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Biologia **72**/8: 831—839, 2017
Section Cellular and Molecular Biology
DOI: 10.1515/biolog-2017-0099

Purification and characterization of α-L-arabinofuranosidases from *Geobacillus stearothermophilus* strain 12

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Abstract: In order to characterize two α-L-arabinofuranosidases (α-L-AFases), Abf1Geo12 and Abf2Geo12, produced by *Geobacillus stearothermophilus* strain 12, the genes (*abf*1 and *abf*2) coding for these enzymes were cloned and sequenced. Based on the protein sequence similarities, approximately 57 kDa two α-L-AFases were assigned to the glycoside hydrolase family 51. To obtain pure enzymes, the *abf*1 and *abf*2 genes were cloned into pET28a+ expression vector and recombinant α-L-AFases were produced in *E.coli* BL21(DE3): pLysS. Characterization of recombinant α-L-AFases revealed that Abf1Geo12 and Abf2Geo12 were active in a broad temperature range from 50 to 85 °C and from 40 to 80 °C, respectively. Also, the Abf1Geo12 was active in a broad pH range from 5.0 to 9.0. The optimum pH and temperature for Abf1Geo12 were determined as pH 6.0 and 65 °C, respectively, whereas the optimum pH and temperature for Abf2Geo12 were determined as pH 5.5 and 60 °C, respectively. Based on characterization studies, it was determined that the Abf1Geo12 was more stable than Abf2Geo12 and previously identified α-L-AFases from *G. stearothermophilus*. Using *p*-nitrophenyl α-L-arabinofuranoside as a substrate, the K_m and V_{max} values for Abf1Geo12 and Abf2Geo12 were determined as 0.31 mM and 290 U/mg for the former enzyme and 0.19 mM and 213.2 U/mg for the latter enzyme, respectively. The activities of Abf1Geo12 and Abf2Geo12 were strongly inhibited by 1 mM Hg^{2+}. Interestingly, Cu^{2+} and Co^{2+} stimulated the activity of Abf1Geo12, but they reduced the activity of Abf2Geo12. The recombinant enzymes released L-arabinose from sugar beet arabinan, arabinobiose, arabinotriose, arabinotetraose and arabinopentaose. Consequently, these characterized two enzymes may be used in industrial fields since they are stable at high temperatures.

Key words: α-L-Arabinofuranosidases; *Geobacillus stearothermophilus*; thermostable enzymes; thermophilic bacteria.

Abbreviations: α-L-AFase, α-L-arabinofuranosidase; CAZy, Carbohydrate-Active Enzymes database; DNS, dinitrosalicylic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GH, glycoside hydrolase; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria Bertani; PAGE, polyacrylamide gel electrophoresis; pNP, *p*-nitrophenyl; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

Introduction

Hemicellulose is the second most abundant polymer in the world (Isikgor & Becer 2015). All plant cell walls are composed of lignocelluloses, such as hemicellulose, cellulose, pectin and lignin (Numan & Bhosle 2006). From cereal crops and crop fibre biomass, the breakdown of hemicelluloses is achieved by enzymes that are increasingly becoming important due to their pivotal role in the utilization of these renewable energy sources (Wagschal et al. 2008). Many enzymes can perform microbial degradation of hemicellulose materials, such as endoxylanases, β-xylosidase, acetylxylan esterases, glucuronidases and α-L-arabinofuranosidase (α-L-AFase). While some of these enzymes, e.g. endoxylanases and β-xylosidase, are responsible for the back-bone degradation, other group of enzymes that are named as accessory enzymes, e.g. α-L-arabinofuranosidase, are responsible for the cleavage of the side chains (Degrassi et al. 2003).

Arabinofuranosidases are hemicellulose degrading enzymes, which cleave L-arabinofuranosyl residues. Within this group of enzymes, the α-L-AFases specifically catalyse the hydrolysis of terminal non-reducing α-L-1,2-, α-L-1,3- and α-L-1,5-arabinofuranosyl residues belonging to different hemicellulosic homopolysaccharides (branched and debranched arabinans) and heteropolysaccharides (arabinoxylans, arabinogalactans, etc.) (Saha 2000; Margolles & de los Reyes-Gavilan 2003; Inacio et al. 2008). Endo-arabinase and α-L-AFase are major hydrolytic enzymes essential to generate L-arabinose from arabinan. To produce arabino-oligosaccharides or L-arabinoses, the arabinan backbone of α-1,5-linked L-arabinofuranosyl should be hydrol-

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ysed by an endo-arabinanase (EC 3.2.1.99). To produce L-arabinoses, arabino-oligosaccharides and arabinan side-chain substitutions should be thoroughly hydrolysed by α-L-AFase (EC 3.2.1.55). In addition, exo-arabinanase (EC 3.2.1.-) is able to release terminal arabinose (non-reducing end with net retention of the anomeric configuration) or arabino-oligosaccharides from arabinan. Based on the amino acid sequence similarities, the endo and exo-arabinanases have been classified in the Carbohydrate-Active Enzymes (CAZy) database (http://www.cazy.org/) into two glycoside hydrolase (GH) families GH43 and GH93, respectively (Cantarel et al. 2009). Besides, α-L-AFases are members of GH families of 3, 43, 51, 54 and 62 (Shi et al. 2014).

The genus *Geobacillus* was separated from the genus of *Bacillus* and the type species of this new genus is *Geobacillus stearothermophilus*. Many kinds of species with thermophilic, acidophilic, alkalophilic and halophilic properties have been included in this genus. Because of thermostable enzymes, such as proteases, amylases, pullulanases, peroxidases, glucose isomerases, lipases, xylanases and DNA restriction enzymes, thermophilic bacilli have became very important in many industrial areas (Canakcıet al. 2007b). In recent years, arabinofuranosidases have become the focus of great interest and have found potential applications in industrial processes. The efficient conversion of hemicellulosic biomass into fuels and chemicals, the improvement in the consistency of beer, clarification of juices, delignification of paper pulp and digestibility enhancement of animal feedstock are examples of these applications (Saha 2000; Canakcıet al. 2007a; Squina et al. 2010). In these processes, thermostable enzymes are more suitable since elevation of temperature increases accessibility and solubility of the substrates (Birgisson et al. 2004).

Until now, α-L-AFases have been studied in several bacteria, fungi and plants. These studies showed that two different arabinofuranosidases in a cell are mostly found in fungi and plants (Filho et al. 1996; Kaneko et al. 1998; Matsuo et al. 2000; Lynette & Cobbett 2003; Sakamoto & Kawasaki 2003). However, in bacteria, a single arabinofuranosidase is mostly found in a cell (Canakcıet al. 2007a,b). In this study, we aimed to purify and characterize two different α-L-AFases (Abf1Geo12 and Abf2Geo12) from a thermophilic bacterium named as *Geobacillus stearothermophilus* strain 12.

Material and methods

Strains, vectors and media

Geobacillus stearothermophilus strain 12 was previously isolated from the Alangullu hot spring in the province of Aydın, Turkey. The strain 12 was identified based on its biochemical, physiological, morphological properties and 16S rRNA gene sequence analysis (Canakcıet al. 2007b). The strains, plasmids and primers used in this study are given in Table 1. All recombinant *Escherichia coli* strains were grown in Luria Bertani (LB) medium supplemented with 50 μg/mL ampicillin or kanamycin at 37 °C. *G. stearothermophilus* strain 12 is publicly-accessible and was deposited at Molecular Biology Laboratory, Department of Biology at Karadeniz Technical University, Trabzon, Turkey.

Detection of abf1 and abf2 genes from G. stearothermophilus 12

Canakcıet al. (2007b) isolated two extracellular α-L-AFases from *G. stearothermophilus* strain 12 when this strain was grown in LB medium containing 1% sugar beet arabinan (Megazyme, Wirklow, Ireland) as a carbon source. Native polyacrylamide gel electrophoresis (PAGE) was used to determine arabinofuranosidase activities using 4-methylumbelliferyl-α-L-arabinofuranoside as a substrate.

To obtain the crude α-L-AFase enzymes from *G. stearothermophilus* 12, 25 μL of overnight culture of the strain 12 was inoculated into fresh LB broth (250 mL) containing sugar beet arabinan and the inoculated medium were incubated at 60 °C for 72 h with shaking (180 rpm). After the incubation period, the culture was centrifuged at 10,000 rpm for 15 min and the obtained supernatant was lyophilized. The lyophilized supernatant was partially purified using CM-Sepharose CL-6B colon according to the study of Kaneko et al. (1998). Based on the sodium dodecyl sulphate (SDS) PAGE analysis of the partial purified supernatant, two different thick bands (∼50 and ∼60 kDa) were detected. These two bands were excised from the gel and sent to the Center of Advanced Proteomics Research Laboratory (University of Medicine and Dentistry of New Jersey) for tryptic digestion and MALDI-TOF analyses. After MALDI-TOF analysis, degenerate primers (ab_Fwd and ab_Rev) were designed and used to amplify *abf1* and *abf2* genes (Table 1). The amplified PCR products were cloned into pGEM-T Easy Vector System (Promega, Madison, WI, USA) for sequencing. The remainders of *abf*1 and *abf*2 genes were obtained by inverse PCR (Ochman et al. 1988).

Cloning, overexpression and purification of α-L-AFases

The *afb*1 and *abf*2 genes were amplified by PCR using Ex-abf1 Fwd-Ex-abf1 Rev and IsabfF-IsabfR primer pairs, respectively. To clone the *abf*1 PCR product into pET28a+ vector, PCR product and the vector were digested with *Nde*I and *Hind*III and then they were ligated. In the same way, the *abf*2 PCR product and the vector were digested with *Nhe*I and *BamH*I and then they were ligated. The recombinant plasmids were named as pES1 and pES2 (Table 1). After that, the pES1 and pES2 plasmids were transformed into *E. coli* BL21 (DE3): pLysS cells. The transformed cells were named as *E. coli* ES1 and *E. coli* ES2 (Table 1).

The *E. coli* ES1 and ES2 cells were grown in LB medium supplemented with 50 μg/mL kanamycin until 0.6 value of the optical density at 600 nm. For overexpression of recombinant proteins, the strains were grown in LB medium for 3 h with induction using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After that, the proteins were purified by MagneHis^{TM} Protein Purification System (Promega, Madison, WI, USA).

Enzyme assay

The enzyme assays were performed based on the hydrolysis of *p*-nitrophenyl (pNP) α-L-arabinofuranoside. α-L-AFase activities were determined in a total volume of 0.5 mL at 60 °C. The reaction mixture contained 0.2 mM pNP α-L-arabinofuranoside, 50 mM potassium phosphate buffer (pH 6.0) and appropriately diluted enzyme solution. The reaction mixture was incubated at 60 °C for 10 min and then

Table 1. The strains, plasmids and primers used in this study.

Strains	Relevant construction, genotype, or sequence (5'–3')[a]	Reference
G. stearothermophilus 12	Wild type	This study
E. coli DH5α	F^- endA1 recA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169	In our lab
E. coli BL21 (DE3): pLysS	F^- ompT gal dcm lon hsdS_B(r_B^- m_B^-)λ(DE3) pLysS(cm^R)	Novagen
E. coli ES1	*E. coli* BL21 (DE3):pLysS; including pES1	This study
E. coli ES2	*E. coli* BL21 (DE3):pLysS; including pES2	This study
Plasmids		
pGEM-T Easy	Cloning vector	Promega
pET28a+	Expression vector	Novagen
pES1	pET28a+, carrying *abf*1 gene of *G. stearothermophilus* 12	This study
pES2	pET28a+, carrying *abf*2 gene of *G. stearothermophilus* 12	This study
Primers		
ab_Fwd	MGN TAY CCN GGN GGN AAY TTY	This study
ab_Rev	CAT YTC RTT NCC NAR RCA C	This study
Abf2-1 Fwd	ggNTgYTTTgCNgAYgARTAYCATTgg	This study
Abf2-1 Rev	ACATVTBRAAYACATgRTANgTHggBgT	This study
Ex-abf1 Fwd	TAACATATGAGTATGAAAAAAGCAACCATG	This study
Ex-abf1 Rev	TATAAGCTTTATTGCTTTGCTAAACGAATG	This study
IsabfF	GGCTAGCGTGAGAAACAGAGCGGTAATAAACG	This study
IsabfR	CGGGATCCCTATCATTGTCGTTCAATCAAAGCCGC	This study

[a] The underlined sequences show multiple cloning sites.

0.5 mL of cold 1 M Na_2CO_3 was added to stop the reaction. The released amount of *p*-nitrophenol was determined by measuring the optical density at 405 nm. One unit of activity was defined as the amount of enzyme, which produces 1 mM *p*-nitrophenol per min (Canakcıet al. 2007b). The endoarabinanase activity of Abf1Geo12 and Abf2Geo12 was tested using red-dyed arabinan (Megazyme, Wirklow, Ireland) as a substrate. The reactions were performed according to the manufacturer's instructions.

Effects of pH and temperature on α-L-AFases activity and the stability

The optimum pH for the activities of Abf1Geo12 and Abf2Geo12 was determined by incubation at 60 °C for 10 min in the pH range from 4 to 10 with pH unit intervals of 0.5. The used buffers were as follows: 50 mM sodium acetate (pH 4.0–5.5), 50 mM potassium phosphate (pH 5.5–7.5), 25 mM Tris-HCl (pH 7.5–9.0) and 50 mM glycine-NaOH (pH 10.0–11.0). The optimum temperature for enzymatic activities was determined in 50 mM potassium phosphate buffer (pH 6.0 for Abf1Geo12 and pH 5.5 for Abf2Geo12) using different temperatures ranging from 40 to 90 °C. The results of optimum temperature and pH were expressed as percentage of the activity.

The thermostability of the purified enzymes was determined in microcentrifuge tubes (0.5 mL) containing 0.5 U of Abf1Geo12 and Abf2Geo12 in 50 mM potassium phosphate buffer (pH 6.0 for Abf1Geo12 and pH 5.5 for Abf2Geo12). These microcentrifuge tubes were incubated at different temperature ranges (from 50 to 70 °C) for different time periods (from 12 to 72 h). Then the samples were removed at the indicated times, cooled on ice bath and assayed for the residual enzyme activities. To determine the effect of pH on the stability of Abf1Geo12 and Abf2Geo12, the microcentrifuge tubes containing 0.5 U of Abf1Geo12 and Abf2Geo12 in aforementioned buffers, which have various pH values, were incubated at the optimum temperatures for 5 h. After the incubation, the samples were assayed for the residual enzyme activities.

Effects of chemical agents and metal cations on α-L-AFases

The effects of metals and chemical agents on the activity of α-L-AFases were determined using 1 mM concentration of several metals ($ZnSO_4$, $HgCl_2$, $CuSO_4$, $MnCl_2$, $MgCl_2$, $CaCl_2$, $CoCl_2$ and NaCl) and different chemical agents [10 mM reducing agent dithiothreitol (DTT), β-mercaptoethanol and chelating agent ethylenediaminetetraacetic acid (EDTA)]. The results were expressed as the residual activity obtained in the absence of the compounds.

Substrate specificity

The substrate specificity of α-L-AFases was tested using the following pNP glycosides (Sigma, St. Louis, MO, USA): pNP α-L-arabinopyranoside, pNP β-D-galactopyranoside, pNP β-D-xylopyranoside, pNP β-L-arabinopyranoside, pNP N-acetyl-β-D-glucosaminide, pNP β-D-fucopyranoside, pNP α-D-glucopyranoside, pNP β-D-glucopyranoside, pNP α-L-rhamnopyranoside, pNP α-D-galactopyranoside, pNP α-D-xylopyranoside and pNP α-L-arabinofuranoside.

Non-chromatic substrates used in this study were arabinooligosaccharides (arabinobiose, arabinotriose, arabinotetraose, and arabinopentaose), oat spelt xylan, larch wood arabinogalactan, rye arabinoxylan and sugar beet arabinan (Megazyme, Bray, Wirklow, Ireland). The reaction mixtures contained 0.5 mL of 2% (w/v) substrate and 0.1 mL of enzymes (1 U) solutions. The mixtures were incubated at optimum temperatures for 12 h. After the incubation, the reactions were stopped by boiling for 5 min. Dinitrosalicyclic acid (DNS) method was used to determine the released L-arabinose (Miller 1959). Thin-layer chromatography (TLC) was used to analyse sugars from arabinan and arabinooligosaccharides. Silica gel 60 F254 plates (Merck) with a solvent system including ethyl acetate, acetic acid and water (2 : 1 : 1) were used for TLC. Plates containing sugars were sprayed with 5% (v/v) sulphuric acid in ethanol and were heated at 120 °C for 10 min.

Table 2. Identification results of the α-L-AFases of *G. stearothermophilus* 12 based on the BLAST search using the complete amino acid sequence.

α-L-AFase	Species	Description	GenBank Acc. No.	Query Coverage (%)	Identity (%)
Abf1Geo12	*Geobacillus* sp. JF8	α-N-arabinofuranosidase	YP008417789	100	98
	Geobacillus vulcani	α-L-arabinofuranosidase	CCF72370	100	97
	Geobacillus sp. C56-T3	α-L-arabinofuranosidase	YP003671207	100	97
	Geobacillus sp. CAMR5420	α-N-arabinofuranosidase	KDE48495	100	97
Abf2Geo12	*Geobacillus* sp. Y412MC61	α-L-arabinofuranosidase	YP003253791	100	99
	Geobacillus stearothermophilus T-6	Arabinofuranosidase	ACE73681	100	99
	Geobacillus sp. WSUCF1	α-L-arabinofuranosidase	WP020755790	100	99
	Geobacillus stearothermophilus	α-N-arabinofuranosidase	KFL17041	100	99

Bioinformatics analyses

All sequencing data were edited by BioEdit (Hall 1999). The obtained sequences were analysed by BLAST searches (Altschul et al. 1990) using the NCBI GenBank database (Benson et al. 2012). The presence of signal peptides for Abf1Geo12 and Abf2Geo12 were investigated by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Homology modelling was performed using Swiss Model server (Schwede et al. 2003).

Nucleotide sequence accession number

The nucleotide sequences of *abf*1 and *abf*2 genes have been deposited with the GenBank database (Benson et al. 2012) under the accession numbers KP113696 and KP113697, respectively.

Results

Determination of α-L-AFases activity

In the study of Canakcıet al. (2007b), two α-L-AFases were determined in the native gel incubated with 4-methylumbelliferyl-α-L-arabinofuranoside under UV light when *G. stearothermophilus* 12 was grown in LB broth containing sugar beet arabinan as a carbon source.

At the beginning of this study, *G. stearothermophilus* 12 was grown in LB broth containing sugar beet arabinan as a carbon source. After that, Abf1Geo12 and Abf2Geo12 were partially purified using CM-sepharose CL-6B column chromatography. The partially purified proteins were loaded onto a PAGE gel and the zymogram analysis was performed using 4-methylumbelliferyl-α-L-arabinofuranoside. After the zymogram analysis, two protein bands were excised from the gel and sent to the Center of Advanced Proteomics Research Laboratory (University of Medicine and Dentistry, New Jersey, USA) for tryptic digestion and MALDI-TOF analysis. For Abf1Geo12, the resulting peptide sequence showed a high similarity with *G. thermoleovorans* α-L-AFase and an approximately 650 bp DNA region was amplified using degenerate primers of ab_Fwd and ab_Rev (Table 1). For Abf2Geo12, the resulting peptide sequence exhibited a high similarity with *G. stearothermophilus* strain T-6 α-L-AFase and an approximately 880 bp DNA region was amplified using degenerate primers of Abf2-1 Fwd and Abf2-1 Rev (Table 1). PCR products were cloned into pGEM-T easy vector and then they were sequenced. BLAST results revealed that the cloned fragments were a part of α-L-AFases. The remainder sequences of *abf*1 and *abf*2 genes were obtained by invers PCR (Ochman et al. 1988). After the full length of the genes were determined, we showed that the 1,509 bp *abf*1 gene encodes a protein with 502 amino acid residues (57 kDa) and the 1,518 bp *abf*2 gene encodes a protein having 505 residues (58 kDa) based on the calculation of ProtParam (http://www.expasy.org/). No signal peptide sequences were determined in both Abf1Geo12 and Abf2Geo12 proteins when analysed by SignalP 4.1.

When the amino acid sequences of Abf1Geo12 and Abf2Geo12 were compared with other amino acid sequences from the GenBank NCBI database, Abf1Geo12 exhibited 98% identity to the α-L-AFase of *Geobacillus* sp. JF8 and 97% identity to the α-L-AFase of *G. vulcani*. Also Abf2Geo12 exhibited 99% identity to the α-L-AFases of *Geobacillus* sp. Y412MC61 and *G. stearothermophilus* T-6 (Table 2).

Based on homology modelling, the Abf1Geo12 should adopt a similar structure to that of *G. stearothermophilus* T-6 α-L-AFase (family GH51) with 96.81% sequence identity (Hovel et al. 2003) and the Abf2Geo12 a structure to that of *Thermobacillus xylanilyticus* arabinofuranosidase (family GH51) and its Glu176Gln mutant (Paes et al. 2008). The nine key conserved residues described previously as responsible for activity were also identified in both Abf1Geo12 and Abf2Geo12 proteins (Fig. 1).

Overexpression and characterization of α-L-AFases

To perform the biochemical characterization of Abf1Geo12 and Abf2Geo12, the cell extracts of *E. coli* ES1 and ES2 harbouring pES1 and pES2 plasmids were obtained by sonication procedure. The overexpressed Abf1Geo12 and Abf2Geo12 proteins were purified using MagneHis Protein Purification System (Promega, Madison, WI, USA). The purified proteins were loaded onto a native gel with extracellular wild-type proteins of *G. stearothermophilus* 12, which were grown in LB broth containing sugar beet arabinan as a carbon source. After electrophoresis, the gel was incubated in 50 mM potassium phosphate buffer containing 4-methylumbelliferyl-α-L-arabinofuranoside for 30 min. The electrophoresis results showed that two different

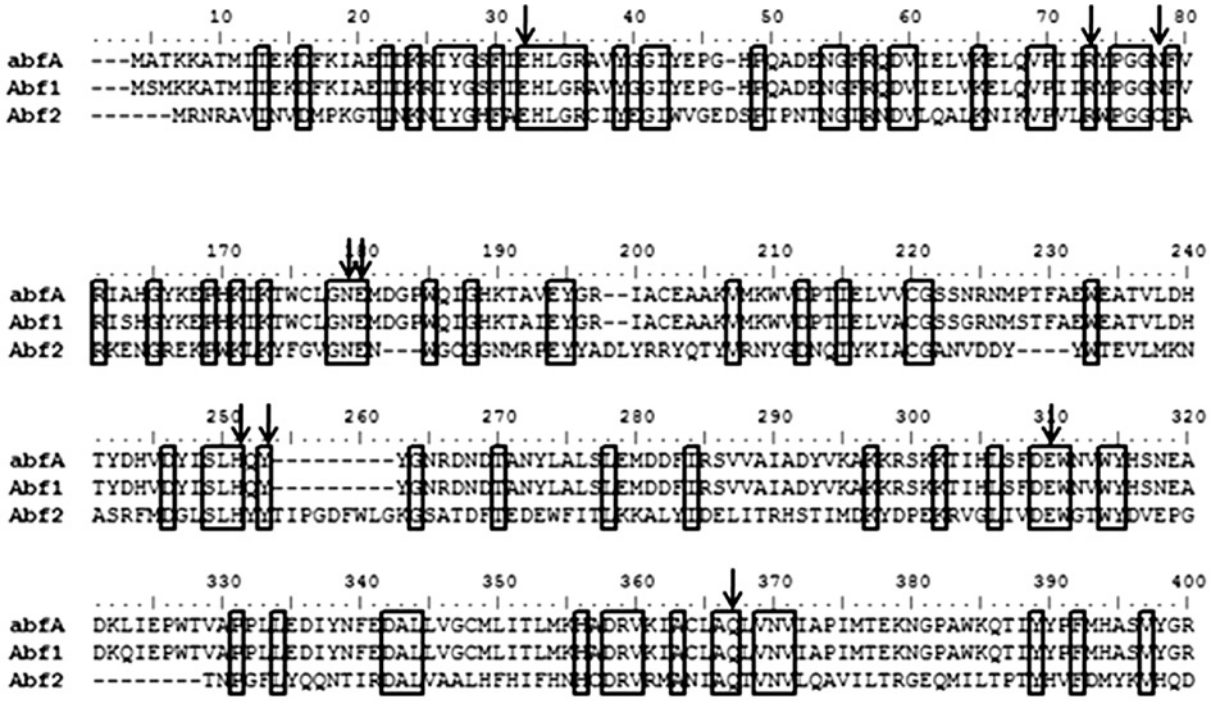

Fig. 1. Comparison of conserved amino acid sequences of α-L-Afases belonging to *G. stearothermophilus* strain 12 (Abf1 and Abf2) and *G. stearothermophilus* strain T-6 (abfA). Black arrows show nine key residues, which are responsible for activity.

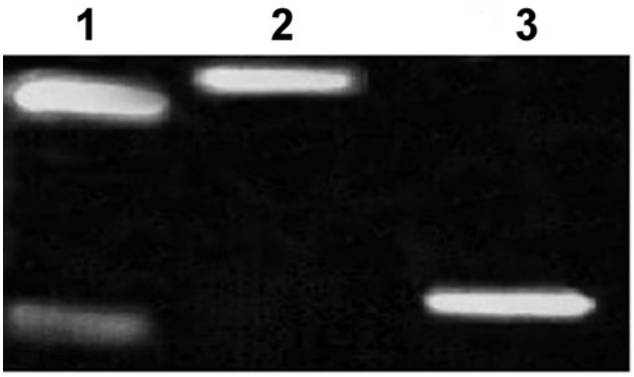

Fig. 2. Native gel electrophoresis of the native and recombinant α-L-AFases. Lane 1: wild type α-L-AFases of *G. stearothermophilus* 12; lane 2: recombinant Abf1Geo12 overexpressed in *E. coli* ES1; lane 3: recombinant Abf2Geo12 overexpressed in *E. coli* ES2. All recombinant enzymes were purified by MagneHis Protein Purification System (Promega; Madison, WI, USA). The gel was incubated in 50 mM potassium phospahate buffer containing 4-methylumbelliferyl-α-L-AF.

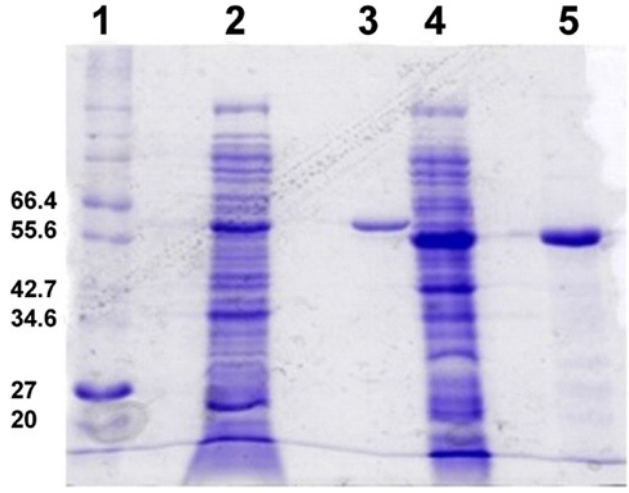

Fig. 3. SDS-PAGE analyses of overexpressed α-L-AFases. Lane 1: protein marker (NEB, Protein marker Broad Range, 2-212 kDa); lane 2: cell extract of *E. coli* ES1 harbouring pES1 that was collected after IPTG induction; lane 3: Abf1Geo12 enzyme purified by Ni-NTA agarose; lane 4: cell extract of *E. coli* ES2 harbouring pES2 that was collected after IPTG induction; lane 5: Abf2Geo12 enzyme purified by Ni-NTA agarose.

α-L-AFases were overexpressed in *E. coli* cells (Fig. 2). The activity staining of the enzymes from native and recombinant cells showed that all cells exhibited the α-L-AFase activity.

Molecular weight

The molecular weight of the histidine-tagged Abf1-Geo12 and Abf2Geo12 proteins were estimated to be around 230 kDa and 60 kDa, respectively, according to the gel filtration chromatography. SDS-PAGE analysis showed that the molecular weights of Abf1Geo12 and Abf2Geo12 proteins were very close to each other (approximately 56 kDa) (Fig. 3).

Effects of pH and temperature on the activity of α-L-AFases and the stability

The effects of pH and temperature on Abf1Geo12 and Abf2Geo12 enzymes were investigated using purified enzymes. The purified enzymes were incubated at different temperature ranges from 40 to 90 ℃ at optimum pH and different pH ranges from 4.0 to 11.0 at optimum temperature. The optimum temperatures for Abf1Geo12 and Abf2Geo12 were determined as 65 ℃ and 60 ℃, respectively (Fig. 4). Abf1Geo12 retained

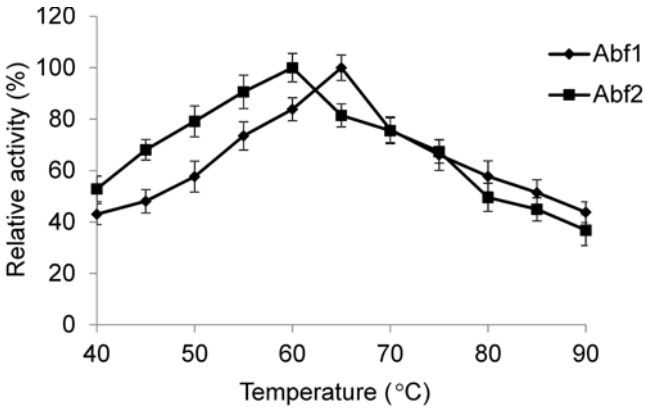

Fig. 4. Optimum temperature values for *G. stearothermophilus* 12 α-L-AFases.

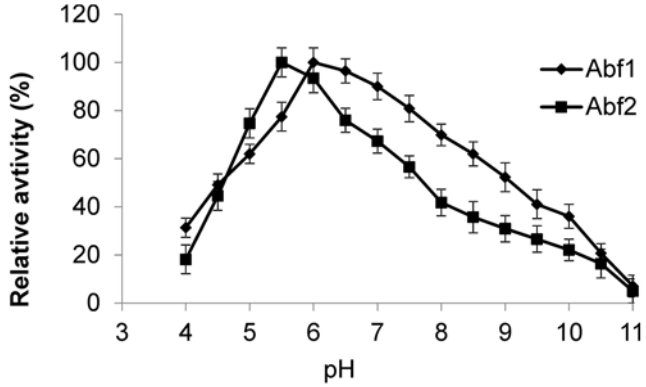

Fig. 5. Optimum pH values for *G. stearothermophilus* 12 α-L-AFases.

over 50% activity at a broad temperature ranges from 50 to 85 ℃. Abf2Geo12 lost 50% of its activity above 80 ℃. The optimum pH values were determined as 6.0 and 5.5 for Abf1Geo12 and Abf2Geo12, respectively. Moreover, Abf1Geo12 and Abf2Geo12 showed over 50% activity at a broad pH ranges from 5.0 to 9.0 and from 5.0 to 7.5, respectively (Fig. 5).

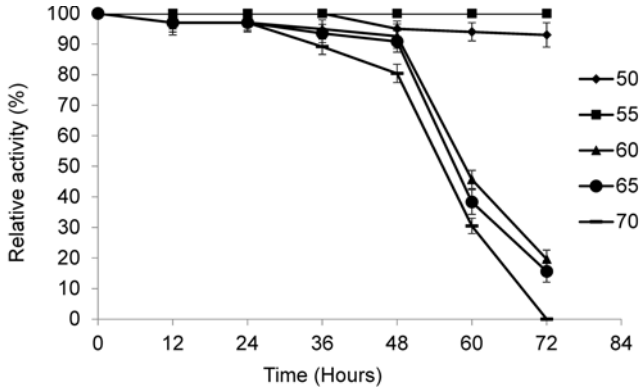

Fig. 6. The stability of Abf1Geo12 enzyme at different temperatures.

The Abf1Geo12 enzyme retained the full activity at 55 ℃ and 93% activity at 50 ℃ for 72h. Abf1Geo12 maintained its full activity at 60, 65 and 70 ℃ for 24 h. The enzyme lost its activity over 50% at 60 ℃, 65 ℃ and 70 ℃ for 60 h (Fig. 6). It was found that Abf2Geo12 was not thermostable.

Effects of chemical agents and metal cations on the activity of α-L-AFases

No effect was determined on the activity of Abf1Geo12 with Mn^{2+}, Zn^{2+}, Ca^{2+} and Na^{2+} metals and DTT, whereas EDTA slightly reduced its activity. The activity of Abf1Geo12 was slightly stimulated by Mg^{2+}, Cu^{2+} and Co^{2+} metals and β-mercaptoethanol (Fig. 7). Also, no effect was determined on the activity of Abf2Geo12 with Zn^{2+}, Ca^{2+}, Na^{2+}, DTT and EDTA, whereas Cu^{2+} and Co^{2+} caused the complete loss of its activity. Mg^{2+} slightly reduced the activity of Abf2Geo12. The activity of Abf2Geo12 was remarkably stimulated by Mn^{2+} and β-mercaptoethanol. Hg^{2+} caused the complete loss of the activity for both Abf1Geo12 and Abf2Geo12 (Fig. 7).

Substrate specificity and kinetic analysis

The K_m and V_{max} values were determined by the

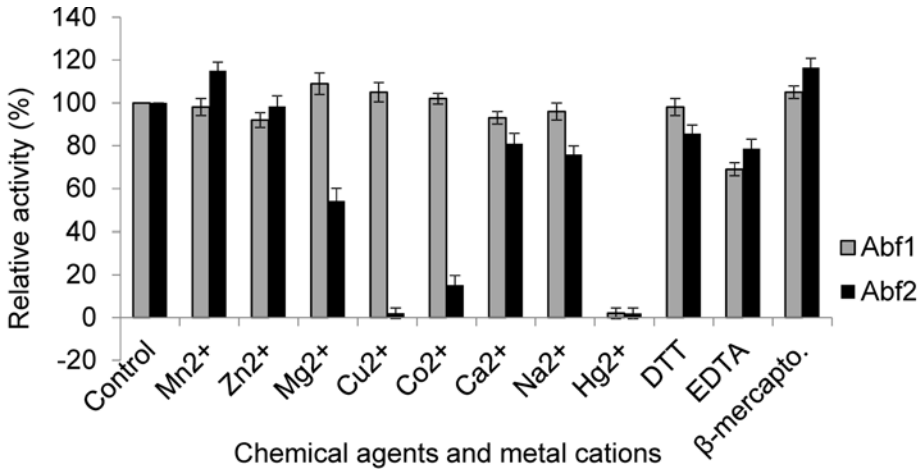

Fig. 7. The effects of chemical agents and metal cations on the activities of Abf1Geo12 and Abf2Geo12 enzymes.

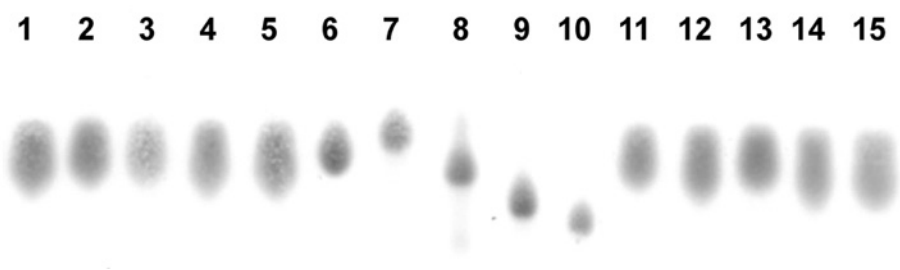

Fig. 8. TLC of hydrolyzed products obtained from sugar beet arabinan and arabinooligosaccharides. Lane 1: sugar beet arabinan; lane 2: arabinobiose; lane 3: arabinotriose; lane 4: arabinotetraose; lane 5: arabinopentaose; lane 6: arabinose; lane 7: arabinobiose; lane 8: arabinotriose; lane 9: arabinotetraose; lane 10: arabinopentaose; lane 11: arabinopentaose; lane 12: arabinotetraose; lane 13: arabinotriose; lane 14: arabinobiose; lane 15: augar beet arabinan. Lanes 1-5: substrates were incubated with 1 U of Abf1Geo12 for 16 h; lanes 6-10: arabinooligosaccharide standards; lane 11- 15: substrates were incubated with 1 U of Abf2Geo12 for 16 h.

hydrolysis of pNP α-L-arabinofuranoside for both Abf1Geo12 and Abf2Geo12. The K_m and V_{max} values for Abf1Geo12 and Abf2Geo12 were determined as 0.31 mM, 290 U/mg and 0.19 mM, 213.2 U/mg, respectively. The substrate specificity of Abf1Geo12 and Abf2Geo12 enzymes was determined using several pNP glycosides. Abf1Geo12 and Abf2Geo12 were not able to hydrolyse all tested pNP glycosides, except for pNP α-L-arabinofuranoside.

To ensure whether Abf1Geo12 and Abf2Geo12 would hydrolyse arabinose-containing oligosaccharides and polysaccharides, the hydrolysis of α-1,5-linked arabinooligosaccharides (containing from two to five arabinose residues) and some arabinose-containing polysaccharides was studied. Abf1Geo12 and Abf2Geo12 were active on sugar beet arabinan and rye arabinoxylan. Abf1Geo12 and Abf2Geo12 also exhibited the activity against α-1,5-linked arabinobiose, arabinotriose, arabinotetraose and arabinopentaose (Fig. 8).

Discussion

The α-L-AFase is an enzyme that degrades hemicellulose containing arabinoxylan, arabinogalactan and L-arabinan residues. L-Arabinose is widely found in nature as a component of biopolymers, such as hemicellulose and pectin. The α-L-AFases specifically catalyse the hydrolysis of terminal non-reducing α-L-1,2-, α-L-1,3- and α-L-1,5-arabinofuranosyl residues from different oligosaccharides and polysaccharides. The α-L-AFases can be found in several plants, fungi and bacteria (Numan & Bhosle 2006). In this study, we isolated two α-L-AFases from a thermophilic bacterium *G. stearothermophilus* strain 12. When the amino acid sequences of Abf1Geo12 and Abf2Geo12 were compared with other sequences in the GenBank NCBI database, we determined that both Abf1Geo12 and Abf2Geo12 were α-L-AFase enzymes. The homology modelling of Abf1Geo12 and Abf2Geo12 showed that they belong to family GH51 in the CAZy database. Based on the amino acid sequence similarity, α-L-AFases have been classified into five GH families – GH3, 43, 51, 54 and 62 (Cantarel et al. 2009). The α-L-AFase of *G. stearothermophilus* T-6 belongs to family GH51 and its crystal structure of revealed nine key residues responsible for catalysis and substrate binding interactions: Glu29, Arg69, Asn74, Asn174, Glu175, His244, Tyr246, Glu294 and Gln351 (Shallom et al. 2002a,b; Hövel et al. 2003). These nine key residues were all found 100% conserved in the Abf1Geo12, but in the Abf2Geo12 one residue (Cys74) was different. These data strongly support that Abf1Geo12 and Abf2Geo12 are members of the family GH51 (Fig. 1).

Several α-L-AFases from thermophilic bacteria that are members of the genus *Geobacillus* have been reported and characterized. In the study recently performed by Rozanov et al. (2015), it has been shown that the bacterium *G. stearothermophilus* 22 VKPM-B-11678 isolated from sediment in Russia had α-L-AFase. In other studies, α-L-AFase enzymes were determined in *G. stearothermophilus* T-6 and L1 (Gilead & Shoham 1995; Bezalel et al. 1993). Also, Canakci et al. (2007a) isolated and characterized a highly thermostable α-L-AFase from *G. caldoxylolyticus*. Moreover, in the study of Canakcıet al. (2007b), it has been detected that some *Geobacillus* species possessed α-L-AFase activity. At the same time, multiple forms of α-L-AFases have mostly been detected in plants (Hirano et al. 1994), fungi (Ramon et al. 1993; Luonteri et al. 1995; Filho et al. 1996; Kaneko et al. 1998) and *Streptomyces* strains (Tajana et al. 1992; Matsuo et al. 2000). However, in this study, we showed that *G. stearothermophilus* 12 produced two types of α-L-AFases showing different physicochemical properties.

In this study, the K_m values of Abf1Geo12 and Abf2Geo12 for the degradation of pNP α-L-arabinofuranoside were determined as 0.31 and 0.19 mM, respectively. In other studies, the K_m values of *G. stearothermophilus* L1 and T6, *G. caldoxylolyticus* TK4 and *Anoxybacillus kestanbolensis* AC26Sari for the degradation of pNP α-L-arabinofuranoside were determined as 0.42, 0.22, 0.17 and 0.139 mM, respectively (Bezalel et al. 1993; Gilead & Shoham 1995; Canakcıet al. 2007a, 2008). These data show that Abf1Geo12 and Abf2Geo12 have higher affinity to pNP α-L-arabinofuranoside than the α-L-AFases of *G. stearothermophilus* T6 and L1.

According to the gel-filtration chromatography, the molecular weight of Abf1Geo12 was estimated to be around 230 kDa and this result clearly showed that

Abf1Geo12 was composed of four identical 56 kDa subunits. The molecular weight of Abf2Geo12 was estimated to be around 60 kDa and this showed that Abf2Geo12 has a monomer structure. For Abf1Geo12, similar results were obtained from previous studies. In the study of Gilead & Shoham (1995), it has been shown that α-L-AFase of *Geobacillus stearothermophilus*
T-6 was a 256 kDa protein that was composed of four identical 64 kDa subunits. In another study, it has been shown that α-L-AFase of *G. caldoxylolticus* was a 236 kDa protein that was composed of four identical 59 kDa subunits (Canakcıet al. 2007a).

In this study, it was determined that the optimum temperatures of Abf1Geo12 and Abf2Geo12 were close to each other (65 ℃ and 60 ℃, respectively). However, based on the stability assays, it was shown that although Abf1Geo12 is thermostable, Abf2Geo12 is not thermostable. Also, in this study, it was found that Abf1Geo12 exhibited the thermostability at 55 ℃ for 72 h, but Abf2Geo12 lost approximately 75% of its activity at 50–55 ° for 4h. Although, the optimum temperatures of Abf1Geo12 and Abf2Geo12 are lower than the α-L-AFases of *G. stearothermophilus* L1 and T6 (70 ℃), Abf1Geo12 is more stable than they both (Bezalel et al. 1993; Gilead & Shoham 1995).

Most of bacterial α-L-AFases are active between pH 5.0 and 7.0 although fungal enzymes have lower optimal pH (2.0–6.0) (Numan & Bhosle 2006). The optimum pH values of α-L-AFases from *G. stearothermophilus* L1 and T6, *G. caldoxylolyticus* TK4 and *A. kestanbolensis* AC26Sari were determined as 6.0, 7.0, 6.0 and 5.5, respectively (Bezalel et al. 1993; Gilead & Shoham 1995; Canakci et al. 2007a, 2008). In our study, the optimum pH was determined as 6.0 for both Abf1Geo12 and 5.5 for Abf2Geo12.

The activities of Abf1Geo12 and Abf2Geo12 were not affected by Zn^{2+}, Ca^{2+} and Na^{2+} as well as by DTT, whereas the activities were slightly reduced by EDTA for both enzymes. While the activity of Abf1Geo12 was not affected by Mn^{2+}, the activity of Abf2Geo12 was remarkably stimulated by Mn^{2+}. Also, although the activity of Abf1Geo12 was stimulated by Mg^{2+}, the activity of Abf2Geo12 was remarkably reduced by Mg^{2+}. Reducing agent β-mercaptoethanol stimulated the activity for both Abf1Geo12 and Abf2Geo12 enzymes. Interestingly, Cu^{2+} and Co^{2+} caused the complete activity loss for Abf2Geo12, but the activity of Abf1Geo12 was stimulated by these metals. Hg^{2+} completely inhibited the activity for both Abf1Geo12 and Abf2Geo12. Hg^{2+} is known to react with sulfhydryl groups of proteins as well as with histidine and tryptophan residues (Margolles & de los Reyes-Gavilan 2003). In all studies performed up to now, all characterized α-L-AFases belonging to the genus *Geobacillus* were inhibited by Hg^{2+} (Gilead & Shoham 1995; Takao et al. 2002; Canakcıet al. 2007a,b;).

Although the purified α-L-AFase enzymes from *G. stearothermophilus* 12 (Abf1Geo12 and Abf2Geo12), *G. stearothermophilus* (T6 and L1) and *A. kestanbolensis* AC26sari (AbfAC26Sari) hydrolysed pNP α-L-arabinofuranoside, they were not able to hydrolyse other pNP glycosides (Bezalel et al. 1993; Gilead & Shoham 1995; Canakcıet al. 2008). In contrast, α-L-AFase (AbfATK4) from *G. caldoxylolyticus* TK4 was able to hydrolyse pNP β-D-xylopranoside and pNP α-L-arabinofuranoside (Canakcıet al. 2007a). In this study, we determined that pNP α-L-arabinopyranoside was not hydrolysed by Abf1Geo12 and Abf2Geo12. This result indicates that Abf1Geo12 and Abf2Geo12 are specifically active toward the furanosidic conformation and α-linkages. Also, Abf1Geo12 and Abf2Geo12 exhibited the activity against sugar beet arabinan (including α-1,3-linkages), rye arabinoxylan and α-1,5-linked oligosaccharides, yielding arabinose as the sole hydrolysis end product. However, Abf1Geo12 and Abf2Geo12 were not able to hydrolyse large wood arabinogalactan and oat spelt xylan. Similarly, α-L-AFase (AbfAC26Sari) from *A. kestanbolensis* AC26sari hydrolysed sugar beet arabinan, rye arabinoxylan and α-1,5-linked oligosaccharides (Canakcıet al. 2008). However, Abf1Geo12 and Abf2Geo12 differ from AbfAC26Sari by hydrolysing oat spelt xylan. Abf1Geo12 and Abf2Geo12 were not able to hydrolyse red-dyed arabinan, a substrate that is cleaved only by endo-1,5-arabinanase activity (Canakci et al. 2007a). Based on all these results, it is possible to say that Abf1Geo12 and Abf2Geo12 are exo-acting enzymes exhibiting hydrolytic activity against α-1,3- and α-1,5-linked non-reducing terminal L-arabinofuranose residues but not against internal α-L-arabinosyl linkages.

In conclusion, we purified and characterized two different α-L-AFases from the species *G. stearothermophilus*. One of these enzymes (Abf1Geo12) is moderately stable and active α-L-AFase. Based on sequence similarities, both enzymes would appear to belong to CAZy database family GH51. This study is the first report showing two different biochemical α-L-AFases from the species *G. stearothermophilus*.

Acknowledgements

This work was supported by The Scientific and Research Council of Turkey (TUBITAK, Grant No.: 211T116).

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Received September 22, 2016
Accepted August 8, 2017