Recombinant Production and Biochemical Characterization of Thermostable Arabinofuranosidase from Acidothermophilic *Alicyclobacillus Acidocaldarius*

Alper Akkaya[1] · Yunus Ensari[2] · Emine Erdogan Ozseker[1] · Ozge Ozsen Batur[3] · Gozde Buyuran[4] · Serap Evran[1]

Abstract

The complete enzymatic degradation of lignocellulosic biomass requires the cooperative action of cellulosic, hemicellulosic, and lignolytic enzymes such as cellulase, xylanase, laccase, galactosidase, and arabinofuranosidase. Arabinofuranosidases (E.C 3.2.1.55), which belong to the glycoside hydrolase family of enzymes, hydrolyze the 1,3- and 1,5-α-arabinosyl bonds in L-arabinose- containing molecules. L-arabinoses are present in hemicellulosic part of lignocellulosic biomass. Arabinofuranosidases also play an important role in the complete hydrolysis of arabinoxylans. Analysis of the genome project and CAZY database revealed two putative arabinofuranosidase genes in the *A. acidocaldarius* genome. The aim of the study was cloning, heterologous expression, purification and biochemical characterization of the arabinofuranosidase enzyme encoded in *A. acidocaldarius* genome. For this purpose, the AbfA gene of the arabinofuranosidase protein was cloned into the pQE-40 vector, heterologously expressed in *E. coli* BL21 GOLD (DE3) and successfully purified using His-Tag. Biochemical characterization of the purified enzyme revealed that *A. acidocaldarius* arabinofuranosidase exhibited activity over a wide pH and temperature range with optimum activity at 45 ºC and pH 6.5 in phosphate buffer towards 4-nitrophenyl-α-L-arabinofuranoside as the substrate. In addition, the enzyme is highly stable over wide range of temperature and maintaining 60% of its activity after 90 min of incubation at 80 ºC. Through the bioinformatics studies, the homology model of *A. acidocaldarius* arabinofuranosidase was generated and the substrate binding site and residues located in this site were identified. Further molecular docking analysis revealed that the substrate located in the catalytically active pose and, residues N174, E175, and E294 have direct interaction with 4-nitrophenyl-α-L-arabinofuranoside. Moreover, based on phylogenetic analysis, *A. acidocaldarius* arabinofuranosidase exists in the sub-group of intracellular arabinofuranosidases, and *G. stearothermophilus* and *B.subtilis* arabinofuranosidases are close relatives of *A. acidocaldarius* arabinofuranosidase. This is the first study to report the gene cloning, recombinant expression and biochemical and bioinformatic characterization of an auxiliary GH51 arabinofuranosidase from an acidothermophilic bacterium *A. acidocaldarius*.

✉ Yunus Ensari
yunusensari@kafkas.edu.tr

1 Faculty of Science, Biochemistry Department, Ege University, Bornova, Izmir 35100, Turkey

2 Faculty of Engineering and Architecture, Bioengineering Department, Kafkas University, Kars 36000, Turkey

3 Faculty of Science, Department of Chemistry, Eskişehir Osmangazi University, Eskişehir 26480, Turkey

4 Vocational School of Health Services, Kırşehir Ahi Evran University, Kırşehir 40100, Turkey

1 Introduction

Enzymes, biological catalysts, have been extensively used in many industrial applications such as; biosynthesis of pharmaceuticals and fine chemicals, food and beverage production, diagnostics and treatment of various diseases due to their superiority over chemical catalysts [1–4]. The successful use of enzymes in industry has made a positive contribution to both the environment and process economics by reducing the amount of energy and chemicals used

in these processes [5]. One of the most important application of enzymes is enzymatic degradation of lignocelluosic biomass.

Enzymatic degradation of lignocellulosic biomass, the most abundant renewable resource in nature, is one of the most important applications of enzymes. Conversion of cellulosic and hemicellulosic components to fermentable sugars by enzymes has significantly improved the overall production cost of ethanol and other value-added products [7, 8]. Many cellulosic, hemicellulosic, and lignolytic enzymes such as cellulase, xylanase, laccase, galactosidase, arabinofuranosidase, etc. are required to achieve complete enzymatic hydrolysis of lignocellulose [9]. Because hemicellulose interacts with cellulose and prevents cellulolytic enzymes from accessing cellulose, it is important to break down hemicellulose to obtain all fermentable sugars from the biomass. Although xylanase and β-xylosidase enzymes are primarily involved in the breakdown of the hemicellulose structure, enzymes such as α-L-arabinofuranosidase, α-glucuronidase, α-galactosidase, acetylxylan esterase and β-mannanase, which are called auxiliary enzymes, are required to hydrolyze the side chains in the hemicellulose structure [10, 11]. The incorporation of lignocellulosic enzymes into many industrial and biotechnological processes has ushered in a new era of more efficient use of cheap agricultural waste [7, 12, 13].

Arabinofuranosidase (E.C 3.2.1.55) involved in the hydrolysis of L-arabinose linkages (1,3- and 1,5-α-arabinosyl) and arabinoxylan, is an important part of the system required for the complete breakdown [13–15]. L-arabinofuranosyl units in xylan chains strongly inhibit xylan degrading enzymes, preventing hydrolysis of the polymer to xylose units. Similarly, the presence of L-arabinofuranosyl units in pectin prevents the degradation of pectin into to its monomers. Arabinofuranosidases hydrolyze hemicellulose and pectin by working synergistically with other hemicellulases and pectinases. Because they enhance the effectiveness of other lignocellulose-converting enzymes, arabinofuranosidases play a cooperative role in biomass breakdown. Arabinofuranosidases act to increase the amount of reducing sugars that are released from lignocellulosic materials [12, 13, 16]. Its synergistic effect with other lignocellulose-converting enzymes has led to the successful use of arabinofuranosidases in a wide range of industrial processes [7, 12, 13].

Arabinofuranosidases were grouped into 9 different glycoside hydrolase classes (GH2, 3, 5, 10, 39, 43, 51, 54, and 62) based on their sequence similarity in the CAZY database (http://www.cazy.org/) [17]. The GH51 family comprises the largest number of arabinofuranosidase enzymes and they catalyze the release of 1,2 and 1,3 α-L-arabinofuranosyl residues in the arabinoxylan structure [9, 18]. Although some plants are capable of producing arabinofuranosidase,

microbial strains are the major producers [9]. Bacteria including Bacillus, Bifidobacterium, Pseudomonas, and, Thermotoga strains, Actinomycetes such as Streptomyces strains and fungi including mainly Aspergillus and Penicillium strains have been reported to produce arabinosfuranosidase with different enzymatic characteristics [9, 17, 19, 20] and recently reviewed by Poria and colleagues [9]. In addition, arabinofuranosidase production has also been shown in the gut metagenome of termites [21].

In addition to the degradation of lignocellulosic biomass for bioethanol production, arabinofuranosidases have many other industrial applications. They have been used as additives in animal feed to improve digestibility, in fruit juice clarification, and in the food industry to improve bread quality. Furthermore, they have applications in pharmaceutical industry such as prebiotics and sweetener production, anticancer and anti-mycobacterial agents [9, 20].

Arabinofuranosidases were produced and secreted along with other lignocellulose-degrading enzymes, resulting in contamination with other enzymes by lignocellulose-degrading organisms, which was undesirable for further industrial applications [11]. The production of arabinofuranosidases as recombinant proteins could overcome this obstacle. In addition, lignocellulose-degrading enzymes derived from thermophilic microorganisms are preferred due to their higher stability [22]. *A. acidocaldarius* DSM 446 is an acidothermophilic bacterium that grows in acidic hot springs with an optimum growth temperature of 60–65 °C. This study aimed at cloning, recombinant expression, purification and biochemical characterization of GH51 arabinofuranosidase encoded in the genome of *A. acidocaldarius* DSM 446 for the first time. The genomic copy of arabinofuranosidase was cloned into the pQE-40 vector, expressed in *E. coli* BL21 GOLD (DE3) and purified by NiNTA affinity chromatography. Subsequently, the purified arabinofuranosidase enzyme was biochemically characterized to elucidate its biotechnological potential. Further bioinformatics studies were performed to generate a homology model and this model was used to identify the substrate binding site and molecular docking, which help to better understanding of the structure-function relationship of the enzyme. Additionally, phylogenetic analysis was performed to elucidate its relationships and similarities with previously identified arabinofuranosidases.

2 Materials and Methods

2.1 Materials

All chemicals were purchased from Sigma-Aldrich (Germany), Merck (Germany), and Biobasic (Canada) if not

stated otherwise. Enzymes were purchased from Thermo-Fisher Scientific if not stated otherwise.

2.2 Strains and Vector

A. acidocaldarius DSM 446 was used to obtain arabinofuranosidase gene; *E. coli* DH5α was used for the cloning, and *E. coli* BL21 GOLD (DE3) was used for the expression of α-N-Arabinofuranosidase; pQE-40 plasmid vector was used for expression of recombinant enzyme. *A. acidocaldarius* DSM 446 was cultivated at 60 ºC in Alicyclobacillus medium and *E. coli* strains were grown at 37 ºC and 160 rpm in LB media.

2.3 Cloning of Arabinofuranosidase Gene

Genomic DNA was isolated from *A. acidocaldarius* DSM 446 using genomic DNA isolation kit according to manufacturer instructions (Thermo Scientific, Lithuania). Genomic DNA was used as template and the gene (NCBI Gene ID: Aaci_2894; Genebank accession number: ACV59898) was amplified by PCR (94°C for 3 min, 1 cycle; 94°C for 40 s/64°C for 40 s/72°C for 90 s, 30 cycles; 72°C for 10 min, 1 cycle) using the forward primer *(5'- ACGCGTCGA-CATGTCCAACCTGAAAGCG-3')* and the reverse primer (*5'-AAGCTTTCAGACGCGAATGCGAATCACG − 3'*). The amplified product was digested with *Sal*I and *Hind*-III and then ligated with T4-DNA-Ligase (Fermentas, Lithuania) into pQE-40 plasmid vector. The ligation products were transformed into *E. coli* DH5α and positive transformants were validated with colony PCR and sequencing.

2.4 Expression of Arabinofuranosidase

The pQE-40 plasmid harboring arabinofuranosidase gene was transformed into *E. coli* BL21 GOLD (DE3). For protein expression, 2% of an overnight culture of *E. coli* BL21 GOLD (DE3) was inoculated into Luria-Bertani (LB) medium (1 L) containing ampicillin (0.1 mg/ml) and tetracycline (12.5 µg/ml) and then cultured at 37 ºC and 160 rpm until the OD_{600} value reached 0.4–0.6. Protein expression was induced by adding 2 ml of 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG) to the culture medium and incubated at 37ºC for 3 h.

2.5 Protein Purification and Quantification

Cells were centrifuged at 4ºC for 40 min at 4000 rpm, and the pellet was resuspended in suspension buffer (pH 7.5 100 mM phosphate buffer) and disrupted by sonication on ice (40%, 20 s interval, 5 min total). The cell suspension was centrifuged at 4ºC and 13,000 rpm for 30 min. The soluble fraction was filtered and equilibrated with buffer (50 mM potassium phosphate, 500 mM NaCl and 10 mM imidazole and pH was adjusted to 7.8) and applied to a His Trap FF crude (GE HEALTHCARE) affinity column. Bound proteins were eluted with elution buffer (50 mM potassium phosphate, 500 mM NaCl and 500 mM imidazole, and pH 7.8).

Purified protein was quantified spectrophotometrically using the Bradford method [23]. 150 µl of sample was added to 3 ml of Bradford reagent. Samples were incubated for 10 min at room temperature. After incubation, the absorbance was measured at 595 nm.

2.6 Activity Assay

Arabinofuranosidase activity was measured as follows; 510 µl of phosphate buffer (50 mM, pH 6.5) and 90 µl of 0.2 mM p-nitrophenyl-α-L-arabinofuranoside were added to the reaction medium and preincubated at 45ºC for 10 min. After preincubation, 200 µl of enzyme solution was added to the reaction medium and incubated at 45ºC for 30 min.

After incubation, 800 µl of 1 M Na_2CO_3 was added to the reaction medium to stop the reaction. The samples were incubated for 10 min at room temperature. After incubation, the absorbance was measured at 405 nm [24]. One unit of enzyme activity was defined as the conversion of 1 µmol of substrate per minute. All measurements were performed in triplicate.

2.7 Characterization of Arabinofuranosidase

Effects of pH and Temperature on Activity Enzymatic reactions were performed in 50 mM Sodium acetate (pH 4.0-4.5-5.0), 50 mM Potassium phosphate (pH 5.5-6.0-6.5-7.0) and 50 mM borate (pH 8.0–9.0) buffers. The effect of temperature on the arabinofuranosidase activity was determined by performing the reaction in pH 6.5 phosphate buffer for 30 min at 20-30-40-45-50-65-85 and 100 °C.

Thermal Stability of Arabinofuranosidase Purified enzyme solutions were incubated at 30-40-50-60-70-80 and 90 °C for 90 min and then kept on ice. Residual arabinofuranosidase activity was measured by a standard assay, as described in Sect. 2.6.

Effects of Different Chemicals on Arabinofuranosidase Activity NaCl, $MgCl_2$, $CaCl_2$, EDTA, $HgCl_2$ and SDS solutions (prepared in pH 6.5 50 mM phosphate buffer) were added to

the reaction medium (the final concentration of the solutions was 10 mM).

2.8 Computational Biology Analysis

Physico-chemical parameters such as MW, theoretical pI, and the aliphatic index were calculated using ProtParam tool (https://web.expasy.org/protparam/). The phylogenetic tree was constructed by the Neighbor-Joining method using MEGA X. The 3D homology model of *A. acidocaldarius* arabinofuranosidase protein (Uniprot ID: C8WV61) was constructed by the SWISS-MODEL server (https://swiss-model.expasy.org/) and validated by QMEAN (https://swissmodel.expasy.org/qmean/), ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php), and SAVES v6.0 (https://saves.mbi.ucla.edu/). The homohexameric structure of arabinofuranosidase was used for substrate docking in AutoDock within YASARA Structure Version 22.9.24. PyMOL was used to prepare all the graphical images.

3 Results and Discussion

3.1 Cloning of Arabinofuranosidase Gene

The coding region of the putative abfA gene was amplified by PCR using genomic DNA as template (Fig. 1A). The 1506 bp. gene fragment was digested with *Sal*I and *Hind*-III and ligated into pQE-40 vector digested with the same restriction enzymes (Fig. 1B). The obtained plasmid construct was transformed into *E.coli* DH5α. Positive transformants were validated by colony PCR (Fig. 1C), restriction digestion, and sequencing. The pQE-40 vector harboring the arabinofuranosidase gene was transformed into *E. coli* BL21 GOLD (DE3) for heterologous expression.

3.2 Expression and Purification of Arabinofuranosidase

A. acidocaldarius arabinofuranosidase protein expressed in *E. coli* BL21 GOLD (DE3) was purified with His-Tag purification by loading cell lysate onto a HisTrap™ FF crude column (Figure S1). Purified protein fractions were analyzed by SDS-PAGE (Fig. 2) with a molecular mass of approximately 56 kDa. The higher purity fractions 6, 7,8 and 13 in Fig. 2 were combined for further analysis.

3.3 Biochemical Characterization of the *A. Acidocaldarius* Arabinofuranosidase Enzyme

For a better understanding of the biochemical properties of recombinant *A. acidocaldarius* arabinofuranosidase, the optimum pH, optimum temperature, thermal stability, substrate spectrum, and kinetic parameters were determined.

3.3.1 Effect of Buffer and pH

Sodium acetate, Potassium phosphate, and borate buffers with different pH values were tested in order to determine the optimal pH value and buffer system. The recombinant *A. acidocaldarius* arabinofuranosidase showed the highest activity at pH 6.5 in Potassium phosphate buffer (Fig. 3). Enzyme activity gradually increased between pH 4.0 and 6.5 and then gradually decreased up to pH 9.0. Arabinofuranosidase showed more than 80% activity between pH 5.0 and pH 7.0. Additionally, the enzyme has approximately 50% of its activity at pH 4.0. A similar pH optimum was reported for α-L-Arabinofuranosidase enzyme from *Bacillus stearothermophilus* No. 236 [25]. The *A. acidocaldarius* arabinofuranosidase enzyme showed activity over a wide pH range (between 4.0 and 9.0). In the literature there are many arabinofuranosidases, obtained from different bacteria such as *Thermobacillus xylanilyticus* [26], *Geobacillus stearothermophilus* [27], *Thermotoga thermarum* [28], *Anoxybacillus kestanbolensis* [24] with activity between pH 4.0 to pH 10, were reported.

3.3.2 Effect of Temperature on Activity

The optimum temperature for the *A. acidocaldarius* arabinofuranosidase was 45 ºC (Fig. 4). However, the arabinofuranosidase enzyme was highly active at all temperatures tested. The arabinofuranosidase exhibited ~90% activity between 20 and 100 ºC which has only been reported for only a few identified and characterized arabinofuranosidases from *Clostridium stercoarium* (temperature range 30–90 ºC) [29] and *Bacillus subtilis* (temperature range 30–80 ºC) [30]. This wide temperature range profile of the enzyme highlights the thermostability of the enzyme and thus increases the industrial potential of the purified enzyme.

3.3.3 Thermal Stability of the Arabinofuranosidase

To examine the thermal stability of the *A. acidocaldarius* arabinofuranosidase, the enzyme was incubated at various temperatures for 90 min. Figure 5 shows that the enzyme is stable at mild temperatures (30–50 ºC). The stability of the enzyme gradually decreases after 50 ºC. However, it still retains 80% activity after 90 min of incubation at 60 and
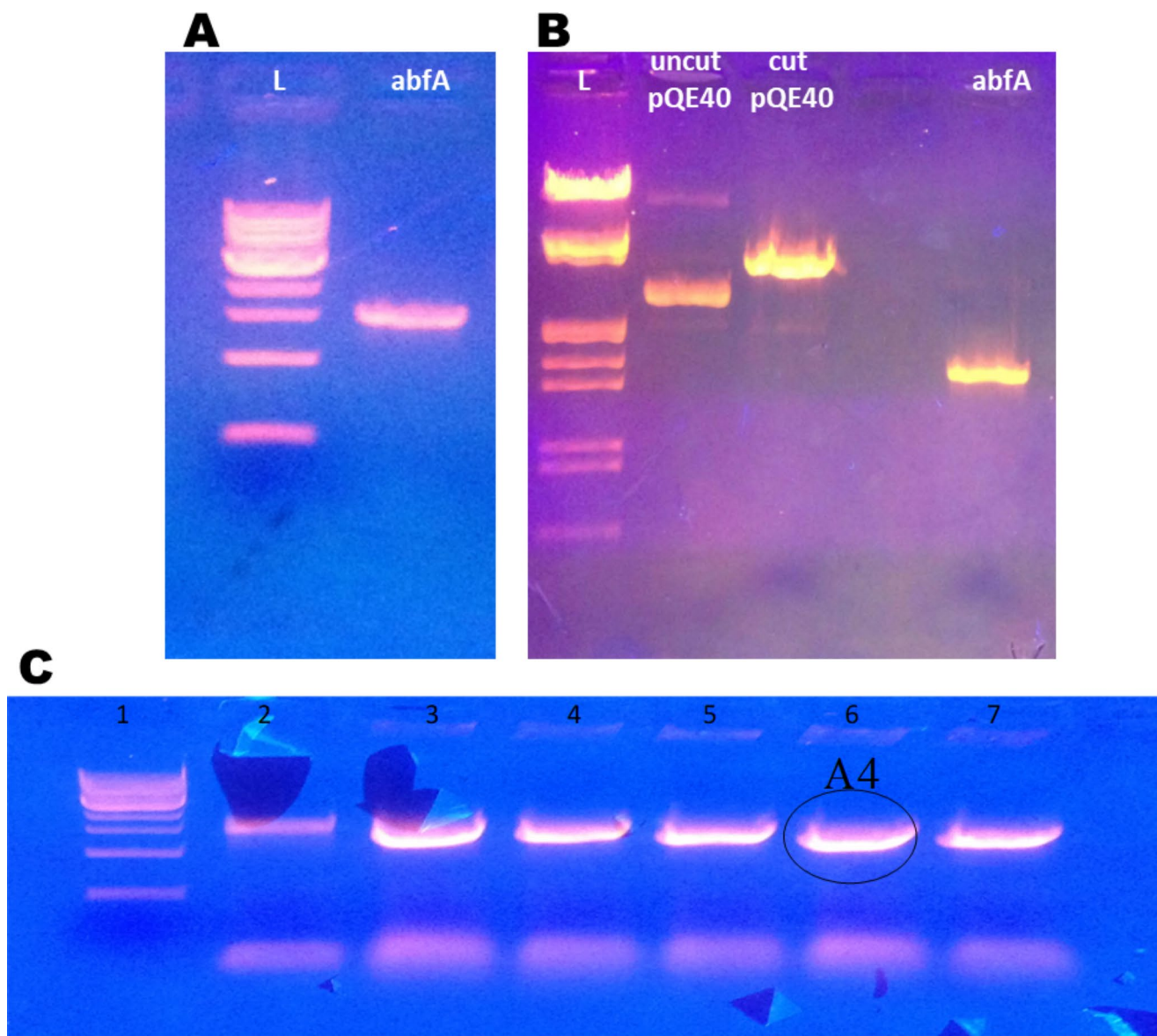
Fig. 1 Cloning of arabinofuranosidase gene into pQE-40 vector. **(A)** PCR amplification of 1506 bp. arabinofuranosidase gene. L; 1 kb DNA Ladder (New England BioLabs), abfA; arabinofuranosidase gene product. **(B)** Restriction digestion of PCR product and vector DNA. L; Lambda DNA EcoRI + HindIII marker. Uncut pQE-40; undigested pQE-40 vector DNA. Cut pQE-40; pQE-40 vector digested with SalI and HindIII enzymes. abfA; arabinofuranosidase PCR product digested with SalI and HindIII enzymes. **(C)** Colony PCR analysis of obtained colonies. 1; 1 kb DNA Ladder (New England BioLabs). 2; Positive control. 3–7; colonies obtained after transformation

70 ºC. Furthermore, it exhibited more than 40% residual activity at 90 ºC after 90 min of incubation. Another α-L-arabinofuranosidase from *Alicyclobacillus* sp. A4, which has 75% sequence identity with *A. acidocaldarius* α-N-Arabinofuranosidase, has 80% of the initial activity at 70 ºC after 60 min. and 30% of the initial activity at 80 ºC after 30 min of incubation [20] which is much lower than the arabinofuranosidase characterized in this study (>50% of the initial activity after 90 min. incubation at 80 ºC). The *Geobacillus stearothermophilus* α-L-arabinofuranosidase enzyme has 50% of the initial activity at 70 ºC after one hour of incubation, which is also less than *A. acidocaldarius* arabinofuranosidase [27]. Other arabinofuranosidases from *Bacillus pumilus* [31], *Bacillus subtilis* [32], *Aureobasidium pullulans* [33], *Streptomyces sp*, [34] have 65% at 75 ºC, 60% at 50 ºC, 70% at 60 ºC, and 45% at 60 ºC, respectively, of their initial activity.

3.3.4 Effect of Chemicals

The effects of chemicals such as NaCl, $MgCl_2$, $CaCl_2$, EDTA, $HgCl_2$ and SDS on enzyme activity were investigated by
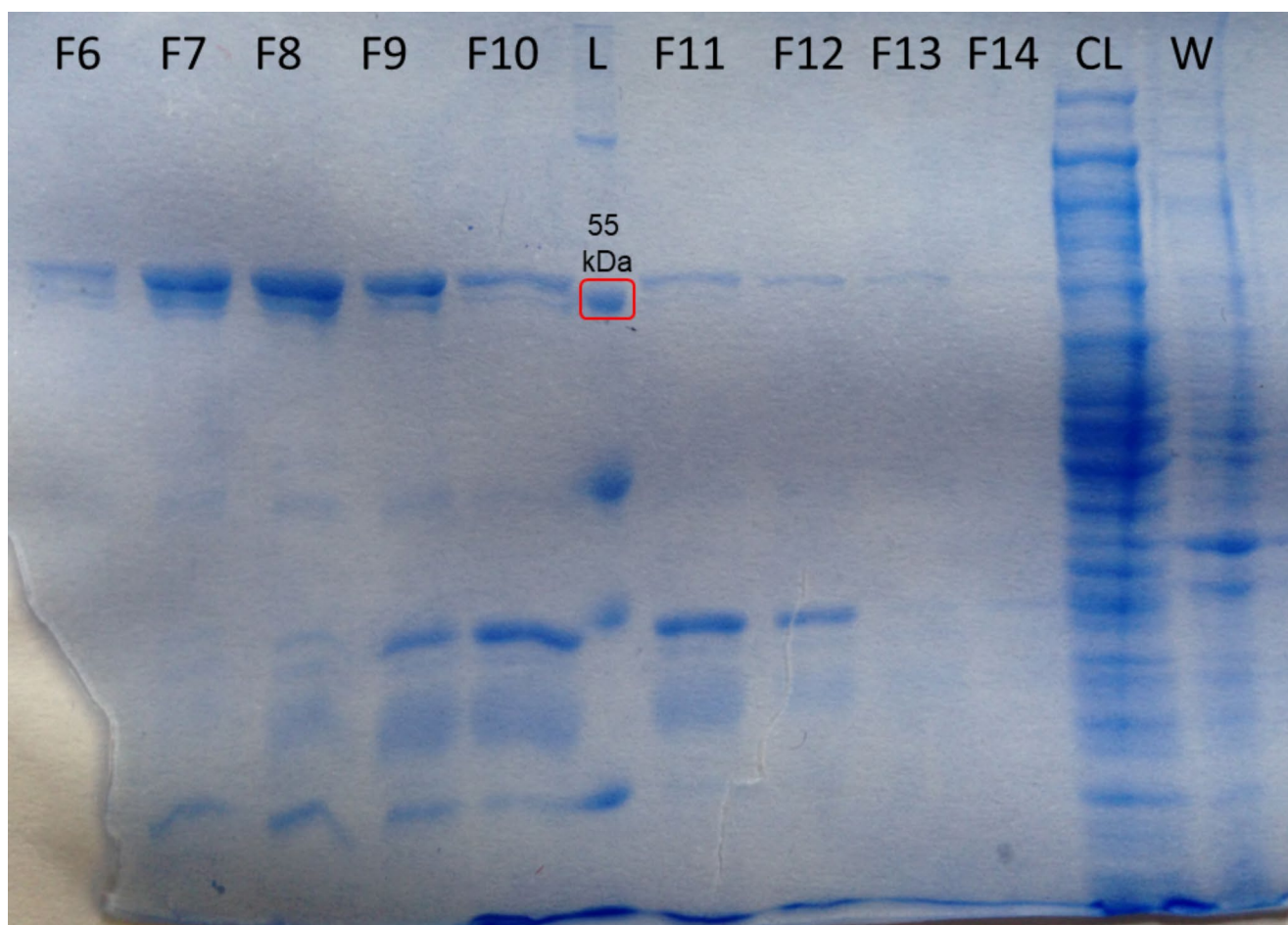
Fig. 2 SDS-PAGE analysis of the fractions obtained from arabinofuranosidase purification. L: Molecular weight reference ladder. F6-F14: Fractions containing arabinofuranosidase protein. CL: Cleared cell lysate before loading on a column. W: Proteins eluted by washing the column

Fig. 3 Effect of buffer and pH on arabinofuranosidase enzyme activity. The activity assays were performed at 45ºC in buffers of pH 4.0–9.0 for 30 min
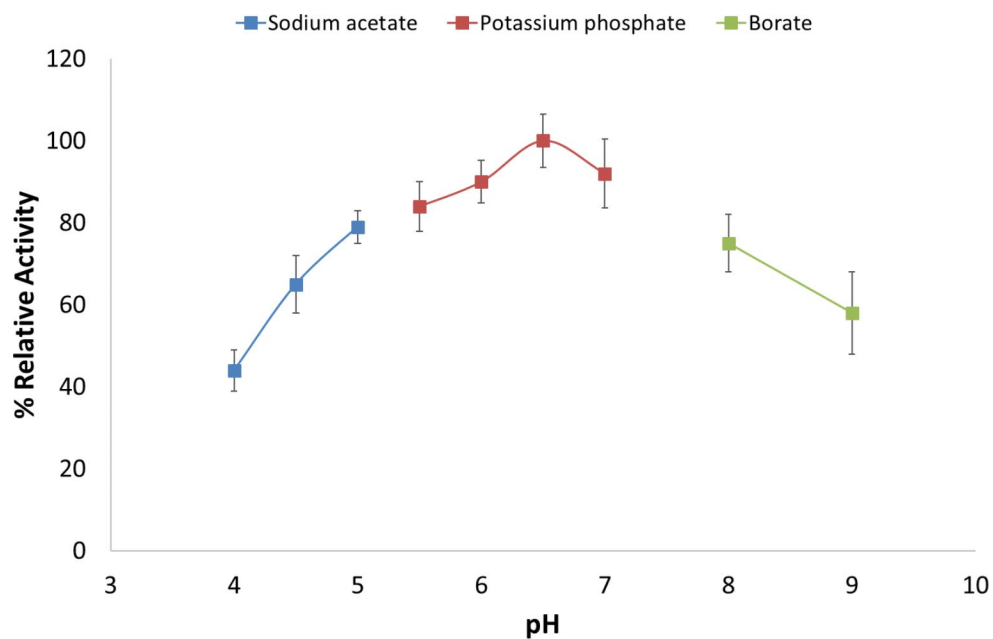
adding them to the reaction medium at a final concentration

Fig. 4 Effect of temperature on arabinofuranosidase enzyme activity. The activity assays were performed reaction in pH 6.5 phosphate buffer at various temperatures for 30 min
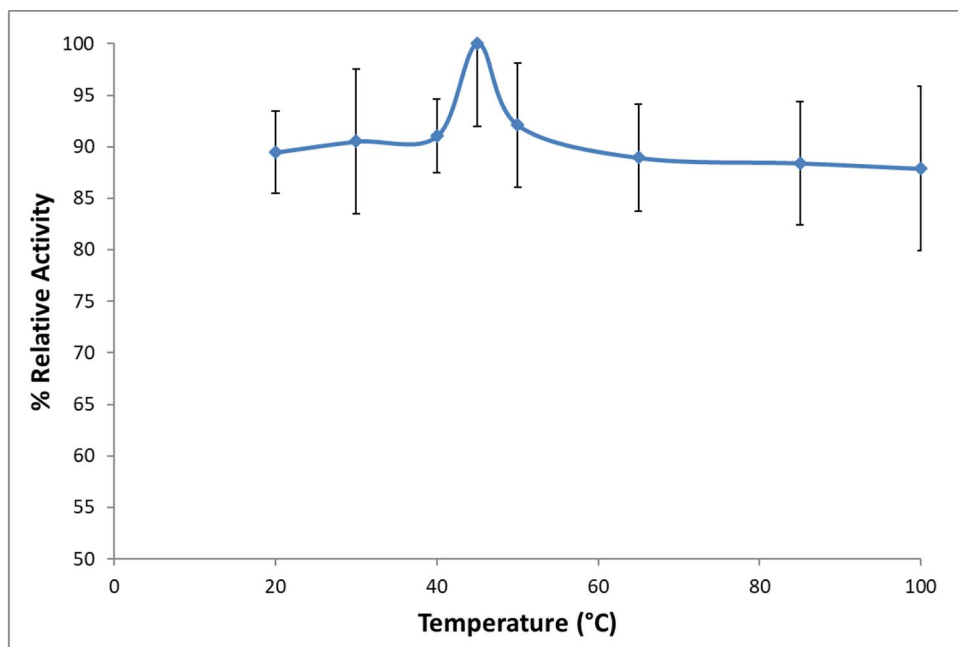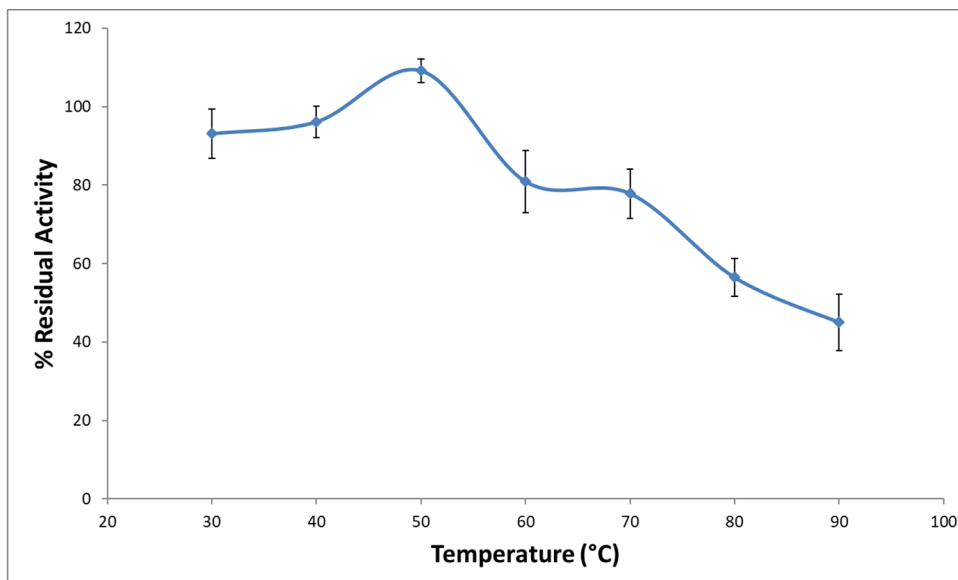

Fig. 5 Thermal stability for recombinant N-Arabinofuranosidase. The standard activity assays were performed after incubation at various temperatures for 90 min

of 10 mM. NaCl and SDS had no significant effect on enzyme activity. While sodium ion had no effect on *Bifidobacterium longum* arabinofuranosidase, it moderately increased *Aspergillus niger* and *Thermomyces dupontii* arabinofuranosidases [35-37Guerfali 2011]. On the other hand, $MgCl_2$, $CaCl_2$, EDTA, and $HgCl_2$ caused a significant decrease in *A. acidocaldarius* arabinofuranosidase activity. While $MgCl_2$ caused to more than 50% loss of activity, $HgCl_2$ caused almost complete inhibition of the enzyme. 10–20% activity was observed when EDTA and $CaCl_2$ were added to the reaction medium. On the contrary, *Geobacillus stearothermophilus* α-L-arabinofuranosidase enzyme was not affected from calcium and magnesium ions. However,

mercury caused 98% activity loss at concentration of 1 mM [27]. Similar inhibitory effect was also observed for *A. niger* arabinofuranosidase [38]. Moreover, EDTA was also inhibited many other arabinofuranosidases from *Clostridium thermocellum*, *Bifidobacterium longum*, *Streptomyces sp.* SWU10, and *Clostridium thermocellum* [39–41].

3.4 Computational Biology Analysis of the *A. Acidocaldarius* Arabinofuranosidase

Bioinformatics analyses including homology modeling, molecular docking and phylogenetic analysis were performed to gain insights into the structure-function

Fig. 6 Effect of chemicals on arabinofuranosidase enzyme activity. Chemicals were added to the reaction medium at a final concentration of 10 mM
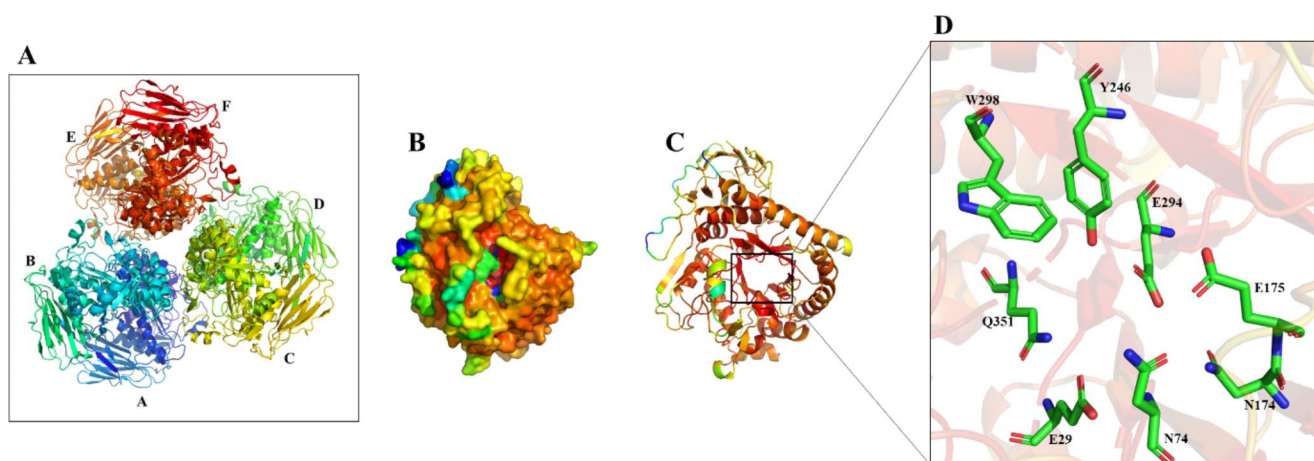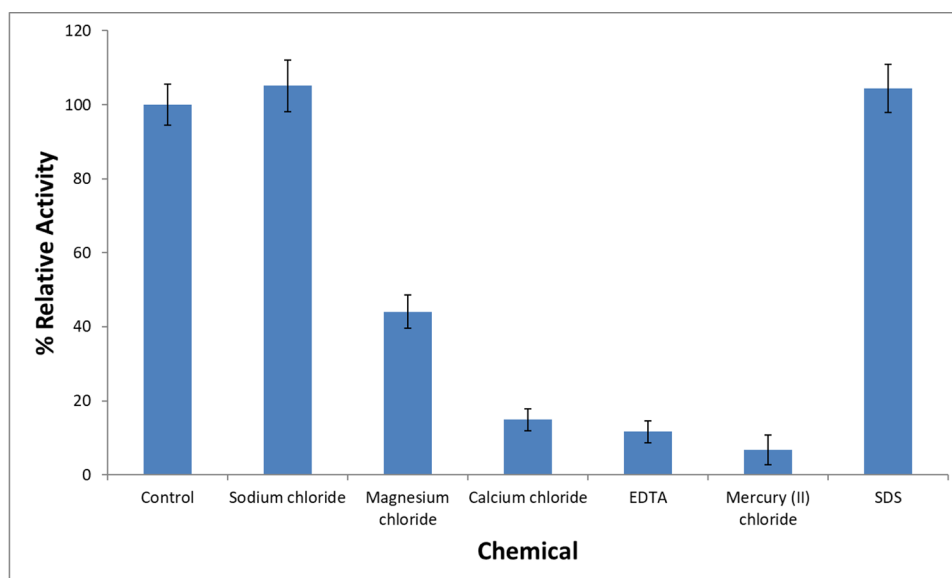

Fig. 7 Homology model of arabinofuranosidase protein. **A;** Homohexameric structure. **B;** Surface representation of 3D monomeric protein. **C;** 3D model of arabinofuranosidase monomer. **D;** Active site residues of arabinofuranosidase enzyme

relationship *A. acidocaldarius* arabinofuranosidase and its similarity to other arabinofuraosidases. The ProtParam tool (https://web.expasy.org/protparam/) was used in order to calculate physicochemical parameters of the 501 amino acid containing *A. acidocaldarius* arabinofuranosidase (UniProt ID: C8WV61). The ProtParam calculated the molecular weight and theoretical pI of the arabinofuranosidase as 56 kDa and 5.40 respectively. Furthermore, aliphatic index which is indicator of thermostability was calculated as 87.56.

The homology model of the 501 aa. *A. acidocaldarius* arabinofuranosidase enzyme was constructed using SWISS-MODEL server and validated using QMEAN, ProSA-web, and SAVES v6.0. SWISS-MODEL yielded a homohexameric 3D structure (Fig. 7) using the *Geobacillus stearothermophilus* α-L-arabinofuranosidase protein as a template (PDB ID: 1QW9). The QMEAN value of the generated 3D

homology model was 0.85 and the ProSA-web tool calculated the Z-score of the model to be -11 (Figure S2), which is in the range of scores typically found for native proteins of similar size. Additionally, a Ramachadran Plot (Fig. 8) was generated by SAVES and it revealed that 90.7% of the residues were in the most favoured regions and 8.9% of the residues were in the allowed regions. The binding site of the *A. acidocaldarius* arabinofuranosidase enzyme was predicted using FTSite binding site prediction tool [42]. The pink mesh represents the predicted binding site (Fig. 8) and the residues within 5 Å of the binding site are F27, E29, R69, G73, N74, F75, W99, N174, E175, W180, S216, Y246, E294, W298, L318, A350, Q351, I356. The active and binding site residues of *Geobacillus stearothermophilus* α-L-arabinofuranosidase were experimentally identified as E29, N74, N174, E175, Y246, E294, W298

Fig. 8 The binding site of the *A. acidocaldarius* arabinofuranosidase enzyme predicted using FTSite. The residues within 5 Å of the binding site (pink mesh) were shown as stick
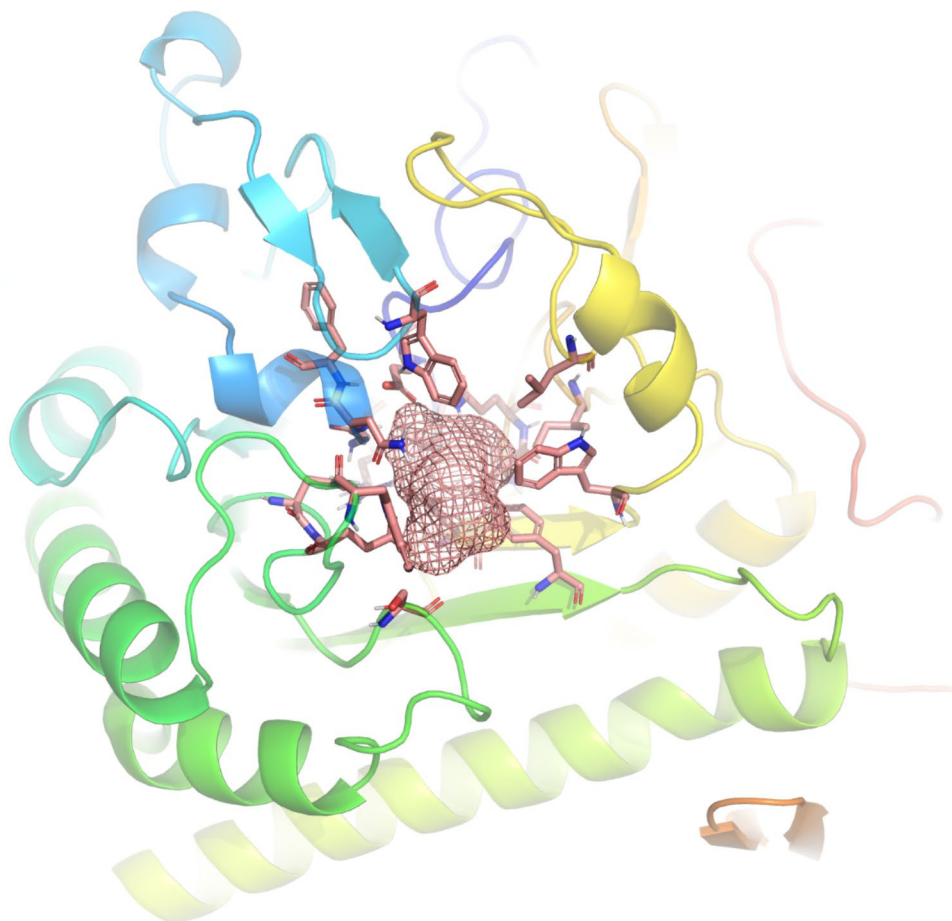

and Q351, all of which are also found in *A. acidocaldarius* arabinofuranosidase.

The docking analysis of arabinofuranosidase with the substrate pNP-α-L-arabinofuranoside was performed using AutoDock within YASARA. 100 runs were performed and docking poses with less than 0.5 Å heavy atom RMSD were superposed and clustered. Hovel et al., reported that the 3.0 Å distance between E175 and the glycosidic oxygen of the substrate is a hydrogen bonding distance and allows the protonation of the exiting aglycon. Additionally, the appropriate distance between E294 and the anomeric carbon is 3.2 Å which allows the nucleophilic attack for the *Geobacillus stearothermophilus* α-L-arabinofuranosidase [43]. The best docking pose is shown in Fig. 9 and it showed that the distances between E294 and the anomeric carbon (D1) of the substrate and the distance between E175 and the glycosidic oxygen (D2) of the substrate and are within the preferred ranges. The docking results showed that the D1 was 3.01 Å and D2 was 2.570 Å with a binding energy of -7.64 kcal/mol. In addition, pNP-α-L-arabinofuranoside has hydrogen bonds with residues E175 and E294 which are catalytic residues and an additional hydrogen bond was also observed with residue N174. This docking result also shows that *A.*

acidocaldarius arabinofuranosidase acts on the arabinofuranosidic linkage on substrates.

The phylogenetic tree was constructed with MEGA X to analyze the phylogenetic relationship between the *A. acidocaldarius* arabinofuranosidase protein and previously characterized arabinofuranosidases. Figure 10 shows that the analyzed proteins were classified into two distinct groups according to the localization of the enzyme. *A. acidocaldarius* arabinofuranosidase was located into same group with the enzymes named as intracellular arabinofuranosidases. In addition, the characterized enzyme was in the same subgroup with the arabinofuranosidases characterized from *Geobacillus stearothermophilus* and *Bacillus subtilis*. Based on this, *A. acidocaldarius* arabinofuranosidase protein sequence was compared with the sequences of *Geobacillus stearothermophilus* and *Bacillus subtilis* arabinofuranosidases by multiple sequence alignment using the Clustal Omega tool. The MSA results (Fig. 11) revealed that *A. acidocaldarius* has 65% sequence identity with *Bacillus subtilis* arabinofuranosidase and 67% sequence identity with *Geobacillus stearothermophilus* arabinofuranosidase protein. In addition, the key residues for substrate binding (E29, N74, N174, Y246 and Q351) and catalytic activity

Fig. 9 The docking analysis of arabinofuranosidase with the substrate pNP-α-L-arabinofuranoside. D1 represents the distance between E294 and anomeric carbon of the substrate and D2 represents the distance between E175 and glycosidic oxygen of substrate
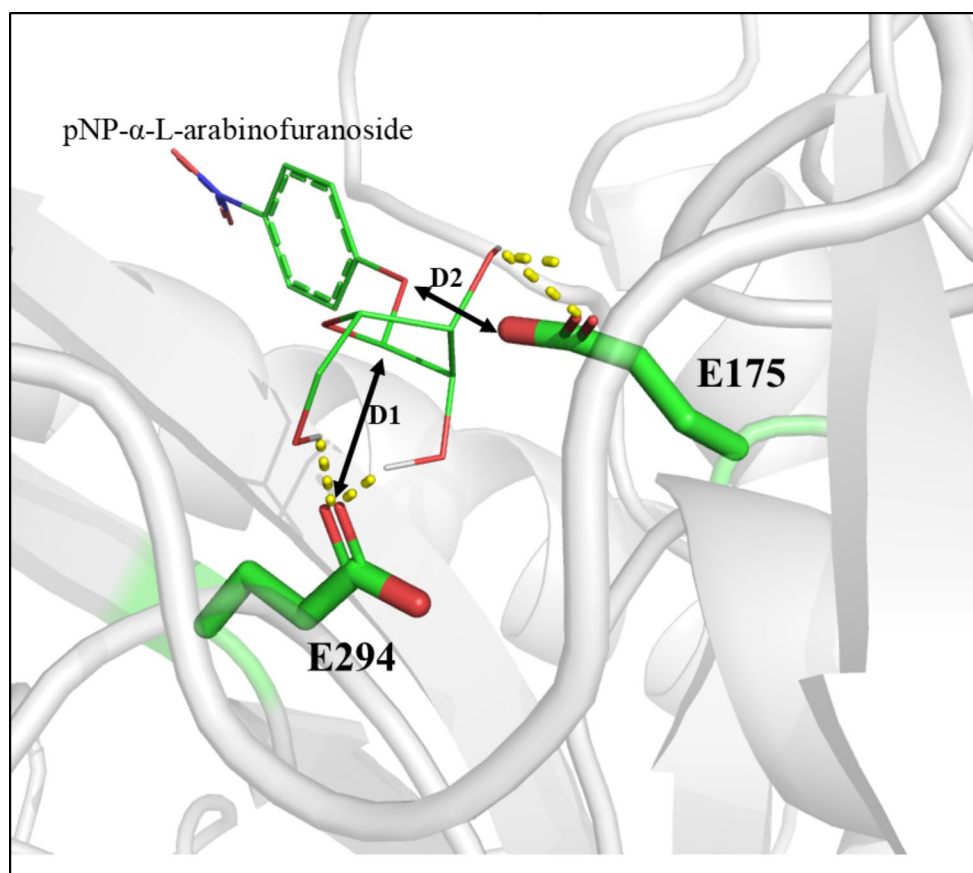
(E175 and E294) are conserved in the *A. acidocaldarius* sequence.

4 Conclusion

L-arabinose units in the form of a side chains linked to hemicelluloses in lignocellulosic biomass and pectic compounds hinder other enzymes such as xylanase, which play a role in the enzymatic hydrolysis of the hemicellulose moiety, and in this case the biomass cannot be fully hydrolyzed to fermentable sugars. Accessory enzymes such as arabinofuranosidase, which belongs to the class of glycoside hydrolyze enzymes, play important role to overcome this obstacle. In this study, the arabinofuranosidase gene from acidothermophilic *A. acidocaldarius* DSM 446 was cloned into pQE-40 plasmid and successfully expressed in *E. coli* BL21 GOLD (DE3). The purified arabinofuranosidase enzyme showed the optimum activity at pH 6.5 and 45 ℃. The enzyme has more than 50% activity over a wide range of pH values from 4.0 to 8.0. It also has more than 85% activity between 20 and 100 ℃. Furthermore, it retains more than 50% its initial activity at 70 ℃ after 90 min of incubation, demonstrating its high stability under extreme conditions. *A. acidocaldarius* DSM 446 is an acidothermophilic bacterium that grows in acidic hot springs with an optimum growth temperature of 60–65 °C. In addition to growth in both acidic and hot springs, having a complete genome project makes the organism an important source of enzymes that are used in harsh industrial conditions.

Computational biology analysis of the binding site revealed that residues F27, E29, R69, G73, N74, F75, W99, N174, E175, W180, S216, Y246, E294, W298, L318, A350, Q351, I356 constitute the binding site of the enzyme. Molecular docking of the pNP-α-L-arabinofuranoside substrate into the active site of the enzyme yielded the catalytically active pose with a free binding energy of -7.64 kcal/mol. In addition, *Geobacillus stearothermophilus* and *Bacillus subtilis* arabinofuranosidases were located in the same group with *A. acidocaldarius* arabinofuranosidase enzyme with sequence identity of 65% and 67%, respectively. In essence, high stability of the produced enzyme at acidic pH and alleviated temperatures may lead to the use of *A. acidocaldarius* arabinofuranosidase enzyme as a potential accessory enzyme for the complete breakdown of lignocellulosic biomass in the production of high value added products.
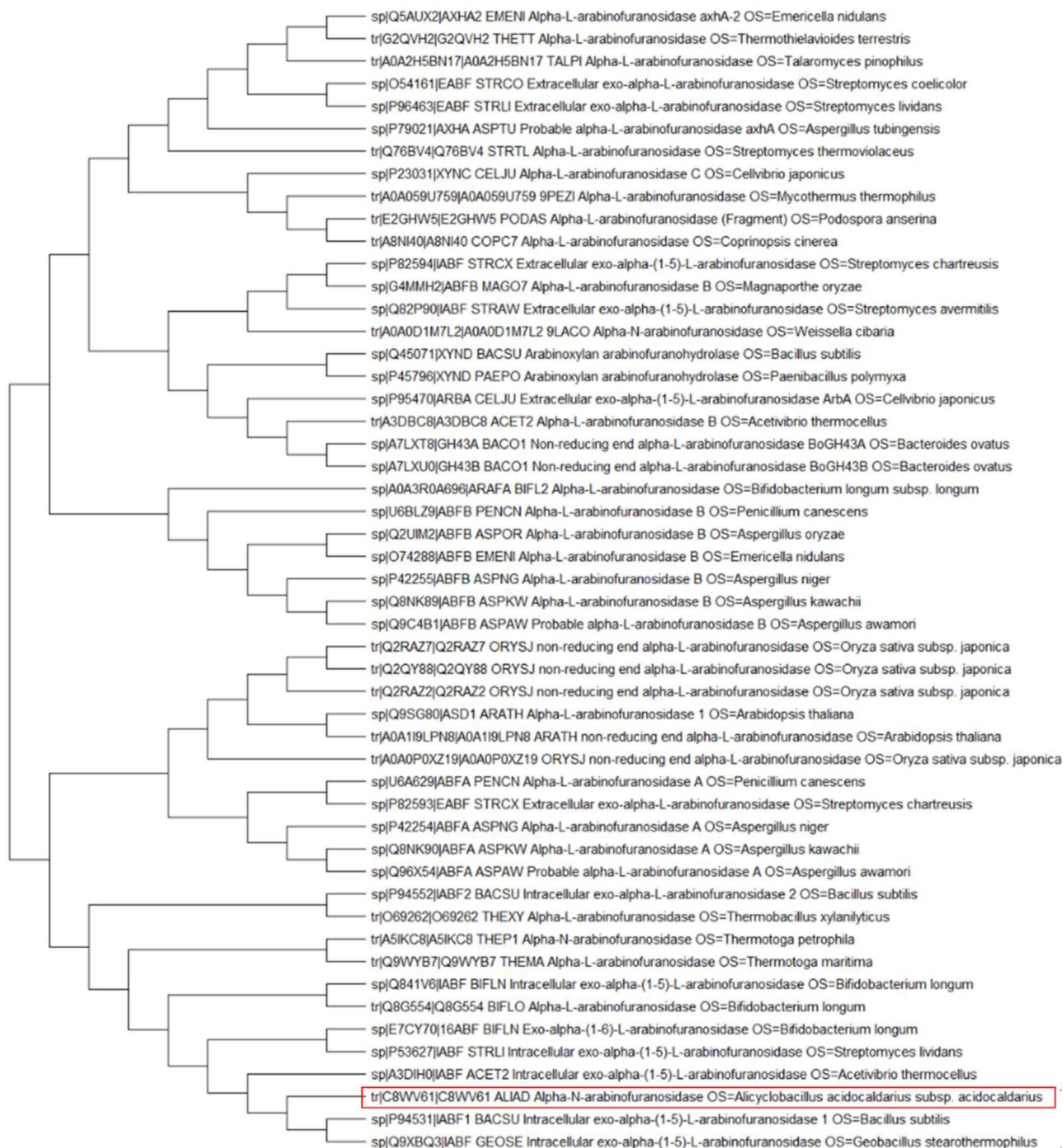
Fig. 10 The phylogenetic analysis of the *A. acidocaldarius* arabinofuranosidase using the neighbor-joining method by MEGA X

Fig. 11 Multiple sequence alignment of the arabinofuranosidases of *(A) acidocaldarius*, *(B) subtilis*, and *G. stearothermophilus*. The star in the black boxes represents the catalytic residues E175 and E294

Author Contributions Alper Akkaya: Conceptualization, Writing, Supervision. Yunus Ensari: Conceptualization, Methodology, Writing. Emine Erdogan Ozseker: Methodology. Ozge Ozsen Batur: Writing. Gozde Buyuran:Methodology. Serap Evran: Conceptualization, Supervision.

Declarations

References

1. Kirk O, Borchert V, Crone Fuglsang T, C (2002) Industrial enzyme applications. Curr Opin Biotechnol 13:345–351
2. Pazarlıoğlu N, Telefoncu A (2010) Biotechnology: Basic Principles and Applications. Birlesik Printing, Izmir

3. Eren K, Çömlekçioğlu Ö, Dostbil U, N (2006) Some microbial enzymes and their usage in industry. K S U Journal of Science and Engineering 9(1):12–19

4. Deveci R (2006) "Investigation and comparison of glucose isomerase enzyme working compressed bed reactors and fluid bed reactors and fuructose production profiles throughout the reactors", Çukurova University, Institute of Science, Chemistry Department (Master Thesis)

5. Demarche P, Junghanns CR, Nair RN, Agathos S (2012) Harnessing the power of enzymes for environmental stewardship. Biotechnol Adv 30:933–953

6. Xu Q, Adney WS, Ding SY, Micheal HE (2007) "Cellulases for biomass conversion, Industrial Enzymes, Springer, Drodrecht, 2007

7. Suwannarangsee S, Bunterngsook B, Arnthong J, Paemanee A, Thamchaipenet A, Eurwilaichitr L, Laosiripojana N, Champreda V (2012) Optimisation of synergistic biomass-degrading enzyme systems for efficient rice straw hydrolysis using an experimental mixture design. Bioresour Technol 119:252–261

8. Viikari L, Vehmaanpera J, Koivula A (2012) "Lignocellulosic ethanol: from science to industry",Biomass and Bioenergy,1–12

9. Poria V, Sainia JK, Singha S, Nainb L, Kuhad RC (2020) Arabinofuranosidases: characteristics, microbial production, and potential in waste valorization and industrial applications. Bioresour Technol 304:123019

10. Broeker J, Mechelke M, Baudrexl M et al (2018) The hemicellulose-degrading enzyme system of the thermophilic bacterium Clostridium stercorarium: comparative characterisation and addition of new hemicellulolytic glycoside hydrolases. Biotechnol Biofuels 11:229

11. Houfani AA, Anders N, Spiess AC, Baldrian P, Benallaoua S (2020) Insights from enzymatic degradation of cellulose and hemicellulose to fermentable sugars– a review. Biomass Bioenergy 134:105481

12. Miyazaki K (2005) Hyperthermophilic alpha-L: -arabinofuranosidase from Thermotoga maritima MSB8: molecular cloning, gene expression, and characterization of the recombinant protein. Extremophiles 9:399–406

13. Numan MT, Bhosle NB (2006) Alpha-L-arabinofuranosidases: the potential applications in biotechnology. J Ind Microbiol Biotechnol 33:247–260

14. Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30:279–291

15. Van den Burg B (2003) Extremophiles as a source for novel enzymes. Curr Opin Microbiol 6:213–218

16. Gonçalves TA, Damásio ARL, Segato F, Alvarez TM, Bragatto J, Brenelli LB, Citadini APS, Murakami MT, Ruller R, Leme P, Prade AF, Squina RA, F.M (2012) Functional characterization and synergic action of fungal xylanase and arabinofuranosidase for production of xylooligosaccharides. Bioresour Technol 119:293–299

17. Wu X, Zhang S, Zhao S, Dai L, Huang S, Liu X, Yu J, Wang L (2022) J Agric Food Chem 70(16):5039–5048

18. Bouraoui H, Desrousseaux ML, Ioannou E, Alvira P, Manai M, Remond C, Dumon C, Fernandez-Fuentes N, O'Donohue MJ (2016) The GH51 α-L-arabinofuranosidase from Paenibacillus sp. THS1 is multifunctional, hydrolyzing main-chain and side-chain glycosidic bonds in heteroxylans. Biotechnol Biofuels 9:140

19. Limsakul P, Phitsuwan P, Waeonukul R, Pason P, Tachaapaikoon C, Poomputsa K, Kosugi A, Ratanakhanokchai K (2021) A novel multifunctional arabinofuranosidase/ endoxylanase/β-xylosidase GH43 enzyme from *Paenibacillus curdlanolyticus* B-6 and its synergistic action to produce arabinose and xylose from cereal arabinoxylan. Appl Environ Microbiol 87:e01730–e01721

20. Yang W, Bai Y, Yang P, Luo H, Huang H, Meng K, Shi P, Wang Y, Yao B (2015) A novel bifunctional GH51 exo-α-l-arabinofuranosidase/endo-xylanase from Alicyclobacillus sp. A4 with significant biomass-degrading capacity. Biotechnol Biofuels 30:8:197

21. Bastien G, Arnal G, Bozonnet S, Laguerre S, Ferreira F, Fauré R, Henrissat B, Lefèvre F, Robe P, Bouchez O, Noirot C, Dumon C, O'Donohue M (2013) Mining for hemicellulases in the fungus-growing termite Pseudacanthotermes militaris using functional metagenomics. Biotechnol Biofuels 14;6(1):78

22. Long L, Sun L, Lin Q, Ding S, St John FJ (2020) Characterization and functional analysis of two novel thermotolerant α-L-arabinofuranosidases belonging to glycoside hydrolase family 51 from Thielavia terrestris and family 62 from Eupenicillium parvum. Appl Microbiol Biotechnol 104:8719–8733

23. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72(1–2):248–254

24. Canakci S, Kacagan M, İnan K, Belduz AO, Saha BC (2008) Cloning, purification, and characterization of a thermostable alpha-L-arabinofuranosidase from Anoxybacillus kestanbolensis AC26Sari. Appl Microbiol Biotechnol 81:61–68

25. Kyoung-Ju K, Kyung-Nam K, Yong-Jin C (2004) Characterization of the arfA gene from Bacillus stearothermophilus No. 236 and its protein product, α-L-Arabinofuranosidase. J Microbiol Biotechnol 143:474–482

26. Debeche T, Cummings N, Connerton I, Debeire P, O'Donohue MJ (2000) Genetic and biochemical characterization of a highly thermostable alpha-L-arabinofuranosidase from Thermobacillus xylanilyticus. Appl Environ Microbiol 66(4):1734–1736

27. Gilead S, Shoham Y (1995) Purification and characterization of alpha-L-arabinofuranosidase from Bacillus stearothermophilus T-6. Appl Environ Microbiol 61(1):170–174

28. Shi H, Zhang Y, Xu B, Tu M, Wang F (2014) Characterization of a novel GH2 family α-L-arabinofuranosidase from hyperthermophilic bacterium Thermotoga thermarum. Biotechnol Lett 36(6):1321–1328

29. Schwarz WH, Bronnenmeier K, Krause B, Lottspeich F, Staudenbauer WL (1995) Debranching of arabinoxylan: properties of the thermoactive recombinant alpha-L-arabinofuranosidase from Clostridium stercorarium (ArfB). Appl Microbiol Biotechnol 43(5):856–860

30. Inácio JM, Correia IL, de Sá-Nogueira I (2008) "Two distinct arabinofuranosidases contribute to arabino-oligosaccharide degradation in Bacillus subtilis.",Microbiology (Reading).;154 (Pt9):2719–2729

31. Pei J, Shao W (2008) Purification and characterization of an extracellular alpha-L-arabinosidase from a novel isolate Bacillus pumilus ARA and its over-expression in Escherichia coli. Appl Microbiol Biotechnol 78(1):115–121

32. Bourgois TM, Van Craeyveld V, Van Campenhout S, Courtin CM, Delcour JA, Robben J, Volckaert G (2007) Recombinant expression and characterization of XynD from Bacillus subtilis subsp. subtilis ATCC 6051: a GH 43 arabinoxylan arabinofuranohydrolase. Appl Microbiol Biotechnol 75(6):1309–1317

33. de Wet BJ, Matthew MK, Storbeck KH, van Zyl WH, Prior BA (2008) Characterization of a family 54 alpha-L-arabinofuranosidase from Aureobasidium pullulans. Appl Microbiol Biotechnol 77(5):975–983

34. Phuengmaung P, Kunishige Y, Sukhumsirichart W, Sakamoto T (2018) Identification and characterization of GH62 bacterial α-l-arabinofuranosidase from thermotolerant Streptomyces sp. SWU10 that preferentially degrades branched l-arabinofuranoses in wheat arabinoxylan. Enzyme Microb Technol 112:22–28

35. Alias N, Mahadi N, Murad A, Bakar F, Rabu A, Illias R (2011) Expression optimisation of recombinant

alpha-L-arabinofuranosidase from Aspergillus niger ATCC 120120 in Pichia pastoris and its biochemical characterization. Afr J Biotechnol 10:6700–6710

36. Lee JH, Hyun YJ, Kim DH (2011) Cloning and characterization of alpha-L-arabinofuranosidase and bifunctional alpha-L-arabinopyranosidase/beta-D-galactopyranosidase from Bifidobacterium longum H-1. J Appl Microbiol 111:1097–1107

37. Guerfali M, Gargouri A, Belghith H (2011) Catalytic properties of Talaromyces thermophilus alpha-L-arabinofuranosidase and its synergistic action with immobilized endo-beta-1,4-xylanase. J Mol Catal B 68:192–199

38. Whitaker JR (1984) Pectic substances, pectic enzymes and haze formation in fruit juices. Enzyme Microb Technol 6:341–349

39. Ahmed S, Luis AS, Bras JL, Ghosh A, Gautam S, Gupta MN, Fontes CM, Goyal A (2013) "A novel alpha-L-arabinofuranosidase of family 43 glycoside hydrolase (Ct43Araf) from Clostridium thermocellum",PLoS ONE, 8, e73575

40. Phuengmaung P, Kunishige Y, Sukhumsirichart W, Sakamoto T (2018) Identification and characterization of GH62 bacterial alpha-L-arabinofuranosidase from thermotolerant Streptomyces sp. SWU10 that preferentially degrades branched L-arabinofuranoses in wheat arabinoxylan. Enzyme Microb Technol 112:22–28

41. Fujita K, Sakamoto A, Kaneko S, Kotake T, Tsumuraya Y, Kitahara K (2019) Degradative enzymes for type II arabinogalactan side chains in Bifidobacterium longum subsp. Longum. Appl Microbiol Biotechnol 103:1299–1310

42. Kozakov D, Grove LE, Hall DR, Bohnuud T, Mottarella SE, Luo L, Xia B, Beglov D, Vajda S (2015) The FTMap family of web servers for determining and characterizing ligand-binding hot spots of proteins. Nat Protoc 10(5):733–755

43. Hövel K, Shallom D, Niefind K, Belakhov V, Shoham G, Baasov T, Shoham Y, Schomburg D (2003) Crystal structure and snapshots along the reaction pathway of a family 51 alpha-L-arabinofuranosidase. EMBO J 22(19):4922–4932

44. Long L, Sun L, Lin Q Characterization and functional analysis of two novel thermotolerant α-L-arabinofuranosidases belonging to glycoside hydrolase family 51 from and family 62 from.,8719–8733