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ORIGINAL ARTICLE

Isolation, Identification and Mutation of a Novel Native Alkaline and Thermophile *Bacillus* Sp. V-41, Containing α-Amylase Activity

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ABSTRACT
Alkaline, halophilic and thermophilic Bacillus sp. strain V-41, which produces extracellular α-amylase was isolated from Van Lake area located in Turkey. Characterization of strain V-41 by 16S rRNA gene analysis found it closest member of Bacillus and related to uncultured bacterium clone HAV7D9G02CMKTO with a similarity of >97%. This bacterial isolate was mutagenized by treatment with Ethidium bromide (EtBr) and 4 mutant variants, V41-M1, V41-M2, V41-M3, and V41-M4 were obtained. The mutant α-amylase from V41-M4 displayed more than 91% activity according to the wild type enzyme. The α-amylase activities from Bacillus sp. V-41, and its mutant variants V41-M1, V41-M2, V41-M3 and V41-M4 were 0.65, 0.84, 0.73, 0.6 and 1.27 U mL^{-1} min^{-1}, respectively. Bacillus sp. wild type strain V-41 and its mutant variant V41-M4 were selected for partial characterization. Maximum α-amylase productions were achieved at the end of 60 and 48 h of growth for V-41 and V41-M4, respectively. The optimum temperatures of the V-41 and V41-M4 α-amylases were found to be 60 and 40 °C, respectively. The optimum pH of the both α-amylases was found to be 7.5. An analysis of the enzyme for molecular mass was carried out by SDS-Starch-PAGE electrophoresis revealed a single band with molecular weight of 70 kDa for wild type and mutant variants.
***Key words**: Isolation, Bacillus, alkaline, α-amylase, Ethidium bromide*

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INTRODUCTION

The use of microbial enzymes in industrial areas increases more and more because of its economical production and immobilization of unsolvable materials in water and durable use in respect to biotechnological activities [1]. Among the microorganisms, *Bacillus* species are good secretors of extracellular enzymes such as amylase, arabinase, cellulase, lipase, protease, and xylanase which play important roles in many biotechnological processes [2]. For applications in industrial processes, the enzymes should be stable at high temperature, pH, presence of salts, solvents, toxicants etc. [3]. α-Amylases (endo-1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) are one of the most important groups of industrial enzymes which hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units [4, 5]. They find applications in the sugar, baking, brewing, paper, textile, distilling industries [6]. The key advantages of using microorganisms for production of amylases are theirs economical huge production capacity and these microbes are also easy to manipulate to obtain enzymes of desired characteristics [7]. α-Amylases have been developed from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have important applications in industrial areas [8]. Nevertheless, bacterial α-amylases particularly *Bacillus* amylases are more desirable according to fungal α-amylases because of their heat stability [9]. The most plentiful used bacterial α-amylases were derived from *B. amyloliquefaciens*, *B. licheniformis* and *B. stearothermophilus* [10]. Most of the strains used for enzyme production have been improved cloning

or mutation methods. UV and chemicals such as ethyl methyl sulfonate (EMS), nitrous acid, N-methyl-N´-nitro-N-nitrosoguanidine (NTG) and Ethidium bromide (EtBr) were found to be suitable mutagens for the improvement of α-amylase production by *Bacillus* and obtained mutants have a higher capacity for amylase production [11, 12, 13]. The aims of this study were isolation, identification of *Bacillus* sp. strain with α-amylase activity and improvement of the enzyme production with mutation by Ethidium bromide.

MATERIALS AND METHODS

Microorganism and culture conditions

Bacillus sp. V-41 was isolated from coast sediment samples collected from Van Lake, Turkey. To select the Gram-positive spore-forming bacteria *Bacillus* sp., soil sample was incubated at 80 °C for 10 min [14]. The isolates were cultivated in LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 9.0) for 24 h at 55 °C with shaking at 200 rpm. The isolates screened for α-amylase activity on LB-agar-starch plates containing (g L^{-1}) tryptone 10, yeast extract 5, NaCl 10, starch 5, agar 15 (pH 9.0) at 55°C [15]. -Amylase positive colonies were selected with iodine staining [16].

Molecular characterization

To determine the 16S rRNA sequences, each strain's genomic DNA was extracted by described previously [17]. To confirm the identities of the isolates, PCR amplification and sequencing of the 16S rRNA gene were done. The 16S rRNA genes were PCR-amplified from the genomic DNA using the bacterial universal primer set of 518F: (5'-CCAGCAGCCGCGGTAATACG-3') and 800R: (5'-TACCAGGGTATCTAATCC-3'), which were also used for sequencing [18, 19]. The PCR reaction mixture included of 5 µL of 10× PCR reaction buffer, 1 µL of 40 mM dNTP mix (200 µM each final), 1 µL each of forward and reverse primers (20 pmol each primer), 0.5 µL of Taq DNA polymerase (5 U/µL), and 3 µL of genomic DNA template in a total volume of 25.0 µl. The following amplification program was used: initial denaturation step at 95 °C for 15 min, which was followed by 30 cycles of denaturation at 95 °C for 35 sec, primer annealing at 55 °C for 35 sec, extension at 72 °C for 35 sec. A final extension step was performed as 72 °C for 5 min. The amplified PCR products were analyzed by 2% (w/v) agarose gel. The sequence of 16S rRNA (1468 bp) was aligned by using the BLAST program to identify the most similar sequence in the database [20]. 16S rDNA sequences of different strains of *Bacillus subtilis* and its phylogenetically related species and genera were downloaded from GenBank database (http://www.ncbi.nlm.nih.gov/entrez) and aligned to construct a neighbor-joining phylogenetic tree using Clustal W algorithm with the help of MEGA software version 4.1 [21].

Mutation procedures

The mutation procedures performed as described by previously [22]: Day 1: The overnight cultured isolate was diluted by sterile deionize water and then spreaded on LB-agar plates by glassy stick. The Ethidium bromide solution (20 mg/ml) was dropped to the pre-marked points on the plate with a micro-pipette. The surfaces of plates were dried for 15-20 minutes and then incubated for overnight at 55°C. Day 2: The colonies around the toxic zones (Ethidium bromide dropped points) were picked onto new LB-agar plates containing soluble starch (0.5% w/v) with sterile toothpicks. The plates were incubated at 55 °C for 24 h. Day 3: The plates were stained by iodine and 4 mutant variants were selected according to activity zone diameters. All mutant variants were stored in sterile glycerol (10% v/v glycerol) at -20 °C for further studies.

Enzyme production

The wild type and mutant variants were grown up in LB medium at 55 °C with shaking at 200 rpm for 24 h. After removal of cells by centrifugation (Hitachi Universal EBA12) (5,000 rpm, 10 min), the supernatant was used for enzyme assay.

Enzyme assay

α-Amylases were assayed by adding 1 mL enzyme to 1 mL soluble starch (%2 w/v) in 50 mM tris buffer (pH 9.0) and incubated at 55 °C for 30 min. The reaction was stopped by adding 3 ml of 3,5-dinitrosalicylic acid reagent and $A_{540\ nm}$ was measured in a Pharmacia spectrophotometer [23]. One enzyme unit is defined as the amount of enzyme releasing 1 mmol of glucose from the substrate in 1 min at 55 °C.

Protein determination

Proteins of wild type and mutant variants were estimated as described by Lowry et al. [24] using bovine serum albumin as the standard.

Effect of incubation period

The effect of incubation period was determined by assaying the enzyme activity in different incubation periods (12, 24, 36, 48, 60, and 72 h).

Effect of pH and temperature on enzyme activity

Temperature and pH effects on enzyme activity were assayed at different temperatures ranging from 30 to 100 ºC and at pH values ranging from 6 to 11 for 30 min. Following buffers were used in the reactions: 50 mM Na-phosphate (pH 6-7) and 50 mM Tris (pH 7-11) [25].

Statistical analyses

The tests were performed in four replications. Standard error was calculated using the statistical software SPSS version 19.0. All analyses were performed at $p \leq 0.05$.

SDS–PAGE and zymogram analysis

SDS-PAGE and SDS-Starch-PAGE (0.2% wt/v starch) were done as described by Laemmli [26] with slab gels (12% wt/v acrylamide). After the electrophoresis, the gel was stained for 1 hour with Coomassie blue R 250 dye in methanol–acetic acid–water solution (4:1:5, by volume) and destained in the same solution without dye [25]. For zymogram of amylase activity by SDS-Starch-PAGE, SDS was removed by washing the gel at room temperature in solutions containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol 40% v/v for 1 h and 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2) for 1 h, respectively. Renaturation of enzyme was carried out by keeping the gel overnight in a solution containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), 5 mM β-mercaptoethanol and 1 mM EDTA at 4 ºC. Gel was then transferred onto a glass plate, sealed with film, and incubated at 55 ºC for 4 h. Gel was stained in a solution of iodine (iodine 5 g/l, KI 50 g/l), for 30 min, clear band indicate the presence of amylase activity [27, 25]. The molecular mass of the enzyme was finally estimated from the position of standard proteins.

RESULTS

The alkaline, halophilic and thermophilic strain *Bacillus* sp. V-41 producing extracellular α-amylase was isolated from Van Lake located in Eastern of Turkey. The bacterium was Gram positive, rod shaped, spore forming and aerobic. The growth observed between pH 6.0-11.0 (with optimal pH of 7.5) in presence of NaCl (5% wt/v) and occurred up to 55 °C.

Identification of the strain and 16S rRNA analysis

The isolate V-41 was identified according to the 16S ribosomal RNA genetic analysis. BLAST analysis of the strain revealed that it had a closest match (97%) with uncultured *Bacillus* sp. strain HAV7D9G02CMKTO (Figure. 1 and Figure 2).

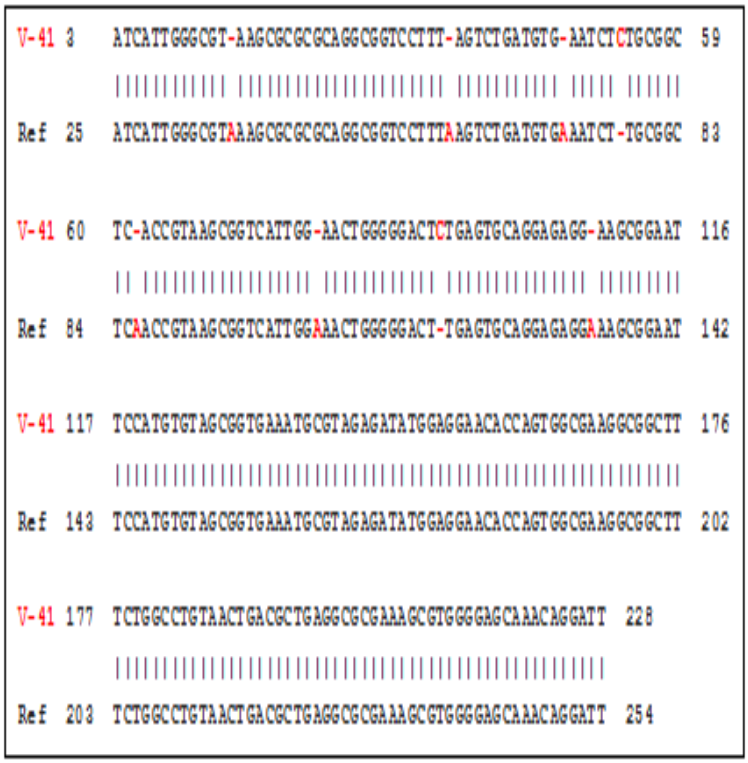

Figure1. Comparison of the 16S rRNA genes from *Bacillus*sp V-41 and uncultured *Bacillus* sp. strain HAV7D9G02CMKTO

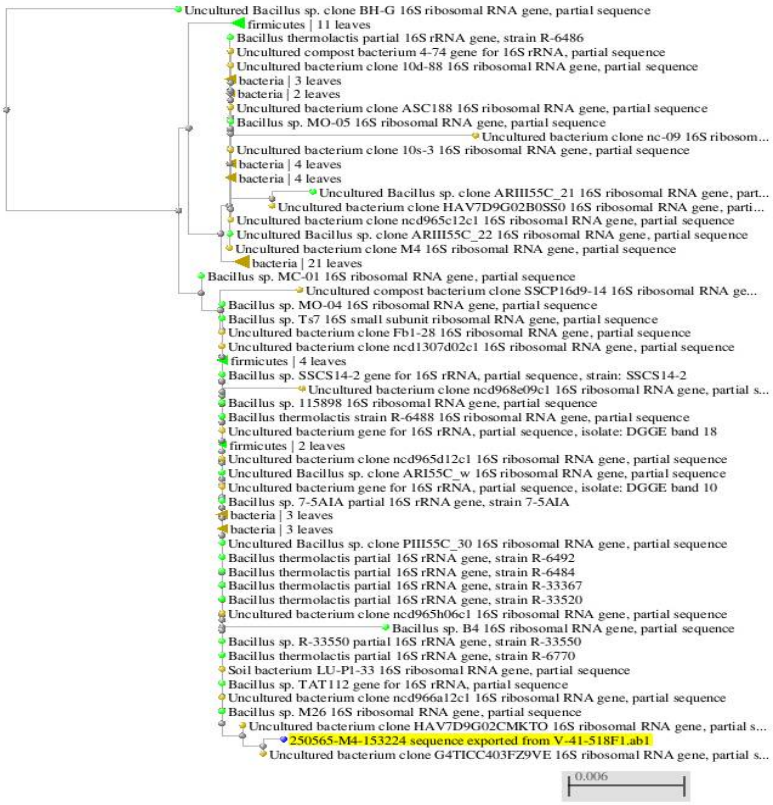

Figure 2.Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of *Bacillus* sp. V-41 and some of its closest phylogenetic relatives.

The mutation tests on the V-41 isolate

After processing of *Bacillus* sp. V-41 by EtBr, four mutant variants (V41-M1, V41-M2, V41-M3, and V41-M4) were obtained. According to diameters of amylase zones on LB-agar plates, mutant variants were compared with wild type strain. With these results, mutant variant V41-M4 was selected for further enzymatic analysis.

Enzyme properties

While specific enzyme activity of *Bacillus* sp. V-41 was 0.65 nmol/mg protein/min, after treatment by EtBr, specific enzyme activities were increased to 0.84, 0.73, and 1.27 nmol/mg protein/min for mutant variants V41-M1, V41-M2, and V41-M4, respectively.

α-Amylase production increased in mutant variants V41-M1, V41-M2, and V41-M4 up to 30.90, 12.73, and 90.90%, respectively (Figure 3). Productions of α-amylases at different time intervals were investigated. The *Bacillus* sp. V-41 and V41-M4 cultures were incubated at 55 °C for 12, 24, 36, 48, 60, and 72 hours. Maximum enzyme productions were recorded after 60 and 48 h at 55 °C for V-41 and V41-M4 α-amylases, respectively (Figure 4).

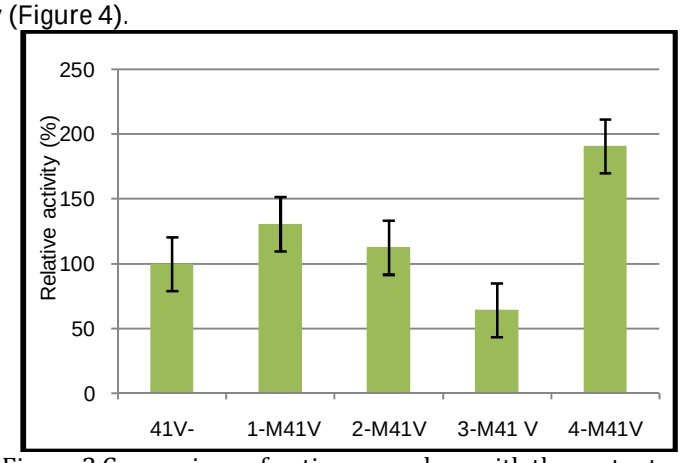

Figure 3.Comparison of native α-amylase with the mutant α-amylases

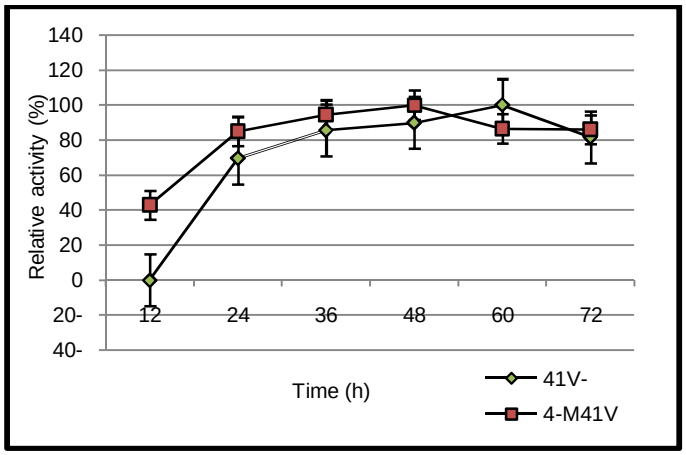

Figure 4.Rate of α-amylase production

The optimum enzyme activities were observed at 60 and 40 °C and at pH 8.0 and 7.5 for V-41 and V41-M4 α-amylases, respectively (Figure 5 and Figure 6). Both enzymes showed similar graphical properties for pH and temperature. Both enzyme activities were decreased after 60 °C after incubation for 30 min.

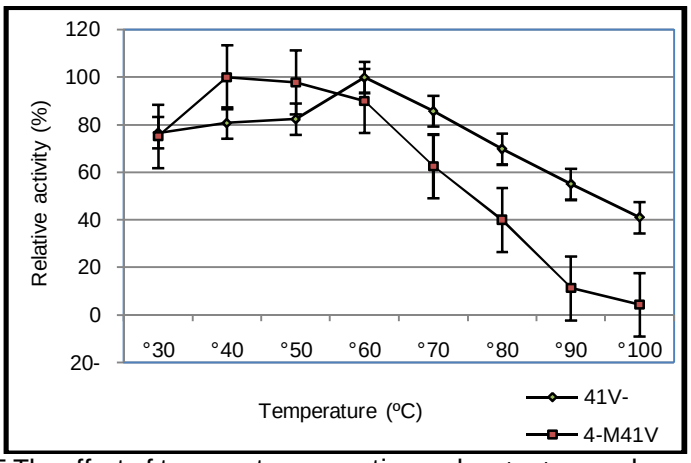

Figure 5.The effect of temperature on native and mutant α-amylases activity

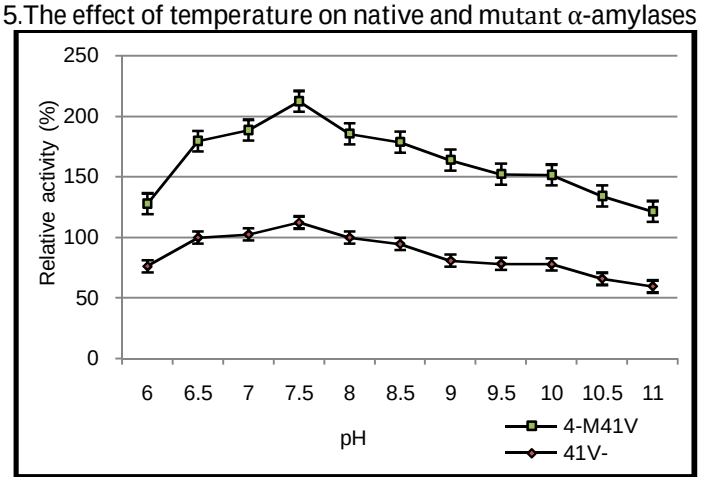

Figure 6. The effect of pH on native and mutant α-amylases activity

Determination of molecular weight

Molecular weights of wild type and mutant α-amylases determined by SDS-Starch-PAGE electrophoresis revealed single band showing α-amylase activity in gel using BioCapt MW software. The molecular mass of bands was 70 kDa (Figure 7).

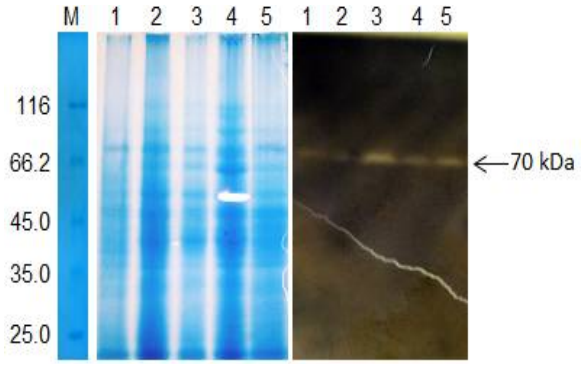

Figure7.Zymogram analysis of α-amylases on SDS-PAGE. The gel was cut into two pieces, the marker and total proteins were visualized with Coomassiebrillant blue staining and the activity of enzyme revealed by iodine (M: Marker, 1: Wild type strain V-41 protein samples, 2-5: Mutant variants protein samples from V41-M1 to V41-M4, respectively).

DISCUSSION

In the present study, a total of 5 soil samples were collected from Van Lake of Turkey and used for isolation of Gram (+), spore forming, and aerobic bacterial strains. About 150 strains were isolated and screened for α-amylase activity. Among these isolates, 50 bacteria showed amylolytic activity on LB-agar plate containing starch. The *Bacillus* sp. isolate V-41 selected for mutation and further studies because of its maximum amylolytic hollow zone around the colony. α-Amylases from alkaline and thermophilic *Bacillus* species were reported previously [28, 29, 30, 31, 25, 32]. Most of the *Bacillus* strains used commercially for the production of α-amylases have an optimum pH between 6.0 and 9.0 for growth and enzyme production [25, 33]. The strain *Bacillus* sp. V-41 was improved for α-amylase production. The chemical mutagen Ethidium bromide (EtBr) was used for mutation of the bacterial strain. The mutant *Bacillus* sp. V41-M4 gave 1.27 U mL^{-1} min^{-1} α-amylase which was around 2 fold higher than the parental strain. Several researchers have employed mutagenesis for α-amylase production by exposing the cultures with UV or chemicals like EMS, nitrous acid and EtBr [34, 35, 36]. The optimum incubation periods for our native enzyme and its mutant variant were 60 and 48 h, respectively. Besides, the native and mutant enzymes showed 70 and 85% of their activity after 24 h, 86 and 95% of their activity after 36 h incubation, respectively. These incubation periods are acceptable short than other bacteria and fungi and they offer unique potential for inexpensive enzyme production. The optimum pH values for native and mutant amylases were 8.0 and 7.5, respectively. Both enzymes were active at slightly acidic and alkaline pH, with a range of pH activity (pH 6.0-11.0). The optimal temperature values for enzyme activity were 60 and 40°C for native and mutant enzymes, respectively. These pH and temperature values are similar to *Bacillus licheniformis* and *Gracilibacillus* [37], *Bacillus* sp. GUF8 [38], *Halomonas* sp. AAD21 [6], *Bacillus cereus* MS6 [40] enzymes. When the enzymatic properties were compared, it was clear that the temperature profile of our mutant enzyme was different from those of the known *Bacillus* α-amylases. The optimal temperatures of most bacterial α-amylases, including those from *Bacillus* sp. V-41 are in the range of 50-90 ºC, but the activity of mutant enzyme is significantly decreased at temperatures lower than 50 ºC. It was reported that the broad range of temperatures and the enzyme's high activity at both moderate and lower temperature values make enzymes highly attractive for both basic research studies and industrial processes [38]. According to the 16S rDNA sequences the strain V-41 belonged to uncultured *Bacillus* strain HAV7D9G02CMKTO with the similarity of 97%. The molecular weight of α-amylase was 70 kDa on SDS-PAGE. Similar findings between 55 kDa and 75 kDa have been reported earlier (32; 41; 42; 43; 44; 45). These differences of molecular weights of α-amylases depend on the genes from the organisms.

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