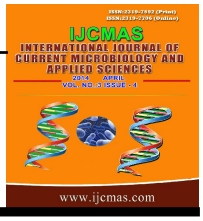
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 4 (2014) pp. 950-960**
http://www.ijcmas.com



Original Research Article

Isolation and Characterization of Alkalin, Halotolerant, Detergent-Stable and Cold-Adaptive α-Amylase from a Novel Isolate *Bacillus* sp. Calp12-7

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

In this study, α-amylase was produced by a novel soil isolate *Bacillus* sp. Calp12-7. The molecular weight of enzyme was estimated to be 114.000 Dalton in SDS-PAGE Zymogram analysis. The enzyme retained more than %90 of its original activity at pH (6.0 -12.0) and conserved up to 98% of its original activity at 5-50°C. The enzyme showed maximum activity at pH 9.0 and 40°C. it retained its activity in the presence of EDTA, $ZnCl_2$, $MgCl_2$, Triton-X-100, Tween 20, Tween 80, 1,10-Phenontroline, PMSF, H_2O_2 and Urea up to 82%, 86%, 97%, 94%, 99%, 99%, 62%, 54%, 26%, and 61%, respectively. However, it was increased in the presence of $CaCl_2$, β-Mercaptoethanol and SDS 12, 27 and 13 %, respectively. It was stable in 3%- 15% of NaCl with more than 80% activity. The enzyme hydrolyzed starch into glucose, maltose, maltotriose, and etc. The results of this study showed that the enzyme is active in wide temperature and pH ranges, in SDS and other detergents (Tween 20, Tween 80, Triton X-100, and etc). Owing to the mentioned characteristics the isolated enzyme can be a good source for detergent additives.

Keywords

Bacillus sp., Cold–adapted, thermo-stabilty, α-amylase, detergent

Introduction

Starch is the most common energy source in the human diet (Van der Maarel et al., 2002). Amylases hydrolyze starch molecules to give diverse products, so they have numerous biotechnological application such as food processing, pharmaceutical and bioremediation (Haki and Rakshit, 2003). α-Amylases are universally distributed throughout the animal, plant and microbial kingdoms. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Pandey et al., 2000). Recently cold active enzymes such as lipase, amylase and etc. attracted interests because they have biotechnological potential, economical and ecological advantages. Using of cold active enzyme can save lots of energy and minimize undesirable chemical reactions that occur at high temperature (Margesin et al., 2002) these enzymes can be useful to prevent any modification of original heat-sensitive substrate and product in

food industry (Cavcchioli, 2002) the enzymes have structural flexibility (particularly around the active site) and high specific activity (Marchi et al., 2007). These properties are of interest in diverse fields such as detergents, textile and food industry, bioremediation and biocatalysts under low temperature conditions. In this study a novel α-amylase producing bacterial strain that was isolated from soil samples was identified, based on morphological, biochemical and molecular tests as a *Bacillus* sp. and called *Bacillus* sp. Calp12-7 and characterized the α-amylase.

Materials and Methods

Isolation and cultivation condition of microorganisms:

Alkaline amylase producing *Bacillus* sp. strains were isolated from alkaline soil samples in Gazi Antep, Turkey and the *Bacillus* sp. Calp12-7 having high activity was screened by employing zone clearing technique using starch agar medium. (Burhan et al., 2003). The strain was identified by studying its morphological and biochemical characteristics (Liu, X.D. and Xu, Y., 2007). Molecular identification of the strain was carried out by analyzing of its 16srDNA sequences by using NCBI, ClustalW sites and MEGA5program (Figure 9.). The sequence was registered in Gen Bank of NCBI with Acession Number of KJ591008.

Enzyme production and Enzyme assay:

Initially, culture conditions such as different temperature, pH and amount of other ingredients of M9 medium were optimized for enzyme production. Subsequently the strain was grown under optimized production condition in 100 ml of M9 minimal medium supplemented by 1% soluble starch with shaking (250 rpm) for 72 hours. The extracellular enzyme solution was partially purified by ethanol precipitation method (McTigue et al., 1995; Arikan., 2008) then the enzyme assay carried out by DNS method (Bernfeld, 1955; Caf et. al., 2013).

Effect of pH and temperature on activity and stability:

Optimal pH on enzyme activity was determined by running the assay activity at different pH ranging from 6.0-12.0 at 40°C for 60 min (Caf et. al., 2013,). Also, the related optimum temperature was determined by varying the incubation temperature from 0°C to 60°C.

Stability of the enzyme in various pH values was measured by pre-incubating the enzyme solution at the pH range of 6.0 to 12.0 for 24 h subsequently; the residual activity was measured by incubating the reaction mixture at optimum pH and temperature for 60 min. Also, In order to ascertain of temperature stability, enzyme solutions were pre-incubated at various temperatures in the range from 0°C to 60°C for 60 min (Carvalho et al., 2008) then remaining activity assayed at optimum pH and temperature as mentioned earlier.

Effect of salt, metal ions and other additives on enzyme activity:

Effect of salt on enzyme activity was assayed in the NaCl concentration range of 3% to 30%. In order to investigate the role of cations, enzyme activity was assayed in the presence of 5mM $CaCl_2$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$. In addition the effects of 10mM SDS, 1mM EDTA, %0.1 PMSF,

1,10-phenantroline, 10mM β-mercaptoethanol, %0.1 TritonX-100, Tween 80, Tween 20 and %1 H_2O_2 were analyzed. (Hmidet et al., 2009; Aygan et al., 2008).

Thin-Layer Chromatography (TLC) Analysis

α-amylase Calp12-7 was incubated with starch (2%) for 2h at optimum pH and temperature. The 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 20% sulfuric acid/ethanol regent and drying in an oven at 120°C for 20 min (Caf et al.,2013; Ksuda et al., 2003 and Rina R.R et al., 2011).

Zymogram analysis

The molecular mass of the enzyme was finally estimated from the position of standard proteins (Sigma M3788 - 205,000 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 asid-water solution (1:1:8), other (having protein bands) was subjected to renaturation solutions followed by incubation at optimum temperature for 2-3h.

For analysis of amylase activity, SDS was removed by washing the gel at room temperature in renaturation solutions containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol 40% for 1 h and 50 mM Na_2HPO_4, 50mM Na_2HPO_4 (pH 7.2) for 1 h, respectively. Renaturation of enzyme proteins was carried out by keeping the gel overnight in a solution 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. After incubation, the gel was stained in a solution of iodine (Iodine 5 g/l, KI 50 g/l), for 15 min (Caf et al., 2013; Hmidet et al., 2009; Burhan et al., 2003).

Results and Discussion

Isolation of *Bacillus* sp. CALP12-7 and enzyme production

The strain grew well between 10-40 ºC and at a wide pH range of 7.0 to 10.0. The optimum temperature and pH for maximum enzyme production was observed at 40ºC and pH 9.0 in the culture medium containing 5% of $NaCl_2$ concentration (Fig.1). According to these results the isolate *Bacillus* sp. Calp12-7 is called cold-adapted alkaliphilic bacterial strain (Ueda et al., 2008; Feller and Gerday, 2003). The optimum incubation period for enzyme production was 72 hour.

Identification of isolated strain

According to morphological and biochemical after analysis the isolated bacterial strain was demonstrated as *Bacillus* sp. Also study of 16srDNA sequence by neighbor-joining method showed that the strain has more than 99% homology and similarity to *Bacillus thuringiensis (Figure.9). the* 16srDNA aequence was registered in Gen Bank of NCBI with Acession Number of KJ591008.

Zymogram analysis

Zymogram analysis showed the presence of single activity band with molecular weights of 114kDa (Fig.1). This result was in agreement with Murakami et al (2007)

who reported two α-amylase from *Bacillus halodurans* 38C-2-1 with molecular weights of 100 kDa and 75 kDa; similarly, Caf et al (2013) have reported two α-amylase from *Bacillus* sp. AC-7 with molecular weights of 174 kDa and 137 kDa.

Temperature and pH optima and stability of the Enzyme

The partially purified enzyme conserved more than %90 and almost 98% of its original activity at pH 6.0 - 12.0 and temperature between 5°C and 50°C, respectively. Also the enzyme showed maximum activity at pH 9.0 (Fig.2) and 40°C (Fig.4) and it produced high volume of oligosaccharides including small amount of maltose and glucose from starch as the end product (Fig.8) (Caf et al., 2013, Roy et al., 2012).

Study thermostability revealed that the enzyme maintained more than 95% of original activity after pre-incubation at (0-50ºC) for 24h. (Fig.5). Although, there are some reports regarding α-amylase enzymes showing cold activity (Caf et al., 2013, Cho et al., 2010, Zhang and Zeng, 2008, Ueda et al., 2008) to our knowledge, up to now Calp12-7 α-amylase is the first enzyme that retaining over 100% of maximum activity after pre incubation at (0-30) °C for 24 hour. Calp12-7 α-amylase retained 88%, 88.%, 90%, 99%, 93%, 44%, 33%, and 24% of original activity after pre-incubation for 24 hour at pH 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 and 13.0, respectively.(Fig.3). These values were in agreement with reports by (Maalej et al. 2013, Ueda et al., 2008) who reported cold-adapted α-amylases (Amy I and Amy II) from *Eisenia foetida* that was stable at pH 7.0–9.0. The enzyme exhibited wide range of pH activity which eliminates the requirement for pH adjustment in its industrial applications. Also, pH stability is an important factor for the conservation and application of enzymes in industry.

Effect of salt, metal ions and other additives on enzyme activity

After pre-incubation for 1 hour in the presence of 1mM EDTA, 5mM $ZnCl_2$, $MgCl_2$, $CaCl_2$ and $MnCl_2$, 10mM SDS, 10mM Urea, 10mM β-mercaptoethanol, 3mM 1,10-phenanthroline %0.1 Triton X-100, %0.1 Tween 20, %0.1 Tween 80, , %0.1 PMSF, and %1 H_2O_2 the enzyme retained its original activity up to 63%, 86%, 97%, 112%, 99%, 113%, 94%, 99%, 100%, 127%, 62% , 54% and %26, respectively (Fig.7). It was stable in 3%-10% concentration of NaCl with more than 71% activity (Fig.6).

The activity was stimulated by Ca^{2+} ions. The result was in agreement with the findings of Ueda et al (2007) and Zhang et al (2008). It has been showed that Ca^{+2} and Cl^{-2} ions are essential for the stability of α-amylases (Viswanathan and Feller, 2001). In presence of Zn^{2+} ions decreased activity of α-amylase Calp12-7. The inhibition by Zn^{2+} (16 %) can be a result of inhibitory effects of heavy metals on enzymes. In another study it was reported that the inhibition of enzymes by Zn is an indication of thermostability for an enzyme (Aygan and Arıkan, 2008). EDTA and 1, 10-phenantroline decreased activity of the α-amylase-Calp12-7 demonstrating that the enzyme is metalloprotein (Sausa et al., 2007). In the presence of 3%-10% NaCl, over 71% of the amylase activity was conserved, this finding is similar to the α-amylase produced by *Bacillus* sp. SMIA-2 which maintained 63% of its original activity in 2 M NaCl after 2 h incubation at 40ºC. (Carvalho et al., 2008).

Figure.1 Analysis of Calp12-7 α-amylase by SDS-PAGE (%10)

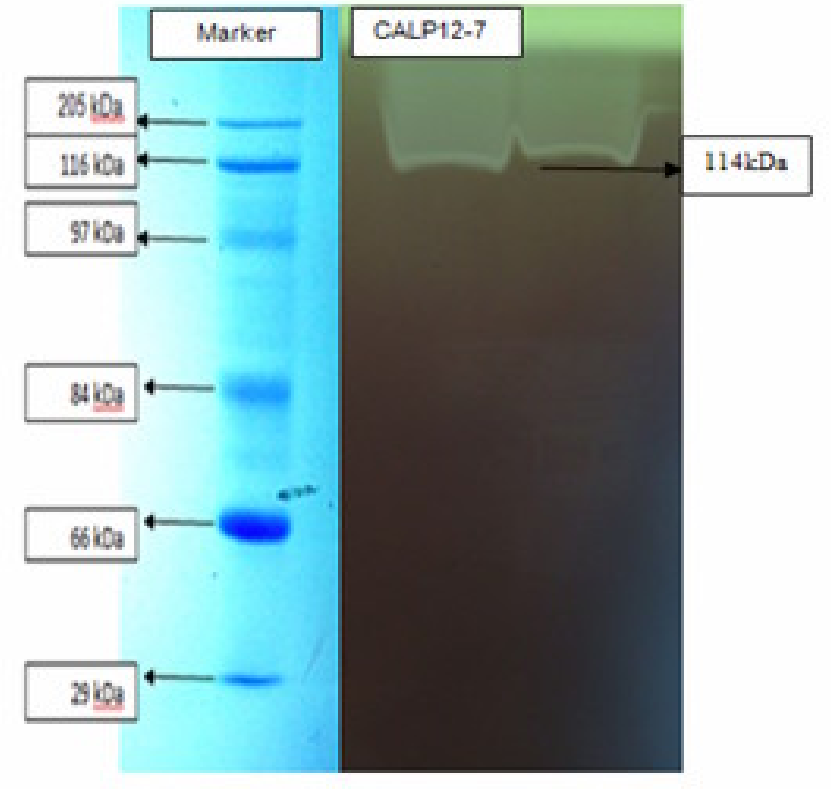


Figure.2 Effect of pH on the activity of α-amylase Calp12-7

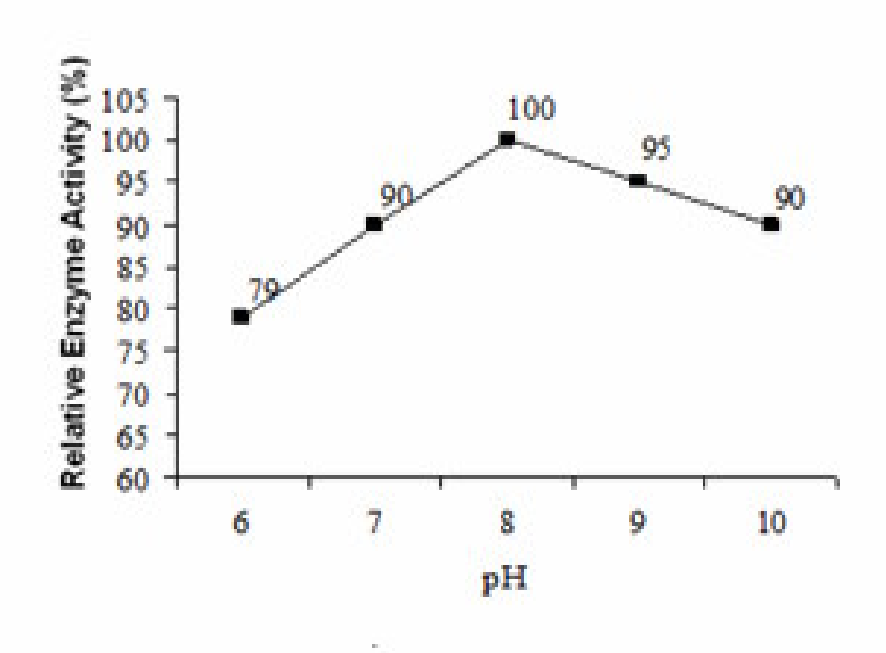


Figure.3 Effect of pH on the stability of α-amylase Calp12-7

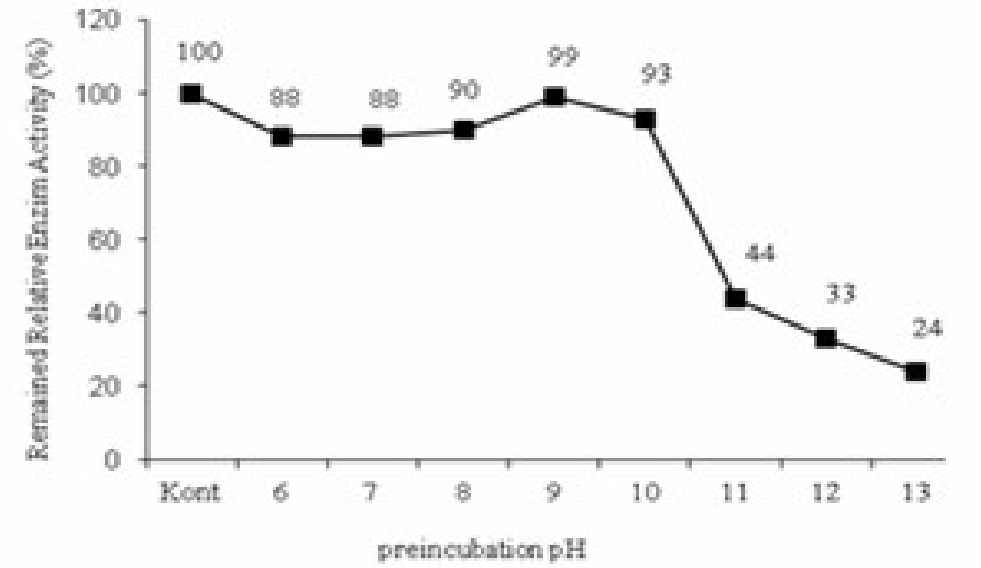


Figure.4 Effect of temperature on the activity of α-amylase Calp12-7

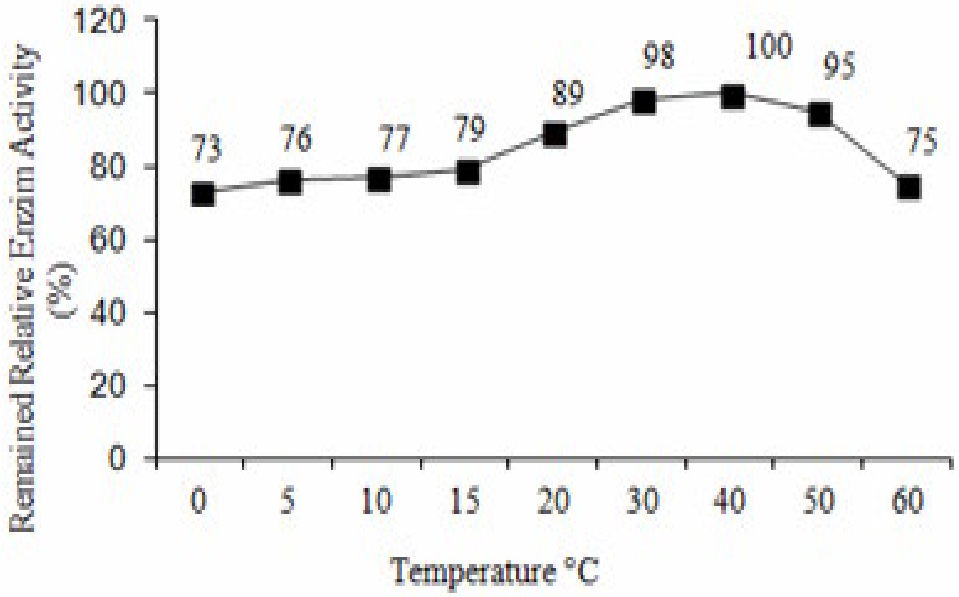


Figure.5 Effect of temperature on the stability of α-amylase Calp12-7

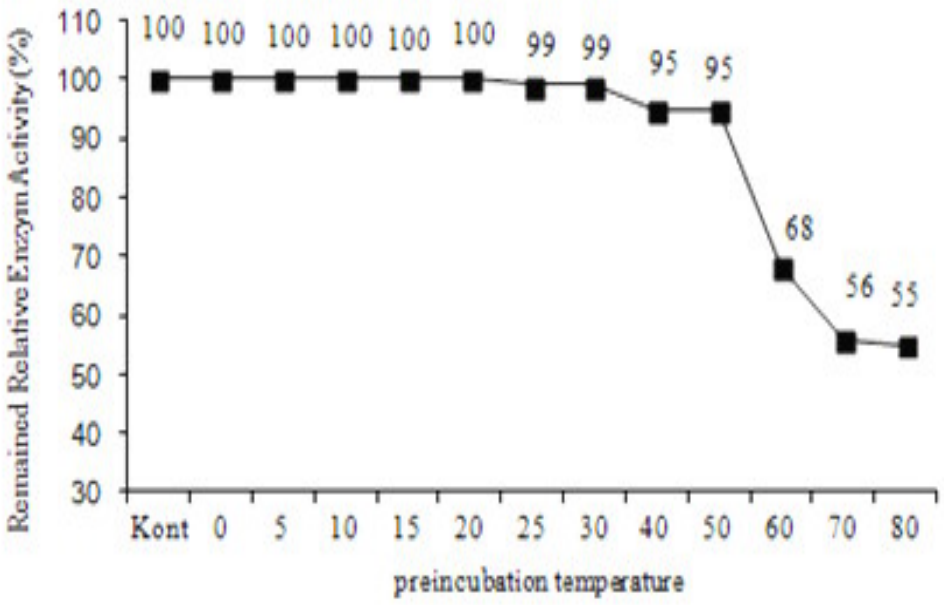


Figure.6 Effect of different NaCl concentration on the activity of α-amylase Calp12-7

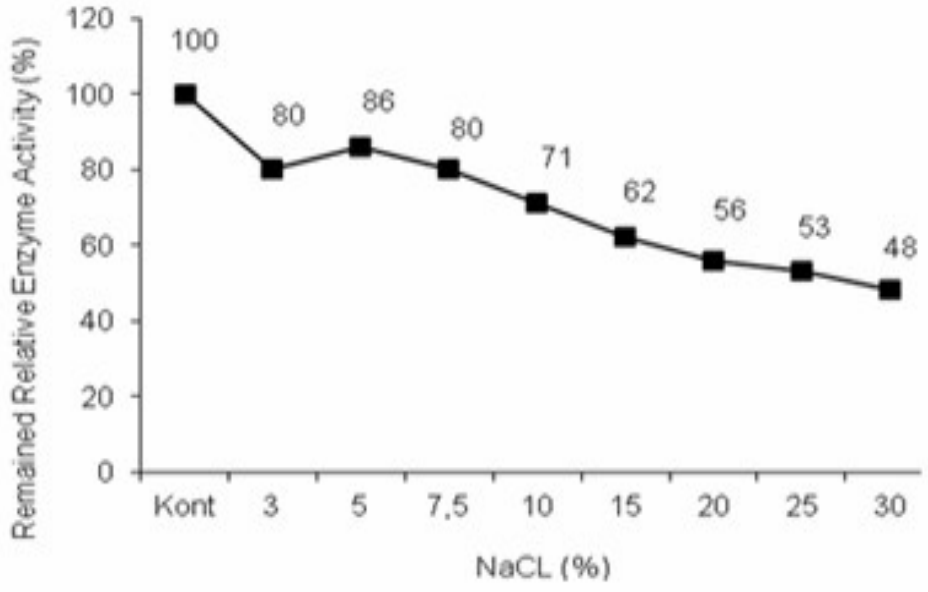


Figure.7 Effect of various effectors with various concentration on the activity of α-amylase Calp12-7

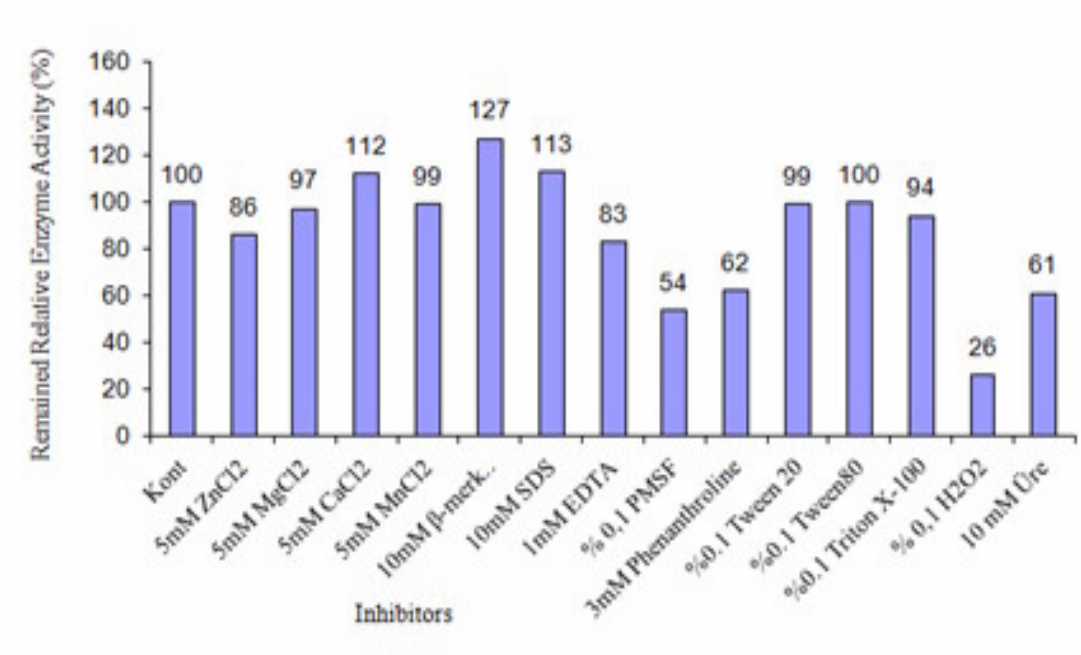


Figure.8 Thin layer chromatography showing the hydrolysed end products of α-amylase Calp12-7

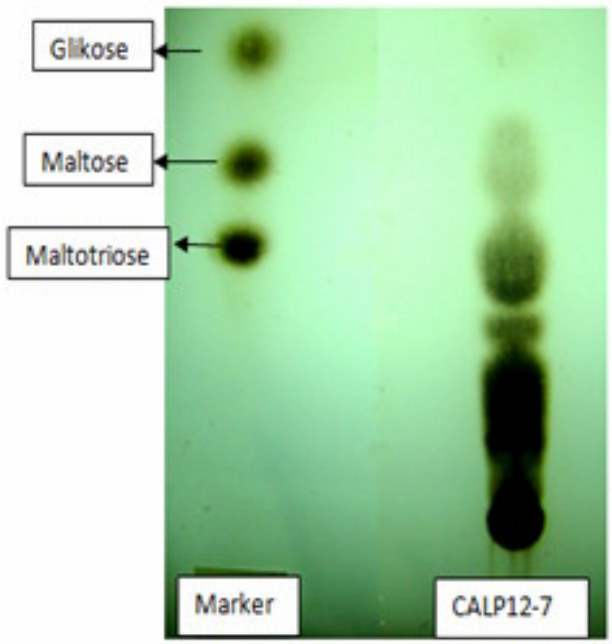
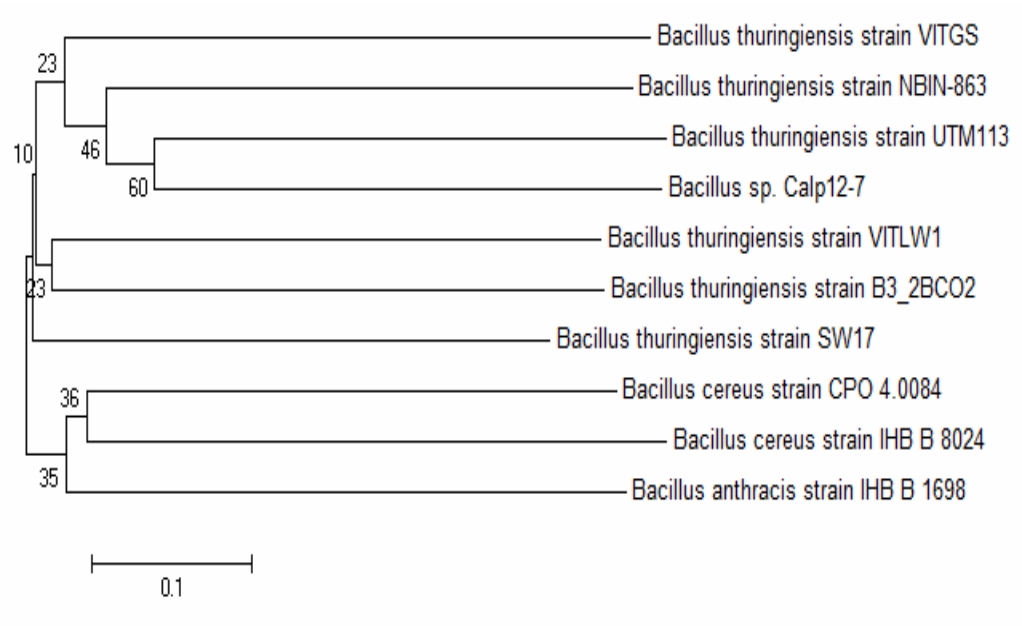


Figure.9 Phylogenetic tree of isolate *Bacillus* sp. Calp12-7 showing the relationship with other members of the genus *Bacillus* sp. using 16S rRNA sequence



Slightly inhibition of α-amylase Calp12-7 was observed in the presence of non-ionic detergent such as %0.1 (v/v) Tween 20, Tween80 and Triton X-100 this result is in agreement with the report of Maalej et al (2013). In contrast to non-ionic detergent, SDS enhanced the enzyme activity (Hauli et al., 2013). On the other hand, it was found that the activity of α-amylase Calp12-7 was strongly inhibited by %1 (v/v) H_2O_2. Similar results have been reported by Carvalho et al (2008).

In Conclusion, the results of this study showed that the enzyme is active in wide temperature and pH ranges, in SDS and other detergents (Tween 20, Tween 80, Triton X-100, and etc). In order to be economic in energy, water having temperature between 0 and 50 °C is suggested for washing. The α-amylase Calp12-7 conserved more than %98 of its original activity in the mentioned temperature ranges. At the other hand all enzymes for detergent use should be able to maintain its activity in presence detergents such as SDS, Tween 20, Tween 80, Triton X-100, and etc. In addition, α-amylases with alkaline pH values have a potential application in dishwasher and laundry detergent formulations (Roy et al., 2012). These results showed that our enzyme is a good source for detergent additive.

Acknowledgement

This research supported by the Cukurova University research found (FEF2012BAP11).

References

Arikan, B., 2008. Highly thermostable, thermophilic, alkaline, SDS and chelators resistant amylase from a thermophilic *Bacillus* sp. isolate A3-15. Bioresource Technology. 99: 3071-3076.

Aygan, A., and B. Arikan. 2009. Production and characterization of multifunctional endoxylanase by *Bacillus* sp. X13. Turk J Biol. 33: 231-237.

Bernfeld, P., 1955. Amylases a and b. In: Methods in Enzymology. (ed.) by Colowick S.B. and Kaplan NO. New York, Academic Press. Vol.1, Pp. 49-53.

Burhan, A., U. Nisa, C. Gokhan, C. Omer, A. Ashabil and G. Osman. 2003. Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. Process. Biochem. 38:1397–1403.

Caf, Y., Y. Maasoglu, E. Valipour and B. Arikan. 2012. Production and characterization of novel cold-active, pH tolerant and detergent-stable: α-amylase from a psychrotrophic bacterium from soil samples, New Biotechnology. 29:23–26.

Carvalho, R.V., T.L.R. Côrrea, J.C.M. Silva, L.R.C. Oliveira Mansur and M.L.L. Martins. 2008. Properties of an amylase from thermophilic *Bacillus* sp. Brazilian. J. of Microbiology. 39: 102-107.

Cavicchioli, R., K.S. Siddiqui, D. Andrews and K.R. Sowers. 2002. Low temperature extremophiles and their applications. Curr. Opin. Biotechnol., 13: 253-261.

Feller. G., and C. Gerday, 2003. Psychrophilic enzymes: hot topics in cold adaptation. Nature Reviews. Microbiology. 1: 201-208.

Haki, G.D., and S.K. Rakshit, 2003. Development in industrially important thermostable enzymes. Review. Biores. Technol.89:17-34.

Hauli, I., B. Sarkar, T. Mukherjee and S.K. Mukhopadhyay. 2013. Isolation and identification of a novel thermo-alkaline, thermostable, SDS and chelator resistant amylase producing Anoxybacillus sp. IB-A from hot spring of Bakreswar, West Bengal (India). First report, Advances in Applied Science Research. 4:202-212.

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

Liu, X. D., and Y. Xu. 2008. A novel raw starch digesting a-amylase from a newly isolated *Bacillus* sp. YX-1: Purification and characterization. Bioresource Technology. 99: 15-20.

Maalej, H., N. Hmidet , O. Ghorbel-Bellaaj and M. Nasri. 2013. Purification and biochemical characterization of a detergent stable α-amylase from Pseudomonas stutzeri AS22, Biotechnology and Bioprocess Engineering. 18: 878-887.

Marchi, P., V. Longhi, S. Zangrossi, E. Gaetani, F. Briani and G. Deho. 2007. Autogenous regulation of Escherichia coli polynucleotide phosphorylase during cold acclimation by transcription termination and antitermination. Mol. Genet. Genomics, 78: 75-84.

Margesin, R., G. Feller, C. Gerday and N.J. Russell. 2002. Cold-adapted microorganisms: adaptation strategies and biotechnological potential. In The Encyclopedia of Environmental Microbiology, Vol. 2. ed. Bitton, G. pp. 871–885. New York: Wiley.

Murakami, S., H. Nishimoto, Y. Toyama, E. Shimamoto, S. Takenaka, J. Kaulpiboon. 2007. Purification and characterization of two alkaline,

thermotolerant α- amylases from *Bacillus halodurans* 38C-2-1 and expression of the cloned gene in *Escherichia coli*. Biosci Biotechnol Biochem. 71: 393–401.

Noomen, H., A. Nedra El-Hadj, H. Anissa, K. Safia, A. Sellami-Kamoun and N. Moncef. 2009. Alkaline proteases and thermostable α-amylase co-produced by *Bacillus* licheniformis NH1: Characterization and potential application as detergent additive. Biochemical Engineering Journal. 47: 71–79.

Pandey, A., P. Nigam, C.R. Soccol, V.Y. Soccol, D. Singh and R. Mohan. 2000. Advances in microbial amylases. Biotechnol. Appl. Biochem. 31: 135-152.

Roy, J. K., S. K. Rai and A. K. Mukherjee. 2012. Characterization and application of a detergent-stable alkaline -amylase from *Bacillus subtilis* strain AS-S01a, International Journal of Biological Macromolecules. 50: 219–229.

Sahnouna, M., S. Bejara, A. Sayaria, M. A. Triki, M. Kriaaa and R. Kammouna. 2012. Production, purification and characterization of two α-amylase isoforms from a newly isolated Aspergillus Oryzae strain S2. Process Biochemistry. 47: 18–25.

Sun, H., P. Zhao, X. Ge, Y. Xia, Z. Hao, J. Liu and M. Peng. 2010. Recent advances in microbial raw starch degrading enzymes. Appl. Biochem. Biotechnol. 160: 988-1003.

Ueda, M., T. Asano, M. Nakazawa, K. Miyatake and K. Inouye. 2008. Purification and characterization of novel raw-starch-digesting and cold-adapted α-amylases from Eisenia foetida. Comp. Biochem. Phys. 150: 125-130.

Van der Maarel, M.J., B. Van der Veen, J.C. Uitdehaag, H. Leemhuis, L. Dijkhuizen. 2002. Properties and applications of starch-converting enzymes of the alpha-amylase family. J Biotechnol. 94: 137-155.

Vihinen, M., and P. Mantsala. 1989. Microbial amylolytic enzymes. Crit. Rev. Biochem. Mol. Biol. 24: 329-418.

Viswanathan, P., and G. Feller. 2001. Structural similarities and evolutionary relationships in chloride-dependent α-amylase. Gene.25: 357-364.

Zhang, J.W., and R.Y. Zeng. 2008. Purification and characterization of a cold-adapted alpha-amylase produced by *Nocardiopsis* sp. 7326 isolated from Prydz Bay, Antarctic. Mar Biotechnol. 10: 75-82.