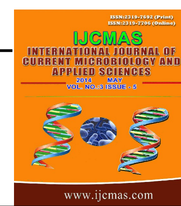


International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 5 (2014) pp. 16-25**
http://www.ijcmas.com

Original Research Article

Study on Cold-Active and Acidophilic Cellulase (CMCase) from a novel psychrotrophic isolat *Bacillus sp*. K-11

Yasemin CAF*, Ebrahim VALIPOUR and Burhan ARIKAN

Biotechnology Department, Institute of Basic and Applied Sciences,
Cukuruva University, Turkey
**Corresponding author*

A B S T R A C T

K-11 cellulase was optimally produced at 15°C and pH 5.0 in a medium containing CMC. Molecular mass Analyses of the partially purified enzyme was carried out by zymogram which revealed three bands as 66, 70 and 81 KDa. The optimum pH of the partially purified enzyme was determined as 4.5 and its optimum temperature as 20°C. The enzyme conserved 100% of its original activity in 10 °C to 40 °C. Also, it was stable at pH from 4.0 to 9.0 with about 92% activity. It retained its activity in the presence of 5mM EDTA, $ZnCl_2$, $MgCl_2$, 3mM PMSF, 0.1 % Tween-20, 0.1 % Tween-80, 1 % SDS, 1 % Triton X-100, 0.1 % H_2O_2 8M urea about 73 %, 105%, 105%, 73%, 79%, 79%, 47%, 79%, 70%, 38%, respectively. However, its activity was increased in the presence of 5mM $CaCl_2$ and β- mercaptoethanol (1%) 64% and 55 %, respectively. The enzyme was stable in different NaCl concentration ranging from 3 to 30 % with more than 76 % activity. TLC analyses of the CMC hydrolysis by the enzyme revealed the presence of maltose and etc.

Keywords

Bacillus sp, Cold–active cellulase, textile, energy saving

Introduction

Cellulose which is the most polymeric and renewable component of plant biomass, is hydrolyzed into soluble sugars by cellulases. Cellulases are accountable to hydrolysis of the β-1,4 glucosidic bonds in cellulose. This cellulolytic activity occurs by the synergistic effect 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) (Das et al., 2010; Liu et al., 2008; Aygan and Arikan, 2008; Cavaco-Paulo, 2001; Bayer et al., 1998).

Cellulases are used in different industrial applications, such as in textile industry for bio-polishing for fabrics and producing stonewashed look of denims, in animal feed industry for improving the nutritional quality and digestibility, in food industry for processing of fruit juices and in baking, in detergent industry for

improving fabric softness and brightness and removing the soil from cotton fibers (Aygan and Arikan, 2008; Cavaco-Paulo, 2001; Hoshino et al., 2000).

Cold-active enzymes compared to mesophilic enzyme are not more attractive only because of their high activity in low temperature which is more preferred in application, so but because, they represent the lower limit of natural protein stability and is a useful tool for studies in the field of protein folding (Wen et al., 2006). The uses of acid cellulases, which are active only at lower pH, are affected by the pH increase that occurs during denim washing due to alkalinity released from the clothes. In the textile industry, there is a need for novel cellulases that are active at broad pH values.

Cold–active enzymes are crucial in saving of energy in textile, detergent, fuel and etc. industrial process (Miettinen-Oinonen et al., 2004). So this study aimed to isolation of acidophilic and cold-active cellulase enzyme from *Bacillus* sp. from soil samples in different regions of Turkey, partial purification and characterization of the enzyme.

Materials and Methods

Isolation of cellulolytic bacteria and culture conditions

Several soil samples were collected from Adana, Turkey. Each sample was incubated at 80°C for 10 min for selection gram-positive spore forming bacteria, *Bacillus* sp. The isolates were screened for cellulase enzyme production on CMC (carboxy methyl cellulose) agar plates containing (gL^{-1}) CMC 10.0; yeast extract 5.0; (NH4)$2SO_4$ 0.5; KH_2PO_4 2.6; Na_2HPO_4 4.4; Agar 15. Its pH was adjusted to 5.0 with 1M HCl. Cellulase positive isolates were selected by staining with Congo Red solution (0.1%). Then protease activity of the selected strains was analyzed and strain with both high cellulase activity and low protease activity was selected for enzyme production and further studies (Voget et al., 2006; Aygan and Arikan, 2008).

Optimization of medium composition

Culture conditions such as different temperature, pH, carbon and nitrogen sources, salt concentration, and substrate (CMC) concentrations were optimized for enzyme production. For this purpose production medium supplemented with different carbon sources (fructose, glucose, maltose and sucrose) and 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) conditions were analyzed for enzyme production by the selected strain *Bacillus* sp.K-11 (Shanmughapriya et al., 2010).

Enzyme Production

Bacillus sp.K-11 was cultivated aerobically in CMC-LB broth containing (g L^{-1}) Tryptone 10, Yeast extract 5, NaCl 5, CMC 4; and pH 5.0, at 15°C for 96 hours in a shaker incubator at 190 rpm. The culture was centrifuged at 8,000 rpm for 20 min using a refrigerated centrifuge; the supernatant was subjected to partially purification by ethanol precipitation, then the partially purified enzyme used for enzyme assays and further characterization (Aygan and Arikan, 2008).

Enzyme assay

CMCase (cellulase) activity was assayed by adding 0.5 mL of enzyme solution to 0.5 mL CMC (1% v/v) in 0.1 M Glisine-NaOH buffer, pH 5.0 and incubating at 20°C for 60 min. The reaction was stopped by the addition of 1 mL of 3,5-Dinitrosalicylic acid (DNS) reagent and its OD_{540} was measured in spectrophotometer (Kowsalya and Gurusamy, 2013; Ferreira et al. 2009).

Influence of pH and temperature on the enzyme activity and stability

The optimum pH for activity of enzyme was determined using different pH buffers: 0,01M citrate phosphate buffer (pH 3.0-5.0), 0,01M sodium phosphate buffer (pH 6.0-8.0) and 0.01M glycine buffer (pH 8.0-10.0). And the optimum temperature was tested at different temperatures 10°C - 50ºC.

For measurement of thermal stability, the enzyme was pre-incubated at temperatures 10-80°C for 60 min also, in order to determine of pH stability, the enzyme was pre-incubated at pH 3.0-10.0 at 25°C for 24 h. After pre-incubation the remaining activity of the enzyme was determined under standard enzyme assay conditions (Zhao et al., 2012).

Effect of different NaCl concentrations on enzyme stability

Effect of NaCl concentrations on enzyme activity was determined by pre-incubating the enzyme in buffer including 3, 5, 7.5, 10, 15, 20, 25 and 30% of NaCl at 25°C for 24 h and remaining activity of the enzyme was determined under standard assay conditions (Zhao et al. 2012; Aygan and Arikan, 2008).

Effect of metal ions, surfactants, chelating agents and inhibitors on enzyme activity

The effects of metal ions, surfactants, chelating agents and inhibitors on cellulolytic activity were determined by pre-incubating the enzyme in the presence of EDTA (5mM), $CaCl_2$ (5mM), $ZnCl_2$ (5mM), $MgCl_2$ (5mM), PMSF (3mM), Tween20 (0,1%), Tween80 (0,1%), 1,10-phenontroline (5mM), SDS (1%), Urea (8M), TritonX-100 (1%), 2-Mercaptoethanol (1%) and H_2O_2 (0.1%) for 60 min at 25°C before adding the substrate. Afterwards, residual activity of the enzyme was measured at optimum activity assay conditions. The control (without metal ions, surfactants, chelating agents and inhibitors) was considered as having 100% activity (Zhao et al. 2012).

Determination of Molecular Weight of the partial purified Enzyme and Zymogram analysis

To determine of molecular weight and active bonds, the enzyme was electrophrosed by using SDS-PAGE (10%) including CMC (0.1%) and 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, one (having marker bands) was stained with 0.1% Coomassie Blue R250 and detected by destaining the gel in methanol-acetic acid-water solution (1:1:8), and the other piece (having protein bands) was subjected to renaturation solutions.

Renaturation of enzyme carried out by incubation of the gel in solution A containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol 40% for 1

h and solution B containing 50 mM Na_2HPO_4, 50mM Na_2HPO_4 (pH 7.2) for 1 h, respectively. After that the gel incubated in solution C containing 50 mM Na_2HPO_4, 50 mM Na_2HPO_4 (pH 7.2), 5 mM β-mercaptoethanol and 1 mM EDTA at 4°C for overnight. Then, the gel was incubated with substrate at 20°C for 5-6 h. After that it was stained with 1% Congo Red solution for 15 min and the activity bands were detected after destaining the gel in 1M NaCl solution for 15 min (Ueda et al., 2010).

Chromatography of the end products of CMC hydrolysis

CMCase was incubated with CMC (2%) at 20°C for 2h. The end products were analyzed (15 µL) by thin layer chromatography. After conducting 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 120°C for 45 min (Ueda et.al., 2010; Kusuda et al., 2003).

Results and Discussion

Isolation of cellulolytic bacteria and culture conditions

The isolate was gram positive, rod shaped, aerobic and spore forming bacterium. According to the results of various morphological and biochemical tests, it was identified as *Bacillus* sp. genus. The strain grew well between 5-30 ºC and at a wide pH range of 5.0 to 9.0 and the optimum enzyme synthesis was occurred at 15ºC and pH 5.0. Optimal concentrations of the production medium components were determined as trypton (10.0 g/l), meat extract (5.0 g/l), NaCl (5.0 g/l) and CMC (6.0 g/l).

Zymogram analysis

The partial purified cellulase appeared as three different active bands on zymogram analysis with molecular masses of 66 kDa, 70 kDa and 81 kDa, respectively (Fig. 1). Similar findings were reported for the cellulases by Liu et al (2013); Zhao et al (2012) and Onsori et al (2005).

Effect of temperature and pH on the enzyme activity and stability

Optimum pH analyses of the K-11cellulase enzyme showed three peaks at pH 4.5, 6.0 and 8.5. But activity of the enzyme at pH 4.5 was higher than other two pH values (Fig.2). These results are supported by the findings of Arikan (Arikan, 2008).The enzyme was almost completely stable at pH ranging from 4.0 to 9.0 for 24 hours with about %92 of residual activity (Fig.3).

The enzyme showed maximum activity at 20°C (Yang and Dang, 2011) (Fig.4). Also, the enzyme was extremely stable at 10 to 40 ºC with almost100% residual activity (Fig.5). These results are in accordance with the reports of Li et al. 2006 and Fan et al. 2007 for acidophilic cellulase.The results showed that the K-11 enzyme was a novel and wonderful lignocellulose-degrading enzyme with important economic advantages.

Effect of various NaCl concentrations, metal ions, and other additives on enzyme activity

In this study k-11 enzyme activity was stimulated by Ca^{2+} ions to 164 %. Before it has been determined that some bacterial enzyme was stimulated by Ca^{2+} which its binding sites contains a number of co-coordinating aspartic acid (Asp), glutamic acid (Glu) residues (Sousa et. al.2007).

Figure.1 SDS-PAGE Analysis of cellulase K-11

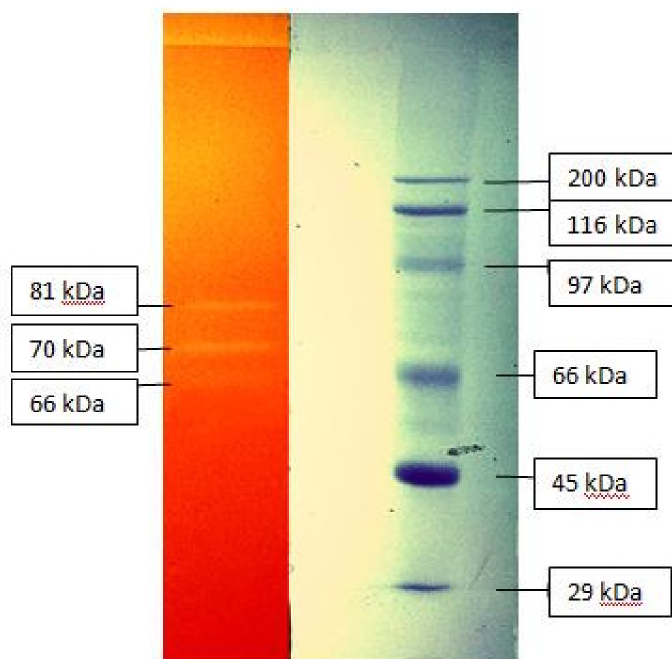


Figure.2 Effect of pH on the activity of cellulase K-11

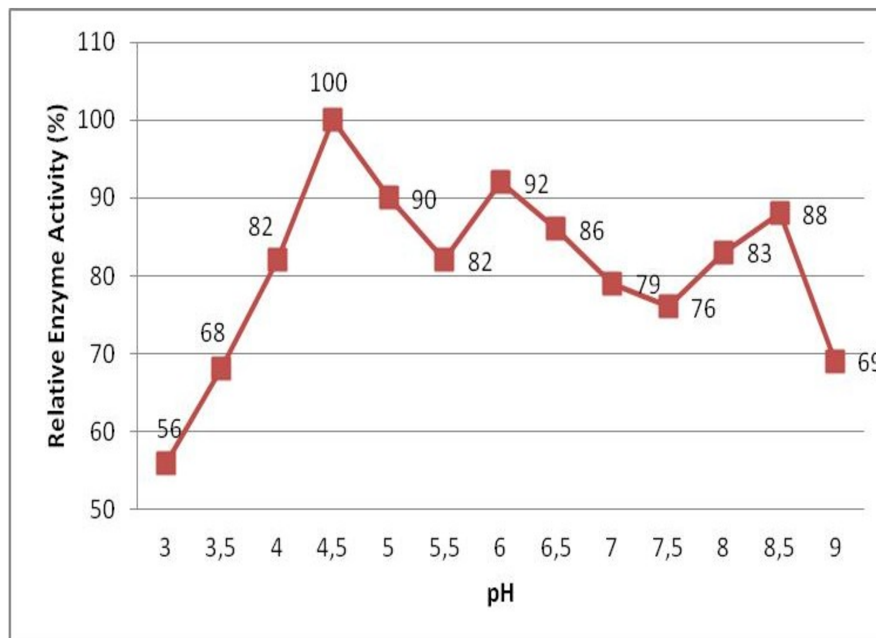


Figure.3 The pH stability of the cellulase by *Bacillus* sp. K-11.

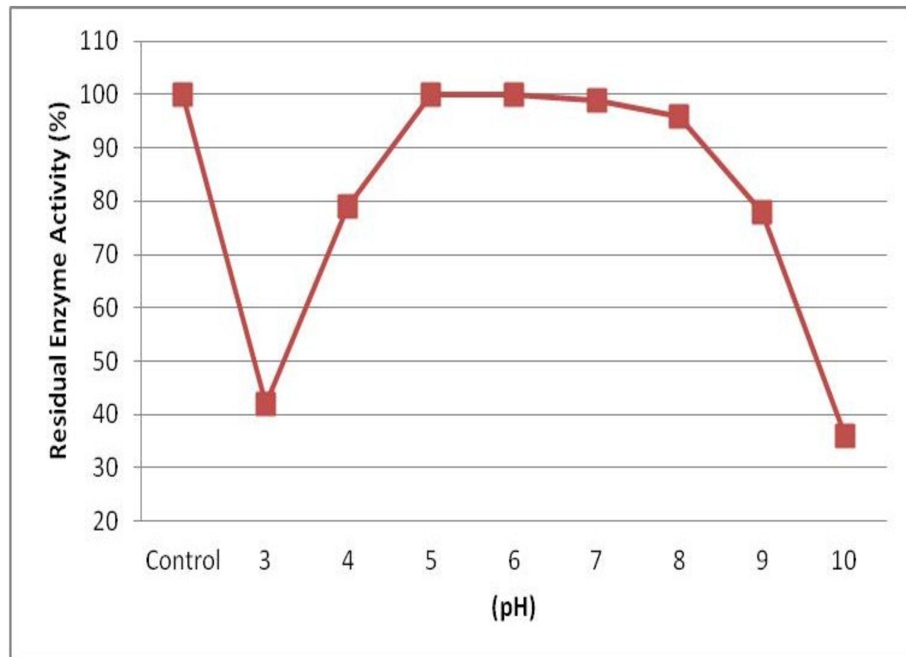


Figure.4 Effect of temperature on the activity of cellulase K-11

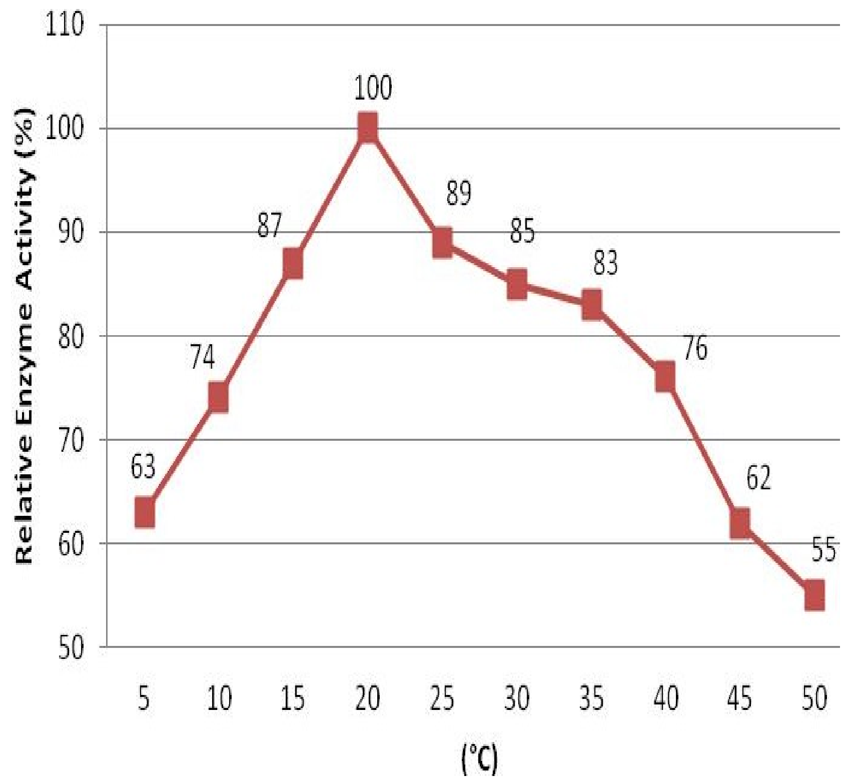


Figure.5 Thermal stability of the cellulase by *Bacillus* sp. K-11.

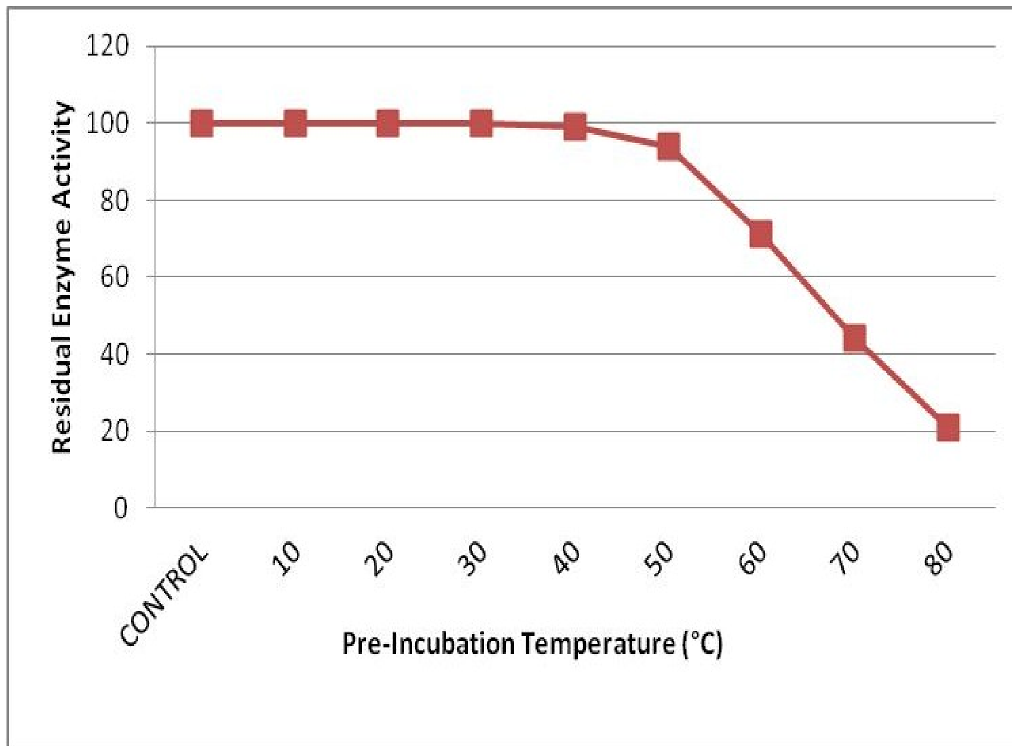


Figure.6 Effect of various additives on the activity of cellulase from *Bacillus* sp. K-11

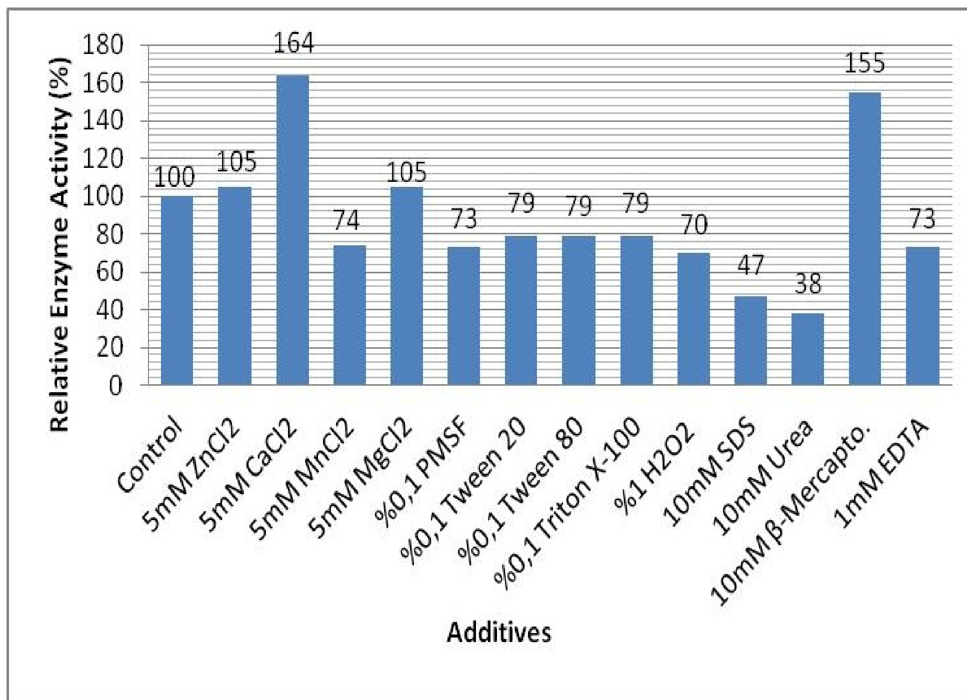


Figure.7 Effect of different NaCl concentration on the activity of cellulase K-11

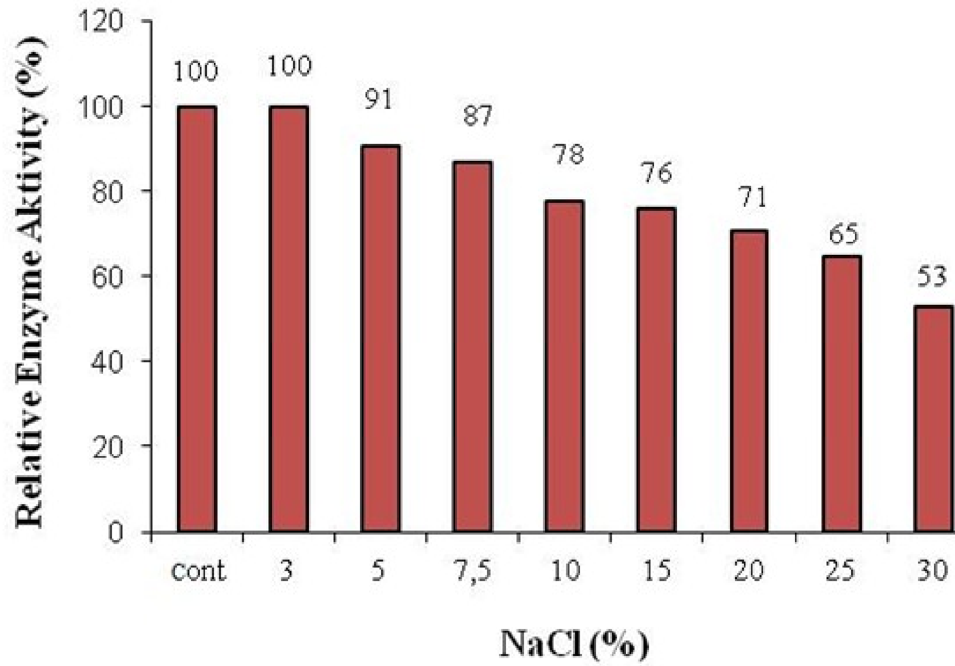
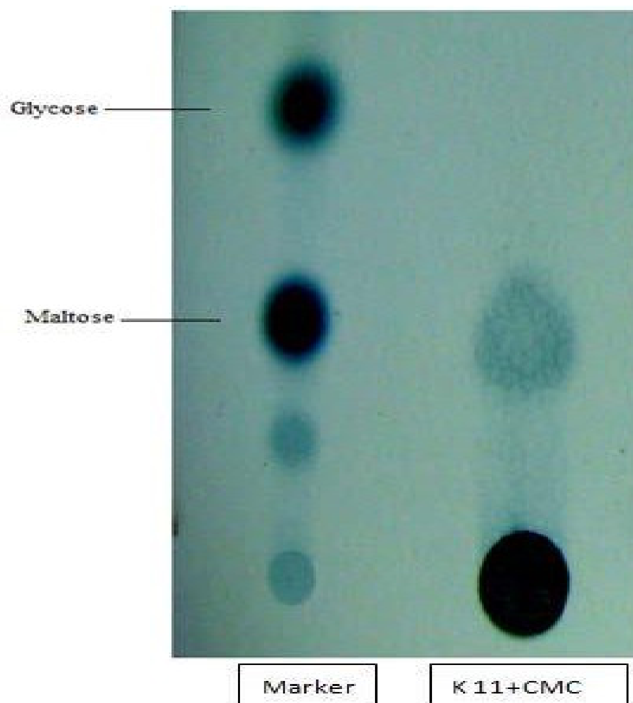


Figure.8 Thin layer chromatography showing the hydrolysed end products



The non-ionic detergents (%0.1 (v/v)) such as Triton X-100, Tween 20, and Tween 80 showed slightly inhibitory effects almost 21, 20 and 24% respectively while %0.1 (w/v) SDS decreased the enzyme activity for 53% . SDS could interact with the hydrophobic group of amino acids, resulting in the decreased enzyme activity (Lucas et al. 2001; Yin et al. 2010).

In this study Zn^{2+} ions didn't affected the activity of K-11 enzyme. In addition, the 1,10-phenantroline and EDTA decreased the activity of K-11 enzyme for about 32% . The high inhibition effect of the chelators suggested that the partial purified K-11 cellulase is a metalloenzyme (Sausa et al., 2007). However, the K-11 cellulase activity was slightly(about13%) inhibited by PMSF showing that the enzyme possesses modification in the serine (ser) residue at active site, because PMSF is known to be a serine protein inhibitor (Aygan and Arikan, 2008; Sausa et al., 2007). On the other hand, it was found that the enzyme activity of K-11 cellulase was slightly induced by H_2O_2 (Fig.6).

Similar results have been reported by Joo and Jang (2005). The enzyme was stable in different NaCl concentrations from 3 to 30 % with more than 76 % of its original activity. This finding is similar to the result of cellulase produced by *Bacillus* sp. C14 which maintained 75% of its original activity in different NaCl concentration from 3 to 30 % after 1 h incubation at 50ºC. (Aygan and Arikan, 2008)

Chromatographic analysis of the end products of CMC hydrolysis

After 2 h incubation of K-11 enzyme-substrate (CMC) reaction mixture the thin layer chromatography revealed the presence of maltose, maltotriose, etc. This result suggested that the K-11 cellulase is a very good producer of maltose (Zhao, 2012) (Fig. 8).

In conclusion, the cellulase enzyme produced by *Bacillus* sp. K-11 is a novel and wonderful source for lignocellulose-degrading enzyme with important economic advantages.

References

Aygan, A., and B. Arikan, 2008. A new halo-alkaliphilic, thermostable endoglucanase from moderately halophilic *Bacillu*s sp.C14 isolated from van soda lake. Int. J. Agri. Biol., 10: 369–74.

Fan, Y.H., W.G. Fang, Y.H., Xiao, X.Y. Yang., Y.J. Zhang, M.J. Bidochka and Y. Pei. 2007. Directed evolution for increased chitinase activity. Appl. Microbiol. Biotechnol., 76(1): 135-139.

Kowsalya, R., and R. Gurusamy. 2013. Isolation, screening and characterization of cellulase producing *Bacillus subtilis* KG10 from virgin forest of Kovai Kutralam, Coimbatore, India, Res. J. Biotechnol., 8 (6). 18-24.

Kusuda, M., M. Nagai, T.C. Hur, M. Ueda, T. Terashita. 2003. Purification and some properties of α-amylase from an ectomycorrhizal fungus, *Tricholoma matsutake*. Mycoscience., 44: 311–317.

Li, Y.H., M. Ding, J. Wang, G.J. Xu and F. Zhao. 2006. A novel thermoacidophilic endoglucanase, Ba-EGA, from a n ew cellulose-degrading bacterium, *Bacillus* sp. AC-1. Appl. Microbiol. Biotechnol., 70(4): 430-436.

Liu, D., J. Li, S. Zhao, R. Zhang, M. Wang, Y. Miao, Y. Shen and Q.

Shen. 2013. Secretome diversity and quantitative analysis of cellulolytic *Aspergillus fumigatus* Z5 in the presence of different carbon sources. Biotechnol Biofuels., 6:149: 1-16.

Lin, L., X. Kan, H. Yan and D.Wang. 2012. Characterization of extracellular cellulose-degrading enzymes from *Bacillus thuringiensis* strains, Electron. J. Biotechnol., DOI: 10.2225/vol15-issue3-fulltext-1.

Lucas, R., A. Robles, M.T. García, G.A. De Cienfuegos and A. Gálvez. 2001. Production, purification, and properties of an endoglucanase produced by the hyphomycete Chalara (Syn.Thielaviopsis) paradoxa CH32. J. Agric. Food Chem., 49(1): 79-85.

Miettinen-Oinonen, A., J. Londesborough, V. Joutsjoki, R. Lantto and J. Vehmaanperä. 2004. Three cellulases from Melanocarpus albomyces for textile treatment at neutral pH. Enzyme Microb. Technol., 34:332–341.

Onsori H., M. R. Zamani, M. Motallebi, and N. Zarghami. 2005. Identification of over producer strain of endo-β-1,4-glucanase in *Aspergillus* Species: Characterization of crude carboxymethyl cellulase. Afr. J. Biotechnol . 4 (1):26-30.

Ramalingam Kowsalya and Ramasamy Gurusamy. 2013. Isolation, screening and characterization of cellulase producing Bacillus subtilis KG10 from virgin forest of Kovai Kutralam, Technology., Res. J. Biotech., 8 (6): 17-24.

Shanmughapriya, S., G. Seghal Kiran, J. Selvin, T. Anto Thomas and C. Rani. 2010. Optimization, Purification, and Characterization of Extracellular Mesophilic Alkaline Cellulase from Sponge-Associated Marinobacter sp. MSI032. Appl Biochem Biotechnol., 162:625–640.

Ueda M., T. Goto, M. Nakazawa, K. Miyatake, M. Sakaguchi and K. Inouye. 2010. A novel cold-adapted cellulase complex from *Eisenia foetida*: Characterization of a multienzyme complex with carboxymethylcellulase, β-glucosidase, β-1,3 glucanase, and β-xylosidase, Comp. Biochem. Physiol., 157: 26–32.

Vıjayaraghavan P., and S.G. Prakash Vincent. 2012. Purification and Characterization of Carboxymethyl Cellulase from *Bacillus* sp. Isolated from a Paddy Field, Pol. J. Microbiol., 1: 51–55.

Yang J., and H. Dang. 2011. Cloning and characterization of a novel cold-active endoglucanase establishing a new subfamily of glycosyl hydrolase family 5 from a psychrophilic deep-sea bacterium., FEMS. Microbiol. Lett., 325(1):71-76.

Yin, L.J., P.S. Huang, and H.H. Lin. 2010. Isolation of cellulase-producing bacteria and characterization of the cellulase from the isolated bacterium *Cellulomonas* sp. YJ5. J. Agric. Food Chem., 58: 9833-9837.

Zeng, R., P. Xiong and J. Wen. 2006. Characterization and gene cloning of a cold-active cellulase from a deep-sea psychrotrophic bacterium *Pseudoalteromonas* sp. DY3 Extremophiles, 10:79–82.

Zhao, K., Z. G. Li and D. L. Wei. 2012. Extracellular Production of Novel Halotolerant, Thermostable, and Alkali-Stable Carboxymethyl Cellulase by Marine Bacterium Marinimicrobium sp. LS-A18, Appl. Biochem. Biotechnol. 168:550-67.