ISOLATION, EXPRESSION AND CHARACTERIZATION OF AN α-L-ARABINOFURANOSIDASE ENZYME FROM *Thermophilic Geobacillus sp*.

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by
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ABSTRACT

ISOLATION, EXPRESSION, CHARACTERIZATION OF AN α-L-ARABINOFURANOSIDASE ENZYME FROM *Thermophilic Geobacillus sp*.

In our study, we have aimed first to isolate an α-L-arabinofuranosidase (ALAF) enzyme, 58.0 kDa, from a thermophilic organism; *Thermophilic Geobacillus* sp. by using molecular cloning techniques, then to characterize this enzyme via biochemical methods.

Throughout the characterization studies, we have investigated the optimum conditions for the highest enzyme activity by means of pH and temperature by using *p*NP-α-L-arabinofuranoside as substrate. Also, effect of various metal ions, some specific chemicals and common organic solvents on enzyme activity was studied. Due to the fact that α-L-arabinofuranosidases mainly hydrolyze α-L-arabinofuranosyl residues of L-arabinose containing polysaccharides, enzyme activity towards sugar beet arabinan was also studied. Our enzyme exhibited activity in a broad pH range between pH 3.0-10.0 at 50˚C and between 30-90˚C in Na-acetate buffer pH 5.0. Optimum activity towards *p*NP-α-L-arabinofuranoside was obtained at pH 5.0 and at 70˚C. Kinetic studies showed that our enzyme has a K_m value as 0.19 mM and V_{max} as 18.6 ΔAbs/min/ml towards *p*NP-α-L-arabinofuranoside and K_m value as 0.1 mM and V_{max} as 8.1 ΔAbs/min/ml towards sugar beet arabinan.

ÖZET

Thermophilic Geobacillus TÜRÜNDEN α-L-ARABİNOFURANOZİDAZ ENZİMİNİN İZOLE EDİLMESİ, İFADELENMESİ VE KARAKTERİZE EDİLMESİ

Bu çalışmada, termofilik bir organizma olan *Thermophilic Geobacillus* türünden, moleküler klonlama teknikleri kullanılarak α-L-arabinofuranozidaz (α-L-AbFase) enziminin izolasyonu ve sonrasında biyokimyasal yöntemlerle karakterizasyonu amaçlanmıştır.

Karakterizasyon çalışmaları sırasında, enzimin *p*NP-α-L-arabinofuranozit substratına karşı en yüksek verimde çalıştığı optimum pH ve sıcaklığı değerlerini araştırdık. Buna ek olarak, çeşitli metal iyonlarının, bazı özel kimyasallar ve yaygın kullanımı olan organik çözgenlerin enzim aktivitesi üzerine olan etkileri çalışıldı. Çalıştığımız enzimin ana olarak L-arabinoze içeren polisakkaritlerde α-L-arabinofuranozil gruplarını parçalamasından dolayı pancar şekerinden elde edilmiş olan arabinan susbtrat olarak kullanılmıştır . Enzimimiz, 50˚C sıcaklıkta geniş bir pH aralığında; pH 3.0-10.0, ve Na-asetat tamponu içinde iken 30 ile 90˚C arasında aktivite göstermiş olup, *p*NP-α-L-arabinofuranozit substratına karşı optimum çalışma koşulları pH 5.0 ve 70˚C olarak saptanmış, kinetik çalışmalar sonrasında enzimin bu substrata karşı K_m ve V_{max} değerleri sırasıyla 14.75 mM ve 1.6 Abs/dak/ml olarak, şeker pancarından elde edilmiş arabinana karşı ise sırasıyla 1.35 mM ve 3.9 ΔAbs/dak/ml olarak hesaplanmıştır.

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LIST OF ABBREVIATIONS

bp	Base pair
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
kb	Kilo base
sp.	Species
kb	Kilobase pairs
l	Liter
M	Molar
mM	Millimolar
µM	Micromolar
ml	Milliliter
µl	Microliter
µg	Microgram
ng	Nanogram
nm	Nanometer
w/v	Weight per volume
rpm	Revolutions per minute
RT	Room temperature
Taq	Thermus aquaticus (DNA polymerase)
UV	Ultraviolet
3-D	Three-Dimensional
X-Gal	5-bromo-4-chloro-3 indolyl-β-D-galactoside
LB	Luria-Bertani
IPTG	Isopropyl-thio-β-D-galactopyranoside
DMSO	Dimethylsulphoxide
TAE	Tris/acetate/ethylenediamine tetra-acetic acid
EDTA	Ethylenediamine tetra acetic acid
TEMED	Tetramethylethylenediamine
ALAF	α-L-arabinofuranosidase
PIPES	Piperazine-N,N'-bis[2-ethanesulfonic acid]
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
DNS	3,5-Dinitrosalicylic Acid
PCR	Polymerase Chain Reaction

CHAPTER 1

INTRODUCTION

Enzymes are said to be catalysts of biological and chemical processes so, they have been used in a wide range of processes in industry and the scientists have studied on different types of enzymes to make use of new ones since the beginning of 1950-60s. The purpose of introducing enzymes into the industry is that not only they are said to decrease the activation energy of the reaction to take place but also they are used to eliminate the use of toxic chemicals and degrade harmful products.

1.1. Thermophiles

Environmental factors such as temperature is important for all living organisms to survive. So, according to their relation to temperature, to classify the living organisms is essential for biological systematics (Kristjansson 1989).

Based on their optimal growth temperatures, microorganisms are divided into three main groups, i.e. psychrophiles (below 20°C), mesophiles (moderate temperatures), and thermophiles (high temperatures, above 55°C) (Brock 1986), hyperthermophiles (above 80°C) (Kristjansson and Stetter 1992). Additional classification was made by Baker et al. (Baker et al., 2001) for the thermophilic organisms and in this case, they divided the thermophiles into three groups based on their minimal and maximal growth temperatures as follows: moderate thermophiles (35-70ºC), extreme thermophiles (55-85ºC) and hyperthermophiles (75-113ºC).

The most common habitats for the thermophilic organisms are geothermally and volcanically heated hydrothermal systems such as solfataric fields, neutral hot springs and submarine saline hot vents (Grant 1998), some of the bacterial and archaeal thermophiles are listed in Table 1.1 and in the Figure 1.1. some of the thermophile sources are given. So, the thermophilic organisms are able to live at high temperatures. Due to the fact that having this property, the proteins or enzymes from such organisms, generally show thermostability/activity at high temperatures.

Table 1.1. Thermophiles and their environments.
(Source: Hough and Danson 1999)

Phenotype	Environment	Typical genera
Thermophilic	55-80ºC	Methanobacterium, Thermoplasma, Thermus*, some Bacillus* species
Hyperthermophilic	80-113ºC	Aquifex*, Archaeoglobus, Hydrogenobacter*, Methanothermus, Pyrococcus, Pyrodictium, Pyrolobus, Sulfolobus, Themococcus, Thermoproteus, Thermotoga*

*Genera of the domain is Bacteria; all others are Archaea.

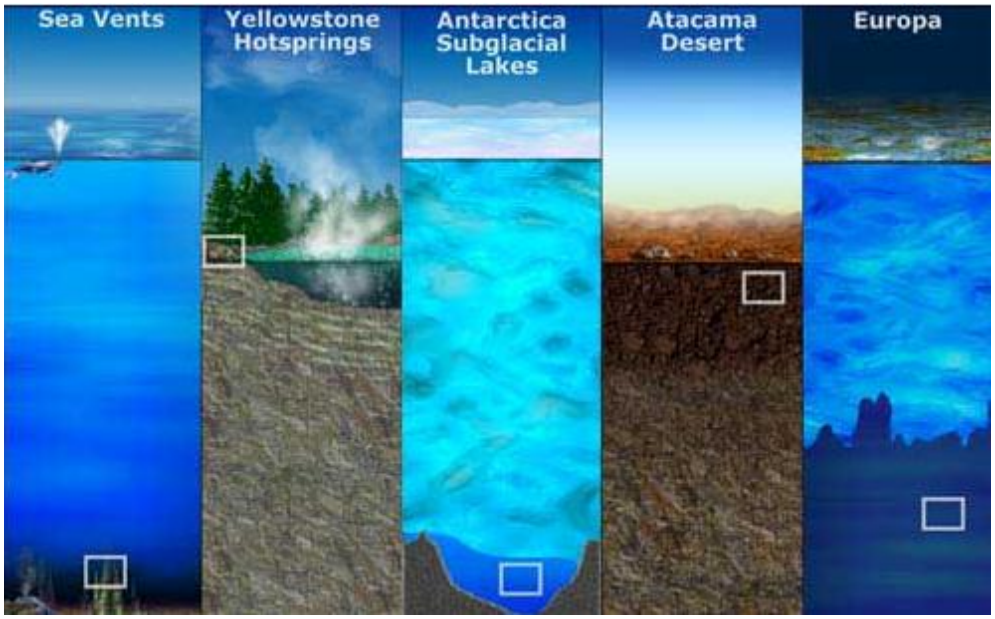

Figure 1.1. Potential sources of the thermophiles.
(Source: www.nsf.gov 2011)

1.2. Thermophilic Enzymes

Thermophilic organisms produces thermostable enzymes and these enzymes find a number of commercial applications in wide range of industry because of their thermostability and thermoactivity (Yavuz et al., 2004). As mentioned previously for the thermophiles, growth temperature is above 55ºC for the thermozymes. Generally, when comparing the thermozymes and the mesozymes, thermozymes are more robust to thermal and chemical denaturation (Zeikus et al., 1998) which makes these enzymes industrially more valuable.

1.3. Applications of Extremozymes

Enzymes from extremophiles also called as 'extremozymes' (Hough and Danson 1999), have potential applications in many areas, either by using the enzymes themselves, or by using them as sources of ideas to modify mesophile-derived enzymes. Extremophilic enzymes have endeared themselves to multibillion-dollar industries, including agricultural, chemical synthesis, laundry detergents (Ito et al., 1998) and pharmaceuticals (Rothschild and Mancinelli 2001). The business markets of these enzymes are growing every year (Zeikus et al., 1998).

Taq polymerase is one of the most popular enzyme isolated from a hot origin in biotechnology is the source of, the enzyme at the crux of the widely used polymerase chain reaction (PCR). DNA polymerases from other thermophiles have been marketed by Promega Corporation as a product for high-fidelity PCR, with each having its own advantages (Mattila et al., 1991; Cariello et al., 1991). There are also many other extremophiles which have industrial applications. Moreover, the properties of the alkaliphilic enzymes fits wells with the requirements to be used in detergents (Ito et al. 1998). The bacteria are used to enrich rotifers, a food organism for larval fish (Williams et al., 1999). For the bioremediation of waters following oil spills, Antarctic bacteria have potential in this process, which is a concern in cold waters (Williams et al., 1999). Also, for the commercial production of β-carotenes, D. salina is widely used, which produces in response to solar radiation, and glycerol, which it produces to counterbalance external osmotic pressure (Neuman et al., 1999). Human health may benefit from extremophiles indirectly through biotechnology and bioremediation;

Table 1.2. Direct uses include marketing of dried Dunaliella as a nutritional supplement, primarily as an antioxidant. Antifreeze proteins show potential as cryoprotectants of frozen organs (Rothschild and Mancinelli 2001).

Table 1.2. Examples of extremophiles in industry and biotechnology.
(Source: Rothschild and Mancinelli 2001)

Industrial process	Biomolecule	Advantages	Source organism
Hydrolysis of starch to produce soluble dextrins, maltodextrins and corn syrups	α-Amylase	High stability, aciduric, bacterial amylase	*Bacillus stearothermophilus* G-ZYME G995 (Enzyme Bio-System Ltd)
Paper bleaching	Xylanases	Decreases amount of bleach needed	Thermophiles
Prevent stalling in range of baked products	α-Amylase	Gives boost to yeast fermentation	Highest-stability bacterial amylase available, G-ZYME G-995
Food processing, baking, brewing, detergents	Proteases	Stable at high temperatures	Thermophiles
PCR reaction	DNA polymerase	No need to add additional enzyme during each cycle	Thermophiles
Cheese maturation, dairy production	Neutral proteases	Stable at low temperatures	Psychrophiles
Degradation of polymers in detergents	Proteases, amylases, lipases	Improved performance of detergent	Psychrophiles
Degradation of polymers in detergents	Cellulases, proteases, amylases, lipases	Stable at high pH	Alkaliphiles
Mariculture	Polyunsaturated fatty acids	Produced in cold temperatures	Psychrophiles
Bioremediation	Reduction of oil spills	Works efficiently in cold waters	Psychrophiles
Pharmaceuticals	Polyunsaturated fatty acids		Psychrophiles
Biosensors	Dehydrogenases		Psychrophiles
Desulphurication of coal	Sulphur oxidation		Acidophiles
Antibiotic production	Antibiotics		Alkaliphiles
Food colouring	Carotene	Inexpensive to produce	Halophiles/ *Dunaliella*
Pharmaceuticals	Glycerol, compatible solutes	Inexpensive to produce	Halophiles
Surfactants for pharmaceuticals	Membranes		Halophiles

1.4. Cell Wall Structure and Hemicellulose

Lignocelluloses of plant cell walls, Figure 1.2, are composed of cellulose, hemicellulose, pectin and lignin which are the key components of lignocelluloses. Hemicelluloses is the most abundant renewable biomass polymer next to cellulose of which about 20–35% is lignocellulosic biomass (Ward and Moo-Young 1989). Some of the hemicelluloses composed of L-arabinosyl residues, such as arabinan, arabinoxylan, gum arabic, and arabinogalactan. The action of α-L-AbFase alone or in combination with other lignocellulose-degrading enzymes represents a promising biotechnological tool as alternatives to some of the existing chemical technologies.

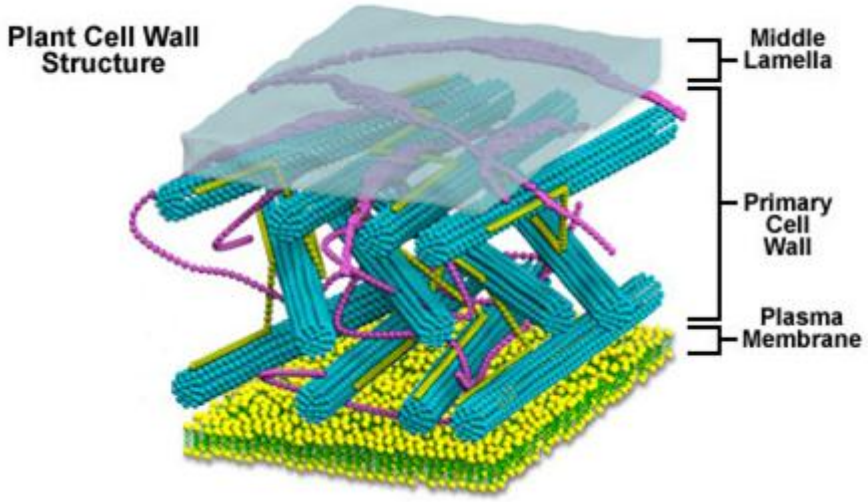

Figure 1.2. Structure of plant cell wall.

1.5. Xylan

Xylan, a xylose polymer having a β(1-4) glycosidic linkage in the main chain, is one of the most important component of hemicellulose in plant cell walls, Figure 1.3. Naturally occurring xylan normally contains L-arabinose and 4-O-methylglucuronic acid as side chains, its hydroxy group often being acetylated, and occasionally contains the β(1-3) glycosidic linkage as minor unit.

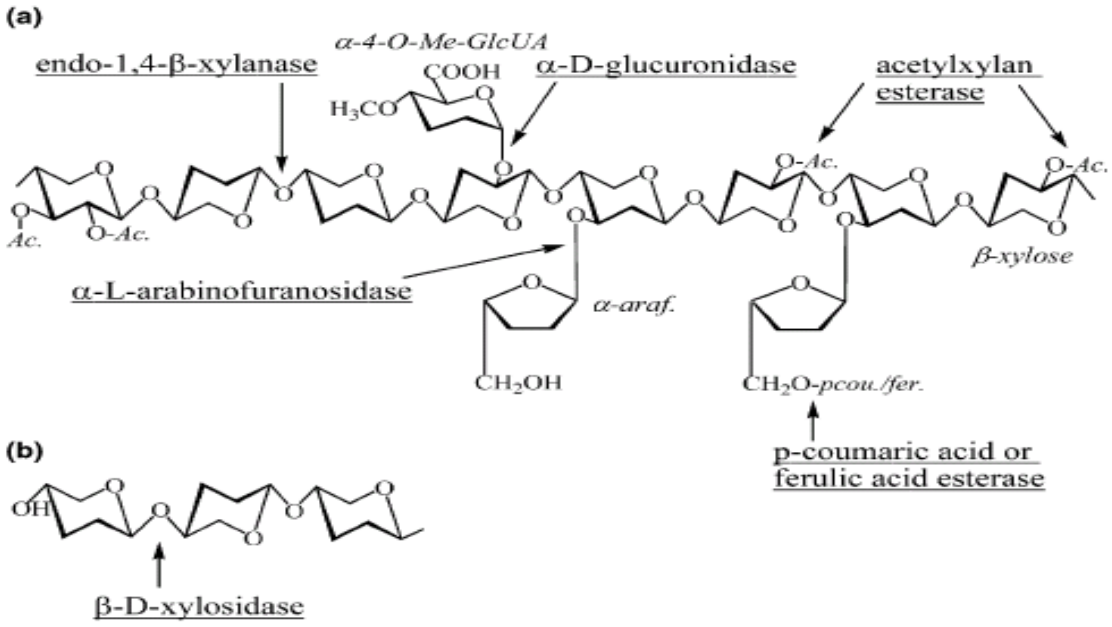

Figure 1.3. **(a)** Structure of xylan and the sites of attack by xylanolytic enzymes. The backbone of the substrate is composed of 1,4-β-linked xylose residues. **(b)** Hydrolysis of xylo-oligosaccharide by β-xylosidase.
(Source: Collins et al. 2005)

1.6. α-L-Arabinofuranosidases

α-L-Arabinofuranosidase (α-L-arabinofuranoside, arabinofuranohydrolase, EC 3.2.1.55, α-L-AbFase) is involved in arabinose release processes from these substrates. In most cases, xylanases, α-L-AbFases and other related enzymes act synergistically for the purpose of degradation xylan, Figure 1.3., and other hemicelluloses to its components (Saha 2000).

α-L-AbFase enzymes are mostly isolated from bacteria, fungi and plants either by classical biochemical methods or molecular cloning techniques. In the Table 1.3, α-L-AbFases from microbial sources and their properties are listed.

Table 1.3. Properties of some microbial α-L-AbFase.
(Source: Numan and Bhosle 2006, modified)

Microorganism	Enzyme	Molecular mass (kDa)	Optimum pH	Optimum temperature (°C)	Polymers attacked	GHs family	Refs
A. oryzae HL15	Afase	60[a] 110[b]	5.5	60	AX, AG, OSX	–	[I]
A. oryzae	abfA	228	5	50	–	51	[II]
A. Kawachii	AkAbfA	80	4	55	AX	51	[III]
	AkAbfB	62					
Fusarium oxysporum f. sp. dianthi (Fod)	abfB	–	4.0	50	OSX	–	[IV]
P. purpurogenum	ABF1	49.6 58	5	50	AX, BX, OSX, IAG, WS, WB	54	[V,VI]
P. chrysogenum	AFQ1	79	4	50	BA, DA, AX, SAG,	51	[VII]
	AFS1	52	3.3–5.0	50	A2, A3		
Rhizomucor pusillus HHT1	AFase	–	–	–	BA, A2, A3, A4, A5	–	[VIII]
B. pumilus PS213	AF	22056[c]	7	55	OSX, AG	51	[IX]
B. breve K-110	AFase	60	–	–	Ginsenoside Rb2 and Rc	51	[X]
Clostridium cellulovorans	rArfA	138	6	40–50	BA, AX	51	[XI]
Bifidobacrerium longum B667	abfB	61°260[b]	6.0	45	BA, AX, A2, A3, A4, A5	51	[XII]
P. cellulosa	abf51A	57	5.5	<55	WA, BA, A2, A3, A4, A5	51	[XIII]
	abf62A	–	–	–	WA, A2, A3, A4, A5	62	
S. chartreusis GS901	AFase I	80	5.5	55	BA, AX, AG, A2, A3	51	[XIV]
	AFase*II*	37	7	50	BA, AX, AG, A2	43	
Streptomyces thermaviolaces OPC-520	STX-IV	37	5	60	AX, OSX	62	[XV]
Thermoanaerobactere ethanolicus JW200	xarB	85	–	65	–	3	[XVI]
Thermobacillus xylaniliticus	AbfD3	56.071	6.2	75	WA, IX, OSX	51	[XVII]
T. fusca		~92	9	65	–	–	[XVIII,XIX]
Bacterium PRI-1686	AraF	350	6	70	BA, DA, OSX	51	[XX]
T. maritima MSB8	AFase	332	7	90	BA, DA	51	[XXI]

[a]Molecular mass determined by SDS-PAGE gel, [b]Molecular mass determined by gel filtration, [c]Molecular mass determined by mass spectrometry. OSX: oat spelt xylan, AG: arabinogalactan, BA: arabinan, BiWX: birchwoodxylan, BeWX: beechwood xylan, WA: wheat arabinoxylan, BX: sugar beet pulp xylan, WBX: wheat bran, IX: larch xylan, SAG: soybean arabinogalactans, IAG: larchwood arabinogalactan, WS: wheat straw, WB: wheat bran, DA: Debranched arabinans, A2: arabinobiose, A3: arabinotriose, A4: arabinotetraose, A5: arabinopentaose. I: Hashimoto and Nakata (2003), II: Matsumura et al. (2004), III: Koseki et al. (2003), IV: Chacòn-Martènez et al. (2004), V: Carvallo et al. (2003), VI: De Ioannes et al. (2000), VII: Sakamoto and Kawasaki (2003), VIII: Rahman et al. (2003), IX: Degrassi et al. (2003), X: Shin et al. (2003), XI: Kosugi et al. (2002), XI: Margolles and de los Reyes-Gavilán (2003), XIII: Beylot et al. (2001), XIV: Matsuo et al. (2000), XV: Tsujibo et al. (2002), XVI: Mai et al. (2000), XVII: Debeche et al. (2000), XVIII: Tuncer (2000), XIX: Tuncer and Ball (2003), XX: Birgisson et al. (2004), XXI: Coen and Dehority (1970).

1.7. Applications of α-L-AbFases in Industry

During the last three decades, xylan-degrading enzymes have received much attention and especially the α-L-AbFase enzymes are the promising tools in different agro-industrial processes (Aryon et al. 1987, Saha 2000) due to the fact that their practical applications in various agro-industrial processes, such as efficient conversion of hemicellulosic biomass to fuels and chemicals, delignification of paper pulp, digestibility enhancement of animal feedstock, production of important medicinal compounds production of bioethanol and the synthesis of oligosaccharides (Saha 2000;

Numan and Bhosle 2006), clarification of juices, and improvement in the consistency of beer (Campbell and Bedford 1992; Viikari et al., 1993; Wong et al., 1988; Zeikus et al., 1991). Tateishi et al. (1996) studied on Japanese pear fruit ripenning and they observed that the α-L-AbFase activity increased importantly during the pocess. In some of these processes α-L-AbFase enzymes are acting synergistically with other lignocellulose degrading enzymes.

Table 1.4. Some of the potential applications of α-L-AbFases.
(Source: Saha 2000)

- Bioconversion of lignocellulosic materials to fermentable products
- Improvement of animal feedstock digestibility
- Delignification of pulp
- Hydrolysis of grape monoterpenyl α-L-arabinofuranosidase
- Clarification of thinning of juices

1.8. The Aim of This Study

As the significance of the α-L-AbFase enzymes explained above, these enzymes have an important role in the degradation of hemicelluloses in the plant cell walls and have many potential applications in industrial processes. In this study, isolation, expression, purification and and characterization of an α-L-AbFase enzyme from a *Thermophilic geobacillus* sp. was aimed.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used in this study were listed in Appendix A.

2.1.2. Medias

Medias were listed in Appendix B.

2.1.3. Reagents and Solutions

Reagents and solutions were presented in Appendix C.

2.2. Methods

2.2.1. DNA Isolation and Gene Expression

2.2.1.1. Growth Condition for Bacteria

All cultures for the purpose of genomic DNA isolation were inoculated at 55˚C and at 200 rpm overnight in liquid LB media.

2.2.1.2. Genomic DNA Isolation

Based on the positive results observed by plate screening, the genomic DNA of the number 90 strain was isolated by using a commercial extraction kit (Genomic DNA Purification Kit, Fermentas). Before the isolation, the strain was incubated in 5.0 ml of liquid LB media without antibiotic in a shaker at 55˚C for 18 hrs. The quantity was again determined by a nano-drop instrument (Nanodrop 1000 Spectrophotometer Thermo Scientific) at 260 nm.

2.2.1.3. Primer Design

The primers were designed based on a previously defined α-L-AbFase coding gene for the α-L-AbFase coding gene amplification of which the accession number in the gene bank is DQ387046, isolated from *Geobacillus thermoleovorans* IT-08 organism. *HindIII* and *NdeI* endonuclease enzymes were used and while designing the primers, 6 nucleotides were added on 5' end of each primer. The following sequences are designed primers (F: forward, R: reverse) and the italic letters correspond to the added 6 nucleotides to reverse primer which *HindIII* recognizes AAGCTT and cleaves between the two adenines. On the other hand, the added 6 nucleotides to forward primer which *NdeI* recognizes CATATG site and cleaves it as; CA-TATG.

- Forward primer: 5' *CATATG*GCTACAAAAAAAGCAACC 3'
- Reverse primer: 5' *AAGCTT*TTATCGTTTTCCTAAACG 3'

2.2.1.4. PCR Amplification

The α-L-AbFase coding gene was amplified under the following conditions:

- ***Reaction Medium***

• dH_2O	13.5µl
• $MgCl_2$	2.0µl
• 10x Tag DNA polymerase buffer including $(NH_4)_2SO_4$	2.5µl
• dNTP mixture	2.5µl
• Forward primer	0.75µl
• Reverse primer	0.75µl
• Template DNA (90 ng/ µl)	2.5µl
• Tag DNA polymerase	0.5µl
Total volume	25.0µl

- ***Reaction Conditions***

• Initial denaturation	94˚C	5 minutes
• Denaturation	94˚C (2+30 cycles)	1 minute
• Annealing	45˚C (2 cycles), 55˚C (30 cycles)	1 minute
• Extension	72˚C (2+30 cycles)	1 minute
• Final extension	72˚C	10 minutes

2.2.1.5. Agarose - Gel Electrophoresis and Gel Extraction/PCR Product Purification from Agarose Gel

PCR products were visualized by agarose gel electrophoresis technique. Briefly, in 70.0 ml 1xTAE buffer 1.0 g of agarose was dissolved in microwave oven until the agarose completely dissolved and the solution was cooled to ~60˚C. 0.7 µl of EtBr (10.0 mg/ml) was added and spread through the solution. Then, it was poured into the horizontal gel apparatus and a special comb was positioned. After solidifying the solution at room temperature, the comb was removed and the gel was repositioned. The tank was filled with 1x TAE electrophoresis buffer just above the gel. The samples were added and mixed with 6x loading dye gently to avoid formation of bubbles. After loading the samples into the wells separately, 100V and 80mA electric field was applied for about 45 minutes. After running the samples on the agarose gel, gel documentation system was used to visualize the samples under the UV light.

2.2.1.6. Protocol for Competent Cell Preparation

2.2.1.6.1. DH5α and BL21(λDE3) Competent Cells

Inoculate the proper cells on LB-agar plates followed by an overnight incubation at 37˚C and after observing the single colonies on plate, culture one of the single colonies into a 5.0 ml of liquid LB media and incubate again for overnight at 37˚C and 180 rpm. The next day in the morning, the cells were poured into 200.0 ml of SOB media and incubated at 10˚C and 37˚C for 2 hours separately and at 10˚C for 16 hours at 100 rpm. The cells were divided into four equal pieces and incubated on ice for 10 minutes. After the centrifugation of the samples at 4˚C and 4,000 rpm for 10 minutes, the supernatants were poured off. 5.0 ml of ice-cold TB solution (Appendix B) was added onto the pellets and the cells were resuspended gently and placed on ice for 10 minutes. Then, the samples were centrifuged at 4˚C and 4,000 rpm for 10 minutes and the supernatant solutions were again poured off. Depending on the amount of the pellet, TB solution was added (~1.0 ml of TB for 1.0 ml of pellet) and resuspended carefully on ice. Before aliquoting the sample, previously filtered (0.2 µm filter) DMSO was added by 7.0% of TB solution. After that step, the aliquots were prepared by dividing the samples as 50.0 or 100.0µl for each previously cooled eppendorfs. Finally, the aliquots were immersed into the liquid nitrogen immediately and stored at -80˚C.

2.2.2. Cloning the α-L-AbFase Enzyme Coding Gene in Cloning Vector; pTZ57R/T

2.2.2.1. Cloning Vector, pTZ57R/T

The purified PCR products, in other word insert, were cloned by using TA cloning vector, (pTZ57R/T) Figure 2.1, included in PCR cloning Kit (Fermentas).

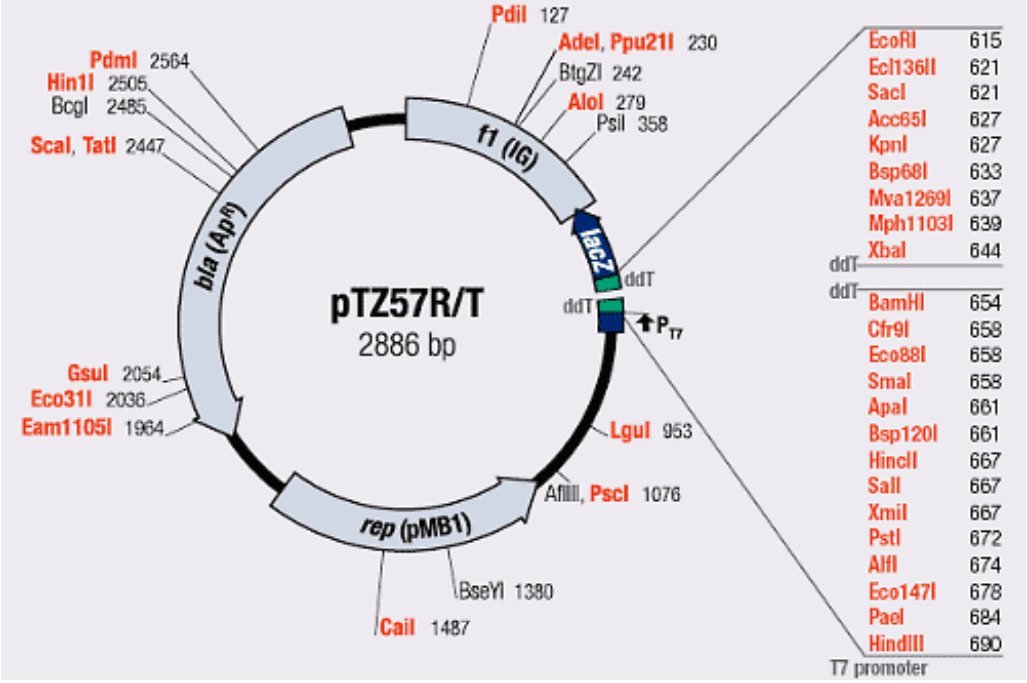

Figure 2.1. pTZ57R/T cloning vector and multiple cloning sites.

All ligation reactions were carried out between (20-25˚C) on bench for 4 hours. Generally, the reaction volume was 20.0 µl which contains 1.0 µl of T4DNA ligase (3.0 Weiss units/µl), 1.0 µl 10x Rapid ligation Buffer of T4DNA ligase, 1.0 µl pTZ57R/T vector (50.0 ng). The reaction volume diluted to 10.0 µl using UP water after addition the proper amount of the insert based on the ratio between the vector and the insert optimized as 1/10 using Equation 2.1.

$$\text{ng of insert} = \frac{\text{ng of vector } x \text{ size of insert (bp)}}{\text{size of vector (bp)}} \; x \; \frac{\text{vector}}{\text{insert}} \qquad 2.1$$

2.2.2.2. Transformation to DH5α Competent Cells

The recombined DNA, composed of our target gene, insert and cloning vector, was transformed throughout the three steps incubation period of 4.0 µl and 100.0 µl of DH5α competent cells, just after melting, as the following:

- 10 min on ice,
- 1 min at 42˚C,
- 2 min on ice.

Then, 400.0 µl of SOC media (Appendix B) or 900 µl LB media (Appendix B) was added and incubated at 37˚C at 200 rpm for 1 hour.

In order to carry out blue/white colony selection on agar plates, on each plate; 40.0 µl of X-gal (20.0 mg/ml), 40.0 µl of IPTG (0.1 M) and 20.0 µl of dH_2O were spread after mixing. These plates were incubated for 1.5-2.0 hours at room temperature. At the next step, 20.0 µl of inoculated cells were spread onto prepared agar plates, and incubated at 37˚C overnight.

Following the overnight incubation, based on the principle of degradation of X-gal by the cells to which the plasmids were not transformed, there on the plates are both blue and white colonies and some of the white colonies were selected and cultured in 10.0 ml of LB-Amp media for plasmid isolation purpose.

2.2.2.3. Plasmid Isolation

Plasmid isolation was carried out the GeneJET Plasmid Miniprep Kit (Fermentas). For this purpose, 5.0 ml of the 10.0 ml inoculated cells were used and following the steps in the kit the plasmids were isolated. The quantities of the isolated plasmids were determined by measuring the absorbance at 260 nm using nanodrop instrument. Next, the sequences of the samples were proved by sequencing.

2.2.2.4. Sequence Analysis and Digestion of α-L-AbFase Gene

All the sequencing experiments were carried out at Biotechnology and Bioengineering Research Laboratory Centre at our institute (16 and 80 capillary, Applied Biosystem, 3130XL). M13 primer was used for the analysis. To get the idea of how the insert is ligated to the vector, the following experiments;

- single digestion using fast-digest *NdeI* endonuclease (Fermentas),
- single digestion using *HindIII* endonuclease (Fermentas) and
- double digestion by using both of these enzymes

were carried out separately and after the samples were run on agarose gel as explained before, visualized under UV light.

2.2.2.5. Phylogenetic Analysis of α-L-AbFase Enzyme

Phylogenetic tree is a way to show the evolutionary relationship of a species or a living organism with the others, between taxonomic groups. In our study, we have collected the data correspond to the other α-L-AbFase enzymes from Protein Data Bank (PDB) and used the ClustalW2 - Multiple Sequence Alignment (Larkin et al., 2007) in order to construct the phylogenetic tree of our protein based on the protein sequences.

2.2.2.6. Expression Vector, pET28a (+)

2.2.2.6.1. Double Digestion of the Insert and Expression Vector

Based on this common property of insert and the pET28a(+) vector, depicted in Figure 2.2 and Figure 2.3, double digestion experiment for both of the vector and the insert was carried out in separate tubes using the followings;

- ***for digestion of insert containing pTZ57R/T***
  - 1.0 µl of fast-digest 10x buffer,
  - 1.0 µl *HindIII* enzyme fast-digest,
  - 1.0 µl *NdeI* enzyme fast-digest,
  - 7.0 µl pTZ57R/T vector,

- ***for digestion of pET28a (+) (without insert)***
  - 1.0 µl of fast-digest 10x buffer,
  - 1.0 µl *HindIII* enzyme fast-digest,
  - 1.0 µl *NdeI* enzyme fast-digest,
  - 7.0 µl pET28a (+) vector.

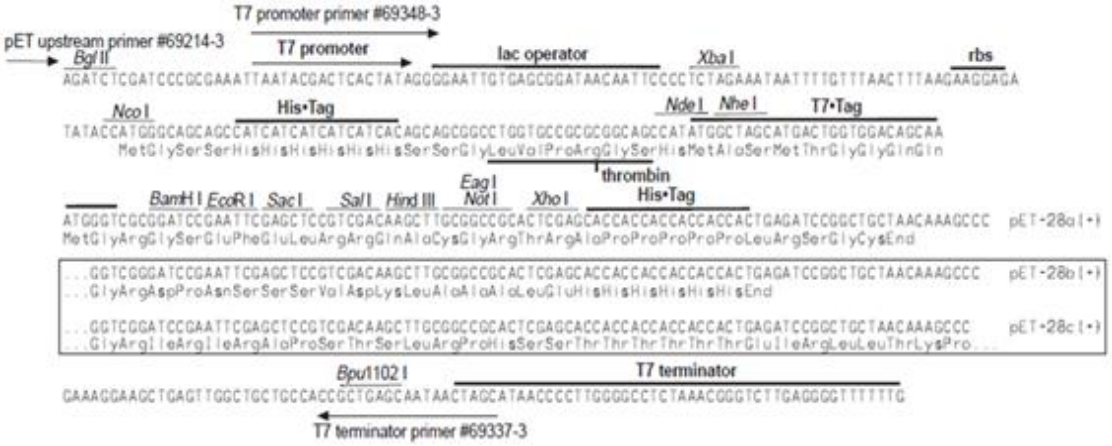

Figure 2.2. pET28a (+) cloning/expression region (Novagen).

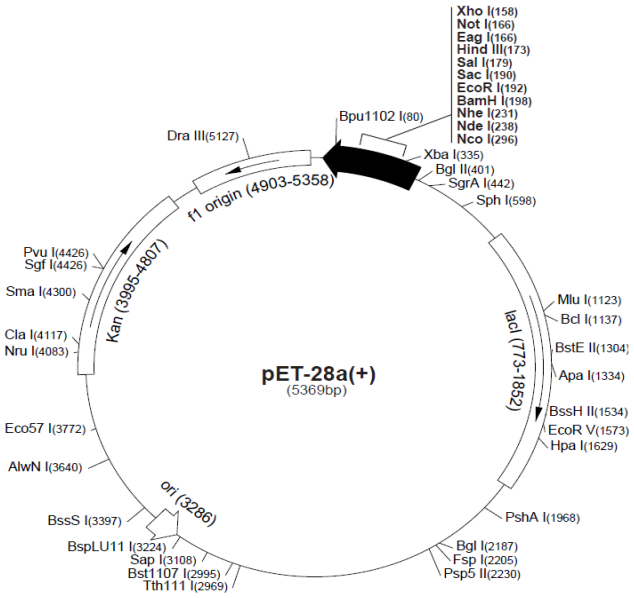

Figure 2.3. pET28a (+) expression vector and multiple cloning sites.

Then, the mixtures were incubated at 37˚C for two hours. The digestion products were run on agarose gel in separate wells. The corresponding bands to insert and vector were cut and extracted from gel using the kit. The quantities of nucleotides were determined by nano-drop instrument, the absorbance values at 260 nm.

2.2.2.6.2. Ligation of the Double Digested Insert and Vector

Double digested insert and double digested pET28a (+) vector were ligated using T4 DNA ligase enzyme which combines the cleaved DNA fragments with either cohesive and blunt ends, using the same insert to vector molar ratio and reaction condition as for ligation of insert and cloning vector.

2.2.2.7. Transformation to BL21 (λDE3) Competent Cells

The transformation of the recombined DNA fragments into the BL21 (λDE3) competent cells, was performed by using;

- 5.0 µl of ligation product and
- 100.0 µl of BL21 (λDE3) competent cells (from -80˚C stock).

They were mixed just after melting the competent cells and incubated;

- on ice for 10 minutes,
- at 42˚C for 45 seconds and
- on ice for 2 minutes.

Then, 400.0 µl of SOC media was added and incubated at 37˚C at 225 rpm for 1 hour. After incubation period, the cells were harvested and the pellet was resuspended in 200.0 µl of the supernatant of which the half amount was spread on LB-Kan agar plate using glass rod. Finally, the plate was incubated at 37˚C in oven for 18 hours.

2.2.3. Protein Expression and Production

2.2.3.1. Effect of Changing Temperature on Induction of α-L-AbFase

Expression of α-L-AbFase gene was carried out after transformation of the pET-28a(+) expression vector into *E. coli* strain BL21(λDE3). The expression vector system works based on bacteriophage T7 RNA polymerase, the BL21(λDE3) host

cells contains a gene encoding the T7 RNA polymerase, under the control of IPTG-inducible promoter and T7 transcriptional promoter. In the pET expression vector, there is a strong phage T7 transcriptional promoter and the α-L-AbFase coding gene was placed downstream from it. The expression of the α-L-AbFase gene is induced after derepression of the lac operator by addition of the lactose analog IPTG, the operon allows the expression of T7 RNA polymerase, which transcribes the α-L-AbFase gene. Because of the fact that the pET expression vector also contains a kanamycin resistant marker, kanamycin (30.0 mg/ml) was added to all media to maintain selection criteria in the following steps for the vector containing cells. First of all, the transformed cells were spread on a LB-Kan agar plate and incubated at 37˚C for 18 hours. Then, from the plate, a single colony was selected and cultured in 30.0 ml of LB-Kan media and incubated at 37˚C and 200 rpm overnight (Sambrook 1989). At that point, in order to investigate the effect of different temperatures on induction/expression level of our protein, the culture was divided into three portions. Each one was diluted to 100.0 ml with fresh LB-Kan media and incubated until the OD (optical density) of the culture is around 0.6 and the cultures were cooled in cold media. To the cultures, IPTG was added to a final concentration of 1.0 mM and incubated overnight. The next day in the morning, the cells were centrifuged at for 15 minutes at 4˚C and 7,000 rpm and resuspended in 50.0 mM Na-P buffer pH 7.0 buffer. Expression level of α-L-AbFase gene was evaluated using SDS-PAGE.

2.2.3.2. Co-expression of α-L-AbFase with Chaperon Proteins

After applying the expression procedure described above, aggregation, Figure 2.4, and inclusion body formation of the target enzyme was observed after checking the pellet for the presence of the enzyme by SDS-PAGE after the cell lysis (sonication).

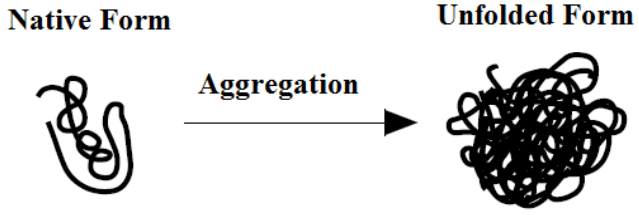

Figure 2.4. Formation of aggregate after protein expression.

In order to co-express the chaperon protein with α-L-AbFase enzyme, first, the BL21(DE3) competent cells were prepared which includes the pET28a(+) plasmids with our gene, then, the chaperon plasmids transformed to these cells using the same procedure as described previously and co-expression was applied based on the procedure explained in the TaKaRa Kit. Briefly, from each plate -additionally, it includes chloramphenicol since the chaperon plasmids are resistant to this antibiotic- prepared after transformation; a single colony was inoculated into LB (Kan + Chloramphenicol) and incubated at 37˚C overnight. The cultures were transferred into fresh LB (Kan + Chloramphenicol), when the OD reached to 0.5, expression of both enzymes is induced by addition of IPTG to a final concentration 1.0 mM at 37˚C and 225 rpm 4 hours. Simply aggregation is depicted in Figure 2.4 and the transformation of the chaperon plasmids into BL21(DE3) competent cells is summarized in Figure 2.5.

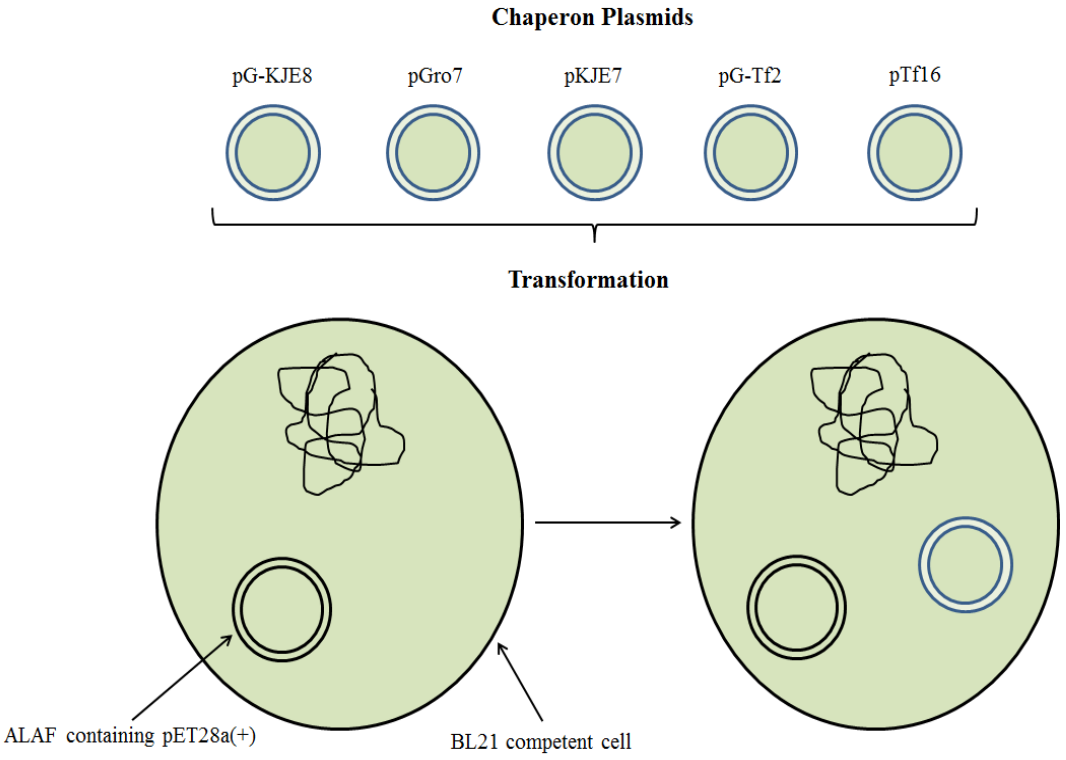

Figure 2.5. Schematic presentation of the transformation of the chaperon plasmids into BL21 competent cells contains ALAF coding gene cloned with pET28a(+) vector.

2.2.4. Total Protein Extraction

After induction of the α-L-arabinofuranosidase enzyme, the cells were harvested by centrifugation at 7,000 rpm at +4˚C for 20 minutes. After resuspension of the cell pellet in 50.0 mM Na-P buffer pH 7.0, cell membranes were disrupted by sonication for 5 minutes on ice (Bandelin, Sonopuls Ultrasonic Homogenizers, HD 2070). Then, the cell debris was discarded by sonication at 13,500 rpm at +4˚C for 15 minutes. For the following analysis, the supernatant was used such as protein purification, SDS-PAGE analysis and etc.

2.2.4.1. Protein Purification

2.2.4.1.1. Affinity Chromatography

A Low Pressure-Liquid Chromatography system (Pro Team LC™ 320, Teledyne Isco.) was used to carry out the purification procedure at room temperature. The supernatant obtained from the sonication step was loaded onto a 2.5 cm x 10 cm His-Taq Nickel Affinity (HIS-Select^{TM} HF Nickel Affinity Gel, Sigma) column which was equilibrated with 20.0 mM Tris-HCl buffer. The column was washed with 100.0 ml of ice cold 20.0 mM Tris-HCl buffer including 0.3 M NaCl and 10.0 mM of imidazole, and the bound proteins eluted with elution buffer including 250 mM imidazole and 0.1 M NaCl in Tris-HCl buffer. The eluted proteins were collected separately as 20 drops in each tube. The fractions were checked for the presence of the target protein by SDS-PAGE analysis. The proper fractions were pooled and concentrated by using a cut-off filter (Amicon® Ultra-4 Centrifugal Filter Unit). The protein was further purified by size-exclusion chromatography using Sepharose G-100 resin or dialysis overnight at +4˚C.

2.2.4.1.2. Size-Exclusion Chromatography

Size-exclusion chromatography was carried out in order to get rid of the imidazole content of the protein containing buffer. For this purpose, briefly, the

column was equilibrated with 50.0 mM Na-P buffer. Then, the concentrated protein solution was loaded onto the column and the sample was eluted through the column with the same buffer with a flow rate as 1.0 ml/min. The presence of the protein was controlled with a detector and the samples were fractioned as 60 drops to each tube. After of all, SDS-PAGE analysis was carried out to check the fractions for the presence and the purity of the protein. The protein containing fractions were pooled and again were concentrated using the same type of filter as defined previously.

2.2.4.1.3. Dialysis

Instead of applying size-exclusion chromatography, in some cases, the sample, in a dialysis tubing (12,000 MW), was dialyzed against 50.0mM sodium phosphate buffer at pH 7.0 in order to get rid of the imidazole that comes from elution buffer used to elute the protein from the column through the affinity chromatography.

2.2.5. SDS-PAGE Analysis

From the expression to the end of purification of the protein at each step, SDS-PAGE analysis was carried out, as the previously defined method by Laemmli (Laemmli 1970), both for checking the presence and the purity of the target protein. In the procedure of the SDS-PAGE analysis, 20.0µl from each samples separately were mixed with 5.0µl of the 5x loading dye and were boiled at 95˚C for 5 minutes. After that the samples were loaded into the wells of the SDS-PAGE gel (10.0%), electric field was applied as 65 V for 30 minutes and 100 V for 2 hours. The gel was placed into coomassie blue dye and heated in microwave oven for 1 minute and cooled to the room temperature on a shaker with 20 rpm. Then, the dye was decanted and the gel was first washed with dH_2O and placed into destaining solution until the gel became transparent.

2.2.6. Activity Determination of the Enzyme

Activity of the enzyme was determined by the method previously described by Canakci et al. (2007) with some modifications. Briefly, the enzyme was assayed using the mixture contained 25.0µl 2.0mM *p*NP-α-L-arabinofuranoside (dissolved in DMSO), 425.0µl of McIlvaine buffer; pH 6.0 and 50.0µl of the diluted enzyme solutions. The hydrolysis reactions were carried out at 50˚C for 10 minutes which were terminated by the addition of 500.0µl of 1.0 M Na_2CO_3 solution. The released *p*NP amount was determined at 420 nm with an extinction coefficient of $1.78x10^4$ $M^{-1}cm^{-1}$. One unit of enzyme activity is defined as the amount of enzyme that releases 1.0µmol of *p*NP per minute.

Sugar beet arabinan (2.0%, w/v) was used as substrate to investigate the ability of the α-L-arabinofuranosidase to release arabinose. For this purpose, the same assay conditions were used as previously described by Canakci et al. (2007), diluted enzyme solution at pH 5.5 and 65˚C. The DNS method (Miller 1959) was performed to investigate the reducing sugar content at the end of the reaction and L-arabinose was used as the standard. One unit of enzyme activity is defined as the amount of 1.0µmol of arabinose that produced by the enzyme activity per minute.

2.2.6.1. Optimization of the Conditions for Enzyme Activity

The activity of the α-L-arabinofuranosidase enzyme was optimized by means of pH and temperature. For this purpose, the activity assay was performed at different pH values using different buffer systems; pH 3.0-6.0 (citrate buffer), pH 7.0 (sodium phosphate buffer), pH 8.0 (Tris-HCl buffer), pH 9.0, 10.0 (Glycine-NaOH buffer) (Appendix C). In order to determine the optimum temperature value, the activity assay was performed at different temperature values between 35-90˚C using the optimum buffer system.

2.2.6.2. Effects of Metal Ions and Chemicals on Enzyme Activity

Effects of different metal ions were determined by applying standard activity assay. In the procedure, the enzyme activity was assayed by the addition of metal ions and chemicals with different final concentrations, for this purpose, $MgSO_4$, $ZnCl_2$, KCl, $CaCl_2$, $MgCl_2$, $CuSO_4$, NaF, AgCl, $CdCl_2$, NaCl and $NiCl_2$ were added to the reaction medium.

To investigate the effects of some detergents, chemical agents and some common organic solvents on enzyme activity again standard assay was carried out as applied for metal ions. The chemicals and solvents were added as the final concentrations 10% by volume in 500.0µl assay mixture. In these experiments, DTT (dithiothreitol), SDS (sodiumdodecylsulfate), methanol, ethanol, ethyl acetate, acetonitrile, acetone, β-ME (β-mercaptoethanol) and EDTA (ethylenediaminetetraacetic acid) were used.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. DNA Isolation and Gene Expression

3.1.1. Genomic DNA Isolation

The genomic DNA of the strain 90 was isolated and served as template in the PCR. The strains were cultivated in LB media without anti-biotic at 55˚C overnight 180 rpm.

3.1.2. Amplification of α-L-AbFase Coding Gene

After that PCR primers were designed according to the sequence of α-L-AbFase enzyme-coding gene, of which the length is 1509 bp. The purpose of choosing this gene is primarily that the source of this gene is also another thermophilic organism, *Geobacillus Thermoleovorans*. Then, PCR was carried out using these primers. Fig 3.1 depicts the image of PCR product on agarose gel after applying electrophoresis.

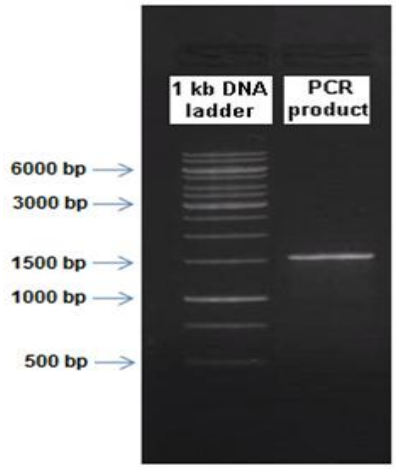

Figure 3.1. Agarose gel image of PCR product.

3.1.3. Cloning the α-L-AbFase Coding Gene in Cloning Vector; pTZ57R/T

α-L-AbFase coding gene was ligated with the pTZ57R/T cloning vector. Then, the ligation products were transformed into DH5α competent cells and cloned in here. Blue/white screening technique was applied to select the right colonies includes our ligation products. It is known that the pTZ57R/T vector has the ampicillin resistant gene, so at this step, the both blue and white colonies include the vector but only the white ones include the proper vectors, properly ligated with insert. Five of the white colonies were selected and after inoculation of these in LB-Amp liquid media, the plasmids were isolated and sequenced. Alignment of the sequencing results showed that the nucleotide sequence overlaps by 91% score with the source gene Q9XBQ3; the gene sequence used to design primers, in Figure 3.2.

```
Source      ATGGCTACAAAAAAAGCAACCATGATCATCGAAAAAGACTTCAAAATTGCTGAAATCGAC 60
α-L-AbFase ATGGCTACAAAAAAAGCACCAATGATCATCGAAAAAGACTTCAAAATCGCTGACATCGAC 60
           ****************** * ************************** ***** ******

source      AAACGCATCTATGGCTCGTTTATTGAACACCTCGGCCGCGCGGTATACGGGGGGATTTAT 120
α-L-AbFase AAACGCATTTATGGTTCTTTTATCGAGCACCTCGGCCGCGCTGTATATGGAGGGATTTAT 120
           ******** ***** ** ***** ** ************** ***** ** *********

source      GAGCCGAGCCATCCGCAGGCCGATGAAAACGGCTTCCGGCAGGATGTCATTGAAATGGTG 180
α-L-AbFase GAGCCGGGCCATCCGCAAGCTGATGAAAACGGGTTCCGGCAAGATGTCATCGAGCTGGTC 180
           ****** ********** ** *********** ******** ******** **  ****

source      AAAGAGTTACAAGTGCCCATTATCCGCTATCCGGGCGGGAATTTTGTGTCCGGTTACAAC 240
α-L-AbFase AAAGAGCTGCAAGTGCCGATCATCCGCTATCCGGGCGGGAATTTTGTGTCCGGTTACAAC 240
           ****** * ******** ** ************************************

source      TGGGAGGACGGAGTCGGGCCAAAAGAAAAGCGGCCGCGGCGGCTTGATTTGGCATGGAAG 300
α-L-AbFase TGGGAAGATGGAGTGGGGCCAAAAGAGCAGCGTCCGCGTCGGCTTGATTTGGCGTGGAAG 300
           ***** ** ***** ***********  **** ***** ************** ******

source      TCAGTGGAAACGAATGAAATTGGCTTGAATGAATTTGTCGATTGGGCCAAGATGGTCGGA 360
α-L-AbFase TCGGTGGAAACGAATGAAATTGGCTTGAATGAATTTATGGATTGGGCCAAGATGGTCGGA 360
           ** ********************************* * ********************

source      GCCGAAGTGAATATGGCCGTCAACTTAGGGACGCGCGGCATTGATGCGGCACGCAACTTG 420
α-L-AbFase GCTGAAGTGAATATGGCCGTCAACTTAGGGACGCGTGGCATTGATGCGGCGCGCAACTTA 420
           ** ********************************* ************** ********

source      GTTGAATATTGCAACCACCCGTCGGGCTCGTATTACAGCGATTTGCGCATTTCCCACGGC 480
α-L-AbFase GTCGAATACTGCAACCACCCGTCGGGCTCCTACTACAGCGATTTGCGCATTGCCCACGGC 480
           ** ***** ******************* ** ****************** ********
```

Figure 3.2. Nucleotide sequence comparison between our protein sequence and the source gene.

(cont. on next page)

```
source      CAAATTGGCCACAAGACAGCCGTTGAGTACGGACGAATCGCTTGTGAAGCGGCCAAAGTG 600
α-L-AbFase CAAATCGGCCATAAGACGGCGGTTGAGTACGGACGAATCGCTTGTGAAGCGGCCAAAGTG 600
           ***** ***** ***** ** ************************************

source      ATGAAATGGGTAGATCCGACCATTGAACTTGTTGCGTGCGGAAGTTCAGGCAGAAATATG 660
α-L-AbFase ATGAAGTGGGTCGACCCGACGATTGAACTTGTTGTGTGTGGAAGCTCAAACCGAAATATG 660
           ***** ***** ** ***** ************* *** ***** ***  * ********

source      CCGACGTTTGCGGAATGGGAAGCGACGGTTCTTGATCACACGTATGAGCATGTCGATTAT 720
α-L-AbFase CCAACGTTTGCGGAATGGGAAGCGACGGTTCTTGACCATACGTATGACCATGTCGACTAT 720
           ** ******************************** ** ******** ******** ***

source      ATTTCCCTCCATCAATACTTTGGAAATCGAGATAATGACACGGCGAATTATTTGGCGCTG 780
α-L-AbFase ATTTCTCTCCATCAATACTATGGAAACCGGGACAATGATACGGCCAATTATTTGGCGTTG 780
           ***** ************* ****** ** ** ***** ***** ************ **

source      TCGCTGGAAATGGATGATTTTATCCGTTCGGTTGTGGCCATTGCCGATTACGTGAAGGCG 840
α-L-AbFase TCGCTGGAAATGGATGATTTTATCCGTTCGGTTGTCGCCATCGCCGATTATGTGAAGGCG 840
           ********************************** ***** ******** *********

source      AAAAAACGAAGCAAGAAGACGATTCATCTGTCGTTTGACGAATGGAACGTATGGTACCAC 900
α-L-AbFase AAAAAACGAAGCAAAAAAACGATCCACCTTTCGTTTGACGAATGGAATGTATGGTACCAC 900
           ************** ** ***** ** ** ***************** ************

source      TCGAATGAGGCGGATAAGCAAATTGAACCGTGGACCGTCGCGCCGCCTTTGTTGGAGGAT 960
α-L-AbFase TCGAATGAGGCAGATAAGTTAATTGAACCATGGACCGTCGCGCCGCCTCTGTTGGAGGAT 960
           *********** ******  ********* ****************** ***********

source      ATTTATAACTTTGAAGATGCGCTACTTGTCGGCTGCATGCTCATTACGCTCATGAAACAT 1020
α-L-AbFase ATTTATAACTTCGAAGATGCGCTTCTTGTCGGCTGCATGCTTATTACGCTCATGAAACAT 1020
           *********** *********** ***************** ******************

source      GCCGATCGGGTGAAAATTGCCTGCTTGGCTCAGTTAGTGAATGTCATTGCACCGATCATG 1080
α-L-AbFase GCCGATCGGGTGAAAATTGCCTGCTTGGCTCAATTAGTGAATGTCATTGCACCGATCATG 1080
           ******************************** ***************************

source      ACGGAACCGAACGGGCCGGCATGGAAGCAAACCATTTACTATCCGTTTATGCATGCCTCG 1140
α-L-AbFase ACGGAAAAGAACGGTCCGGCATGGAAGCAAACGATTTACTATCCGTTTATGCATGCCTCG 1140
           ******  ****** ***************** ***************************

source      GTTTACGGCAGAGGGGTGGCGTTGCACCCAGTTATTTCAAGCCCGAAATACGACAGCAAA 1200
α-L-AbFase GTTTACGGCAGAGGAGTGGCGTTGCACCCAGTCATTTCAAGTCCGAAATACGACAGCAAA 1200
           ************** ***************** ******** ******************

source      GACTTCACAGATGTTCCGTATTTAGAGTCGATCGCTGTTTACAATGAAGAAAAAGAAGAA 1260
α-L-AbFase GACTTCACAGATGTTCCGTATTTAGAGTCGATCGCTGTTTACAATGAAGAAAAAGAAGAA 1260
           ************************************************************

source      GTGACGATTTTTGCGGTCAACCGTGATATGGACGATTCGTTATTGCTTGAATGCGATGTC 1320
α-L-AbFase GTGACGATTTTTGCGGTCAACCGTGATATGGAAGACGCGTTGTTGCTGGAATGCGATGTT 1320
           ******************************** **  **** ***** ***********

source      CGCCATTTTGACGATTATCGCGTTATTGAACATATCGTATTGGAACATGAAAACGTGAAA 1380
α-L-AbFase CGCAGCTTTGAAGACTACCGTGTCATTGAACATATCGTTTTGGAACATGACAACGTGAAA 1380
           ***   ***** ** ** ** ** ************** *********** *********
source      CAAACGAATTCCGCGCAATCTTCCCCGGTCGTTCCGCACCGCAACGGCGATGCTCAACTA 1440
α-L-AbFase CAAACGAATTCGGCCCAATCTTCCCCGGTTGTCCCACATCATAACGGCGATGCCCAACTG 1440
           *********** ** ************** ** ** ** *  *********** *****

source      TCCGGCGGGAAAGTGTCGGCGACGTTGTCGAAGTTATCGTGGAATGTGATTCGTTTAGGA 1500
α-L-AbFase TCCGGTGGGAAAATGTCGGCCATGTTGCCGAAGTTATCGTGGAATGTTATTCGTTTAGGA 1500
           ***** ****** ******* * **** ****************** ************
```

Figure 3.2. (Cont.)

(cont. on next page)

```
source     AAACGATAA 1509
α-L-AbFase AAACGATAA 1509
           *********
```

Figure 3.2. (Cont.)

Based on the nucleotide sequencing result, the protein sequence of our enzyme matches up by 95% with the source gene and 98% score, in Figure 3.3, with another α-L-AbFase gene, DQ387046, identified by Hövel et al. (2003).

```
α-L-AbFase MATKKAPMIIEKDFKIADIDKRIYGSFIEHLGRAVYGGIYEPGHPQADENGFRQDVIELV 60
DQ387046   MATKKATMIIEKDFKIAEIDKRIYGSFIEHLGRAVYGGIYEPGHPQADENGFRQDVIELV 60
source     MATKKATMIIEKDFKIAEIDKRIYGSFIEHLGRAVYGGIYEPSHPQADENGFRQDVIEMV 60
           ******.**********:**********************.**************:*

α-L-AbFase KELQVPIIRYPGGNFVSGYNWEDGVGPKEQRPRRLDLAWKSVETNEIGLNEFMDWAKMVG 120
DQ387046   KELQVPIIRYPGGNFVSGYNWEDGVGPKEQRPRRLDLAWKSVETNEIGLNEFMDWAKMVG 120
source     KELQVPIIRYPGGNFVSGYNWEDGVGPKEKRPRRLDLAWKSVETNEIGLNEFVDWAKMVG 120
           ****************************:**********************:*******

α-L-AbFase AEVNMAVNLGTRGIDAARNLVEYCNHPSGSYYSDLRIAHGYKEPHKIKTWCLGNEMDGPW 180
DQ387046   AEVNMAVNLGTRGIDAARNLVEYCNHPSGSYYSDLRIAHGYKEPHKIKTWCLGNEMDGPW 180
source     AEVNMAVNLGTRGIDAARNLVEYCNHPSGSYYSDLRISHGYKEPHKIKTWCLGNEMDGPW 180
           ************************************:**********************

α-L-AbFase QIGHKTAVEYGRIACEAAKVMKWVDPTIELVVCGSSNRNMPTFAEWEATVLDHTYDHVDY 240
DQ387046   QIGHKTAVEYGRIACEAAKVMKWVDPTIELVVCGSSNRNMPTFAEWEATVLDHTYDHVDY 240
source     QIGHKTAVEYGRIACEAAKVMKWVDPTIELVACGSSGRNMPTFAEWEATVLDHTYEHVDY 240
           ******************************.****.******************:****

α-L-AbFase ISLHQYYGNRDNDTANYLALSLEMDDFIRSVVAIADYVKAKKRSKKTIHLSFDEWNVWYH 300
DQ387046   ISLHQYYGNRDNDTANYLALSLEMDDFIRSVVAIADYVKAKKRSKKTIHLSFDEWNVWYH 300
source     ISLHQYFGNRDNDTANYLALSLEMDDFIRSVVAIADYVKAKKRSKKTIHLSFDEWNVWYH 300
           ******:*****************************************************

α-L-AbFase SNEADKLIEPWTVAPPLLEDIYNFEDALLVGCMLITLMKHADRVKIACLAQLVNVIAPIM 360
DQ387046   SNEADKLIEPWTVAPPLLEDIYNFEDALLVGCMLITLMKHADRVKIACLAQLVNVIAPIM 360
source     SNEADKQIEPWTVAPPLLEDIYNFEDALLVGCMLITLMKHADRVKIACLAQLVNVIAPIM 360
           ****** ***************************************************

α-L-AbFase TEKNGPAWKQTIYYPFMHASVYGRGVALHPVISSPKYDSKDFTDVPYLESIAVYNEEKEE 420
DQ387046   TEKNGPAWKQTIYYPFMHASVYGRGVALHPVISSPKYDSKDFTDVPYLESIAVYNEEKEE 420
source     TEPNGPAWKQTIYYPFMHASVYGRGVALHPVISSPKYDSKDFTDVPYLESIAVYNEEKEE 420
           ** ***************************************************

α-L-AbFase VTIFAVNRDMEDALLLECDVRSFEDYRVIEHIVLEHDNVKQTNSAQSSPVVPHHNGDAQL 480
DQ387046   VTIFAVNRDMEDALLLECDVRSFEDYRVIEHIVLEHDNVKQTNSAQSSPVVPHRNGDAQL 480
source     VTIFAVNRDMDDSLLLECDVRHFDDYRVIEHIVLEHENVKQTNSAQSSPVVPHRNGDAQL 480
           **********:*:******** *:************:****************:******

α-L-AbFase SGGKMSAMLPKLSWNVIRLGKR 502
DQ387046   SDRKVSATLPKLSWNVIRLGKR 502
source     SGGKVSATLSKLSWNVIRLGKR 502
           *. *:** *.************
```

Figure 3.3. Protein sequence comparison between our protein, DQ387046 and the source gene.

3.1.4. Phylogenetic Analysis of α-L-AbFase Enzyme

Phylogenetic tree of α-L-AbFase enzyme was constructed using ClustalW2 program (http://www.genome.jp/tools-bin/clustalw), based on the protein sequences of the α-L-arabinofuranosidases recorded in the uniprot, protein data bank (http://www.uniprot.org/) in Figure 3.4.

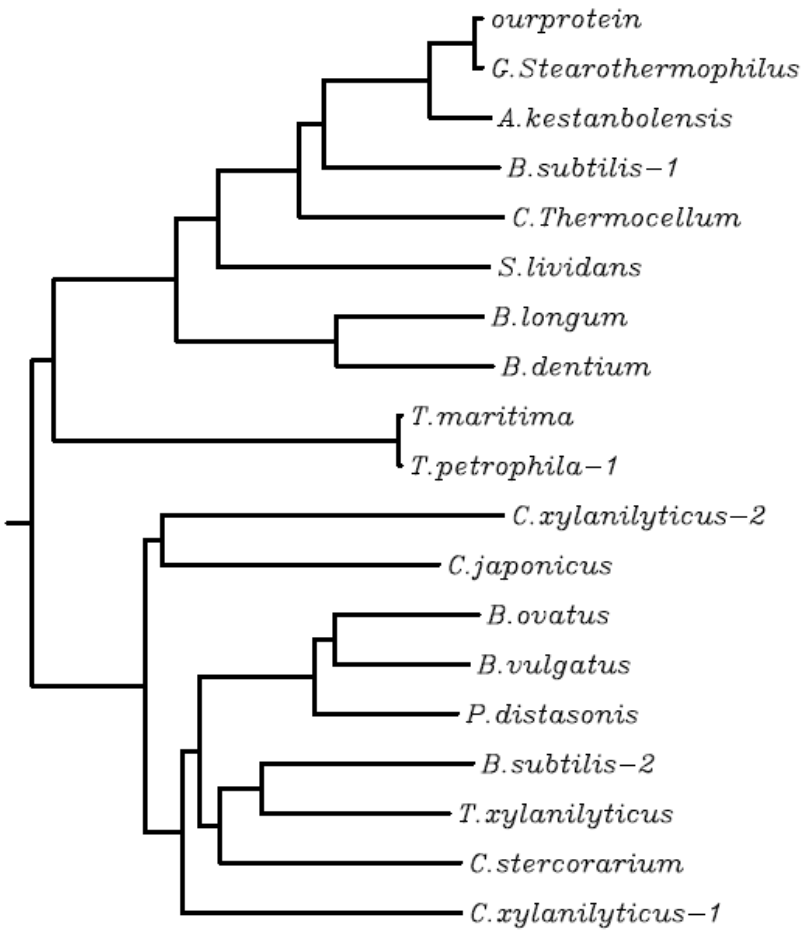

Figure 3.4. Phylogenetic tree of α-L-Abfase.

3.1.5. Protein Expression, Production and Purification

α-L-AbFase coding-gene was ligated with expression vector; pET28a(+), following the double-digestion with the endonucleases; *NdeI* and *HindIII*. Then, the ligation products were transformed into protein expression strain, BL21(DE3). At this step, the presence of vectors was tested via colony PCR and to the positive colonies

also, sequence analysis was carried out. Based on the sequencing results, proper colonies were used for the protein production. The molecular weight of the protein was calculated not only theoretically but also by SDS-PAGE analysis as to be 58.0 kDa.

After applying the SDS-PAGE analysis to the fractions collected at each step such as; before sonication, after sonication, after Ni-affinity chromatography and size-exclusion/dialysis, we observed that there was much of our protein in the pellet obtained after sonication. We assumed that the reason for this may have caused by the improper folding of our protein.

Although it is not so common observation in related experiments in the literature, improper folding may be resulted by expression under stress conditions and / or being not in its original host strain. So, we decided to use another method; chaperon plasmids to recover the protein and to decrease the amount of protein loss. For this purpose, as explained above in the Materials and Methods section, firstly, competent cells were prepared including the expression vector ligated with α-L-AbFase coding gene. Next, co-expression of the chaperon proteins with our protein was done separately and after sonication the protein amounts in the soluble fractions were checked via SDS-PAGE analysis. The 10% SDS-PAGE gel image is shown in Figure 3.5. Based on the SDS-PAGE analysis results, the amount in the CE (co-expressed protein cell lysate) line is much more than the amount in SE (Cell lysate of single expressed protein) line. At the end, we have obtained nearly 100.0 mg of protein in 500.0 ml culture media from CE system whereas from SE system, we have only obtained not more than 5.0 mg of protein.

The protein samples were purified by applying two different techniques which are Ni-affinity chromatography and dialysis or size-exclusion chromatography and then, concentrated using 30,000MW cutoff centrifugal filters.

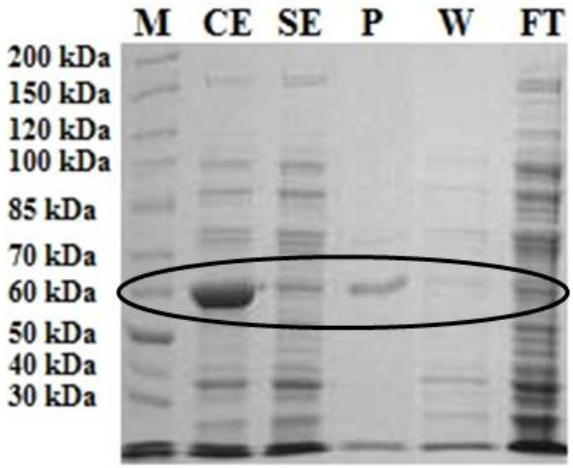

Figure 3.5. M: Protein marker, CE: Co-expressed protein cell lysate, SE: Cell lysate of single expressed protein, P: Purified protein using Ni-affinity chromatography, W: Fraction pooled with washing buffer, FT: Flow through of Ni-affinity chromatography.

3.2. Protein Characterization

3.2.1. Optimum pH and Temperature

Activity measurements of the enzyme; optimum pH and temperature values, pH and temperature tolerance, kinetic parameters were determined by applying the activity assay explained by Canakci et al. (2007). Based on these results, we can say that our enzyme is active in a broad pH and temperature interval; showed activity from pH 3.0 to pH 10.0 and active between 30 to 90˚C. The optimum pH and temperature values were obtained as pH 5.0 (Na-citrate buffer) and 70˚C. Relative enzyme activities at different pH values are depicted in Figure 3.7 and enzyme activities at different temperature values are in Figure 3.8. Most of the α-L-AbFases have optimum temperatures between 50-60˚C and also, there are some exceptions from thermophilic organisms such as *Thermotoga maritime* 90˚C, *Geobacillus caldoxylolyticus* TK4 75-80˚C in pH 6.0 (Canakci et al. 2007), G. *stearothermophilus* T6 (Gilead and Shoham 1995) and G. *stearothermophilus* L1 (Bezalel et al., 1993) both have optimal temperature as 70˚C, *Thermobacillus xylanilyticus* 75˚C (Debeche et al., 2000). Similar things can be said about the optimum pH values. Since the most of α-L-

AbFases have optimum pH values between pH 3.0 to 6.0 but there are also some exceptions which are again thermophiles having optimum pH values above 6.0.

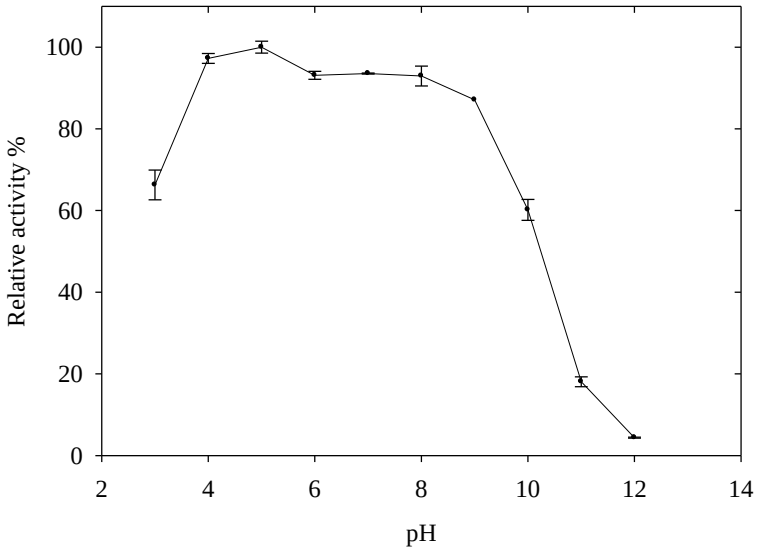

Figure 3.6. Relative activity of enzyme at different pH values.

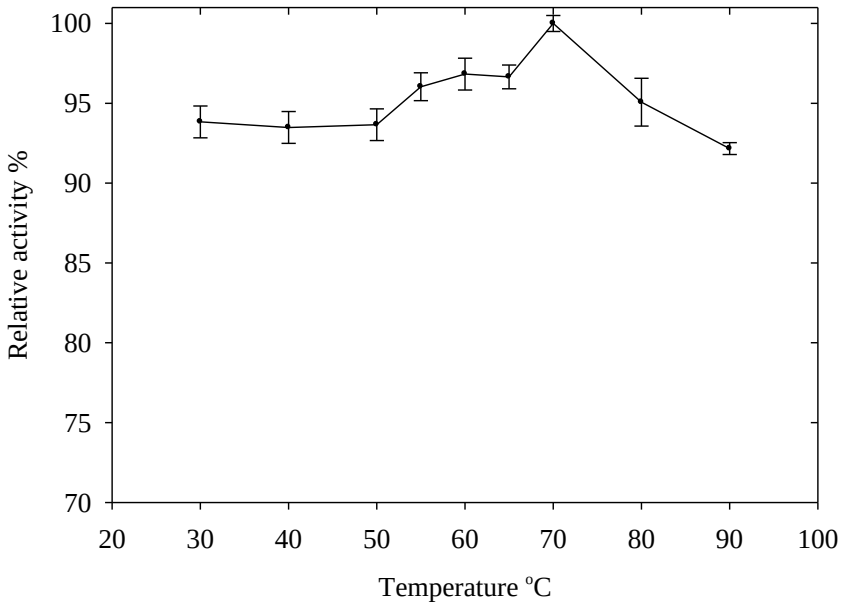

Figure 3.7. Relative activity of enzyme at different temperature values.

3.2.2. pH and Temperature Stabilities

We also have investigated the stability of the enzyme at different pH and temperature values for different time intervals. Temperature stability of the enzyme

was studied for 15 minutes, 0.5 hour, 1 hour and 2 hours and for the pH stability tests were carried out after incubation of enzyme with the buffer at 55˚C for 0.5 hour, 1 hour and 2 hours. The results for temperature and pH stabilities are shown in Figure 3.9 to Figure 3.12 and Figure 3.13 to Figure 3.15 separately.

The response of the α-L-AbFases enzymes to the changing pH and temperature strongly depends on the source from which the enzyme is isolated (Numan and Bhosle 2006). α-L-AbFase from *Thermotoga maritima* MSB8 showed the highest temperature stability, this enzyme has the optimum temperature value of 90˚C at pH 7.0. Furthermore, under these conditions, the enzyme stayed stable for 24 hours and also at 100˚C for 20 minutes it retained 50% of its activity (Miyazaki 2005). Another α-L-AbFase isolated from *Rhodothermus marinus* showed stability at 85˚C for 8.3 hours in a pH range of 5.0–9.0 (Gomes et al. 2000). Canakci et al. (2007) worked on another α-L-AbFase and in this case, the enzyme exhibited full activity at 60–65°C for 96 hours, at 70°C for 48 hours, and 75°C for 12 hours. There is no activity loss after 10 minutes at 80°C, but the residual activity was 90% after 30 minutes at 80°C.

When comparing with the literature works, our enzyme is fairly good since it retained at least 97% of its activity after 2 hour incubation in Na-acetate buffer pH 5.0 at 70˚C, 94% of its activity after 1 hour incubation at 65˚C, full activity after 1 hour and 97% after 2 hours at 55˚C. Also, our enzyme conserved at least 70% of its activity after incubation at pH 4, 5, 6, 8 and 9 at 55˚C.

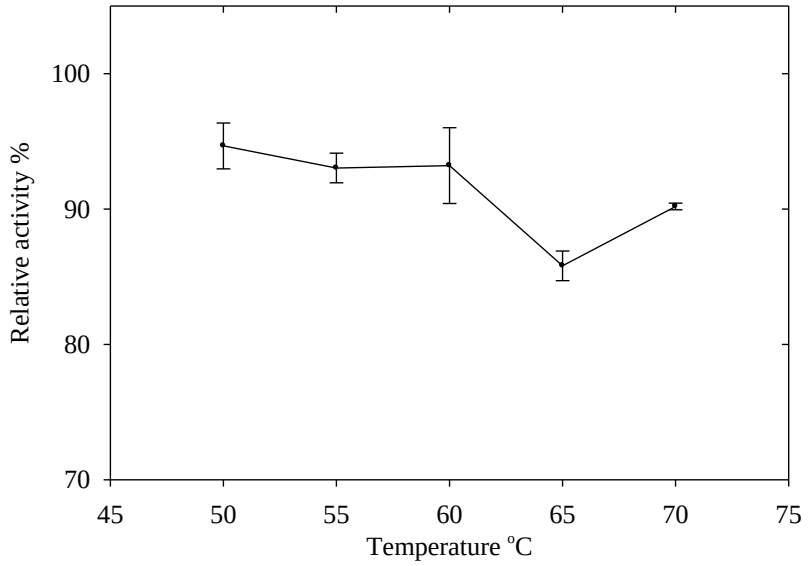

Figure 3.8. Stability profile of the enzyme at different temperature values after 15 minutes.

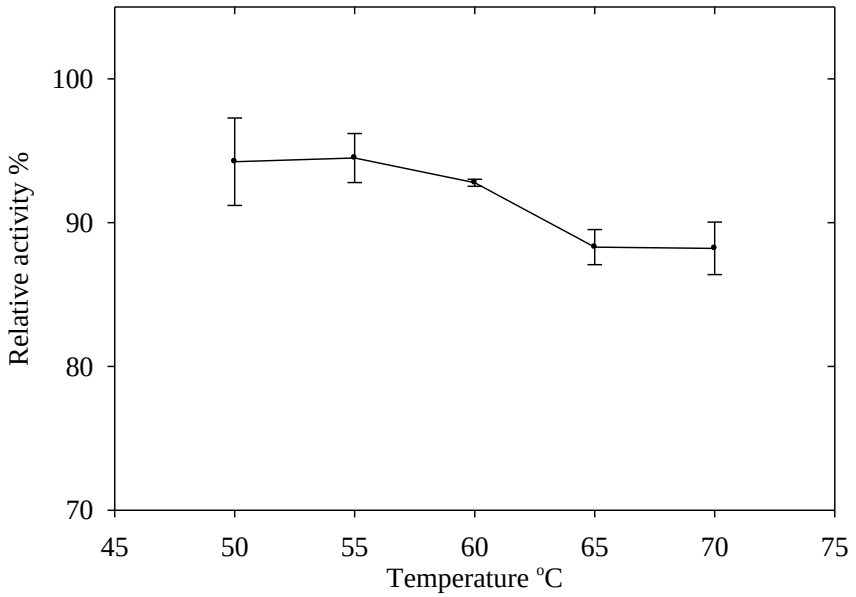

Figure 3.9. Stability profile of the enzyme at different temperature values after 30 minutes.

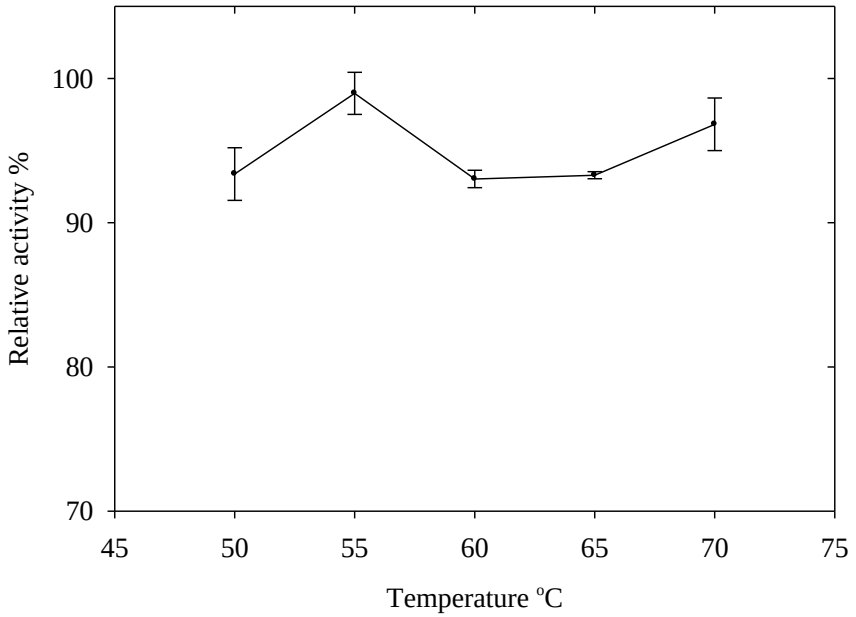

Figure 3.10 Stability profile of the enzyme at different temperature values after 60 minutes.

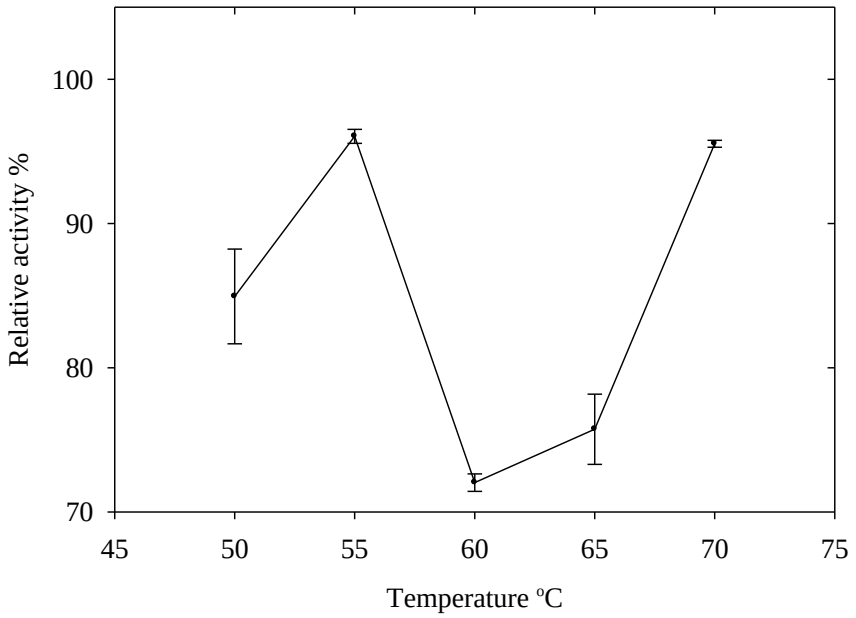

Figure 3.11 Stability profile of the enzyme at different temperature values after 120 minutes.

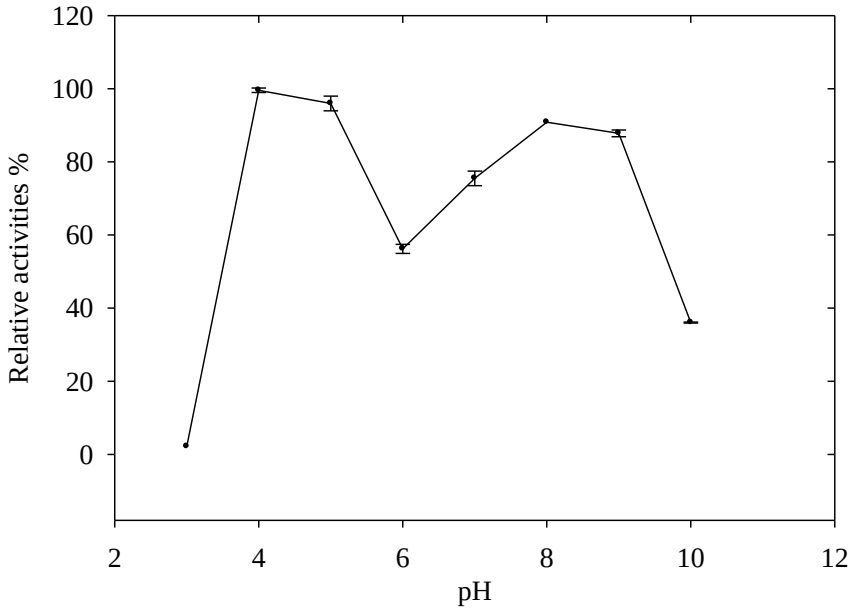

Figure 3.12. Stability profile of the enzyme at different pH values after 30 minutes.

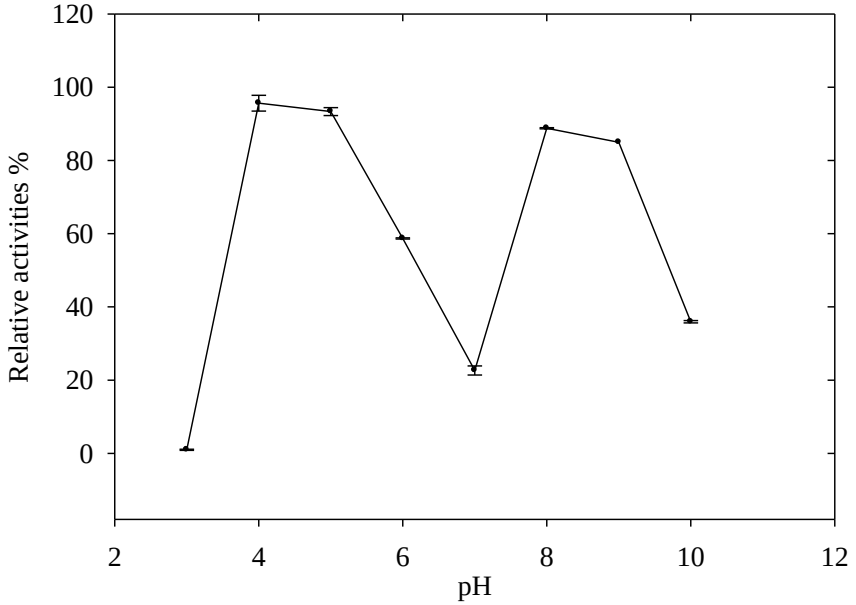

Figure 3.13. Stability profile of the enzyme at different pH values after 60 minutes.

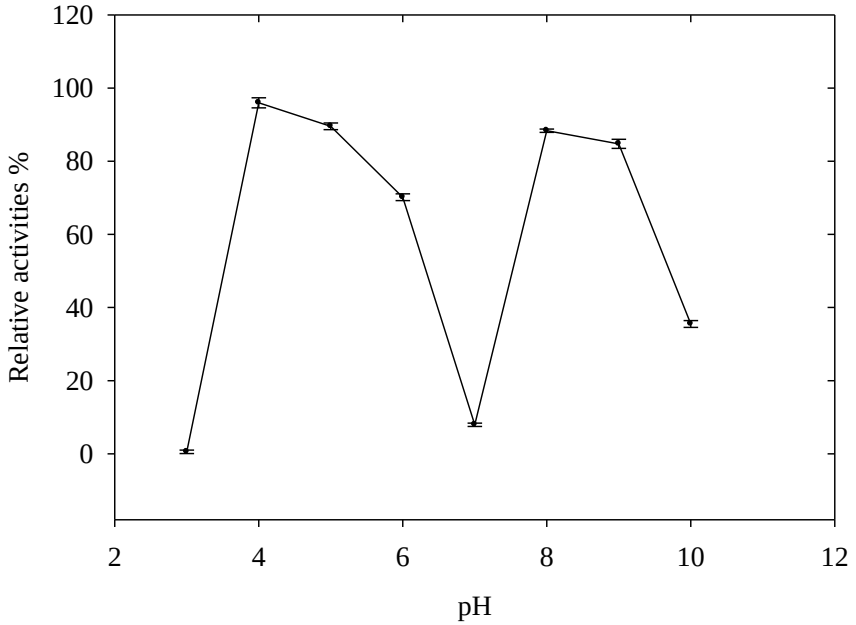

Figure 3.14. Stability profile of the enzyme at different pH values after 120 minutes.

3.2.3. Effect of Metal Ions and Chemicals on Enzyme Activity

It is known that additional metal ions and/or chemicals can improve the enzyme activity or substrate binding capacity etc. For this purpose, we have screened

the effects of some metal ions and some common chemicals via standard enzyme activity assay. In Table 3.1, the results of these experiments are shown. From the Table 3.1, Ag^{+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, Cd^{2+} and SDS have negative effects whereas NaF, KCl and EDTA have positive effects on enzyme activity. Also, effect of 2.0 mM Ni^{2+} and 3.0 mM DTT (data not shown) both increased the enzyme activity by 15%. It is hard to say the same thing for NaCl and Ca^{2+} since at some certain level, they increase the enzyme activity; Table 3.1 and Table 3.2.

In some cases, the activities of α-L-AbFase enzymes are affected by metal ions, ionic and nonionic detergents, and chelating and reducing agents depending on the enzyme and concentration of the agent used (Hespell and O'Bryan 1992; Margolles and de los Reyes-Gavilán 2003). For instance, unlike the cases of α-L-AbFase enzymes from *Bifidobacterium longum* B667, α-L-AbFase from *Thermobacillus xylanilyticus* D3 and *Thermotoga maritime* (Miyazaki 2005) where the enzyme activity was not affected by the addition of EDTA and DTT, in our case both of them increased the enzyme activity more than 10%, but similarly were affected by Cu^{2+} ions (Debeche et al., 2000; Margolles and de los Reyes-Gavilán 2003). Like the previous studies, metal ions such as Ag^{+}, Zn^{2+} (5.0 µM, data not shown) and Cd^{+2} had an inhibitory effect on our enzyme (Margolles and de los Reyes-Gavilán 2003, Sakamoto and Kawasaki 2003, Tsujibo et al., 2002).

Table 3.1. Effects of metal ions at low concentrations on enzyme activity.

	1.0mM	2.5mM	5.0mM
None	100.0	100.0	100.0
NaCl	101.3 ± 0.0	100.4 ± 2.5	97.1 ± 0.3
$CdSO_4$	106.1 ± 0.3	126.7 ± 0.9	60.5 ± 3.1
NaF	105.8 ± 0.0	108.5 ± 1.3	103.4 ± 1.6
$CoCl_2$	119.7 ± 1.9	117.3 ± 0.3	117.3 ± 0.3
$CaCl_2$	109.9 ± 0.0	119.3 ± 2.5	120.2 ± 1.9
KCl	115.9 ± 0.9	115.2 ± 0.6	115.7 ± 1.3
$CuSO_4$	117.5 ± 1.3	115.7 ± 1.9	115.9 ± 2.2
$MnCl_2$	188.6 ± 2.2	140.4 ± 1.9	164.1 ± 1.2
$MgCl_2$	104.3 ± 1.6	106.1 ± 0.3	105.8 ± 0.0
$NiCl_2$	113.9 ± 1.5	114.1 ± 0.0	97.6 ± 0.4
DTT	112.8 ± 0.0	112.5 ± 0.4	112.7 ± 0.2
EDTA	118.0 ± 1.3	114.8 ± 0.3	114.7 ± 0.0

Table 3.2. Effects of metal ions at high concentrations on enzyme activity.

	25.0 mM	50.0 mM	100.0 mM
None	100.0	100.0	100.0
AgCl	6.5 ± 0.6	7.3 ± 0.9	6.5 ± 1.2
NaCl	98.9 ± 1.2	101.9 ± 1.5	98.5 ± 0.0
$CdCl_2$	78.7 ± 0.6	43.4 ± 0.9	36.1 ± 0.4
NaF	111.0 ± 1.3	111.4 ± 0.3	112.7 ± 0.7
$CaCl_2$	75.3 ± 0.3	122.2 ± 1.5	49.5 ± 0.8
KCl	108.4 ± 0.0	109.7 ± 1.3	112.3 ± 1.8

3.2.4. Effect of Organic Solvents on Enzyme Activity

We have screened the activity profile of the enzyme after treatment with various common organic solvents by using again the same activity assay. Additionally, here we have added organic solvents depending on the final concentration (volume/volume) by decreasing the amount of buffer solution. As can be seen in the Table 3.3, in most cases, enzyme retained its 85% activity even the amount of solvents

is 5% by volume. Up to a certain concentration level, ethyl acetate addition enhanced the activity, whereas hexane, ethanol, methanol and acetonitrile, even not too much, decreased the activity. Moreover, interestingly, acetone addition increased slightly and β-mercaptoethanol has no effect on enzyme activity. All in all, we know that it is significant to obtain enzymatic activity in organic solvents for some industrial applications (Aehle 2007), it makes possible for us to introduce our enzyme to the industrial applications.

Table 3.3. Relative enzyme activity after treatment with organic solvents

	1.0 % (v/v)	2.5 % (v/v)	5.0 % (v/v)
None	100.0	100.0	100.0
Hexane	86.9 ± 0.3	90.8 ± 0.6	86.9 ± 0.6
EtOAc	120.0 ± 0.6	99.8 ± 0.9	96.8 ± 0.3
EtOH	94.6 ± 0.9	92.9 ± 0.0	92.9 ± 0.0
MeOH	86.5 ± 0.2	86.5 ± 0.0	86.5 ± 0.1
Acetone	100.2 ± 0.9	103.2 ± 0.3	106.2 ± 0.9
Acetonitrile	85.2 ± 0.0	94.6 ± 1.8	93.8 ± 0.3
BME	101.1 ± 0.6	100.2 ± 0.2	100.6 ± 0.1

3.2.5. Analysis of Kinetic Parameters

In this section, kinetic parameters of the protein though *p*NP-α-L-arabinofuranoside were calculated Lineweaver–Burk plots; Figure 3.15 and 3.16. K_m was calculated as 0.19 mM and V_{max} was found as 18.6 ΔAbs/min/ml. For the sugar beet arabinan, the K_m and V_{max} values were calculated as 0.1 mM and 8.1 ΔAbs/min/ml, respectively.

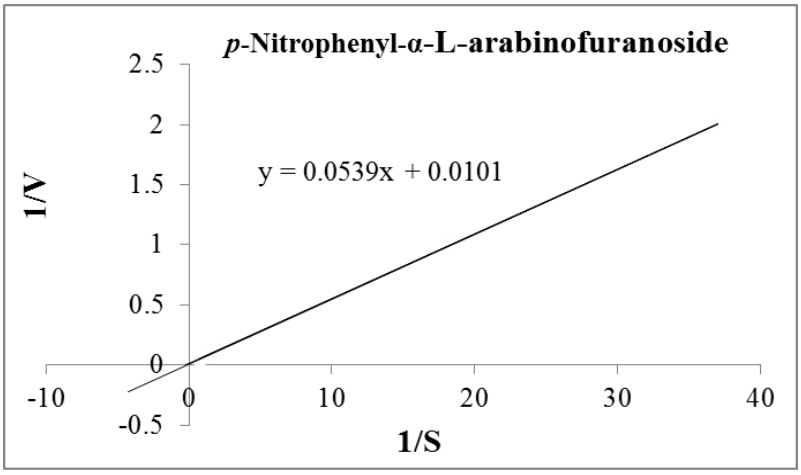

Figure 3.15. Kinetic parameters against *p*NP-α-L-arabinofuranoside.

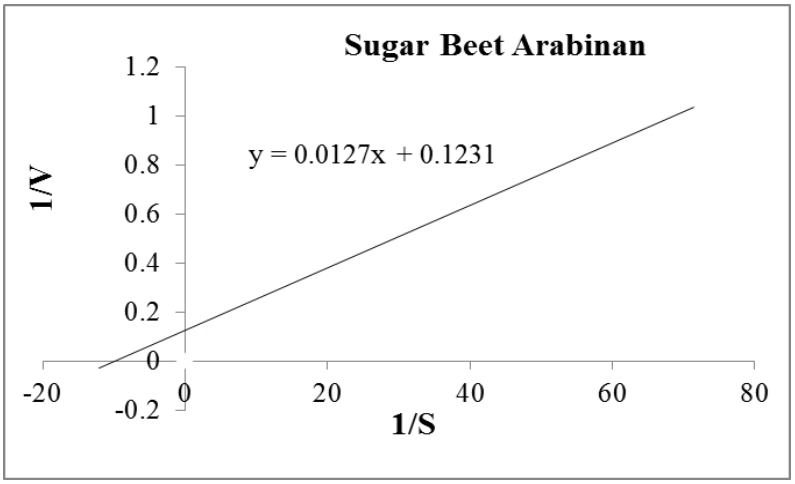

Figure 3.16. Kinetic parameters against sugar beet arabinan.

3.3. Structural Analysis

Protein sequence alignment analysis of the GH 51 family member α-L-AbFases showed that apart from Glu294 which is known to be the catalytically active residue (Hövel et al. 2003); there are 51 residues conserved in all of them. Also, based on the 3D structural analysis of these conserved residues using the predicted structure of our protein (SWISS-MODEL) (Guex and Peitsch 1997) and the structure of another α-L-AbFase resolved previously by Hövel et al. (Hövel et al. 2003), Glu29, Asn74, Asn174 and Tyr246, are all conserved and also located around Glu294 residue in Figure 3.16, can be assumed to be important for enzyme activity. Distances between

these residues and Glu294 is not more than 11.8Å which may be used for further explanation of the catalytic mechanism.

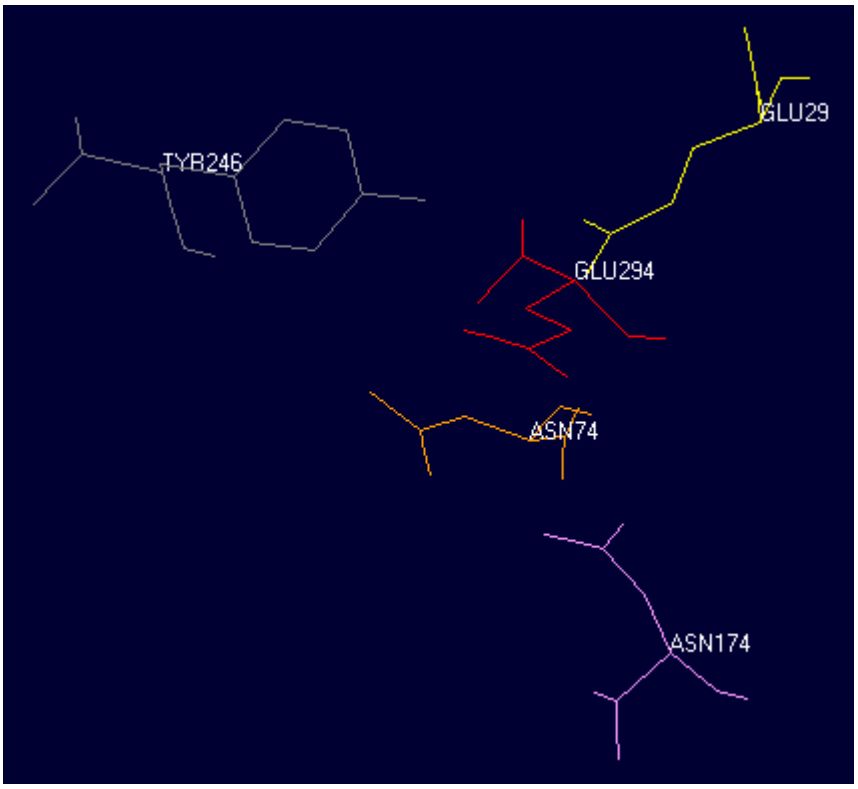

Figure 3.17. Amino acid residues located around the Glu294.

CHAPTER 4

CONCLUSION

In our study, we have mainly aimed to isolate and characterize an α-L-AbFase enzyme by using molecular cloning techniques from a thermophilic organism; *Thermophilic geobacillus* sp.

For this purpose, first we have isolated an α-L-AbFase enzyme and characterized it by means of optimum pH and temperature values etc. The stability profiles of the enzyme in different buffer systems at 55˚C and at different temperatures in Na-acetate buffer at pH 5.0 were investigated. According to the results obtained from profiling experiments, enzyme retained most of its activity after 2 hours incubation in different buffers except for the pH 3.0 and pH 7.0, and retained more than 95% of its activity even after 2 hours incubation at 55˚C and 70˚C. Also, we have tested the effects of metal ions, some specific chemicals and common organic solvents on enzyme activity. Briefly, Ag^{+}, Zn^{2+}, Cu^{2+}, Mg^{2+} and SDS strongly shielded, while Ni^{2+}, NaF, KCl, EDTA and DTT improved the enzyme activity. Enzyme activity was not affected significantly by the addition of 10% of its activity organic solvents. For the kinetic analysis of the enzyme with pNP-α-L-arabinofuranoside at 55˚C, Km and Vmax were obtained as 0.19 mM and 18.6 ΔAbs/min/ml, and Km 0.1 Vmax 8.1 with sugar beet arabinan.

As a conclusion, we have successfully cloned, expressed and characterized an α-L-AbFase enzyme.

APPENDIX A

CHEMICALS USED IN EXPERIMENTS

Agar- Agar

Bacteriological peptone (from casein)

Tryptone

Yeast Extract

Glycerol

NaCl

Na_2HPO_4

$MgSO_4.7H_2O$

Ammonium sulfate

NaH_2PO_4

Calcium chloride

Nutrient broth

Sodium carbonate

Tris Base

EDTA

Isopropanol

Ethidium bromide

Taq DNA polymerase

dNTP set

Nde I

Hind III

Bromophenol blue

Standard agarose

APPENDIX B

MEDIAS

Luria Bertani (LB) broth, per liter

- 10.0 g of tryptone,
- 5.0 g of yeast extract and
- 5.0 g of NaCl

were dissolved in one liter dH_2O and sterilized.

Luria Bertani (LB) agar, per liter

- 10.0 g of tryptone,
- 5.0 g of yeast extract,
- 5.0 g of NaCl and
- 15.0 g of agar

were dissolved in one liter dH_2O and sterilized.

SOC Medium, per 100.0 ml

- 2.0 g Tryptone,
- 0.5 g of Yeast Extract,
- 1.0 ml of 1.0 M NaCl,
- 0.25 ml of 1.0 M KCl,
- 1.0 ml of 2.0 M Mg^{2+}

and 1.0 ml of 2.0 M Glucose were mixed and dissolved in dH_2O up to 100.0 ml and sterilized.

SOB Medium, per 100.0 ml

- 2.0 g of Tryptone,
- 0.5 g of Yeast Extract,
- 1.0 ml of 1.0 M NaCl,
- 0.02 g of KCl,
- 1.0 ml of 1.0 M $MgCl_2$ and
- 1.0 ml of 1.0M $MgSO_4$

Were mixed and dissolved in deionized water and diluted up to 100.0 ml and sterilized.

TB Medium, per 100.0 ml

0.3 g PIPES, 3.0 ml 1.0 M $CaCl_2$ and 1.85 g KCl were dissolved in 100.0 ml deionized water. The pH of the solution was adjusted to 6.7 with 5.0M KOH. Then 1.4 g $MnCl_2$was added. Finally, the solution was filter-sterilized using 0.2µm filter.

APPENDIX C

REAGENTS AND SOLUTIONS

Citrate Buffer; pH 3.0, pH 4.0, pH 5.0, pH 6.0

Citrate buffer (Gomori 1955) stock solutions: A: 0.1 M citric acid; B: 0.1 M sodium citrate. Using the following amounts from these stock solutions and diluting them to 100.0 ml with 50.0 ml dH_2O, the following buffer systems were prepared. Finally, to obtain 50.0 mM concentration, one more 1:2 dilutions were applied.

Volume of 0.1 M Citric acid, ml	**Volume of 0.1 M Sodium citrate, ml**	**Desired pH**
46.5	3.5	3.0
33.0	17.0	4.0
20.5	29.5	5.0
9.5	40.5	6.0

Sodium Phosphate Buffer, 0.1 M; pH 7.0

Indicated amounts of stock solutions were mixed and diluted as 1:2 to obtain 50.0 mM buffer system at pH 7.0.

Volume of 1.0 M Na_2HPO_4, ml	**Volume of 1.0 M NaH_2PO_4, ml**	**Desired pH**
57.7	42.3	7.0

Tris-Cl Buffer, 1.0 M; pH 8.0

121.1 g of Tris base was dissolved in 700.0 ml of dH_2O. Concentrated HCl was added until the pH reached to 8.0. The solution was filled up to 1.0 L with dH_2O. Finally, the stock solution was diluted to 50.0 mM with dH_2O.

Glycine-NaOH Buffer; pH 9.0, pH 10.0

0.2 M glycine and 0.2 M NaOH were prepared and 25.0 ml of glycine stock solution was mixed with defined amounts of 0.2 M NaOH and then, diluted to 100.0 ml with dH_2O (Pearse 1980).

Volume of 0.2 M NaOH, ml	Desired pH
4.4	9.0

1.0 M Glycine solution was prepared and before the final dilution, the pH was adjusted to 10 using 6.0 M NaOH.

95% Ethanol/Water, 70% Ethanol/Water

Ultra-pure H_2O was used to dilute the 100% (molecular biology grade) ethanol to get required concentration as mentioned above and the solutions were stored at room temperature.

10.0% (w/v) Ammonium Persulfate

0.1 g of ammonium persulfate was dissolved in 1.0 ml dH_2O.

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