

# Recombinant Production and Biochemical Characterization of Thermostable N-arabinofuranosidase from Acidothermophilic Alicyclobacillus acidocaldarius

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## Research Article

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# Abstract

Enzymes are successfully used in many industrial process, such as food and paper industries. Enzymes, which are derived from microorganisms living under mild conditions, are not suitable for industrial processes performed at high temperature, acidic or basic reaction conditions. Thus, enzymes derived from extremophilic microorganisms are needed for these hard industrial conditions. *Alicyclobacillus acidocaldarius* DSM 446 with an optimum growth temperature of 60–65°C, is an acidothermophilic bacterium that grows in acidic hot springs. In addition to growth in both acidic and hot springs, having a complete genome project makes the organism an important source for enzymes that are used in harsh industrial conditions. Arabinofuranosidases (E.C 3.2.1.55; AFases) belong to glycoside hydrolase family enzymes. AFases hydrolyzes 1,3- and 1,5- $\alpha$ -arabinosyl bounds of L-arabinose containing molecules. L-arabinoses are present in hemicellulosic part of lignocellulosic biomass. AFases 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. In this study, we aimed cloning, heterologous expression, purification and biochemical characterization of the  $\alpha$ -N-Arabinofuranosidase enzyme encoded in *A. acidocaldarius* genome. For this purpose, the gene of  $\alpha$ -N-Arabinofuranosidase protein was cloned into pQE-40 vector and expressed heterologously in *E. coli* BL21 GOLD (DE3). Biochemical characterization of the purified enzyme revealed that *A. acidocaldarius*  $\alpha$ -N-Arabinofuranosidase enzyme showed optimum activity at 45 °C and pH 6.5 in phosphate buffer. Furthermore, enzyme has 60% activity after 90 min. incubation at 80 °C. *A. acidocaldarius*  $\alpha$ -N-Arabinofuranosidase enzyme has high potential to be used as accessory enzyme for the complete degradation of arabinoxylans.

## Introduction

Enzymes, which are biological catalysts, have been used extensively in the preparation of many foods and beverages, biosynthesis of pharmaceuticals and fine chemicals, diagnostics and treatment of various diseases, and as additives in detergents and other cleaning agents [1–2]. Nowadays, enzymes, which have very important metabolic functions in the cells, have entered into daily and economic life to use for various purposes [3]. Due to their superiority over chemical catalysts previously used in industrial processes, enzymes have successfully replaced these catalysts. Enzymes belonging to all enzyme classes are widely used in many fields such as food, clinic, pharmaceutical and molecular biology [4]. The successful use of enzymes in the industry has contributed positively to both the environment and the process economy by reducing the amount of energy and chemicals previously used in these processes [5]. Following the widespread use of enzymes in various biotechnological and industrial processes, enzyme technology (White Biotechnology) is one of the most popular and interesting areas of biotechnology with advances in recombinant DNA technology. The majority of the enzymes used in the industry belong to the hydrolase class [1, 6]. 59% of commercially used enzymes are proteases, 28% carbohydrases, 3% lipases and 10% are other enzymes [3].

Lignocellulosics are the most abundant renewable resources in nature and they can be used as carbon sources in the production of both biofuel and other high value-added microbial products. The conversion of cellulosic and hemicellulosic components to fermentable sugars are one of the most important steps to improve total production costs obtain through ethanol or other high value-added chemicals by using lignocellulosic biomass. For example, in recent years, a significant decrease has been observed in the production costs of ethanol using enzymatically hydrolyzed biomass as feedstock [7–8]. Many cellulosic, hemicellulosic, and lignolytic enzymes such as cellulase, xylanase, laccase, galactosidase, arabinofuranosidase, and so on, are needed in order to achieve full enzymatic hydrolysis of lignocellulose [9]. It is important to degrade hemicellulose which surrounds and interacts with cellulose in order to obtain all the fermentable sugars from biomass. Since hemicellulose hinders the access of cellulolytic enzymes to cellulose and thus decreases the fermentable sugar yield. Although endoxylanase and  $\beta$ -xylosidase enzymes are mainly involved in the degradation of the hemicellulose structure, enzymes such as  $\alpha$ -L-arabinofuranosidase,  $\alpha$ -glucuronidase,  $\alpha$ -galactosidase, acetylxyloxyesterase and  $\beta$ -mannanase, which are called auxiliary enzymes, are needed in the hydrolysis of the side chains in the hemicellulose structure [10, 11]. Implication of lignocellulosic enzymes in many industrial and biotechnological processes led to start a new era that allows for more efficient usage of cheap agricultural waste [7, 12, 13].

Arabinofuranosidases (E.C 3.2.1.55) involved in hydrolysis of L-arabinose bonds (1,3- and 1,5- $\alpha$ -arabinosyl) and arabinoxylan is an important part of the system required for the complete disintegration [13–15]. L-arabinofuranosyl units in xylan chains strongly inhibit xylan degrading enzymes and this prevents hydrolysis of the polymer to xylose units. Similarly, the presence of L-arabinofuranosyl units in pectin prevents the degradation of this polymer to its monomers. Arabinofuranosidases perform hydrolysis of hemicellulose and pectin by working synergetically with other hemicellulases and pectinases. Because of they increase the efficacy of other lignocellulosic enzymes, these enzymes have a cooperative role in biomass conversion. Arabinofuranosidases have an effect that increases the release of reducing sugars from lignocellulosic materials [12, 13, 16]. They have a synergistic effect with other lignocellulosic enzymes, and this enzyme has been used successfully in many industrial processes [7, 12, 13].

In this study, we aimed cloning, recombinant expression, purification and biochemical characterization of L- arabinofuranosidase encoded of in the genome of *A. acidocaldarius* DSM 446. Herein, the genomic copy of  $\alpha$ -N- arabinofuranosidase was cloned into pQE-40 vector, expressed in *E. coli* BL21 GOLD (DE3) and purified by using NiNTA affinity chromatography. Subsequently, purified  $\alpha$ -N-arabinofuranosidase enzyme was biochemically characterized in order to elucidate full potential to be used in lignocellulosic biomass degradation.

## Materials And Methods

### Strains and Vector

*A. acidocaldarius* DSM 446 was used to obtain  $\alpha$ -N-Arabinofuranosidase gene; *E. coli* DH5 $\alpha$  was used for the cloning, and *E. coli* BL21 GOLD (DE3) was used for the expression of  $\alpha$ -N-Arabinofuranosidase; pQE-40 plasmid vector was used for expression of recombinant enzyme.

### **Cloning of Arabinofuranosidase gene**

Genomic DNA was isolated from *A. acidocaldarius* DSM 446 using genomic DNA isolation kit according to manufacturer instructions (Thermo Scientific). Genomic DNA was used as template and the gene (NCBI Gene ID: Aaci\_2894; Genbank 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'-ACGCGTCGACATGTCCAACCTGAAAGCG-3') and the reverse primer (5'-AAGCTTTCAGACGCGAATGCGAATCACG-3'). The amplified product was digested with *Sa*I and *Hind*-III and then ligated with T4-DNA-Ligase (Fermentas) into pQE-40 plasmid vector. The ligation products were transformed into *E. coli* DH5 $\alpha$  and positive transformants were validated with colony PCR and sequencing.

### **Expression of Arabinofuranosidase**

pQE-40 plasmid harboring  $\alpha$ -N-Arabinofuranosidase gene was transformed into *E. coli* BL21 GOLD (DE3). For the expression of protein, 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  $\mu$ g/ml) and then cultured at 37°C and 160 rpm until OD<sub>600</sub> value reaches 0.4-0.6. Protein expression was induced by supplementing 2 ml of 0.5 mM isopropyl- $\beta$ -thiogalactopyranoside (IPTG) to culture medium and performed at 37°C for 3 hours.

### **Protein Purification and Quantification**

Cells were centrifuged at 4°C and 4000 rpm for 40 min and pellet was resuspended in suspension buffer (pH 7.5 100 mM phosphate buffer) and disrupted by sonication on ice (40 %, 20 second interval, totally 5 min). Cell suspension was centrifugated at 4°C and 13000 rpm for 30 min. 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 loaded onto 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 Bradford Method [17]. 150  $\mu$ l of sample was added to 3 ml of Bradford Reagent. Samples were incubated at room temperature for 10 min. After incubation, absorbance was measured at 595 nm.

### **Activity Assay**

510  $\mu$ l of phosphate buffer (50 mM, pH 6.5) and 90  $\mu$ l of 0.2 mM p-Nitrophenyl- $\alpha$ -L-arabinofuranoside were added to reaction medium and incubated at 45°C for 10 min and pre-incubation was performed.

After pre-incubation, 200 µl of enzyme solution was added to reaction medium and incubated at 45°C for 30 min.

After incubation, 800 µl of 1 M Na<sub>2</sub>CO<sub>3</sub> was added to reaction medium to stop the reaction. Samples were incubated at room temperature for 10 min. After incubation, absorbance was measured at 405 nm [18]. One unit of enzyme activity was defined as the conversion of 1 µmole of substrate per minute.

## Characterization of Enzymes

**Effects of pH and temperature on activity;** The enzymatic reactions were carried out in 50 mM acetate (pH 4.0-4.5-5.0), 50 mM 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. The residual arabinofuranosidase activity was measured by standard assay as described above.

**Effects of different chemicals on Arabinofuransidase activity;** NaCl, MgCl<sub>2</sub>, CaCl<sub>2</sub>, EDTA, HgCl<sub>2</sub> and SDS solutions (prepared in pH 6,5 50 mM phosphate buffer) were added to reaction medium (final concentration of solutions was 10 mM).

## Computational Biology Analysis

Physico-chemical parameters such as MW, theoretical pI, and aliphatic index was 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* α-N-Arabinofuranosidase protein (Uniprot ID; C8WV61) was constructed by SWISS-MODEL server (<https://swissmodel.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 α-N-Arabinofuranosidase was used for substrate docking in AutoDock within YASARA Structure Version 22.9.24. PyMOL was used to prepare all the graphical images.

# Results And Discussion

## Expression and purification of α-N-Arabinofuranosidase

*A. acidocaldarius* α-N-Arabinofuranosidase protein expressed in *E. coli* BL21 GOLD (DE3) was purified with His-Tag purification by loading cell lysate to HisTrap™ FF crude column (Figure S1). Purified protein fractions were analyzed with SDS-PAGE (Figure 2) with a molecular mass of approximately 56 kDa.

## Figure 2

## Biochemical Characterization of the *A. acidocaldarius* $\alpha$ -N-Arabinofuranosidase Enzyme

For better understanding of the biochemical properties of recombinant *A. acidocaldarius*  $\alpha$ -N-Arabinofuranosidase, optimum pH, optimum temperature, thermal stability, substrate spectrum, and kinetic parameters were determined.

### ***Effect of Buffer and pH***

Aspartate, 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*  $\alpha$ -N-Arabinofuranosidase showed the highest activity at pH 6.5 in phosphate buffer (Figure 3). Enzyme activity increased gradually between pH 4.0 to 6.5 and then decreased gradually until pH 9.0.  $\alpha$ -N-Arabinofuranosidase showed more than 80 % activity between pH 5.0 to pH 7.0. Additionally, enzyme has around 50 % activity at pH 4.0. A similar pH optima was reported for *Bacillus stearothermophilus* No. 236  $\alpha$ -L-Arabinofuranosidase enzyme [19]. The *A. acidocaldarius*  $\alpha$ -N-Arabinofuranosidase enzyme exhibited activity at wide pH range (between 4.0 to 9.0). In the literature there are many arabinofuranosidases, obtained from different bacteria such as *Thermobacillus xylanilyticus* [20], *Geobacillus stearothermophilus* [21], *Thermotoga thermarum* [22] (DOI 10.1007/s10529-014-1493-6), *Anoxybacillus kestanbolensis* [18] with activity between pH 4.0 to pH 10, were reported.

### **Figure 3**

### ***Effect of Temperature on Activity***

The optimal temperature for the *A. acidocaldarius*  $\alpha$ -N-Arabinofuranosidase was 45 °C (Figure 4). However, the  $\alpha$ -N-Arabinofuranosidase enzyme was highly active at tested all temperatures.  $\alpha$ -N-Arabinofuranosidase exhibited 90 % activity between 20-100 °C which was reported before for only a few identified and characterized arabinofuranosidases from *Clostridium stercoarium* (temperature range 30-90 °C) [23] and *Bacillus subtilis* (temperature range 30-80 °C) [24]. This wide range temperature profile of the enzyme highlights the thermostability of the enzyme hence increases the industrial potential of the purified enzyme.

### **Figure 4**

### ***Thermal Stability of the $\alpha$ -N-Arabinofuranosidase***

To examine the thermal stability of the *A. acidocaldarius*  $\alpha$ -N-Arabinofuranosidase, enzyme was incubated at various temperatures for 90 min. Figure 5 shows that the enzyme is stable at mild temperatures (30-50 °C). Stability of the enzyme gradually decreases after 50 °C. However, it still retains 80 % activity after 90 min. incubation at 60 and 70 °C. Furthermore, it exhibited more than 40 % residual activity at 90 °C after 90 min. incubation. Another  $\alpha$ -L-arabinofuranosidase from *Alicyclobacillus* sp. A4, which has 75 % sequence identity with *A. acidocaldarius*  $\alpha$ -N-Arabinofuranosidase, has 80 % of the initial activity at 70 °C after 60 min. and 30 % of the initial activity at 80 °C after 30 min. incubation [25] which

is much more less than arabinofuranosidase characterized in this study (>50 % of the initial activity after 90 min. incubation at 80 °C). *Geobacillus stearothermophilus* α-L-arabinofuranosidase enzyme has 50 % of the initial activity at 70 °C after 1-hour incubation, that is also less than *A. acidocaldarius* α-N-Arabinofuranosidase [21]. Other arabinofuranosidases from *Bacillus pumilus* [26], *Bacillus subtilis* [27], *Aureobasidium pullulans* [28], *Streptomyces* sp. [29] have 65 % at 75 °C, 60 % at 50 °C, 70 % at 60 °C, and 45 % at 60 °C of their initial activity respectively.

## Figure 5

### *Effect of Chemicals*

The effects of chemicals such as NaCl, MgCl<sub>2</sub>, CaCl<sub>2</sub>, EDTA, HgCl<sub>2</sub> and SDS on enzyme activity were investigated by adding them into reaction medium at 10 mM final concentration. NaCl and SDS did not show any significant effect on enzyme activity. On the other hand, MgCl<sub>2</sub>, CaCl<sub>2</sub>, EDTA, and HgCl<sub>2</sub> led to significant decrease in *A. acidocaldarius* α-N-Arabinofuranosidase activity. While MgCl<sub>2</sub> caused to more than 50 % activity loss, HgCl<sub>2</sub> caused almost complete inhibition of the enzyme. 10-20 % activity was observed when EDTA and CaCl<sub>2</sub> were added into reaction medium.

## Figure 6

### **Computational Biology Analysis of the *A. acidocaldarius* α-N-Arabinofuranosidase**

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

## Figure 7

The homology model of the 501 aa. *A. acidocaldarius* α-N-Arabinofuranosidase enzyme was constructed with SWISS-MODEL server and validated with QMEAN, ProSA-web, and SAVES v6.0. SWISS-MODEL yielded a homohexameric 3D structure (Figure 7) using *Geobacillus stearothermophilus* α-L-arabinofuranosidase protein as template (PDB ID: 1QW9). The QMEAN value of the generated 3D homology model was 0.85 and ProSA-web tool calculated the Z-score of the model as -11 (Figure S2) which is in the range of scores typically found for native proteins of similar size. Additionally, Ramachandran Plot (Figure 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* α-N-Arabinofuranosidase enzyme was predicted using FTSite binding site prediction tool [30]. The pink mesh represents the predicted binding site (Figure 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.

## Figure 8

The docking analysis of  $\alpha$ -N-Arabinofuranosidase with the substrate pNP- $\alpha$ -L-arabinofuranoside was performed with AutoDock within YASARA. 100 runs were performed and docking poses 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 glycosidic oxygen of substrate is a hydrogen bonding distance and allows the protonation of the departing aglycon. Additionally, the appropriate distance between E294 and anomeric carbon is 3.2 Å which allows the nucleophilic attack for the *Geobacillus stearothermophilus*  $\alpha$ -L-arabinofuranosidase [31]. The best docking pose was shown in Figure 9 and it revealed that the distances between E294 and anomeric carbon (D1) of the substrate and the distance between E175 and glycosidic oxygen (D2) of 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 -7.64 kcal/mol binding energy.

## Figure 9

The phylogenetic tree was constructed with MEGA X to analyze phylogenetic relationship between *A. acidocaldarius*  $\alpha$ -N-Arabinofuranosidase protein with previously characterized arabinofuranosidases. Figure 10 showed that analyzed proteins were classified into two distinct groups according to localization of the enzyme. *A. acidocaldarius*  $\alpha$ -N-Arabinofuranosidase 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*  $\alpha$ -N-Arabinofuranosidase protein sequence was compared with the sequences of *Geobacillus stearothermophilus* and *Bacillus subtilis* arabinofuranosidases by multiple sequence alignment using Clustal Omega tool. MSA results (Figure 11) revealed that *A. acidocaldarius* has 65 % sequence identity with *Bacillus subtilis* arabinofuranosidase and 67 % sequence identity with *Geobacillus stearothermophilus* arabinofuranosidase protein. Moreover, the key residues for substrate binding (E29, N74, N174, Y246 and Q351) and catalytic activity (E175 and E294) are conserved in the sequence of *A. acidocaldarius*.

## Figure 10

## Figure 11

# Conclusion

L-arabinose units in the form of a side chain 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 part, and in this case, the biomass cannot be fully hydrolyzed into fermentable sugars. Accessory enzymes such as arabinofuranosidase belonging to the glycoside hydrolyze class enzymes play important role to overcome this obstacle. In this study, the  $\alpha$ -N-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  $\alpha$ -N-Arabinofuranosidase enzyme showed the

optimum activity at pH 6.5 and 45 °C. However, enzyme has more than 50% activity between wide range of pH values between 4.0 to 8.0 and more than 85% activity between 20 and 100 °C. Furthermore, it retains more than 50% its initial activity at 70 °C after 90 min. incubation that represent its high stability under extreme conditions. *A. acidocaldarius* DSM 446 is an acidothermophilic bacteria that grows in acidic hot springs with an optimum growth temperature of 60–65°C. In essence, high stability of the produced enzyme at acidic pH and alleviated temperatures may lead to use of *A. acidocaldarius*  $\alpha$ -N-Arabinofuranosidase enzyme as a potential accessory enzyme for the complete breakdown of lignocellulosic biomass in production of high value added products.

## Declarations

The authors declare no competing financial interest.

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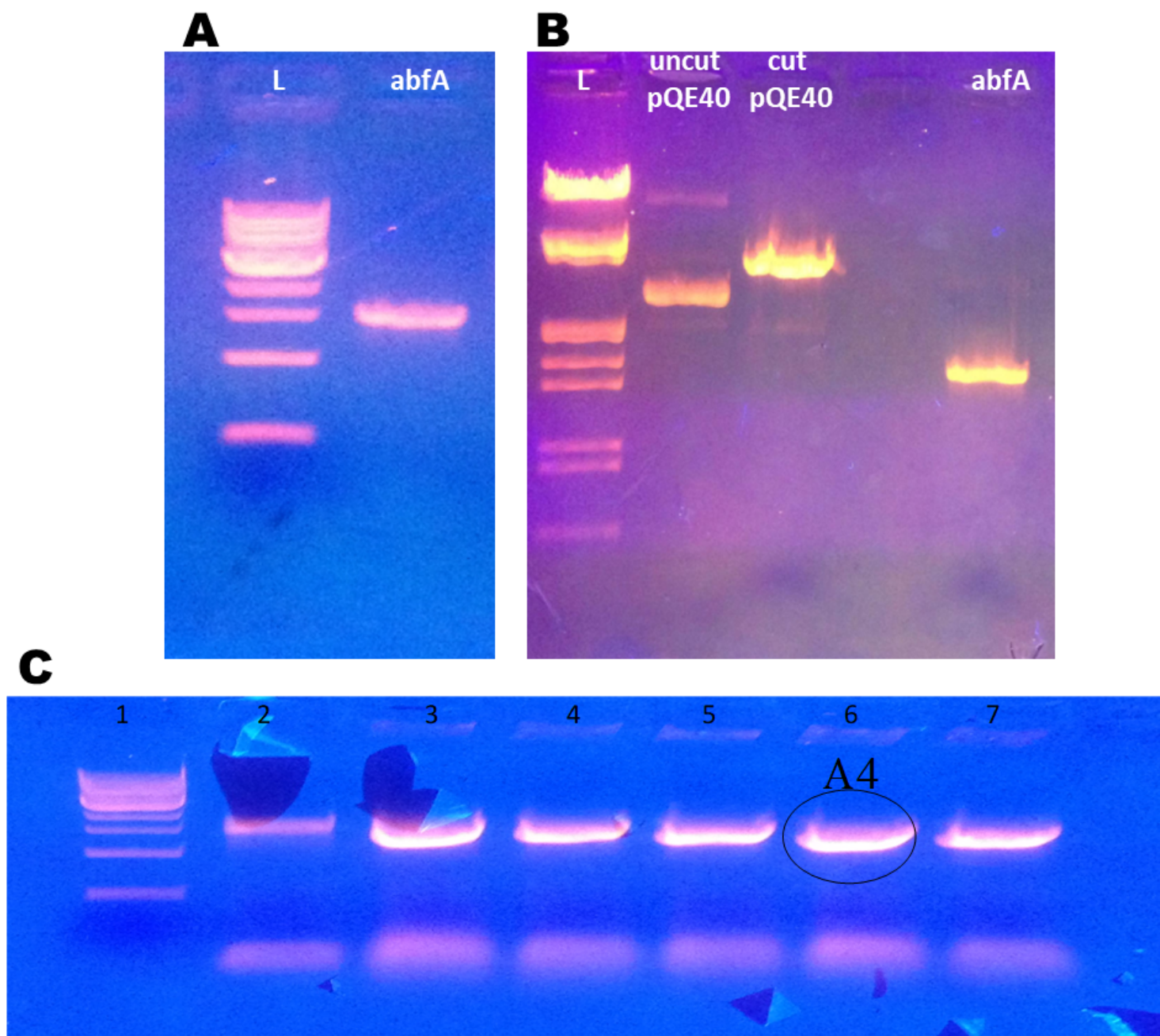
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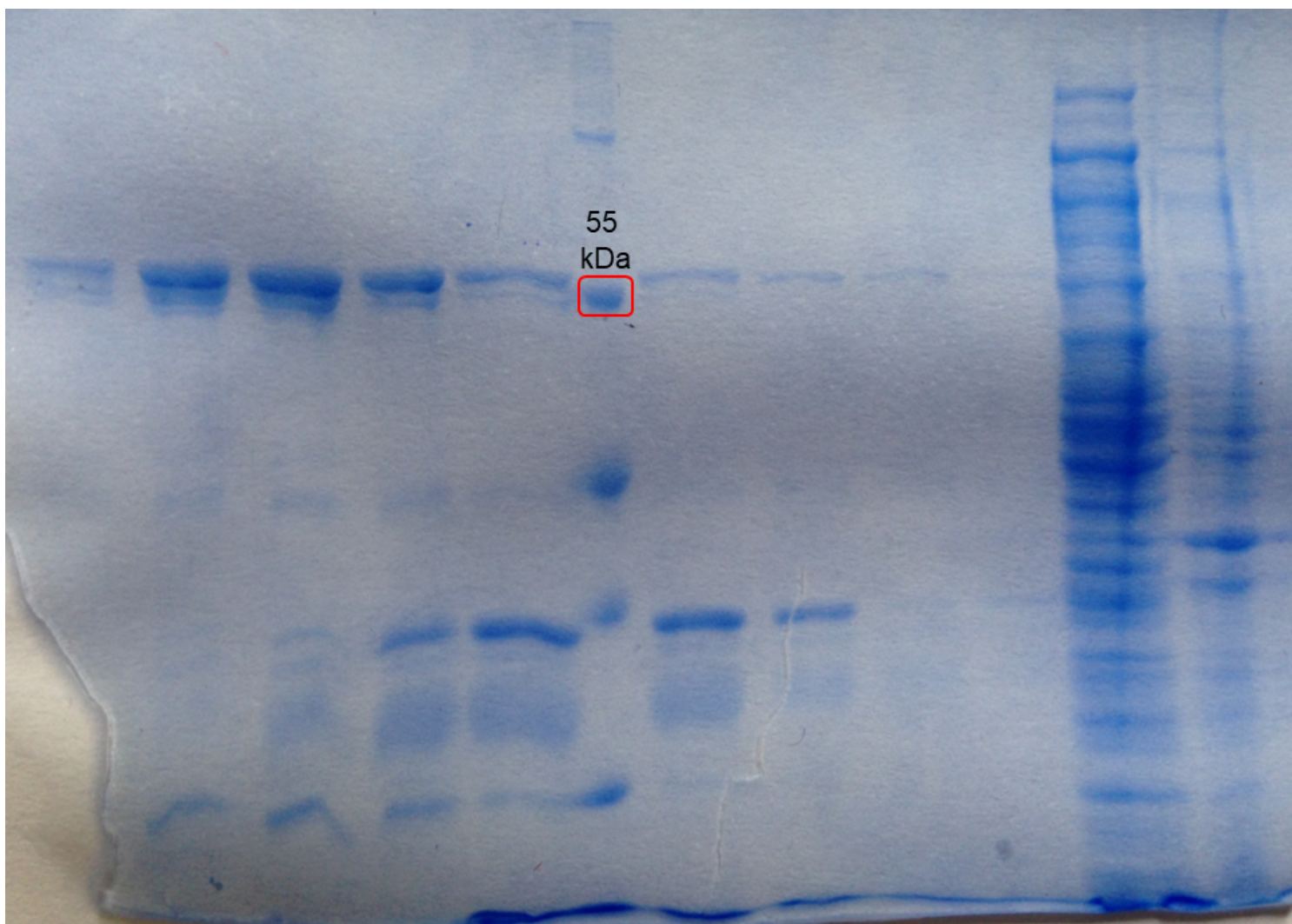
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## Figures



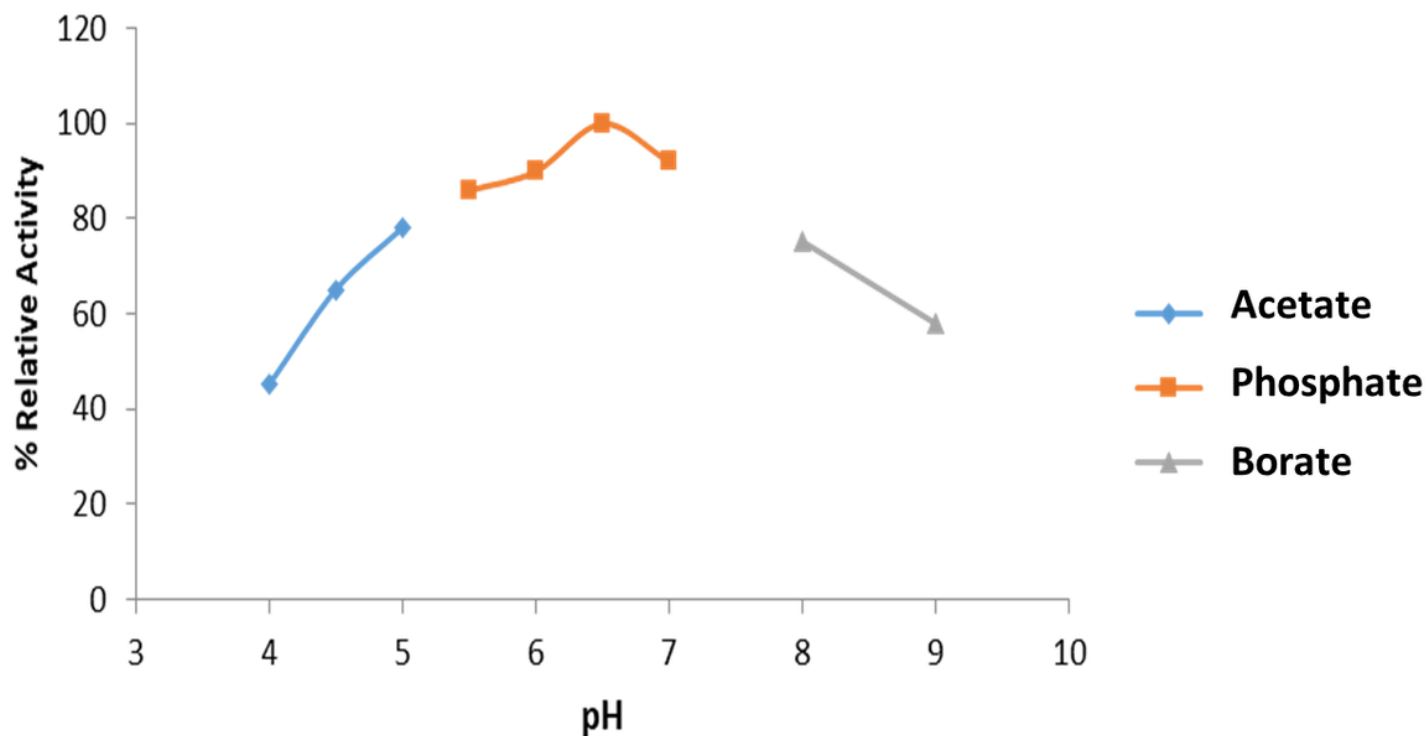
**Figure 1**

Cloning of  $\alpha$ -N-Arabinofuranosidase gene into pQE-40 vector. **A)** PCR amplification of 1506 bp.  $\alpha$ -N-Arabinofuranosidase gene. L; 1 kb DNA Ladder (New England BioLabs), abfA;  $\alpha$ -N-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 Sall and HindIII enzymes. abfA;  $\alpha$ -N-Arabinofuranosidase PCR product digested with Sall 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.



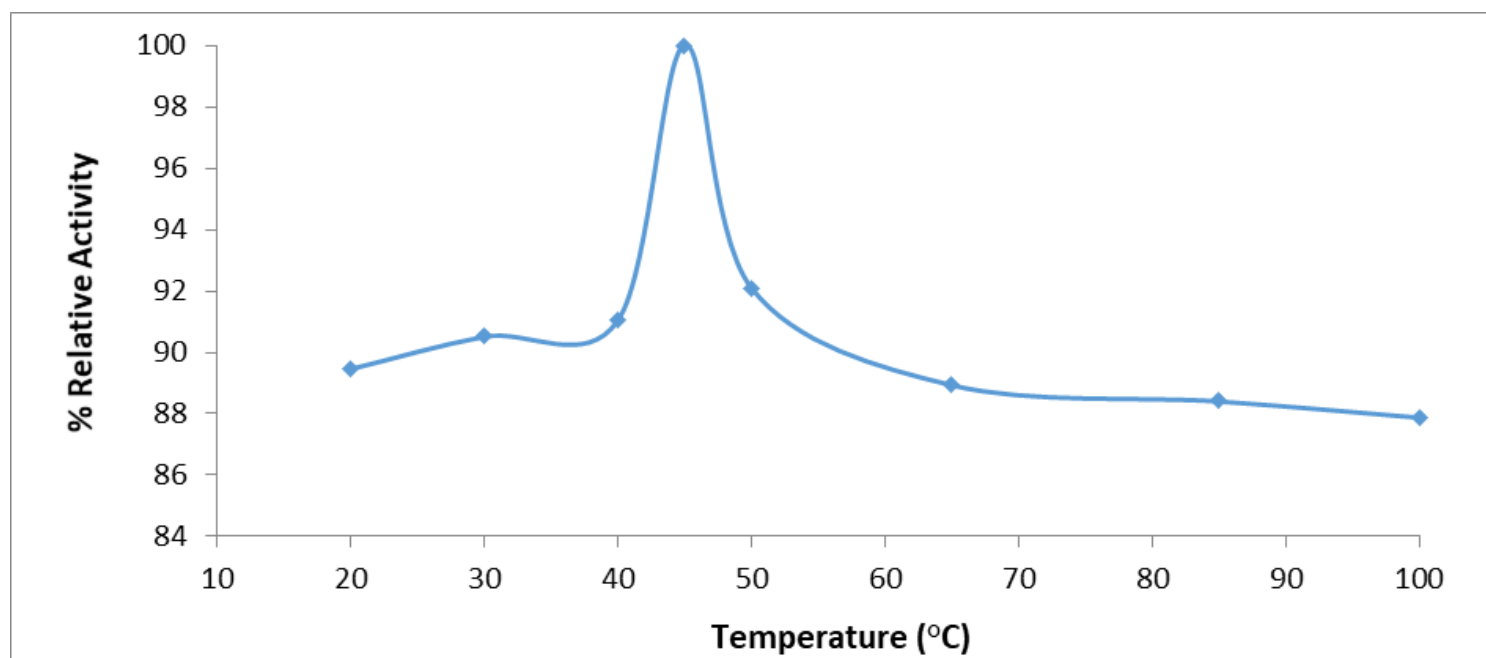
**Figure 2**

SDS-PAGE analysis of the fractions obtained from  $\alpha$ -N-Arabinofuranosidase purification.



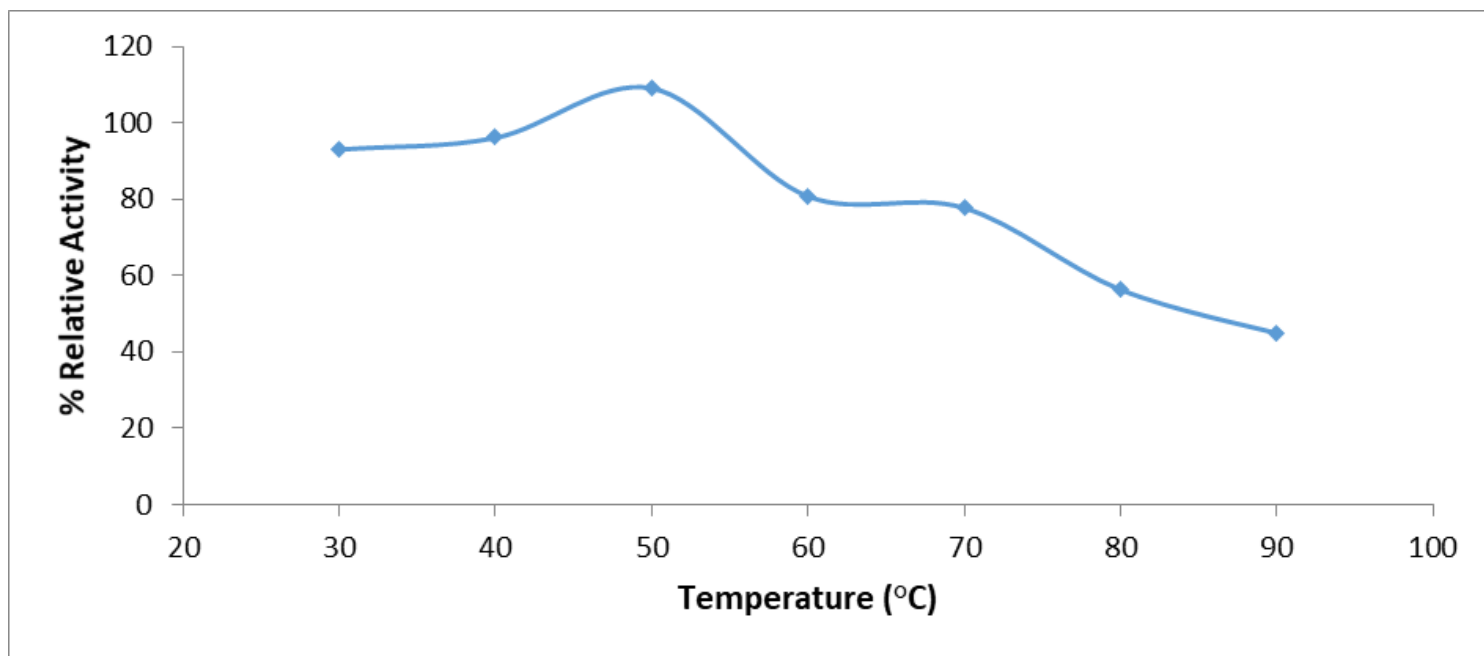
**Figure 3**

Effect of buffer and pH on  $\alpha$ -N-Arabinofuranosidase enzyme activity.



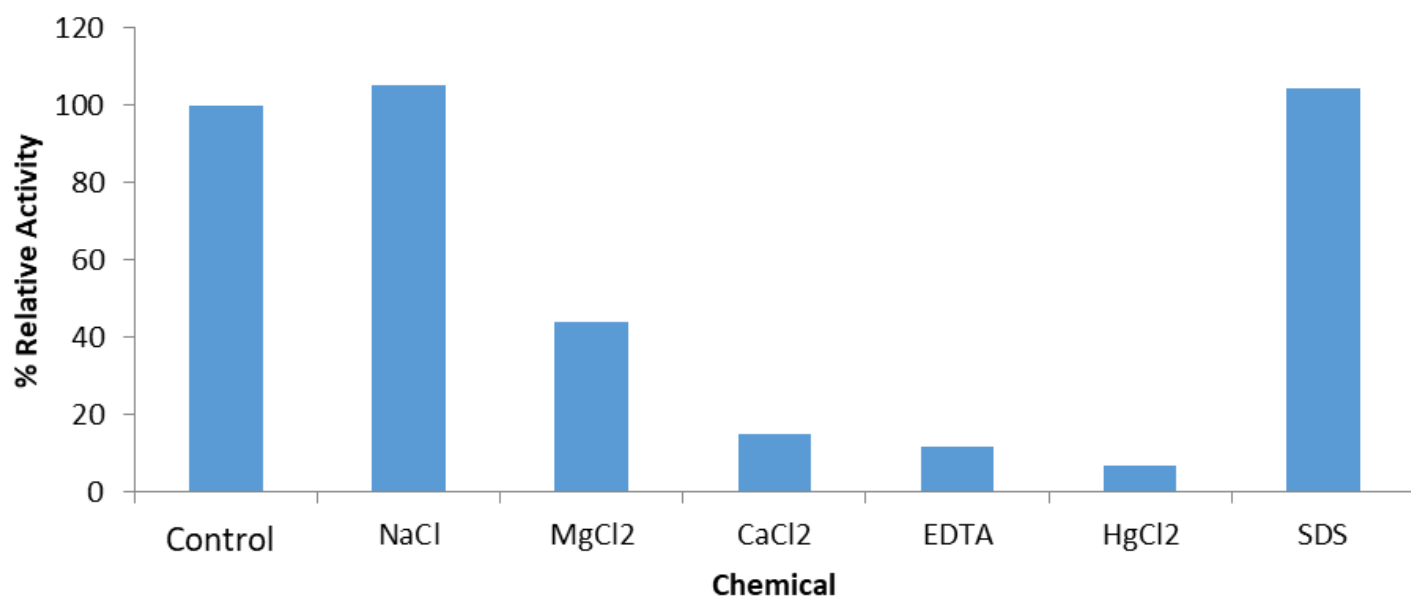
**Figure 4**

Effect of temperature on  $\alpha$ -N-Arabinofuranosidase enzyme activity.



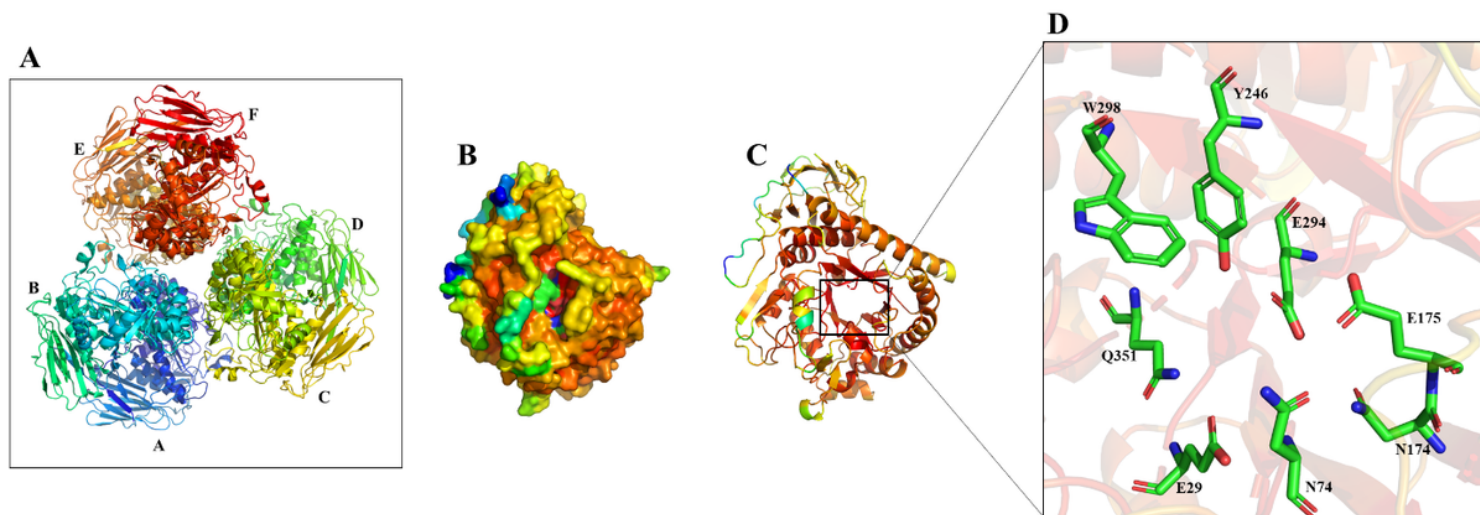
**Figure 5**

Thermal stability for recombinant N-Arabinofuranosidase.



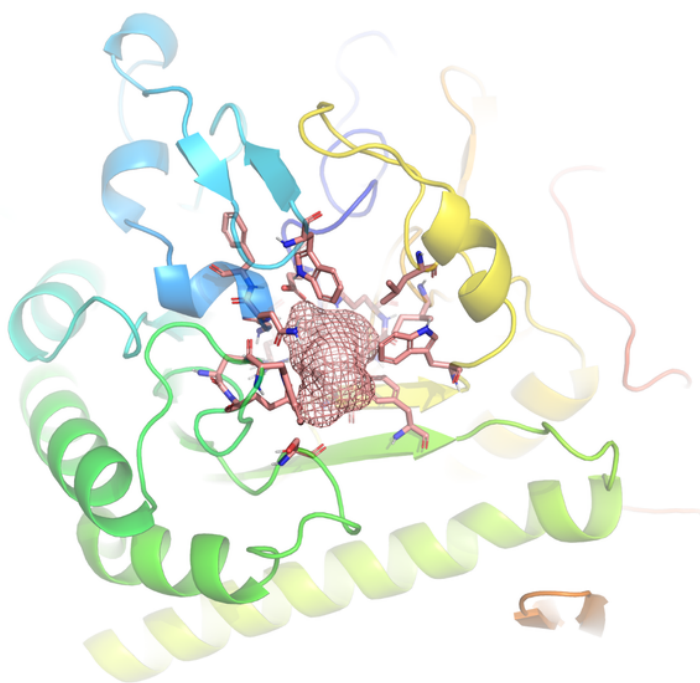
**Figure 6**

Effect of chemicals on α-N-Arabinofuranosidase enzyme activity.



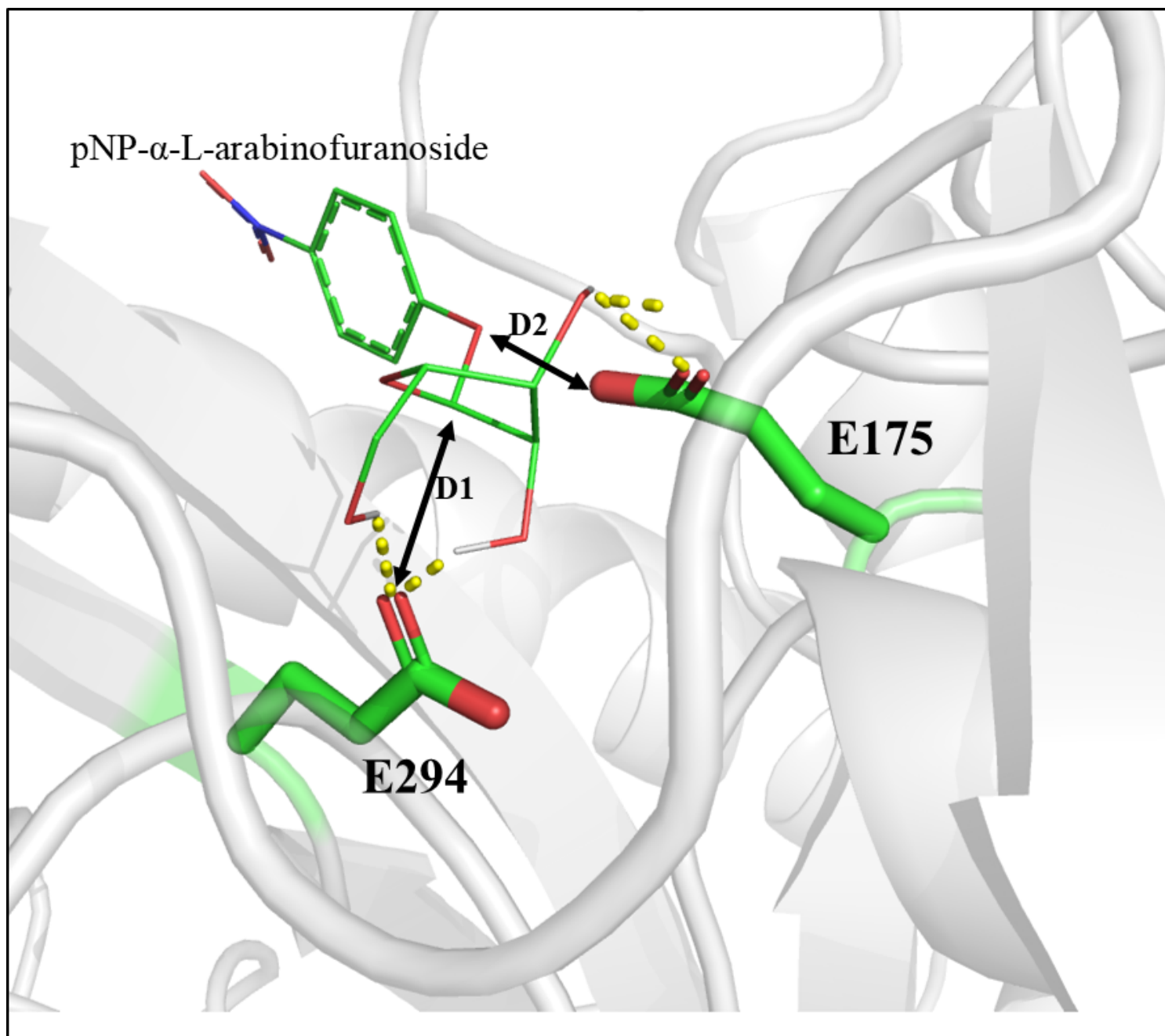
**Figure 7**

Homology model of  $\alpha$ -N-Arabinofuranosidase protein. A; Homohexameric structure. B; Surface representation of 3D monomeric protein. C; 3D model of  $\alpha$ -N-Arabinofuranosidase monomer. D; Active site residues of  $\alpha$ -N-Arabinofuranosidase enzyme.



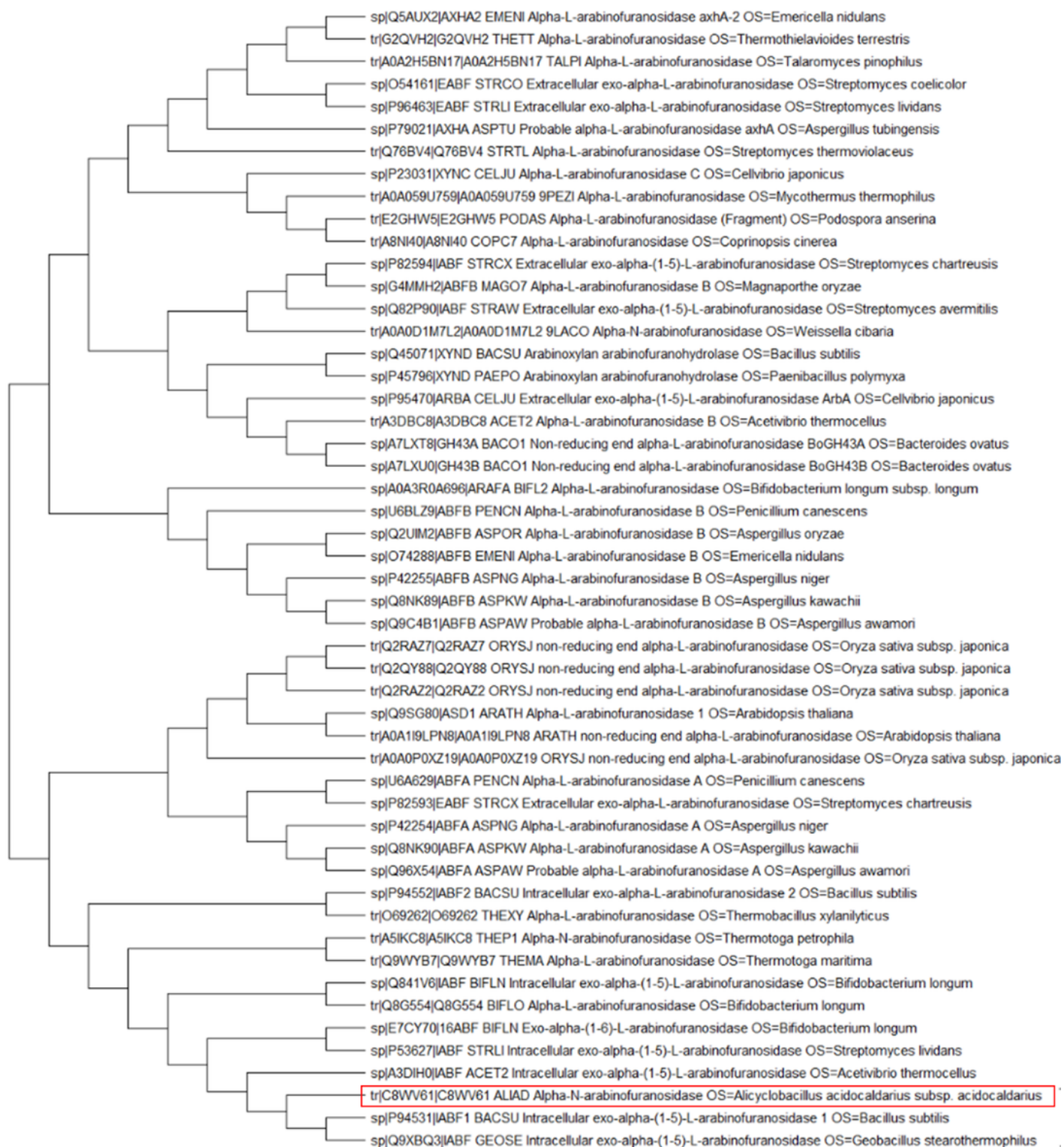
**Figure 8**

The binding site of the *A. acidocaldarius*  $\alpha$ -N-Arabinofuranosidase enzyme predicted using FTSite. The residues within 5 Å of the binding site (pink mesh) were shown as stick.



**Figure 9**

The docking analysis of  $\alpha$ -N-Arabinofuranosidase with the substrate pNP- $\alpha$ -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.



**Figure 10**

The phylogenetic analysis of the *A. acidocaldarius*  $\alpha$ -N-Arabinofuranosidase using the neighbor-joining method by MEGA X.

|                            |                                                                   |     |
|----------------------------|-------------------------------------------------------------------|-----|
| tr C8WV61 Alicyclobacillus | MSNLKARMTIDPAYRLAETDPRIYGSFIEHLGRAVYGGIYDPSHPTADEDGFRQDVIDL       | 60  |
| sp P94531 Bacillus         | --MKKARMIVDKKEYKIGEVDKRIYGSFIEHMGRAVYEGIYEPDHPEADEDGFRKDVQSLI     | 58  |
| sp Q9XBQ3 Geobacillus      | MATKKATMIEKDFKIAEIDKRIYGSFIEHLGRAVYGGIYEPGHPQADENGFRQDVIELV       | 60  |
|                            | * * * : : : * * * * * : * * * * * : * * * * * : * * * * *         |     |
| tr C8WV61 Alicyclobacillus | KELNVPIVRYPGGNFVSGYRWEDGVGPVEQRPVQLDLAWRSLEPNRVGLNEFARWAKKAN      | 120 |
| sp P94531 Bacillus         | KELQVPIIRYPGGNFLSGYNWEDGVGPVENRPRRLDLAWQTETNEVGTNEFLSWAKKVN       | 118 |
| sp Q9XBQ3 Geobacillus      | KELQVPIIRYPGGNFVSGYNWEDGVGPKEQRPRLDLAWKSVETNEIGLNEFMDWAKMVG       | 120 |
|                            | * * * : * * * * * : * * * * * : * * * * * : * * * * * : * * * * * |     |
| tr C8WV61 Alicyclobacillus | SQVMMAVNLGTRGIEEAKQIVEYCNHPGGSYWSDLRRKHGIEQPHGIRVWCLGNEMDGPW      | 180 |
| sp P94531 Bacillus         | TEVNMMAVNLGTRGIDAARNLVEYCNHPKGSYWSDLRRSHGYEQPYGIKTWCLGNEMDGPW     | 178 |
| sp Q9XBQ3 Geobacillus      | AEVNMMAVNLGTRGIDAARNLVEYCNHPSGSYYSDLRIAHGYKEPHKIKTWCLGNEMDGPW     | 180 |
|                            | : * * * * * : * * * * * : * * * * * : * * * * * : * * * * *       |     |
| tr C8WV61 Alicyclobacillus | QIGHKTADEYGRLAQEAAKVMKMWDPSELVACGSSGSKMATFPDWERIVLEHAYDEVDDY      | 240 |
| sp P94531 Bacillus         | QIGHKTADEYGRLAETAKVMKMWDPSELVACGSSNSGMPFIDWEAKVLEHTYEHVDY         | 238 |
| sp Q9XBQ3 Geobacillus      | QIGHKTADEYGRIACEAAKVMKMWDPTELVVCGSSNRMPTFAWEATVLDHTYDHDY          | 240 |
|                            | * * * * * : * * * * * : * * * * * : * * * * * : * * * * *         |     |
| tr C8WV61 Alicyclobacillus | LSLHTYYGNRDGLANFLACSLDMDAFIRAVVATCDFVRAKKRSNKTIYLSFDEWVWFH        | 300 |
| sp P94531 Bacillus         | ISLHTYYGNRDNNLPNYLARSMDLDFIKSVAATCOYVKAATRSKKTINLSLDEWVWFH        | 298 |
| sp Q9XBQ3 Geobacillus      | ISLHQYYGNRDNDTANYLALSLEMDDFIRSVVAIADYVKAATRSKKTINLSLDEWVWFH       | 300 |
|                            | : * * * * * : * * * * * : * * * * * : * * * * * : * * * * *       |     |
| tr C8WV61 Alicyclobacillus | SNEADKQVEPWQVGPPLLEDVYTMEDALVVGCLITLLKHADRVRIACLAQLVNVIAPIIM      | 360 |
| sp P94531 Bacillus         | SNEADKKVEPWITARPILEDIYNFEDALLVGSLLITMLQHADRVKIACLAQLVNVIAPIIM     | 358 |
| sp Q9XBQ3 Geobacillus      | SNEADKLIETVAPPLLEDIYNFEDALLVGCMLITLMKHADRVKIACLAQLVNVIAPIIM       | 360 |
|                            | * * * * * : * * * * * : * * * * * : * * * * * : * * * * *         |     |
| tr C8WV61 Alicyclobacillus | TENGGPSNRQTFYFPAHASNLAHGVVLYAPVESPKYDSKDFTDVPYLEAVPVWNEAAGE       | 420 |
| sp P94531 Bacillus         | TEKGGEAWRQPIFYPMHASVYGRGESLKLPISSPKYDCSDFTDVPYVDAVVYSEEEET        | 418 |
| sp Q9XBQ3 Geobacillus      | TEKNGPAWKQTIYPPMHASVYGRGVALHPVISSPKYDSKDFTDVPYLESAIVYNEEKEE       | 420 |
|                            | * * * * * : * * * * * : * * * * * : * * * * * : * * * * *         |     |
| tr C8WV61 Alicyclobacillus | MVLLAVNRA-EEPLALDVLDRGFNARSEEHIVLTHPNMKAVNTKERPNVVPQKRSIGA        | 479 |
| sp P94531 Bacillus         | LTIFAVNKA-EDQMETEISLRGFESYQIAEHIVLEHQDIKATNQHNK-NVVPHSGSSS        | 476 |
| sp Q9XBQ3 Geobacillus      | VTIFAVNRDMEDALLLECDVRSFEDYRVIEHIVLEHDNVKQTNSAQSS-PVPHRNGDAQ       | 479 |
|                            | : : : * * * : * * : : : * * * : * * : * * * : * * : * * * : * * * |     |
| tr C8WV61 Alicyclobacillus | VDAGRLAVELPALSWNVIRIRV--                                          | 501 |
| sp P94531 Bacillus         | VSENGLTAHFTPLSWNVIRLKKQS                                          | 500 |
| sp Q9XBQ3 Geobacillus      | LSDRKVSATLPKLSWNVIRLGKR-                                          | 502 |
|                            | : : : : : * * * * * :                                             |     |

**Figure 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.

# Supplementary Files

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