INVESTIGATIONS ON THE N-LINKED GLYCOSYLATION OF THE *ASPERGILLUS NIGER* LIPASE IN *PICHIA PASTORIS*

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Submitted to Graduate School of Engineering and Natural Sciences

in partial fulfillment of the requirements for the degree of

Master of Science

Sabancı University

August 2014

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DATE OF APPROVAL: August 6, 2014

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BIO, MSc Thesis, 2014

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Keywords: Protein Engineering, Lipase, N-Linked Glycosylation, Thermostability,

Site-directed Mutagenesis.

Abstract

Aspergillus niger lipase naturally exists as an extracellular 1,3-specific lipase with molecular weight of 35-40 kDa. The optimal temperature for ANL activity lies between 30°C-50°C, while the optimal pH is between 5.0-6.0. As the native *Aspergillus niger* lipase contains sugar modified residues, here the aim is to determine the impacts of N-linked glycosylation of ANL in *Pichia pastoris*. ANL variants generated in this study are single or double combination of asparagine mutations which as N59Q, N150Q, N269Q are referred to ANL1, ANL2 and ANL3 respectively. These asparagine sites were predicted using glycolsylation servers NetNGlyc and GlycoEP. Native ANL, as well as two other mutants (ANL2, ANL1-2) out of three mutants (ANL1, ANL2 and ANL3) are obtained in high purity through routine molecular biology and protein engineering tools including site-directed mutagenesis, ligation-independent cloning, heterologous protein expression and affinity purification methods. For characterization of glycosylation patterns, native and mutant ANL were treated with EndoHf and analyzed with SDS-PAGE. The native ANL and the mutants were also used in lipase assays to determine thermostability. The results indicated that the mutations possess differential glycosylation patterns than those of the native lipase. According to the SDS-PAGE analysis, native ANL was observed at 35-40 kDa with a smear appearance, while the mutants that contain N150Q were between 30-35 kDa and the other mutants did not change the native features. This result indicates that mutation in the 150th aminoacid has significant effect to alter the glycosylation of ANL. After EndoHf treatment, the smear appearances of the native the mutants disappeared, and all of the proteins were observed as single band at 35 kDa. The thermostability results showed that all of the lipases including the native ANL had the maximal activity at 30-45°C, indicating that the mutations did not have any effect on thermostability of the native ANL until 50°C. N-linked glycosylation site (N150Q) in ANL had a major impact in glycosylation of ANL in *P. pastoris*, while the other two mutations (N59Q, N269Q) were not included in glycosylation mechanism. Finally, the scope of this thesis could be used to generate mutations with altered post-translational modifications and thus altered stability. As a result, the findings of this thesis would impose implications in enzymology which aims to design enzymes with optimal features.

ASPERGILLUS NIGER LIPAZ'IN PICHIA PASTORIS'DEKI N-BAĞLI GLIKOZILASYON ILE ILGILI ARAŞTIRMA

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BIO, Yüksek Lisans Tezi, 2014

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Anahtar Kelimeler: Protein Mühendisliği, Lipaz, N-Bağlı Glikozilasyon, Termoaktivite, Yönlendirilmiş Mutagenez

Özet

Aspergillus niger lipazı doğal olarak 35-40 kDa moleküler ağırlığında olan 1,3 özlü ekstraselüler bir enzimdir. ANL'nin sıcaklığa karşı optimum aktivitesi 30^{o}C-50^{o}C aralığında ve ANL'nin pH'a karşı optimum aktivitesi 5.0-6.0'dır. Asıl *Aspergillus niger* lipazı şeker içermektedir; bu sebepten dolayı bizim buradaki amaçımız N-bağlı glikozilasyonın *Pichia pastoris*'deki etkilerini belirlemektir. Bu araştırma içerisinde tekli ve çiftli asparajinin mutasyonuna (ANL1, ANL2 ve ANL3) bağlı olarak değişiklik gösteren ANL'ler üretilmiştir. Bu asparajin lokasyonları NetNGlyc 1.0 sunucusu ve GlycoEP sunucusu aracılığıyla elde edilmiştir. ANL ile birlikte üç mutant ANL'nin ikisi (ANL2, ANL1-2), protein mühendisliği rutin yöntemlerinden olan yönlendirilmiş mutagenez, ligasyondan bağımsız klonlama, heterolog protein ekspresyonu, afinite purifikasyonu kullanılarak yüksek saflıkta elde edilmiştir. Glikozilasyon desenlerinin karakterizasyonu için, asıl ve mutant ANL'ler EndoHf ile işlenmiş olup analizleri SDS-PAGE ile yapılmıştır. Ayrıca asıl ve mutant ANL'lerin termostabilitesi denemeleri yapılmıştır. Sonuçlara göre mutasyona uğrayan ANL'lerin glikozilasyon desenleri asıl ANL'den farklılık göstermektedir. SDS-PAGE analizlerine göre, asıl ANL bulanık bir görüntüyle 35-40 kDa arasında görünmekte olup, N150Q mutasyonuna sahip olan ANL bulanık bir görüntüyle 30-35 kDa arasında görünmektedir. Diğer mutasyona sahip ANL örnekleri asıl ANL'den farklılık göstermemektedir. Bu sonuç 150. aminoacidin ANL'nin glikozilasyonunu değiştirerek etki ettiğini göstermektedir. EndoHf işleminden sonra, bulanık olan görüntü kaybolmakta ve bütün proteinler tek bir şerit halinde 35 kDa olarak gözlenmiştir. Termostabilite sonuçlarına göre asıl ANL'de dahil olmak üzere tüm lipazlar maksimum aktiviteye 30-45^{o}C arasında sahiptir. Bu durumda mutasyonların 50 dereceye kadar termostabiliteye bir etkisi olmadığı görülmektedir. P. *pastoris*'deki ANL'nin içerisindeki N-bağlı glikozilasyon yerinin (N150Q) glikozilasyona etkisi büyük olup, diğer iki mutasyonun glikozilasyon mekanizmasında önemi yoktur. Son olarak, bu çalışma translasyon sonrası modifikasyonları içeren dolayısıyla stabilite değişimini sağlayacak mutasyonların üretimini kapsamaktadır. Böylelikle bu çalışma, optimal özellikteki enzimlerin tasarımını hedefleyerek, enzimoloji dalındaki uygulamalara katkı sağlayacaktır.

Acknowledgements

Foremost, I would like to express my sincere gratitude to my advisor Prof. Dr. Uğur Sezerman for continuous support of my Ms. study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research, rehab, and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ms. study.

Besides my advisor, I also express my gratitude to my thesis jury members; Prof. Dr. Selim Çetiner, Asst. Prof. Dr. Alpay Taralp. I am grateful to all my thesis jury for accepting to be part of my committee. I also convey my eternal gratitude to Dr. Emel Durmaz Timuçin who stayed by me whenever I needed help not only during my thesis period but also all my education and academic life.

My sincere thanks also go to Prof. Dr. Yury Chernoff and Dr. Denis Kiktev, for offering me the summer intership opportunities in their groups and leading me working on diverse exciting projects.

I express my sincere thanks to my friends: Batuhan Orbay Yenilmez, Deniz Adalı, Duygu Soysal, Hazal Yılmaz, Edip Çoban and for their precious thoughts and emotional support during my thesis process.

Last but not the least, I would like to thank my sister and my parents who supported me in every step of my life and reminded me that no matter what happens, they will always be there for me.

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List of Symbols and Abbreviations

4MU-cap	4-Methylumbelliferyl caprylate
ANL	*Aspergillus Niger* lipase
ANL1-2	*Aspergillus Niger* lipase with mutations N59Q and N150Q
ANL2	*Aspergillus Niger* lipase with mutation N150Q
ANL2-3	*Aspergillus Niger* lipase with mutations N150Q and N269Q
N59Q	Asparagine to glutamine mutation at residue 59
N150Q	Asparagine to glutamine mutation at residue 150
N269Q	Asparagine to glutamine mutation at residue 269
SDS-PAGE	Sodium doedecyl sulphate-polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
WT	Wild type which is ANL

1 INTRODUCTION

1.1 Lipases

1.1.1 Background

Lipases are metabolic enzymes that are found in every domain of life. Various types of lipases are obtained from animals, plants and microorganisms. Lipases were discovered in the early 20th century by Eijkmann while he was simply observing the several bacteria which are able to produce and secrete lipases and the degradation of lipids by the help of these produced enzymes. Microbial enzymes are one of the most prominent and the largest classes of enzymes because of the diverse variety of microbes which are identified and studied in detail [1, 2].

Lipases or triacylglycerol acylhydrolases (EC 3.1.1.3) are hydrolytic enzymes that catalyze reversibly the cleavage of ester bonds of triglycerides on water-insoluble substrates. During the digestion, transportation and processing of triglycerides lipases have a crucial role. Bacteria, fungi and yeast are the organisms which take a high responsibility to produce lipases. Since lipases come from different sources, they can operate in wide range of environmental conditions (pH and temperature) [3, 4].

1.1.2 Reactions

Lipase is the enzyme which hydrolyzes triacylglycerols (TAG) to free fatty acids (FA), diacylglycerols (DAG), monoacylglycerols (MAG) and glycerol [5]. Hydrolysis is an equilibrium reaction; therefore concentration of reactants and products may lead to change and affect the reaction. In this equilibrium one reactant is water; therefore, changing the hydrolytic conditions of the reaction shifts the equilibrium from forward to reverse or vice versa [6].

Apart from hydrolysis, the reverse reaction perfoming (esterification, transesterification and its sub-divides such as acidolysis, interesterification, alcoholysis) can be catalyzed by lipases in anhydrous organic solvents, biphasic systems and micellar solution (Figure 1.1) [7.8].

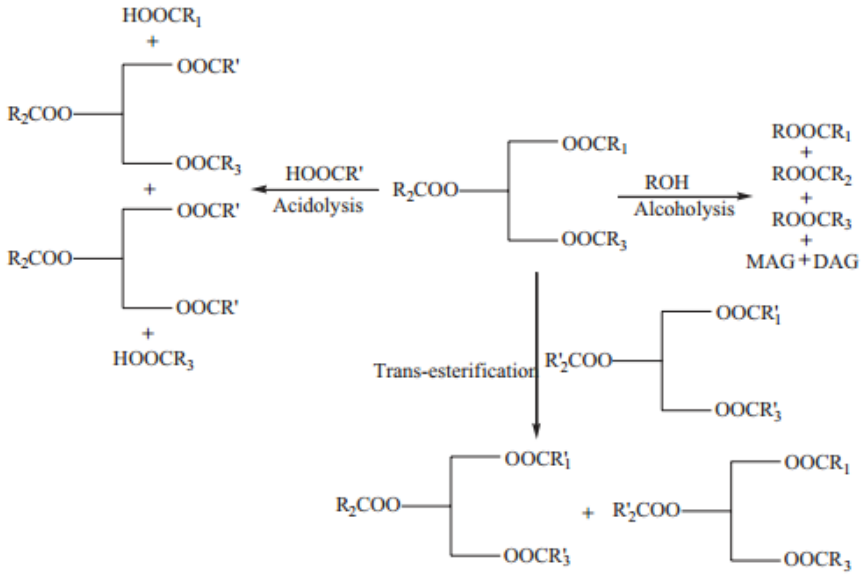

Figure 1.1: Different Lipase-catalyzed Reactions in Aqueous and Non-aqueous Solutions.

In aqueous solutions, the equilibrium moves towards ester hydrolysis, and in non-aqueous solutions the equilibrium moves towards ester synthesis. Lipases are capable of catalyzing acyl transfer reactions to synthesize new esters in organic solvents [7].

Lipases can be seen in different roles during hydrolysis reaction or esterification. During esterification lipases are in the habit of forming carboxylic ester bonds and catalyzing acyl transfer reactions whereas during hydrolysis reaction lipases break carboxylic ester bonds. The natural substrates of the lipases are the triacylglycerols [9, 10]. The process is followed as formation of an unstable acyl-enzyme intermediate, breakdown of free enzyme and acid in hydrolysis or to free enzyme and an ester in esterification and transesterification.

As mentioned earlier, chemical properties of the reactants and presence of water can affect the results of reactions in the lipase-catalyzed reactions. When there is limited amount of accessible water the reaction, the reaction may produce carboxyl/thiolester or amide. The ester group is able to form the acyl-enzyme intermediate due to releasing an acid by the acyl donor (water as acyl acceptor) or forming a new ester [9].

Transesterification is the conversion of a carboxylic acid ester into a different carboxylic acid ester. The most common method of transesterification is the reaction of the ester with an alcohol in the presence of an acid catalyst as shown in Figure 1.1.

Apart from, the hydrolysis and the synthesis of carboxylic esters, lipases can also utilize compounds excluding water and alcohol as nucleophiles through various reactions including aminolysis, thiotransesterification and oximolysis in organic solvents with selectivity [7].

1.1.3 Mechanism

Eventhough lipases catalyze diverse set of reactions; the fact that each reaction mechanism has its own properties means that lipases are distinctive from one another. Catalytic machinery is conserved for all lipases and is composed of serine, histidine and aspartate/glutamate aminoacids [11]. In order to lower the pKa of the serine hydroxyl, histidine and aspartate/glutamate are aligned. Thus nucleophilic attacks on the ester bond can be carried out by the serine. The acyl donor, the substrate, interacts with the active site of the lipase, forming the enzyme-substrate (ES) complex. In the catalytic triad, histidine activates the hydroxyl group of serine which leads to serine to carry out a nucleophilic attacks on the carbonyl carbon of the substrate. Thus the formation of the first tetrahedral intermediate is demonstrated. The main-chain amide groups of two residues create a hole which stabilizes the negative charge on the oxyanion. On the other hand the positive charge on the histidine is stabilized by the aspartate/glutamate. Another intermediate is determined by the first leaving group of the substrate after the decomposition of tetraheadral intermediate. However due to the formation of the acyl enzyme intermediate, a second tetrahedral intermediate is formed. This formed intermediate has the highest energy barrier in the reaction. In order to yield the deacylated-free form of the enzyme and the hydrolysis of the second substrate, an acid, this intermediate is also collapsed. There is a transfer of the proton from the substrate to histidine during the deacylation step.

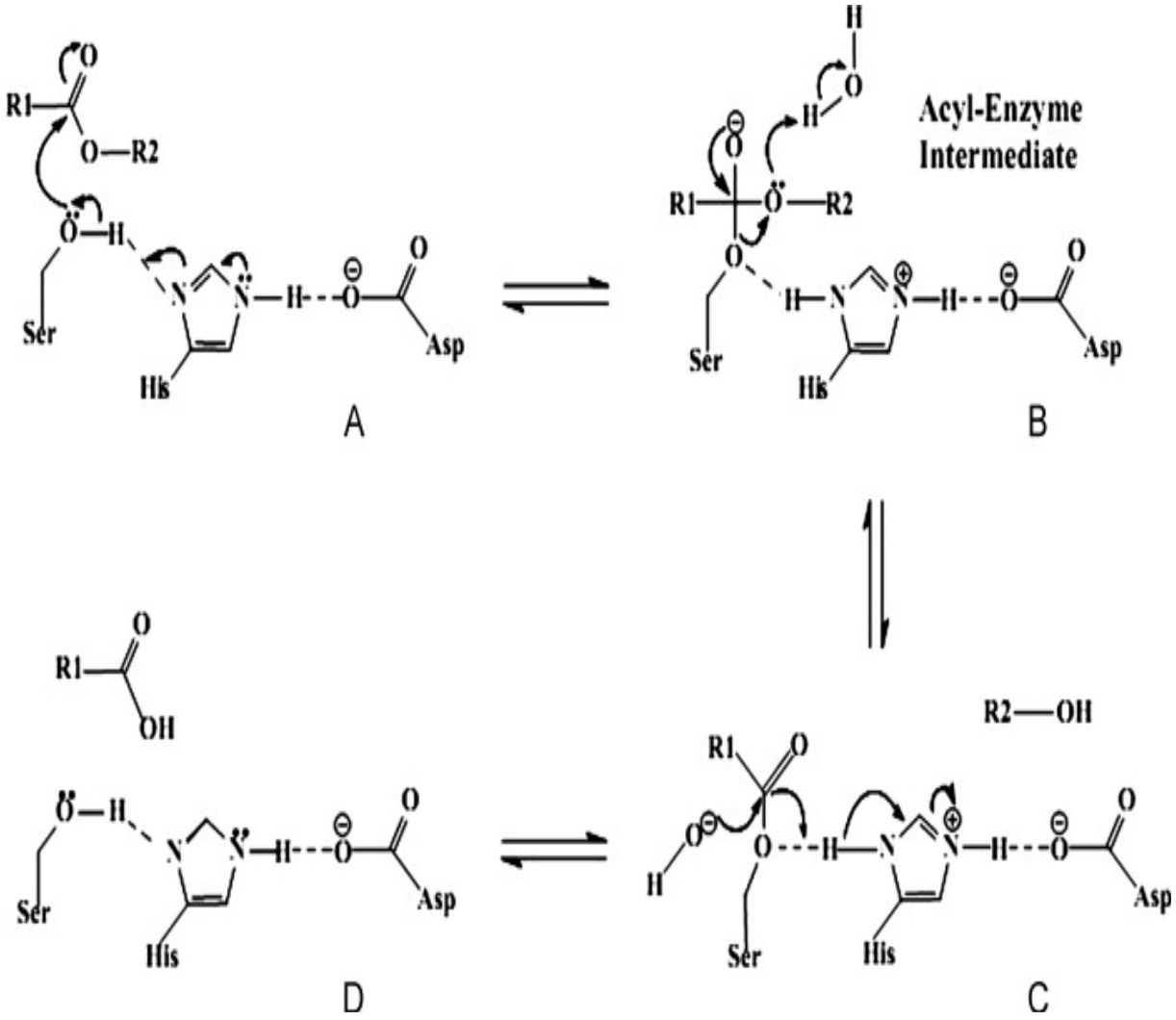

Figure 1.2: Mechanism of hydrolysis by lipases.

During Step A, His residue acts as a general base and removes a proton from the active site of Ser. In Step B, an acyl-enzyme intermediate is formed, followed by the deacetylation (Step C). With a nucleophile attacking the acetylated enzyme, the catalytic site is regenerated and a long-chain fatty acid is formed as a product (Step D). [7]

1.1.4 Substrate Selectivity

Lipases show different selectivity towards their ligands (certain fatty acids or groups of fatty acids) such as the type and chain-length of the acyl group in their substrates. To illustrate that a lipase called porcine has specificity to cis-2 over cis-7 octadecenoyl moiety [2] and also the *Aspergillus flavus* lipase exhibits higher selectivity for tricaprin than the triolein [16]. Furthermore, primary alcohols are mostly preferred by lipases, but tertiary alcohols are least preferred by lipases [5]. Lipases can accommodate not only triglycerides and aliphatic esters in their catalytic pockets but also the different compounds such as alicylic, bicyclic and aromatic esters as well as thioesters and activated amines, suggesting that they possess a preference over a wide range of substrate molecules [14–16]. Many studies in the literature focus on the chain-length selectivity of lipases [17-20]. The range of the chain-length which is preferred by lipases changes from short (C4) to is a range of medium (C8) to long (C16) chain-length of fatty acids [2]. Apart from all of these general knowledge there are some exceptions can be seen such as *R. miehei* lipase can hydrolyze esters of long chain fatty acids as long as C22 [21].

1.1.5 Structure

The first lipase structure has been crystallized by Brady in 1990 [11]. Nowadays it is possible to find more than one hundred crystallized lipase structures in Protein Data Bank (PDB). Careful studies of these structures revealed several common features of lipases. The first common feature is that all of the lipases are members of "α/β-hydrolase fold" family which has a core structure composing of parallel β strands which are surrounded by α helices [22-25]. The second common feature is that lipases have an active site which is formed by a catalytic triad composing of serine, histidine and aspartic acid/glutamic acid amino acids. Although chemically similar catalytic machinery is shared by lipases and proteases, structurally different active sites are possessed by lipases due to the distinct orientation of the seryl hydroxyl group to give rise to an inverted stereochemistry of catalytic triad in lipases [25-27]. The third common feature is that all of the lipases have a catalytic serine residue

which is found in a hairpin turn between an α-helix and α-helix/β-sheet in a highly conserved motif of the pentapeptide G-X-S-X-G. This sequence forms a specific β-turn-α motif which is called the "nucleophilic elbow" [22, 23, 25]. The fourth common feature is that four binding pockets form the catalytic cleft of the lipases; three binding pockets hold the *sn*-1, *sn* -2 and *sn* -3 acyl chains of triacylglycerol as well as an oxyanion hole which is formed by two backbone amides of two residues located in the N-terminal region of the lipase and the C-terminal of the catalytic serine [29, 30]. The last common feature is that they all have a lid or flap which consists of two amphiphilic helices [14] which serves to illustrate that the lipase of guinea-pig has a "mini-lid" composed of five amino acids [28].

According to Pleiss et al. (1998) from the point of geometry, the catalytic cleft of lipases can be catagorized into three groups which are hydrophobic crevice-like, tunnel-like and funnel-like binding sites [31]. The binding pocket variability of lipases may contribute to different substrate selectivities and also determine stereoselectivityby means of the possible steric interaction in the cleft.

1.1.6 Lipases and Industry

Microbial lipases are considered as a significant group of enzymes which have biotechnological impact not only due to their versatile properties such as selectivity but also to the ability of the mass production with relatively low costs via fermentation [32]. In addition, microbial lipases can operate in a large variety of environmental conditions. These characteristics make them favorable for industrial applications. Most detergent and cosmetics markets, for example, depend on the specific selectivity feature of lipase. Lipases therefore are seen as one of the most important groups of commerical enzymes [33].

Catalytic reactions of lipases occur in aqueous and organic media; therefore they are mostly attractive for solving the challenging synthesis of organic reactions [34]. Due to the broad use of lipases in industry lipases, they are seen as an attractive candidate for the application of protein engineering. Microbial lipases are a favored source for many industrial applications due to their extracellular nature since they can be isolated and purified relatively easily and thus produced at large quantities. According to scientific articles and reviews about lipases not requiring cofactors, stability in organic solvents, possessing wide substrate selectivity at

high temperatures and ionic strengths, as well as high enantioselectivity are the main reasons for use in industrial applications [45].

In the food processing industry modified fats and oils, key food constituents, are one of the important fields of biotechnology. Lipases can be easily modified due to their lipid properties through changing the fatty acid chain locations in the glyceride or changing the location of one or more of these with novel lipases. Therefore, relatively low-priced and less favorable fats could be modified to a fat which has higher value [36]. To illustrate lipases are used by various modification of the fatty acid chain lengths to hydrolyze the milk fat and to enhance the flavor of cheese flavor [1]. Another example of the usage of lipases in the food industry is in the flavor enhancement of the bakery products by freeing short chain fatty acid chains using transesterification and prolonging the shelf lives. Table 1.1 depicts the other industrial areas where the lipases are mostly used.

Industry	Effect	Product
Bakery	Flavour improvement and shelf-life prolongation	Bakery products
Beverages	Improved aroma	Beverages
Chemical	Enantioselectivity	Chiral building blocks and chemicals
Cleaning	Synthesis Hydrolysis	Chemicals Removal of cleaning agents like surfactants
Cosmetics	Synthesis	Emulsifiers, moisturising agents
Diary	Hydrolysis of milk fat Cheese ripening Modification of butter fat	Flavour agents Cheese Butter
Fats and oils	*Trans*-esterification Hydrolysis	Cocoa butter, margarine Fatty acids, glycerol, mono- and diglycerides
Food dressing	Quality improvement	Mayonnaise, dressings and whippings
Health food	*Trans*-esterification	Health food
Leather	Hydrolysis	Leather products
Meat and fish	Flavour development and fat removal	Meat and fish products
Paper	Hydrolysis	Paper products
Pharmaceuticals	*Trans*-esterification Hydrolysis	Speciality lipids Digestive aids

Table 1.1: List of the industrial areas that lipases are used and their effects [1]

1.1.7 Engineering Lipases

In the application of chemical catalysis there is a certain pattern should be followed by all enzyme catalysis. Therefore, chemical catalysis might be seen as inferior to enzyme catalysis under the circumstances that means of less efficiency and specificity of chemical catalysis. Specific products are produced in the selective nature of reactions for enzymes via applying enzymes as catalysts. Therefore, use of the selective nature of an enzyme might decrease waste products or energy consumption.

It is the general knowledge that the function ability of enzymes and proteins depends on the lock and key theory. Therefore the structure and function of the lipases also depends on this theory. Under these circumstances protein engineering takes a crucial place leading to generate new lipases with altered substrate selectivities and improved stabilities. As mentioned in earlier sections, lipases are used in many industrial fields; therefore, lipases that are obtained via protein engineering research for harsh conditions such as showing activity at various pH scale(s), at high temperatures might breakdown the limitations of lipases if their native cannot cope with harsh conditions [37].

1.2 Protein Engineering

1.2.1 Background

Protein engineering can be defined as the design and construction of new proteins or enzymes with novel or desired functions by modifying primary structure of protein by using recombinant DNA technology [38]. The major principles of the protein engineering are rational approach using three-dimensional (3D) models and random approach using directed evolution [39]. By applying protein engineering, it is easy to manipulate and clarify the folding of protein and stability or structure-function relationships [40]. Thus protein engineering is an important tool with crucial impact over industrial and research applications. In order to design the drugs for treatments, for example, protein engineering has a great impact in therapeutics research, whereas it also has an effect on industrial enzymes [38].

1.2.2 Random and Rational Approach

Design, mutagenesis and production are three major steps of protein engineering methodology. To start protein engineering, prior information about the structure and sequence of the protein or gene of interest can be required. In a rapid changing biological world, different methods of protein engineering are applied such as a random approach or rational approach to obtain desired proteins [38]. Nowadays, the most common method is known as recombinant DNA technologies.

The random approach is the selection of improved proteins/enzymes from a large number of randomly generated variants. Generation of these variants can be obtained by either random aminoacid substitutions or selection/identication of these mutations and then can be screened according to their specific properties [41]. Random approach is also known as directed evolution due to the way of generation - the generation of large numbers of DNA fragments and library of these mutants. In a random approach, the design steps and mutagenesis are continued simultaneously.

The rational approach is the guided design of novel proteins/enzymes with improved and desirable properties [38]. Apart from the random approach, the steps of design and mutagenesis are not continued simultaneously in a rational approach. With the help of three-dimensional (crystal structure) structures of the proteins, the rational approach is determined such as site-directed mutagenesis or site-saturation mutagenesis. These methods of a rational approach are preferred according to the properties of protein of interest [42]. Thus the knowledge of the protein structure, which can lead to determine the specific sites for mutations to consider improved qualities and novel functions, is used by rational approach. In addition if there is only knowledge about the primary structure of the protein, then random mutagenesis and selection can be required for the desired protein properties [43].

At the end approaches that are random and rational also show two different outcomes. The random approach mostly determines the outcomes whereas information about the structure-function relations of the proteins can be provided by rational approach. After the design step, the progress is carried out with a mutagenesis step such as heterologous protein expression, purification of altered protein, and characterization of altered protein.

1.2.2.1 Mutagenesis

As mentioned under previous subtitles, a rational approach is mostly used to manipulate DNA for protein engineering. In order for my research to start and continue, the best approach might be the rational approach, the site-directed mutagenesis.

Site-directed mutagenesis is *in-vitro* in that it depends on replacing the certain length of the aminoacids into the target site or gene by applying the PCR-based technique. To carry out the site-directed mutagenesis, there are possible methods such as the whole plasmid single-round PCR method and the overlap extension PCR (OE-PCR). Two primers with mutations, that should be complementary to opposite strands of the DNA template plasmid, can be required for whole plasmid single-round PCR. During the whole plasmid single-round PCR, the replication of two strands of the template should result in the generation of mutated plasmid without overlapping breaks. In order to obtain circular or nicked vector that contains the mutant gene there is a step of selective digestion. After the selective digestion, transformation of the vector can be carried out to repair the nick in the DNA that is the way of obtaining the mutated plasmid [43]. Another method is the overlap extension PCR. During the application of this method two primer pairs (one of the each primer pairs possesses the mutant codon as the mismatched sequence) can be used. The overlap extension PCR depends on two different PCRs. First of all two double-stranded DNA products can be obtained by using required primers. Generations of two heteroduplexes that have mutated sequences are obtained after the process of denaturation and annealing steps. With the help of DNA polymerase the missing parts of the DNA length can be completed the overlapping 3' and 5' ends of the heteroduplexes. In order to amplify the mutatagenic DNA products, the second PCR of the overlap extension PCR can be applied with the usage of the nonmutated primer pairs [42]. Figure 1.3 represents the overall process of the OE-PCR.

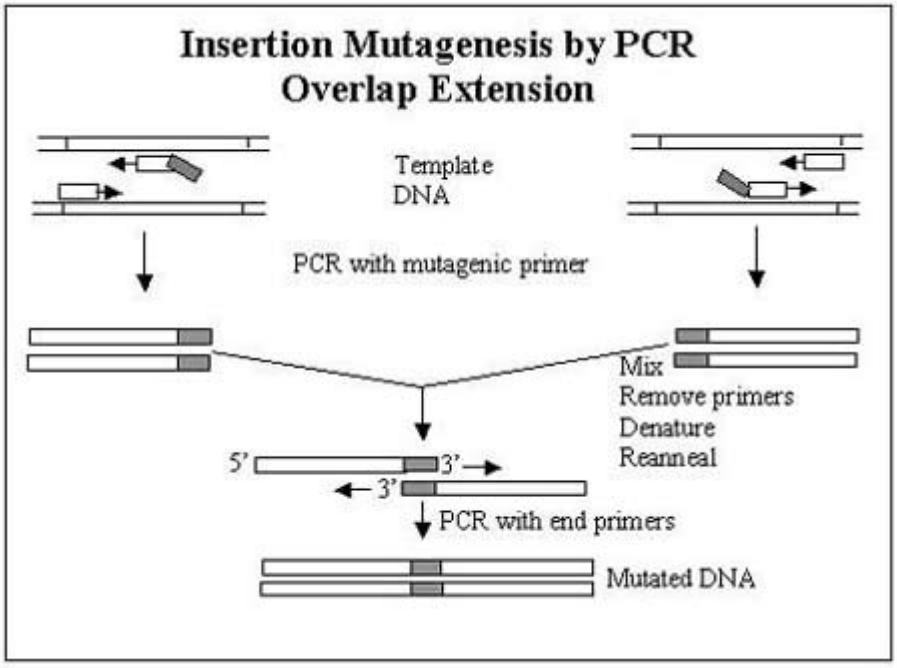

Figure 1.3: Principle of OE-PCR in Site-directed Mutagenesis.

Lines indicate the double-stranded DNAs with arrows representing the 5' to 3' orientation. The small black rectangle is the site of the mutagenesis. Lower case letters indicate the primers; upper case letters indicate PCR products. Annealing of the denaturated fragments and DNA polymerase enzyme extend the overlapping 3' and 5' ends of the heteroduplexes in the boxed portion of the figure. Mutant product is further amplified by additional primers.

1.2.2.2 Production

In the rational approach the following step of the mutagenesis is the production of the recombinant proteins. During the step of production of protein, desired and undesired proteins are totally expressed, a process which is called heterologous protein expression in various expression systems (in model organisms) such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Pichia pastoris*. Therefore purification of desired protein takes a key role in this process.

According to the aim of the research one of the expression systems can be chosen. In order to deal with post-translational modifications such as phosphorylation or glycosylation on the recombinant protein, the best option for eukaryotic proteins can be working with yeast systems. The logic behind of using *Pichia pastoris* is that it has a high growth rate and can grow on a simple, inexpensive medium. In addition, the production of *Pichia pastoris* can be done in either shake flasks or a fermenter depending on the scale of the production. AOX1 and AOX2, that are the two alcohol oxidase genes, are a strongly inducible promoter.

Methanol, that is the carbon and energy source for *Pichia pastoris,* induces the AOX promoters. Introduction of the desired protein can be done under the control of the AOX1 promoter. Therefore, the production of desired protein can be induced by the addition of methanol. In the yeast system, secretion of the desired protein in a growth medium highly facilitates protein purification. pPICZα and pPICZαA are the commercial plasmids of yeast systems [49].

Another expression system, which is most widely used system for decades in the production of recombinant proteins, is protein expression in *E. coli.* The logic behind using *E. coli* is that *E. coli* genetics are well understood that it is possible to generate almost unlimited quantities of proteins. In addition, the culturing time of the *E. coli* is shorter than other expression systems and also the expression of more than one protein is possible in *E. coli* [45]. The promoter (T7 promoter) that can be used in *E. coli* is different than the promoter of *Pichia pastoris* (AOX promoters).

According to required amount of protein the scale of productions might be changed. Therefore various production methods can be applied such as bench-scale production, fermentation, and a cellular production. After the production of the recombinant proteins, purification is the most important step of the rational approach because of the aim of obtaining only the desired proteins in order to characterize and analyze the mutant proteins.

The aim of the thesis depends on investigation of licked glycosylation; therefore, the expression system of *Pichia pastoris* is chosen in this thesis.

1.3 *Aspergillus Niger* lipase

Aspergillus niger lipase is an extracellular 1,3-specific lipase with a molecular weight of 35.5 kDa and an isoelectric point of 4.4. In addition the optimal activity of the ANL is between 35^{o}C-55^{o}C for temperature whereas the optimal activity of the ANL is between 5.0-6.0 for pH[46]. The native *Aspergillus niger* lipase contains sugar content but according to previous reports the percentage of sugar content is variable from 2.8% to 7% [46].

Aspergillus niger lipase is a 891-bp DNA fragment and the lipase is 297 amino acids in length. Although the full-length lipase gene and protein product is known, the 3D structure of ANL remains to be elusive. Yet in the study of Zhengyu Shu, ANL was obtained through homology modeling using the BioEdit software, lipase engineering database, PSIPRED and SwissModel servers [47]. According to this model, the ANL has an α/β hydrolase fold with GXSXG motif containing the catalytic serine. According to this model, ANL structure is composed of at least 30% helical and 20% sheet secondary structures [46]. The predicted structure of the ANL in the study of Shu is represented in the Figure 1.4 [47]

It has been noted that ANL is of great importance in biocatalysis performed in the food industry, in which the lipase has been utilized as food and as a detergant additive like in cellulose acetylation [47]. Moreover the usage of ANL is safe in the food and pharmaceutical industry [48].

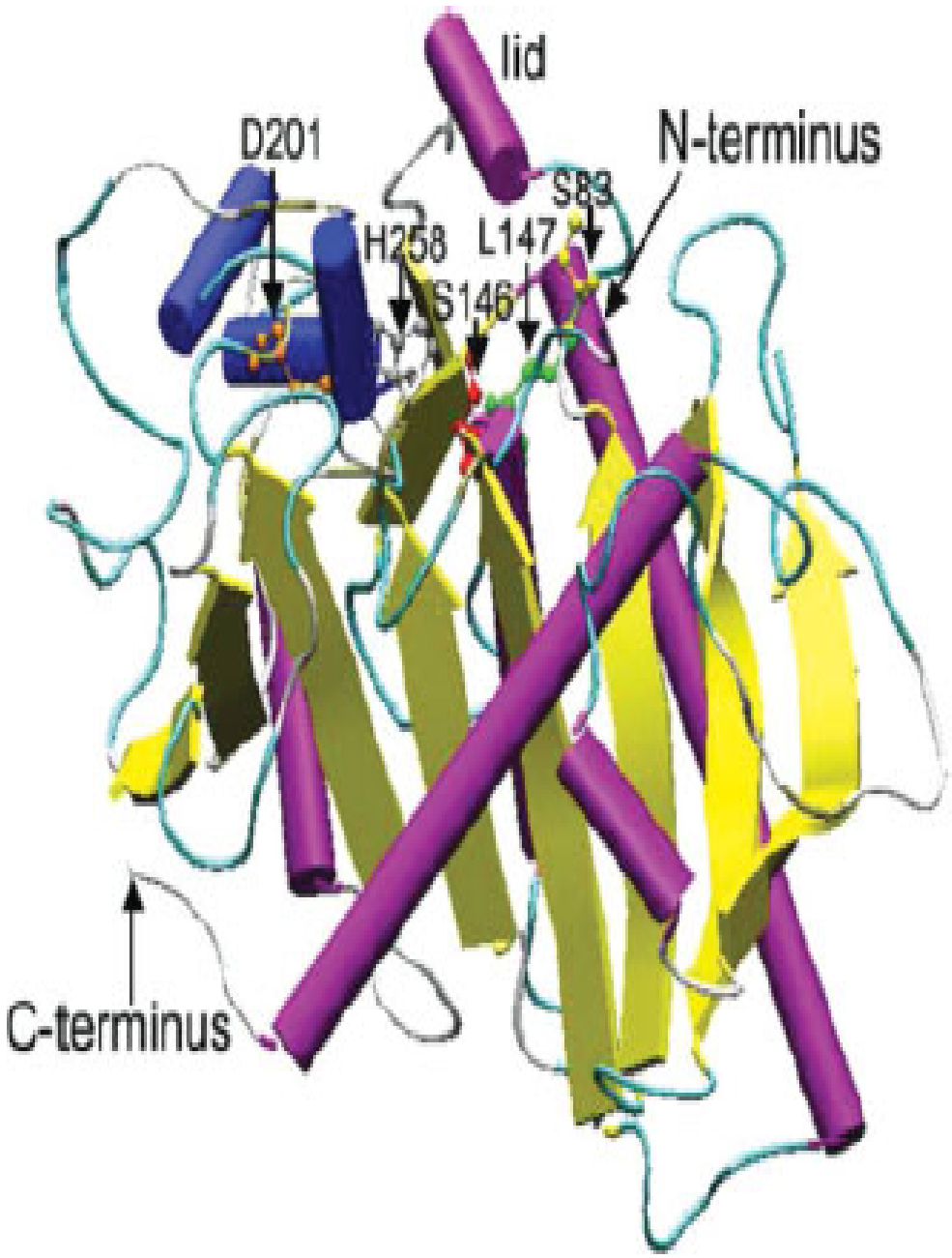

Figure 1.4: Structure of *Aspergillus niger* lipase by homology modelling [47].

1.3.1 Prospective Applications of *Aspergillus Niger* lipase

Aspergillus niger lipase is one of the chief producers of commercial lipases due to its low cost of extraction, thermal and pH stability, substrate specificity, and activity in organic solvents. Most lipases are suitable as a detergent additive due to having properties of their thermostabilities, high activities at alkaline pH, and stabilities towards anionic surfactants. In addition most lipases have a role as biocatalysts and therefore novel reactions and a wide of variety transformations such as chemo-, region-, and enantioselective can be carried out in both aqueous and nonaqueous media; because they possess broad substrate tolerance, high stability towards temperatures and solvents, and high enantioselectivity they can be chosen by organic chemists.

Nowadays, due to the catalytic behavior of lipases applications of lipases, which can be found in various fields of industrial microbiology and biotechnology, can be documented by numbers of research investigations. To illustrate, *Aspergillus niger* lipase has already been used for detergents, the oleochemical industry, synthesis of triglycerides, synthesis of surfactants, synthesis of ingredients for personal care products, pharmaceuticals and agrochemicals. Therefore, there is a lot of scope to search for newer lipases with desired selectivity and substrate tolerance.

1.3.2 N-Linked Protein Glycosylation

One of the fundamental and extensive post-translational modification is the asparagine (N)-linked glycosylation of proteins which can result in the covalent attachment of an oligosaccharide onto asparagine residues of polypeptide chains. N-linked glycosylation has been known as a modification that can be seen in eukaryotes, but according to recent studies it can be also seen in prokaryotes [50]. The earlier studies in model organisms indicate that all three domains of life reveal that the core of N-glycosylation can be represented by three homologous processes. First of all glycosyltransferases can assemble the glycan from nucleotide-activated building blocks on a lipid anchor through the stepwise incorporation of monosaccharides [50]. The lipid-linked oligosaccharide is then re-oriented from the cytosolic to the luminal side of the eukaryotic ER membrane or of the plasma membrane in

prokaryotes, where it serves as donor for glycosylation. After flipping to the luminal side of the ER, the lipid-linked oligosaccharide can be further extended in many eukaryotes. Secondly proteins with the consensus sequence for glycosylation N-X-S/T where X is not Pro; note that Thr is more common than Ser when translocated to the ER lumen serve as acceptors [50]. Thirdly the *en bloc* transfer of the oligosaccharide to the asparagine side chain of the acceptor polypeptides can be catalyzed by the oligosaccharyltransferase [50].

After being covalently linked to proteins, the N-glycan can be altered in eurkaryotes. This sequential processing is coupled to the secretory pathway and results in a species-specific or even cell type-specific diversity of N-linked glycans [50]. A functional view of eukaryotic N-glycan structure is represented in Figure 1.5.

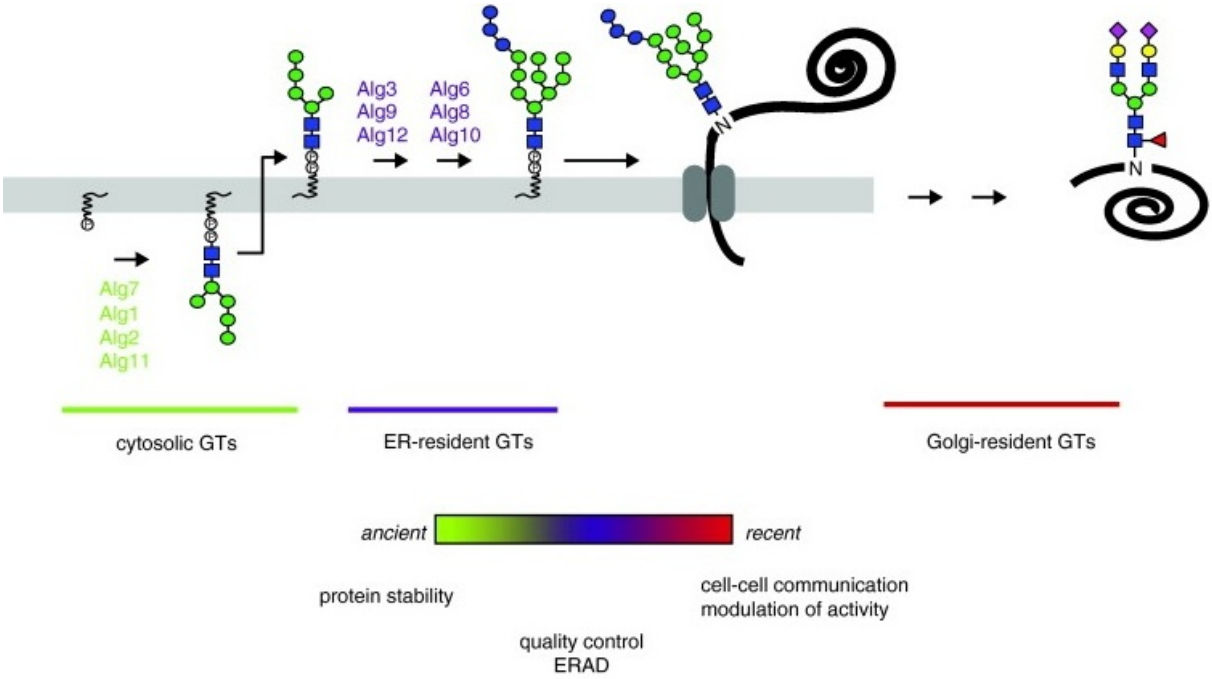

Figure1.5: A functional view of eukaryotic N-glycan structure [50]

The functions of N-glycosylation can be divided into groups which are extrinsic and intrinsic. The intrinsic functions of the N-glycosylation are that providing structural components to the cell wall and extracellular matrix and modifying protein properties such as stability to high temperature or pH. On the other hand the extrinsic functions of N-glycosylation are that directing trafficking of glycoproteins and mediating cell signaling such as cell-cell or cell-matrix interactions [51].

Glycosylation may alter the structure of the proteins; therefore it may cause altering the function of the protein. According to previous studies due to the binding of the sugar to

protein, the structure of protein might be changed which is associated with intracellular of folding; therefore, it may increase the stability of the protein and the activity of N-glycan protein [50]. According to most previous studies, the elimination of the glycosylation site of the protein causes the decrease in the activity level of protein [54]. In the literature, contrary experimental data can also be seen. The research of Variketta, for example, indicated that the content of sugar was completely removed by endoglycosidase F treatment, but the deglycosylated enzyme retained full activity in *Aspergillus niger* lipase [46]. Apart from all of that the mutations in eighteen genes involved in N-linked glycosylation result in a variety of diseases which are mostly associated with nervous system [52].

N-linked glycosylation also has a key role in the production of therapeutic proteins in health industry because most of the antibodies are the N-linked glycoproteins such as Etanercept and Rituximab.

2 METHODS

2.1 Prediction of N-linked Glycosylation Sites

In order to determine the possible glycosylation sites, two N-glycosylations predictors are used which are NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) and GlycoEP (http://www.imtech.res.in/raghava/glycoep/submit.html). First of all NetNglyc 1.0 server was used, there was a limitation with this server; because the NetNglyc server predicts N-Glycosylation sites in human proteins using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr sequons. Therefore we decided to use another N-linked glycosylation predictor which was GlycoEP. According to GlycoEp information, GlycoEp webserver is trained and optimized using exclusively eukaryotic glycosites's datasets and it can perfom predictions with very high accuracy as well as stringency for three glycosylation types in eukaryotes. These two predictors indicated the same glycosylation sites. The data is represented under the result section.

2.2 Molecular Cloning

A 891-bp DNA fragment indicating the *Aspergillus Niger* lipase (ANL) gene was amplified from the mature lipase clone (pPICZαA – ANL) DNA through ligation independent cloning. The primer sets contain EcoRI and SalI restrictions sites for directional cloning ligation and have sequences of; for forward (CCGGAATTCATGTTCTCTGGACGGTTT) and for reverse (CCGGTCGACTAGCAGGCACTCGGAAAT). The PCR condition for amplification of the ANL gene is given in Table 2.1.

Step	ºC	min	Cycle
Initial Denaturation	94	3	1
Denaturation	94	0.5	35
Annealing	54	0.5	
Extension	72	0.75	
Final Extension	72	7	1
Hold	4		

Table 2.1: PCR Profile for ANL

4 µg of pPICZαA, the expression vector for P. Pastoris (see Appendix A1 for the map), was linearized by the restriction enzymes EcoRI and SalI in the reaction mixture shown in the Table 2.2.

pPICZαA	50 µl
EcoRI and SalI	2 µl
Green Buffer	6 µl
ddH2O	2 µl
$Volume_{final}$	60 µl

Table 2.2: pPICZαA EcoRI and *SspI* Digestion

Agarose gel electrophoresis was carried out for linearized vector and PCR products at 100 Volt for 20 minutes, in 1.2% agarose gels by using TBE (tris-borate EDTA) buffer system. Each of the fragments was extracted from the agarose gel using QIAquick Gel Extraction Kit following the manufacturer's instructions (see Appendix A2). The extracted DNA fragments were ligated using T4 DNA Ligase. The ligation reaction was carried out at 25°C for overnight. The reaction mixture is as indicated in Table 2.3.

Vector	Volume	**PCR Product**	Volume
ddH2O	2	ddH2O	2 µl
5X Buffer	14 µL	5X Buffer	14 µl
T4 Polymerase	2 µL	T4 Polymerase	2 µl
dGTP	2 µL	dCTP	2 µl
DNA	50 µL	DNA	50 µl
Vfinal	70 µL	Vfinal	70 µL

Table 2.3: T4 Polymerase Reaction

Before the setting up the ligation reaction, DNA fragments were extracted using phenol-chloroform and precipitated using ethanol with the following protocol:

- Product of T4 DNA polymerase reaction for vector and insert (70 µl) are completed up to 100 µl with ddH_2O.
- Addition of 1:1 ratio phenol/ chloroform (100 µl) into both tubes.
- Vortex thoroughly.
- 5 minutes of centrifugation at 13,200 rpm.
- Collect the upper (aqueous) phase.
- Addition of 4 µL NaOAc, 10 µl LPA and 250 µl EtOH (%100).
- Keep the tubes at -80°C for 20 minutes.
- 15 minutes of centrifugation at 13,200 rpm.
- Discard the supernatant.
- Addition of 250 µl EtOH (%70) onto the pellet.
- 10 minutes of centrifugation at 13,200 rpm.
- Discard the supernatant.
- Resuspend the pellet with 10 µl of ddH_2O.

Chemically competent *E. coli* (XL21-Blue) cells prepared and transformation of the ligation reaction mixture to the *E.coli* XL21-Blue cells was carried out as following protocol:

- Add the annealing mixture onto 200 µl XL21-Blue competent cell.
- Keep the mixture on ice for 20 minutes.
- Heat shock for 1 minute at 42°C.

- Transfer the mixture on ice and incubate for 10 minutes.
- Add 800 µl of super optimal broth with catabolite repression (SOC) onto the cells.
- Incubate the cells at 37°C with 250 rpm shaking for 60 minutes.
- Centrifuge the cells at 7000 rpm for 2 minutes.
- Discard the supernatant.
- Resuspend the pellet in approximately 100 µl of the remaining supernatant.
- Spread the cells on LB agar plate with the appropriate antibiotic using glass beads.
- Incubate the plates at 37°C for overnight (16-18 hours).

Colony PCR was performed to confirm the cloning with Table 2.4. Single colonies in the transformation plates were selected and used as template in the PCR that had the same cycling profile in Table 2.1. Also the same primers in the cloning PCR were used.

Forward Primer	1 µl
Reverse Primer	1 µl
Taq Polymerase Master Mix	10 µl
ddH_2O	8 µl
V_{final}	20 µl

Table 2.4: Colony PCR Reaction

The results of the colony PCR was evaluated in 1.2% agarose gel using GeneRuler^{TM} 1 kb DNA Laddder SM0311 (Fermantas, see Appendix A3 for Electrophoresis Marker Legends). Plasmid purifications were carried out from the colony PCR colonies according to the instructions given in the Qiagen Plasmid Purification Kit (see Appendix A4 for the protocol). For two set of colonies EcoRI and SalI digestion was performed as a confirmation such as recommended by Fermentas. AOX1 Forward (GACTGGTTCCAATTGACAAGC) and AOX1 Reverse (GCAAATGGCATTCTGACATCC) were used for sequencing by Molecular Cloning Laboratories (MCLAB).

2.3 Site-directed Mutagenesis

Three mutations which are N59Q (Asparagine to Glutamine at residue 59), N150Q (Asparagine to Glutamine at residue 150), and N269Q (Asparagine to Glutamine at residue 269) were generated using Overlap Extension PCR (OE-PCR). The primers of the mutation are demonstrated in Table 2.5. For whole mutants (N59Q, N150Q, and N269Q), two PCR reactions were prepared by using primers F_ANL_LIC as forward and reverse primers of the mutant; and forward primers of the mutant and R_ANL_LIC as reverse. These DNA segments were applied gel extraction from 1.2% agarose gel and used to obtain full-length DNA fragment by Overlap Extension PCR. The initial 15 cycles were carried out without any primers and the consecutive 15 cycles were performed with ANL_LIC primers. The PCR profile of the OE-PCR and PCR mixtures are indicated Table 2.6 and Table 2.7.

Mutation	Direction	5'-3' Sequence
N59Q	forward	CATCCCCACGCGCCGGTGATGCACCCATCGT
	reverse	TTATCCACTTCCAATGCCAGGGCAGCAGCAC
N150Q	forward	ACCCATCGCATCCCCACGCGCCCAGGATGCACCCATCGT
	reverse	CCTGGGACGATGGGTGCATCCTGGGCGCGTGGGGATG
N269Q	forward	GCGGCATCCCCATGCTGCAATGATGCACCCATCGTGCTT
	reverse	TGCCAGGGCAGCAGCACGCCAACTGCTCGGCAAGTCG

Table 2.5: Primer Sequences for Mutagenesis

	Q1/G1	**Q2/G2**
ddH_2O	9,5 µl	9,5 µl
2X pwo MasterMix	12,5 µl	12,5 µl
Forward Primer	1.25 µl	1.25 µl
Reverse Primer	1.25 µl	1.75 µl
Template	0.5 µl	0.5 µl
V_{final}	25 µl	25 µl

Table 2.6: 1st Reaction of OE-PCR

For the 1st reaction of the OE-PCR for Q1 and G1 primer combinations, initial denaturation (3 minutes at 94°C), denaturation (30 seconds at 94°C), annealing (30 seconds at 54°C), extension (45 seconds at 72°C) and the final extension (7 min at 72°C) are applied as 35 cycle for denaturation, annealing and extension. For Q2 and G2 primer combinations, initial denaturation (3 minutes at 94°C), denaturation (30 seconds at 94°C), annealing (30 seconds at 54°C), extension (45 seconds at 72°C) and the final extension (7 min at 72°C) are applied as 35 cycle for denaturation, annealing and extension.

Q1/G1	5-10 µl
Q2/G2	5-10 µl
2X pwo Master Mix	25 µl
ddH_2O	10 µl

Table 2.7: 2nd Reaction of OE-PCR

After 15 cycles which was adjusted as 3 minutes at 94°C for initial denaturation, 30 seconds at 94°C for denaturation, 30 seconds at 54°C for annealing and 45 seconds at 72°C for extension; 2.5 µl F_ANL_LIC and 2.5 µl R_ANL_LIC primers were added to the reaction. 30 seconds at 94°C for denaturation, 45 seconds at 53°C for annealing and 1 minute at 72°C for extension were performed as 30 cycles and 7 minutes at 72°C as final extension. The mutant DNA fragments were cloned into pPICZαA vector through the procedure given above for ANL in the section 2.1.1.

2.4 Lipase Expressions and Purification

After the sequence confirmations, the positive colonies of *Pichia Pastoris* cells were selected for lipase expressions. All mutants (ANL1-2, ANL2, and ANL2-3) and WT (ANL) were started in 5 ml cell culture in YPD at room temperature shaker. After two days they were transferred into 50 ml of BMG buffer (5 ml of 10X YNB, 5 ml 10X Phosphate-Buffer, 5 ml of 10X Glycerol, 100 µl of 500X Biotin and ddH_2O) and left at 30°C shaker with 250 rpm for three days. Then all of them were harvested by 10 minutes of centrifugation at 4000 rpm to obtain the cell pellet containing gene of interest. Each of the pellet was resuspended into 5 ml of BMM buffer (100 mM potassium phosphate buffer pH 6.0, 13.4 g/L yeast nitrogen base,

0.00004% biotin) supplemented with 1% sorbitol and methanol. After the resuspending step, each flask was filled up with the BMM buffer until the final volume was 50 ml and once again left at 30^{o}C shaker with 250 rpm shaking. All mutants and WT were expressed in 50 ml cell culture using methanol as the inducer and sorbitol as the non-repressive carbon source. The expressions were carried out for five days by sampling every day by centrifugation at 13,200 rpm for 2 minutes. After determining the time point for the maximal protein yield through analytical methods such as lipase assay and SDS-PAGE, expressions were carried out at higher volumes. The expression medium was similarly separated from the cells by centrifugation and the secretion medium was concentrated using Vivaspin 6, MWCO 10000 concentrator. Bradford protein assays were used to determine the quantity of the lipases by absorbance measurements at 595 nm. The protein solutions were kept at 4^{o}C.

2.5 SDS-PAGE

SDS-PAGE (sodium dodecyl polyacrylamide electrophoresis) was carried out using the stacking gel at 4% and separating gel at 12.5%. Before loading, the samples were mixed with SDS sample buffer and boiled at 95^{o}C for 10 minutes. The gel was run for 2 hours at 120 Volts and the protein bands were visualized after coomassie staining.

2.6 Fluorescent Lipase Assays

Lipase activity was measured with fluorescent assays in 96-well black micro-titer plate by using substrate and 4MU-caprylate. Different concentrations of purified lipases were assayed in a reaction medium which contains 100 mM Tris-Cl as a buffer at ph 7.25. 4MU-fluorescence was measured by Fluoroskan Ascent (Thermoscientific) with 355 nm excitation wavelength and 460 nm emission wavelength every minute for 1 hour in a kinetic manner. The software of the fluorometer was used to determine the initial velocities and the measurements were performed in duplicates.

2.6.1 Thermostability Assay

Purified proteins were used in a series of enzymatic assays to profile their thermostability by quantifying residual activity. For this purpose, the lipases were incubated at 35-55°C for 30 minutes. The amount of the lipase that has a linear relationship with relative fluorescence was determined prior to assays and 10 nM of lipases were used in the final reaction mixture. The residual activity was quantified in enzyme assays using 4MU-caprylate as the substrates. The enzyme activities were recorded from duplicate measurements and the assays were performed in 0.1 mM Tris-Cl pH 7.25 at room temperature using 250 µM substrate. The percent activity was calculated by adjusting the maximum activity to 100%.

2.7 Enzymatic Cleavage of Glycosylation

Two commercially available enzymes, Endo Hf (New England Biolabs) and PNGase F (New England Biolabs) were used for enzymatic degradation of glycosyl moieties of the modified residues.

For the treatment of EndoHf, 1-20 µg of glycoprotein was combined with 1 µl of 10X Glycoprotein Denaturing Buffer and then this mixture (glycoprotein) was denatured by heating reaction at 100^{o}C for 10 minutes. After this step, the previous product, and 2 µl of 10X G5 Reaction Buffer, 2 µl of Endo Hf and 6 µl of ddH_2O were combined. This reaction was left for 1 hour at 37^{o}C.

For the treatment of PNGase F, 1-20 µg of glycoprotein was combined with 1 µl of 10X Glycoprotein Denaturing Buffer and then this mixture (glycoprotein) was denatured by heating reaction at 100^{o}C for 10 minutes. After this step, the previous product, and 2 µl of 10X G7 Reaction Buffer, 2 µl of PNGase, 2 µl of %10 NP and 4 µl of ddH_2O were combined. This reaction was left for 1 hour at 37^{o}C.

Furthermore SDS-PAGE (sodium dodecyl polyacrylamide electrophoresis) was carried out for these samples to confirm whether a difference exists. Stacking gel contained 4% polyacrylamide and separating gel contained 12.5% polyacrylamide. The buffer exchange of the lipases against deionized water was carried out by 10 kDa filter concentrators (Millipore).

3 RESULTS

3.1 Prediction of N-linked Glycosylation Sites

In order to determine the possible glycosylation sites, two N-glycosylations predictors are used which are NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) and GlycoEP (http://www.imtech.res.in/raghava/glycoep/submit.html).

The result of NetNGlyc is represented in Figure 3.1 whereas the result of GlycoEP is represented in Figure 3.2.

First of all NetNglyc 1.0 server was used yet with a limitation because the NetNglyc server predicts N-Glycosylation sites in human proteins using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr sequons. Therefore we decided to use another N-linked glycosylation predictor which was GlycoEP. According to GlycoEp information, GlycoEp webserver is trained and optimized using exclusively eukaryotic glycosites's datasets and it can predict with very high accuracy as well as with high stringency for three glycosylation types in eukaryotes. These two servers predicted the same glycosylation sites.

According to both results, N-linked glycosylation sites were found in motif sequences of Asn-Xaa-Ser/Thr sequons. We believed that GlycoEP is more reliable than the NetNGlyc 1.0 server; because GlycoEP is the predictor which examines the yeast systems. After obtaining these data, we designed our primers to eliminate the possible glycosylation sites positions 59, 150, 269. Asparagine is the aminoacid that takes a place at each position and this aminoacid was substituted with glutamine. Because the biochemical structures of asparagine and glutamine are very similar, it would be a complementary mutation for the overall structure yet a loss of function regarding glycosylation.

(Threshold=0.5)

SeqName	Position	Potential	Jury agreement	N-Glyc result
Sequence	59 NVTC	0.6952	(9/9)	++
Sequence	150 NLTS	0.7088	(8/9)	+
Sequence	269 NSTA	0.3552	(8/9)	-

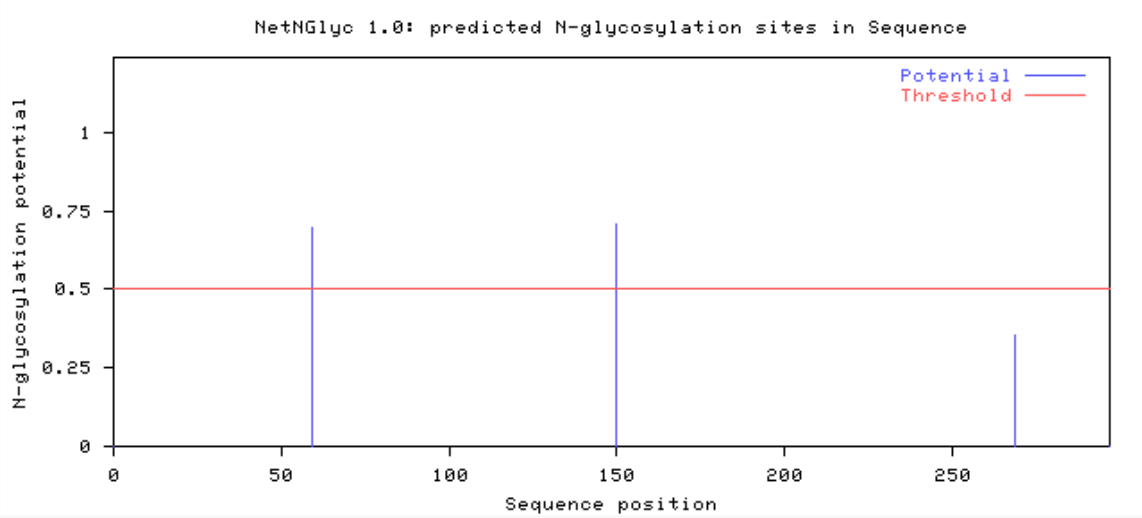

Figure 3.1: The results of NetNGlyc 1.0 server

According to Figure 3.1 the position of 59th aminoacid, the position of 150th aminoacid and the position of 269th aminoacid are the crucial point for the rest of this research. In Figure 3.1 the blue lines indicate the potential sites of glycosylation, whereas the red line indicates the threshold which means that there is a low possibility if the blue lines are under this red line. Therefore the position of 59th aminoacid is the most probable site to be glycosylated while the position of 269th aminoacid is the least.

Position	Residue	Score	Prediction
51	NNI	-0.67175493	Non-glycosylated
52	NID	-1.0666679	Non-glycosylated
59	NVT	1.2758118	Potential Glycosylated
85	NNF	-0.83737803	Non-glycosylated
86	NFG	-1.0955384	Non-glycosylated
98	NTN	-1.0615909	Non-glycosylated
100	NKR	-0.97900916	Non-glycosylated
115	NWI	-1.0882451	Non-glycosylated
127	NDD	-1.1742797	Non-glycosylated
150	NLT	0.78057089	Potential Glycosylated
188	NDG	-0.60385996	Non-glycosylated
205	NYA	-1.1103765	Non-glycosylated
220	NFR	-1.3746577	Non-glycosylated
227	NDI	-1.031342	Non-glycosylated
269	NST	0.96019863	Potential Glycosylated
274	NAG	-0.87228554	Non-glycosylated

Figure 3.2: The results of GlycoEP

According to Figure 3.2 the position of 59th aminoacid, the position of 150th aminoacid and the position of 269th aminoacid are the crucial point for the rest of this research. In Figure 3.2 the highlighted green colored text indicates predicted glycosylation sites. Therefore the position of 59th aminoacid and the position of 150th aminoacid 269th aminoacid are selected for mutagenesis studies.

3.2 Molecular Cloning of ANL2, ANL1-2, ANL2-3 Mutations

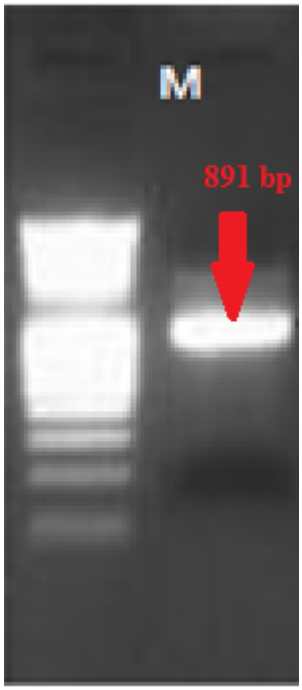

Figure 3.3: Agarose Gel Electrophoresis Results of amplifications of ANL mutants which has 891 base-pairs and amplified by using ANL_LIC primer pairs. Analysis was made in agarose gel under 100V for 20 minutes.

Mutant ANL genes and the linearized expression vector pPICZαA were separated in agarose gel electrophoresis according to their sizes (Figure 3.3). The results indicated the presence of linearized pPICZαA expression vector at 3593 bp and the amplified ANL mutants at 891 bp. Prior to annealing reactions, another agarose gel was used to visualize the T4 DNA polymerase treated samples to determine the appropriate amounts of pPICZαA vector and ANL mutants for annealing reaction.

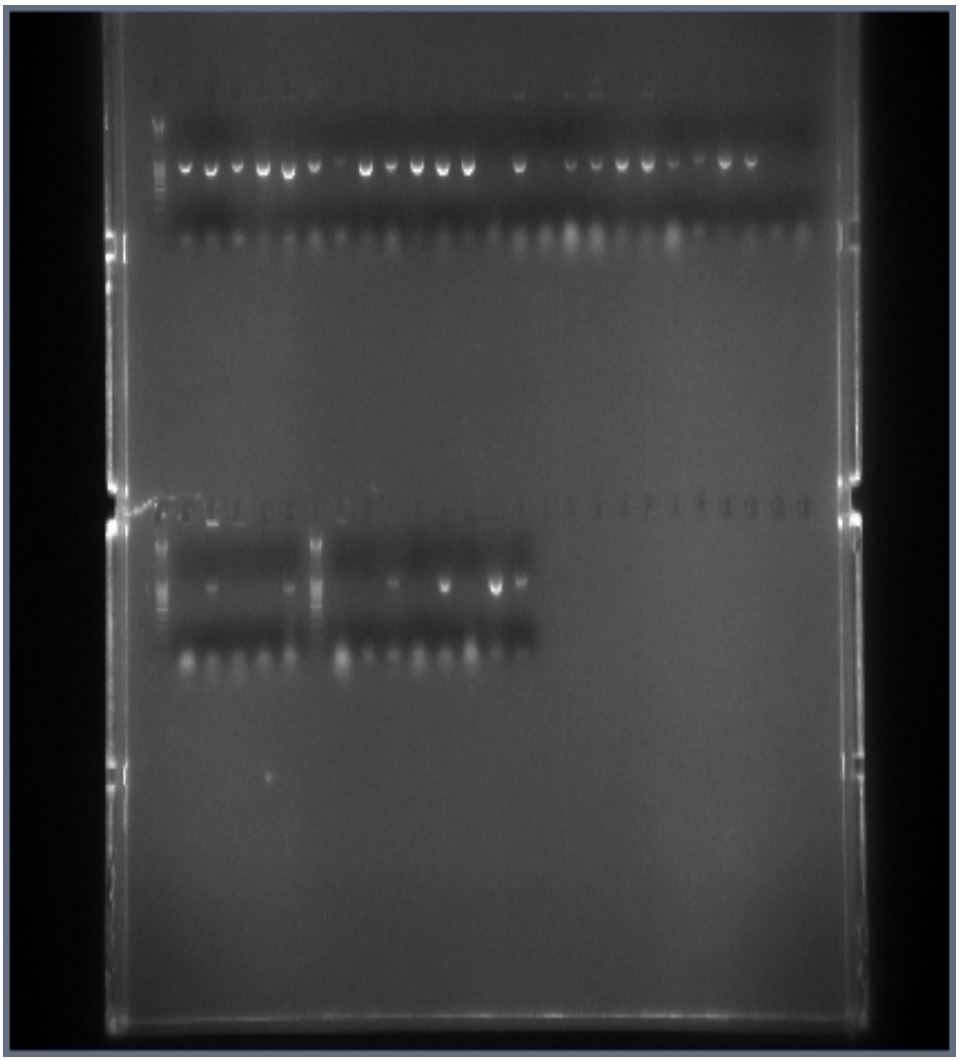

Figure 3.4: Cloning Confirmations of ANL2, ANL1-2, and ANL2-3 Mutants by Colony PCR.

Colony PCR was performed using F-ANL-Eco and R-ANL-Sal primers to forty single colonies obtained from the transformation plates. Analysis was made in agarose gel under 100V for 20 minutes.

40 single colonies were selected from the transformation plates and colony PCR was performed. 28 of 40 colonies indicated the presence of the insert and confirmed to contain the mutant gene (Figure 3.4).

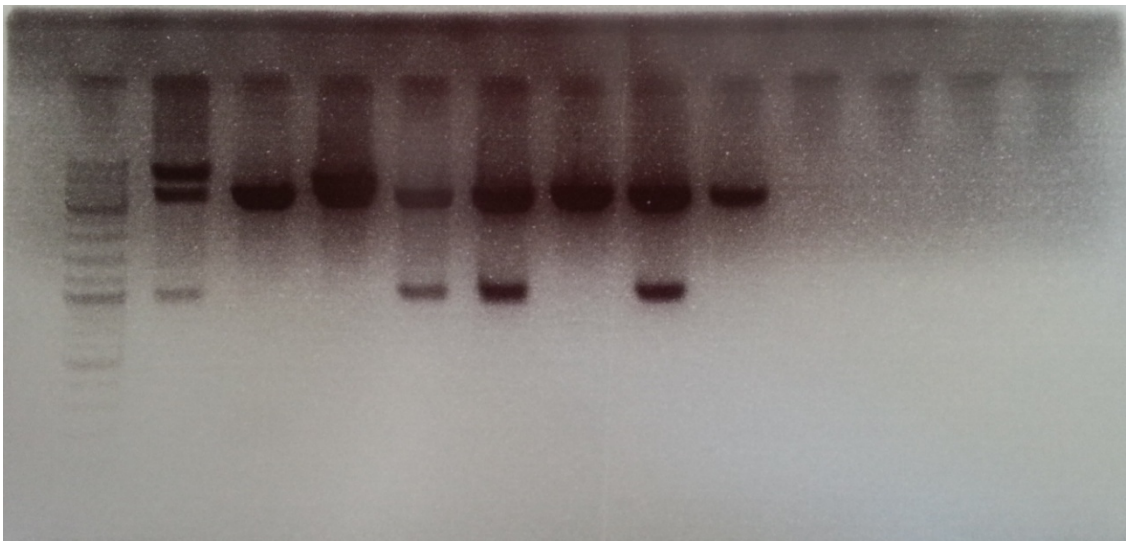

Figure 3.5: Cloning Confirmations of ANL Mutants by Double-Digest.

Confirmation of the pure plasmids of ANL2, ANL1-2, ANL2-3 mutations are performed using *EcoRI* and *SalI* digestion enzymes and the analysis were made in agarose gel under 100V for 20 minutes.

Double digestion of recombinant plasmids was performed using *EcoRI* and *Sal*I restriction enzymes and confirmed the presence of mutant ANL genes (Figure 3.5).

Figure 3.6: Cloning Confirmations of ANL1 Mutant by Sequencing

Reverse and forward readings are performed and mutated sites are indicated by black brackets.

The sequencing result for the ANL1 mutant confirmed the cloning by site directed mutagenesis. Asparagine amino acid at residue 59 was converted to glutamine amino acid which is indicated in Figure 3.6 with a black bracket.

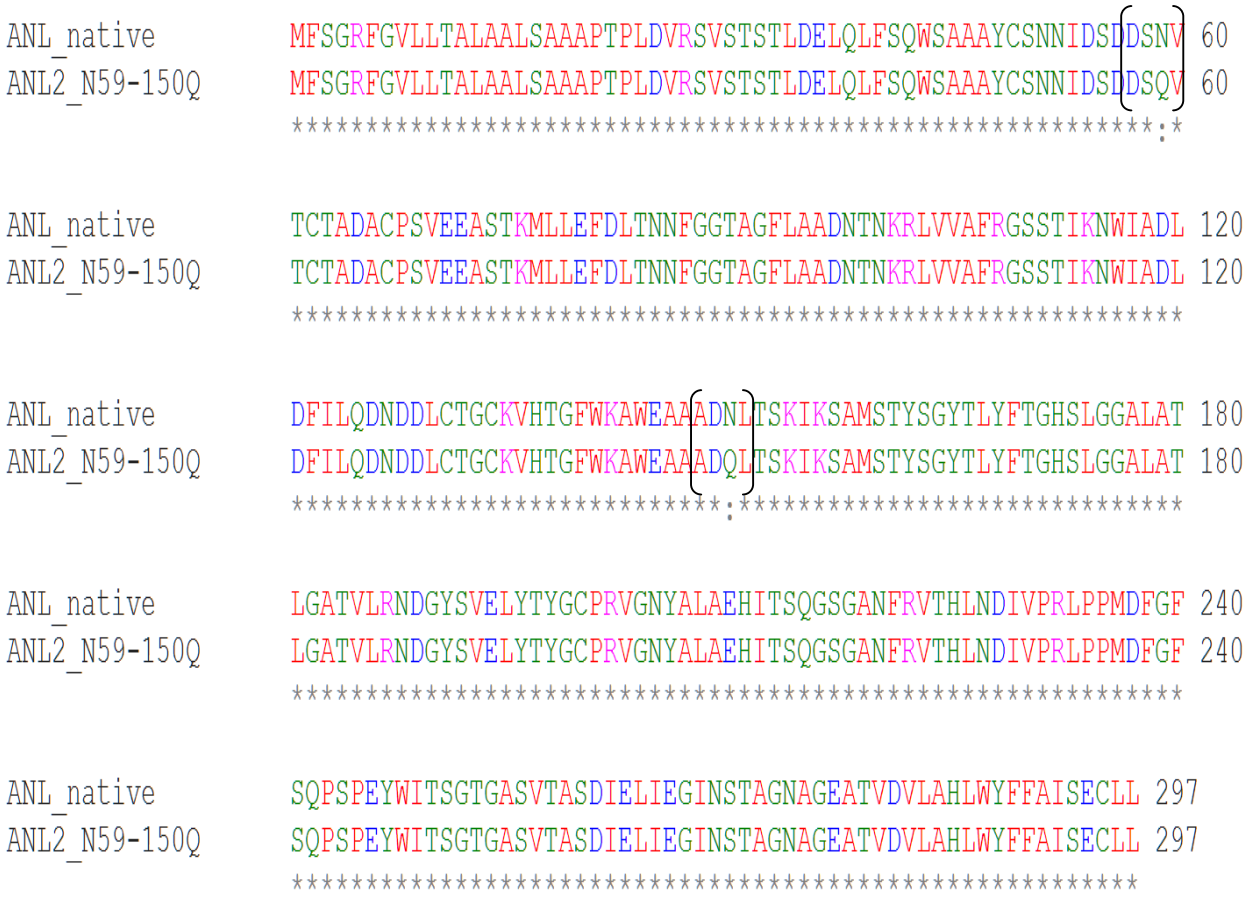

Figure 3.7: Cloning Confirmations of ANL1-2 Mutant by Sequencing

Reverse and forward readings are performed and mutated sites are indicated by black brackets.

The sequencing result for the ANL1-2 mutant confirmed the cloning by site directed mutagenesis. Asparagine amino acids at residue 59 and 150 were converted to glutamine amino acids which are indicated in Figure 3.7 with a black bracket.

Figure 3.8: Cloning Confirmations of ANL3Mutant by Sequencing

Reverse and forward readings are performed and mutated sites are indicated by black brackets.

The sequencing result for the ANL2-3 mutant confirmed the cloning by site directed mutagenesis. Asparagine amino acids at residues 150 and 269 were converted to glutamine amino acids which are indicated in Figure 3.8 with a black bracket.

3.3 Expressions and Purifications of ANL (WT), ANL2, ANL1-2 and ANL2-3 Mutant Lipases

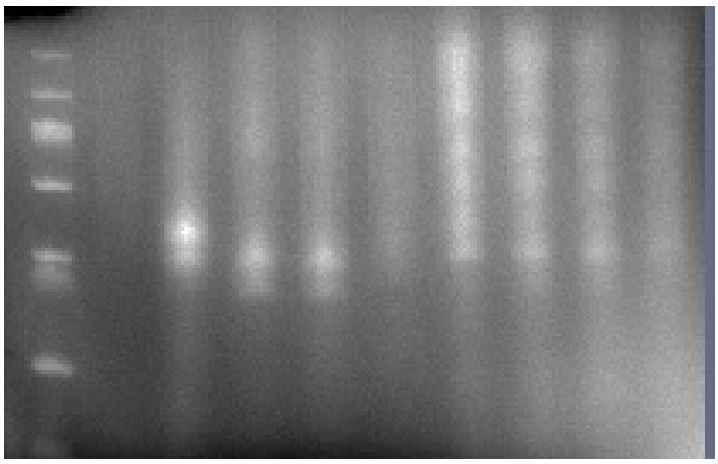

Figure 3.9: Expressions of ANL (WT), ANL2, ANL1-2, and ANL2-3 Mutations. Whole cell-fraction intervals were analyzed in 12% SDS-PAGE which was stained with coomassie dye. The gel indicates: Marker, WT t_0, purified WT, purified ANL2, purified ANL1-2, purified ANL2-3, WT, ANL2, ANL1-2, and ANL2-3 samples

Aspergillus niger lipase is 35-40 kDa protein and P. pastoris expression of ANL, ANL2, ANL1-2 confirmed the presence of the recombinant ANL around 30-35 kDa on the SDS-PAGE (Figure 3.9). All of the expressions were performed with shake flasks. Although the expression was repeated more than twice, it was observed that the expression levels of the recombinant ANL often fluctuated, suggesting that shake flask expressions were not consistent. Although there were variations between two identical expression trials, the recombinant ANL was able to be produced at high quantity. Such variations could be highly similar due to the shake flask system itself. Unlike to the controlled environments like fermentation systems, shake flask systems are prone to produce variations in the pH and temperature of expression media. Nevertheless ANL, ANL2, ANL1-2 were successfully expressed. Yet expression levels of ANL2-3 were too low compared with the other three lipases.

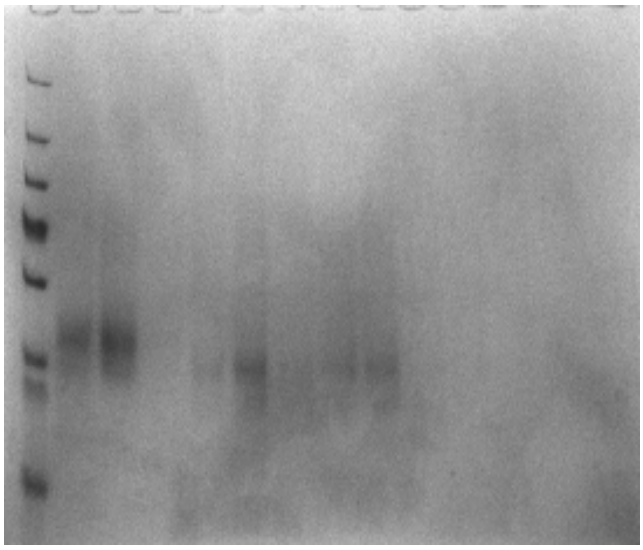

Figure 3.10: Column Purification for the ANL (WT), ANL2, ANL1-2, and ANL2-3 Mutation.

Marker / ANL E1 / ANL E2/ ANL E3/ ANL2 E1/ ANL2 E2/ ANL2 E3/ ANL1-2 E1/ ANL1-2 E2/ ANL1-2 E3/ ANL2-3 E1/ ANL2-3 E2/ ANL2-3 E3

The purification gel for the ANL mutants confirmed the protein expression, which was shown in the input protein and purely, eluted proteins (30-35 kDa) whereas the purification gel for the ANL confirmed the protein expression, which was shown in the input protein and purely, eluted proteins (35-40 kDa) in the elution steps (Figure 3.10).

3.4 EndoHf Treatment of ANL (WT), ANL2, ANL1-2 and ANL2-3 Mutant Lipases

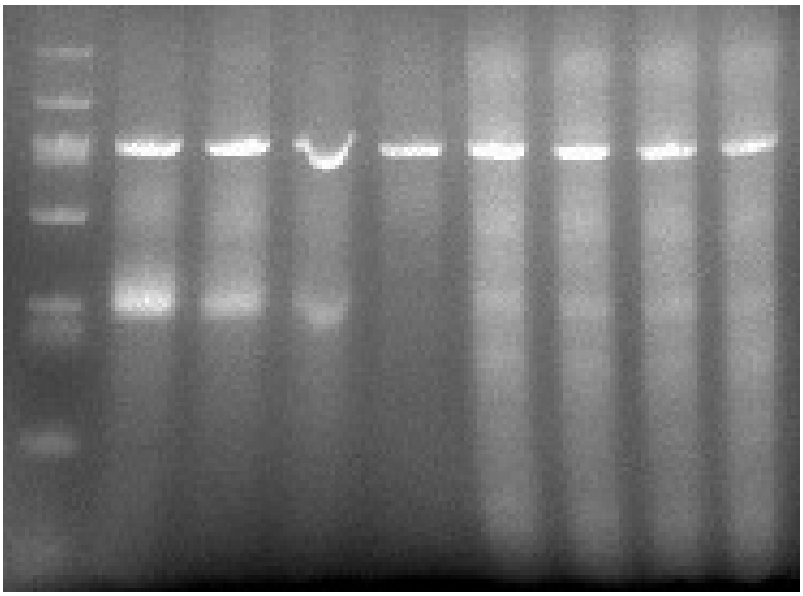

Figure 3.11: Expressions of ANL (WT), ANL2, ANL1-2, and ANL2-3 Mutations after EndoHf treatment. Whole cell-fraction intervals were analyzed in 12% SDS-PAGE which was stained with coomassie dye. The gel indicates: Marker, WT t_0, purified WT, purified ANL2, purified ANL1-2, purified ANL2-3, WT, ANL2, ANL1-2, and ANL2-3 samples

After the EndoHf treatment the regular pattern of the ANL proteins were lost and the size of protein bands was moved to 40 kDa. Moreover the upper bands which are around 70 kDa correspond to the EndoHf enzyme in the Figure 3.11.

3.5 Characterization of ANL2, ANL1-2, and ANL2-3 Mutants Lipases

3.5.1 Determination of the Linear Range for Fluorescent Lipase Assays

ANL is 39 kDa and its molar concentration is equal to 25 µM at 1mg/ml. Fluorescent lipase assay by using 4MU-caprylate as substrate was performed to diluted proteins at pH 7.25 and room temperature. Linear range was determined between 1 µg/ml and 0.1 µg/ml, which correspond to 25 nM and 2.5nM, respectively. Therefore, 10 nM, which corresponds to 390 ng/ml, was used in the fluorescent lipase assays. 20 µl of enzyme solutions were used in all assays.

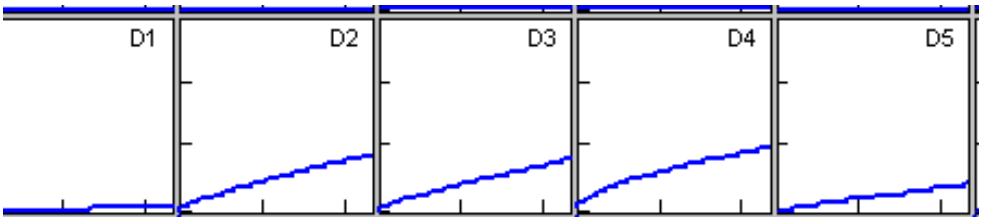

Figure 3.12: The result of enzyme activity assay.Order: Blank, WT, ANL2, ANL1-2, ANL2-3

Average rate: Unit: /min, Ignore first n: 0, Ignore last n: 70

Blank	WT	ANL2	ANL1-2	ANL2-3
2,922	32,51	29,17	39,48	14,54

Figure 3.13: The result of enzyme activity assay.Order: Blank, WT, ANL2, ANL1-2, ANL2-3

Bradford protein assays were applied to determine the concentration of the wild type ANL, and mutations of ANL2, ANL1-2, and ANL2-3 proteins, which were measured with spectrophotomer (ELISA reader) at 595 nm. The concentration of wild type ANL protein was measured as 25 mg/ml; ANL2 protein was 56 mg/ml; ANL1-2 was 81 mg/ml; and ANL2-3 protein was 63 mg/ml. All proteins were diluted to 0.39 µg/ml to reach the linear range in fluorescent lipase assays.

3.5.2 Thermostability Assay

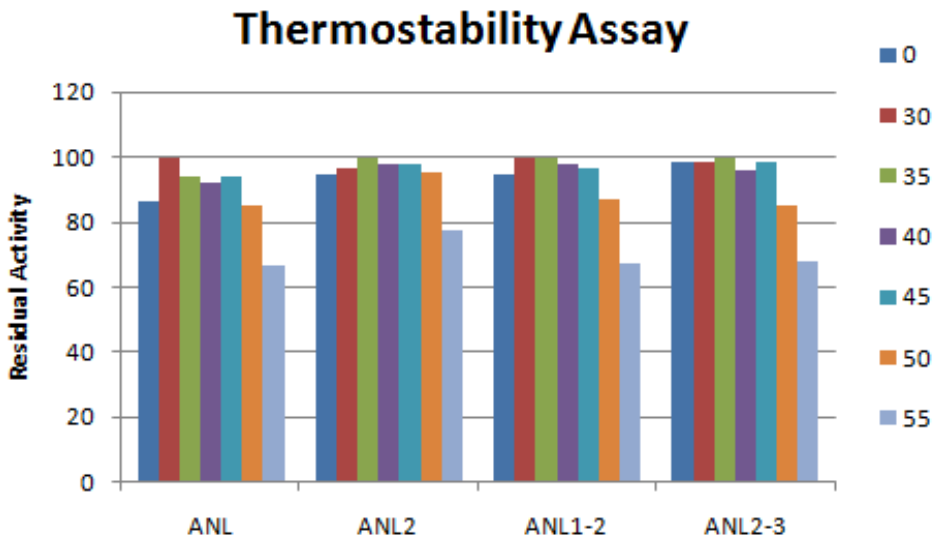

Figure 3.12: Thermostability Assay for WT, ANL2, ANL1-2 and ANL2-3.

Thermostability assay was performed in 0.1 mM Tris-Cl, pH 7.25 at given temperatures (30ºC-55ºC) using 250 µM substrate (4MU-caprylate). Percent activity was demonstrated and the highest activity for each mutant was set to 100%.

Results of the thermostability assay indicated that the wild type ANL shows the highest activity at 30ºC and the percent hydrolysis is between 66-93% at other temperatures. However, mutants of ANL2, ANL1-2 and ANL2-3 show lower thermostability at 30ºC compared to wild type ANL. Mutants of ANL2, ANL1-2 and ANL2-3 show the highest activity at 30ºC and the percent hydrolysis is between 67-98% at other temperatures. Mutant ANL1-2 has higher thermostability compared to WT and other mutants (Figure 3.12). On the other hand thermostability assay shows that mutant ANL2 can conserve its thermostability more than WT and other mutants at various temperatures. Overall, although slight changes were observed in thermostability of ANL mutants with respect to the native ANL, the overall thermostability of the native ANL was not affected at all by any of these mutations. In other words, all of the lipases showed the highest activity between 30-45°C.

4 DISCUSSION AND CONCLUSION

As with many recombinant proteins produced in eukaryotic hosts, glycosylation of proteins is an important issue with respect to the fact that this kind of modification in the protein sequences might affect functionality and stability [57]. Taking the advantage of being an eukaryotic species, *P. pastoris* also possesses protein synthesis pathway with the performance of higher eukaryotic protein modifications like glycosylation [58, 59]. N-glycosylation is the attachment of oligosaccharides to specific asparagine residues within the consensus sequence Asn-X-Ser/Thr. *Aspergillus niger* lipase (ANL) is an important biocatalyst in the food processing industry [46]. ANL displays particularly positional selectivity toward the one- and three- positions of the glycerol moiety. It is also regarded as safe by the Food and Drug Administration of the United States of America, which makes it a conventional food additive in the food processing industry. Recently, ANL has also been used as a detergent additive, and used many other organic synthesis reactions [55, 56]. Much research on ANL has been concentrated on its production, purification, characterization [46, 47, 55, 56]. Here we aimed to determine the impacts of N-linked glycosylation of ANL in *Pichia pastoris*.

First of all possible glycosylation sites were determined via NetNGlyc 1.0 server and GlycoEP server. These two servers indicated the same locations (positions 59, 150, and 269) for N-linked glycosylation which were facilitator to determine the possible sites. From this point forth we decided to create primers to alter the asparagine to glutamine in order to investigate the effect of glycosylation while preserving the structure of the protein.

In order to obtain and characterize proteins which carry our desired sequences, the steps were followed such as site-directed mutagenesis, lipase heterologous lipase expression, lipase column purification via nickel-coated beads, fluorescent lipase assays and EndoHf treatment.

The results indicated that mutations possess glycosylation patterns different than the native lipase. According the SDS-PAGE analysis, native ANL was observed at 35-40 kDa with a smear appearance, while the mutant that contains N150Q mutation was between 30-35 kDa and the other mutants did not change the native features. Incomplete separation of the components can lead to overlapping bands which is known as smear appearance. According to literature, the native ANL has various lengths; therefore it is highly possible to have smear appearance even after purification procedure. This result indicates that mutation in the 150th aminoacid has a significant effect to alter glycosylation of ANL. After EndoHf treatment, the

smear appearances of the native the mutants were disappeared, and all of the proteins were observed as single band at 35 kDa. According to enzyme activity assay, at the same concentration of enzyme was applied for an analysis. The results showed that activities of ANL2 and the wild type were equal to each other whereas the activity of ANL1-2 was higher (1.5 fold) than the activity of wild type. The thermostability results showed that all of the lipases including the native ANL had the maximal activity at 30-45°C, indicating that the mutations did not have any effect on thermostability of the native ANL until 50^{o}C; but thermostability of ANL-2 mutant was higher than the wild type ANL at 55^{o}C. This situation might stem from the similar biochemical properties of asparagine and glutamine, both of which are polar amino acids. N-linked glycosylation site (N150Q) in ANL had a major impact in glycosylation of ANL in *P. pastoris*, while the other two mutations (N59Q, N269Q) were not included in glycosylation mechanism. As predicted (Figure 3.1) using glycosylation servers, this site was the most promising site to be glycosylated in *P. pastoris*. In line with this expectation, we observed that the mutants containing N150Q substitution showed limited glycosylation compared with the native lipase. On the other hand, two other predicted sites (N59Q and N269Q) did not have any impacts on the glycosylation of native ANL. Considering that the sites were predicted less confidently than N150Q, it was indeed expected that N59Q and N269Q did not show any significant effects on glycosylation of native ANL. Basically, these results confirm that the native ANL is prone to glycoslyation at N150Q, while it is not at N59Q and N269Q.

The commercial exploitation of lipases in particular has traditionally been hampered by their inadequate stability and the limitations imposed by reaction conditions (neutral buffers, moderate temperature and pH stability, and so on) [60]. However, the recent developments in biocatalysis in unconventional media, especially organic solvents [6], transformed the industrial perception of enzymes as very delicate catalysts, generally unsuitable for large-scale chemical synthesis. Most of the commercially available detergents have proteases as additives [61]. The resistance of our enzyme toward proteases is another property which may be of industrial importance as there is a growing interest to engineer lipases to be protease-resistant for use in detergents [61]. The lipase purified by us may be suitable for some of the above biotransformations. Finally, the scope of this thesis could be used to generate mutations with altered post-translational modifications and thus altered stability. As a result, the findings of this thesis impose would impose implications in enzymology which aims to design enzymes with optimal features.

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A APPENDIX

A.1 Expression Vector Maps

A.2 QIAquick Gel Extraction Kit

A.3 Electrophoresis Marker Legends

A.4 QIAGEN Plasmid Purification Kit

A.1 Expression Vector Maps

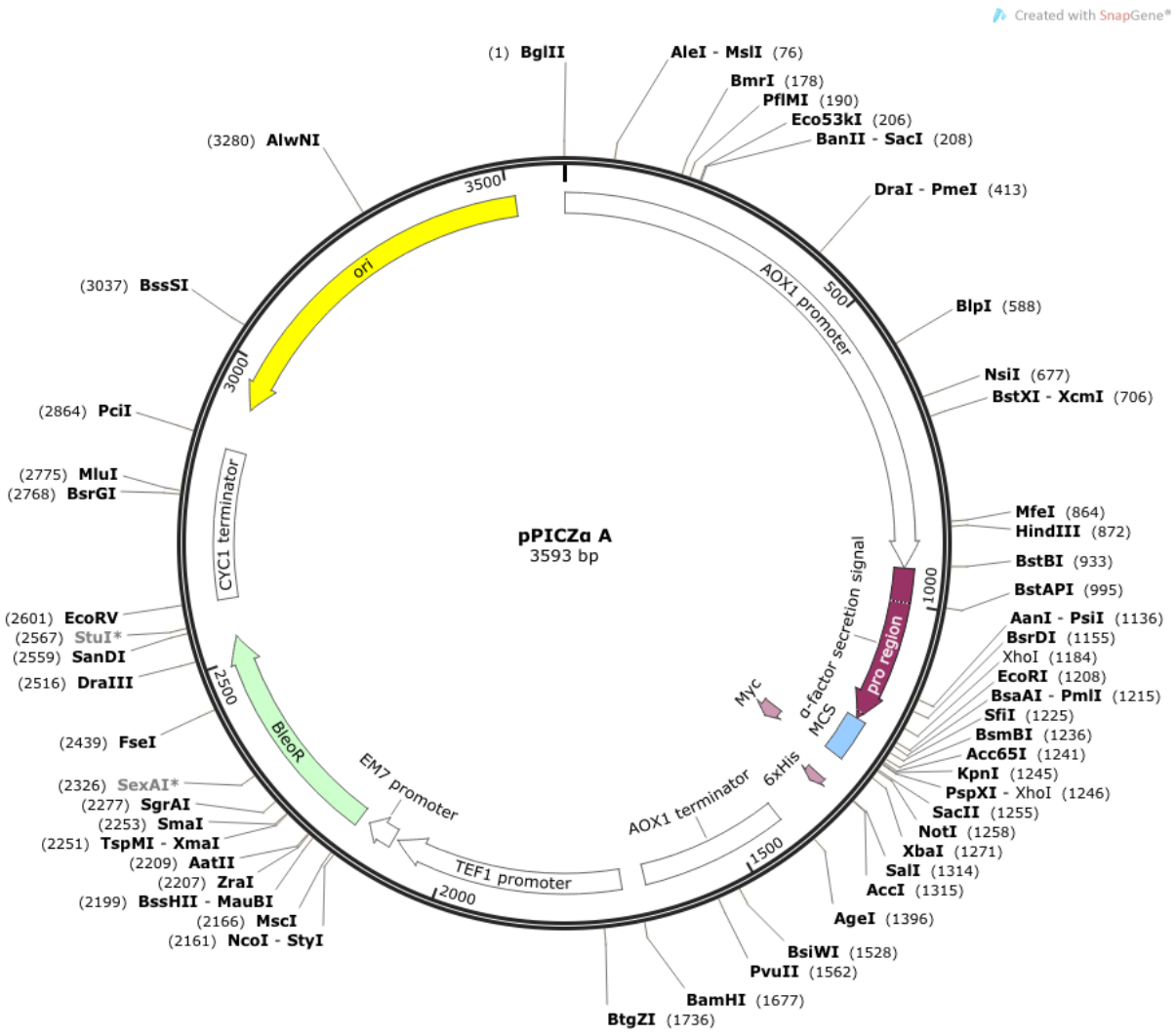

Figure A.1: Expression Vector Map

A.2 QIAquick Gel Extraction Kit

QIAGEN- QIAquick Gel Extraction Kit Protocol:

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg ~ 100 µl). For >2% agarose gels, add 6 volumes Buffer QG.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.

5. Add 1 gel volume of isopropanol to the sample and mix.

6. Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold.

7. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 µl, load and spin/apply vacuum again.

8. If the DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 0.5 ml Buffer QG to the QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.

9. To wash, add 0.75 ml Buffer PE to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.

Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt-ended ligation), let the column stand 2–5 min after addition of Buffer PE.

10. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min at 17,900 x*g* (13,000 rpm) to remove residual wash buffer.

11. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

12. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl Buffer EB to the center of the QIAquick membrane let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.

13. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

A.3 Electrophoresis Marker Legends

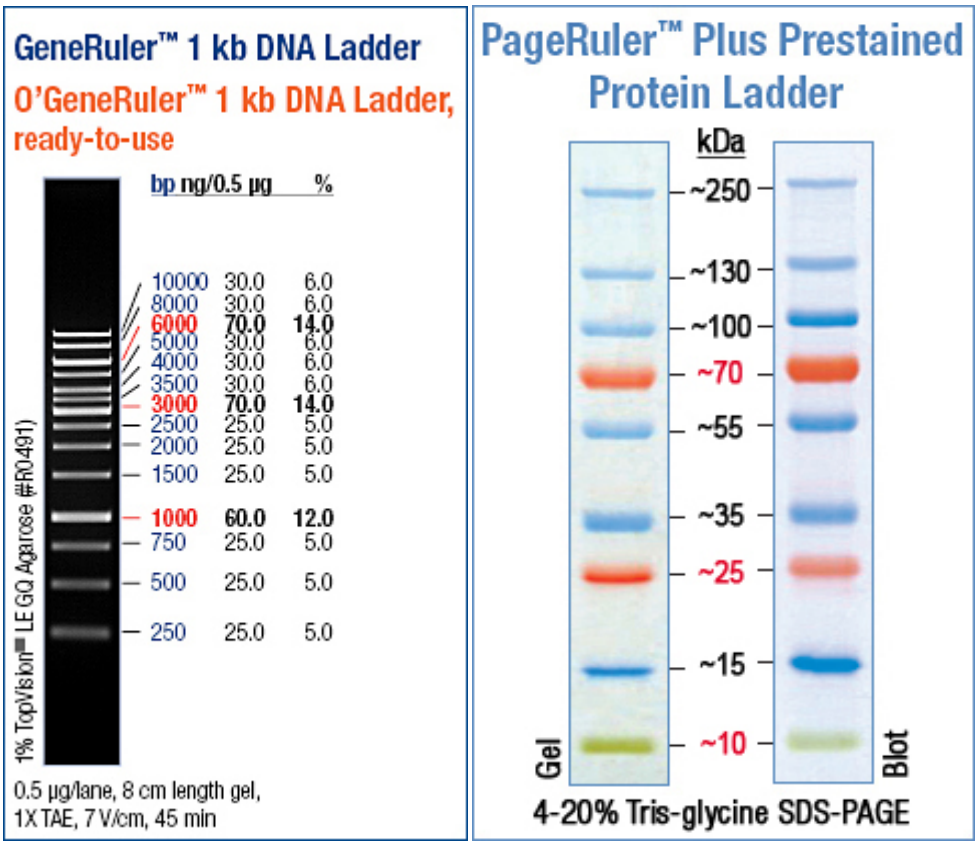

Figure A.3: Electrophoresis Marker Legends

A.4 QIAGEN Plasmid Purification Kit

QIAGEN- Plasmid DNA Purification Kit Protocol:

1. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a 1.7ml microcentrifuge tube.

No cell clumps should be visible after resuspension of the pellet. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.

Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min. If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

3. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4 – 6 times. Keep on ice for 10 minutes.

To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. The solution should become cloudy. If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless.

4. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.

5. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.

6. Centrifuge for 30–60 s. Discard the flow-through.

7. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60s.

8. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.

Important: Residual wash buffer will not be completely removed unless the flowthrough is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

9. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.