INVESTIGATION OF ALKALINE AND THERMAL STABILITY OF ALPHA-L-ARABINOFURANOSIDASE PRODUCED BY DIRECTED EVOLUTION

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ABSTRACT

INVESTIGATION OF ALKALINE AND THERMAL STABILITY OF ALPHA-L-ARABINOFURANOSIDASE PRODUCED BY DIRECTED EVOLUTION

Alpha-L-arabinofuranosidase (Abf) is a type of glycoside hydrolase that cleaves the α-L-arabinofuranosidic bonds in the polysaccharides with arabinose. There are many ranges of biotechnological application fields of this enzyme such as pulp and paper industry. The purpose of this study is to identify *Geobacillus vulcani* GS90 by 16S rRNA analysis and to investigate the alkaline and thermal stability of Abf and its mutants produced by error-prone PCR.

During this study, firstly, partial 16S rDNA gene was amplified by using universal primers, cloned and sequenced by Sanger method. The partial 16S rDNA sequence was analyzed by BLAST and phylogenetic tree was constructed. Secondly, *abf* and its mutants were cloned and 73 mutants were screened for functional analysis in terms of total proteins. After purification of Abf and its functional mutant enzymes, they were analyzed in terms of stability and activity against three different conditions. They were 70^{o}C- pH 5.0, 71^{o}C- pH 5.0 and 70^{o}C- pH 9.6.

It was detected that *G. vulcani* 3S-1 was the closest strain of *G. vulcani* GS90. In addition, it could be deduced that L307S and Q90H/L307S mutants were more stable than Abf at 71^{o}C- pH 5.0 and less stable at 70^{o}C- pH 9.6. According to SWISS MODEL analysis, the surrounding residues of 90th and 307th amino acid constructed no hydrogen bonds in Abf, two hydrogen bonds in Q90H/L307S and three hydrogen bonds in L307S. Moreover, it was detected that these mutants had both a longer β-strain and more number of β-strains than Abf. Finally, the predicted solvent accessibilities of Abf, Q90H/L307S and L307S were investigated and it was deduced that relocations of R447 and some asparagines could have affected the alkaline stability of them.

ÖZET

YÖNLENDİRİLMİŞ EVRİM YOLUYLA ÜRETİLEN ALFA-L-ARABİNOFURANOZİDAZ'IN TERMAL VE ALKALİ KARARLILIĞININ ARAŞTIRILMASI

Alfa-L-arabinofuranozidaz (Abf), arabinozu içeren polisakkaritlerde α-L-arabinofuranozitik bağlarını kesen bir çeşit glikozit hidrolazdır. Bu enzimin, kağıt hamuru ve kağıt endüstrisi gibi bir çok biyoteknolojik uygulama alanı mevcuttur. Bu çalışmanın amacı, *Geobacillus vulcani* GS90'nı 16S rRNA analizi ile tanımlamak ve hataya eğilimli PCR ile oluşturulan Abf ve mutantlarının alkali ve termal kararlılıklarını araştırmaktır.

Bu çalışma sırasında, ilk olarak, kısmi 16S rDNA geni evrensel primerler kullanılarak çoğaltıldı, klonlandı ve Sanger metodu ile dizilendi. Kısmi 16S rDNA dizisi, BLAST ile analiz edildi ve filogenetik ağaç oluşturuldu. İkinci olarak ise, *abf* ve onun mutant genleri, klonlandı ve 73 mutant, total proteinleri kullanılarak fonksiyonel analiz için tarandı. Abf ve onun mutant enzimlerinin saflaştırılmasından sonra, bunlar üç farklı koşulda kararlılıkları ve aktiviteleri test edildi. Bu koşullar, 70^{o}C- pH 5.0, 71^{o}C- pH 5.0 ve 70^{o}C- pH 9.6'dır.

G. vulcani 3S-1'in, *G. vulcani* GS90'nın en yakın suşu olduğu belirlendi. Bunun yanı sıra, Abf'ye nazaran L307S and Q90H/L307S mutantlarının 71^{o}C- pH 5.0 koşulunda daha kararlı ve 70^{o}C- pH 9.6 koşulunda ise daha az kararlı olduğu sonucu çıkarılabildi. SWISS MODEL analizine göre, 90. ve 307. amino asitlerin çevresindeki amino asitler, Abf'de hiç hidrojen bağı oluşturmazken, Q90H/L307S'de iki ve L307S'de üç hidrojen bağı oluşturuldu. Üstelik, bu mutantların Abf'ye kıyasla hem daha uzun bir β-tabakaya hem de daha fazla sayıda β-tabakalara sahip oldukları belirlendi. Son olarak, Abf, Q90H/L307S ve L307S enzimlerinin öngörülmüş çözgen ulaşılabilirliği araştırıldı ve R447 ve bazı asparaginlerin yerini değiştirmelerinin alkali kararlılığa etki etmiş olabileceği sonucu çıkarıldı.

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CHAPTER 1

INTRODUCTION

1.1. Extremophiles

Microorganisms have a great range of living area. While some of them adapt to moderate conditions, the others attune into the extreme conditions in terms of temperature, pH, pressure and salinity in addition to the other physical conditions (van den Burg, 2003). Extremophiles are classified in four basic groups depending on their optimal living temperature. They are psychrophile (below 15^{o}C), mesophile between 15^{o}C and 60^{o}C, thermophile more than 60^{o}C and hyperthermophile (above 80^{o}C). Thermophilic microorganisms that are seemed more common than hyperthermophiles are divided into two domains called as bacteria and archaea in addition to eukaryotes such as fungi, algae and protozoa. Not only photosynthetic, thionic and entero bacteria in bacterial species but also pyrococcus, thermococcus, sulfolobus and methanogens in archaea are expressed as thermophiles (Rothschild and Mancinelli, 2001&Vieille and Zeikus, 2001) (Figure 1.1). Nowadays, at least 70 species as thermophiles have been known to exist in the world and the majority of them are archaea (Kashefi and Lovley, 2003). Also, thermophilic microorganisms are inhabited in geothermal site locations such as hot springs, deep sea hydrothermal vent and sediments of hot springs (Mamo and Gessese, 1999& Sebastien et al., 2001& Badal et al., 1989).

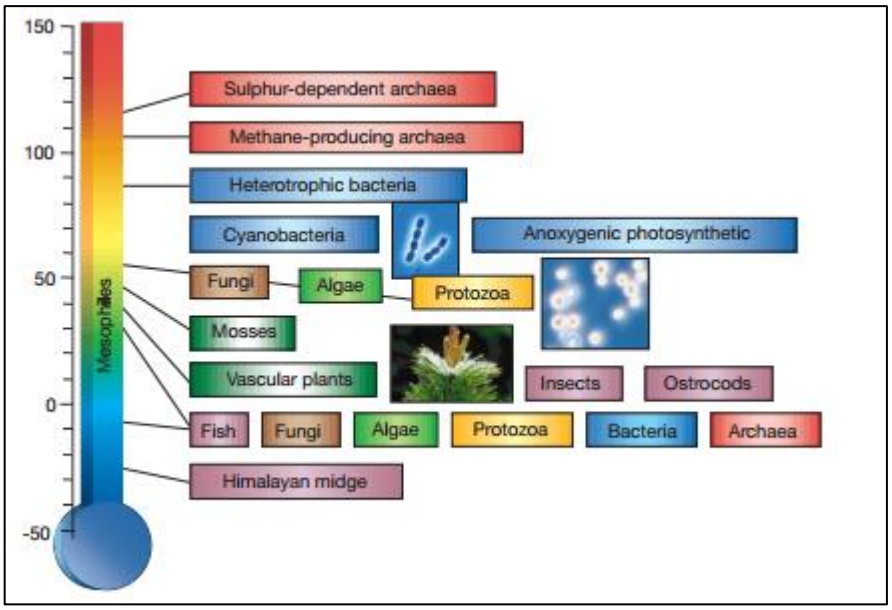

Figure 1.1. Different temperature ranges of thermophilic organisms
(Source: Rothschild and Mancinelli, 2001)

1.2. Thermophilic Enzymes

Thermopilic microorganisms contain thermostable cellular components such as proteins and nucleic acids. Thermostable enzymes obtained from thermopilic organisms are utilized in many ranges of industrial fields such as pulp and paper, leather, detergent industries. An example of thermostable enzymes is xylanases that converts the craft pulp into xylan and lignin and they have usually the optimal temperature between 45 and 65^{o}C depending on what type of source they are obtained. Xylanases from Thermotoga species are the exception because they work even at 105^{o}C (Table 1.1) (Haki and Rashit, 2003). Thermostable enzymes are used in these industrial fields effectively by fermentation processes. In general, fermentation conditions are be adjusted to minimum 60^{o}C because they eliminate contamination problems and provide the fluidic medium and soluble substrate (Morozkinaa et al, 2010).

Table 1.1. Thermophilic enzymes and their applications
(Source: Haki and Rashit, 2003)

Enzyme	Temperature range (°C)	Bioconversions	Applications
α-Amylase (bacterial)	90–100	Starch → dextrose syrups	Starch hydrolysis, brewing, baking, detergents
α-Amylase (fungal)	50–60	Starch → dextrose syrups	Production of maltose
Pullulanase	50–60	Starch → dextrose syrups	Production of glucose syrups
Xylanase	45–65, 105	Craft pulp → xylan + lignin	Pulp and paper industry
Chitinase	65–75	Chitin → chitobiose	Food, cosmetics, pharmaceuticals, agrochemicals
		Chitin → *N*-acetyl glucosamine (chitibiase)	
		N-acetyl glucosamine → glucosamine (deacetylation)	
		Chitin → chitosan (deacetylase)	
Cellulase	45–55, 95	Cellulose → glucose	Cellulose hydrolysis, polymer degradation in detergents
Protease	65–85	Protein → amino acids and peptides	Baking, brewing, detergents, leather industry
Lipase	30–70	Fat removal, hydrolysis, interesterification, alcholysis, aminolysis	Dairy, oleo chemical, detergent, pulp, pharmaceuticals, cosmetics and leather industry
DNA polymerase	90–95	DNA amplification	Genetic engineering/PCR

1.3. The Structure of Plant Cell Wall

Plant cells are enclosed by cell wall like fungi, prokaryotes and algae unlike animal cells. The cell wall in these organisms has two common functions although chemical compositions differ from each other. These functions are that they control the cell bulk and give the cell shape (Taiz and Zeiger, 2010).

Plant cell wall is prevalently composed of primary and secondary cell walls. In the primary cell wall, cellulose microfibrils are buried in two basic polysaccharides and structural proteins that constitute the hydrated matrix. Cellulose is consisted of linearly long chain D-glucoses that link to each other by β-1,4-glucosidic bonds. While hemicelluloses contain mainly xyloglucan, glucuronoarabinoxylan and glucomannan, pectins include homogalacturonan and rhamnogalacturonan with side chains such as arabinan, galactan or arabinogalactan (Figure 1.2) (Taiz and Zeiger, 2010).

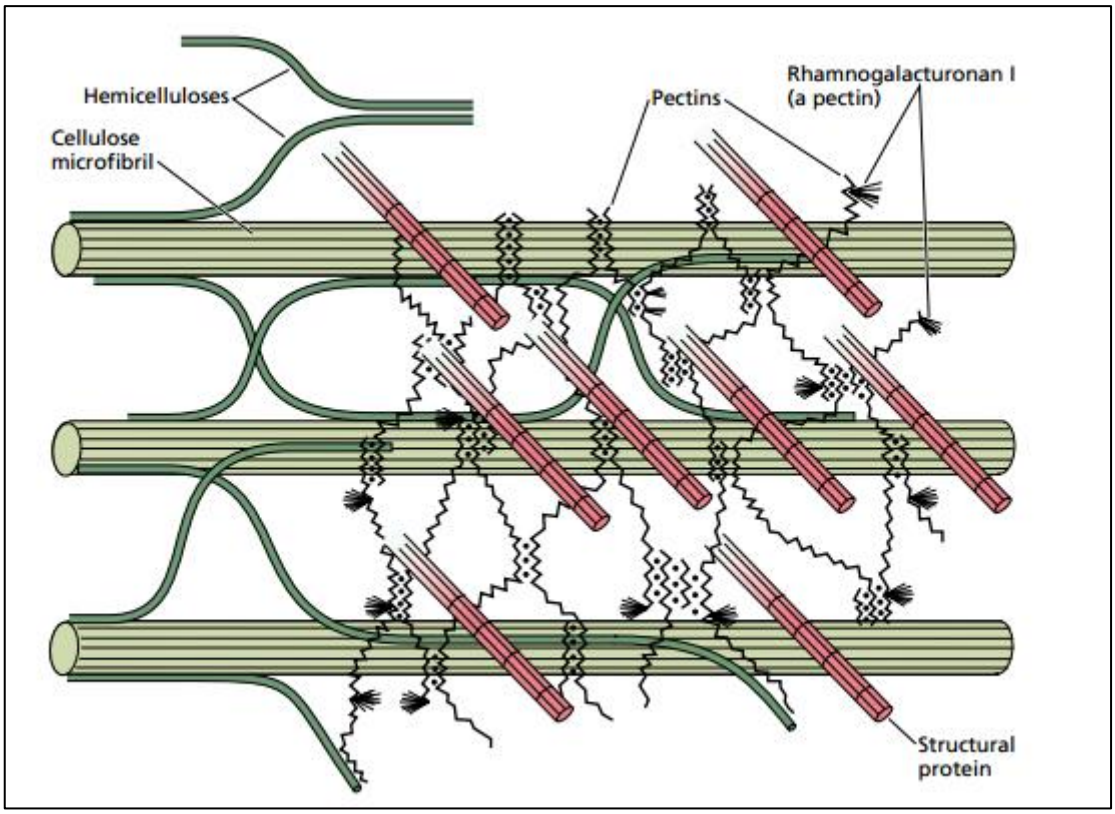

Figure 1.2. The primary cell wall structure
(Source: Taiz and Zeiger, 2010)

The secondary cell wall that is called as lignocellulose is composed of cellulose, hemicellulose and lignin (Howard et al., 2003). Hemicellulose interacts with both cellulose and lignin. While hemicellulose forms hydrogen bonds with cellulose, it binds to lignin covalently and lignocellulose is built as a complicated structure (Shallom and Shoham, 2003) (Figure 1.3).

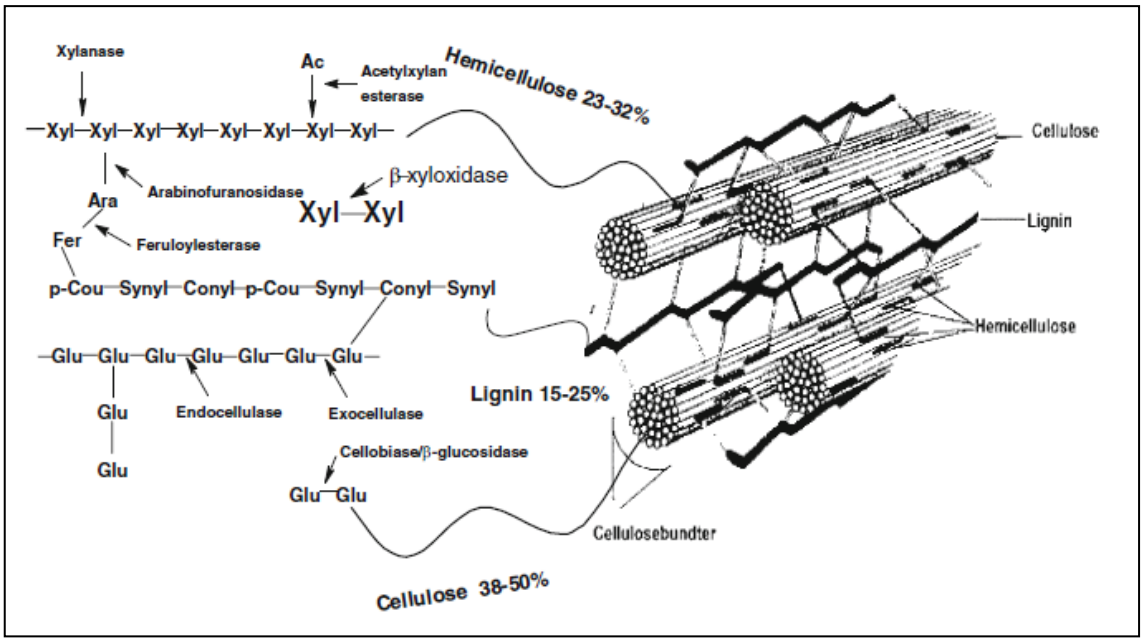

Figure 1.3. The secondary cell wall structure and composition
(Source: Khandeparker and Numan, 2008)

The composition of plant cell wall can vary depending on different plant sources. The primary cell wall has pectins in ratio between 10 to 35%, secondary cell wall contains 15-25% lignins. In addition, while 20-25% cellulose is found in the primary cell wall, this ratio is 38 to 50% in the secondary cell wall. When it comes to the percent of hemicellulose, it shows large differences between 25 to 85% in the primary cell wall while the secondary cell wall has 23 to 32% (Taiz and Zeiger, 2010& Khandeparker and Numan, 2008).

Hemicelluloses that are found in both primary and secondary cell walls contain pentoses such as D-xylose, L-arabinose, hexoses such as D-mannose, D-glucose, D-galactose and sugar acids. Unlike xyloglucan in the backbone of primary cell wall, hemicelluloses in the secondary cell wall are mainly composed of xylan structure (Taiz and Zeiger, 2010& McMillan, 1993).

1.4. Xylanolytic Enzymes

Although xylan contents differ from each other depending on sources such as hardwood, softwood or grasses (Puls and Schuseil, 1993), overall contents involve D-xylopyranose ring in the backbone, α-O-methyl-D-glucuronic acid ring, L-arabinofuranose, ferulic acid and acetyl xylan in the side chains (Beg et al., 2001). All

of the xylanolytic enzymes are necessary for degradation of xylan completely (Subramaniyan and Prema, 2002). These enzymes are endo-1,4-β-xylanase, 1,4-β-D-xylosidase, α-L-arabinofuranosidase, α-D-glucuronidase, acetyl xylan esterase and feruloyl xylan esterase (Beg et al., 2001). While endo-1,4-β-xylanase degrades the backbone of xylan in a random way, 1,4-β-D-xylosidase uses the xylobiose as a substrate. They are the main enzymes because they split off the backbone of xylan. In addition, α-L-arabinofuranosidase, α-D-glucuronidase, acetyl xylan esterase and feruloyl xylan esterase enzymes attack to the side chains of xylan (Biely et al., 1986). That is why they are called as accessory enzymes (Wood et al., 1992). Synergistic action takes place between all of these enzymes for complete degradation of xylan structure (Biely et al., 1986) (Figure 1.4).

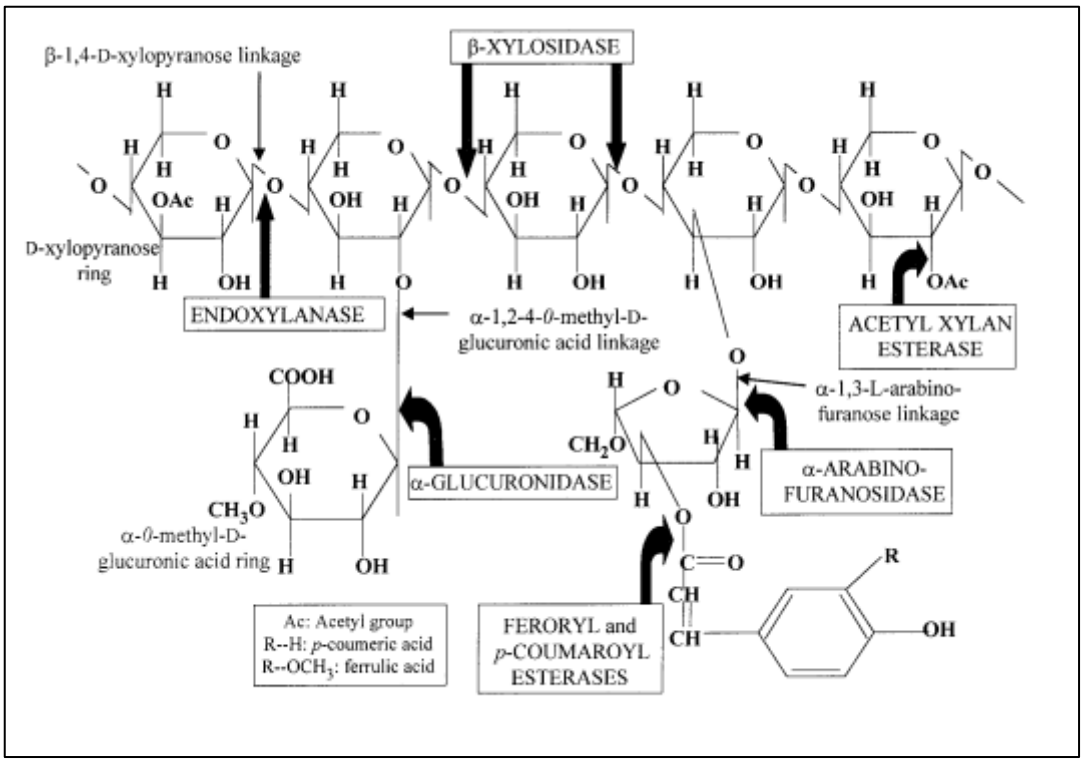

Figure 1.4. Xylan degrading enzymes and their cleavege position
(Source: Beg et al., 2001)

1.5. Alpha-L-Arabinofuranosidase (Abf)

Alpha-L-arabinofuranosidase (Abf) (E.C.3.2.1.5) that breaks into pieces the terminal and non-reducing α-1,2-, α-1,3-, and/or α-1,5-L-arabinofuranoside substitutions in α-L-arabinosides such as arabinoxylan, L-arabinan and the other

polysaccharides that include arabinose (Kaji, 1984). Although Abf enzymes that are a kind of glycoside hydrolase (GH) are divided into five families that are called as 3, 43, 51, 54 and 62, they belong to GH51 in most cases (Coutinho and Henrissat, 1999). This classification is based on the similarities of amino acid sequences (Henrissat and Davies, 1997).

Many ranges of organisms in three kingdoms that include bacteria, plant and fungi are utilized as a source in order to produce Abf in different kind of families for providing more knowledge about these enzymes through characterization (Canakci et al., 2008& Hata et al., 1992& de Wet et al., 2008). In order to eliminate the lack of the structural knowledge that cannot be obtained by characterization studies of Abf, some crystallographic studies related to different type of families that include the Abf have been performed (Hövel et al., 2003& Miyanaga et al., 2004& Fujimoto et al., 2010). The most elaborate study is about Abf in GH51 family in *Geobacillus stearothermophilus* T6. This study has shown that AbfA that is a kind of Abf is a hexamer and while Glu175 is the acid/base residue that is linked to substrate by hydrogen bond, Glu294 is the nucleophile which carries out the nucleophilic attack to catalyze the substrate (Hövel et al., 2003). On the other hand, Abf in GH54 family includes Glu221 as nucleophile and Asp297 as acid/base residue (Miyanaga et al., 2004).

Abf enzymes are a part of L-arabinan utilization system to obtain L-arabinose so that it is used as the sources of carbon and energy by passing through Pentose Phosphate Pathway (PPP). This system has been elaborately examined in *Geobacillus stearothermophilus* T-6. This system depends on whether L-arabinose concentration is high or very low. At very low free L-arabinose concentration, four different operons play a role in the process from the sensation of free L-arabinose to final degradation of L-arabinan. First operon which is called as araPST is for sensing the L-arabinose from outside and initiates a cascade of signal transductions. AraP that is a sugar-binding lipoprotein binds to L-arabinose and presents the sugar to AraS that is a histidine sensor kinase and phosphorylates the AraT which is a response regulator. AraT activates the expression of second operon that is araEGH encoding the ABC transport system by binding to the promoter that is araE. After L-arabinose is transported from outside to inside the cell via ABC transport system, AraR that is a repressor protein regulates negatively the other two operons called as araDBA-abp-abnB and abnEFJ-abnA-abfBA-araJKLMN. These repressor protein is inactivated by binding to L-arabinose that plays a

role as molecular inducer. Because of inactivation of AraR, these two operons are expressed. The latter called as L-arabinan utilization system encodes extracellular endo-1,5-α-arabinanase (AbnA) to degrade the main backbone of arabinan in order to form short branched arabino-oligosaccharides, the specific ABC transport system (AbnEFJ) to pass into the cell and intracellular α-L-arabinofuranosidases (AbfBA) to catalyze α-1,2 and α-1,3-arabinofuranosidic bonds. The final operon called as L-arabinose utilization system encodes the β-L-arabinopyranosidase (Abp), intracellular arabinanase (AbnB) and some enzymes (AraDBA) to convert the arabinose into xylulose-5-phosphate that can go into PPP (Figure 1.5) (Shulami et al., 2011).

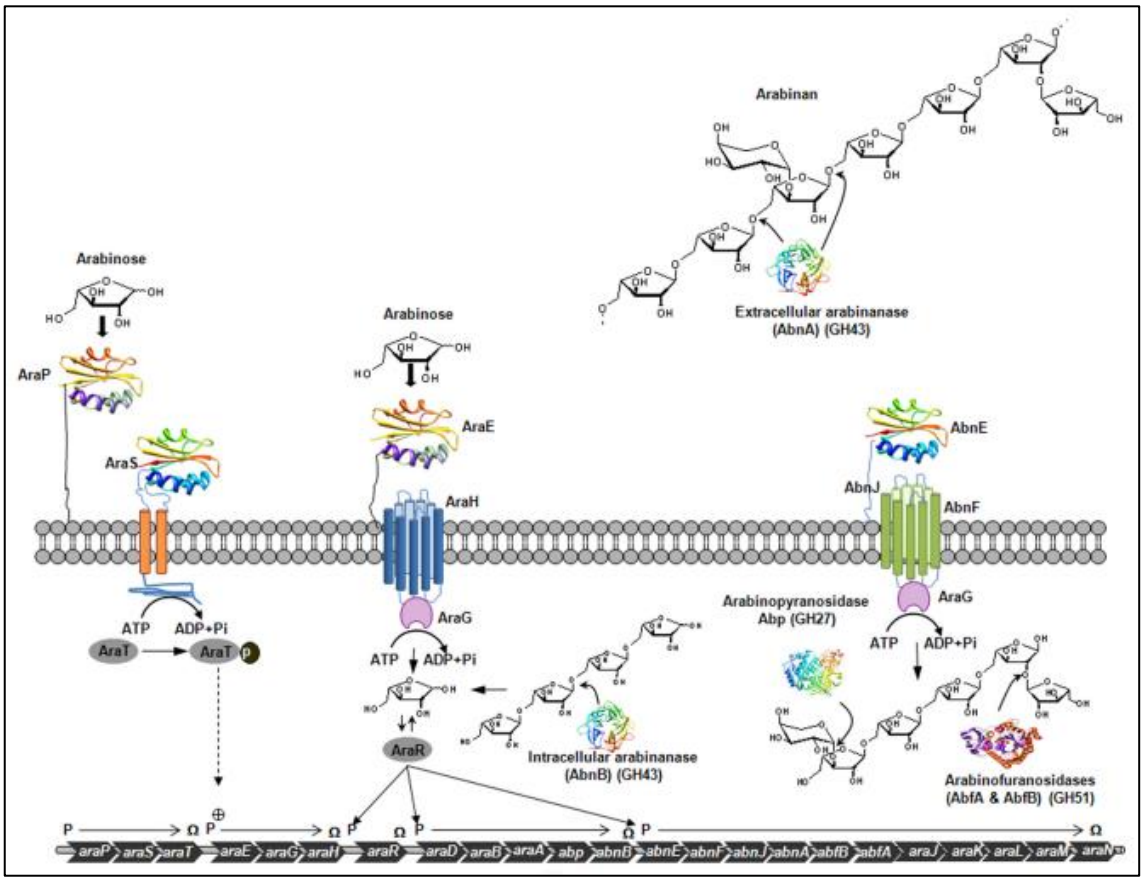

Figure 1.5. The operon systems including *abfBA* gene and their regulation
(Source: Shulami et al., 2011)

In this system, two Abf enzymes are synthesized called as AbfA and AbfB. While one of them degrades the α-1,2-arabinofuranosidic bond, the other hydrolyzes the α-1,3-arabinofuranosidic bond in order to form L-arabinose and short arabino-oligosaccharides. Shulami and collegues (2011) have shown that when the culture is

grown on arabinose compared to xylose and glucose, the expression level of AbfA has increased up to 28 to 35 fold.

1.6. Industrial Application of Abf Enzymes

Recently, hemicellulases have attracted attention in a wide range of industrial fields. Abf and the other hemicellulases which work in the process of agro-industrial play a promising role in a synergistic manner (Aryon et al., 1987& Saha, 2000). These processes include the manufacture of medicinal substances, wine, acetic acid for bread quality, paper from pulp, feedstock for animals, fruit juice and fermentable sugar for bioethanol (Numan, and Bhosle, 2006).

L-arabinose that is released by Abf might be potentially used as antiglycemic agent. This compound represses sucrase in intestine of animals and decreases glycemic response. This is an example of medicinal substances (Seri et al., 1996& Numan, and Bhosle, 2006). In addition, monoterpenols that gives the aromas to wine have aromatic structure and links to α-L-arabinofuranose sugar. Monoterpenols are released by Abf enzymes and wine flavour is increased by free monoterpenols (Biskup et al., 1993& Mateo and Jimanez, 2000). When it comes to acetic acid production for bread quality, the purpose of this is to enhance shelf life of bread. In order to perform this, pentosan is added into the dough and xylanolytic enzymes containig Abf enzymes that are found in some specific microorganisms degrade the pentosans to produce free pentoses such as arabinose and xyloses. These microorganisms use these pentoses in fermentation processes in order to form the acetic acid that provides to postpone the staling of the bread (Gobbetti et al., 2000). In pulp and paper industry, even though molecular chlorine or chlorine dioxide is used for effective bleaching agents, organochlorine compounds are formed since they react with lignin. These are toxic, persistence to degrade, tend to accumulate in the environment and transfer along food chain (Solomon, 1996). In order to eliminate these problems, the biological tools, especially hemicellulases including Abf as enzymes, are used for bleaching purpose in the high temperature and alkaline pH (Bezalel et al., 1993; Wang and Xia, 2008). Abf enzymes were also utilized to increase the digestibility of animal feedstocks by cleaving the hemicellulose including the arabinose (Morrison, 1982& Greve et al., 1984), to clarify the fruit juice by degrading the 1,5-L-arabinans (Saha, 2000) and to produce the

bioethanol from hemicellulosic materials that can be degraded and fermented by certain microorganisms (Sørensen et al., 2005).

1.7. Directed Evolution

Darwinian evolution is significant to form the life including alive organisms. The driving force of evolution is the repetitive mutation and selection for adaptation purpose of fittest form of living organisms. While useful mutations are passed from generation to generation, the hosts that include the harmful ones progressively become extinct (Jackel et al., 2008). Directed protein evolution is a technique that simulates the Darwinian evolution in laboratory scale in order to improve the protein stability and biochemical function. It is based on random mutagenesis for forming the protein diversity for improvement purpose (Kaur and Sharma, 2006). This job is fulfilled by applying the mutation and selection that is carried out by repetition rounds (Cobb et al, 2012& Bloom and Arnold, 2009).

Directed protein evolution can be applied in vitro and in vivo. In vivo methods can be classified based on the use of prokaryote and eukaryote as host. The screening and gene transfer techniques that are applied in vivo evolution system differ from each other depending on which the type of the host is used. While gene transfer job is achieved in prokaryotic host by transformation, transfection is performed for eukaryotic host. In addition, host cell is screened by FACS and drug resistance in eukaryotic host system although it is carried out by colony color and drug resistance in prokaryotic host system. On the other hand, there are in vitro methods including in vitro only and semi-in vitro. While in vitro only is performed completely independent on cell whatever the steps such as mutagenesis, translation or screening are, both in vivo and in vitro conditions are carried out in semi-in vitro process that includes in vitro mutation and in vivo screening. The difference between these techniques are screening at which in vitro only contains just binding affinity while semi-in vitro is based on binding affinity, colony color and drug resistance. Another difference is that transformation is used as a step which cell utilizes in semi-in vitro methods. (Figure 1.6) (Blagodatski and Katanaev, 2011).

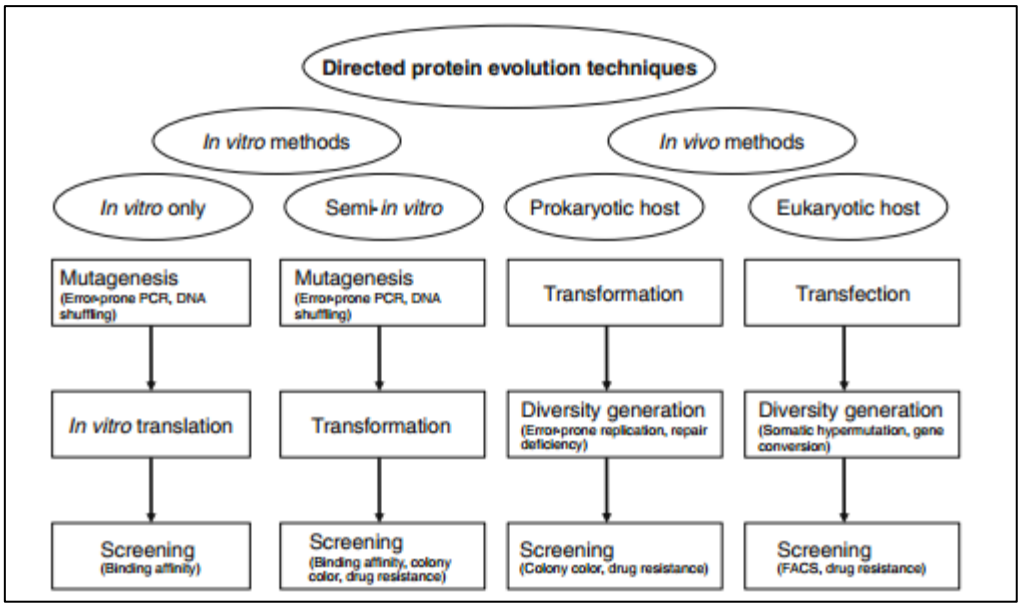

Figure 1.6. The classification of directed protein evolution techniques (Source: Blagodatski and Katanaev, 2011)

The most widely used techiques in in vitro evolution is the error-prone PCR and DNA shuffling. The error-prone PCR is based on random mutagenesis technique that increases the error rate of taq DNA polymerase during PCR (Kaur and Sharma, 2006). The fidelity of this enzyme is $1x10^{-4}$ to $2x10^{-4}$. This frequency is enhanced to $1x10^{-3}$ after 25 cycles of PCR (Cadwell and Joyce, 1994). As well as $MgCl_2$ concentration is increased, $MnCl_2$ is used to incorporate the base mispairing by conformational changes in substrate, enzyme and template during error-PCR (Beckman et al., 1985). On the other hand, the other technique called as DNA shuffling is based on random recombination that can be applied generally among parent genes with at least 70% homology (Miyazaki, Arnold, 1999). In this technique, parent genes are randomly splitted to fragments by DNase I and PCR with primers are carried out after self-priming PCR (Zhang et al., 1997).

1.8. The Aim of This Study

Abf enzymes have a significant role for the pulp and paper industry, especially in high temperature and alkaline pH conditions. Therefore, the aim of this study is to identify the isolate # 90 that is known as *Geobacillus sp.* and to investigate the stability of Abf and its mutants against high temperature and basic pH conditions.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals utilized in this study were indicated in Appendix A.

2.1.2. Media

Media were shown in Appendix B.

2.1.3. Reagents and Solutions

Reagents and solutions were listed in Appendix C.

2.2. Methods

2.2.1. Molecular Identification

2.2.1.1. Organism and Culture Conditions

Bacterial isolate called as number 90 (# 90) was gathered from Balçova Geothermal Water region and was grown on LB agar without antibiotic at 55^{o}C for 18 hours. A single colony was selected, inoculated into 5 ml of LB broth and grown at 55^{o}C by shaking at 200 rpm for chromosomal DNA isolation purpose.

2.2.1.2. Chromosomal DNA Extraction

Chromosomal DNA was obtained by using Genomic DNA Extraction Kit (Fermentas) from an overnight culture according to manufacturer's recommendation. The amount of chromosomal DNA was measured by Nanodrop 1000 Spectrophotometer Thermo Scientific.

2.2.1.3. 16S rRNA Analysis

2.2.1.3.1. Cloning of Partial 16S rDNA Fragment

Chromosomal DNA from isolate # 90 was used for molecular identification by 16S rRNA analysis. Polymerase Chain Reaction (PCR) which was the first step of 16S rRNA analysis was applied by using chromosomal DNA as template, Taq polymerase and PCR master mix which was prepared by 5 µl of 10X Taq buffer, 5 µl of 10X dNTPmix (2 mM), 0.75 µl of forward primer (10 µM), 0.75 µl of reverse primer (10 µM), 34 µl of ddH_2O and 2.5 µl of $MgCl_2$ (25 mM). Universal bacterial primers which were E334F (5'CCAGACTCCTACGGGAGGCAG'3) as forward primer and E1115R (5'CAACGAGCGCAACCCT'3) as reverse primer were designed as specific to partial 16S rDNA fragment. PCR amplification conditions were consisted of initial denaturation step at 94^oC for two minutes, the steps with 25 cycles in the brink of denaturation at 94^oC for 30 seconds, annealing at 62^oC for 30 seconds and extention steps at 72^oC for one minute and final extention step at 72^oC for five minutes. After PCR amplification, partial 16S rDNA fragment was run on 0.8% agarose gel about an hour. PCR product was purified from agarose gel by Silica Bead DNA Gel Extraction Kit (Fermentas) according to manufacturer's recommendation.

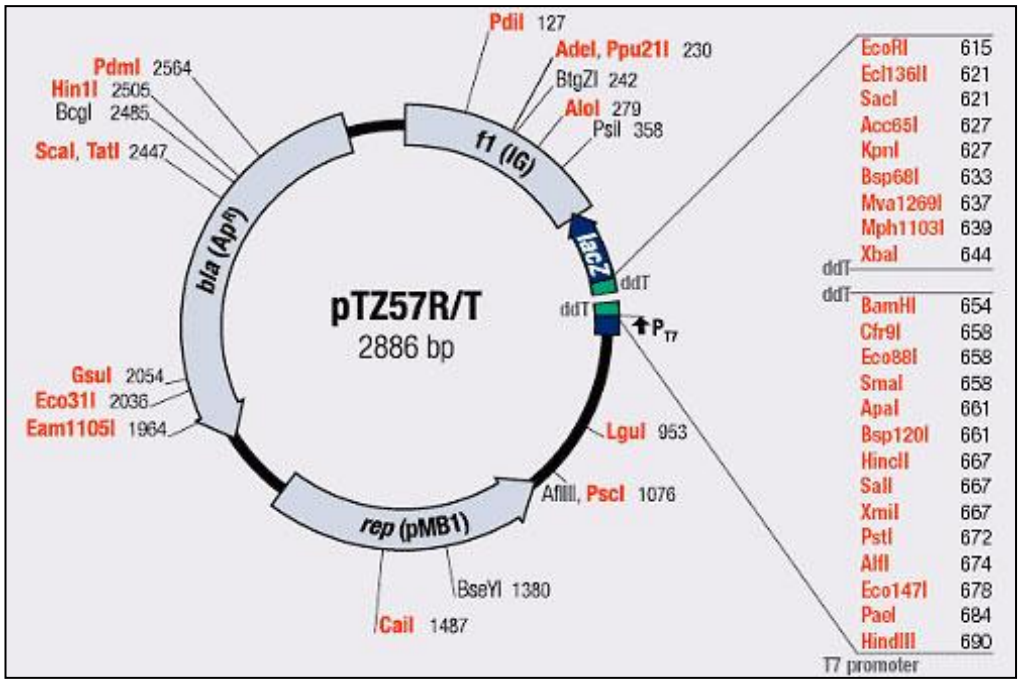

Figure 2.1. Restriction map of pTZ57R/T that is cloning vector

Partial 16S rDNA fragment was ligated into cloning vector called as pTZ57R/T (Fermentas) (Figure 2.1). Ligation was carried out by using 2 µl of 10X T4 DNA ligase buffer, 1 µl of pTZ57R/T (55 ng/µl), 1 µl of T4 DNA ligase (1 weiss U/µl), ddH_2O and purified DNA fragment that was called as insert. The amount of insert was calculated by equation below:

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

According to this equation, molar ratio of vector and insert was taken as 1/10. This ligation mix was incubated at room temperature for overnight. 5 µl of ligation mix was transformed into 50 µl of *Escherichia coli* DH5α (*E. coli* DH5α) by heat shock method and waited in turn for 10 minutes on ice, 45 seconds in 42°C and 2 minutes on ice again. After transformants were diluted in 250 µl of SOC medium, they were grown at 37°C by shaking for an hour. 200 µl of culture was cultivated into LB agar with ampicillin (100 µg/ml) and blue-white screening mix for selection of recombinant colonies and waited at 37°C for overnight. 40 µl of 0,1 M IPTG (Isopropyl β-D-1-thiogalactopyranoside), 40 µl of 20 mg/ml X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) and 20 µl of ddH_2O for each petri dish were mixed to carry out blue-white colony screening method. According to this method, pTZ57R/T carries *lacZ* gene

encoding β-galactosidase enzyme. The *lacZ* deletion mutant gene found in *E. coli* DH5α host is complemented by this gene found in multiple cloning site of pTZ57R/T and fully active β-galactosidase enzyme is produced by metabolizing X-gal that gives blue color to the colonies because of product of X-gal. However, when the insert ligates into the plasmid, *lacZ* gene is disrupted, active β-galactosidase enzyme cannot be produced and so white colonies are formed instead of blue ones. These white colonies are called as recombinant colonies. After overnight incubation, a single white colony was selected and transferred into the 5 ml of LB broth in order to grow at 37^{o}C, 200 rpm for 18 hours. Recombinant plasmid with partial 16S rDNA was isolated from this culture by GeneJET Plasmid Miniprep Kit (Fermentas) according to manufacturer's recommendation.

2.2.1.3.2. Sequencing of Partial 16S rDNA Fragment

The sequencing analyses were performed at Biotechnology and Bioengineering Research Laboratory Center at İzmir Institute of Technology (İzmir, Turkey) using 16 and 80 capillary (Applied Biosystem, 3130XL). This instrument was based on Sanger method. For this purpose, M13 was used as primers. Finch TV 1.4.0 version was applied as a sequence analysis program.

2.2.1.3.3. Phylogenetic Analysis of # 90 Strain

The partial sequence of 16S rDNA was used for construction of phylogenetic tree. MEGA 5 (Molecular Evolutionary Genetics Analysis) program was selected for this purpose. In this program, closest species was found out by using partial sequence of 16S rDNA input belonging to # 90 strain and added into the program as FASTA format of 16S rDNA sequences of these species. All of the sequences were aligned by ClustalW and phylogenetic tree was constructed by Maximum Likelihood (ML) method.

2.2.2. Cloning of the *abf* Gene and Its Mutants

2.2.2.1. *abf*-specific Primer Design

abf-specific primers were designed by using HE653772 accession number found in NCBI database. Forward and reverse primers were 5'GGAACTTGCA-TATGGCTACAAAAAAAGCAACC'3 (primer F) and 5'GGAACATGA-AGCTTTTATCGTTTTCCTAAACG'3 (primer R), respectively. Adaptors recognized by *Nde*I and *Hind*III restriction endonucleases were added to 5' end of each primer which was 32 bp. The palindromic sequences recognized by *Nde*I and *Hind*III are CA-TATG and A-AGCTT, respectively. There were overhang sequences 10 bp for *Nde*I and 9 bp for *Hind*III.

2.2.2.2. Error-prone PCR (EpPCR) Amplification and Agarose Gel Electrophoresis

Two different PCR amplification reactions were set up. While one of them was error-prone PCR (epPCR) for generating mutant *abf*, another one was PCR for amplifying the wild type *abf*. In order to apply epPCR, 10X Mutagenic PCR Buffer, 10X Mutagenic dNTP mix and $MnCl_2$ were prepared and used for increasing the error rate of Taq polymerase. While 10X Mutagenic PCR Buffer was included 70 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3) and 0.01% (weight/volume) gelatin, 10X Mutagenic dNTPmix had 2 mM dATP, dGTP and 5 mM dCTP, dTTP. This procedure was adapted from Cadwell and Joyce methodology (Cadwell and Joyce, 1994). As difference from PCR for *abf*, $MnCl_2$, different concentrations of dNTPs and high concentration of $MgCl_2$ were utilized for epPCR. All of the PCR amplification reaction mixtures and conditions were carried out as shown in Table 2.1, 2.2 and 2.3.

Table 2.1. PCR reaction mixture for *abf* gene

10X Taq Buffer with $(NH_4)_2SO_4$	5 µl
10X dNTP mix (2 mM)	5 µl
Primer F (10 µM)	0.75 µl
Primer R (10 µM)	0.75 µl
Template DNA	1 µl
$MgCl_2$ (25 mM)	3 µl
ddH_2O	34 µl
Taq Polymerase (5 U/µl)	0,5 µl

Table 2.2. PCR reaction mixture for *abf* mutants

10X Mutagenic Taq Buffer	5 µl
10X Mutagenic dNTP mix	5 µl
Primer F (10 µM)	0.75 µl
Primer R (10 µM)	0.75 µl
Template DNA	1 µl
$MnCl_2$ (1 mM)	2.5 µl
ddH_2O	34,5 µl
Taq Polymerase (5 U/µl)	0,5 µl

Table 2.3. Conditions for all PCR reactions

Step	Temperature	Time	Cycle
Initial Denaturation	95^oC	3 minutes	1
Denaturation	95^oC	30 seconds	35
Annealing	55,9^oC	45 seconds	
Extention	72^oC	90 seconds	
Final Extention	72^oC	10 minutes	1

In agarose gel electrophoresis, firstly, 0.8% agarose gel was prepared by mixing 0.8 gram agarose and 100 ml 1X TAE buffer, following by boiling this mixture in microwave oven about two minutes. When the solution was cooled to ~60˚C, 0.5 µl of ethidium bromide (10 mg/ml) was added and gently shaked to obtain homogen mix. After the gel cassette and comb were positioned into horizontal agarose gel electrophoresis system, agarose mixture was poured off on cassette and waited for drying about 30 minutes. 1X TAE buffer was filled into cassette in the way of overlying. PCR products were loaded on agarose gel after mixing with 6X DNA loading dye and run in 100 volt, 85 watt about an hour. As soon as the bands were displayed, they were cut by lancet and purified from agarose gel by Silica Bead DNA Gel Extraction Kit (Fermentas) acording to manufacturer's recommendation.

2.2.2.3. Double Digestion of PCR Products and Agarose Gel Electrophoresis

In order to set up the double digestion reaction, 2 µl of 10X FastDigest Buffer, purified PCR product up to 200 ng, ddH2O, 1 µl of *Nde*I and 1 µl of *Hind*III fast digest enzymes were utilized for each reaction and it was incubated in waterbath at 37^{o}C. These restriction enzymes had 1 FDU/µl concentration. One FDU (FastDigest Unit) defines as the amount of the enzyme required to digest 1 µg of lambda DNA in 5 minutes at 37^{o}C in 1X FastDigest Buffer (Fermentas). The reaction time was optimized as four hours. In this process, sequential reaction was carried out. According to this, *Nde*I was added into reaction and waited for two hours, following by putting on *Hind*III for two hours. 0.8% agarose gel was prepared, these double digested PCR products were run on gel and purified as explained above by Silica Bead DNA Gel Extraction Kit (Fermentas) according to manufacturer's recommendation.

2.2.2.4. Ligation of Purified Double Digested PCR Product into Expression Vector pET28a(+)

Purified double digested PCR product was ligated into the expression vector that is called as pET28a(+) including multiple cloning site, kanamycin resistant gene, T7 promotor and region encoding 6 unit histidine tags in order to able to purify the

recombinant protein by Nickel affinity chromatography (Figure 2.2). In multiple cloning site, this vector has also *Nde*I and *Hind*III recognition sites. In order to perform the ligation reaction, in addition to purified double digested PCR product and pET28a(+), 10X T4 DNA ligase buffer, ddH_2O and T4 DNA ligase were added into the reaction mixture and incubated at room temperature for overnight.

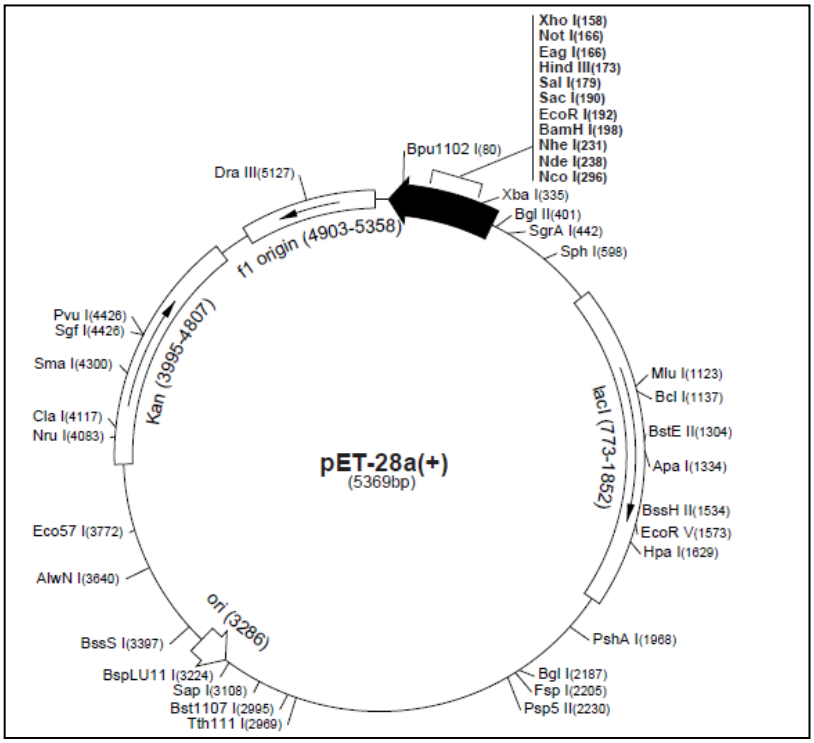

Figure 2.2. Restriction map of pET28a(+) that is expression vector

2.2.3. Construction of Mutant Gene Libraries

2.2.3.1. Competent Cell Preparation

Both competent *E. coli* DH5α and *E. coli* BL21 (λDE) strains were prepared as follows:

After a single colony was taken from LB agar into 5 ml of LB broth, the cells were shaked at 37°C and 275 rpm for 6 hours. At the last of this time, 250 µl of culture were transferred into 250 ml of fresh broth and shaken at 20°C and 180 rpm for overnight. Optic density at 600 nm was read in regular time periods and when absorbance reached to 0.55, culture was incubated in ice for ten minutes. The cells were

centrifuged at 2500 g and +4^{o}C for ten minutes. The medium was poured off and the remaining drops were taken by micropipette. After the pellet was resuspended by 80 ml of ice cold Inoue transformation buffer, the cells were centrifuged at 2500 g and +4^{o}C for ten minutes, the medium was poured off and the remaining drops were taken by micropipette again. The pellet was resuspended in 20 ml of ice cold Inoue transformation buffer and 1,5 ml of DMSO (Dimethyl sulfoxide) was added into this solution. The bacterial solution was gently mixed and stored on ice for ten minutes. Aliquots of 50 and 100 µl were divided into microcentrifuge tubes cooled previously by chilling in liquid nitrogen and these samples were stored at -80^{o}C (Sambrook et al., 1989).

2.2.3.2. Transformation of Ligation Product into *E. coli* DH5α and Plasmid Isolation

Ten µl of ligation products were transferred into 100 µl of competent *E. coli* DH5α and followed heat shock transformation procedure as explained above. The colonies formed were inoculated into LB broth and grown at 37^{o}C by shaking at 200 rpm for 17 hours. pET28a(+) with *abf* and mutant genes were isolated from the cells by GeneJET Plasmid Miniprep Kit (Fermentas) according to manufacturer's recommendation and measured the concentrations of plasmids by nanodrop apparatus.

2.2.3.3. Transformation of pET28a(+) with Insert into *E. coli* BL21 (λDE3)

Five µl of each plasmid was transformed into 50 µl of competent *E. coli* BL21 (λDE3) by heat shock method and the plates were incubated at 37^{o}C for overnight.

2.2.4. Expression of *abf* Gene and Its Mutants

When expressed protein was exceeded 2% of total cellular proteins, it was leaded to inclusion body formation. In order to decrease level of inclusion body, chaperon plasmids were expressed together with *abf* gene while the expression of *abf*

gene was initiating (Singh and Panda, 2005). For this purpose, pG-Tf2 (TaKaRa Kit) as chaperon plasmid encoding chaperon protein which was a type of heat shock protein assisting to solubilize the expressed Abf enzyme was utilized. This plasmid has tig region encoding TF (Trigger Factor) and GroEL-GroES encoding the chaperon protein. In addition, not only it is a tetracycline inducible promoter (Kohara et al., 1987) but also it has chloramphenicol resistance gene for using in selective medium (Figure 2.3). *E. coli* BL21(λDE3) competent cell including pG-Tf2 plasmid was prepared as describe above. It was proved how inclusion body was decreased and solubilized Abf was increased by using chaperon plasmid 4 called as pG-Tf2 (İlgü, 2011). For this purpose, this procedure was used.

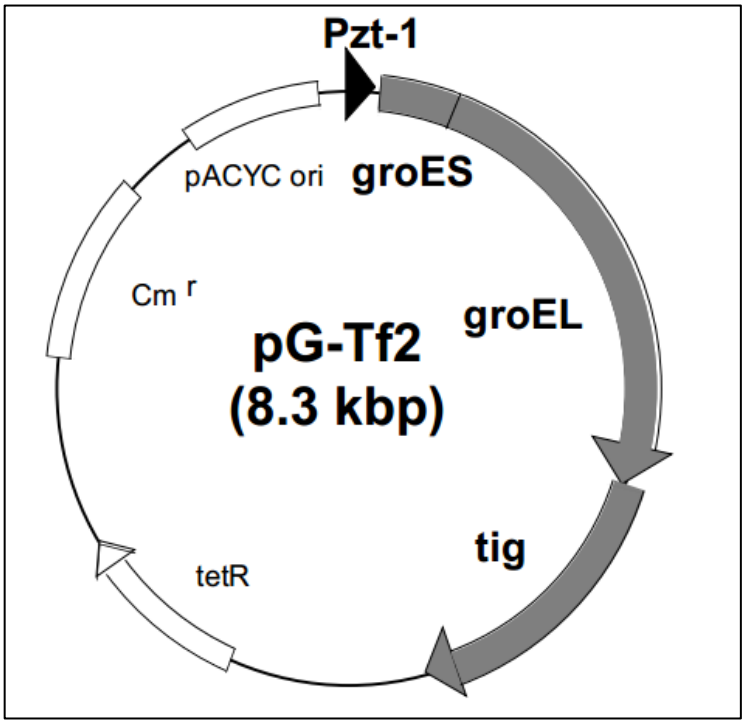

Figure 2.3. Sheme of pG-Tf2 that is chaperon plasmid

A single colony was selected from each wild type and mutants and inoculated into 5 ml of LB broth with kanamycin (50 µg/ml) and chloramphenicol (20 µg/ml) as antibiotic following by waiting at 37^{o}C, 200 rpm for overnight. After that time, 0.5 ml of this culture was taken and transferred into 10 ml of fresh liquid medium with kanamycin (50 mg/ml) and chloramphenicol (20 mg/ml). After the Optic density was followed until 0.7 absorbance was obtained, 1 mM IPTG as final concentration was

added into culture to provide overexpressed protein and incubated at 37^{o}C and 225 rpm for four hours.

2.2.5. Total Protein Extraction

Total protein extraction was performed in three steps: In first step, *E. coli* BL21 (λDE) liquid culture that included overexpressed Abf was centrifuged at 5100 rpm for 20 minutes at +4^{o}C and the supernatant was discarded. In the second step, pellet was dissolved in breaking buffer (Stephens et al., 2009) and sonicated to degrade the cell wall and membrane for five minutes on ice by sonicator (Bandelin, Sonopuls Ultrasonic Homogenizers, HD 2070). In the last step, these samples were centrifuged in 14000 rpm for 20 minutes at +4^{o}C and supernatant was taken into micro centrifuge tubes as total protein.

2.2.6. Screening of Functional Mutant Abf Enzymes

In order to ensure which mutant Abf is functional, activity assay was performed by spectrophotometry. According to this assay, 5 µl of 2 mM *pNP-α*-L-arabinofuranoside as substrate, 85 µl of Na-acetate (pH 5.0) as buffer and 10 µl of total protein were used for initiating the reaction and incubated for ten minutes at 70^{o}C. At the end of the process, this reaction was stopped by adding 100 µl of 1 M Na_2CO_3 and absorbance at 420 nm was measured. Screening assay was studied as dual repetition in 96- well plate. This experiment was applied twice for each sample.

2.2.7. Large-Scale Production of Abf and Its Functional Mutant Enzymes

In order to produce Abf and its mutant enzymes in large scale, a single colony was taken from each related gene libraries and was inoculated into 40 ml of LB broth for overnight. These cultures were transferred into one liter of fresh LB broth with related antibiotics and shaked in 230 rpm. After the O.D. was reached to 0.7 absorbance, 1 mM IPTG as final concentration was added into culture to provide

overexpressed protein and incubated at 37^{o}C and 225 rpm for four hours. Total protein extraction was performed as described above in detail except sonication time was applied as ten minutes.

2.2.8. Purification of Abf and Its Functional Mutant Enzymes

2.2.8.1. Affinity Chromatography

In order to purify Abf and its mutant enzymes that were found in supernatant called as total protein, the Low Pressure-Liquid Chromatography system (Pro Team LC^{TM} 320, Teledyne Isco.) was utilized at +4^{o}C. After 2.5 cm x 10 cm His-Taq Nickel Affinity (HIS-Select^{TM} HF Nickel Affinity Gel, Sigma) column was equilibrated by two column volume of 6X His Wash Buffer including 20 mM Tris-HCl (pH 8.0), 300 mM NaCl and 1 mM imidazole, total protein was loaded into the column and it was poured off by the tap. One volume column of 6X His Wash Buffer was passed through column and protein was eluted drop by drop by 28 ml of 6X His Elution Buffer including 20 mM Tris-HCl (pH 8.0), 300 mM NaCl and 250 mM imidazole in a way that each test tube was consisted of 20 drops. The concentration of each fraction was measured by nanodrop and fraction concentration - tube number graphics were drawn. Certain tubes were selected and collected into same tube.

2.2.8.2. Dialysis

In order to remove imidazole and NaCl from enzymes, dialysis process was applied at +4^{o}C. Fort this purpose, the collected enzyme was placed into dialysis membrane (12,000 MW) by clamping both ends. Then, enzyme in dialysis membrane were placed into two liters of 20 mM Tris-HCl (pH 8.0) buffer. The dialysis process was run by eight hours on magnetic stirrer and incubation was repeated again for overnight.

2.2.9. Determination of Enzyme Concentration

The enzyme concentrations were determined by Bradford Method (Bradford, 1976) which was based on binding the arginine, lysine in primary way, and histidine, aromatic residues in lesser degree (Compton and Jones, 1985& Congdon et al., 1993). Together with blank, all of protein concentration determinations were carried out in 96-well plate in a way with dual repetition. In order to carry out the drawing of standard curve, 100 mg/ml Bovine Serum Albumin (BSA) was prepared as the main stock solution by ddH_2O and it was serially diluted into three intermediate stocks that were 10 mg/ml, 2 mg/ml and 500 µg/ml. Seven samples which were 0, 10, 25, 50, 75, 100, 150 and 200 µg/ml BSA were obtained by using serial dilution and 500 µg/ml BSA. 0 referred to blank composed of 10 µl of ddH_2O. In order to determine the protein concentration, together with blank, 190 µl of 1X Bradford Reagent was added into 10 µl of protein sample and incubated in darkness for ten minutes. The absorbance value at 595 nm was measured in spectrophotometer.

2.2.10. Determination of Activity and Stability Profiles of Abf and Its Functional Mutant Enzymes

The activity and stability profiles were generated at high temperature. Three types of conditions were applied for Abf and its mutant enzymes. First one was optimum pH and temperature which were 5.0 and 70^{o}C, respectively (İlgü, 2011). In this condition, the activity and stability assay were performed for 0, 20, 40, 60, 80 and 100 minutes. The second one was pH 5.0 and 71^{o}C. This condition contained 0, 10, 20, 30 and 40 minutes. The third one was pH 9.6 and 70^{o}C in the 0, 20, 40, 60 and 80 minutes. In order to achieve this study, the purified enzymes were diluted four times into related pH by certain buffer called as 50 mM Na-acetate pH 5.0. These diluted enzymes were incubated for the specified times and 20 µl of incubated enzymes were transferred into microcentrifuge tubes and then, they were stored on ice until overall process was completed. These samples were used to measure the absorbance in 420 nm by using optimum conditions as described before. This experiment was carried out as dual repetition and repeated three times. Activity profile of each enzyme was performed using the ratio of incubated enzyme to unincubated enzyme in the specified times. In

addition, stability profiles were performed using the ratio of incubated enzymes to unincubated Abf in the specified times.

2.2.11. SDS-PAGE Analysis

Vertical SDS-PAGE system was used for separating the proteins based on molecular weights. In this system, 12% separating and %4 stacking gels were prepared (Laemmli, 1970). SDS-PAGE analysis was used to confirm the existence of pure Abf and its mutants. According to this method, equal concentrations of the pure Abf and its mutants were mixed by 5X protein loading dye. Together with protein marker, they were boiled for denaturation of proteins at 95^{o}C for 5 minutes. These proteins were loaded and run on gel at 65 V for three hours. Gel was transferred into the container which included the coomassie blue staining solution by shaking at 60 rpm for overnight. As soon as staining process was completed, staining solution was poured down and the gel was cleaned by dH_2O for sending away the remaining dye. Destaining solution was added into the container and the gel was incubated about an hour by shaking at 60 rpm. This gel was displayed by VersaDoc gel displaying system.

2.2.12. Sequence Analysis and Structural Studies

abf and its mutant genes were sequenced by 16 and 80 capillary, Applied Biosystem, 3130XL at Biotechnology and Bioengineering Research Laboratory Center in İzmir Institute of Technology. This system was based on Sanger method. For this purpose, *abf*-specific PCR and T7 primers were used to obtain the complete sequence of the genes. Finch TV 1.4.0 version was utilized as a sequence analysis program.

Nucleotide sequences of *abf* and its mutant genes were translated into the amino acid sequences by reading the triple codon to an amino acid. Three dimensional (3-D) structures were predicted by I-Tasser program which were improved by Zhang Laboratory using these amino acid sequences (Zhang, 2008). These predicted structures were analyzed by PyMol and SWISS-MODEL programs to be displayed the 3-D structures of proteins. In this program, the interactions between ligand and Abf were investigated and Abf and its mutant enzymes were compared with each other in terms of β-strains, hydrogen bonds and predicted solvent accessibilities.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Molecular Identification

3.1.1. Chromosomal DNA Extraction

The concentration of chromosomal DNA which was isolated by Genomic DNA Extraction Kit (Fermentas) was determined as 70.2 ng/µl.

3.1.2. 16S rRNA Analysis

3.1.2.1. Cloning of Partial 16S rDNA Fragment

The first step of cloning which was PCR amplification was performed and E334F and E1115R as universal primers were used for this purpose. The length of DNA region that was amplified on partial 16S rDNA was 781 bp as depicted in Figure 3.1.

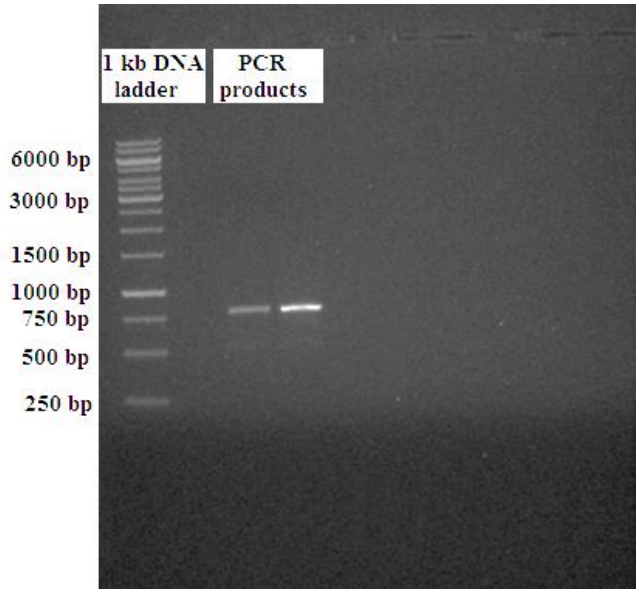

Figure 3.1. The agarose gel display of partial 16S rDNA

After the purification of PCR product from 0.8% agarose gel, the concentration of purified gene was measured as 13.2 ng/µl. This gene was ligated into pTZ57R/T (Fermentas) vector as 1/10 molar ratio of insert/vector. Insert amount that was needed for ligation was calculated as 148.8 ng and 11.3 µl from the gel purified stock was added into ligation mix for cloning of the partial 16S rDNA. After the ligation process, this ligation product was transferred into *E. coli* DH5α host cells by heat shock protokol and grow on LB agar with ampicillin. After 18 hours incubation at 37^{o}C, one white colony was selected to inoculate in 5 ml of LB broth. At the last step of cloning, pTZ57R/T with partial 16S rDNA was purified by GeneJET Plasmid Miniprep kit (Fermentas) and the concentration of the DNA was measured as 40 ng/µl.

3.1.2.2. Sequencing of Partial 16S rDNA Fragment

pTZ57R/T with partial 16S rDNA fragment was sequenced by Sanger method and M13 forward and reverse primers were used through double read. Sequences were obtained by forward and reverse reads and displayed by Finch TV 1.4.0. The overlapped regions were determined by using ClustalW2 in order to form overall partial 16S rDNA fragment sequence. BLAST (Basic Local Aligment Search Tool) in NCBI was applied to the sequence and 16S ribosomal RNA sequences (Bacteria and Archaea) were selected in Blastn. Consequently, *Geobacillus vulcani* (*G. vulcani*) 3S-1 was found out as the closest strain to # 90 showing as query by 99.7% identity. The alignment results were depicted in Figure 3.2. These result suggested that # 90 was a strain of *G. vulcani*. In addition, *Geobacillus thermoleovorans* (*G. thermoleovorans*) and *Geobacillus kaustophilus* (*G. kaustophilus*) strains located in the alignment order following *G. vulcani* by showing 99.6% and 99.5% identity, respectively. This result was supported by Nazina and colleagues (2004). According to their study, it was shown that the closest species of *G. vulcani* were *G. kaustophilus* and *G. thermoleovorans* based on 16S rRNA analysis with 99.3% and 99.1% sequence similarity, respectively (Nazina et al., 2004).

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Query  1     GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACC  60
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Sbjct  1045  ACCTGTCACCCTGTCCCCCCGAAGGGGGAACGCCCAATCTCTTGGGTTGTCAGGGGATGT  986

Query  121   CAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTG  180
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Query  781   G  781
             |
Sbjct  325   G  325
```

Figure 3.2. Alignment between # 90 and *G. vulcani* 3S-1. Query indicates # 90 and Sbjct indicates *G. vulcani* 3S-1

3.1.2.3. Phylogenetic Analysis of # 90 Strain

The phylogenetic tree was constructed by maximum likelihood (ML) method which was found in bootstrap test. As shown in BLAST, the closest strain of # 90 was confirmed as *G. vulcani* 3S-1 and # 90 strain was named as *G. vulcani* GS90. *Streptomyces coelicolor* A3(2) that was a kind of actinobacteria was used as output group in this study. The number on the left of species name was accesion number. The numbers that specify under and top of the lines indicate the branch length and the percentage of same results during repetitions, respectively. According to this analysis, the same result was taken between *G. vulcani* GS90 and *G. vulcani* 3S-1 at 82% of 500 repetitions. In addition, it was concluded that *G. vulcani* GS90 may be evolved from the same ancestor with *G. vulcani* 3S-1 (Figure 3.3).

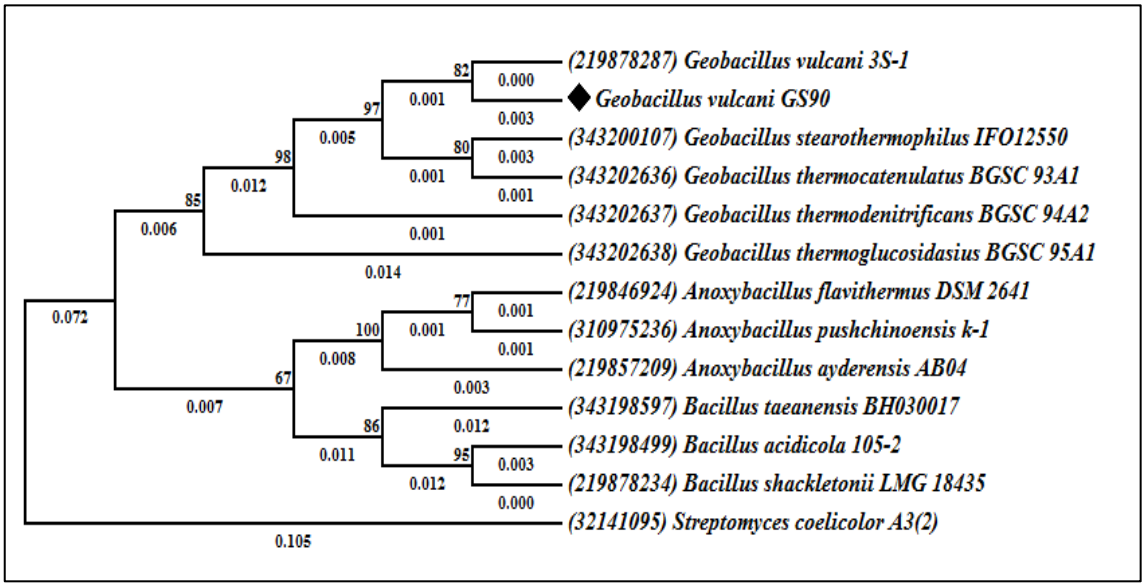

Figure 3.3. Phylogenetic Tree for *G. vulcani* GS90

3.2. Cloning of the *abf* Gene and Its Mutants

3.2.1. Error-Prone PCR

PCR reactions for *abf* and its mutants were performed by amplifying the *abf* gene. Mutagenesis conditions were constructed for increasing the error rate during the amplification of *abf* mutants. The length of *abf* gene and its mutants was 1509 bp (İlgü, 2011). When adaptors from primers were added into the *abf* gene, the length of these PCR products were 1529 and displayed on agarose gel as shown in Figure 3.4.

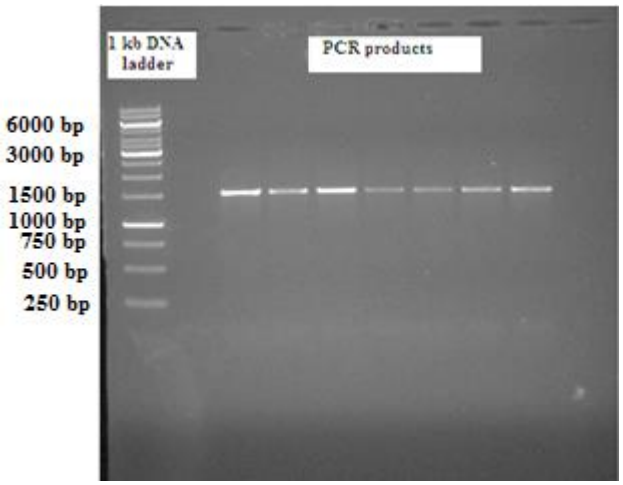

Figure 3.4. Agarose gel display of *abf* gene and its mutants

abf and its mutant PCR products were digested by *Nde*I and *Hind*III to ligate into the pET28a(+) which was expression vector as described in Materials and Methods. The PCR products carrying mutations from different points of this gene were separated from each other by ligating into the one pET28a(+). In transformation, because one expression vector with mutant gene was transferred into *E. coli* DH5α host cells, each colony was assumed that it had a mutant enzyme. Mutant gene found in each colony was confirmed by colony PCR method after transformation as presented in Figure 3.5. A total of 73 clones were isolated from these colonies.

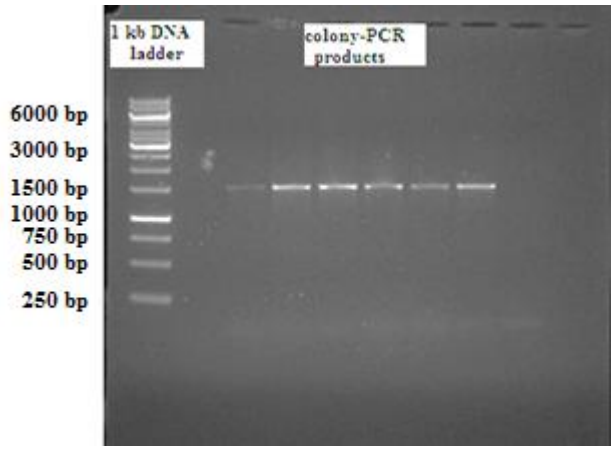

Figure 3.5. The agarose gel display of colony PCR of *abf* gene and its mutants

3.2.2. Screening of Functional Mutant Abf Enzymes

Each clone was transformed into *E. coli* BL21 (λDE3) including pG-Tf2 for overexpression purpose. The small scale production of the total protein of each colony was used for screening of Abf mutant enzyme function. As a result of activity assay, seven Abf enzyme mutants were detected. About 90% of the clones didn't display any Abf enzyme activity. Ebrahimi (2010) studied xylanase enzyme by error-prone PCR and observed that 33% of 200 clones lost their activity. In this study, Ebrahimi used 20 times more $MnCl_2$ concentration than what we used in our study. (Ebrahimi, 2010).

Two of the seven mutant enzymes displayed more stability than Abf in the screening assay. Therefore, Abf and the two functional mutants were overexpressed in large scale and purified via affinity chromatography. These two mutants were named as Q90H/L307S and L307S according to their sequence data. The pure enzyme concentrations were determined by Bradford Method. The concentrations of these enzymes were detected by constructing the standard curve with BSA as shown in Figure 3.6. The concentration of Abf, Q90H/L307S and L307S were obtained as 1.324, 0,693 and 0,681 mg/ml, respectively.

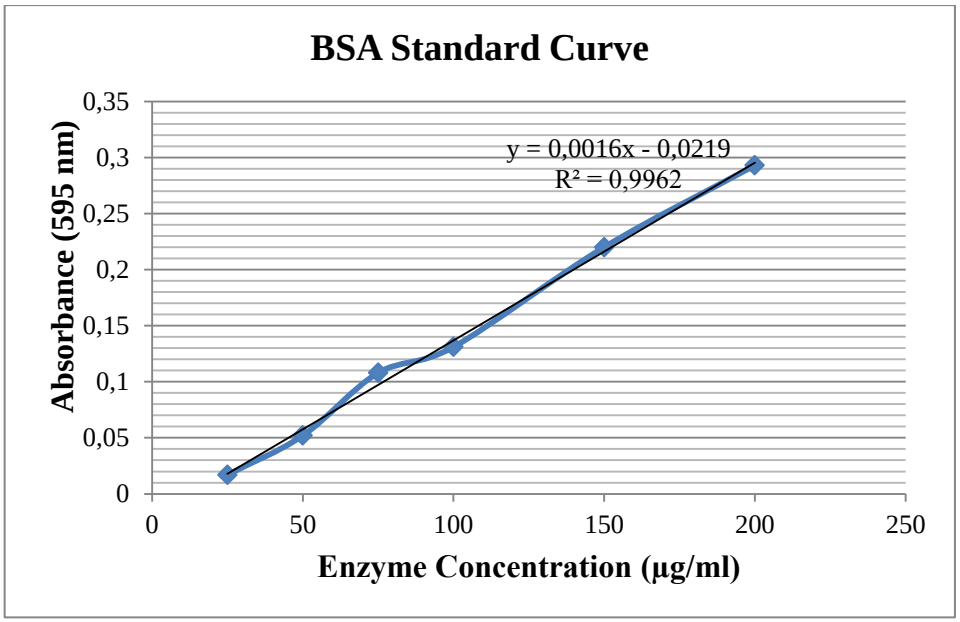

Figure 3.6. BSA standard curve for determination of enzyme concentrations

3.2.3. Stability Profiles of Abf and Its Functional Mutant Enzymes

Stability assays were fulfilled in three different conditions. The first condition was 70^{o}C and pH 5.0 that were optimum temperature and pH as characterized by Hüseyin İlgü (İlgü, 2011). The second condition was 71^{o}C and pH 5.0 that were high temperature and optimum pH conditions. The third condition was 70^{o}C and pH 9.6 that were optimum temperature and basic pH conditions. Two mutants, Q90H/L307S and L307S, showed higher thermal stability compared to Abf. When Figure 3.7 was examined for the first condition, it was observed that Abf has lost 78.4% of its activity after 100 minutes. It was calculated as 70.8% and 69.4% for Q90H/L307S and L307, respectively. It was concluded that these mutants could have slightly higher stability than Abf.

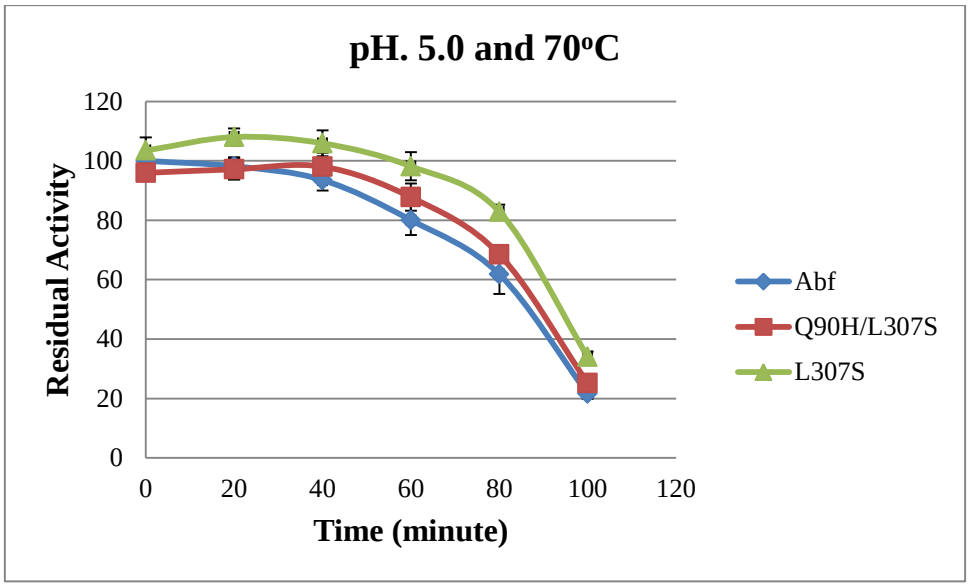

Figure 3.7. Stability profiles of Abf, Q90H/L307S and L307S at 70°C and pH 5.0

Figure 3.8 shows the stability profiles of the three enzymes for the second condition which was pH 5.0 and 71°C. It was calculated that Abf has lost 82.5% of its activity after 40 minutes and this value was 64.1% and 57.9% for Q90H/L307S and L307, respectively. It could be concluded that L307 had higher thermal stability than Q90H/L307S and Abf, while Q90H/L307S was better than Abf in terms of thermostability.

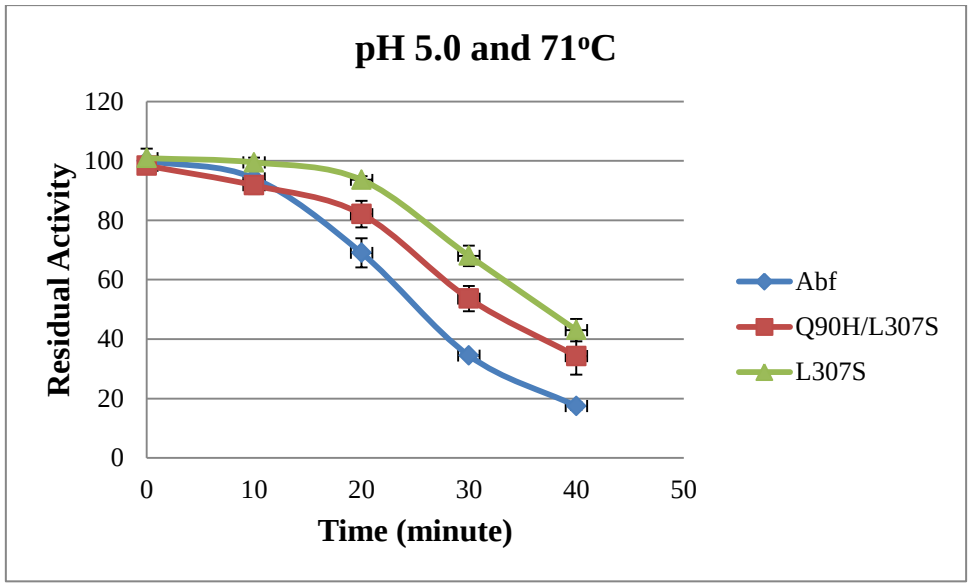

Figure 3.8. Stability profiles of Abf, Q90H/L307S and L307S at 71°C and pH 5.0

Conversely to thermal stability, alkaline stability of variants were surprisingly different when the alkaline stability in optimum temperature was investigated. At this condition, it was observed that Abf has lost 72% of its activity after 80 minutes. This value was 95% and 90% for Q90H/L307S and L307S, respectively (Figure 3.9). It could be concluded that Abf had higher alkaline stability than Q90H/L307S and L307S while L307S was slightly better than Q90H/L307S.

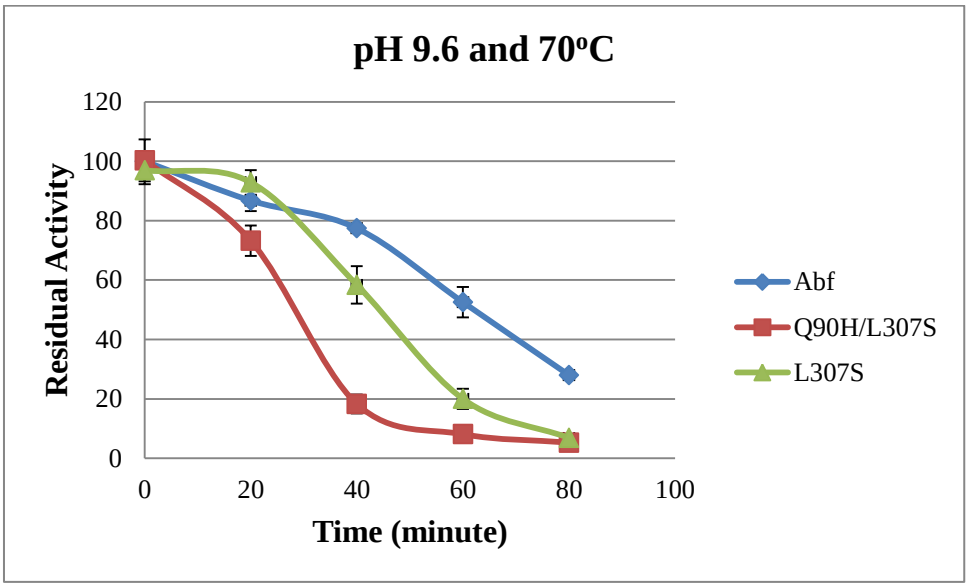

Figure 3.9. Stability profiles of Abf, Q90H/L307S and L307S at 70°C and pH 9.6

It was concluded that thermal stability might not to be shown parallel with alkaline stability. In other words, although Abf might has the lowest thermostability, it might has the highest alkaline stability in the same temperature. Stephen et al. (2009) has shown the similar results in xylanase about thermal and alkaline stability. According to this study, G53 had the lowest stability at high temperature and neutral pH even though the highest stability belonged to the same mutant at lower temperature and basic pH (Stephens et al., 2009).

Table 3.1 indicates the changes of stabilities of Abf, Q90H/L307S and L307S in 40 minutes for all of the conditions. In the first condition, the residual activity of Abf slightly decreased by comparing to its maximal activity. It was concluded that the dramatical change might not be occured in the stability of Abf. On the other hand, the residual activity of Q90H/L307S and L307S slightly exhibited an increase by comparing to maximal activities of these mutants. The stabilities of Q90H/L307S and L307S were not affected in this condition during this time. It was deduced that

Q90H/L307S and L307S mutants could adapt to this condition better than Abf. In the second condition, the residual activity of Abf dramatically declined when compared with Q90H/L307S and L307S. It was concluded that Abf might have the lowest stability than these mutants while the highest stability could belong to L307S during this time. In the third condition, the residual activity of Q90H/L307S decreased in a dramatical way when compared with Abf and L307S. In addition, the changes of residual activity of Abf and L307S slightly differed from each other and Abf was conserved the its residual activity compared to L307S. It was deduced that Abf could have the highest stability and Q90H/L307S could have the lowest stability in this condition. Moreover, it was deduced that the stabilities of Abf and L307S might be closer than the stability differences between Abf and Q90H/L307S (Table 3.1). Mchunu et al. (2007) have studied the directed evolution on xylanase enzyme. According to their study, when one round error-prone PCR was performed on wild type xylanase (XylA), they produced the D2 variants. D2 has have high activity and low thermostability compared to XylA (Mchunu et al., 2009).

Table 3.1. The changes of stabilities of Abf, Q90H/L307S and L307S in 40 minutes

	Abf	**Q90H/L307S**	**L307S**
First condition	-6.4%	+2.1%	+2.4%
Second condition	-82.5%	-64.1%	-57.9%
Third condition	-22.6%	-82%	-38.6%

3.2.4. Activity Profiles of Abf and Its Functional Mutant Enzymes

Activity assays were performed in three different conditions. They were 70^{o}C and pH 5.0 as first condition that were optimum temperature and pH as characterized by Hüseyin İlgü (İlgü, 2011). The second condition was 71^{o}C and pH 5.0 that were high temperature and optimum pH. The final condition was 70^{o}C and pH 9.6 that were optimum temperature and basic pH. When Figure 3.10 was examined, the remaining activity of Abf was 21.6% while the residual activity after 100 minutes were 26.2% in Q90H/L307S and 32.9% in L307S. It could be concluded that these values slightly different from each other and the highest remaining activity was belong to L307S. In

addition, the difference between Abf and L307S has shown an gradual increase until 80 minutes. Moreover, it could be deduced that the half-lives of all the enzymes might be at intervals between 80th and 100th minutes (Figure 3.10).

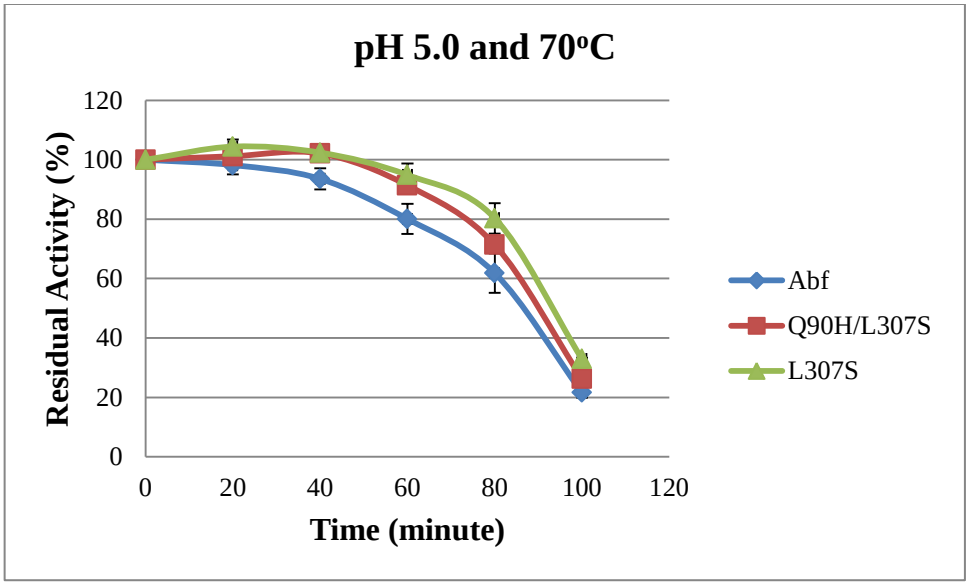

Figure 3.10. Activity profiles of Abf, Q90H/L307S and L307S at 70°C and pH 5.0

When the second condition was investigated, L307S with 42.7% had the highest value of residual activity while Abf with 17.5% was the lowest value after 40 minutes. On the other hand, residual activity for Q90H/L307S was 34.8%. While the difference of residual activity between L307S and Abf was 25.3%, it was 17.3% between Q90H/L307S and Abf. It could be concluded that the highest activity was belong to L307S which was higher than Abf and Q90H/L307S. In addition, while these variants had similar to each other as residual activity in 10th minute, it was observed that difference among all of the variants have enhanced after 10th minute. Moreover, the half-life of the Abf was between 20th and 30th minute, while half-lives in mutants were between 30th and 40th minute. It could be concluded that L307S had higher enhanced activity when compared with Q90H/L307S and Abf (Figure 3.11).

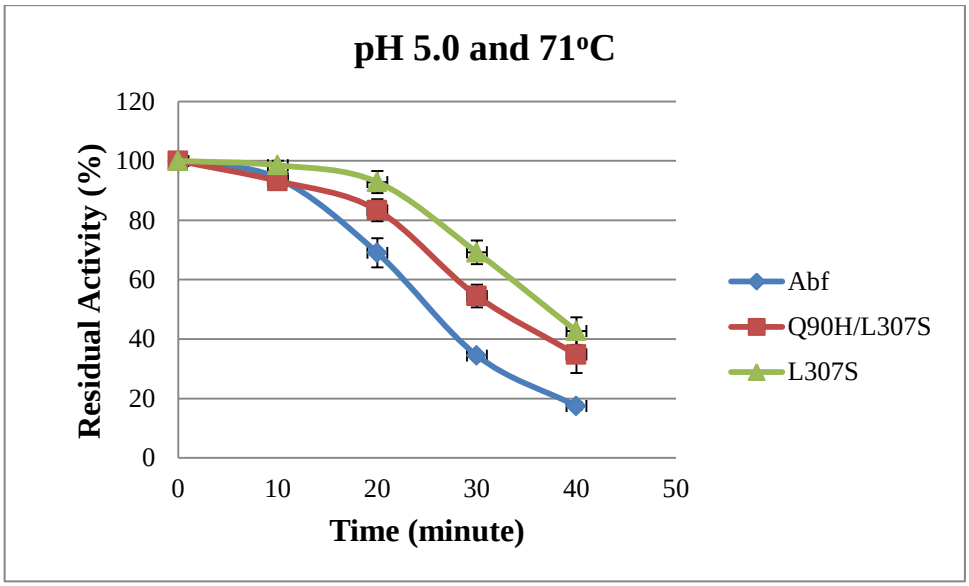

Figure 3.11. Activity profiles of Abf, Q90H/L307S and L307S at 71°C and pH 5.0

As shown in Figure 3.12, the residual activity of Abf was 28% when compared to Q90H/L307S and L307S that had the residual activity of 5.3% and 7.1%, respectively after 80 minutes at the third condition. According to these results, it was observed that the activities of two mutants have become lower than Abf in alkaline conditions. It could be concluded that the highest alkaline activity was belong to Abf. In addition, the half-lives of Abf, L307S and Q90H/L307S were between 60-80 minutes, 40-60 minutes and 20-40 minutes, respectively. It could be concluded that conformational structures of two mutants declined faster than Abf. Moreover, L307S had the highest residual activity until 20th minute of incubation compared to Abf. Abf has started to have the highest activity after 20 minutes (Figure 3.12).

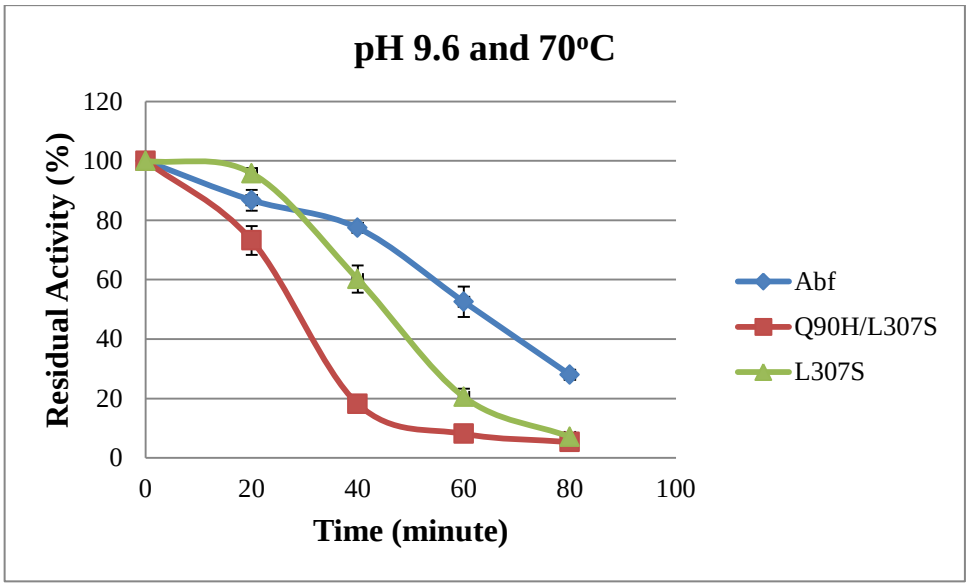

Figure 3.12. Activity profiles of Abf, Q90H/L307S and L307S at 70°C and pH 9.6

SDS-PAGE electrophoresis was carried out in order to confirm the molecular weight of Abf and its mutants that were determined as 58 kDa by İlgü (2011). In this assay, 3 µg from each dialyzed enzymes were loaded on 12% SDS gel and run approximately 3 hours in total. After staining and destaining steps, the gel was displayed as shown in Figure 3.13. Circularized bands indicate Abf, Q90H/L307S and L307S, respectively. Lane 1 was protein marker. According to this analysis, the bands belonging to enzymes were closer to 60 kDa band in protein marker. Therefore, the same result with İlgü (2011) was obtained by determining the molecular weight of enzyme as 58 kDa.

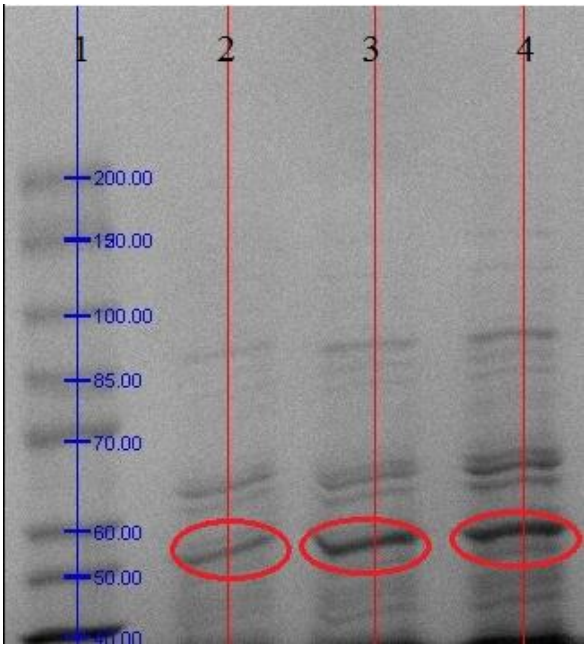

Figure 3.13. SDS-PAGE analysis: lane 1 (Protein Marker), lane 2 (Abf), lane 3 (Q90H/L307S) and lane 4 (L307S)

3.2.5. Sequence Analysis of *abf*, *Q90H/L307S* and *L307S* Genes and Their Enzymes

Nucleotide sequences of *abf, Q90H/L307S* and *L307S* genes were obtained by using the Sanger Method and aligned by Clustal W2 program. According to *Q90H/L307S* nucleotide sequence compared to *abf* gene sequence, four substitutions were observed as A21C, G270C, T920C and C972T. While two of them were the transition mutations, T920C and C972T, the remainders were transversion mutations, A21C and G270C. In addition, the epPCR conditions have provided around three and four mutations in *abf* gene. In other words, error rate was approximately determined as 3/1000. On the other hand, *L307S* gene had three substitutions as A21C, T920C and C972T (Table 3.1). Two of them were transition mutations that were composed of T920C and C972T while another one was transversion mutation that was consisted of A21C. In total, three mutations were obtained in the gene. Thus, the error rate was around 2/1000. It was concluded that both *Q90H/L307S* and *L307S* might be taken place in the same PCR reaction because of the same mutations in the certain positions (Figure 3.14). It was constructed the same PCR condition for error-prone PCR by Ruller and colleagues (2008). According to their study, it was concluded that 3-4 base substitutions could occur per one kb (Ruller et al., 2008).

```
L307S           ATGGCTACAAAAAAAGCACCCATGATCATCGAAAAAGACTTCAAAATCGCTGACATCGAC 60
Q90H/L307S      ATGGCTACAAAAAAAGCACCCATGATCATCGAAAAAGACTTCAAAATCGCTGACATCGAC 60
Abf             ATGGCTACAAAAAAAGCACCAATGATCATCGAAAAAGACTTCAAAATCGCTGACATCGAC 60
                ********************* ****************************************

L307S           AAACGCATTTATGGTTCTTTTATCGAGCACCTCGGCCGCGCTGTATATGGAGGGATTTAT 120
Q90H/L307S      AAACGCATTTATGGTTCTTTTATCGAGCACCTCGGCCGCGCTGTATATGGAGGGATTTAT 120
Abf             AAACGCATTTATGGTTCTTTTATCGAGCACCTCGGCCGCGCTGTATATGGAGGGATTTAT 120
                ************************************************************

L307S           GAGCCGGGCCATCCGCAAGCTGATGAAAACGGGTTCCGGCAAGATGTCATCGAGCTGGTC 180
Q90H/L307S      GAGCCGGGCCATCCGCAAGCTGATGAAAACGGGTTCCGGCAAGATGTCATCGAGCTGGTC 180
Abf             GAGCCGGGCCATCCGCAAGCTGATGAAAACGGGTTCCGGCAAGATGTCATCGAGCTGGTC 180
                ************************************************************

L307S           AAAGAGCTGCAAGTGCCGATCATCCGCTATCCGGGCGGGAATTTTGTGTCCGGTTACAAC 240
Q90H/L307S      AAAGAGCTGCAAGTGCCGATCATCCGCTATCCGGGCGGGAATTTTGTGTCCGGTTACAAC 240
Abf             AAAGAGCTGCAAGTGCCGATCATCCGCTATCCGGGCGGGAATTTTGTGTCCGGTTACAAC 240
                ************************************************************

L307S           TGGGAAGATGGAGTGGGGCCAAAAGAGCAGCGTCCGCGTCGGCTTGATTTGGCGTGGAAG 300
Q90H/L307S      TGGGAAGATGGAGTGGGGCCAAAAGAGCACCGTCCGCGTCGGCTTGATTTGGCGTGGAAG 300
Abf             TGGGAAGATGGAGTGGGGCCAAAAGAGCAGCGTCCGCGTCGGCTTGATTTGGCGTGGAAG 300
                ****************************** ******************************

L307S           TCGGTGGAAACGAATGAAATTGGCTTGAATGAATTTATGGATTGGGCCAAGATGGTCGGA 360
Q90H/L307S      TCGGTGGAAACGAATGAAATTGGCTTGAATGAATTTATGGATTGGGCCAAGATGGTCGGA 360
Abf             TCGGTGGAAACGAATGAAATTGGCTTGAATGAATTTATGGATTGGGCCAAGATGGTCGGA 360
                ************************************************************

L307S           GCTGAAGTGAATATGGCCGTCAACTTAGGGACGCGTGGCATTGATGCGGCGCGCAACTTA 420
Q90H/L307S      GCTGAAGTGAATATGGCCGTCAACTTAGGGACGCGTGGCATTGATGCGGCGCGCAACTTA 420
Abf             GCTGAAGTGAATATGGCCGTCAACTTAGGGACGCGTGGCATTGATGCGGCGCGCAACTTA 420
                ************************************************************

L307S           GTCGAATACTGCAACCACCCGTCGGGCTCCTACTACAGCGATTTGCGCATTGCCCACGGC 480
Q90H/L307S      GTCGAATACTGCAACCACCCGTCGGGCTCCTACTACAGCGATTTGCGCATTGCCCACGGC 480
Abf             GTCGAATACTGCAACCACCCGTCGGGCTCCTACTACAGCGATTTGCGCATTGCCCACGGC 480
                ************************************************************

L307S           CAAATCGGCCATAAGACGGCGGTTGAGTACGGACGAATCGCTTGTGAAGCGGCCAAAGTG 600
Q90H/L307S      CAAATCGGCCATAAGACGGCGGTTGAGTACGGACGAATCGCTTGTGAAGCGGCCAAAGTG 600
Abf             CAAATCGGCCATAAGACGGCGGTTGAGTACGGACGAATCGCTTGTGAAGCGGCCAAAGTG 600
                ************************************************************

L307S           ATGAAGTGGGTCGACCCGACGATTGAACTTGTTGTGTGTGGAAGCTCAAACCGAAATATG 660
Q90H/L307S      ATGAAGTGGGTCGACCCGACGATTGAACTTGTTGTGTGTGGAAGCTCAAACCGAAATATG 660
Abf             ATGAAGTGGGTCGACCCGACGATTGAACTTGTTGTGTGTGGAAGCTCAAACCGAAATATG 660
                ************************************************************

L307S           CCAACGTTTGCGGAATGGGAAGCGACGGTTCTTGACCATACGTATGACCATGTCGACTAT 720
Q90H/L307S      CCAACGTTTGCGGAATGGGAAGCGACGGTTCTTGACCATACGTATGACCATGTCGACTAT 720
Abf             CCAACGTTTGCGGAATGGGAAGCGACGGTTCTTGACCATACGTATGACCATGTCGACTAT 720
                ************************************************************

L307S           ATTTCTCTCCATCAATACTATGGAAACCGGGACAATGATACGGCCAATTATTTGGCGTTG 780
Q90H/L307S      ATTTCTCTCCATCAATACTATGGAAACCGGGACAATGATACGGCCAATTATTTGGCGTTG 780
Abf             ATTTCTCTCCATCAATACTATGGAAACCGGGACAATGATACGGCCAATTATTTGGCGTTG 780
                ************************************************************
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Figure 3.14. Alignment of *L307S*, *Q90H/L307S* and *abf* gene sequences

(cont. on next page)

```
L307S           ATTTCTCTCCATCAATACTATGGAAACCGGGACAATGATACGGCCAATTATTTGGCGTTG 780
Q90H/L307S      ATTTCTCTCCATCAATACTATGGAAACCGGGACAATGATACGGCCAATTATTTGGCGTTG 780
Abf             ATTTCTCTCCATCAATACTATGGAAACCGGGACAATGATACGGCCAATTATTTGGCGTTG 780
                ************************************************************

L307S           TCGCTGGAAATGGATGATTTTATCCGTTCGGTTGTCGCCATCGCCGATTATGTGAAGGCG 840
Q90H/L307S      TCGCTGGAAATGGATGATTTTATCCGTTCGGTTGTCGCCATCGCCGATTATGTGAAGGCG 840
Abf             TCGCTGGAAATGGATGATTTTATCCGTTCGGTTGTCGCCATCGCCGATTATGTGAAGGCG 840
                ************************************************************

L307S           AAAAAACGAAGCAAAAAAACGATCCACCTTTCGTTTGACGAATGGAATGTATGGTACCAC 900
Q90H/L307S      AAAAAACGAAGCAAAAAAACGATCCACCTTTCGTTTGACGAATGGAATGTATGGTACCAC 900
Abf             AAAAAACGAAGCAAAAAAACGATCCACCTTTCGTTTGACGAATGGAATGTATGGTACCAC 900
                ************************************************************

L307S           TCGAATGAGGCAGATAAGTCAATTGAACCATGGACCGTCGCGCCGCCTCTGTTGGAGGAT 960
Q90H/L307S      TCGAATGAGGCAGATAAGTCAATTGAACCATGGACCGTCGCGCCGCCTCTGTTGGAGGAT 960
Abf             TCGAATGAGGCAGATAAGTTAATTGAACCATGGACCGTCGCGCCGCCTCTGTTGGAGGAT 960
                ******************* ******************************************

L307S           ATTTATAACTTTGAAGATGCGCTTCTTGTCGGCTGCATGCTTATTACGCTCATGAAACAT 1020
Q90H/L307S      ATTTATAACTTTGAAGATGCGCTTCTTGTCGGCTGCATGCTTATTACGCTCATGAAACAT 1020
Abf             ATTTATAACTTCGAAGATGCGCTTCTTGTCGGCTGCATGCTTATTACGCTCATGAAACAT 1020
                *********** ************************************************

L307S           GCCGATCGGGTGAAAATTGCCTGCTTGGCTCAATTAGTGAATGTCATTGCACCGATCATG 1080
Q90H/L307S      GCCGATCGGGTGAAAATTGCCTGCTTGGCTCAATTAGTGAATGTCATTGCACCGATCATG 1080
Abf             GCCGATCGGGTGAAAATTGCCTGCTTGGCTCAATTAGTGAATGTCATTGCACCGATCATG 1080
                ************************************************************

L307S           ACGGAAAAGAACGGTCCGGCATGGAAGCAAACGATTTACTATCCGTTTATGCATGCCTCG 1140
Q90H/L307S      ACGGAAAAGAACGGTCCGGCATGGAAGCAAACGATTTACTATCCGTTTATGCATGCCTCG 1140
Abf             ACGGAAAAGAACGGTCCGGCATGGAAGCAAACGATTTACTATCCGTTTATGCATGCCTCG 1140
                ************************************************************

L307S           GTTTACGGCAGAGGAGTGGCGTTGCACCCAGTCATTTCAAGTCCGAAATACGACAGCAAA 1200
Q90H/L307S      GTTTACGGCAGAGGAGTGGCGTTGCACCCAGTCATTTCAAGTCCGAAATACGACAGCAAA 1200
Abf             GTTTACGGCAGAGGAGTGGCGTTGCACCCAGTCATTTCAAGTCCGAAATACGACAGCAAA 1200
                ************************************************************

L307S           GACTTCACAGATGTTCCGTATTTAGAGTCGATCGCTGTTTACAATGAAGAAAAAGAAGAA 1260
Q90H/L307S      GACTTCACAGATGTTCCGTATTTAGAGTCGATCGCTGTTTACAATGAAGAAAAAGAAGAA 1260
Abf             GACTTCACAGATGTTCCGTATTTAGAGTCGATCGCTGTTTACAATGAAGAAAAAGAAGAA 1260
                ************************************************************

L307S           GTGACGATTTTTGCGGTCAACCGTGATATGGAAGACGCGTTGTTGCTGGAATGCGATGTT 1320
Q90H/L307S      GTGACGATTTTTGCGGTCAACCGTGATATGGAAGACGCGTTGTTGCTGGAATGCGATGTT 1320
Abf             GTGACGATTTTTGCGGTCAACCGTGATATGGAAGACGCGTTGTTGCTGGAATGCGATGTT 1320
                ************************************************************

L307S           CGCAGCTTTGAAGACTACCGTGTCATTGAACATATCGTTTTGGAACATGACAACGTGAAA 1380
Q90H/L307S      CGCAGCTTTGAAGACTACCGTGTCATTGAACATATCGTTTTGGAACATGACAACGTGAAA 1380
Abf             CGCAGCTTTGAAGACTACCGTGTCATTGAACATATCGTTTTGGAACATGACAACGTGAAA 1380
                ************************************************************

L307S           CAAACGAATTCGGCCCAATCTTCCCCGGTTGTCCCACATCATAACGGCGATGCCCAACTG 1440
Q90H/L307S      CAAACGAATTCGGCCCAATCTTCCCCGGTTGTCCCACATCATAACGGCGATGCCCAACTG 1440
Abf             CAAACGAATTCGGCCCAATCTTCCCCGGTTGTCCCACATCATAACGGCGATGCCCAACTG 1440
                ************************************************************

L307S           TCCGGTGGGAAAATGTCGGCCATGTTGCCGAAGTTATCGTGGAATGTTATTCGTTTAGGA 1500
Q90H/L307S      TCCGGTGGGAAAATGTCGGCCATGTTGCCGAAGTTATCGTGGAATGTTATTCGTTTAGGA 1500
Abf             TCCGGTGGGAAAATGTCGGCCATGTTGCCGAAGTTATCGTGGAATGTTATTCGTTTAGGA 1500
                ************************************************************

L307S           AAACGATAA 1509
Q90H/L307S      AAACGATAA 1509
Abf             AAACGATAA 1509
                *********
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Figure 3.14. (cont.)

When aminoacid sequences of Abf and Q90H/L307S were compared, it was observed that half of the mutations were the silent mutations that corresponded to P7P and F324F. The remainders were the missense mutations that were Q90H and L307S (Table 3.2). According to these substitutions, Glutamine and leucine were converted into histidine and serine in the position 90 and 307, respectively. It was observed that the former substitution was exchanged from polar uncharged aminoacid to positively charged residue while hydrophobic aminoacid was converted into polar uncharged aminoacid in latter substitution. On the other hand, when the aminoacid sequence of L307S was investigated, similar results to Q90H/L307S were observed as two silent mutations in the same positions while it had only one missense mutation that was L307S (Table 3.1). According to this substitution, leucine that was hydrophobic residue was converted into serine in position 307 that was polar uncharged residue (Figure 3.15).

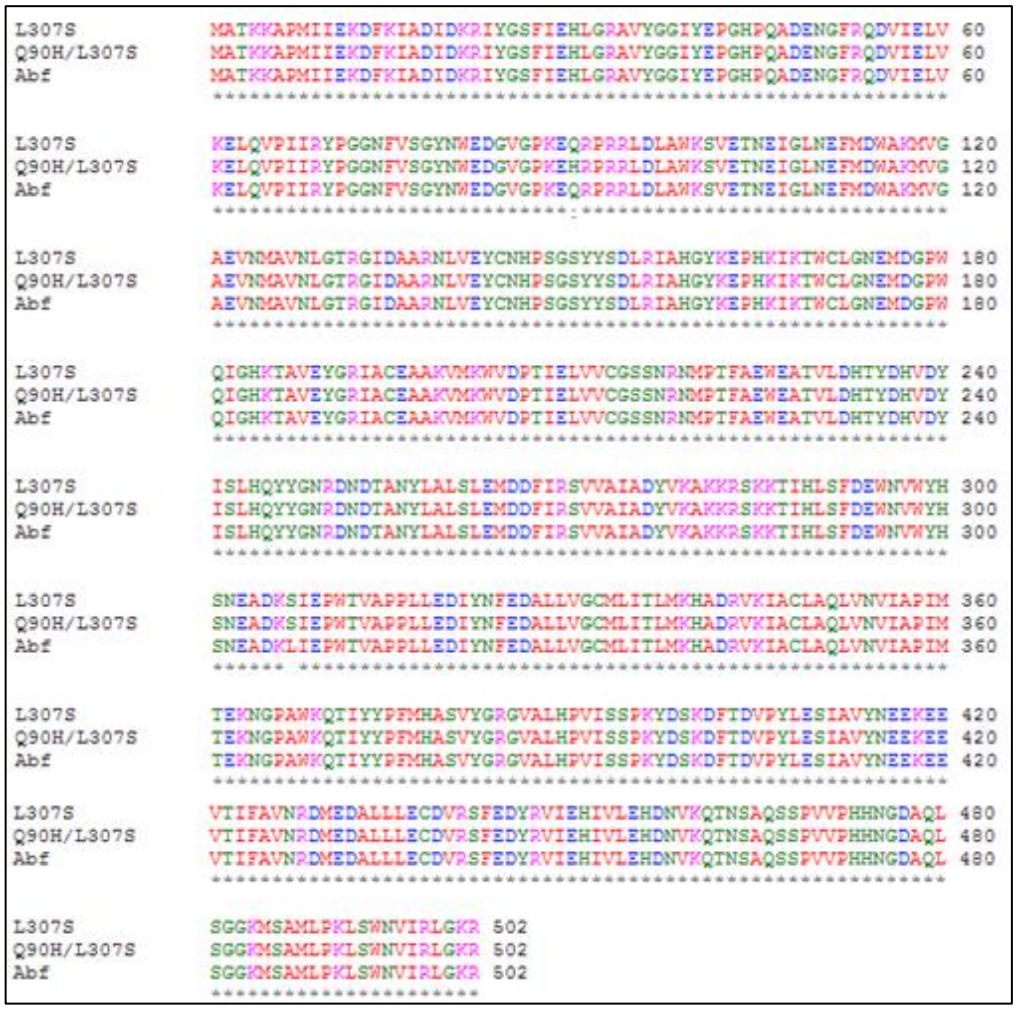

Figure 3.15. Alignment of L307S, Q90H/L307S and Abf aminoacid sequences

Table 3.2. Nucleotide and amino acid substitutions of Q90H/L307S and L307S mutants

Abf mutants	Nucleotide substitution	Aminoacid substitution
Q90H/L307S	A21C, G270C, T920C and C972T	P7P, Q90H, L307S, and F324F
L307S	A21C, T920C and C972T	P7P, L307S, and F324F

3.2.6. Protein Modeling

The predicted 3D structures of Abf, Q90H/L307S and L307S were constructed by I-Tasser (Zhang, 2008). This analysis was divided into two parts. The first part contained the comparison between Abf and *α*-L-arabinofuranosidase enlightened the 3-D structure by x-ray chrystalography in terms of predicted substrate binding site. The second part included the analysis of the mutations in Q90H/L307S and L307S by comparing with Abf. Experimental conditions such as certain pH and temperature were ignored during these analysis because all of the predicted 3D structures were constructed in an experimental-free way. Abf had eight residues (E29, G73, N74, W99, N174, Y246, E294 and Q351) in predicted substrate binding site for ligand called as *pNP-α*-L-arabinofuranoside and abbreviated as AHR-1. It was observed that only one residue from predicted binding site was bound to AHR-1 (Figure 3.16). This residue was E294 and it could be suggested that it may have a key role in binding and catalysis of this substrate. Hövel and colleagues (2003) have shown in their study that E294 was nucleophilic residue. It may carry out nucleophilic attack to substrate by binding and forming the structure called as covalent intermediate that occured between arabinofuranose and enzyme (Hövel et al., 2003). It could be deduced from this part of analysis that E294 may play a catalytic nucleophile role.

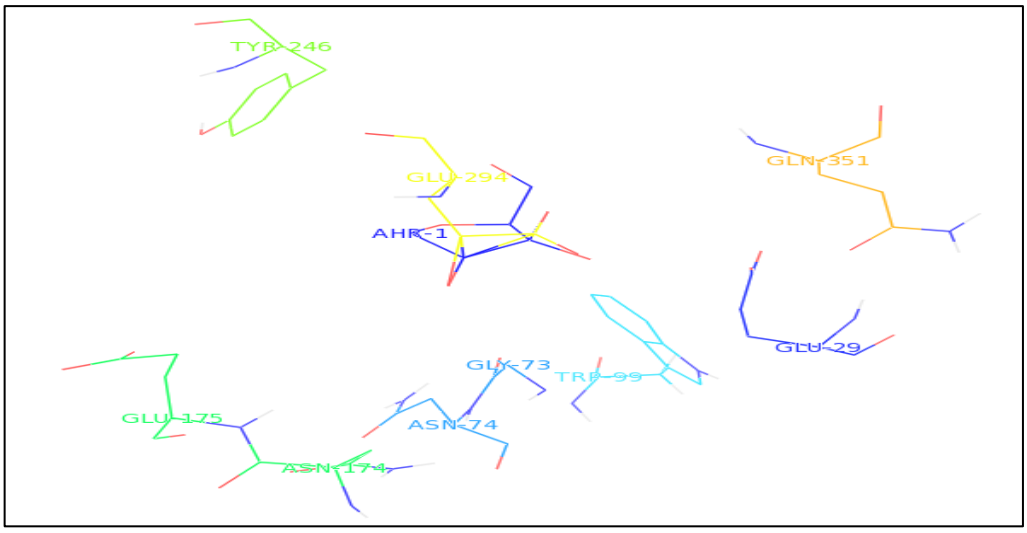

Figure 3.16. The residues in predicted binding site of Abf with ligand

The second part of this analysis was the comparison between Abf, Q90H/L307S and L307S in the course of structure. Each variant was analyzed in PyMOL in terms of overall structure in order to analyze a number and the size of β-strains. According to this analysis, it was observed that five extra β-strains were found in Q90H/L307S and L307S when compared with Abf. In addition, these mutants contained one longer β-strain (Figure 3.17). This one was shorter in Abf. Hakulinen and colleagues (2003) have shown that a number and the size of β-strains could positively affect the stability It could be concluded that these mutants were better than Abf in the course of thermal stability (Hakulinen et al., 2003). It could be concluded that Q90H/L307S and L307S had higher stability than Abf. This result could support the experimental studies about thermostability.

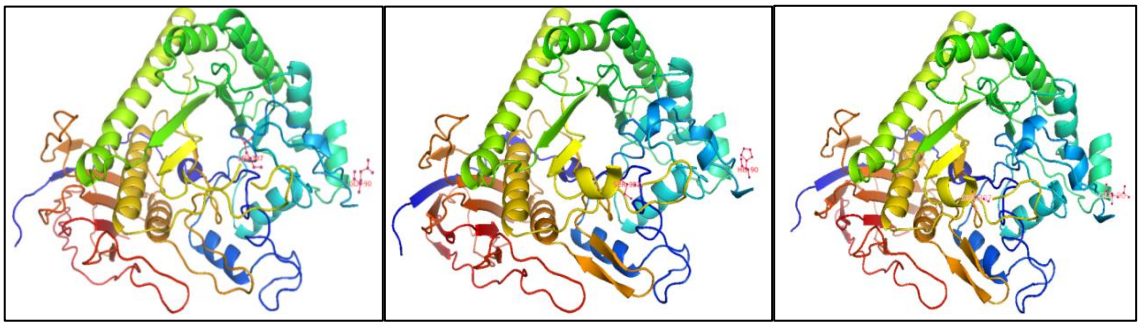

Figure 3.17. The predicted three dimensional structures. Abf (left), Q90H/L307S (middle) and L307S (right)

Together with aminoacids around residues 90 and 307 in folded situation of variants were analyzed by SWISS-MODEL. According to this analysis, when Q90 and L307 residues in Abf were compared with substitutions in Q90H/L307S and L307S, three hydrogen bonds were observed in L307S although it had only one substitution (Figure 3.20). In the light of this data, it could be deduced that L307S substitution has provided to get close not only S307 to E309 and A304 but also Q90 without change to E89 and P87. In addition, two hydrogen bonds in Q90H/L307S (Figure 3.19) were observed even though Abf had no hydrogen bond (Figure 3.18). This result in Q90H/L307S showed that Q90H and L307S substitutions have provided to be shorter distance between S307 and two residues calles as E309 and A304, although together with H90 and its around residues could not be constructed any hydrogen bonds. This data has shown that it could be parallel with the experimental studies about thermostability.

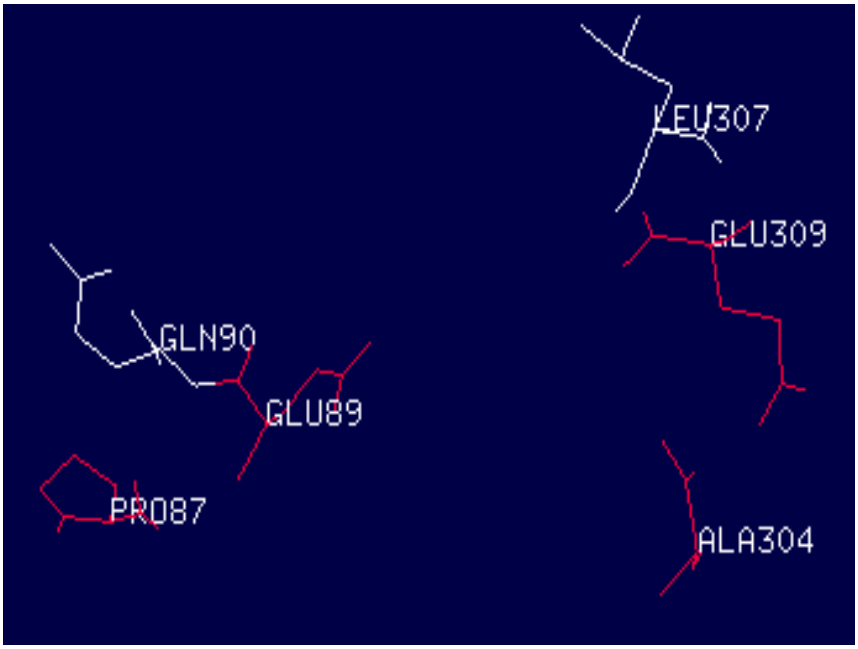

Figure 3.18. Localization of certain residues in Abf before mutation

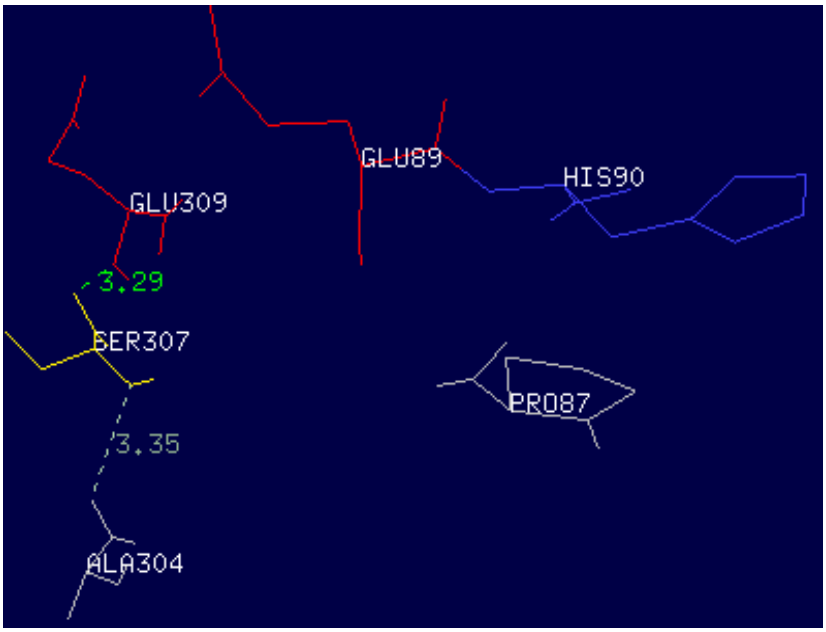

Figure 3.19. Relocalization of residues of mutation regions in Q90H/L307S

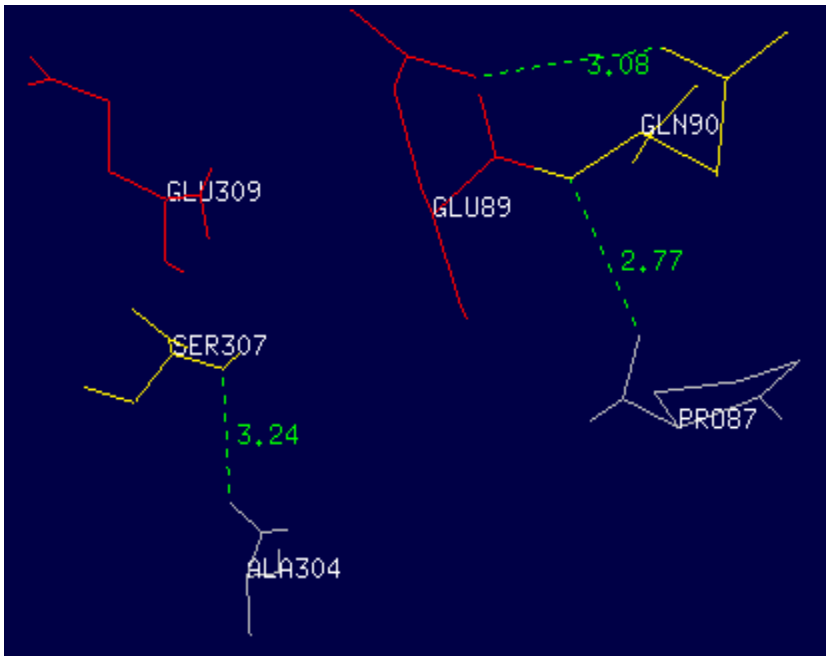

Figure 3.20. Relocalization of residues of mutation regions in L307S

The predicted solvent accessibilities of secondary structures of Abf and its mutants were investigated. According to this assay, it was observed that R447 was closer to enzyme surface in Abf when R447 was examined in Q90H/L307S and L307S mutants. Sokalingam and colleagues (2012) have shown that the stability of protein under alkaline conditions could increase when arginine residues located on the near of

protein surface. The reason of it was that arginine residues on protein surface could induce more electrostatic interactions (Sokalingam et al., 2012). Therefore, Abf might be more alkaline stability than Q90H/L307S and L307S. In addition, N105, N249 and N252 residues were farther and N302 residue was closer to enzyme surface in Abf when they were examined in Q90H/L307S and L307S mutants. In total, Asparagine residues moved away from enzyme surface to interior. Manikandan and colleagues (2006) have shown that the stability of protein under alkaline conditions could increase when asparagine residues located on the interior of protein. The reason was that deamination of asparagine residues could be caused (Manikandan et al., 2006). Hence, Abf might be more alkaline stability than Q90H/L307S and L307S. These data showed that it could be parallel with the experimental studies about alkaline stability.

CHAPTER 4

CONCLUSION

In this study, the principal objectives were to perform molecular identification of Abf by 16S rRNA analysis and to investigate the stability of Abf after the application of one round epPCR in the condition of basic pH and high temperature.

Firstly, partial 16S rDNA from *Geobacillus sp*. was cloned into pTZ57R/T, transformed to *E. coli* DH5α, sequenced by Sanger method, aligned by BLAST and performed the phylogenetic tree by MEGA 5. The result showed that the closest species was *G. vulcani* 3S-1 and named as *G. vulcani* GS90.

Secondly, PCR was carried out for Abf and its mutants, PCR products were cloned into pET28a(+) and 73 mutant colonies in total were screened in terms of total protein. In addition, seven colonies were found as functional and analyzed for their stabilities after purification. It was observed that two of them called as Q90H/L307S and L307S could be more stable than Abf against high temperature. Therefore, the stability profiles were constructed for Abf and these mutants. The nucleotide sequences of *abf* and these mutants and their aminoacid sequences were obtained, aligned with each other and constructed the predicted 3D structure in order to compare the changes in certain positions and the structure. The results showed that both two mutants could be more stable in optimum pH and high temperature when compared with Abf. In contrast, when the basic pH and optimum temperature were investigated, it was observed that these mutants could have less stable than Abf. The experimental results about thermostability and alkaline stability could be parallel with protein modelling studies

REFERENCES

Aryon, A. P., Wilson, B., Strauss, C. R., and Williams, P. J. 1987. The Properties of Glycosidases of Vitis vinifera and a Comparison of Their E-glucosidase Activity with that of Exogenous Enzymes. An Assessment of Possible Applications in Enology. *American Journal of Enology and Viticulture*. 38: 182–188.

Badal, C., Gwo-Jenn, S., Kailash, C., Lloyd, W., and Gregory, Z. 1989. New Thermostable α-Amylase-like Pullulanase from Thermophilc *Bacillus* sp. 3183. *Enzyme and Microbial Technology*. 11: 760–764.

Beckman, R. A., Mildan, A. S., and Loeb, L. A. 1985. On the Fidelity of DNA Replication: Manganese Mutagenesis in vitro. *Biochemistry*. 24: 5810-5817.

Beg, Q. K., Kapoor, M., Mahajan, L., and Hoondal, G. S. 2001. Microbial Xylanases and Their Industrial Applications: A Review. *Appied Microbioogy and Biotechnology*. 56: 326–338.

Betts, W. B., Dart, R. K., Ball, A. S., and Pedlar, S. L. 1991. Biosynthesis and Structure of Lignocellulose. In: Betts, W. B. (Ed). *Biodegradation: Natural and Synthetic Materials*. pp 139–155. Springer-Verlag, Berlin, Germany.

Bezalel, L., Shoham, Y., Rosenberg, E. 1993. Characterization and Delignification Activity of a Thermostable α-L-Arabinofuranosidase from *Bacillus stearothermophilus*. *Applied Microbiology and Biotechnology.* 40: 57-62.

Biely, P., Mackenzie, C. R., Puls, J., and Schneider, H. 1986. Cooperativity of Esterases and Xylanases in the Enzymatic Degradation of Acetyl Xylan. *Biotechnology*. 4: 731–733.

Biskup, S. E., Intert, F., Holthnujzen, J. H., Stengele, M., Schultz, G. 1993. Glycosically bound Volatiles-A review 1986–1991. *Flavour and Fragrance Journal*. 8: 61–80.

Blagodatski, A., and Katanaev, V. L. 2011. Technologies of Directed Protein Evolution in vivo. *Cellular and Molecular Life Sciences*. 68: 1207–1214.

Bloom, J. D., and Arnold, F. H. 2009. In the Light of Directed Evolution: Pathways of Adaptive Protein Evolution. *PNAS*. 106(1): 9995-10000.

Bradford, M. M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding. *Analytical Biochemistry*. 72: 248–254.

Cadwell, R. C., and Joyce, G. F. 1994. Mutagenic PCR. *Genome Research*. 3: 136–140.

Canakci, S., Kacagan, M., Inan, K., Belduz, A. O., and Saha, B. C. 2008. Cloning, Purification, and Characterization of a Thermostable α-L-arabinofuranosidase

from *Anoxybacillus kestanbolensis* AC26Sari. *Applied Microbiology and Biotechnology*. 81: 61–68.

Cobb, R. E., Sun, N., and Zhao, H. 2012. Directed Evolution as a Powerful Synthetic Biology Tool. *Methods*. Article in press.

Compton, S. J. and Jones, C. G. 1985. Mechanism of Dye Response and Interference in the Bradford Protein Assay. *Analytical Biochemistry*. 151: 369–374.

Congdon, R. W., Muth, G. W., and Splittgerber, A. G. 1993. The Binding Interaction of Coomassie Blue with Proteins. *Analytical Biochemistry*. 213: 407–413.

Coutinho, P. M., and Henrissat, B. 1999. Carbohydrate-Active Enzymes server at http://afmb.cnrs-mrs.fr/_/cazy/CAZY/index.html.

de Wet, B. J. M., Matthew, M. K. A., Storbeck, K. H., van Zyl, W. H., and Prior, B. A. 2008. Characterization of a Family 54 α-L-arabinofuranosidase from *Aureobasidium pullulans*. *Applied Microbiology and Biotechnology*. 77: 975–983.

Ebrahimi, M. 2010. Engineering Thermostable Xylanase Enzyme Mutant from *Bacillus halodurans*. *African Journal of Biotechnology*. 9 (47): 8110-8117.

Gilead, S., and Shoham, Y. 1995. Purification and Characterization of Alpha-L-arabinofuranosidase from *Bacillus stearothermophilus* T-6. *Applied and Environmental Microbiology*. 61: 170–174.

Greve L. C., Labavitch, J. M., Hungate, R. E. 1984. Alpha-L-Arabinofuranosidase from *Ruminococcus albus* 8: Purification and Possible Roles in Hydrolysis of Alfalfa Cell Wall. *Applied and Environmental Microbiology*. 47: 1135–1140.

Gobbetti, M., Lavermicocca, P., Minervini, F., De Angelis, M., and Corsetti, A. 2000. Arabinose Fermentation by *Lactobacillus plantarumin* Sourdough with Added Pentosans and Alpha-L-arabinofuranosidase: A Tool to Increase the Production of Acetic Acid. *Journal of Applied Microbiology*. 88: 317–324.

Haki, G. D., and Rakshit, S. K. 2003. Developments in Industrially Important Thermostable Enzymes: A Review. *Bioresource Technology*. 89: 17–34.

Hakulinen, N., Turunen, O., Janis, J., Leisola, M., and Rouvinen, J. 2003. Three-dimensional Structures of Thermophilic β-1,4-Xylanases from *Chaetomium thermophilum* and *Nonomuraea flexuosa*: Comparison of Twelve Xylanases in Relation to Their Thermal Stability. *Eur J Biochem*. 270: 1399-1412.

Hata, K., Tanaka, M., Tsumuraya, Y., and Hashimoto, Y. 1992. α-L-Arabinofuranosidase from Radish (*Raphanus sativus* L.) Seeds. *Plant Physiology*. 100: 388-396.

Henrissat, B., and Davies, G. 1997. Structural and Sequence-based Classification of Glycoside Hydrolases. *Current Opinion in Structural Biology*. 7: 637–644.

Howard, R. L., Abotsi, E., Jansen van Rensburg, E. L., and Howard, S. 2003. Lignocellulose Biotechnology: Issues of Bioconversion and Enzyme Production. *African Journal of Biotechnology*. 2 (12): 602-619.

Hövel, K., Shallom, D., Niefind, K., Belakhov, V., Shoham, G., Baasov, T., Shoham, Y., and Schomburg, D. 2003. Crystal Structure and Snapshots along the Reaction Pathway of a Family 51 α-L-arabinofuranosidase. *The EMBO Journal*. 22 (19): 4922-4932.

İlgü, H. 2011. *Isolation, Expression and Characterization of an α-L-Arabinofuranosidase Enzyme from Thermophilic Geobacillus sp*. İzmir Institute of Technology. Graduate School of Engineering and Science. Unpublished Master Thesis: İzmir.

Jackel, C., Kast, P., and Hilvert, D. 2008. Protein Design by Directed Evolution. *Annual Review of Biophysics*. 37: 153-173.

Kaji, A. 1984. L-Arabinosidases. *Advances in Carbohydrate Chemistry and Biochemistry*. 47: 383–394.

Kashefi, K., and Lovley, D. R. 2003. Extending the Upper Temperature Limit for Life. *Science*. 301:934.

Khandeparker, R., and Numan, M. T. 2008. Bifunctional Xylanases and Their Potential Use in Biotechnology. *Journal of Industrial Microbiology and Biotechnology*. 35: 635-644.

Kohara, Y., Akiyama, K., and Isono, K. 1987. The Physical Map of the Whole E. coli Chromosome: Application of a New Strategy for Rapid Analysis and Sorting of a Large Genomic Library. *Cell*. 50: 495–508.

Kaur, J., and Sharma, R. 2006. Directed Evolution: An Approach to Engineer Enzymes. *Critical Reviews in Biotechnology*. 26: 165–199.

Mamo, G., and Gessese, A. 1999. A Highly Thermostable Amylase from a Newly Isolated Thermophilic *Bacillus sp*. WN11. *Journal of Applied Microbiology*. 86: 557–560.

Manikandan, K., Bhardwaj, A., Gupta, N., Lokanath, N. K., Ghosh, A., Reddy, V. S., and Ramakumar, S. 2006. Crystal Structures of Native and Xylosaccharide-bound Alkali Thermostable Xylanase from an Alkalophilic *Bacillus sp.* NG-27: Structural Insights into Alkalophilicity and Implications for Adaptation to Polyextreme Conditions. *Protein Science*. 15: 1951-1960.

Mateo, J. J., Jimènez, M. 2000. Monoterpenes in Grape Juice and Wines. *Journal of Chromatography A*. 881: 557–567.

Mchunu, N. P., Singh, S., and Permaul, K. 2009. Expression of an Alkalo-tolerant Fungal Xylanase Enhanced by Directed Evolution in *Pichia pastoris* and *Escherichia coli*. *Journal of Biotechnology*. 141: 26-30.

McMillan, J. D. 1993. Pretreatment of Lignocellulosic Biomass. In: Himmel, M. E., Baker, J. O., Overend, R. P., editors. *Enzymatic Conversion of Biomass for Fuel Production*. American Chemical Society. Washington, DC:.

Miyazaki, K, and Arnold, F. H. 1999. Exploring Nonnatural Evolutionary Pathways by Saturation Mutagenesis: Rapid Improvement of Protein Function. *Journal of Molecular Evolution*. 49: 716-720.

Morrison, I. M. 1982. The Degradation of Isolated Hemicellulose and Lignin-Hemicellulose Complexes by Cell Free, Rumen Hemicellulase. *Carbohydrate Research*. 101: 93-100.

Morozkinaa, E. V., Slutskaya, E. S., Fedorova, T. V., Tugay, T. I., Golubeva, L. I., and Koroleva, O. V. 2010. Extremophilic Microorganisms: Biochemical Adaptation and Biotechnological Application (Review). *Applied Biochemistry and Microbiology*. 46 (1): 1-14.

Nazina, T. N., Lebedeva, E. V., Poltaraus, A. B., Tourova, T. P., Grigoryan, A. A., Sokolova, D. Sh., Lysenko, A. M., and Osipov, G. A. 2004. *Geobacillus gargensis sp*. nov., a Novel Thermophile from a Hot Spring, and the Reclassification of *Bacillus vulcani* as *Geobacillus vulcani* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*. 54: 2019–2024.

Numan, M. T., and Bhosle, N. B. 2006. Alpha-L-Arabinofuranosidases: The Potential Applications in Biotechnology. *Journal of Industrial Microbiology and Biotechnology*. 33 (4): 247-260.

Puls, J., and Schuseil, J. 1993. Chemistry of Hemicelluloses: Relationship between Hemicellulose Structure and Enzyme Required for Hydrolysis. In: Coughlan, M. P,, Hazlewood, G. P. (eds). *Hemicellulose and Hemicellulases*. Portland Press, London, pp 1–28.

Rothschild, L. J., and Mancinelli, R. L. 2001. Life in Extreme Environments. *Nature*. 409: 1092-1101.

Ruller, R., Deliberto, L., Ferreira, T. L., and Ward, R. J. 2008. Thermostable Variants of the Recombinant Xylanase A from *Bacillus subtilis* Produced by Directed Evolution Show Reduced Heat Capacity Changes. *Proteins: Structure, Function and Bioinformatics*. 70: 1280-1293.

Saha, B. 2000. Alpha-L-Arabinofuranosidases - Biochemistry, Molecular Biology and Application in Biotechnology. *Biotechnology Advances*. 18: 403 – 423.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd Edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sebastien, Z., Jean-Luk, R., Dider, F., Yannik, G., Joseph, B., and Jacques, D. 2001. Characterization of Highly Thermostable Alkaline Phosphatase from the

Euryarchaeon *Pyrococcus abyssi*. *Applied and Environmental Microbiology*. 67: 4504–4511.

Seri, K., Sanai, K., Matsuo, N., Kawakubo, K., Xue, C., and Inoue, S. 1996. L-arabinose Selectively Inhibits Intestinal Sucrase in Uncompetitive Manner and Reduces Glycemic Response After Sucrose Ingestion in Animals. *Metabolism*. 45: 1368–1374

Shallom, D., and Shoham, Y. 2003. Microbial Hemicellulases. *Current Opinion in Biotechnology*. 6: 219-28.

Shulami, S., Raz-Pasteur, A., Tabachnikov, O., Gilead-Gropper, S., Shner, I., and Shoham, Y. 2011. The L-Arabinan Utilization System of *Geobacillus stearothermophilus*. *Journal of Bacteriology*. 193 (11): 2838-2850.

Singh, S. M., and Panda, A. K. 2005. Solubilization and Refolding of Bacterial Inclusion Body Proteins. *Journal of Bioscience and Bioengineering*. 99: 303-310.

Sokalingam, S., Raghunathan, G., Soundrarajan, N., and Lee, S-G. 2012. A Study on the Effect of Surface Lysine to Arginine Mutagenesis on Protein Stability and Structure Using Green Fluorescent Protein. *PLoS ONE*. 7 (7): 1-12.

Solomon, K. R. 1996. Chlorine in the Bleaching of Pulp and Paper. *Pure and Applied Chemistry*. 68 (9): 1721-1730.

Sørensen, H. R., Pedersen, S., Viksø-Nielsen, A., and Meyer, A.S. 2005. Efficiencies of Designed Enzyme Combinations in Releasing Arabinose and Xylose from Wheat Arabinoxylan in an Industrial Ethanol Fermentation Residue. *Enzyme and Microbial Technology*. 36: 773–784.

Stephens, D. E., Singh, S., and Permaul, K. 2009. Error-prone PCR of a Fungal Xylanase for Improvement of Its Alkaline and Thermal Stability. *FEMS Microbiol Letters*. 293: 42–47.

Subramaniyan, S., and Prema, P. 2002. Biotechnology of Microbial Xylanases: Enzymology, Molecular Biology and Application. *Critical Reviews in Biotechnology*. 22 (1): 33-46.

Taiz, L., and Zeiger, E. 2010. Cell Wall: Structure, Biogenesis and Expansion. Fifth eds. Plant Physiology.

van den Burg, B. 2003. Extremophiles as a Source for Novel Enzymes. *Current Opinion in Microbiology*. 6: 213-218.

Vieille, C., and Zeikus, G. J. 2001. Hiperthermophilic Enzymes: Sources, Uses, and Molecular Mechanisms for Thermostability. *Microbiology and Molecular Biology Reviews*. 65: 1-43.

Wang, Q., and Xia, T. 2008. Enhancement of the Activity and Alkaline pH Stability of *Thermobifida fusca* Xylanase A by Directed Evolution. *Biotechnology Letters*. 30: 937-944.

Wood, T. M., Castanares, A., Smith, D. C., McCrae, S. I., and Brown, J. A. 1992. Xylan-Degrading Enzyme Systems of the Fungi Aspergillus Awamori and *Penicillium pinophilum*: Studies on the Isolation of Overproducing Mutant Strains and Aspects of the Enzymology. In: Visser, J., Beldman, G., Kusters-Van, Someren, M. A, Voragen, A. G. J, editors. *Xylans and Xylanases*. Amsterdam, The Netherlands: Elsevier Science Publishers. pp. 187–202.

Zhang, Y. 2008. I-TASSER Server for Protein 3D Structure Prediction. *BMC Bioinformatics*. 9: 40-48.

Zhang, J. H., Dawes, G., and Stemmer, W. P. 1997. Directed Evolution of a Fucosidase from a Galactosidase by DNA Shuffling and Screening. *PNAS*. 94: 4504-4509.

APPENDIX A

CHEMICALS AND ENZYMES

Agar

Trypton

Yeast Extract

NaCl

dNTP set

$MgCl_2$

Glycerol

Commasie brilliant blue G-250

PIPES

Taq DNA polymerase

Commasie brilliant blue R-250

EDTA

Tris base

Glacial acetic acid

Etidium bromide

Bromophenol blue

DNA ladder

Nde I

Hind III

T4 DNA ligase

pTZ57R/T

pET28a(+)

pG-Tf2

DNA ladder

pNP-α-L-arabinofuranoside

Sodium carbonate

APPENDIX B

MEDIA

Luria Bertani (LB) broth, per liter

- Tryptone (10 g)
- Yeast extract (5 g)
- NaCl (5 g)

were dissolved in one liter dH2O and autoclaved at 121^{o}C 15 min.

Luria Bertani (LB) agar, per liter

- Tryptone (10.0 g)
- Yeast extract (5.0 g)
- NaCl (5.0 g)
- Agar (15.0 g)

were dissolved in one liter dH_2O and autoclaved at 121^{o}C 15 min.

SOC medium, per 100 ml

- Tryptone (2.0 g)
- Yeast extract (0.5 g)
- 1.0 M NaCl (1.0 ml)
- 1.0 M KCl (0.25 ml)
- 2.0 M Mg^{2+} (1.0 ml)
- 2.0 M Glucose (1.0 ml)

were mixed, dissolved in 100 ml dH_2O and autoclaved at 121^{o}C 15 min

APPENDIX C

REAGENTS AND BUFFERS

Citrate Buffer; pH 5.0

In order to prepare the 50 mM citrate buffer pH 5.0, 20.5 ml 0.1 M citric acid and 29.5 ml 0.1 M sodium citrate was mixed and diluted by dH_2O in equal amount to obtain 50 mM solution. pH. 5.0 was checked by pH meter.

Tris-Cl Buffer, 1.0 M; pH 8.0

121.1 g Tris base was dissolved in 750 ml of dH_2O. pH of buffer was adjusted to 8.0 by concentrated HCl. This solution was completed into 1 liter by dH_2O. Finally, stock solution was diluted to 20 mM by dH_2O.

Glycine-NaOH Buffer; pH 9.6

0.2 M glycine and 0.2 M NaOH were prepared and 25.0 ml from glycine stock solution was mixed with 11.2 ml 0.2 M NaOH and pH 9.6 was checked by pH meter. Finally, diluted by dH_2O in order to obtain 50 mM buffer.