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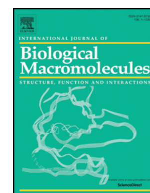
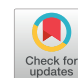


# Identification and characterization of novel thermostable α-amylase from *Geobacillus* sp. GS33



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ABSTRACT

In this study, the heterologous expression and biochemical characterization of a thermostable α-amylase from *Geobacillus* sp. GS33 was investigated. The recombinant α-amylase was overexpressed in *Escherichia coli* BL21 (λDE) and purified via anion exchange and size-exclusion chromatography. The purified α-amylase had a molecular weight of about 60 kDa, and was active in a broad range of pH 3–10 and temperature (40–90 °C) with maximum activity at pH 7–8 and 60 °C. The enzyme retained 50% residual activity at 65 °C, but only 20% at 85 °C after 16 h. At pH 9 and pH 7, the residual activity at 65 °C was 50% and 30%, respectively. The enzyme was remarkably activated by $Co^{2+}$, $Ca^{2+}$, $Mg^{2+}$, PMSF, DTT, and Triton X-100, but partially inhibited by $Cu^{2+}$, methanol, hexane, ethanol, acetone, SDS, and Tween 20. A molecular phylogeny analysis showed that the enzyme's amino acid sequence had the closest connection with an α-amylase from *Geobacillus thermoleovorans* subsp. stromboliensis nov. 3D-structure-based amino acid sequence alignments revealed that the three catalytic residues (D217, E246, D314) and the four $Ca^{2+}$ ion coordination residues (N143, E177, D186, H221) were conserved in α-amylase from *Geobacillus* sp. GS33. The temperature stability and neutral pH optimum suggest that the enzyme may be useful for industrial applications.

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## 1. Introduction

Starch, the most abundant carbon energy source on earth, is composed of amylose with mostly α-1,4-linked glucose residues and amylopectin with 95% α-1,4-linkages and 5% α-1,6-branch linkages [1]. α-Amylase (α-1-4 D-glucan glucanohydrolase) (EC 3.2.1.1) hydrolyzes the α-1-4 glycosidic bonds of starch and produces glucose-containing oligosaccharides [2]. The α-amylases have attracted great attention, because they are necessary for a variety of biotechnological applications. They are used for increasing bread quality in baking, starch liquefaction, starch saccharification, fiber and cotton desizing, biofuel production, the sizing during paper manufacture, and improving digestibility of animal feed. They are also used in the brewing, detergent and pharmaceutical industry [3–5].

α-Amylases are widely produced by animals, plants, and microorganisms [6–11]. Especially, α-amylases, which are active in the presence of denaturant and also in a wide range of temperature and pH, are preferred for industrial applications since it lowers the cost, enhances substrate solubility, and lowers contamination originated from mesophilic microorganisms. These types of enzymes are also produced by extremophiles, which are microorganisms residing in harsh conditions [12].

Recently, much effort has been devoted to the identification of α-amylases from extremophilic microorganisms such as bacteria and fungi [13,14]. Thermostable α-amylases are present in certain thermophilic bacillus species of bacteria, such as *Bacillus*, *Geobacillus*, and *Anoxybacillus* genera [15]. Among thermophilic bacillus species, there have been many recent studies of the *Geobacillus* genus which is an α-amylase producer [16–26].

*Geobacillus* species, which are gram-positive and endospore-forming bacteria, can thrive at high temperatures in the range of 37 to 75 °C where many other species cannot survive [27]. Various enzymes produced by *Geobacillus* species have thermal stability and are resistant to extreme conditions in terms of pH, organic solvents, chemical denaturants, and detergents [28]. Recently, the identified strain *Geobacillus vulcani* GS90 [29] isolated from Balçova geothermal region, İzmir, Turkey [30], has been successfully studied as a producer of another thermostable enzyme (α-L-arabinofuranosidase), which is highly active in a wide range of pH and temperature [31]. Since α-amylase is used in various industrial applications, it has been aimed to investigate a novel thermostable α-amylase with convenient biochemical properties from *Geobacillus* source.

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Here, we cloned, heterologously expressed, and characterized a novel thermostable α-amylase of another *Geobacillus* strain (*Geobacillus* sp. GS33) from Balçova geothermal region, İzmir, Turkey. Besides, we performed a phylogenetic analysis based on the amino acid sequence and evaluated the three-dimensional structure of the novel thermostable α-amylase obtained from homology modeling.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Bacterial strains and growth conditions

The environmental isolate (R33) obtained from re-injection water (the returning point of the heating system at 65 °C and pH 8.5) isolates of Balçova Geothermal region of İzmir, known as thermophilic *Geobacillus* sp. [30] and called as *Geobacillus* sp. GS33 in this study, was used as a source of the α-amylase gene. Luria-Bertani (LB) agar (1% (w $v^{-1}$) tryptone, 0.5% (w $v^{-1}$) yeast extract, 1% (w $v^{-1}$) sodium chloride, 1.5% (w $v^{-1}$) agar) was used to obtain a single colony of *Geobacillus* sp. GS33 strain. The growth condition of this strain was 55 °C and 200 rpm. Beside this, *E. coli* DH5α and *E. coli* BL21 (λDE3) were grown at 37 °C, 200 rpm, using LB media.

#### 2.1.2. Screening of thermostable α-amylase enzyme

Thermophilic *Geobacillus* sp. GS33 culture was checked for amylase activity, using the starch-iodine test [32]. The strain was grown on starch agar at 55 °C for 16 h. The solid culture was dyed with 0.1% iodine solution containing 2 g potassium iodide and 1 g of iodine per liter.

#### 2.1.3. Cloning of the thermostable α-amylase gene

Genomic DNA was isolated from the culture of thermophilic *Geobacillus* sp. GS33 by genomic DNA mini kit (Invitrogen PureLink), according to the manufacturer's instructions. The amplification of the thermostable α-amylase gene was performed using forward (5′-CATATGGAAATGGGGAACCGGCTCTTTATG-3′) and reverse (5′-AAGCTTTTATTCATTGATCCGTTTTGCCCG-3′) primers restricted with the *Nde*I and *Hind*III sites (underlined), respectively. The amplified thermostable α-amylase gene product was cloned into pTZ57R/T vector (Fermentas-Life Science Technologies, Lithuania). The new plasmid construct was then transformed into *E. coli* DH5α. The strain was grown in LB medium including ampicillin (100 μg $mL^{-1}$). After the plasmid isolation from the *E. coli* DH5α culture, it was restricted by *Nde*I and *Hind*III enzymes. The restricted fragment was purified from agarose gel and cloned into the pET-28a(+) expression vector (Novagen), which has restriction ends of *Nde*I and *Hind*III. The nucleotide sequence of the thermostable α-amylase gene was determined and deposited in the EMBL Nucleotide Sequence Databases with the assigned GeneBank accession number of MT427399.

### 2.2. Expression and purification of the thermostable α-amylase enzyme

The co-expression of the thermostable α-amylase with chaperone protein pKJE7 (Chaperone Plas-mid, TAKARA Bio Inc., Japan) was performed to enhance the amount of properly folded target enzyme, using *Escherichia coli* BL21 (λDE) with the IPTG-inducible plasmid pET-28a(+) encoded for thermostable α-amylase. In line with this purpose, the strain was cultivated in 100 mL of LB medium and incubated at 200 rpm, 37 °C in the presence of kanamycin (30 μg $mL^{-1}$), chloramphenicol (20 μg $mL^{-1}$). Overexpression of α-amylase was carried out by adding a final concentration of 1 mM IPTG at an OD600 of 0.4, and the cells were further grown at 200 rpm, 37 °C for 4 h. The cells were centrifuged at 4 °C and 5000 rpm for 10 min and the pellets were resuspended in lysis buffer with 50 mM Tris-Cl (pH 8.0) containing 10% glycerol, 0.1% Triton X-100, 100 μg $mL^{-1}$ lysozyme, 1 mM PMSF and 2 mM $MgCl_2$. The disruption of the cell membranes was performed by sonication for 6 × 20 s on ice (Bandelin, Sonopuls Ultrasonic Homogenizers, HD 2070). The cell debris were removed by centrifugation at 4 °C and 100,00*g* for 20 min. The supernatant was loaded on an anion exchange DEAE-cellulose column (2.5 cm × 10 cm) equilibrated by 50 mM Tris-Cl buffer (pH 7.2) and the column was washed with this buffer. Elution of the proteins was done using a salt gradient of 0–2 M NaCl in 50 mM Tris-HCl, pH 7.2. Protein fractions were collected and protein concentration was determined at 280 nm using a Nanodrop ND1000. Further purification of the collected fractions was performed by size-exclusion chromatography using Sepharose G-100 resin. To check the purity of the target protein, the purified sample was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis (12%) [33]. For this analysis, protein molecular weight marker was used as the standard, ranging from 14.4 to 116 kDa (ThermoFisher Scientific). Measurement of protein concentration was quantitatively performed at 595 nm by Bradford method, using bovine serum albumin (BSA) as a standard protein [34].

The enzyme activity was confirmed by the dropping of crude protein extract and purified enzyme on 1% starch agar plate, which is then dyed with 0.1% iodine solution at 55 °C for 10 min.

### 2.3. Identification of thermostable α-amylase by mass spectrometric analysis

Thermostable α-amylase was identified by mass spectrometric analysis, using in-gel digestion adapted from [35]. Briefly, the protein spots were cut from SDS-PAGE gel and gel pieces were incubated into wash solution (50% (v $v^{-1}$) methanol and 5% (v $v^{-1}$) acetic acid) overnight. Dehydration of the gel was performed using 200 μL of acetonitrile for 3 min, 30 μL of 10 mM DTT for 30 min, and 30 μL of 100 mM iodoacetamide for 30 min at room temperature, respectively. Then, rehydration was performed by incubating the sample for 10 min in 200 μL of 100 mM ammonium bicarbonate solution at room temperature. The samples were again dehydrated by 200 μL of acetonitrile at room temperature for 5 min. The samples were dried in a vacuum centrifuge at room temperature for 3 min, and then rehydrated using 30 μL of 20 ng $mL^{-1}$ trypsin solution upon incubation of 10 min on ice. After centrifugation of the samples, the pellets were resuspended in 5 μL of 50 mM ammonium bicarbonate, the mixture was vortexed, centrifuged for 30 s, the supernatant was discarded and the pellet was incubated overnight at 37 °C for digestion. The samples were dissolved in 30 μL of 50 mM ammonium bicarbonate, incubated for 10 min, and centrifuged for 30 s. 30 μL of extraction buffer (50% (v $v^{-1}$) acetonitrile and 5% (v $v^{-1}$) formic acid) was added to the supernatant and the mixture was incubated for 10 min and centrifuged for 30 s. This step was repeated once more. Then, the supernatant was evaporated by a vacuum centrifuge to reduce the volume to less than 20 μL. Subsequently, the supernatant was analyzed for protein identification by MALDI-TOF-TOF Mass Spectrometry that uses a matrix of α-cyano-4-hydroxycinnamic acid (CHCA).

### 2.4. Biochemical characterization of thermostable α-amylase

#### 2.4.1. Standard activity assay

The activity of the α-amylase enzyme was investigated by DNS (dinitrosalycilic acid) assay, as previously described with some modifications [36]. Briefly, the enzyme activity was determined using 25 μL of purified enzyme solution and 50 μL of 1% starch solution, and 20 min of incubation at 55 °C. 100 μL of DNS reagent was then added to the mixture for the end of the reaction. The mixture was boiled for 5 min and 825 μL of deionized water ($dH_2O$) was added after cooling to the room temperature. OD540 value was measured to detect the reducing sugar content where D-glucose (0–2 mg $mL^{-1}$) was used as the standard. One unit of enzyme activity was defined as the amount of 1 μM of D-glucose released by the enzyme per minute under the standard activity assay conditions.

*2.4.2. Influence of pH and temperature*

The effect of pH on enzyme activity was investigated using buffers at different pH, ranging from 3 to 10; Na-citrate buffer (pH 3–6), sodium phosphate buffer (pH 7–8), Tris-HCl buffer (pH 8), and glycine-NaOH buffer (pH 9–12). Also, the optimum temperature of the amylase was investigated in a variety of temperatures (40–90 °C). The analysis for optimum temperature and pH were performed under standard assay conditions.

*2.4.3. Thermal and pH stability of the thermostable α-amylase*

The characterization of recombinant thermostable α-amylase was performed in terms of thermal and pH stability. To study thermal stability, the enzyme was incubated in Tris-HCl (pH 8) at 65 °C and 85 °C for 1, 2, 3, 4, 5, 6, 12, 16, and 24 h and the residual enzyme activity was determined by the standard activity assay. For pH stability, the amylase was incubated for 6, 16, and 24 h at 60 °C in buffers of pH 7 and 9. Then, the residual enzyme activity was measured using the standard activity assay.

*2.5. Effect of metal ions and chemical reagents on thermostable α-amylase*

To investigate the effect of metal ions ($CaCl_2$, NaCl, $MgCl_2$, $CuSO_4$, $CoCl_2$, and KCl) at 10 and 25 mM concentrations and chemical reagents (10% hexane, 10% methanol, 10% ethanol, 10% acetone, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM 1,4-dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), 1% Tween 20, 1% Triton X-100 and 1% β-mercaptoethanol (BME)), thermostable α-amylase was incubated in the presence of these chemicals at 60 °C for 20 min. The reaction was carried out by the addition of 1% starch to the purified enzyme solution including each above-mentioned chemical and then the residual enzyme activity was measured using standard activity assay.

*2.6. Data presentation and statistical analysis*

Unpaired and two-tailed *t*-test was carried out for statistical data analysis using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA) (www.graphpad.com). All experiments were carried out at least in duplicate.

*2.7. Bioinformatic analysis*

The gene sequence coding for thermostable α-amylase was obtained by the dideoxynucleotide chain termination method by 16 and 80 capillary, 3130XL (Applied Biosystem, CA/USA) [37]. Finch TV 1.4.0 version was applied for gene sequence analysis. Also, amino acid sequences were aligned using Clustal Omega [38] and BLAST algorithms for protein sequence [39,40] to evaluate the sequence similarity of the thermostable α-amylase with the other amylase enzymes obtained from various sources. Molecular evolutionary relationship analysis based on amino sequence data was performed by MEGA X version 10.1 BETA [41]. Besides, the predicted three-dimensional (3D) structure of the amylase was performed using homology modeling by I-Tasser online server [42–44]. These were analyzed by the PyMOL Molecular Graphics System, Version 2.0 (Schrödinger, LLC). The alignment of amino acid sequences in thermostable α-amylase and templates from the PDB library was carried out using Clustal Omega [38].

## 3. Results and discussion

α-Amylase hydrolyzes the α-1-4 glycosidic bonds of starch and produces glucose-containing oligosaccharides [2]. The α-amylases have attracted great attention, because they are necessary for many industrial applications [3–5]. Here, we heterologously expressed and characterized an α-amylase from thermophilic bacterium *Geobacillus* sp. GS33.

*3.1. Screening of α-amylase*

*Geobacillus* sp. GS33 culture was grown on the starch agar at 55 °C during 16 h and then treated with an iodine solution. The activity screening result showed that white zones formed around each colony of *Geobacillus* sp. GS33 strain (Suppl. data 1), indicating that the strain can degrade the starch.

*3.2. Amino acid sequence of novel thermostable α-amylase*

The α-amylase gene was obtained from the chromosomal DNA of *Geobacillus* sp. GS33 and sequenced using the dideoxynucleotide chain termination method. Its size was found as 1542 bp encoding 513 amino acids. Novel thermostable α-amylase enzyme shares 93.76% amino acid sequence identity with the α-amylase from *Geobacillus thermoleovorans* subsp. stromboliensis nov. (strain Pizzo[T]) [26]. Also, the enzyme showed a sequence identity of 90.45% and 88.06% with α-amylase from *G. kaustophilus* HTA426 and *G. stearothermophilus*, respectively. However, there was a poor identity between our enzyme and α-amylase from *Virgibacillus salinus* by 38.37% (Suppl. data 2). The phylogenetic tree was constructed using the Maximum Likelihood (ML) method to determine the evolutionary connection of novel thermostable α-amylase. Obviously, α-amylase from *Geobacillus* sp. GS33 had the closest connection with α-amylase from *Geobacillus thermoleovorans* subsp. stromboliensis nov. (strain Pizzo[T]) (Fig. 1).

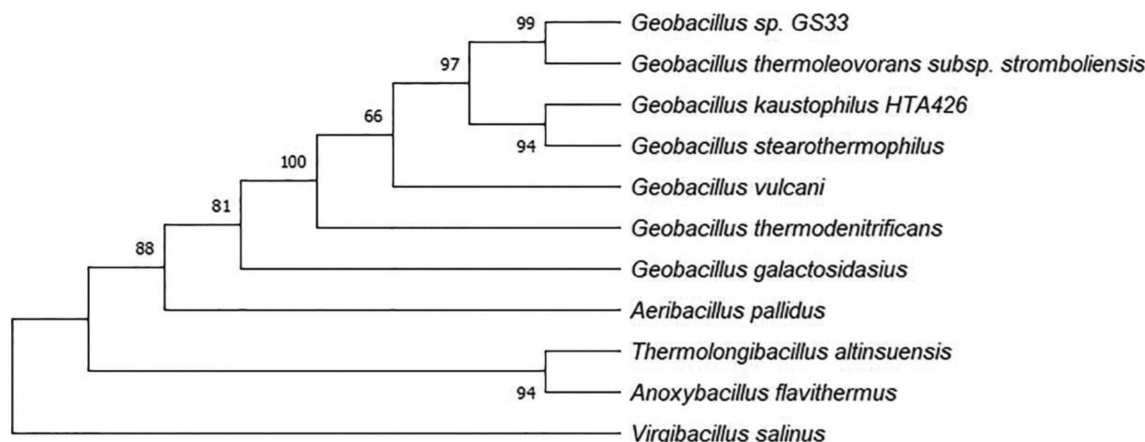


**Fig. 1.** The phylogenetic tree showing the relation of α-amylases of *Geobacillus* sp. GS33 with the other amylases from different sources.

### 3.3. Overexpression and purification of thermostable α-amylase

Thermostable α-amylase was overexpressed with chaperone protein pKJE7. The amylase was purified by anion exchange purification and then size-exclusion chromatography (Suppl. data 3). The specific activity of novel α-amylase was found as 4953.65 U $mg^{-1}$ and the enzyme purity was checked by SDS-PAGE analysis, indicating its molecular weight of about 60 kDa (Fig. 2). Most of the microorganisms produce thermostable α-amylases that are 21 to 160 kDa of molecular weight [45]. Recently, some studies of the *Geobacillus* genus have reported α-amylases with similar molecular weight to that of *Geobacillus* sp. GS33 [17,18,22,23,26]. An example of this is that the molecular weight of α-amylase from strain Pizzo$^T$ is estimated to be 58 kDa [26].

A novel thermostable α-amylase was identified using MALDI-TOF-TOF mass spectrometry (Suppl. data 4). Based on this analysis result, the best peptide match of thermostable α-amylase was with the α-amylase from *Geobacillus thermodenitrificans* NG80-2, indicating 91% similarity, using NCBInr database. Besides, theoretical isoelectric point (pI) and a nominal mass of thermostable α-amylase were found as 5.66 and approximately 59.8 kDa, respectively.

### 3.4. Biochemical characterization of novel thermostable α-amylase

The purified novel thermostable α-amylase was characterized in various conditions (pH, temperature etc.) and the analysis results are summarized in Fig. 3 and Table 1. The activity of thermostable α-amylase was assessed in the temperature ranges of 40–90 °C and pH range of 3–10. The amylase exhibited hydrolytic activity in a broad range of pH and temperature, and the optimum conditions were found at 60 °C and pH 7–8 (Fig. 3a and b). Recently, there have been many studies in the literature about the characterization of α-amylase of *Geobacillus* strains isolated from different environments. Accordingly, thermostable α-amylase of *Geobacillus* SBS-4S isolated from hot spring present in Northern areas of Pakistan had a maximum activity of 55 °C and pH 8–9 [21]. Similarly, thermostable α-amylase of *Geobacillus* bacterium (K1C) from hot springs, India, kept its maximal activity at 55 °C and pH 9 [22], whereas optimum values of thermostable and alkalitolerant amylase from *Geobacillus stearothermophilus* HP 3 were 55 °C and pH 9 [19]. Also, α-amylase from *Geobacillus stearothermophilus* SR74 had an optimum temperature and pH of 65 °C and pH 7.0, respectively [17]. In line with this, α-amylase of a deep-sea thermophile, *Geobacillus* sp. 4j had a maximum activity at 60–65 °C and pH 5.5 [23], while α-amylases from *Geobacillus* sp. D413 and *G. stearothermophilus* ATCC 12980 had a maximum activity at 65 °C, and pH 7.5–9, respectively [16]. The other thermostable α-amylases from different *Geobacillus* sp. optimally works at 70 to 75 °C at the interval of pH 5–9, except α-amylase of *Geobacillus* sp. Iso5 isolated from geothermal spring keeping its maximum activity at 90 °C [18,20,24–26,46]. Beside *Geobacillus* genus, different thermophilic *Bacillus* sp. sources of α-amylase had a similar optimum temperature and pH values to thermostable α-amylase [47–51].

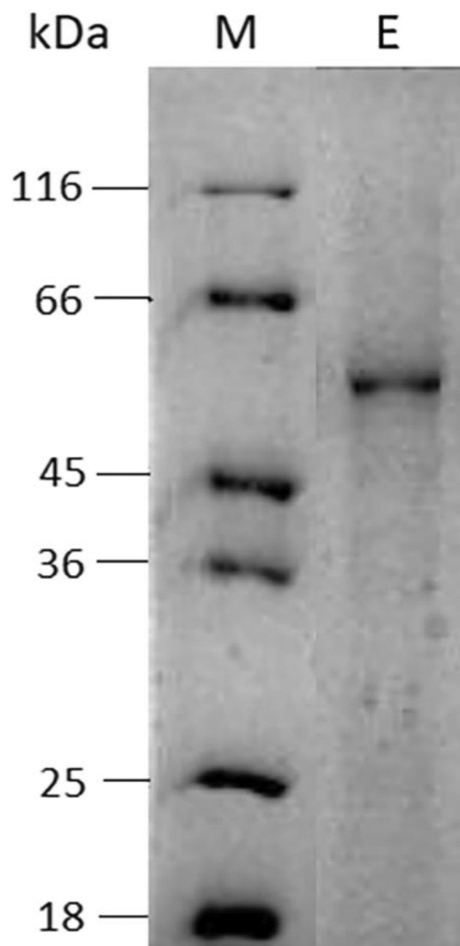


**Fig. 2.** SDS-PAGE display of the purified α-amylase from *Geobacillus* sp. GS33.

Various temperature and pH effects on novel thermostable α-amylase stability were investigated. The thermal stability analysis results showed that the enzyme retained 80% and 50% of residual activity at 65 °C upon incubation of 12 h and 24 h, respectively. Also, the residual activity of the enzyme at 85 °C was higher than 50% after 4 h of incubation and then dropped to the levels of 20% upon 6 h of incubation (Fig. 3c). Many recent studies of the α-amylase from *Geobacillus* species have shown lower thermostability, compared to the α-amylase from *Geobacillus* sp. GS33. Accordingly, Sudan et al. (2018) have shown that α-amylase from *Geobacillus* bacterium (K1C) had a residual activity of about 50% at 70 °C and 80 °C upon incubation of 9 h and 2 h, respectively [22]. Also, the residual activities of α-amylases from *Geobacillus* sp. D413 and *G. stearothermophilus* ATCC 12980 were 65% and 54% at 75 °C upon incubation of 10 min [16]. Besides, α-amylase from *Geobacillus stearothermophilus* SR74 retained its activity below 20% at 65 °C after 90 min [17], whereas α-amylase from thermophilic *Geobacillus stearothermophilus* decreased below 50% at 70 °C after 2 h [18]. To our knowledge, only two recent studies have reported comparable results of α-amylase thermostability to that of α-amylase from *Geobacillus* sp. GS33. Accordingly, the residual activity of α-amylase from *Geobacillus* sp. 4j was higher than 50% at 80 °C after 4 h [23], whereas α-amylase from *Bacillus subtilis* had more than 50% of residual activity at 80 °C after 5 h [46]. pH stability analysis results for α-amylase from *Geobacillus* sp. GS33 showed that the enzyme conserved partial activity even after 24 h at pH 7 and 9. The enzyme was relatively more stable at pH 9, comparing to the pH 7, indicating that α-amylase from *Geobacillus* sp. GS33 was an alkalitolerant enzyme (Fig. 3d).

The effects on novel α-amylase were investigated in the presence of different metal ions and chemical reagents on the starch hydrolysis. The analysis results showed that some metal ions ($Co^{2+}$, $Ca^{2+}$, and $Mg^{2+}$) with 10 and 25 mM concentrations remarkably stimulated the activity of α-amylase activity, whereas 10 and 25 mM $Cu^{2+}$ ion strongly reduced its activity (Table 1). Also, some chemical reagents such as methanol, hexane, ethanol, acetone, SDS, and Tween 20 reduced, to some extent, the amylase activity, whereas PMSF, DTT, and Triton X-100 induced the enzyme activity in a significant level (Table 1). In literature, there have been some recent studies about the effect of different metal ions and chemicals on α-amylase activity. Accordingly, α-amylase activity from *Geobacillus* SBS-4S was increased in the presence of all the tested metal ions and chemicals including $Mg^{2+}$, $Ca^{2+}$, $Co^{2+}$, and Triton X-100 [21]. Also, several metal ions such as $Mg^{2+}$, $Ca^{2+}$, and $Co^{2+}$ increased α-amylase activity from *Geobacillus* sp. 4j [23]. In contrast, α-amylase activity from *Geobacillus* bacterium (K1C) was

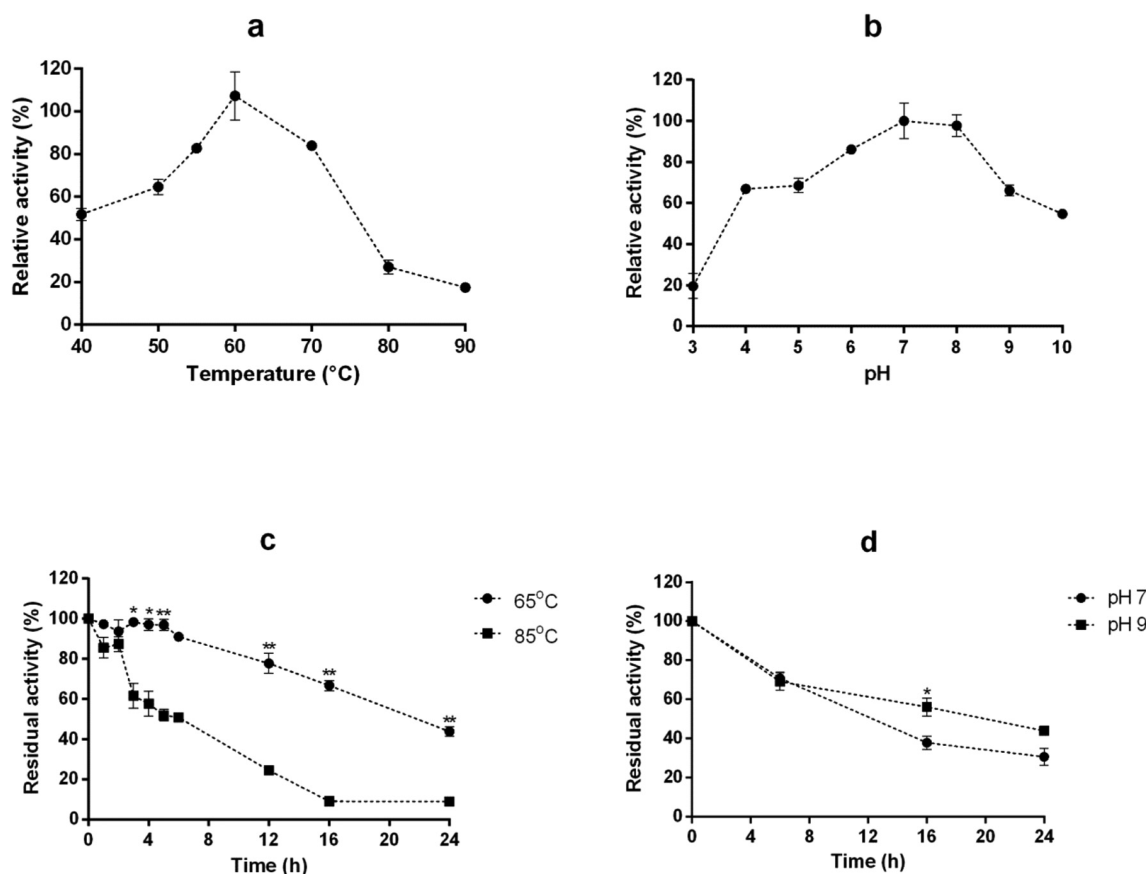


**Fig. 3.** Temperature and pH effects on the activity and stability of the purified α-amylase from *Geobacillus* sp. GS33. a) pH effect. b) Temperature effect. c) Temperature stability. d) pH stability. Statistical significance indicated $^*p < 0.05$, and $^{**}p < 0.01$.

inhibited by most of the tested metal ions and chemicals including $Cu^{2+}$, $Mg^{2+}$, $Ca^{2+}$, and TritonX [22]. Furthermore, the activity of α-amylase from *Geobacillus* sp. nov. was slightly increased in the presence of $Cu^{2+}$, $Mg^{2+}$, and $K^{+}$ even though $Ca^{2+}$ decreased its activity [20]. Besides this, α-amylase activity from *Geobacillus* sp. TF14 was activated only in the presence of $Ca^{2+}$; however, the other tested chemicals significantly inhibited its activity [24]. There have been some recent reports about the effects of metal ions and chemicals on the α-amylase activity in different thermophilic microorganisms from *Geobacillus* species. Accordingly, α-amylase activity from *Thermomyces dupontii* was stimulated by $Na^{+}$, $Ca^{2+}$, and $Co^{2+}$, Triton X-100. The other tested metal ions and chemicals such as $Mg^{2+}$, $Cu^{2+}$, and DTT inhibited its activity [52]. Also, α-amylase activity from thermophilic *Anoxybacillus flavithermus* sp. nov. SO-19 was significantly stimulated by only two metal ions $Mg^{2+}$ and $Co^{2+}$ [53]. Besides, α-amylase activity from a thermophilic *Anoxybacillus thermarum* A4 strain was increased in the presence of several metal ions such as $Cu^{2+}$, $Ca^{2+}$, $Co^{2+}$, and Triton X-100; however, its activity was gradually decreased by an increase of $Mg^{2+}$ concentration [54]. All the tested metal ions including $Ca^{2+}$ also decreased the catalysis of α-amylase from thermophilic actinobacterium *Laceyella sacchari* TSI-2 [55].

**Table 1**
Effect of metal ions and chemical reagents on α-amylase from *Geobacillus* sp. GS33.

| Chemical reagents | Residual activity (%) | Metal ions | Residual activity (%) |
|---|---|---|---|
| Control | 100 | $Ca^{2+}$ (10 mM) | 149 ± 0.4 |
| Acetone (10%) | 58 ± 5.9 | $Ca^{2+}$ (25 mM) | 146 ± 1.6 |
| Methanol (10%) | 66 ± 5.6 | $Na^{+}$ (10 mM) | 95 ± 1.04 |
| Ethanol (10%) | 65 ± 1.0 | $Na^{+}$ (25 mM) | 93 ± 0.7 |
| Hexane (10%) | 82 ± 11.7 | $Cu^{2+}$ (10 mM) | 78 ± 0.8 |
| PMSF (10%) | 145 ± 1.4 | $Cu^{2+}$ (25 mM) | 38 ± 0.7 |
| DTT (1 mM) | 124 ± 0.7 | $Co^{2+}$ (10 mM) | 172 ± 1.8 |
| EDTA (1 mM) | 97 ± 14.1 | $Co^{2+}$ (25 mM) | 171 ± 5.2 |
| SDS (1%) | 36 ± 1.5 | $Mg^{2+}$ (10 mM) | 125 ± 1.9 |
| Tween 20 (1%) | 66 ± 1.4 | $Mg^{2+}$ (25 mM) | 120 ± 2 |
| Triton X-100 (1%) | 128 ± 3.2 | $K^{+}$ (10 mM) | 96 ± 2.6 |
| BME (1%) | 90 ± 14.9 | $K^{+}$ (25 mM) | 89 ± 0.2 |

### 3.5. Homology modeling on novel thermostable α-amylase

LOMETS, which is a meta-server threading program, identified the template structures with the highest statistical significance from PDB library and used many templates of GH13 family proteins for alignments in the I-Tasser modeling of novel α-amylase. As a result, four PDB hits were found out by this program, and sequence identity values were determined by Blastp: 4E2O from *Geobacillus thermoleovorans* CCB_US3_UF5 [56], 5A2B from *Anoxybacillus ayderensis* [57], 2WKG from *Nostoc punctiforme* PCC 73102 [58] and 2DIJ from *Bacillus circulans* [59]. Among these enzymes, 4E2O (referred also as GTA) had the highest amino acid sequence identity with the thermostable novel α-amylase (amino acid sequence identity of 92.95%) (Suppl. data 5). Besides this, the protein structure alignment result exhibited high similarity and great compatibility in all folding patterns between the thermostable α-amylase model and GTA (Fig. 4b).

GTA is designated by creating a truncated form of α-amylase (513 residues in length) from *Geobacillus thermoleovorans* CCB_US3_UF5 a hot spring in Ulu Slim, Malaysia and it optimally works at 70 °C and pH 6. The recombinant GTA lacks N-terminal and C-terminal transmembrane regions [56]. This enzyme shows a 100% amino acid sequence identity with the extracellular α-amylase from strain Pizzo$^{T}$ [26]. The phylogenetic analysis based on amino acid sequence data showed that novel α-amylase had the closest connection with α-amylase from strain Pizzo$^{T}$ (Fig. 1). As in the other α-amylase members of the GH13 family,

a

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α-amylase   MEMGNRLFMLLVLPFLLFYAMPAAAAEKEERTWEDEAIYFIMVDRFNNMDPTNDQNVNVN   60
GTA         --------------------------EKEERTWQDEAIYFIMVDRFNNMDPTNDQNVNVN   34

α-amylase   DPKGYFGGDLKGVTAKLDYIKEMGFTALWVTPIFKNMPGGYHGYWIEEFYQVHPHFGTLG   120
GTA         DPKGYFGGDLKGVTAKLDYIKEMGFTAIWLTPIFKNMPGGYHGYWIEDFYQVDPHFGTLG   94

α-amylase   DLKKLPKKTHKRDMKGILEFVANHGGYNHPWVHDPTKKKWFLPKKENFYWDDPTPLENGW   180
GTA         DLKTLVKEAHKRDMKVILDFVANHVGYNHPWLHDPTKKDWFHPKKEIFDWNDQTQLENGW   154

α-amylase   VYGLPDLAQENPEVQTYLIDAAQWWIKETDIDAYRLDTVRHVPKSFWQEFVKENKSVKKD   240
GTA         VYGLPDLAQENPEVKTYLIDAAKWWIKETDIDGYRLDTVRHVPKSFWQEFAKEVKSVKKD   214

α-amylase   FFLLCEVWSDDPRYIADYGKNGIDGFVDYPLYGAVKQSLARRDASPPPLYDVWEYNKTVY   300
GTA         FFLLGEVWSDDPRYIADYGKYGIDGFVDYPLYGAVKQSLARRDASLRPLYDVWEYNKTFY   274

α-amylase   DRPHLLASFLDNHDTVRFTKLAIDNRNNPISRIKLAMTYLFTAPGIPIMYYGTEIAMNGG   360
GTA         DRPYLLGSFLDNHDTVRFTKLAIDNRNNPISRIKLAMTYLFTAPGIPIMYYGTEIAMNGG   334

α-amylase   QDPDNRRLMDFRADPEIIDYLKKIGPLRQELPSLRRGDFTLLYEKDGMAVLKRQYQDETT   420
GTA         QDPDNRRLMDFRADPEIIDYLKKIGPLRQELPSLRRGDFTLLYEKDGMAVLKRQYQDETT   394

α-amylase   VIAINNTSETQHVHLTNDQLPKNKELRGFLLDDLVRGDEDGYDLVLDRETAEVYKLREKT   480
GTA         VIAINNTSETQHVHLTNDQLPKNKELRGFLLDDLVRGDEDGYDLVLDRETAEVYKLREKT   454

α-amylase   GINIPFIAAIVSVYVLFLLFLYLVKKRAKRINE   513
GTA         ---------------------------------   454
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b

D186 N143 D217 D314 E246 E177 H221

**Fig. 4.** Amino acid sequence alignment (a) and structural alignment (b) of the novel $\alpha$-amylase model (violet) and template GTA (orange) using PyMOL Molecular Graphic System. Red amino acids refer to catalytic residues and blue amino acids refer to $Ca^{2+}$ ion coordination residues.

GTA structurally has three domains as catalytic domain A (extended between residues 30–140 and 191–397), domain B (residues 141 to 190) and domain C (residues 398 to 480). The catalytic domain of GTA is consisted of $(\beta/\alpha)_8$ barrel and has three conserved catalytic residues as D217, E246, and D314, which make some interactions with the ligand [56]. The analysis result of amino acid sequence and structural alignments indicated that three catalytic residues and catalytic domain as $(\beta/\alpha)_8$ barrel were conserved in $\alpha$-amylase (Fig. 4a and b).

Furthermore, GTA binds to one $Ca^{2+}$ ion, coordinating with the residues N143, E177, and D186 in domain B and residue H221 in domain A, as well as three water molecules [56]. The amino acid sequence analysis result revealed that $Ca^{2+}$ ion coordination residues were also conserved in novel α-amylase from *Geobacillus* sp. GS33 (Fig. 4a). It is well known that α-amylase members from GH13 are generally metalloenzymes dependent on $Ca^{2+}$ ion [60]. The enzyme activity of GTA does not show an increase in the presence of $CaCl_2$, although its thermal stability considerably increases [56]. The present study showed that the activity of novel α-amylase was significantly enhanced by 10 and 25 mM $Ca^{2+}$ levels (Table 1).

To conclude, we characterized a novel thermostable α-amylase of *Geobacillus* sp. GS33 isolated from Balçova Geothermal region, İzmir, Turkey. With the highest activity at 60 °C and pH 7–8, this enzyme possesses some preferable properties as a thermostable α-amylase. The enzyme works in a wide range of temperatures (40–90 °C) and pH (3 −10). Also, it had a high thermostability, exhibiting activity even at 85 °C throughout 16 h. and it is highly tolerant of $Co^{2+}$, $Ca^{2+}$, $Mg^{2+}$, PMSF, DTT, and Triton X-100. Those characteristics make novel α-amylase a valuable candidate for some industrial applications.

## Author statement

The research project was conducted under the supervision of Dr. Gülşah Şanlı-Mohamed.

Methodology, experimental part, writing and draft preparation were performed by Tülin Burhanoğlu.

Data curation, visualization and writing - reviewing and editing were done by Yusuf Sürmeli.

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## Appendix A. Supplementary data

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