activity staining gel resulting a single band, which is similar to the monomeric endoglucanase reported by many researchers (Hakamada *et al*., 1997; Christakopoulos *et al*., 1999; Endo *et al*., 2001).

The *Bacillus* sp. C14 endoglucanase enzyme showed optimal activity at pH 11, which was higher than that of *Bacillus* sp. KSMN 252 (Endo *et al*., 2001) and *Bacillus* sp. KSM-635 (pH 10) (Ito *et al*., 1989). Even enzyme stability at pH 12 is higher than those by having retaining activity over 64% (Fig. 4) as in *Bacillus* sp HSH-810 (Kim *et al*., 2005). Most alkaline cellulases from *Bacillus* sp. reveal an optimum activity from 40 to 60ºC (Hakamada *et al*., 1997; Ito, 1997; Christakopoulos *et al*., 1999). *Bacillus* sp. JB-99 was also able to grow over a wide range of pH (6-12) and it requires an alkaline pH of 8-10 for growth and enzyme secretion (Johnvesly & Naik, 2001). Endo *et al*. (2001) reported that a novel alkaline endogluconase from an alkaline *Bacillus* isolate, optimal pH for activity was as high as 10 and the optimal temperature 55ºC. The enzyme C14 also conforms to these results with its optimum pH 11 and temperature 50ºC (Fig. 2 & 3). The optimal temperature of C14 endoglucanase enzyme activity was also similar to the

Fig. 5. Thermal stability of *Bacillus* sp. C14 endoglucanase. The enzyme was pre-incubated at temperatures between 20 to 90 ^{o}C for 15(●), 30(■) and 60(▲) min at optimum pH

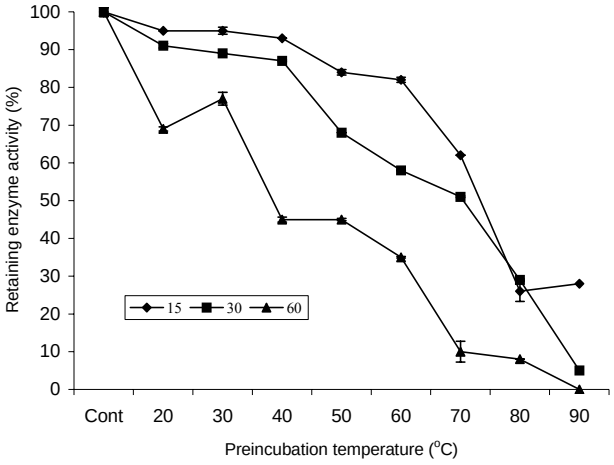
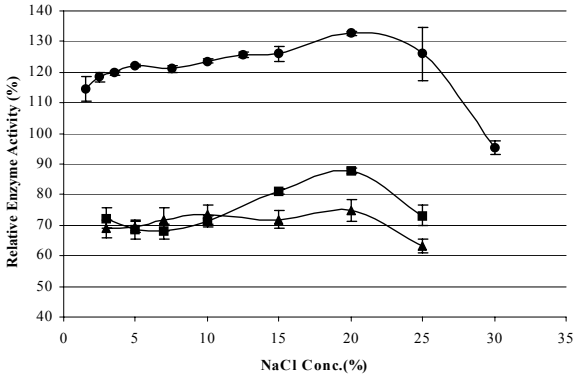


Fig. 6. Effect of salt concentration on the enzyme activity and stability of *Bacillus* sp. C14 endoglucanase Enzyme was preincubated for 1 (■)and 6 hours (▲)at desired salt concentration



result of Oyekola *et al*. (2007). The highest mean activity of *Bacillus* sp. C14 endoglucanase enzyme was observed as 88 and 96% between 20 and 60ºC and pH 8 to 12, respectively. Endo *et al*. (2001) have also reported that more than 80% of endoglucanase enzyme activity was present between pH 8 and 11.

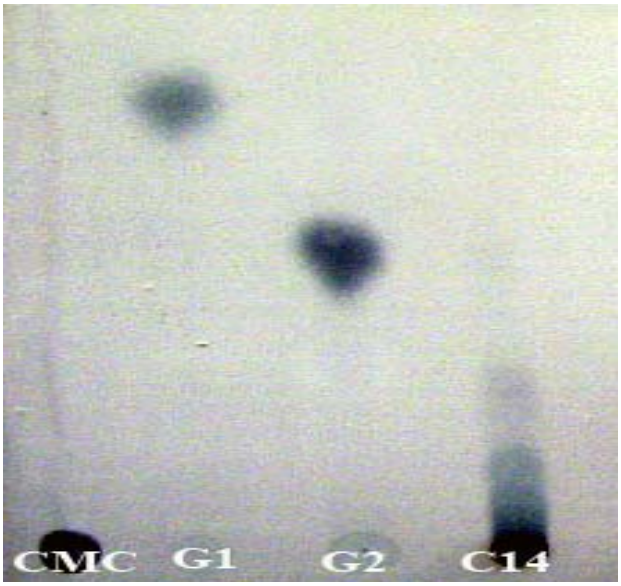
On the other hand, the C14 enzyme presents a thermal stability average 71 and 60% for 15 and 30 min, respectively between 20 to 90ºC (Fig. 5). Some reports (Oyekola *et al*., 2007) show that cell-free endogluconase enzyme lost original activity within 20 min. According to these findings the enzyme was with thermophile, thermostable, pH stable and highly alkaline properties, which indicated it to be a good additive for enzyme-based detergent applications.

The C14 endoglucanase enzyme was not completely

Table I. Effect of different metals ions, surfactants, chelating agents and inhibitor on endoglucanase from *Bacillus* sp. C14

Chemicals	Concentrations	Relative enzyme activity (%)
Control	none	100
EDTA	5 mM	93
$CaCl_2$	5 mM	132
Na_2SO_3	5 mM	102
$ZnCl_2$	5 mM	77
KCl	5 mM	40
β-Mercaptoethanol	1%	69
Triton X-100	1%	52
SDS	1%	81
PMSF	5 mM	43
Urea	8 m	42

Fig. 7. Thin layer chromatography of the end product of endoglucanase from *Bacillus* sp.C14. CMC: 5µL Untreated sample for negative control; G1: 5 µL of Glucose (4% w/v); G2: 5 µL of Maltose, (4% w/v); C14: 5µL of enzyme subsrate mixture. Samples migration was carried out from buttom to top of chromatography plate



inhibited by most of the effectors (Table I). However, the enzyme was resistant to chelating agent EDTA and SDS by preserving original enzyme activity around 93 and 81%, respectively. Slight inhibition of the enzyme was observed with EDTA (7%), therefore the enzyme is not classified as a metallo-enzyme. Other researchers have also reported similar finding (Ito *et al*., 1989; Sharma *et al*., 1990; Hakamada *et al*,. 1997; Mawadza *et al*., 2000). Our enzyme was inhibited at proportion 58% by 8 M urea (Table I). Alkaliphilic properties of the enzyme were also supported by the inhibitory effect of urea 6 and 8 M (Horikoshi, 1999; Burhan *et al*., 2003). The metal ions K^+ and Zn^{2+} inhibited the enzyme activity by a variable degree 60 and 23%, respectively. On the other hand, a stimulated activity was observed in the presence of Ca^{2+} (132%) and Na_2SO_3 (102%) (Table I). Ca^{2+} ions have earlier been reported to be required by cellulosome enzymes with the former enhancing the substrate binding affinity of the enzyme and stabilizing the conformation of the catalytic site (Mansfield *et al*., 1998). These results suggest that the metal ions such as Ca^{2+}, $Na^{+1}$and Zn^{2+} apparently protected the enzyme against thermal denaturation and played a key role to continue the active conformation of the enzyme at high temperatures (Donagy & Mckay, 1993). Many reports are available about different activation and the inhibition pattern of the cellulases. Most metal ions such as K^+, Na^+, Ca^{2+}, Zn^{2+} etc., did not actually influence the enzyme activity (Mawadza *et al*., 2000) but Singh *et al*. (2001 & 2004) reported an increased enzyme activity in the presence of Na^+. The inhibition of C14 by Zn^{2+} (27%) concur with other work as a result of inhibitory effects of heavy metals on enzymes. Since thiol groups are targets for the heavy metals, this suggests that these groups are present at the active site of the enzyme (Murashima *et al*., 2002). The obstructive activity of Zn^{2+} was also reported by Voget *et al*. (2006) and Huang and Monk (2004). The earlier reports suggested that the inhibiton of enzymes by Zn^{2+} is an indication of thermostability for an enzyme (Mamo & Gessesse, 1999).

The decreased activity with 5 mM KCl (60%) indicated that this endoglucanase is halophilic, since most halophilic cellulases are inactivated at less than 2 M NaCl or KCl (Madern *et al*., 2000). C14 endoglucanase enzyme was inhibited by 57% with 5 mM PMSF (Table I). Nearly complete loss of enzyme activity with 1-2 mM PMSF concentrations supported the fact that modification of a serine residue or thiol group at the active site (Kambourova *et al*., 2003). This finding substantiated that heavy metals inhibit our enzyme (C14) endoglucanases by interacting with potential thiol groups at the active site.

When *Bacillus* sp. C14 endoglucanase enzyme pre-incubated with SDS (1%) for 30 min, the activity was 81% of the original activity. Saxena *et al*. (2007) reported that highly thermostable and alkaline amylase enzyme stability was 86.36 % after 1 h incubation with SDS. Our results indicated that C14 enduglucanase enzyme was highly stable (81%) with 1% SDS for 30 min. This resistance, which is essential requirements, suggests that the enzyme may be used as an effective additive in detergents.

Although increased enzyme activity was evident in the presence of NaCl, our enzyme presented a maximal activity at 20% (3.4 M) NaCl concentration. Another α-amylase from a halophilic archeon has also showed a salt requirement for stability and activity, being stable from 2 to 4 M NaCl with maximal activity at 3 M NaCl (Pomares *et al*., 2003). As a result C14 has halophilic properties.

CONCLUSION

The enzyme C14 is a halophilic, highly alkaline and thermostable, pH stable, highly resistant to SDS and chelators. Most of thermophiles, thermostable and inhibitors

resistant enzymes have considerable potential in different industrial process and laundry application such as textile, starch and cellulose hydrolyzing. The ability of C14 endoglucanase to show optimum activity at 50ºC and pH 11, with a good stability at pH 6 to 12 for 24 h, high resistance to SDS and optimum activity with 20% NaCl (132%) suggested that the enzyme has a potential for use in textile industry, hydrolysing of cellulosic materials, beverage and detergent industry.

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