© by PSP Volume 31– No. 06/2022 pages 5750-5757 Fresenius Environmental Bulletin

ISOLATION AND PARTIAL CHARACTERIZATION OF ALKALINE TOLERANT ALPHA-AMYLASE FROM *Bacillus pacificus* FEB5

Bahri Devrim Ozcan[1,*], Elif Dikkaya[1], Makbule Baylan[2], Gamze Mazi[2]

[1]Animal Biotechnology and Genetic Engineering Laboratory, Department of Animal Science, Faculty of Agriculture, University of Cukurova, 01330 Saricam, Adana, Turkey
[2]Department of Basic Sciences, Faculty of Aquaculture, University of Cukurova, 01330 Saricam, Adana, Turkey

ABSTRACT

In this study, α-amylase producing *Bacillus* strain FEB5 was isolated from the soil samples collected from Çukurova University campus area. The isolate was identified as *Bacillus pacificus* according to 16S rDNA nucleotide sequence analysis. *B. pacificus* FEB5 α-amylase showed its maximum activity at pH 11.0 and 40 °C. The enzyme retained all its activity after 15 minutes of pre-incubation at 40 °C, but above this temperature, it lost its activity gradually until 70 °C, but rapidly at the temperatures above 70 °C. FEB5 α-amylase reached its highest activity level at 24 hours after inoculation. While the enzyme reached higher activity levels compared to the control group in the presence of SDS and Co; $CaCl_2$, $MgCl_2$, and EDTA showed inhibitory effects on the enzyme. SDS-PAGE and zymogram analysis revealed two α-amylase bands of 16.5 and 8.3 kDa.

KEYWORDS:
Bacillus pacificus, Isolation, Alkaline Tolerant, α-Amylase, Characterization

INTRODUCTION

Amylases are widely distributed all over the world and have an important several industrial potential. These enzymes hydrolyze starch molecules to dextrins and other smaller polymers composed of glucose units [1, 2, 3]. Endoamylases and exoamylases are two subcategories of amylases. Endoamylases hydrolyze starch from the interior of the molecule to produce linear and branched oligosaccharides of various chain lengths, whereas exoamylases act from the nonreducing end, resulting in short products [4, 5]. Alpha-amylase (EC 3.2.1.1; α-1,4-glucan glucano-hydrolase) is an endoamylase that hydrolyzes starch molecules from the α-1,4-glycosidic bonds [1].

Although α-amylases are produced from various species of plants, animals, and microorganisms [6, 7], industrially important α-amylases are obtained from microorganisms. Among microorganisms, fungi and bacteria have been the most popular amylase producers [6]. *Bacillus* species such as *B. subtilis*, *B. megaterium*, *B. licheniformis*, *B. circulans*, *B. amyloliquefaciens*, and *B. stearothermophilus* are common bacterial sources for wide-scale enzyme production in industrial applications [8]. Different bacterial strains have different growth conditions and enzymatic production profile [9]. Due to this reason, the studies on isolation and characterization of new microorganisms producing amylase enzymes important.

In this study, α-amylase producer *Bacillus* strains were isolated and the enzymes were partially characterized.

MATERIALS AND METHODS

Isolation and identification of isolate. *B. pacificus* FEB5 was isolated from the soil sample collected from Çukurova University campus area in Adana, Turkey. The sample was collected into previously sterilized and labelled screw-caped bottles and transferred to the lab. One g (1 g) of the soil sample was dissolved in 9 ml of distilled and sterilized water. After waiting for 30 min at room temperature in laminar flow, 500 µl of the diluted sample was transferred into the sterile microsantrifuge tube and incubated at 80 ℃ for 10 min for the selection of Gram-positive and spore-forming *Bacillus* strains. *Bacillus* spores were germinated overnight at 37 ℃ for 24 h in LB broth containing tryptone (10 g/l), yeast extract (5 g/l), and NaCl (10 g/l) (pH 7.5). The bacterial culture was diluted up to10^{-6} times and then 100 µl of 10^{-5} and 10^{-6} diluted samples spread onto the LB-agar (LB broth supplemented with 15 g/l agar) plates. After 24 h incubation at 37 ℃, the colonies were picked up with sterile toothpicks and transferred onto the LB-starch-agar (LB-agar supplemented with 5 g/l soluble starch) for detection for α-amylase activity. Alpha-amylase producing colonies with clear activity zones were determined by iodine staining and stored at 4 ℃ in the refrigerator until required for use.

Isolate was identified by sequencing the 16S rDNA gene. The 16S rDNA gene sequence was blasted with the genbank nr database. Based on the

maximum identity score, the first 10 sequences were selected and aligned using multiple sequence alignment software (Clustal W). Phylogenetic tree was constructed using MEGA 11.0 software.

Enzyme experiments. *B. pacificus* FEB5 was cultured in a LB broth at 37 ℃ with average shaking for 24 h. After removing the bacterial cells by centrifugation (Hettich Universal EBA12) (4500 rpm, 10 min), the cell-free supernatants were used in the enzyme experiments. Alpha-amylase activities were assayed by adding 1 ml enzyme to 1 ml substrate solution (soluble starch, 2%, w/v) in 100 mM sodium phosphate buffer (pH 7.5) and incubating for 30 min under assay conditions. The reactions were stopped by addition of 3 ml of 3,5-dinitrosalicylic acid reagent and measured at A_{540} nm using a spectrophotometer (Pharmacia) [10].

Effects of temperature and pH on the enzyme activities. The effects of temperature and pH on the enzyme activity were assayed at various temperatures ranging from 30 to 100 °C and at pH values ranging from 4.0 to 12.0 for 30 min. The buffers used in pH reactions were 100 mM Na-acetate (pH 4.0-6.0), 100 mM Na-phosphate (pH 6.0-8.0), and 100 mM Tris (pH 8.0-12.0) [11].

Thermal stabilities of the enzyme. The maintained enzyme activities were determined under standard enzyme assay conditions after pre-incubation of the enzyme at the temperatures ranging from 30-100 ℃ for 15 min.

Effect of chemicals. The effects of $CaCl_2$, $MgCl_2$, Co, SDS, and EDTA (5 mM each) on the enzyme activity were determined by pre-incubating the extracellular enzyme in the presence of these chemicals for 30 min at optimum temperature value before adding the substrate [12]. Relative enzyme activities were measured under standard enzyme assay conditions. The control group, which did not contain any additive, was taken as 100% activity.

Time dependent enzyme activity. Enzyme activities were assayed at various time intervals of bacterial growth process ranging from 24 to 96 h. Relative enzyme activities of the enzyme were measured under standard enzyme assay conditions. The time periods with the highest enzyme activities were taken as 100% activity.

Electrophoretic analysis of extracellular proteins. After 48 h cultivation in LB broth, the isolate was centrifuged, and then the culture supernatant was mixed with equal volume of Trichloroacetic acid (TCA, 20% (w/v)) for precipitation of denatured proteins. The mixture was incubated at room temperature for overnight and denatured protein patterns obtained by centrifuging at 5000 rpm for 10 min. Supernatant was removed and protein pellet was air-dried for 60 min. Tris-HCl buffer (0.1 M, pH 8.0) was used for dissolving the protein pellet and then applied to SDS-PAGE and SDS-Starch-PAGE (0.2% starch) according to Laemmli [13] with slab gels (12% w/v acrylamide). Activity staining (zymogram) of SDS-Starch-PAGE was performed according to Arikan *et al*. [11] and Ikeda *et al*. [14].

RESULTS

In the study, Gram-positive, spore forming, and aerobic *Bacillus* strain FEB5 was isolated. The isolate was identified as *Bacillus pacificus* according to 16S rDNA nucleotide sequence analysis (Figure 1) with the sequence similarity of 99.16%, and therefore the isolate was assigned as *Bacillus pacificus* FEB5 strain (Figure 2). The isolate showed its optimum growth at pH 7.5 in LB medium at 37 ℃. Alpha-amylase activity of the isolate was observed on LB-agar plate containing soluble starch as substrate by iodine staining.

1	CGGCCCCGAC	TCCTACGGGA	GGCAGCAGTA	GGGATTTTTC	CGCATGGACG	AAAGTTCGAC	60
61	GGAGCAACGC	CGCCTGAGTG	ATGAAGGCTT	TCGGGTCGTA	AAACTTTGTT	GTTAGGGAAG	120
121	AACAAGTGCT	AGTTGAATAA	GCTGGCACCT	TGACGGTACC	TAACCAGAAA	GCCACGGCTA	180
181	ACTACGTGCC	AGCAGCCGCG	GTAATACGTA	GGTGGCAAGC	GTTATCCGGA	ATTATTGGGC	240
241	GTAAAGCGCG	CGCAGGTGGT	TTCTTAAGTC	TGATGTGAAA	GCCCACGGCT	CAACCGTGGA	300
301	GGGTCATTGG	AAACTGGGAG	ACTTGAGTGC	AGAAGAGGAA	AGTGGAATTC	CATGTGTAGC	360
361	GGTGAAATGC	GTAGAGATAT	GGAGGAACAC	CAGTGGCGAA	GGCGACTTTC	TGGTCTGTAA	420
421	CTGACACTGA	GGCGCGAAAG	CGTGGGGAGC	AAACAGGATT	AGATACCCTG	GTAGTCCACG	480
481	CCGTAAACGA	TGAGTGCTAA	GTGTTAGAGG	GTTTCCGCCC	TTTAGTGCTG	AAGTTAACGC	540
541	ATTAAGCACT	CCGCCTGGGG	AGTACGGCCG	CAAGGCTGAA	ACTCAAAGGA	ATTGACGGGG	600
601	GCCCGCACAA	GCGGTGGAGC	ATGTGGTTTA	ATTCGAAGCA	ACGCGAAGAA	CCTTACCAGG	660
661	TCTTGACATC	CTCTGAAAAC	CCTAGAGATA	GGGCTTCTCC	TTCGGGAGCA	GAGTGACAGG	720
721	TGGTGCATGG	TTGTCGTCAG	CTCGTGTCGT	GAGATGTTGG	GTTAAGTCCC	GCAACGAGCG	780
781	CAACCCTTGA	TCTTAGTTGC	CATCATTAAG	TTGGGCACTC	TAAGGTGACT	GCCGGTGACA	840
841	AACCGGAGGA	AGGTGGGGAT	GACGTCAAAT	CATCATGCCC	CTTATGACCT	GGGCTACACA	900
901	CGTGCTACAA	TGGACGGTAC	AAAGAGCTGC	AAGACCGCGA	GGTGGAGCTA	ATCTCATAAA	960
961	ACCGTTCTCA	GTTCGGATTG	TAGGCTGCAA	CTCGCCTACA	TGAAGCTGGA	ATCGCTAGTA	1020
1021	ATCGCGGATC	TAGCATGCAC	GCGGTGAATA	CGTTCCCGGG	CCTTGTACAC	ACC	1073

FIGURE 1
16S rDNA nucleotide sequence of *Bacillus pacificus* FEB5 strain

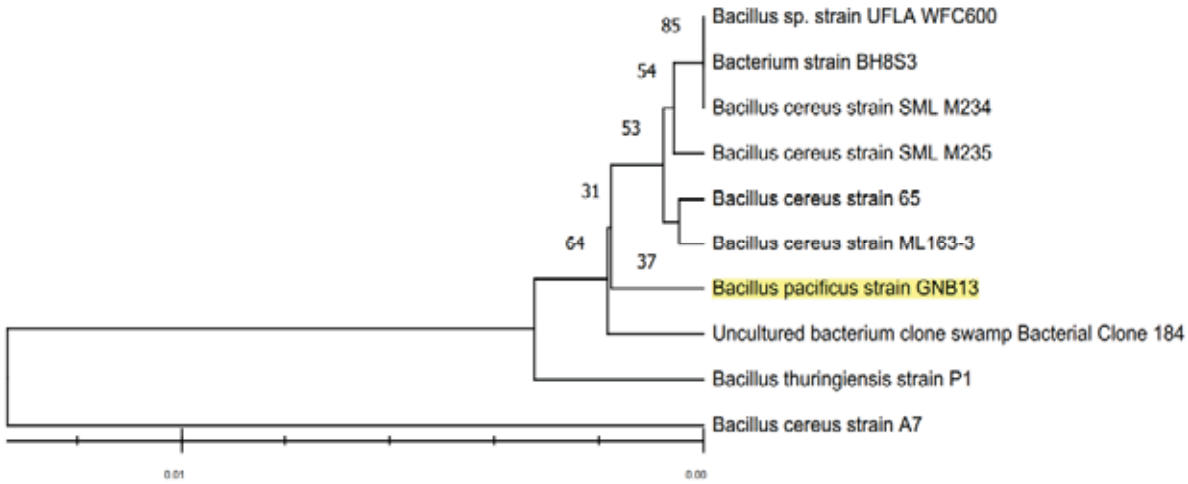


FIGURE 2
Phylogenetic tree derived from 16s rDNA sequence alignment, showing the location of strain FEB5. According to the tree, it belongs to *Bacillus pacificus*

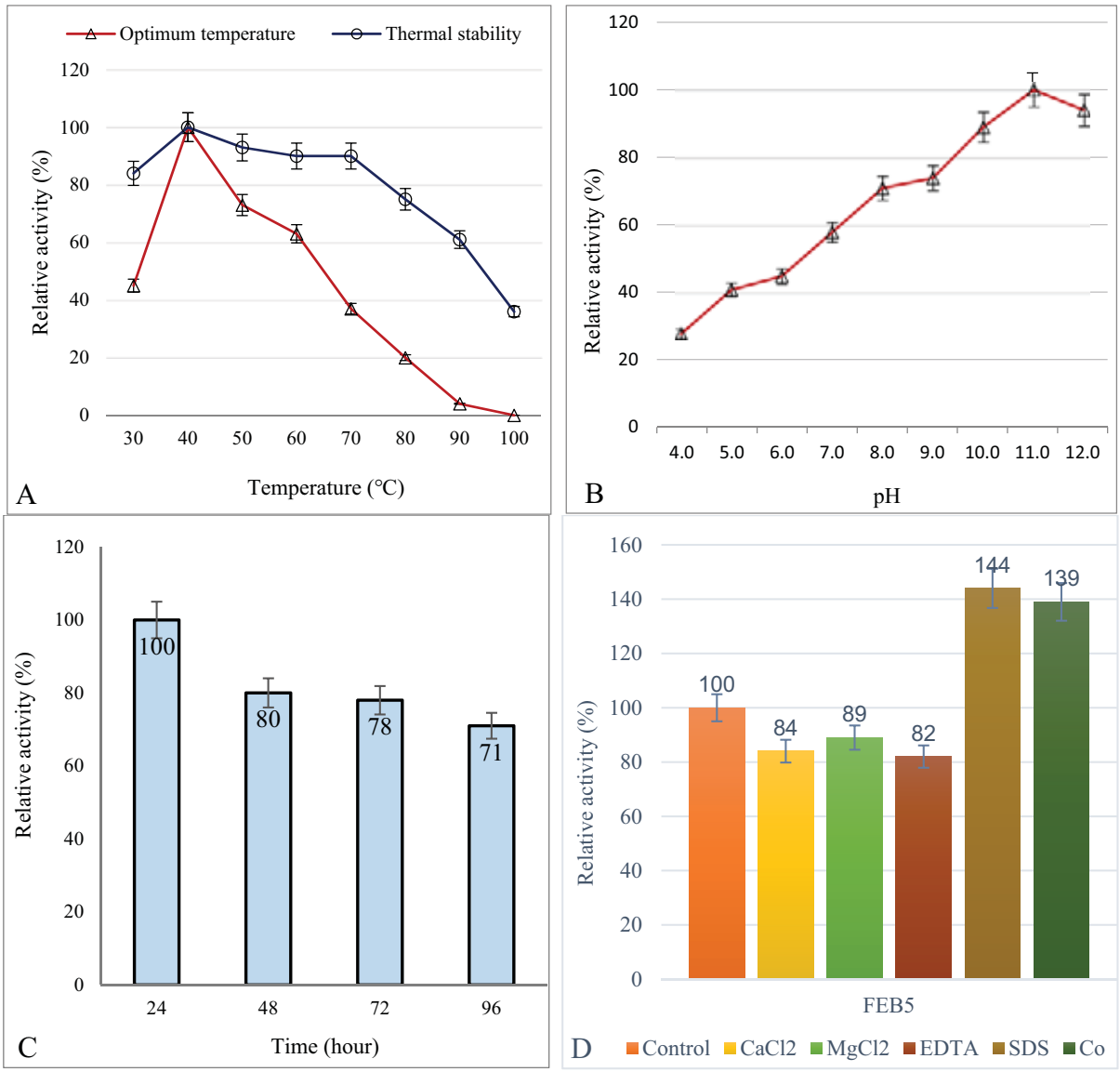


FIGURE 3
The effect of some factors on *B. pacificus* FEB5 α-amylase activity (A: Effect and stability of temperature, B: Effect of pH, C: Time dependent enzyme activity, D: Effect of various chemicals on the enzyme)

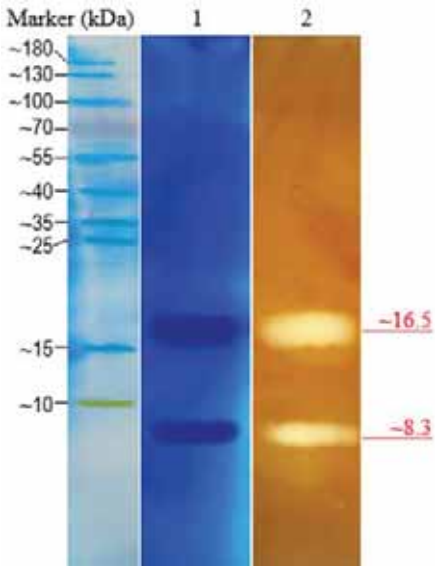


FIGURE 4
Comparison of the total proteins of isolates on SDS-PAGE and the zymogram analysis of the α-amylases (Marker: ThermoFisher Scientific, 26616)

B. pacificus FEB5 α-amylase showed its optimum activity at 40 °C (Figure 3A). Above this temperature, the enzyme activity significantly decreased and remained at the level of 73% and 63% at 50 and 60 °C, respectively. The relative activities at 70, 80 and 90 °C were 37%, 20% and 4%, respectively. The mean relative activity between 40 and 60 ℃ was calculated as 78.7%. While *B. pacificus* FEB5 α-amylase completely retained its activity after 15 min of pre-incubation at 40 °C, it showed 84, 93, 90, 90 and 75% remaining activity after 15 min of pre-incubation at 30, 50, 60, 70 and 80°C, respectively. The enzyme also showed 61% and 36% residual activity after pre-incubation at 90 and 100 ℃, respectively (Figure 3A). The mean relative activity between 30 and 80 ℃ was calculated as 88.7%.

B. pacificus FEB5 α-amylase showed its optimum activity at pH 11.0 (Figure 3B). While FEB5 α-amylase showed 94% relative activity at pH 12.0, it lost its activity rapidly at neutral and acidic pH values. The mean relative activity of the enzyme between pH 8.0 and 12.0 was calculated as 85.6%. On the other hand, the mean relative activity at the acidic pH values (pH 4.0-6.0), calculated as 38%.

B. pacificus FEB5 showed the maximum enzyme production at the inoculation time of 24 hours (Figure 3C). The α-amylase activity of the isolate was 80, 78 and 71% at 48, 72 and 96 hours, respectively.

SDS and Co were activators for both enzymes. SDS and Co increased FEB5 α-amylase by 44% and 39%, respectively. $CaCl_2$, $MgCl_2$, and EDTA showed inhibitory effects on the enzyme. While $CaCl_2$, $MgCl_2$, and EDTA partially inhibited FEB5 α-amylase by 16, 11, and 18%, respectively (Figure 3D).

The extracellular total proteins of *B. pacificus* FEB5 were studied by SDS-PAGE and SDS-Starch-PAGE. After being checked by SDS-PAGE and the zymogram analysis, the extracellular proteins exhibited two α-amylase bands (Isoform I and Isoform II) with the molecular weights of 16.5 and 8.3 kDa (Figure 4).

DISCUSSION

Strains of many mesophilic and thermophilic *Bacillus* species including *B. subtilis*, *B. amyloliquefaciens*, *B. stearothermophilus*, *B. licheniformis*, *B. cereus*, *B. thuringiensis*, *B. macerans*, *B. megaterium*, *B. coagulans*, *B. circulans*, *B. alvei*, and *B. globisporus* are α-amylases producers and they were well-studied [15]. Among these microorganisms, *B. stearothermophilus* and *B. amyloliquefaciens* bacteria have been well studied and characterized because they have been used in a wide range of industries as α-amylase source [16].

B. pacificus FEB5 which was isolated in the present study, was aerobic, spore-forming, and wild-type strain. The isolate was identified as genus *Bacillus* because it was germinated from spore forms under aerobic conditions [17].

The isolate showed its optimum activity at 40 ℃. It is known that bacterial amylases show variations in term of optimum temperature. The optimum temperature value of FEB5 α-amylase can be considered as a mesophilic value. *Bacillus* FEB5 is a mesophilic bacterium and showed its best growth at 37 ℃. Therefore, it should be considered normal for a mesophilic bacterium to produce enzymes with mesophilic character. However, in another study, it was reported that *Bacillus pacificus* α-amylase (amB.p) showed significant activity between 45-60 ℃, with maximal activity at 55 ℃, and at 80℃ the enzyme lost most of its activity [18]. Nevertheless, the number of studies on *B. pacificus* α-amylase is quite limited. Therefore, it will not be sufficient to compare our findings with those obtained from a single literature. Nusrat and Rahman [19] reported production of three amylases and growth of their producer *Bacillus* isolates, *B. subtilis*, *B. amyloliquefaciens* and *Bacillus* sp., both decreased drastically above 40 ℃, in a way that supports our finding. Several other investigators also reported similar findings, maximum α-amylase production from *Bacillus* strains at 35-37 ℃ [20, 21].

B. pacificus FEB5 α-amylase showed a significant residual activity after 15 min of pre-incubation between 30 and 90 °C. After pre-incubation at 90 ℃, the enzyme only 39% of its activity. Thermal stability is desirable in the industrial areas where heat treatment processes are used. Although it depends on the duration of the heat treatment, it is desirable for the enzymes to maintain their activity for a period of time under high temperature conditions. It was reported that half-lives of *Bacillus* α-amylases at 90 ℃

greatly increase in the series from *B. amyloliquefaciens* to *B. stearothermophilus* to *B. licheniformis* [22]. It was also obtained that presence of high concentrations of salts diminish the difference in thermostability between thermophilic and mesophilic α-amylases [22]. Another finding is NH_2 group existence in the amino acid sequence critical for thermal stability. In relation to that, findings of a previous study showed the lysine residues existence in the thermophilic but not in the mesophilic enzyme [22]. Determining the amino acid sequence of *B. pacificus* FEB5 α-amylase will help us understand the thermostability mechanism of the enzyme.

B. pacificus FEB5 showed its maximum activity at pH 11.0. This result shows that the enzyme has a highly alkali tolerance. An alkaline character α-amylase from *B. pacificus* also obtained with Alonazi et al. [18]. Pure amB.p was reported to be active in the pH range of 6.0-12.0, with maximal activity at pH 9.5 [18]. However, similar findings with our result were also reported for α-amylases from bacilli isolate [23], *Saccharoplyspora* A9 [24], *Bacillus* sp. isolate A3–15 [25], *Bacillus* sp. TSCVKK [26]. Different optimum pH values were reported for α-amylase, such as 5.0-8.0 [27], 6.0 [28], 6.0-9.0 [29], 8.0-11.0 [30], and 5.5-9.5 [31]. Isolation of microorganisms producing the alkaline enzymes are very important because their tremendous potentiality in several industries such as detergent [24]. Therefore, α-amylase from *B. pacificus* FEB5 strain may have wide spread applications in detergent, food and other industrial processes.

The production of α-amylase by the isolate starts at the beginning of the inoculation time reaching the maximum level after 24 h, and after that enzyme level decreased linearly from 24 h to 96 h. Our finding was in accordance to that reported by Abate et al [32] who obtained maximum α-amylase production after 24 h incubation of *B. amyloliquefaciens.* Alonazi et al [18] also reported the maximum α-amylase activity of *B. pacificus* at 24 h of incubation time but decreasing enzyme activity dramatically after that time. A possible reason for enzyme inactivation after 24 h might be due to release of intracellular proteases in the culture medium at the end of exponential phase [19].

$CaCl_2$, $MgCl_2$ end EDTA inhibited *B. pacificus* FEB5 α-amylase by 16, 11 and 18%, respectively. Ca^{2+} is necessary for the catalytic activities of α-amylase enzymes, but its binding strength to the enzyme varies depending on the enzyme source [33]. Although calcium is used to sustain or increase catalytic activities of α-amylases under extreme conditions, studies showing an inhibition effect on enzyme activity have also been reported. Alpha-amylases from *Geobacillus thermoleovorans* [34], *B. coagulans* B 49 [35], *B. licheniformis* AI20 [36], *Bacillus* sp. GA4 [37], and *Bacillus* strains ZF4 and A4 [38] were inhibited in the presence of calcium. Besides the primary Ca^{2+} binding sites, calcium ions also bind to the secondary sites, which involves catalytic residues (Asp and Glu) located at the bottom of substrate binding cleft [39]. It was reported that the presence of these secondary Ca^{2+} binding sites cautions the inhibition of α-amylase by Ca^{2+} at higher concentration [35]. Inhibition of α-amylases in the presence of $MgCl_2$ and EDTA were also reported by [31, 34, 37, 40]. However, Özdemir and Özcan [41] reported that $MgCl_2$ stimulated α-amylase activity by 13%, while EDTA inhibited it by 33%. The inhibition effect of EDTA on enzyme activities suggests that ZF4 and A4 α-amylases may be metalloenzymes [42]. Co and SDS stimulated *B. pacificus* FEB5 α-amylase by 39 and 44%, respectively. Xian et al. [40] reported that $CoCl_2$ with the concentrations of 1, 2.5, and 5 mM stimulated TpAA α-amylase at rates of 156.46%, 146.60, and 126.99%, respectively. Literature reviews indicate that increasing metal ion concentration causes a decrease in stimulation effect. The findings of Xian et al. [40] were confirmed our results. However, in another study, *Bacillus* sp BKL20 α-amylase was inhibited by 12 and 60% at the Co^{2+} concentrations of 1 mmol/L and 10 mmol/L, respectively [34]. This can probably be explained by the fact that the binding strength of the metal ion to the enzyme differs depending on the enzyme source, and the catalytic activities of the enzymes produce different results. The previous results from other researchers reveal that SDS usually shows inhibitory effect on α-amylases. The inhibitory effect of SDS on the enzyme may be due to the local conformational changes on the active site, partial reversible unfolding, and subsequent inactivation [43]. However, a SDS concentration of 0.1 stimulated the α-amylase activity of *B. subtilis* Clb-34 strain by 20% [44]. In two other studies, it was also reported that the use of 10 mM SDS stimulated α-amylase activity by 13% [45] and 110.39% [46]. These literature reports are similar to our results.

Zymogram analysis showed that *B. pacificus* FEB5 α-amylase produced two activity bands: Isoform-I and Isoform-II. The molecular weights of Isoform-I and Isoform-II were calculated as approximately 16.5 and 8.3 kDa, respectively. The molecular weight of bacterial alpha-amylases shows wide variation. In previous studies, it has been reported that the molecular weight of *Bacillus* sp. CT2 α-amylase is 38 kDa [47], *B. subtilis* α-amylase 67 kDa [44], *Nesterenkonia* sp F α-amylase 100 and 106 kDa, [48], and *Pediococcus ethanolidurans* α-amylase ~150 kDa [49]. Febriani et al [50] reported the molecular weight of α-amylase from *Geobacillus* sp. nov. as 12.2 kDa which very similar to our findings. Beside, Maitiy et al [51], and Sahnoun et al [52] were reported isoforms of α-amylases from *B. thuringiensis* CKB19 and *Aspergillus oryzae* S2 strains, respectively. Ballschmiter et al [53] also reported the segregation of two different α-amylases from *Anaerobranca gottschalkii*, having different optimal temperatures and pH. The appearance of two bands with

different molecular weights in the zymogram analysis can be explained either by the presence of two different amylase genes in the genome of the microorganism [51], or by the proteolytic degradation of a single enzyme [52]. This can only be revealed by detailed analysis of two separate fractions.

CONCLUSION

In the present study, mesophilic *Bacillus* sp. FEB5, which is capable of producing α-amylase, was isolated and studied. According to phylogenetic tree, it belongs to *Bacillus pacificus*. Research findings suggest that highly alkali tolerant enzyme could be good candidate for applications in detergent, food and other industrial processes.

ACKNOWLEDGEMENTS

The authors are grateful to the Scientific Projects Coordination Unit of Çukurova University (BAP Project No: FBA-2019-10474) for funding program.

REFERENCES

[1] Yamashita, I. (1994) Starch-processing enzymes produced by recombinant yeasts and fungi. In: Recombinant Microbes for Industrial and Agricultural Applications, Murooka, Y. and Imanaka, T. (Eds). Marcel Dekker Inc., New York. 341-357.

[2] Nielsen, J.E., Borchert, V. (2000) Protein engineering of bacterial α-amylases. Biochimica et Biophysica Acta. 1543, 253-274.

[3] Asgher, M., Asad, M.J., Rahman, S.U., Legge, R.L. (2007) A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. Journal of Food Engineering. 79, 950-955.

[4] Yamagata, H., Udaka, S. (1994) Starch-processing enzymes produced by recombinant bacteria. In: Recombinant Microbes for Industrial and Agricultural Applications, Murooka, Y. and Imanaka, T. (Eds). Marcel Dekker Inc., New York. 325-340.

[5] Haki, G.D., Rakshit, S.K. (2003) Developments in industrially important thermostable enzymes: A review. Bioresource Technology. 89, 17-34.

[6] Adeyanju, M.M., Agboola, F.K., Omafuvbe, B.O., Oyefuga, O.H., Adebawo, O.O. (2007) A thermostable extracellular α-amylase from *Bacillus licheniformis* isolated from cassava steep water. Biotechnology. 6(4), 473-480.

[7] Hagenimana, V., Vezina, L.P., Simard, R.E. (1992) Distribution of amylase within sweet potato (*Ipomea batatas* L.) root tissue. Journal of Agricultural and Food Chemistry. 40, 1777-1783.

[8] Demirkan, E. (2011) Production, purification, and characterization of α-amylase by *Bacillus subtilis* and its mutant derivates. Turkish Journal of Biology. 35, 705-712.

[9] Luang-In, V., Yotchaisarn, M., Saengha, W., Udomwong, P., Deeseenthum, S., Maneewan, K. (2019) Isolation and Identification of Amylase-Producing Bacteria from Soil in Nasinuan Community Forest, Maha Sarakham, Thailand. Biomedical and Pharmacology Journal. 12(3), 1061-1068.

[10] Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 31, 426-428.

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

[12] Lo, H.F., Lin, L.L., Chen, H.L., Hsu, H.H., Chang, C.T. (2001) Enzyme properties of a SDS-resistant *Bacillus* sp. TS-23 α-amylase produced by recombinant *Escherichia coli*. Process Biochemistry. 36, 743-750.

[13] Laemmli U.K. (1970) Cleauage of structural proteins during the assembly of the head of the bacteriophage T4. Nature. 227, 680-685.

[14] Ikeda T., Yamazaki H., Yamashita K., Shinke R. (1992) The tetracycline inducible expression of alpha-amylase in *Bacillus subtilis*. Journal of Fermentation and Bioengineering. 74, 58-60.

[15] Claus, D., Berkeley, R.C.W. (1986) The genus *Bacillus*. In: Bergey's Manual of Systematic Bacteriology, Sneath, P.H.A., Mair, N.S., Sharpe, M.E., Holt, J.G. (Eds). Williams and Wilkins, Baltimore. 1105-1139.

[16] Ozcan, B.D., Baylan, M., Ozcan, N., Tekdal, D. (2010) Characterization of thermostable α-amylase from thermophilic and alkaliphilic *Bacillus* sp. Isolate DM-15. Journal of Biological Sciences. 5(1), 118-124.

[17] Remize, F. (2017) Spore-forming bacteria. In: The Microbiological Quality of Food. Bevilacqua, A., Corbo, M.R., Sinigaglia, M. (Eds). Elsevier. ISBN: 978-0-08-100502-6.

[18] Alonazi, M., Karray, A., Badjah-Hadj-Ahmed, A.Y., Bacha, A.B. (2021) Alpha amylase from *Bacillus pacificus* associated with brown algae *Turbinaria ornata*: Cultural conditions, purification, and biochemical characterization. Processes. 9(16), 1-13.

[19] Nusrat, A., Rahman, S.R. (2007) Comparative studies on the production of extracellular α-amylase by three mesophilic *Bacillus* isolates. Bangladesh Journal of Microbiology. 24(2), 129-132.

[20] Bajpai, P., Bajpai, P.K. (1989) High temperature alkaline α-amylase from *Bacillus licheniformis* TCRDC-B13. Biotechnology and Bioengineering. 33(1), 72-78.

[21] Sarikaya, E., Gürgün, V. (2000) Increase of the α-amylase yield by some *Bacillus* strains. Turkish Journal Biology. 24, 299-308.

[22] Tomazic, S.J., Klibanov, A.M. (1988) Why is one *Bacillus* α-amylase more resistant against irreversible thermoinactivation than another? The Journal of Biological Chemistry. 263(7), 3092-3096.

[23] Akkaya, B., Yenidunya, F.Y., Akkaya, R. (2012) Production and immobilization of a novel thermoalkalophilic extracellular amylase from bacilli isolate. International Journal of Biological Macromolecules. 50(4), 991-995.

[24] Chakraborty, S., Khopade, A., Biao, R., Jian, W., Liu, X.-Y., Mahadik, K., Chopade, B., Zhang, L., Kokare, C. (2011). Characterization and stability studies on surfactant, detergent and oxidant stable α-amylase from marine haloalkaliphilic *Saccharopolyspora* sp. A9. Journal of Molecular Catalysis B: Enzymatic. 68(1), 52–58.

[25] Arikan, B. (2008). Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic *Bacillus* sp. isolate A3-15. Bioresource Technology. 99(8), 3071–3076.

[26] Kondepudi, K.K., Chandra, T.S. (2008). Production of surfactant and detergent-stable, halophilic and alkalitolerant α-amylase by a moderately halophilic *Bacillus* sp. strain TSCVKK. Applied Microbiology and Biotechnology. 77(5), 1023–1031.

[27] Whitford, J. (1998) Mechanisms and applications of enzymatic thermostability. WWU Honors Program Senior Projects. 333. https://cedar.wwu.edu/wwu_honors/333 (Accessed date: 18 December 2021).

[28] Mamo, G., Gashe, A., Gessese, A. (2001) A highly thermostable amylase from a newly isolated thermophilic *Bacillus* sp. WN11. Journal of Applied Microbiology. 86(4), 557-560.

[29] Dash, B.K., Rahman, M.M., Sarker, P.K. (2015) Molecular identification of a newly isolated *Bacillus subtilis* BI19 and optimization of production conditions for enhanced production of extracellular amylase. BioMed Research International. 9.

[30] Mathew, C.D., Rathnayake, S. (2014) Isolation and characterization of alpha amylase isolated from a hot water spring in Sri Lanka. International Research Journal of Microbiology. 5(4), 50-61.

[31] de Carvalho, R.V., Correa, T.L.R., da Silva, J.C.M., de Oliveira Mansur, L.R.C., Martins, M.L.L. (2008) Properties of an amylase from thermophilic *Bacillus* sp. Brazilian Journal of Microbiology. 39, 102-107.

[32] Abate, C.M., Castro, G.R., Sineriz, F., Callieri, D.A.S. (1999) Production of amylolytic enzymes by *Bacillus amyloliquefaciens* in pure culture and in co-culture with *Zymomonas mobilis*. Biotechnology Letters. 21, 249-252.

[33] Deljou, A., Arezi, I. (2016) Production of thermostable extracellular a-amylase by a moderate thermophilic *Bacillus licheniformis*-AZ2 isolated from Qinarje Hot spring (Ardebil prov. of Iran). Periodicum Biologorum. 118(4), 405-416.

[34] Kubrak, A.I., Storey, J.M., Storey, K.B., Lushchak, V.I. (2010) Production and properties of a-amylase from *Bacillus* sp. BKL20. Canadian Journal of Microbiology. 56, 279–288.

[35] Mehta, D., Satyanarayana, T. (2013) Biochemical and molecular characterization of recombinant acidic and thermostable raw starch hydrolysing α-amylase from an extreme thermophile *Geobacillus thermoleovorans*. Journal of Molecular Catalysis B: Enzymatic. 85–86, 229–238.

[36] Babu, K.R., Satyanarayana, T. (1993) Extracellular calcium-inhibited α-amylase of *Bacillus coagulans* B 49. Enzyme and Microbial Technology. 15, 1066–1069.

[37] Abdel-Fattah, Y.R., Soliman, N.A., El-Toukhy, N.M., El-Gendi, H., Ahmed, R.S. (2013) Production, purification, and characterization of thermostable *α*-amylase produced by *Bacillus licheniformis* Isolate AI20. Journal of Chemistry. 2013, 11.

[38] Ozdolap, M., Ozcan, B.D. (2022) Partial characterization of alpha-amylase enzymes produced by *Bacillus* isolates. Fresen. Environ. Bull., 31, 2259-2266.

[39] Mehta, D., Satyanarayana, T. (2016) Bacterial and archaeal α-amylases: Diversity and amelioration of the desirable characteristics for industrial applications. Frontier in Microbiology. 7, 1129.

[40] Xian, L., Wang, F, Luo, X., Feng, Y-L., Feng, J-X. (2015) Purification and characterization of a highly efficient calcium-independent α-amylase from *Talaromyces pinophilus* 1-95. PLoS One. 10(3), e0121531.

[41] Özdemir, G., Özcan, B.D. (2020) Isolation of thermostable α-amylase producing mesophilic bacterium and partial characterization of the enzyme. OKU Journal of Natural and Applied Sciences. 3(2), 75-82.
[42] Fincan, S.A., Enez, B., Özdemir, S., Bekler, F.M. (2014) Purification and characterization of thermostable -amylase from thermophilic *Anoxybacillus flavithermus*. Carbohydrate Polymers. 102, 144-150.
[43] Sahoo, S., Roy, S., Maiti, S. (2016) A high salt stable α-amylase by *Bacillus* sp. MRS6 isolated from municipal solid waste; purification, characterization and solid state fermentation. Enzyme Engineering. 5(3), 152.
[44] Tiwari, S., Gaur, R. (In press) A surfactant, oxidant and inhibitor compatible thermo-solvent-tolerant amylase from a novel extrimophilic *Bacillus subtilis* strain Clb-34 Mk443366: Study on purification and characterization. Research Square.
[45] Caf, Y., Valipour, E., Arikan, B. (2014) Isolation and characterization of alkalin, halotolerant, detergent-stable and cold-adaptive α-amylase from a novel isolate *Bacillus* sp. Calp12-7. International Journal of Current Microbiology and Applied Sciences. 3(4), 950-960.
[46] Hauli, I., Sarkar, B., Mukherjee, T., Mukhopadhyay, S.H. (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(5), 202-212.
[47] Türker, C., Özcan, B.D. (2015) Isolation of alpha-amylase producing thermophilic *Bacillus* strains and partial characterization of the enzymes. Turkish Journal of Agriculture - Food Science and Technology. 3(6), 387-393.
[48] Shafiei, M., Ziaee, A-A., Amoozegar, M.A. (2010) Purification and biochemical characterization of a novel SDS and surfactant stable, raw starch digesting, and halophilic a-amylase from a moderately halophilic bacterium, *Nesterenkonia* sp. strain F. Process Biochemistry. 45, 694-699.
[49] Iuchi, A., Haruguchi, S., Mongkolthanaruk, W., Arima, J., Nagase, M., Khanh, H.Q., Ichiyanagi, T., Yamaguchi, T., Shimomura, N., Aimi, T. (2012) Characterization of novel amylase from amylolytic lactic acid bacteria *Pediococcus ethanolidurans* isolated from Japanese pickles (Nuka-zuke). Food Science and Technology Research. 18(6), 861-867.
[50] Febriani, Rayyana, Ulya, M., Oesman, F., Akhmaloka, Iqbalsyah, T.M. (2019) Low molecular weight alkaline thermostable α-amylase from *Geobacillus* sp. nov. Heliyon. 5(7), e02171.
[51] Maity, C., Samanta, S., Halder, S.K., Das Mohapatra, P.K., Pati, B.R., Jana, M., Mondal, K.C. (2011) Isozymes of α-amylases from newly isolated *Bacillus thuringiensis* CKB19: Production from immobilized cells. Biotechnology and Bioprocess Engineering. 16, 312-319.
[52] Sahnoun, M., Jemli, S., Trabelsi, S., Ayadi, L., Bejar, S. (2016) *Aspergillus oryzae* S2 α-amylase domain C involvement in activity and specificity: In vivo proteolysis, molecular and docking studies. PLos One. 11(4), e0153868.
[53] Ballschmiter, M., Armbrecht, M., Ivanova, K., Antranikian, G., Liebl, W. (2005) AmyA, an α-Amylase with β-cyclodextrin-forming activity, and AmyB from the thermoalkaliphilic organism *Anaerobranca gottschalkii*: Two α-amylases adapted to their different cellular localizations. Applied and Environmental Microbiology. 71(7), 3709-3715.

Received: **22.12.2021**
Accepted: **24.02.2022**

CORRESPONDING AUTHOR

Bahri Devrim Ozcan
Animal Biotechnology and Genetic Engineering Laboratory,
Department of Animal Science,
Faculty of Agriculture,
University of Cukurova,
01330 Saricam Adana – Turkey

e-mail: bdozcan@gmail.com