

Full Length Research Paper

Alkaline thermostable and halophilic endoglucanase from *Bacillus licheniformis* C108

Ashabil Aygan^{1*}, Lutfiye Karcioglu¹ and Burhan Arikan²

¹Kahramanmaraş Sutcu Imam University, Faculty of Sciences and Letter,
Department of Biology- K.Maras-Turkey.

²Cukurova University, Faculty of Sciences and Letter, Department of Biology, Molecular Biology and Biotechnology
Laboratory- Adana- Turkey.

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An endoglucanase was purified from halophilic alkaline *Bacillus licheniformis* isolated from soils of Lake Van in Turkey. The optimal pH and temperature of the endoglucanase produced by *B. licheniformis* C108 were 10.0 and 30°C, respectively. The enzyme was highly stable up to 100°C at pH 10.0 and the enzyme retained its complete activity for 6 h in 7 to 10% of NaCl. The activity of the enzyme was significantly inhibited by sodium dodecyl sulfate (SDS), Triton X-100, zinc chloride (ZnCl₂), phenylmethanesulfonyl fluoride (PMSF) and Urea. The partially purified enzyme revealed that, products of carboxymethylcellulosic hydrolysis were glucose, cellobiose and other longer cellooligosaccharides. Thermostability, alkalinity, halostability and high hydrolytic capability make this enzyme a potential candidate for environmental bioremediation and bioethanol production processes from cellulosic biomasses as well as waste treatment processes.

Key words: Cellulose, *Bacillus licheniformis*, CMCase, endoglucanase, halostable.

INTRODUCTION

Cellulose is the most available sources of alkaline on earth and it is produced continuously as a result of agricultural, industrial and forestry product. Its abundance and renewability, makes it a great candidate for energy sources and feedstock (Fan et al., 1982). The increasing concern about fossil fuel shortage makes researchers work on to search new possible substances that can be used for the production of bio-ethanol from cellulosic materials. Cellulosic material is available in nature as stalks, stems, husks as well as waste paper produced industrially (Immanuel et al., 2007). This unbranched biopolymer is usually found in company with hemicellulose, lignin and some other polysaccharides. Therefore, bio-conversion of this type of materials needs different novel enzymes to produce soluble sugar (Alam et al., 2004).

The cellulase enzyme complex consists of different enzymes acting synergistically on cellulose for complete degradation (Bhat and Bhat, 1997). Endoglucanases

(1,4-(D-glucan-4-glucanohydrolase; EC 3.2.1.4) attack cellulose chains in a random manner. The potential applications of cellulases are in food, textile, detergent and the pulp and paper industry (Ikeda et al., 2006; Cavaco-Paulo, 1998; Tolan and Foody, 1999). Another exceptional use area of the cellulases are the bioconversion of cellulosic material for bioethanol production while enormous amount of agricultural and industrial cellulosic wastes have been produced annually (Cherry and Fidantsef, 2003; Kim et al., 2003). Therefore, this enzyme plays a key role for achieving huge benefits for effective resource utilization (Wen et al., 2005). Surely, microbial cellulases are economic, important and environmentally safe (Percival-Zhang et al., 2006). A good deal of fungi and bacteria were isolated, identified as a cellulose utilizing organism and were investigated (Juhász et al., 2005; Jorgensen and Olsson, 2006; Bischoff et al., 2007; Kang et al., 2007; Kaur et al., 2007; Lee et al., 2008; Comlekcioglu et al., 2008). Alkaliphilic bacteria can grow in alkaline condition and produce different enzymes that are active and suitable for harsh condition compared to their counterparts. Among the bacteria, the genus *Bacillus* is an attractive industrial organism due to growth

*Corresponding author. E-mail: ashabil@ksu.edu.tr. Tel: +90-344-2191299. Fax: +90-344-2191042.

rate, extracellular secretion and biosafety capacity (Schallmeyer et al., 2004).

Although, the alkaline cellulases have been studied widely, limited reports are available about halotolerant alkaline cellulases. In this study, alkaliphilic halotolerant microorganism, *Bacillus licheniformis* C108, from soil in Turkey, producing endoglucanase was isolated and some enzymic characterization of alkaline endoglucanase was determined from it.

MATERIALS AND METHODS

Isolation and selection of microorganism

B. licheniformis C108 was isolated from soils of Lake Van, situated in Eastern Anatolia in Turkey. Selection of gram positive and sporulating bacteria, *Bacillus* sp., was carried out by pasteurizing the samples at 80°C for 10 min (Ozcan et al., 2010). Bacterial isolates were screened for carboxymethylcellulase (CMCase) production on CMC-agar plates (Singh et al., 2001). CMCase positive isolates were selected by flooding the agar plates with Congo red solution (0.1%). CMCase positive strains were stored at +4°C on agar slope until enzyme production processes.

Random amplified polymorphic DNA (RAPD) sequencing and computer analysis

Identification of the microorganism was accomplished by studying 16S rDNA sequence analysis beside its morphological and biochemical properties such as colony morphology, gram staining, spore possession, motility and catalase production and acid production from glucose, xylose and manitol. The 16 s rDNA gene was amplified from the isolated *Bacillus* sp. genomic DNA by using polymerase chain reaction amplification (PCR) method and PCR products were recovered from agarose gels using Nucleospin® Extract Kit (Macherey-Nagel GmbH & Co.). The PCR product was sequenced on both strands by a commercial company (Refgen, Ankara, Turkey) using automatic sequencer. The analyses of the nucleotide sequence were performed by Clone Manager 5 and homology search was carried out by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequence of the isolate C108 has been registered in the GenBank under accession number EU878306. The sequences belonging to the same or closely related species available through NCIB gene bank were aligned.

Enzyme production

B. licheniformis C108 was cultured in a medium that contained carboxymethylcellulose (10 g/l), pepton (5 g/l), yeast extract (5g/l), KH₂PO₄ (1 g/l) and NaCl (10%, w/v). The initial pH of the medium was adjusted to 10.0 after autoclaving with 10% Na₂CO₃ (Singh et al., 2001). Cultures were grown up until the beginning of stationary phase at 37°C with shaking at 200 rpm. After removing the cells by centrifugation (Hettich Universal 30 RF) (11200 g, 20 min) at 4°C, the supernatant was used for further work.

Partial purification of endoglucanase

Supernatant was subjected to previously chilled ethanol (96%) at 4°C with continuous stirring and the mixture was left at -30°C overnight. The enzyme was recovered by resuspending the precipitate in 100 mM sodium phosphate buffer at pH 7.6 after centrifugation at

11200 g for 20 min at 4°C.

Enzyme assay

Carboxymethylcellulase (CMCase) activity was assayed by adding equal amount of enzyme solution to CMC substrate solution (1%, w/v) in 100 mM Glycine-NaOH buffer, pH 10.0 and incubation at 30°C for 30 min. The reaction was stopped by the addition of 3,5-dinitrosalicylic acid reagent and finally, absorbance was measured in a Cecil 5500 spectrophotometer (A₅₅₀) (Bernfeld, 1955). One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol of reducing sugars per minute under the standard assay conditions.

Effect of pH, temperature and NaCl concentration on enzyme activity and stability

The effects of pH and temperature on enzyme activity were assayed at different temperatures that ranged from 4 to 100°C and at pH values from 6.0 to 12.5 for 30 min. The following buffers were used in the reactions: 100 mM Na-Phosphate buffer (pH 6.0 to 8.0), 100 mM Glycine-NaOH buffer (pH 8.5 to 10.5) and 100 mM Borax-NaOH buffer (pH 11.0 to 12.5). To determine the temperature stability, the enzyme was pre-incubated at various temperatures between 20 and 100°C for 15 min at optimum pH and the remaining activity was determined under standard enzyme assay condition. For the determination of pH stability, the enzyme was pre-incubated at pH that ranged from 6.0 to 11.0 at 30°C for 60 min and 24 h. The effect of NaCl concentration on enzyme activity and stability was also tested. It was determined under standard enzyme assay condition by including NaCl into the substrate. The stability of the enzyme against NaCl was assayed by pre-incubating the enzyme in 100 mM Glycine-NaOH buffer pH 10.0 including different NaCl concentration at optimum temperature (30°C) for 60 min and then, the remaining activity at standard assay condition was measured. The activity of the enzyme stored at +4°C was used as the control groups. The experiments were repeated three times and mean values were taken.

Effect of additives on enzyme activity

The effect of metal ions and chemicals, including chelators and inhibitors on CMCase activity, was tested by preincubating the enzyme in the presence of substances of 5, 8 mM or 1% concentration at 30°C (Egas et al., 1998; Lo et al., 2001). All metals are used in chloride form. The activity in the absence of the additives was taken as 100%.

Chromatography of the end products of CMC hydrolysis

CMC (1%) was digested with partially purified CMCase C108 at 30°C for 60 min incubation. The end products were analysed by silica gel 60 (GF₂₅₄) (Merck) thin-layer chromatography. After developing the products with a solvent system of chloroform-acetic acid-distilled water (6:7:1 v/v/v), the spots were visualized by spraying of the solution of Aniline (1%, v/v), Diphenilamine (1%, w/v) and Orthophosphoric acid (10%, v/v) in acetone and baking in an oven at 160°C for 30 min (Singh et al., 2004).

RESULTS

Isolation and identification of microorganism

A total of 226 bacterial isolates were screened for

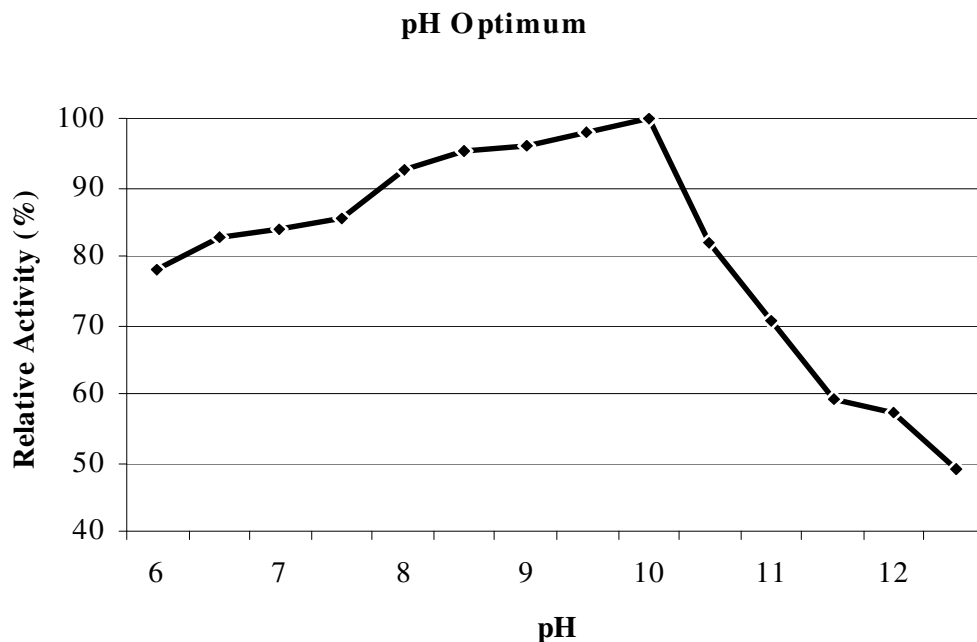


Figure 1. Effect of pH on the activity of *B. licheniformis* C108 endoglucanase. The buffers used were Na-Phosphate buffer (pH 6.0 to 8.0), Glycine-NaOH buffer (pH 8.5 to 10.5), and Borax-NaOH buffer (pH 11.0 to 12.0).

CMCase production on CMC-agar plates (Singh et al., 2001). Among the isolated strains, a total of 33 CMCase positive strains were selected by exposing the agar plates to Congo red solution (0.1%). The strains were aerobic, gram positive, rod, motile and spore forming. The highest CMCase producer of the isolate C108 was chosen for further investigation and the growth observed was between pH 7.0 and 12.0 in the presence of NaCl (10%), and up to 45°C. Enzyme synthesis occurred between 25 and 45°C and optimum pH 9.0. The optimum temperature for enzyme production and growth was 37°C and the NaCl concentration was 3 and 5%. Growth was not observed in the presence of 15 and 20% NaCl, which indicates that, the strain *B. licheniformis* C108 could possibly be presumed as moderately halophilic according to Ventosa et al. (1998).

The nucleotide sequence for the 16s rDNA gene of the isolate C108 was also determined. The sequence had 903 bp lengths and the guanine-cytosine (GC) content of the sequence was found as 54.8%. The 16S rDNA sequence analysis of the strain C108 revealed that, the organism shared 97% of its identity with different *B. licheniformis* 16S rDNA sequences available in Genbank databases (Accession number: EU878306).

Effect of pH, temperature and NaCl concentration on enzyme activity and stability

Although the optimum temperature was observed at 30°C, the enzyme had a wide range activity from 20 to

80°C with an average of 85%. Between the temperatures +4 to 90°C, the highest mean enzyme activity was 80%. The lowest activity rate (53%) was obtained at 100°C, which is highly an acceptable value for most industrial application. The enzyme also showed a significant relative activity over 90% between pH 6.5 and 10.5 with an optimum of 10, but more than 95% activity was obtained in between pH 8.5 and 10.0. Although the highest activity was at pH 10.0, there was a sharp decrease at pH 10.5 and so on (Figure 1).

For thermal stability estimation, the CMCase was preincubated at temperatures between 20 and 100°C for 15 min at optimum pH and the remaining activity was determined (Figure 2). The enzyme was highly active from 20 to 70°C for 15 min with a remaining activity over 75%. The enzyme preserved 100% of the original activity at 20°C and gradual loss of activity was obtained. Despite the gradual reduction in activity, the enzyme preserved 63% of the original activity at 100°C. The pH stability of enzyme was determined by pre-incubation at 30°C for 60 min and 24 h. It seemed that, the highest stability of the enzyme was at pH 7.0 by preserving original activity around 70% for 60 min (Figure 3). Loss of activity was obtained gradually with an increased pH values. At pH 11.0, only the 30% of original activity remained. At the end of the 24 h, the remaining activity was steadily reduced from pH 6.0 (65%) through pH 10.0 (20%).

Endoglucanase activity was increased in the presence of NaCl from 3.5 to 15% and the maximal activity was obtained in the presence of 10% NaCl (106.4%) (Figure 4a). To test the effect of salt concentration for endo-

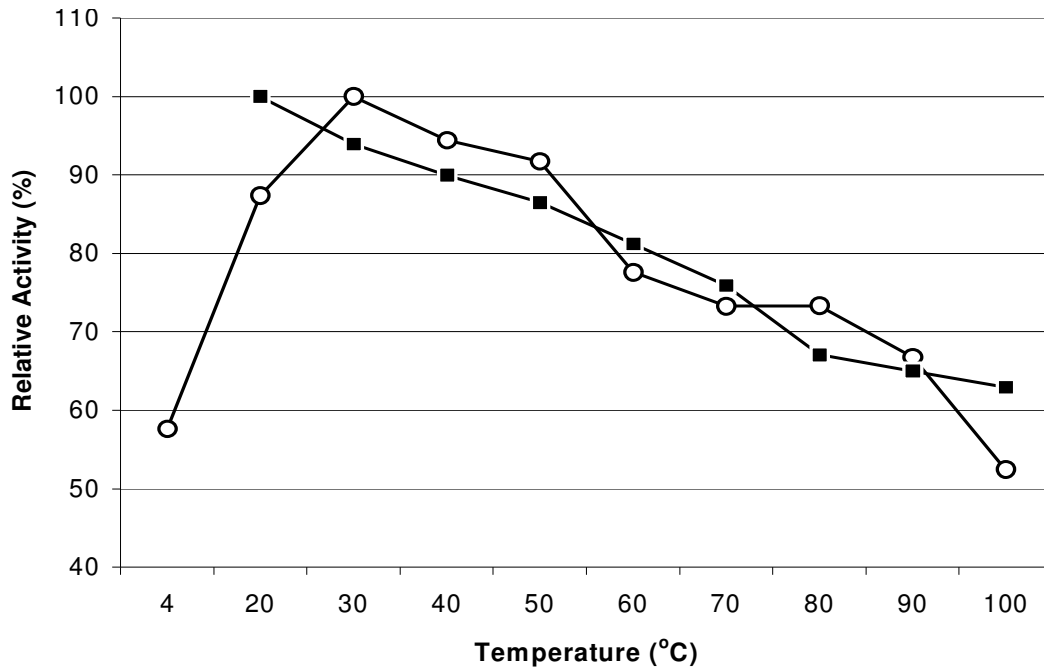


Figure 2. Effect of temperature on the activity of *B. licheniformis* C108 endoglucanase. The reaction mixture contained 0.5 ml substrate (CMC (1%) in Glycine-NaOH buffer, pH 10.0) and 0.5 ml of enzyme for optimum temperature (○). The mixture was incubated for 30 min at 4 to 100°C under standard enzyme assay condition. For thermal stability (■) of *B. licheniformis* C108 endoglucanase, the enzyme was pre-incubated at temperatures for 15 min at optimum pH (10.0) and the remaining activity was determined under standard enzyme assay condition.

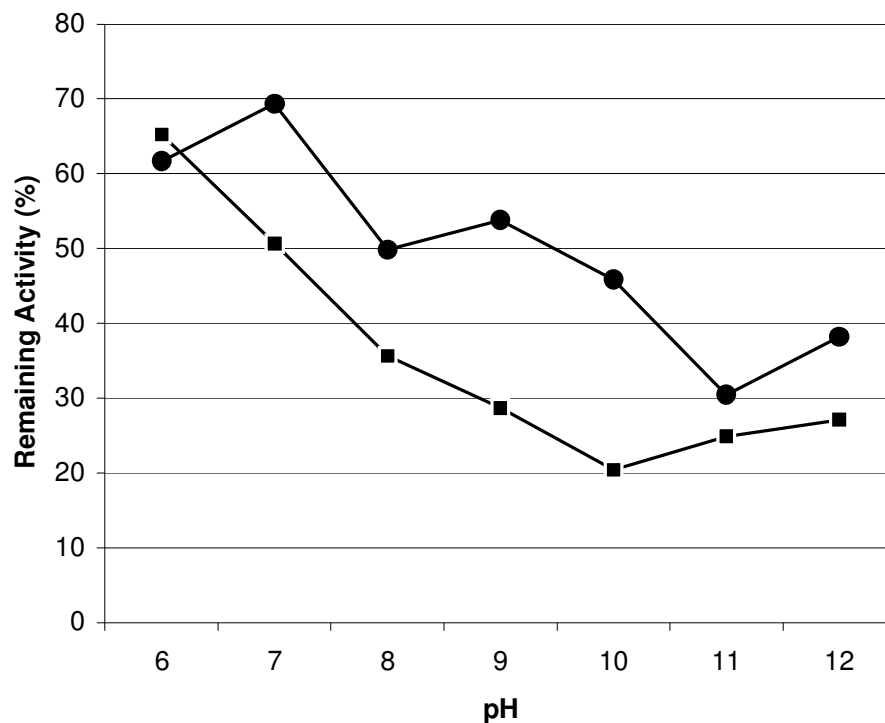


Figure 3. Effect of pH on the stability of *B. licheniformis* C108 endoglucanase. For determination of pH stability of CMCase, the enzyme was pre-incubated in buffers at 30°C for 60 min (●) and 24 h (■). The buffers used were sodium-phosphate buffer (pH 6.0 to 8.0), Glycine-NaOH buffer (pH 9.0 to 10.0), and Borax-NaOH buffer (pH 11.0).

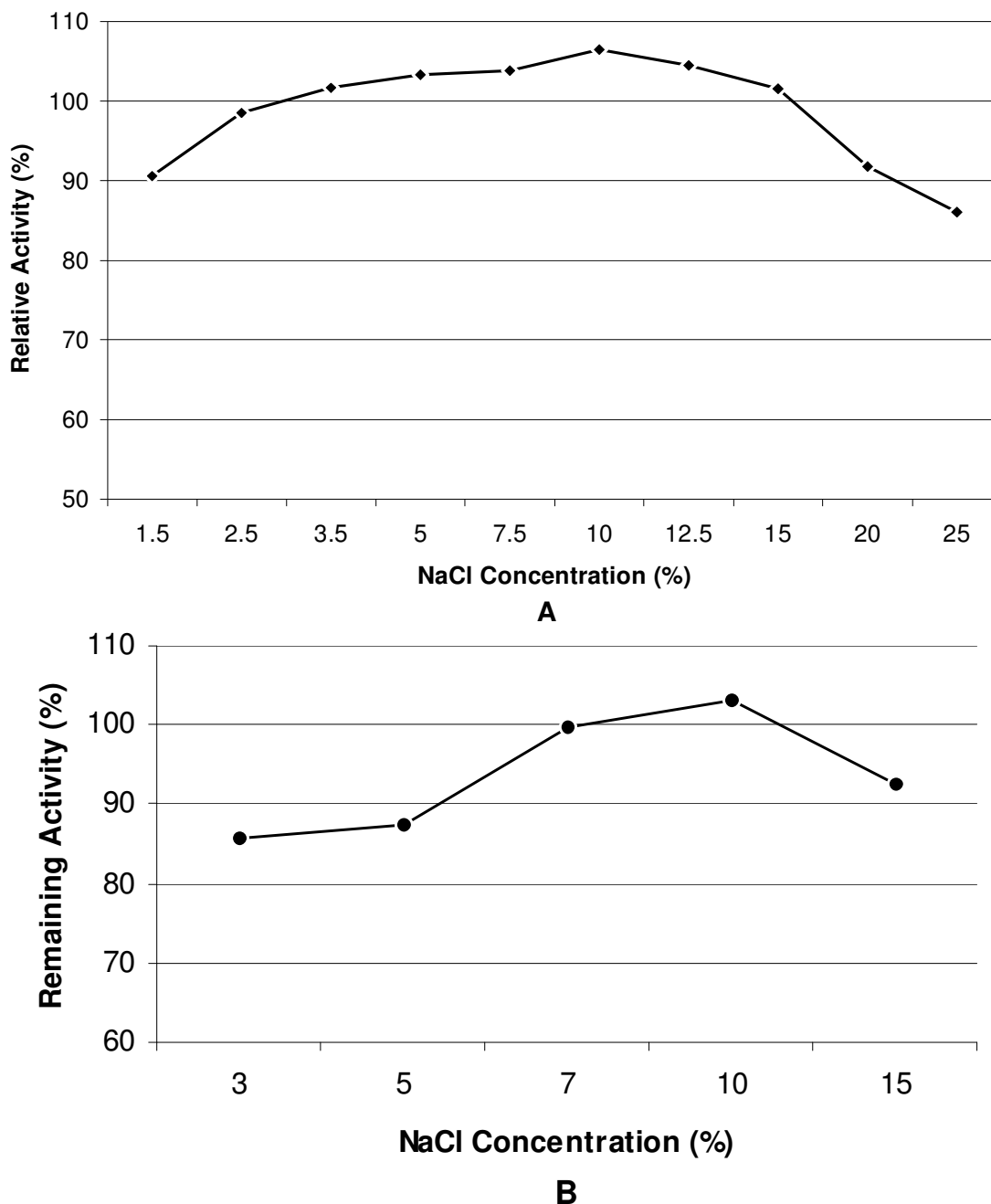


Figure 4. Effect of NaCl on activity of *B. licheniformis* C108 endoglucanase (a) and salt concentration on the stability of *B. licheniformis* C108 endoglucanase (b). Enzyme was pre-incubated for 60 min in different NaCl concentration and the remaining activity was determined under standard enzyme condition.

glucanase stability, different NaCl concentrations were used (Figure 4 b). Cellulase stability was tested by pre-incubating the enzyme at 30°C, pH 10.0 for 60 min at determined salt concentration. The enzyme retained its activity over 80% in 3 and 5% NaCl concentration. The enzyme was not affected by 7% NaCl whereas little activatory effect was obtained with 10% NaCl. The enzyme retained its activity around 9% with 15% of NaCl which was higher than that of 3 and 5% NaCl.

Effect of additives on enzyme activity

The enzyme was incubated at 30°C for 15 and 60 min at different concentration of the metal ions and various chemicals prior to standard enzyme assay condition. The activities measured were expressed as remaining activity as shown in Table 1. The activity of the enzyme was reduced to 62% by EDTA (5 mM) within 15 min. Enzymatic activity was highly reduced (23%) when incubation lasted

Table 1. Effect of additives on the activity of CMCase produced by *B. licheniformis* C108.

Effector	Concentration	Relative enzyme activity (%)	
		15 min	60 min
EDTA	5 mM	53.42	22.89
SDS	1%	20.53	11.58
Triton X-100	1%	28.95	13.95
CaCl ₂	5 mM	42.11	56.58
ZnCl ₂	5 mM	20.53	20.26
KCl	5 mM	17.63	32.11
Na ₂ SO ₃	5 mM	47.37	39.47
PMSF	5 mM	12.89	7.89
2-Merk	1%	76.32	0.00
Urea	8 M	19.74	3.95

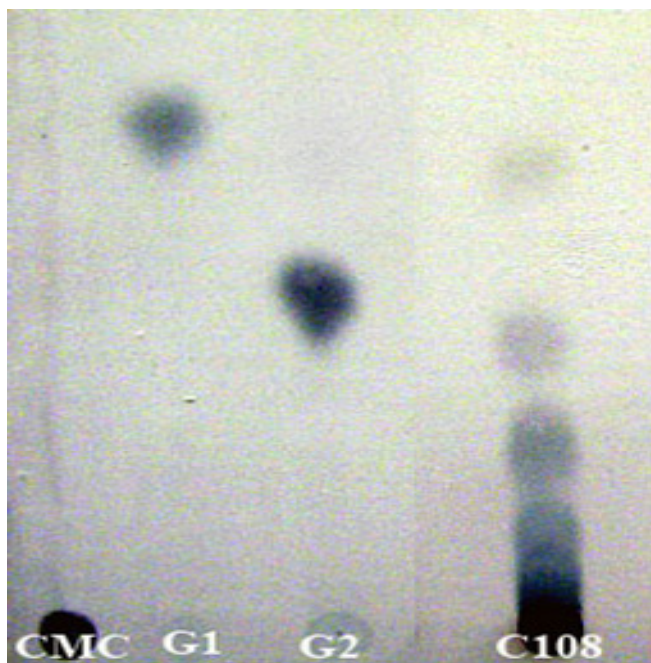


Figure 5. Thin layer chromatography of the end product of endoglucanase from *B. licheniformis* C108. CMC; untreated sample for negative control; G1, glucose; G2, maltose; C108, enzyme substrate mixture.

for 60 min. The enzyme was highly affected by SDS (1%) and Triton X-100, ZnCl₂, PMSF, β- mercaptoethanol and urea. On the other hand, an increasing retained activity by time was obtained with CaCl₂ and KCl.

Analysis of the end product

After 60 min incubation of enzyme and substrate mixture at 30°C, glucose was formed from CMC to some extent. But majority of the hydrolyzed products were cellobiose and the longer oligosaccharides (Figure 5).

DISCUSSION

Several bacterial strains were isolated that produced alkaline cellulases. One of these strains *Bacillus* sp. 1139 was reported by Horikoshi (1999) as, producing a cellulase with an optimum activity at pH 9.0. The enzyme was stable between the pH ranges of 6.0 and 11.0 at 4°C overnight (Horikoshi, 1999). *B. licheniformis* C108 showed a good growth between pH 7.0 and 12.0 in the presence of various NaCl concentration that ranged from 3 to 15%. Therefore, *B. licheniformis* C108 strain could be described as moderately halophilic.

B. licheniformis C108 endoglucanase enzyme was optimally active at pH 10.0 which is similar to that of *Bacillus* sp. KSMN 252 (Endo et al., 2001), *Bacillus* sp. SM-635 (pH 10.0) (Ito et al., 1989) and *Bacillus* sp. HSH-810 (Kim et al., 2005). Mainly, the reported alkaline cellulases from *Bacillus* sp. present an optimum activity from 40 to 70°C (Kim et al., 2005; Hakamada et al., 1997; Ito, 1997; Christakopoulos et al., 1999; Mawadza et al., 2000). Within 30 min, the optimum temperature of the C108 was 30°C, which is one of the lowest temperatures reported to date, and it is lower than the cellulases from fungi (Murashima et al., 2002; Saha, 2004). The highest mean activity of *B. licheniformis* C108 endoglucanase enzyme was observed as 90% both between 20 and 60°C and pH 6.5 to 10.5. Endo et al. (2001) have also indicated that, over 80% of endoglucanase enzyme activity was observed between pH 8.0 and 11.0. The temperature study projected that, the C108 enzyme was highly stable even at 100°C with 62% of the remaining activity presenting better stability than that of Hakamada et al. (1997, 2002), Singh et al. (2001) and Endo et al. (2001) findings. The remaining activity was almost 100% with 7 to 15% NaCl. Therefore, C108 endoglucanase might be called halophilic because the halophilic enzymes are usually inactive when the NaCl or KCl concentration is less than 2 M (Madern et al., 2000). Another α-amylase from a halophilic archeon has also displayed a salt demand for its stability and activity from 2 to 4 M

NaCl with an optimal at 3 M NaCl (Pomares et al., 2003). This halophilic stability and adaptation of C108 enzyme suggested that, more acidic surface and less hydrophobic core is probable (Wejse et al., 2003). C108 endoglucanase could be a good choice for some biotechnological application requiring high halostability and activity.

The enzyme activity was inhibited completely by β -mercaptoethanol and urea within 60 min and strongly inhibited by SDS, Triton X-100, $ZnCl_2$ and PMSF. EDTA and $CaCl_2$, results pointed out that the enzyme is a metallo enzyme and needs some metal ions for its activity (Table 1) (Kim et al., 2001). As a result, C108 is highly alkaline, thermostable and halostable endoglucanase with 30°C optimum temperature. Therefore, the enzyme has the potentials to be used in ethanol production processes from cellulosic biomass or waste treatment (Karam and Nicell, 1997).

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REFERENCES

- Alam MZ, Manchur MA, Anwar MN (2004). Isolation, purification, characterization of cellulotic enzymes produced by the isolate *Streptomyces omiyaensis*. Pak. J. Biol. Sci. 10: 1647-1653.
- Bernfeld P (1955). Amylases α - and β -methods. Enzymology, 1: 149-158.
- Bhat MK, Bhat S (1997). Cellulose degrading enzymes and their potential industrial applications. Biotechnol. Adv. 15: 583-620.
- Bischoff KM, Liu S, Hugfes R (2007). Cloning and characterization of a recombinant family 5 endoglucanase from *Bacillus licheniformis* strain B-41361. Process Biochem. 42: 1150-1154.
- Cavaco-Paulo A (1998). Mechanism of cellulase action in textile processes. Carbohydr. Polym. 37: 273-277.
- Cherry JR, Fidantsef AI (2003). Directed evolution of industrial enzymes: An update. Curr. Opin. Biotechnol. 14: 438-443.
- Christakopoulos P, Hatzinikolau DG, Fountoukidis G, Kekos D, Claeyens M, Macris BJ (1999). Purification and mode of action of an alkali-resistant endo-1, 4- β -glucanase from *Bacillus pumilus*. Arch. Biochem. Biophys. 364: 61-66.
- Comlekcioglu U, Akyol I, Ozkose E, Kar B, Ekinci MS (2008). Carboxymethylcellulase production by the anaerobic rumen fungus *Neocallimastix* sp. GMLF7. Ann. Microbiol. 58(1): 115-119.
- Egas MCV, Dacosta MS, Cowan DA, Pires EMV (1998). Extracellular α -amylase from *Thermus filiformis* Ork A2: purification and biochemical characterization. Extremophiles, 2: 23-32.
- Endo K, Hakamada Y, Takizawa S, Kubota H (2001). A novel alkaline endoglucanase from an alkaliphilic *Bacillus* isolate: enzymatic properties and nucleotide and deduced amino acid sequences. Appl. Microbiol. Biotechnol. 57: 109-116.
- Fan LT, Lee Y, Gharpuray MM (1982). The nature of lignocellulosics and their pretreatment for enzymatic hydrolysis. Adv. Biochem. Symp. 7: 45-64.
- Hakamada Y, Koike K, Yoshimatsu T, Mori H, Kobayashi T, Ito S (1997). Thermostable alkaline cellulase from an alkaliphilic isolate, *Bacillus* sp. KSM-S237. Extremophiles, 1: 151-156.
- Hakamada Y, Endo K, Takizawa S, Kobayashi T, Shirai T, Yamane T, Ito S (2002). Enzymatic properties, crystallization and deduced amino acid sequence of an alkaline endoglucanase from *Bacillus circulans*. Biochim. Biophys. Acta. 1570: 174-180.
- Horikoshi K (1999). Alkaliphiles: Some Applications of Their Products for Biotechnology. Microbiol. Mol. Biol. Rev. 63: 735-750.
- Ikeda Y, Park EY, Okida N (2006). Bioconversion of waste office paper to gluconic acid in a turbine blade reactor by the filamentous fungus *Aspergillus niger*. Bioresour. Technol. 97: 1030-1035.
- Immanuel G, Bhagavath CMA, Raj PL, Esakiraj P, Palavesam A (2007). Production and partial purification of cellulase by *Aspergillus niger* and *A. fumigatus* fermented in coir waste and sawdust. Internet J. Microbiol. 3(1).
- Ito S, Shikata S, Ozaki K, Kawai S, Okomoto K, Inoue S, Takai A (1989). Alkaline cellulase for laundry detergent: production by *Bacillus* sp. KSM-635 and enzymatic properties. Agric. Biol. Chem. 53: 1275-1281.
- Ito S (1997). Alkaline cellulase from alkaliphilic *Bacillus*: enzymatic properties, genetics and application to detergents. Extremophiles, 1: 61-66.
- Jorgensen H, Olsson L (2006). Production of cellulases by *Penicillium brasilianum* IBT 20888- Effect of Substrate on hydrolytic performance. Enzyme Microb. Technol. 38: 381-190.
- Juhász T, Szengyel Z, Reczey K, Sika-Aho M, Viikari L (2005). Characterization of cellulases produced by *Trichoderma reesei* on various carbon sources. Process Biochem. 40: 3519-3525.
- Kang HJ, Uegaki K, Fukada H, Ishikawa K (2007). Improvement of the enzymatic activity of the hyperthermophilic cellulase from *Pyrococcus horikoshi*. Extremophiles, 11: 251-256.
- Karam J, Nicell JA (1997). Potential Applications of Enzymes in Waste Treatment. J. Chem. Tech. Biotechnol. 69: 141-153.
- Kaur J, Chadha BS, Kumar BA, Saini HS (2007). Purification and characterization of two endoglucanases from *Melanocarpus* sp. MTCC 3922. Bioresour. Technol. 98: 74-81.
- Kim JY, Hur SH, Hong JH (2005). Purification and characterization of an alkaline cellulase from a newly isolated alkaliphilic *Bacillus* sp. HSH-810. Biotechnol. Lett. 27: 313-316.
- Kim KC, Yoo SS, Oh Young A, Seong-Jun K (2003). Isolation and characterization of *Trichoderma harzianum* FJ1 producing cellulase and xylanase. J. Microbiol. Biotechnol. 13: 1-8.
- Kim SS, Kim YJ, Rhee I-K (2001). Purification and characterization of a novel extracellular protease from *Bacillus cereus* KCTC 3674. Arch. Microbiol. 175: 458-461.
- Lee YJ, Kim BK, Lee BH, Jo LI, Lee NK, Chung CH, Lee YC, Lee JW (2008). Purification and characterization of cellulase produced by *Bacillus amyloliquefaciens* DL-3 utilizing rice hull. Bioresour. Technol. 99: 378-386.
- Lo HF, Lin LL, Chen HL, Hsu HH, Chang CT (2001). Enzymatic properties of a SDS-resistant *Bacillus* sp. TS-23 α -amylase produced by recombinant *E. coli*. Process Biochem. 36: 743-750.
- Madern D, Ebel C, Zaccari G (2000). Halophilic adaptation of enzymes. Extremophiles, 4: 91-98.
- Mawadza C, Hatti-Kaul R, Zvauya R, Mattiason B (2000). Purification and characterization of cellulases produced by *Bacillus* Strain. J. Biotechnol. 83: 177-187.
- Murashima K, Nishimura T, Nakamura Y, Kuga J, Moriya T, Simuda N (2002). Purification and characterization of new endo-1, 4- β -D-glucanases from *Rhizopus oryzae*. Enzyme. Microb. Biotechnol. 30: 319-326.
- Ozcan BD, Baylan M, Ozcan N, Tekdal D (2010). Characterization of Thermostable α -amylase from Thermophilic and Alkaliphilic *Bacillus* sp. isolate DM-15. Res. J. Biol. Sci. 1: 118-124.
- Percival-Zhang YH, Himmel ME, Mielenz JR (2006). Outlook for cellulase improvement: Screening and selection strategies. Biotechnol. Adv. 24: 452-481.
- Pomares FP, Bautista V, Ferrer J, Pire C, Marhuenda-Egae FC, Bonete MJ (2003). α -Amylase activity from the halophilic archeon *Haloferax mediterranei*. Extremophiles, 7: 299-306.
- Saha BC (2004). Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*. Process Biochem. 39: 1871-1876.
- Schallmey M, Singh A, Ward OP (2004). Developments in the use of *Bacillus* species for industrial production. Can. J. Microbiol. 50: 1-17.
- Singh J, Batra N, Sobti RC (2001). A highly thermostable, alkaline CMCase produced by a newly isolated *Bacillus* sp.VG1. World J. Microbiol. Biotechnol. 17: 761-765.
- Singh J, Batra N, Sobti RC (2004). Purification and characterization of

- alkaline cellulase produced by a novel isolate, *Bacillus sphaericus* JS1. J. Ind. Microbiol. Biotechnol. 31: 51-56.
- Tolan JS, Foody B (1999). Cellulase from submerged fermentation. In: Tsao GT (ed.). Recent Progress in Bioconversion of Lignocellulosics. Springer-Verlag, Berlin. Adv. Biochem. Eng. Biotechnol. pp. 41-67.
- Ventosa A, Nieto JJ, Oren A (1998). Biology of moderately halophilic aerobic bacteria. Microbiol. Mol. Biol. Rev. 62: 504-544.
- Wejse PL, Ingvorsen K, Mortensen KK (2003). Xylanase production by a novel halophilic bacterium increased 20-fold by response surface methodology. Enzyme Microb. Technol. 32: 721- 27.
- Wen Z, Liao W, Chen S (2005). Production of cellulase by *Trichoderma reesei* from dairy manure. Bioresour. Technol. 96: 491-499.