Improvement of Enzyme Activity of a Novel Native Alkaline and Thermophile Bacillus sp. CU-48, Producing α-Amylase and CMCase by Mutagenesis

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ABSTRACT
Alkaline, halophilic and thermophile Bacillus sp. strain CU-48, which produces extracellular α-amylase was isolated from Çukurova University campus area located in Turkey. Characterization of strain CU-48 by 16S rRNA gene analysis found it closest member of Bacillus thermolactis with a similarity of >98%. This bacterial isolate was mutagenized by treatment with Ethidium bromide (EtBr) and 4 mutant variants, CU48-M1, CU48-M2, CU48-M3, and CU48-M4 were obtained. According to the results, all mutant variants lost CMCase activities in compare to wild type strain. The mutant α-amylase from CU48-M2 displayed more than 101% activity according to the wild type enzyme. The specific α-amylase activities from Bacillus sp. CU-48, and its mutant variants CU48-M1, CU48-M2, CU48-M3, and CU48-M4 were 1.67, 3.15, 4.4, 1.95 and 2.02 U mL⁻¹ min⁻¹, respectively. Bacillus sp. wild type strain CU-48 and its mutant variant CU48-M2 were selected for partial characterization. Maximum α-amylase productions were achieved at the end of 24 and 12 h of growth for CU-48 and CU48-M2, respectively. The optimum temperature and pH values of the CU-48 and CU-48-M2 α-amylases were found to be 60 and 50 °C, and 7.0 and 8.0, respectively. An analysis of the enzyme for molecular mass was carried out by zymogram analysis revealed a single band with molecular weight of 45 kDa for wild type and mutant variants.

Keywords: Bacillus, thermophile, alkaline, halophile, α-amylase, carboxymethylcellulase, isolation, characterization, mutagenesis, Ethidium bromide

INTRODUCTION
The use of microbial enzymes in industrial areas increases more and more because of its economical production and immobilization of unsoluble 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. amyloquefaciens, B. licheniformis and B.
_Bacillus_ sp. strain with α-amylase and cellulase activities and improvement of the enzyme production with mutation by Ethidium bromide.

**MATERIALS AND METHODS**

**Microorganism and culture conditions**

_Bacillus_ sp. CU-48 was isolated from coast sediment samples collected from Van Lake, Turkey. To select the Gram-positive spore-forming bacteria _Bacillus_ sp., soil samples was incubated at 80 °C for 10 min [14]. The isolates were cultivated in LB medium (10 g tryptone, 5 g yeast extract, 50 g NaCl, pH 9.0) for 24 h at 55°C with shaking at 200 rpm. The isolates screened for α-amylase and CMCase activities on LB-agar plates containing starch (0.5%) and CMC (0.1%), respectively, at 55°C [15]. To detection of α-amylase activity, the starch containing plates were stained with iodine [16]. Cellulolytic isolates were selected by flooding the agar plates with Congo-red solution (0.1%) [17].

**Molecular characterization**

To determine the 16S rRNA sequences, each strain's genomic DNA was extracted as described previously [18]. 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 universal primer set of 518F: (5’-GGACGAGCCGGTAAATACG-3’) and 800R: (5’-TACCAAGGTTACTCTAATCC-3’), which were also used for sequencing [19, 20]. The PCR reaction mixture included 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 (5U/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 at 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 [21]. 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 [22].

**Mutation procedures**

The mutation procedures performed as described by previously [23]:

Day 1: The overnight cultured isolate was diluted by sterile deionized 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) and CMC (0.1%) with sterile toothpicks. The plates were incubated at 55°C for 24 h.

Day 3: The plates were screened and selected for α-amylase and CMCase activities according to zone diameters. All mutant variants were stored in sterile glycerol (10% v/v) 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 (Hettich Universal EBA12, 5,000 rpm, 10 min), the supernatants were used for enzyme assays.

**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 A540 nm was measured in a Pharmacia spectrophotometer [24]. 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. [25] using bovine serum albumin as the standard.

**Effect of incubation period**

The effect of incubation period was determined by assaying the enzyme activities 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 °C 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 mMTris (pH 7-11) [26].
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 \( p < 0.05 \).

SDS–PAGE and zymogram analysis
SDS-PAGE and SDS-Starch-PAGE (0.2% wt/v starch) were done as described by Laemmli[27] with slab gels (12% wt/v acrylamide). After the electrophoresis, the gels were 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 [26]. For zymogram of amylase activities by SDS-Starch-PAGE, SDS was removed by washing the gel at room temperature in solutions containing 50 mM Na_{2}HPO_{4}, 50 mM NaH_{2}PO_{4} (pH 7.2), isopropanol 40% v/v for 1 h and 50 mM Na_{2}HPO_{4}, 50 mM NaH_{2}PO_{4} (pH 7.2) for 1 h, respectively. Renaturation of enzymes were carried out by keeping the gel overnight in a solution containing 50 mM Na_{2}HPO_{4}, 50 mM NaH_{2}PO_{4} (pH 7.2), 5 mM β-mercaptoethanol and 1 mM EDTA at 4 °C. Gel was then transferred onto glass plate, sealed with film, and incubated at 55 °C for 4 h. The SDS-Starch-PAGE 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 [26, 28].

RESULTS AND DISCUSSION
The alkaline, halophilic and thermophilic strain *Bacillus* sp. CU-48 producing extracellular α-amylase and CMCase was isolated from Çukurova University located in Adana of Turkey. The bacterium was Gram positive, rod shaped, spore forming and aerobic. The optimal growth observed at pH 9.0 in presence of NaCl (5% wt/v) and occurred up to 55 °C.

Identification of the strain and 16S rRNA analysis
The isolate *Bacillus* sp. CU-48 was identified according to the 16S ribosomal RNA genetic analysis. BLAST analysis of the strain revealed that it had a closest match (98%) with *Bacillus thermolactis* (Fig. 1, 2).

The mutation tests on CU-48 isolate
After processing of *Bacillus* sp. CU-48 by EtBr, four mutant variants (CU48-M1, CU48-M2, CU48-M3, and CU48-M4) were obtained. According to diameters of amylase zones on LB-agar plates, mutant variants were compared with wild type strain. The mutant variant CU48-M2 was selected for further enzymatic analysis. No CMCase activity was observed in all mutant variants after mutagenesis of the wild type strain.

Enzyme properties
The specific α-amylase activities from *Bacillus* sp. CU-48 and its mutant variants CU48-M1, CU48-M2, CU48-M3, and CU48-M4 were found to be 1.67, 3.15, 4.4, 1.95 and 2.02 U mL^{-1} min^{-1}, respectively. α-Amylase production increased in mutant variants CU48-M1, CU48-M2 and CU48-M4 up to %52.98, %101.32, and 4.63%, respectively (Fig. 3). In spite of that, α-amylase production of mutant variant CU48-M3 was decreased (7.62%). Productions of α-amylases at different time intervals were investigated. The *Bacillus* sp. CU-48 and CU48-M2 cultures were incubated at 55 °C for 12, 24, 36, 48, 60, and 72 hours. Maximum enzyme productions were recorded after 24 and 12 h at 55 °C for CU-48 and CU48-M2 α-amylases, respectively (Fig. 4).
Figure 2. Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of Bacillus sp. CU-48 and some of its closest phylogenetic relatives.

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 50 °C and at pH 7.0 and 8.0 for CU-48 and CU48-M2 α-amylases, respectively (Fig. 5, 6). Both enzymes showed similar graphical properties for pH and temperature. Both enzyme activities were decreased after 60 °C after 30 min of incubation.}

**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 bands showing α-amylase activity in gel using BioCapt MW software. The molecular mass of bands was 45kDa (Fig. 7).

In the present study, soil samples were collected from Çukurova University of Turkey and used for isolation of Gram (+), spore forming, and aerobic bacterial strains. Among the isolates, *Bacillus* sp. CU-48with α-amylase and cellulase activities was selected for mutation and further studies because of its maximum amylolytic and cellulase activity hollow zones around the colony on LB-agar plates. *Bacillus* sp. CU-48 was showed the maximum growth with the conditions of 5% w/v NaCl at 55°C and pH 9.0. These values indicate that our isolate is moderately halophilic according to the classification of Kushner [29], haloalkaliphile which require both an alkaline pH (>pH 9) and high salinity (up to 33% wt/v NaCl) [30], and thermophile [31]. α-Amylases from alkaline and thermophilic *Bacillus* species were reported previously [26, 32-36]. 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 [26,37].

**Figure 7.** Zymogram analysis of α-amylases on SDS-PAGE. The gel was cut into two pieces, the marker and total proteins were visualized with Coomassie brilliant blue staining and the activity of enzyme revealed by iodine (M: Marker, 1: Wild type strain CU-48 protein samples, 2-5: Mutant variants protein samples from CU-48-M1 to CU-48-M4, respectively).

Also CMCase from alkaline and thermophilic *Bacillus* species were reported previously [38-42]. Most of the *Bacillus* strains used commercially for the production of cellulase have an optimum pH between 6.0 and 10.0 for growth and enzyme production [26, 38-41,43].

The strain *Bacillus* sp. CU-48 was improved for α-amylase production. The chemical mutagen Ethidium bromide (EtBr) was used for mutation of the bacterial strain. According to the results all of the mutant variants lost CMCase activities in compare to wild type strain. Ethidium bromide (EtBr) has led to frameshift mutations. α-Amylase-positive *Bacillus* sp. ORB (1) strain was converted to a negative strains by Ethidium bromide [23].

The mutant *Bacillus* sp. CU48-M2 gave 4.4 U mL⁻¹ min⁻¹ α-amylase which was around 2.63 fold higher than the parent strain. Several researchers have employed mutagenesis for α-amylase production by exposing the cultures with UV or chemicals like EMS, nitrous acid and EtBr [43-45].

http://ijcns.aizeonpublishers.net/content/2014/2/ijcns97-103.pdf
The optimum incubation periods for our native enzyme and its mutant variant CU48-M2 were 24 and 12 h, respectively. Besides, the native enzyme showed 48% activity after 36 h and mutant variant showed 76% activity after 24 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 amyloses were 7.0 and 8.0, 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 50°C for native and mutant enzymes, respectively. These pH and temperature values are similar to Bacillus licheniformis and Gracilicibacillus [46], Bacillus sp. GUF8 [47], Halomonassp. AAD21 [6], Bacillus cereus MS6 [48] 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-amyloses. The optimal temperatures of most bacterial α-amyloses, including those from Bacillus sp. CU-48 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 [47].

The molecular weight of α-amylose was 45kDa on SDSPAGE. Similar findings between 35 kDa and 55 kDa have been reported earlier [49-53]. These differences of molecular weights of α-amylases depend on the genes from the organisms.

According to the 16S rDNA sequences the strain CU-48 belonged to Bacillus thermostaticus strain with the similarity of 98%. Coorevits et al [54] isolated 18 B. thermostaticus species from dairy environment (according to 93.9% 16S rRNA gene sequence similarity). They described that, all their isolates Gram-positive, rod-shaped cells, growth occurs at pH 7 but not at pH 6 or 8, and 40-60 ºC (optimally at 50 ºC), but does not occur at 30 or 70 ºC, does not tolerate 1% NaCl (w/v) for growth. In spite of that, our isolate CU-48 was cultured in LB medium with 5% NaCl at pH 9. To our knowledge, there are no other comparative studies on B. thermostaticus isolate.

CONCLUSION

In this study we isolated alkaline, halophile and thermophile wild type Bacillus strain CU-48 producing thermostable α-amylose and cellulasefem Çukurova University soil samples. After mutation by EtbR, the obtained mutant variant CU48-M2 was found to be more potent for α-amylose production and so it can be further characterized for further studies.

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