# MODIFICATIONS ON PROTEIN TERMINI OF BACILLUS THERMOCATENULATUS LIPASE AND THEIR IMPACTS ON ACTIVITY AND STABILITY

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# Modifications on Protein Termini of *Bacillus thermocatenulatus* Lipase and Their Impacts on Activity and Stability

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## Abstract

*Bacillus thermocatenulatus* lipase (BTL2) is a thermostable enzyme with a known three dimensional structure (PDB ID: 2W22). The N- and C- termini of protein backbone in this structure seated very close to each other (<5 Å), unlike to other lipase structures having their termini located fairly apart from each other. For other proteins that possess circular backbone, the close proximity of the protein termini has been shown to contribute to thermal stability. From this perspective, the protein termini and particularly its impacts on lipase stability are investigated in this thesis. During these investigations, three BTL2 variants are used and explicitly these are N7G, N7Q and R5C-A6C-N5C-S386C-L387C-R388C. The closest contact of the backbone termini is a hydrogen bond formed by the side chain of 7th asparagine and the main chain of L387. In the first two mutations N7G and N7Q, the impact of this hydrogen bond is investigated, while in the third mutation three consecutive residues from the N-terminus (5-6-7) and from the C-terminus (386-387-388) are substituted with cysteines aiming to induce a disulfide bond in the third mutant. Along with the native BTL2, three mutants are obtained in high purity via application of various molecular biology and protein engineering routines including site-directed mutagenesis, ligation-independent cloning, heterologous protein expression and affinity purification methods. The native BTL2 and the mutants are subsequently characterized in enzyme activity assays to determine their thermoactivity, thermostability and substrate selectivity profiles. Furthermore the far-UV circular dichorism (CD) spectra are collected for all lipases to analyze their secondary structure and melting temperatures. The results indicated that all three mutations did not have any significant effects on thermal stability, thermoactivity and substrate selectivity of native BTL2 suggesting that the modification of the hydrogen bond at the lipase termini is not related to the integrity and thermal stability of the catalytic domain. Different from the hydrogen bond mutants, the third mutant showed significant decrease in the thermoactivity and thermal stability of the native BTL2. This particular finding suggested that the cysteine substitutions at the termini caused destabilization of the active site and overall structure of the lipase. Considering the possible implications of the modification of the protein backbone such as generation of protein analogues with optimal stabilities, this thesis aimed to analyze the impacts of the modifications of the termini on the lipase characteristics. Overall, it has been concluded that such modifications of the termini would be useful in generation of lipase variants with optimal features without affecting the core domains.

# *Bacillus thermocatenulatus* Lipazı Protein Uçlarının Modifikasyonu ve Bu Modifikasyonların Aktivite ve Stabiliteye Olan Etkisi

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## Özet

*Bacillus thermocatenulatus* lipazı (BTL2) üç boyutlu yapısı bilinen (PDB ID: 2W22) termokararlı bir enzimdir.Amino ve karboksi uçları birbirinden oldukça uzakta bulunan diğer lipaz yapılarına kıyasla *Bacillus thermocatenulatus* lipazının amino ve karboksi uçları birbirine yakın bir mesafede (<5 Å) bulunmaktadır.Dairesel protein omurgasına sahip diğer proteinler için protein uçlarının birbirine yakınlığının termal stabiliteye olan katkısı gösterilmiştir.Bu açıdan yola çıkarak, bu tez protein uçlarının lipaz stabilitesine olan etkisini araştırmaktadır.Bu araştırma için N7G, N7Q ve R5C-A6C-N5C-S386C-L387C-R388C olarak üç farklı BTL2 mutantı kullanılmıştır.BTL2'nin protein omurgası ucundaki en yakın teması yan zincirdeki N7 ve ana zincirdeki L387 rezidüsü arasındaki hidrojen bağı oluşturmaktadır. N7G ve N7Q mutasyonlarında hidrojen bağının etkisi araştırılırken, diğer mutasyonda ise amino ucundan ardışık üç rezidü (5-6-7) ve karboksi ucundan ardışık üç rezidü (386-387-388) sistein amino asitine çevrilerek uçlar arası disülfit bağı kurulması amaçlanmıştır.BTL2 ile birlikte üç mutant BTL2 proteini, protein mühendisliği rutin yöntemlerinden olan yönlendirilmiş mutagenez, ligasyondan bağımsız klonlama, heterolog protein ekspresyonu ve afinite purifikasyonu kullanılarak yüksek saflıkta elde edilmiştir.Doğal BTL2 ve mutant proteinlerin termoaktivite, termostabilite ve subsrat seçiciliği profilleri enzim aktivite analizleri ile karakterize edilmiştir.Ayrıca bütün lipazların ikincil yapıları ve erime sıcaklıkları CD spektroskopi yöntemi ile belirlenmiştir.Sonuçlar, üç mutasyonun da termostabiliteye, termoaktiviteye ve subsrat seçiciliğine büyük bir etkisi olmadığı yönündedir.Ayrıca protein uç kısmındaki hidrojen bağı mutasyonlarının, protein bütünlüğüyle ve protein aktif bölgesinin termal stabilitesiyle ilgisi olmadığı gösterilmiştir. Hidrojen bağı mutasyonlarından farklı olarak üçüncü mutant, BTL2'nin termoaktivitesini ve termal stabilitesini düşürmüştür. Bu bulgu, protein ucundaki sistein amino asitlerinin aktif bölge ve lipaz yapısının destabilizasyonuna neden olduğunu göstermektedir.Optimum stabiliteye sahip protein analogları üretmek gibi protein omurgasında yapılan modifikasyonların olası etkileri düşünüldüğünde, bu tez protein uçlarındaki modifikasyonların lipaz karakteristiğine olan etkisini analiz etmeyi amaçlamıştır.Genel olarak, protein uçlarındaki modifikasyonlar protein aktif bölgesini etkilemeden optimum özelliklere sahip lipaz değişkenleri üretmek için kullanışlı olacaktır.

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## List of Symbols and Abbreviations

| | |
|---|---|
| 4MU-C2 | 4-Methylumbelliferyl acetate |
| 4MU-C3 | 4-Methylumbelliferyl propionate |
| 4MU-C4 | 4-Methylumbelliferyl butyrate |
| 4MU-C6 | 4-Methylumbelliferyl caproate |
| 4MU-C7 | 4-Methylumbelliferyl enanthate |
| 4MU-C8 | 4-Methylumbelliferyl caprylate |
| 4MU-C12 | 4-Methylumbelliferyl laurate |
| 4MU-C16 | 4-Methylumbelliferyl palmitate |
| 4MU-C18 | 4-Methylumbelliferyl elaidate |
| BTL2 | *Bacillus thermocatenulatus* lipase 2 |
| BTL2_cys | BTL2 with mutations R5C-A6C-N5C- S386C-L387C-R388C |
| CD | Circular dichorism |
| N7G | Asparagine to glycine mutation at residue 7 |
| N7Q | Asparagine to glutamine mutation at residue 7 |
| SDS-PAGE | Sodium doedecyl sulphate-polyacrylamide gel electrophoresis |
| PCR | Polymerase Chain Reaction |
| WT | Wild type |

# 1 INTRODUCTION

## 1.1 Lipases

### 1.1.1 Background

Microbial enzymes are one of the prominent and the largest classes of enzymes because of the large variety of microbes which are known and widely studied. Among microbial enzymes, lipases constitute a significant place owing to their ability to perform biocatalysis.

Lipases are ubiquitously produced by animals, plants and microorganisms. They are not only found in every domain of life but also are widely used enzymes in organic synthesis [1, 2]. Lipases are first discovered by Christiaan Eijkman in 1900s after his observation of particular bacteria that produces and secretes lipases into the extracellular environments to degrade lipids. Lipases (EC 3.1.1.3, triacyl-glycerol lipase) belong to hydrolases and are used for promising protein engineering studies. Since lipases have been broadly investigated; their reactions, mechanism of action, selectivity and structure have already been elucidated and this knowledge is very important in terms of protein engineering [3, 4].

### 1.1.2 Reactions

Lipases are ubiquitous enzymes which are responsible for catalyzing the breakdown of triacylglycerols (TAG) releasing free fatty acids (FA), diacylglycerols (DAG), monoacylglycerols (MAG) and glycerol [5]. This hydrolysis is an equilibrium reaction; hence change in the concentration of reactants and products can disturb the reaction. Moreover, one of the reactants of this hydrolysis reaction is water; hence altering the hydrolytic conditions of reaction mixture changes the equilibrium between forward (hydrolysis) and reverse (synthesis) reactions [6].

Lipases can also catalyze the reverse reaction performing esterification, transesterification which can be subdivided as acidolysis, interesterification, alcoholysis; aminolysis, oximolysis and thiotransesterification in anhydrous organic solvents, biphasic systems and micellar solution (Figure 1.1) [7, 8].

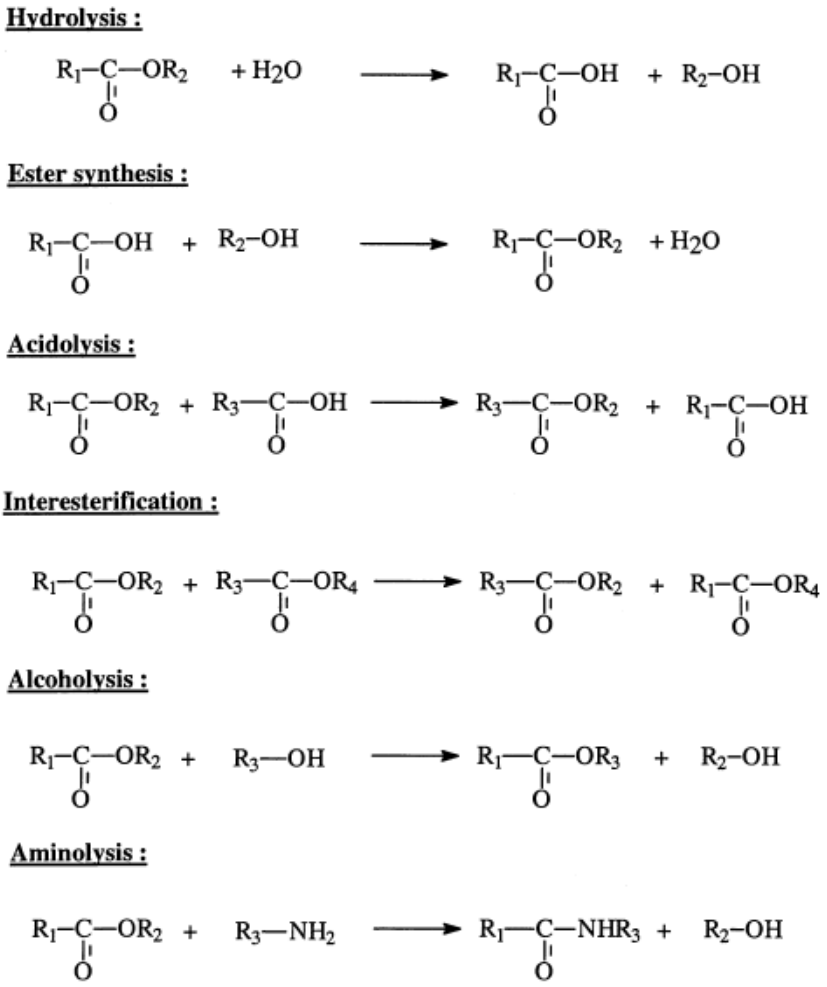



**Figure 1.1:**

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].

In hydrolysis reaction of lipases, they act on carboxylic ester bonds to break them and in esterification, they act on carboxylic ester bonds to form them and catalyze acyl transfer reactions. Triacylglycerols are the natural substrates of the lipases [9, 10]. Initially an unstable acyl-enzyme intermediate is formed, which then collapses to free enzyme and

an acid in hydrolysis or to free enzyme and an ester in esterification and transesterification.

In lipase-catalyzed reactions according to the chemical properties of the reactants and water presence in the media, potential outcomes can be different. Under low water conditions, a carboxyl/thiolester or amide can be produced as well. The acyl-enzyme intermediate can be formed by an ester group as the acyl donor through releasing an acid (water as acyl acceptor) or forming a new ester (alcohol/thiol/amine as acyl acceptor) [9].

Transesterification is defined as the exchange of acyl radicals between an ester and an acid (acidolysis) or an alcohol (alcoholysis) or an ester (interesterification), as shown in Figure 1.1.

Apart from, the hydrolysis and the synthesis of carboxylic esters, lipases can 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

Despite the versatility of the lipase-catalyzed reactions, the mechanism of the lipase action is unique. The catalytic machinery is preserved in all lipases and is comprised of three residues which are serine, histidine, and aspartate/glutamate. This catalytic triad is identical to serine proteases [11]. Therefore, it is accepted that the mechanism of the lipases are the same with the serine protease catalysis [12]. Reaction mechanism of lipases in hydrolysis of triacylglycerols is demonstrated in Figure 1.2. The mechanism occurs by the alignment of the histidine and the aspartate/glutamate residues to decrease the pKa of the serine hydroxyl that makes serine to perform a nucleophilic attack on the ester bond [13].

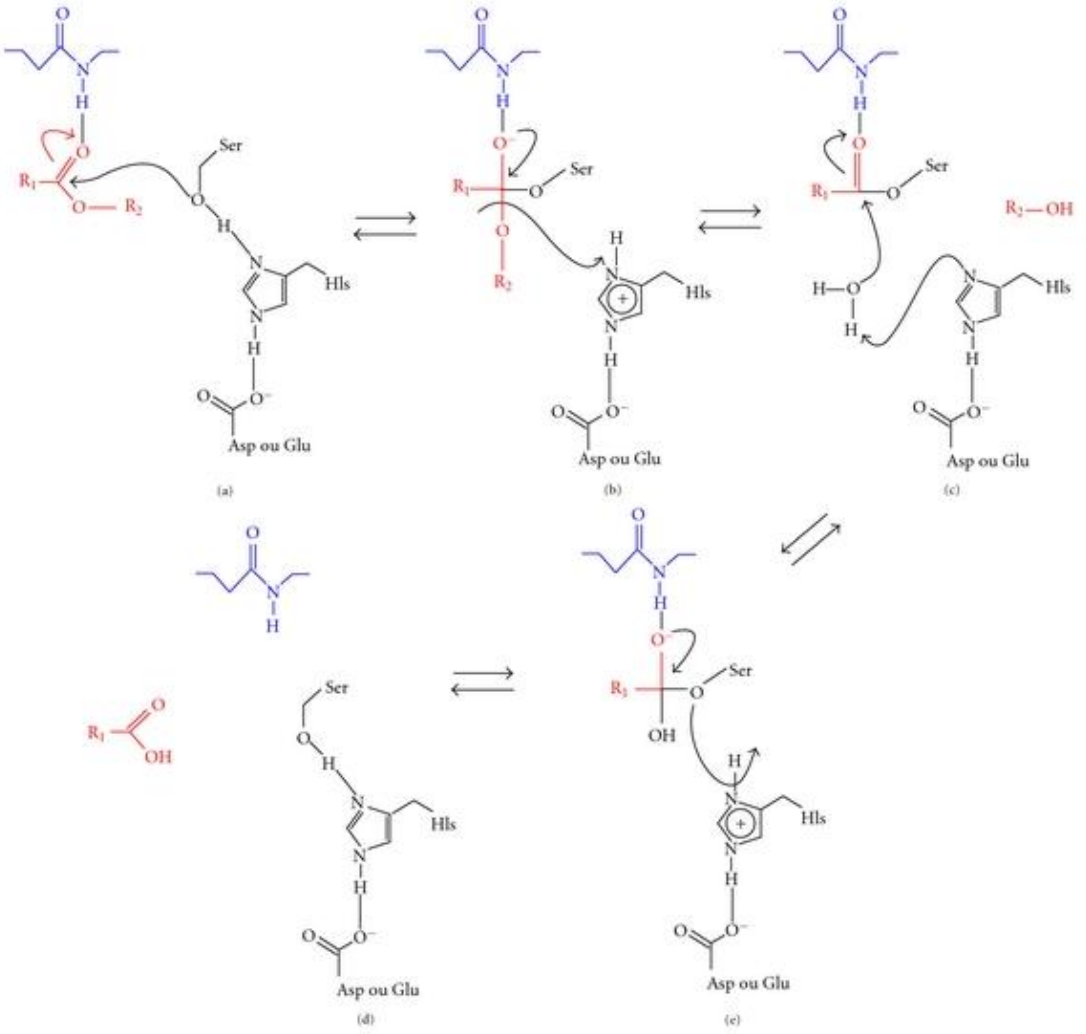



**Figure 1.2:**

Mechanism of the Hydrolysis Reaction of Triacylglycerols and Ester Bonds by Lipases. The catalytic triad and water are indicated as black. The oxyanion hole residues are indicated as blue and the substrate is indicated as red. "a" represents the nucleophilic attack of the serine hydroxyl on the carbonyl carbon of the ester bond which is susceptible to nucleophilic attack. "b" indicates the tetrahedral intermediate. "c" represents the acyl-enzyme intermediate and nucleophilic attach which is carried out by water. "d" indicates the tetrahedral intermediate. "e" represents the free enzyme [14].

The mechanism of the lipase catalysis includes the nucleophilic attack of hydroxyl group of serine residue which is found in the active site, on the carbon from the ester bond of the substrate resulting in the formation of the tetrahedral intermediate. Then the negative charge on the substrate is stabilized in the oxyanion hole which is formed by the main-chain amide groups of two residues. Histidine possesses a positive charge

which is stabilized by the aspartate/glutamate of the catalytic triad. The tetrahedral intermediate loses an alcohol molecule to form an acyl-enzyme intermediate. Later, the acyl-enzyme intermediate is hydrolyzed by the nucleophilic attack of a water molecule to form the second tetrahedral intermediate which eventually loses an acid molecule resulting in the generation of the enzyme in its native form [14].

### 1.1.4 Substrate Selectivity

Lipases show selectivity towards fatty acids regarding the type, the chain length and the presence of alcohol moieties in the substrates [15]. Therefore, lipases can have preference towards a particular fatty acid or a group of fatty acids. For instance, the *Aspergillus flavus* lipaseexhibits higher selectivity for tricaprin than the triolein, while the *Candida rugosa*. Furthermore the *Rhizomucor miehei* lipase has higher preference for oleic acid than eladic acid whereas the lipase A of *Candida antartica* has higher selectivity for elaid acid than oleic acid [16, 17]. Additionally lipase activities may differentiate according to the different classes of alcohols such that primary alcohols have greater preference than the secondary alcohols and the secondary alcohols have greater preference than tertiary alcohols for lipases [18]. Also, it is indicated that tertiary alcohols and their esters act as poor substrates to lipases [19, 20].

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 [21].

Lipases also show chain length selectivity and in terms of the chain length of fatty acids, most of the lipases have greater selectivity towards medium (C6) to long (C16) chain lengths [2]. Nevertheless, few exceptions can be counted such as *Penicillium roquefortii* and *Bacillus thermocatenulatus*lipase which hydrolyze esters of short chain (C4) instead of medium and long chain fatty acids [22]. On the other hand, *R. miehei* lipase can hydrolyze esters of long chain fatty acids as long as C22 [23].

### 1.1.5 Structure

The lipase structure has first been found in 1990 by Brady (REF). Following this structure of the *R. miehei* lipase, the three dimensional structures of various lipases have been revealed by X-ray crystallography [11]. Depending on these structural studies, the following characteristics can be considered as common to all lipases:

(1) All of the lipases are members of "α/β-hydrolase fold" family which has a core structure composing of parallel β strands which surrounded by α helices [24-27].
(2) 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 Gly-X-Ser-X-Gly. This sequence forms a specific β-turn-α motif which is called "nucleophilic elbow" [24, 25, 27].
(3) Lipases have an active site which is formed by a catalytic triad composing of serine, histidine and aspartic acid/glutamic acid amino acids. Although lipases and proteases share chemically similar catalytic machinery, they possess structurally different active sites due to the distinct orientation of the seryl hydroxyl group to give rise to an inverted stereochemistry of catalytic triad in lipases [27-29].
(4) Active site of the lipases is covered by a lid or a flap which consists of two amphiphilic helices [30]. The lid structure can vary among lipases by means of size and composition. For example, the lipase of guinea-pig has a "mini-lid" composed of five amino acids [31]. However, *Bacillus thermocatenulatus* lipase has two α-helices which constitute the 20% of the structure of the lipase.
(5) 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 and an oxyanion hole which is formed by two backbone amides of two residues located in N-terminal region of the lipase and the C-terminal of the catalytic serine [32, 33].

Lipases are divided into three subgroups in terms of geometry of the catalytic cleft according to Pleiss et al. (1998). These subgroups are hydrophobic crevice-like, funnel-like and tunnel-like binding sites [34]. 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 [35]. Substrate selectivity is among one of the most important characteristics of the microbial lipases that makes them favorable for industrial applications such as detergent and cosmetics markets. Therefore, lipases belong to the third largest group of commercial enzymes [36].

One of the significant fields in food processing industry is modification of the fats and oils which are major food constituents. Lipases enable the modification of lipid properties through changing the fatty acid chain locations in the glyceride and relocating one or more of these with novel ones. Therefore, relatively low-priced and less favorable fats could be modified to a fat which has higher value [37]. Moreover, esterification and transesterification provide production of value added products hence, higher industrial potential compared to production of fatty acids through hydrolysis.

Lipases are able to catalyze esterification, interesterification and transesterification in non-aqueous media that gives rise to versatility of lipase reactions resulting in being a potential application tool for food, detergent, pharmaceutical, leather, textile, cosmetic and paper industries [38]. Although the major fields of the lipases are food processing and detergent industry; by the help of new biotechnological applications, lipases not only can be used in synthesis of biodiesel, agrochemicals, drug intermediates, amino acid derivatives, polymers, and flavor compounds; but also can be applied in biosensor and bioremediation systems [39, 40].

Lipases are broadly used in dairy industry for the purpose of milk fat hydrolysis and enhancement of cheese flavors via modification of the fatty acid chain lengths [41]. Also, flavor enhancement of the bakery products by freeing short chain fatty acid chains using transesterification and prolonging the shelf lives are another applications of the lipases. Besides, lipases are used in production of detergents as in oleochemistry and several ester syntheses as in organic synthesis.

Lipases are utilized in pharmaceutical applications by enriching poly unsaturated fatty acids (PUFAs) from animal and plant lipids that are used in production of various

pharmaceuticals. PUFAs have metabolic benefits in terms of being crucial for normal synthesis of lipid membranes and prostaglandins. Microbial lipases are efficient in obtaining PUFAs from plant and animal lipids including menhaden oil, tuna oil and borage oil [37]. In addition to that, liposomes are used medically to optimize drug actions by having role in their transporting to target areas. A class of non-steroidal anti-inflammatory drugs called profens is produced through hydrolysis and esterification reactions of lipases [42].

Lipases have large numbers of application potentials since they display region, substrate and stereo-specificity. Besides, as compared to other enzymes, lipases are not only more stable in organic solvents, at high temperatures and ionic strengths, but also do not need cofactors in their reactions [43].

**Table 1.1:** Industrial Applications of Lipases

| **Industry** | **Action** | **Product/Application** |
|---|---|---|
| Detergents | Hydrolysis of fats | Removal of oil stains from fabrics |
| Dairy foods | Hydrolysis of milk fat, cheese ripening, modification of butter fat | Development of flavoring agents |
| Bakery foods | Flavor improvement | Shelf-life prolongation |
| Food dressings | Quality improvement | Mayonnaise, dressing, and whippings |
| Health foods | Transesterification | Health foods |
| Meat and fish | Flavor improvement | Meat and fish product, fat removal |
| Fats and oils | Transesterification; hydrolysis | Cocoa butter, margarine, fatty acids, glycerol, monoglycerides and diglycerides |
| Chemicals | Enantioselectivity, synthesis | Chiral building blocks, chemicals |
| Pharmaceuticals | Transesterification; hydrolysis | Specialty lipids, digestive aids |
| Cosmetics | Synthesis | Emulsifiers, moisturizers |
| Leather | Hydrolysis | Leather products |
| Paper | Hydrolysis | Paper with improved qualities |
| Cleaning | Hydrolysis | Removal of fats |
| Beverages | Improved aroma | Beverages |

### 1.1.7 Engineering Lipases

All enzyme catalysis must follow the rules that apply to chemical catalysis. However, enzyme catalysis is superior to chemical catalysis by means of efficiency and specificity. Selective nature of reactions for enzymes makes the production of particular products via applying enzymes as catalysts. If the selective nature of enzymes is used in industrial production, energy consumption and waste products may be decreased. Catalysis of enzymes can be considered more important compared to chemical catalysis resulting that enzymes are being more prominent for industrial field.

Utilization of protein engineering methods has increased the information about structure-function relationship that leads to generate new lipases with altered substrate selectivities and improved stabilities. In this sense, variable lipases can be generated to develop efficiency in industrial biocatalysis. Therefore, in protein engineering research, lipases are one of the most popular classes of enzymes. However, harsh conditions in industry cause limitations toward lipase use by damaging the protein nature of lipases [44]. Therefore, thermostable lipases are considered as a solution to cope with these particular problems.

## 1.2 Protein Engineering

### 1.2.1 Background

Protein engineering can be defined as the design of novel proteins with improved and desirable functions and/or properties. It basically relies on the application of recombinant DNA technology to alter amino acid sequences [45]. The engineering approaches are based on distinctive principles which are rational approach using three-dimensional (3D) models and random approach using directed evolution [46]. Protein engineering is an important tool to elucidate the protein folding and stability or structure-function relationships [47]. Therefore, protein engineering has broad applications varying from industry to basic sciences. Increasing quality of vaccines, development in therapeutics and improved properties of industrial enzymes are achieved via protein engineering [45].

### 1.2.2 Random and Rational Approach

Protein engineering methodology is constituted of three steps which are the design, the mutagenesis and the production. The whole process may require some prior information such as the structure or sequence of the protein of interest. Various protein engineering methods are applicable today since the rapid advancements of biological sciences; more importantly recombinant DNA technologies. Nevertheless, the engineering rationale depends on either random approach which is the selection of improved proteins/enzymes from randomly generated variants or rational approach which is the guided design of novel proteins/enzymes with improved and desirable properties [45].

The random approach is performed by random mutagenesis and selection which is applied by generating a large number of enzyme variant with random amino acid substitutions and selection/identification of these mutations by their favorable properties by screening methods [48]. Random approach is also referred as directed evolution because of random generation of large numbers of DNA fragments and library of these mutants. Design and mutagenesis steps are carried out concurrently in random approach through directed evolution, domain-swapping which is defined as shuffling of multiple genes or circular permutation which refers to shuffling of protein termini [49].

Design and mutagenesis steps are carried out separately in rational approach. Design step of the rational approach is determined by the three-dimensional structures of the enzymes/proteins. Site-directed mutagenesis, in which amino acids of the selected sites are substituted with desired amino acids, is generally used as mutagenesis step of the rational approach [50]. Also, site-saturation mutagenesis which is the replacement of a single amino acid within a protein with each of the other amino acids can be carried out in mutagenesis of rational approach. This method gives information about all possible variations at that specific site. Therefore, rational approach uses the knowledge of the enzyme structure to determine the specific sites for mutations considering improved qualities and novel functions.

Rational approach is preferable if the structure and the mechanism of the protein of interest are known such as known crystal structure. On the contrary, if the knowledge of the protein of interest is limited like knowing only the primary sequence, evolutionary methods including random mutagenesis and selection is used for the desired protein properties [51]. The consequences of the two approaches are different. Rational

approach provides information about structure-function relations of the enzymes. However, screening method of the random approach determines the outcomes.

In both of the approaches, mutagenesis step is followed after the design step. Heterologous protein expression and purification of the engineered proteins are carried out for obtaining enzyme analogues and enzyme characterization which provides the evaluation of the intended improved properties.

In this thesis, rational approach is chosen since the three dimensional structure of the enzyme is available on the Protein Data Bank. Selection. The critical sites is determined according to the protein visualization tools using VMD (Visual Molecular Dynamics). As methodological overview, mutagenesis and production steps of the rational approach are explained in the following subsections.

#### 1.2.2.1 Mutagenesis

As a protein engineering method, rational approach involves mutagenesis which is one of the most popular DNA manipulation techniques. There are different mutagenesis approaches such as chemical, oligonucleotide-, polymerase chain reaction (PCR)-dependent and cassette mutagenesis [45]. Rational approach generally involves site-directed mutagenesis which is used in my research.

Site-directed mutagenesis is an *in-vitro*, PCR-based technique that introduces specific amino acids into predetermined target site or gene. Two common methods are used for site-directed mutagenesis and these are overlap extension PCR (OE-PCR) method and the whole plasmid single-round PCR method. Overlap extension PCR method makes use of two primer pairs; one of the each primer pairs possesses the mutant codon as the mismatched sequence. First polymerase chain reaction of the OE-PCR which contains two PCRs uses these four primers resulting two double-stranded DNA products. After denaturation and annealing of these double-stranded DNAs, two heteroduplexes are generated and each strand of the heteroduplex contains mutated sequences. DNA polymerase enzyme in the reaction completes the overlapping 3’ and 5’ ends of the heteroduplexes. For the amplification of the mutagenic DNA products, second PCR of the OE-PCR is carried out using the nonmutated primer pairs which is demonstrared in Figure 1.3 [50].

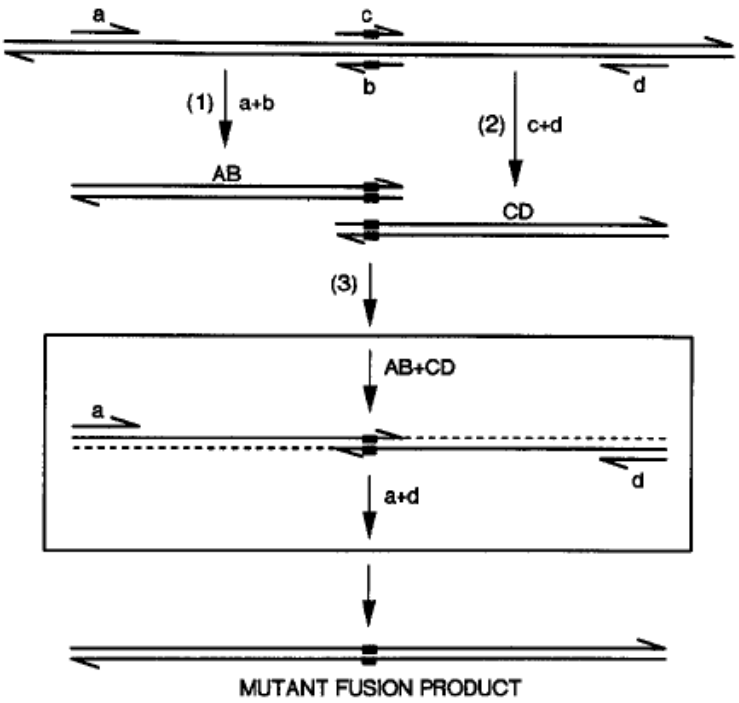



**Figure 1.3:**

Principle of OE-PCR in Site-directed Mutagenesis.
Lines indicate the double-stranded DNAs with arrows representing the 5' to 3' orientation. 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 extends the overlapping 3' and 5' ends of the heteroduplexes in the boxed portion of the figure. Mutant product is further amplified by additional primers [50].

Whole plasmid single round PCR is another site-directed mutagenesis method which requires two primers with mutations. These primers are complementary to the opposite strands of a double-stranded DNA template plasmid. When the polymerase reaction is performed, two strands of the template can be replicated resulting generation of mutated plasmid without overlapping breaks. Selective digestion is used for getting circular, nicked vector which contains the mutant gene. Transformation of this vector makes the repairing of the nick in the DNA and mutated plasmid is obtained [52].

#### 1.2.2.2 Production

Production of the recombinant proteins is the next step following mutagenesis in the rational approach. Heterologous protein expression and purification are used for the production purposes since different expression systems are available such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Pichia pastoris*. Expression system of *E. coli* is chosen in this thesis.

Protein expression in *E. coli* is the most widely used system in producing recombinant proteins for more than two decades. The reasons can be counted as *E. coli* is a well-established host since the recombinant technology is founded on this organism, culturing time of the *E. coli* is shorter compared to other expression systems, genetic manipulation is easier for being a prokaryotic organism and media required for the expression is cheaper than the other medias. Besides, it is possible to express more than one protein with *E. coli* [53]. T7 promoter system is mostly used in *E. coli* expression in which gene of interest is carried by an expression vector cloned downstream of the T7 promoter and this promoter is introduced into T7 expression host. Choice of the promoter and strain is also important parameters for choosing the expression system.

Preferring one of the expression systems is also associated with the protein of interest. Yeast systems are chosen for the eukaryotic proteins since yeast is able to achieve post-translational modifications on the recombinant protein. Therefore, if the emphasis of the research is relevant with the phosphorylation or glycosylation, yeast expression systems could be used.

The production scale is determined with regard to the required amount such as cellular production for cell biology studies, bench-scale production for characterization studies and fermentation for industrial applications.

Production of the recombinant proteins by heterologous expression and purification is the last step of the rational approach which is resulted by the characterization of the mutant proteins such as protein size, concentration and enzyme activity.

## 1.3 Bacillus thermocatenulatus lipase

### 1.3.1 Lipase family 1.5

Bacterial lipases are classified as eight lipase families with regard to their peptapeptide motif containing catalytic serine [54]. Family 1 has six subfamilies and five of them have Gly-X-Ser-X-Gly pentamer motif. However, Ala-X-Ser-X-Gly pentamer is found in the members of Lipase 1.5. Despite the fact that some properties of Family 1.5 are different from other subfamilies, Lipase Family 1.5 is known to posses high sequence identity among its identified members and as a result they share common biochemical and structural properties. Lipases of this family show higher activity at high pHs and high temperatures since they are originated from thermoalkalophilic species [55, 56]. In addition to that, members of this lipase family are larger lipases like more than 40 kDa compared with other subfamilies. Extra domain which is not seen in other lipase families is found in members of Family 1.5 for zinc coordination which is the reason for the increased thermostability [57]. *Bacillus thermocatenulatus* lipase (BTL2) which would the lipase of interest in this thesis is also member of the Lipase Family 1.5.

### 1.3.2 Biochemistry

*Bacillus thermocatenulatus* lipase (BTL2) is coded by 1167 bp fragment and composed of 389 residues. The predicted molecular weight of the enzyme is 43 kDa. Lipase stability is preserved at pH range of 9-10 and in the temperature range of 60-70ºC and also in various organic solvents and detergents [58]. At pH 8-9 and 60 ºC temperatures, BTL2 shows optimum activity [59]. BTL2 prefers to catalyze *sn*-1/3 acyl chain in triglycerides that is common for microbial lipases. In terms of chain selectivity, BTL2 does not show a wide range of chain-length selectivity such that it shows activity towards short (C4) and medium (C8) chains. Among these, short chains (C4) are the most preferable chain-length for BTL2. However, in long chains of triglycerides which refer to C10 and longer chain length, BTL2 shows only lower activity [56].

### 1.3.3 Structure

The crystal structure of BTL2 is solved at 2.2 Å. Three-dimensional structure of BTL2 contains 389 residues and an irregular α-β hydrolase fold which is formed by a central β-sheet of seven strands which is defined as β3 to β9 and surrounded by α-helices which are defined as α1 and α13 on one side and α2, α4 and α10 on the other side [27]. An

extra zinc domain is found in BTL2 that is formed by helices α3 and α5 and strands b1 and b2 [59]. Coordination bonds of zinc are generated with His-82 and His-88 from the extra domain and Asp-62 and Asp-239 from the core domain. BTL2 structure is elucidated in the active (open) form with two molecules of Triton X-100 which is located at the active site [27]. VMD representation of the BTL2 structure is demonstrated in Figure 1.4.

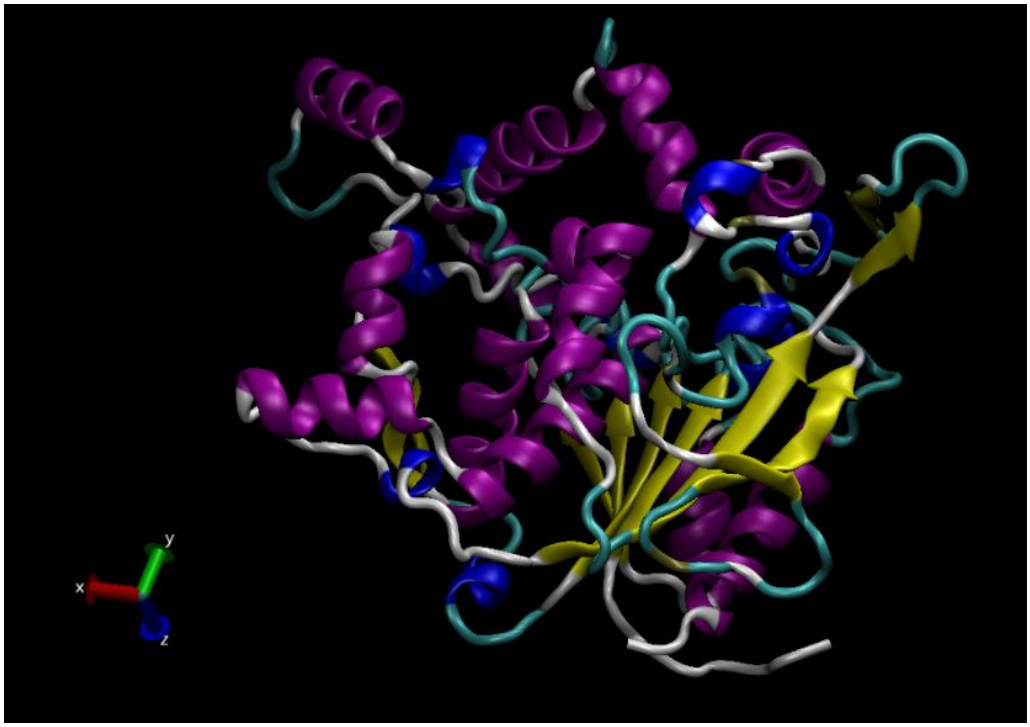



**Figure 1.4:**

VMD Representation of Open Conformation of *Bacillus thermocatenulatus* Lipase. Figure indicates α-β hydrolase core; cap domain, and extra zinc domain.

The catalytic machinery of BTL2 is formed by catalytic triad residues which are Ser-114, His-359 and Asp-318 -indicated in Figure 1.5- and found in α-β hydrolase fold, and the oxyanion hole and its mechanism is like most of the lipases explained in the "1.1.3 Mechanism" section. Catalytic serine of the BTL2 has Ala-X-Ser-X-Gly motif [60]. Conformation of the catalytic serine changes from closed to open formation as well as the hydrogen bonding patterns in the BTL2.

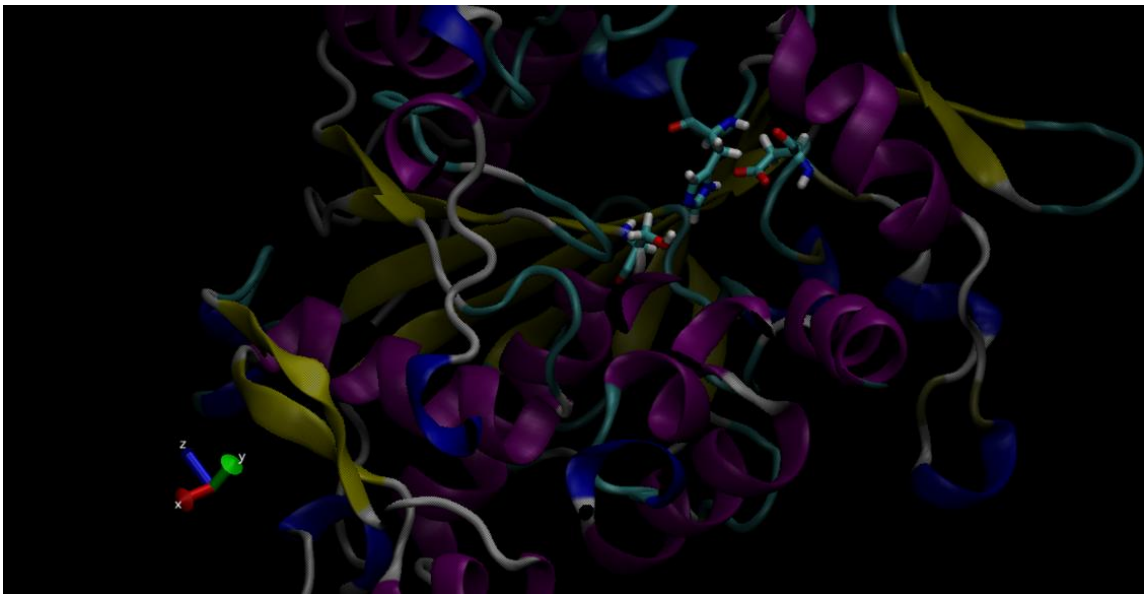


**Figure 1.5:**

VMD Representation of Catalytic Triad Residues in Open Conformation of *Bacillus thermocatenulatus* Lipase.
Figure indicates catalytic triad residues which are Ser-114, His-359 and Asp-318 (from left side to right side) and found in α-β hydrolase fold

Two molecules of Triton X-100 in hydrophobic active cleft utilize its identification. The cleft is 14-Å deep with an ovoid shape and its dimensions are 18×25 Å. Hydrophobic and aromatic side chains cover the walls of the cleft. These side chains which are A241, I320, L171, L184, L189, L209, L245, L360, L57, M174, F17, F182, F291, P165, Y30, V172, V175, V188, V234, V295, V321 and V365, utilize perfect stabilization of the substrate. F17 aromatic side chain is located at the base of the cleft and separates it to two sites. Moreover, four binding pockets, an oxyanion hole and three pockets for various branches of the TAG substrate are found in the active cleft of BTL2 [27].

Like the most of the lipase, as a structural element BTL2 has a highly mobile lid domain at the entrance of the active cleft to control the access to the active site. Enzyme and lipid aggregates interactions give rise to lid displacement which makes the active site available for the substrate by increasing its catalytic activity. This phenomenon is defined as interfacial activation [61]. In BTL2, α6 and α7 helices form the lid structure which is found as linked to core and has high flexibility. In contrast to closed (inactive) form of the BTL2 in which two helices forms the lid structure, C termini of α6 and α7 helices move about 20 Å away from the active cleft entrance in open form of BTL2

[57]. From close to open conformations, the second α helix moves away from the opening of the cleft by moving its mass center approximately 20 Å and induces disruption of the first α helix in the lid. Therefore, the first α helix of the closed conformation generates two smaller α helices which enables substrates to reach the catalytic serine [27].

### 1.3.4 Prospective Applications of *Bacillus thermocatenulatus* lipase

*Bacillus thermocatenulatus* lipase (BTL2) is a thermoalkalophilic lipase belonging to the Lipase Family 1.5 and an enantioselective biocatalyst which makes it an important alternative to its counterparts in industry since its catalytic activity towards different chiral substrates [62]. 95% sequence identity is found in members of Lipase Family 1.5 which means new discoveries about BTL2 likely accounts for whole family [54]. Apart from that, BTL2 has high resistance to extreme conditions and inactivation agents such as heat, elevated temperatures, pH and organic solvents [38]. Therefore, BTL2 is considered as a significant research target due to being a thermostable enzyme and its potential to overcome harsh conditions in industry.

BTL2 would also be a potential catalyst for enrichment of long chain triglycerides. Also, in terms of chain length specificity, BTL2 shows highest specific activity towards short chains (C4) of fatty acids among other lipases, which makes it favorable for production of short chain fatty acids [56, 58]. Highest specific activity of BTL2 towards tributyrin makes it a perfect candidate for dairy industry such as preparation and enhancement of cheese flavors; and production of milk fat. Due to the potential of BTL2 in industrial applications, the properties of this enzyme can be enhanced and improved via protein engineering studies.

## 1.4 Circular proteins

Circular proteins have the advantage over ancestral linear proteins and preserve the intrinsic biological properties and functions of those linear proteins as well. Termini of the linear peptide chains often show flexibility that makes them convenient targets for attack of the proteolytic enzymes. Advantages of the circular proteins involve higher resistance to proteolytic cleavage and increased stability. The cyclotides are one of the

important families of circular proteins that contain cyclic cysteine knot motif which provide additional stability and complexity among members of the family [63].

Despite the field of circular proteins is still in its infancy, the circular shape of proteins appears to be involved in improving stability without comprising the biological activity compared to linear backbone [64]. Overall, whilst the role of circular backbone is not fully understood, the circular backbone is likely to be accounted for thermostability in the structure [65, 66].

The protein of interest, BTL2; having its termini seated close to each other (~5 Å), is a candidate to generate a circular backbone and investigate its affects on thermal stability and activity. Formation of disulfide bridges at the termini by addition of cysteine residues by mutagenesis might have an effect on thermostability. Previous mutagenesis strategies to obtain higher thermostable lipase variants have included forming extra disulfide bonds via cysteine knot motifs that provided additional stability [63]. The decreased conformational chain entropy of the denatured protein stems from the introduction of disulfide bonds which makes significant contributions to protein stability. In order to enhance the stability of proteins, several attempts to introduce novel disulfide bonds have done. However, some of the attempts did not result in stabilization. Even some experiments lead to the destabilization of protein when compared to the nativeenzymes. One of the reasons for the drawbacks of disulfide bond introduction is the existence of steric contacts of the strain since the introduced disulfide bond precluded required stereochemistry [65]. In addition, substitution of a residue with cysteine may cause loss of favorable interactions [67]. Determination of a strain with a suitable stereochemistry may not negatively affect after the substitution of cysteine residues but may increase thermostability of the protein.

In BTL2, closest contact of the backbone termini is a hydrogen bond which is formed by the side chain of 7th asparagine and the main chain of leucine at residue 389 [27]. Mutagenesis strategies can be used to elucidate the effect of this hydrogen bond in the termini whether this bond contributes to thermostability. Conversion of asparagine at residue 7 to glycine by mutagenesis eliminates the formation of hydrogen bonding and conversion of asparagine at residue 7 to glutamine adds extra carbon chain to the site of hydrogen bonding. Investigation of these two mutations reveals the role of hydrogen bonding between the termini of BTL2.

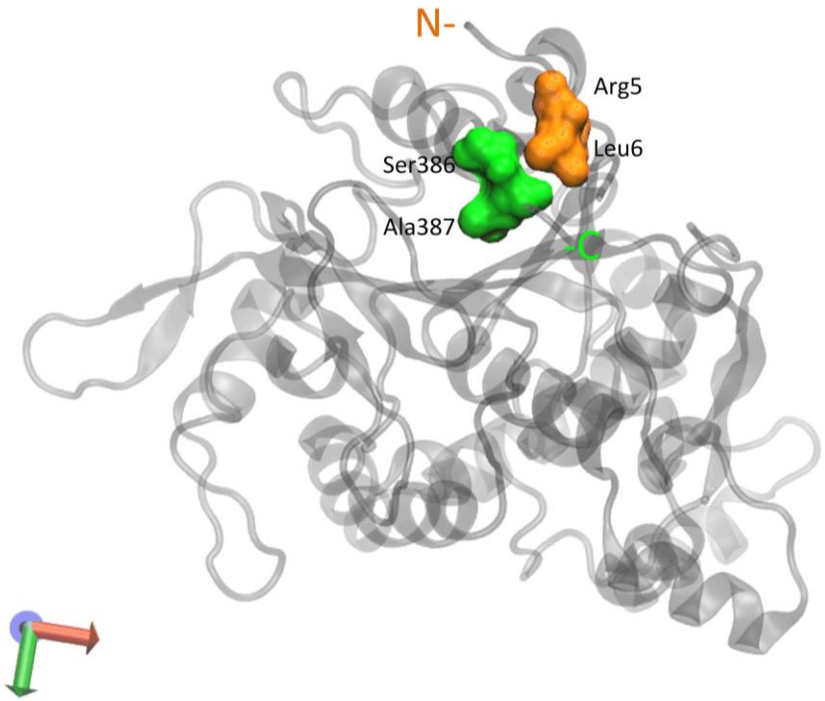



**Figure 1.6:**

VMD Representation of Termini of *Bacillus thermocatenulatus* Lipase.
Figure indicates the proximity of N and C termini and mutation locations for disulfide bonds.

# 2 EXPERIMENTAL

## 2.1 Methods

### 2.1.1 Molecular Cloning

A 1,167-bp DNA fragment indicating the *Bacillus thermocatenulatus* lipase (BTL2) gene was amplified from the mature lipase clone (pPICZαA – BTL2) DNA through ligation independent cloning. The primer sets contain ligation-independent cloning (LIC) sites; (**bold**) for forward (**TACTTCCAATCCAATGAA**GCGGCATCCCCACGCG) and for reverse (**TTATCCACTTCCAATGAA**AGGCCGCAAACTCGCCAA). PCR condition is demonstrated as below.

**Table 2.1:** PCR Profile for BTL2

| Step | ºC | min | Cycle |
|---|---|---|---|
| Initial Denaturation | 94 | 3 | 1 |
| Denaturation | 94 | 0.5 | |
| Annealing | 52 | 0.5 | 35 |
| Extension | 72 | 1 | |
| Final Extension | 72 | 7 | 1 |
| Hold | 4 | | |

4 µg expression vector which is pMCSG7 (N-terminal polyhistidine bacterial expression vector) was linearized by using the restriction enzyme *Ssp*I(see Appendix A1 for expression vector map) according to Table 2.2.

**Table 2.2:** pMCSG7 *SspI* Digestion

| | |
|---|---|
| pMCSG-7 | 50 µl |
| *Ssp*I | 2 µl |
| Green Buffer | 6 µl |
| ddH2O | 2 µl |
| $Volume_{final}$ | 60 µl |

Agarose gel electrophoresis was carried out for linearized vector and PCR products at 100 Volt in 1.2% agarose gels by using TBE (tris-borate EDTA) buffer for 20 minutes. Each of the fragments was extracted from the agarose gel according to the instructions of the QIAquick Gel Extraction Kit (see Appendix A2). The extracted DNA fragments were treated with T4 DNA Polymerase. The polymerization activity of T4 DNA Polymerase was terminated by adding excess amount of dGTP for the linear vector and dCTP for the PCR products. T4 DNA Polymerase reaction was carried out at 20ºC for 60 minutes and 75ºC for 20 minutes. The reaction mixture is as indicated in Table 2.3.

**Table 2.3:** T4 Polymerase Reaction

| **Vector** | Volume | **PCR Product** | Volume |
|---|---|---|---|
| ddH2O | 1 | ddH2O | 1 µl |
| 5X Buffer | 14 µL | 5X Buffer | 14 µl |
| T4 Polymerase | 3 µL | T4 Polymerase | 3 µl |
| dGTP | 2 µL | dCTP | 2 µl |
| DNA | 50 µL | DNA | 50 µl |
| Vfinal | 70 µL | Vfinal | 70 µL |

DNA fragments which are treated with T4 Polymerase were extracted using phenol-chloroform and precipitated using 2-proponal 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 -80ºC 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 $ddH_2O$.

Vector pMCSG7 and PCR products were annealed at 22ºC for overnight in which 150 ng vector and 100 ng PCR products were used. Chemically competent *E. coli* (Shuffle, NEB)cells prepared and transformation of the annealing reaction mixture to the *E.coli* Shuffle cells was carried out as following protocol:

- Add the annealing mixture onto 200 µl Shuffle 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 according to 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.

**Table 2.4:** Colony PCR

| | |
|---|---|
| Forward Primer | 0.5 µl |
| Reverse Primer | 0.5 µl |
| Taq Polymerase Master Mix | 5 µl |
| $ddH_2O$ | 4 µl |
| $V_{final}$ | 10 µl |

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 *Bam*HI and *Kpn*I digestion was performed as a confirmation such as recommended by Fermentas. T7 Terminator (GCTAGTTATTGCTCAGCGG) and T7 Promoter (TAATACGACTCACTATAGGG) were used for plasmid sequencing by Molecular Cloning Laboratories (MCLAB).

### 2.1.2 Site-directed Mutagenesis

Two mutations which are N7G (Asparagine to Glycine at residue 7) and N7Q (Asparagine to Glutamine at residue 7) were generated using Overlap Extension PCR (OE-PCR). The primers of the mutation are demonstrated in Table 2.5. For both mutants (N7G and N7Q), two PCR reactions were prepared by using primers F_BTL2_LIC as forward and reverse primers of the mutant; and forward primers of the mutant and R_BTL2_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 BTL2_LIC primers. The PCR profile of the OE-PCR and PCR mixtures are indicated Table 2.6, Table 2.7, and Table 2.8.

**Table 2.5:** Primer Sequences for Mutagenesis

| Mutation | Direction | 5'-3' Sequence |
|---|---|---|
| N7G | forward | CATCCCCACGCGCCGGTGATGCACCCATCG |
| | reverse | CGATGGGTGCATCACCGGCGCGTGGGGATG |
| N7Q | forward | CATCCCCACGCGCCCAGGATGCACCCATCGT |
| | reverse | ACGATGGGTGCATCCTGGGCGCGTGGGGATG |
| BTL2_cys | forward | TACTTCCAATCCAATGCCGCGGCATCCCCATGCTGCAATGATGCACCCATCGTGCTT |
| | reverse | TTATCCACTTCCAATGCCAGGGCAGCAGCACGCCAACTGCTCGGCAAGTCG |

OE-PCR Primer Combinations

Q1: F_BTL2_LIC / R_mutant (N7Q)

Q2: R_BTL2_LIC / F_mutant (N7Q)

G1: F_BTL2_LIC / R_mutant (N7G)

G2: R_BTL2_LIC / F_mutant (N7G)

**Table 2.6:** 1st Reaction ofOE-PCR

| | **Q1/G1** | **Q2/G2** |
|---|---|---|
| $ddH_2O$ | 11.5 µl | 5.5 µl |
| 2X pwo MasterMix | 15 µl | 7.5 µl |
| Forward Primer | 1.5 µl | 0.75 µl |
| Reverse Primer | 1.5 µl | 0.75 µl |
| Template | 0.5 µl | 0.5 µl |
| $V_{final}$ | 30 µl | 15 µl |

For the 1st reaction of the OE-PCR for Q1 and G1 primer combinations, initial denaturation (3 minutes at 94°C), denaturation (20 seconds at 94°C), annealing (20 seconds at 55°C), extension (20 seconds at 72°C) and the final extension (3 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 55°C), extension (1 min at 72°C) and the final extension (5 min at 72°C) are applied as 35 cycle for denaturation, annealing and extension.

**Table 2.7:** 2nd Reaction of OE-PCR

| | |
|---|---|
| Q1/G1 | 20 µl |
| Q2/G2 | 1 µl |
| 2X pwo Master Mix | 25 µl |
| $ddH_2O$ | 4 µl |

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_BTL2_LIC and 2.5 µl R_BTL2_LIC primers were added

to the reaction. 30 seconds at 94°C for denaturation, 30 seconds at 53°C for annealing and 45 seconds at 72°C for extension were performed as 30 cycles and 7 minutes at 72°C as final extension.

For the mutant BTL2_cys, PCR was performed to obtain the mutant fragment according to the table below. The PCR profile was the same as the Table 2.1.

**Table 2.8:** PCR for BTL2_cys Mutant

| | |
|---|---|
| Template (150 ng) | 0.5 µl |
| Forward Primer | 2.5 µl (0.5 mM) |
| Reverse Primer | 2.5 µl (0.5 mM) |
| PWO (2X) | 25 µl |
| ddH2O | 19.5 µl |
| Vfinal | 50 µl |

The mutant DNA fragments were cloned into pMCSG7 vector through the procedure given above for BTL2 in the section 2.1.1.

### 2.1.3 Lipase Expressions

After the sequence confirmations, the positive colonies of Shuffle *E.coli* cells were selected for lipase expressions. All mutants (N7Q, N7G and BTL2_cys) were expressed in 20 ml cell culture using 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) as the inducer which was added to the culture when the optical density of the cells reached to a value between 0.5-1 at 600 nm. The expressions were carried out for eight hours by taking samples in every one hour as $t_0$, $t_1$, $t_2$, $t_3$, $t_4$, $t_5$, $t_6$, $t_7$ and $t_8$. Each sample was harvested by 5 minutes of centrifugation at 13,200 rpm to obtain the cell pellet containing protein of interest. The cells were lysed by adding 100 µl B-PER (Thermoscientific) to pellets. Further centrifugation was performed to B-PER solubilized samples for fractionation of the soluble lysate. Enzyme activity of the soluble fraction was measured using the fluorescent substrate 4- methylumberrilferone (4MU) caprylate in 0.1 M Tris-Cl at pH 7.25. Furthermore, 20 µl of the soluble fractions were analyzed by SDS-PAGE (sodium dodecyl polyacrylamide electrophoresis) and the gels were stained with Coomassie-Blue. The highest expression interval was determined by comparing $t_0$ with $t_1$, $t_2$, $t_3$, $t_4$, $t_5$, $t_6$, $t_7$ and $t_8$ to carry out another expression under the same experimental conditions at higher volumes (~150 ml)

for purification. The expression was terminated after 8-hours of incubation by centrifugation at 10,000 rpm for 15 minutes at 4°C. The pellets were stored at -20°C until purification.

### 2.1.4 Lipase Purifications

The pellets of mutant cells cultures (N7Q, N7G, and BTL2_cys) were lysed with B-PER treatment and fractionated at 10,000 rpm for 30 minutes at 4°C. The supernatants were resuspended in 20 mM sodium phosphate buffer containing 50 mM imidazole for binding to nickel-coated beads of poly-histidine tagged proteins (GenScript) and transferred to the purification column. Binding of the mutant proteins to the beads were carried out by overnight binding at 4°C (or 1-hour incubation at room temperature). Consecutive three washing steps (W1, W2, and W3) were performed by washing the column by 1 ml of 20 mM sodium phosphate buffer containing 50 mM imidazole and three elution steps (E1, E2, and E3) were performed by 300 µl of 20 mM sodium phosphate buffer containing 500 mM imidazole. SDS-PAGE (sodium dodecyl polyacrylamide electrophoresis) was carried out for these samples to confirm the presence and assess the purity of the lipases. Stacking gel contained 4% polyacrylamide and separating gel contained 12.5% polyacrylamide. Buffer exchange of the purified lipases against deionized water was carried out by 10 kDa filter concentrators (Millipore). Bradford protein assays were applied to determine the quantity of the lipases by adding 200 µl of Bradford reagent to diluted protein samples and measured with spectrophotomer (ELISA reader) at 595 nm absorbance.

### 2.1.5 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 Gemini XS (Molecular Device) with 355 nm excitation wavelength and 460 nm emission wavelength every minute for 1 hour in a kinetic manner. SoftMaxPro Software was used to determine the initial velocities and the measurements were performed in duplicates.

#### 2.1.5.1 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 20°C, 30°C, 40°C, 50°C, 60°C and 70°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%.

#### 2.1.5.2 Thermoactivity Assay

Thermoactivity assay was performed to determine the optimal temperature of the lipases in the temperature range of 40-80°C. The mixture containing 3 µl of concentrated hydrochloric acid (HCl) and 97 µl of deionized water was added to the plate prior to measurements. Thermomixer was set to the given temperature and 25 µl 0.1 mM Tris-Cl pH 7.25 and 64 µl dd$H_2O$ were added in 1.5 ml Eppendorf tube in the thermomixer. When the mixture reached the given temperature, firstly the enzyme, secondly 250 µM of substrate were added and immediately 100 µl of reaction mixture was transferred to the 96-well black micro-titer plate, which was the time zero measurement. For the other time points, similarly 100 µl of mixture was taken and put into the 96-well black micro-titer plate. This procedure was repeated for the temperatures 50°C, 60°C, 70°C and 80°C and the resulting fluorescence was measured in the Gemini XS (Molecular Device).

#### 2.1.5.3 Substrate Selectivity Assay

Substrate selectivity assay was performed using 4MU-based substrates; 4MU-acetate (C2), 4MU-propionate (C3), 4MU-butyrate (C4), 4MU-caproate (C6), 4MU-enanthate (C7), 4MU-caprylate (C8), 4MU-laurate (C12) and 4MU-palmitate (C16). The assays were performed in 0.1 mM Tris-Cl pH 7.25 at room temperature using 50 µM from each substrate in the final reaction.

#### 2.1.5.4 Circular Dichorism Spectroscopy

Far-UV circular dichorism (CD) spectra were collected using J-815 spectropolarimeter (Jasco) in $N_2$ atmosphere equipped with thermostatically controlled cuvette with 1.0 mm path length at a scanning speed of 100 nm/min. Three scans were averaged to obtain final spectra of 0.1-0.5 mg/ml of wild-type and mutant lipases in water, which was corrected for the background. Mean residue ellipticity [θ] is calculated from the equation:

$$[\theta] = \theta \times \mathrm{M}/(c \times \mathrm{l} \times \mathrm{n_R})$$

*M* is the molecular mass in g/mol and *c* is the concentration in mg/ml, *l* is the cell length in centimeters, and $n_R$ is the number of residues. The thermal denaturation profiles were determined by tracing ellipticity at 222 nm at a 5°C/min heating rate from 30°C to 90°C and the $T_m$ values were calculated from the midpoint of the transition curves between folded and unfolded states of the lipases.

# 3 RESULTS

## 3.1 Molecular Cloning of BTL2_cys, N7G and N7Q Mutations

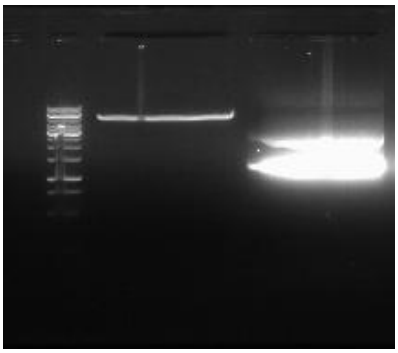


**Figure 3.1:** Agarose Gel Electrophoresis Results of the vector pMCSG-7 which has 5286 base-pairs and PCR amplification of BTL2_cys which has 1167 base-pairs and amplified by using BTL2_LIC_cys primer pairs. Analysis was made in agarose gel under 100V for 20 minutes.

The amplified BTL2_cys gene and the linearized expression vector pMCSG-7 were size separated via agarose gel electrophoresis (Figure 3.1). The results indicated the presence of linearized pMCSG-7 expression vector at 5286 bp and the amplified BTL2_cys at 1167 bp. Prior to annealing reactions another agarose gel was used to visualize the T4 DNA polymerase treated samples to determine the appropriate amounts of pMCSG-7 vector and BTL2_cys for annealing reaction (Figure 3.2).

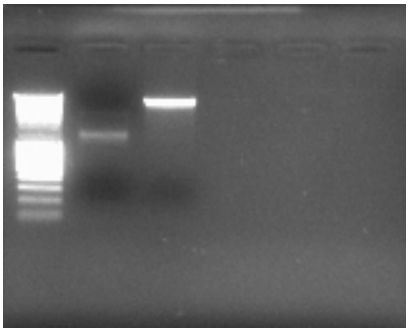


**Figure 3.2:** Agarose Gel Electrophoresis before annealing reaction of pMCSG-7 vector and BTL2_cys after phenol-chloroform extraction. The wells of the gel indicates in order: Marker / BTL2_cys / pMCSG-7

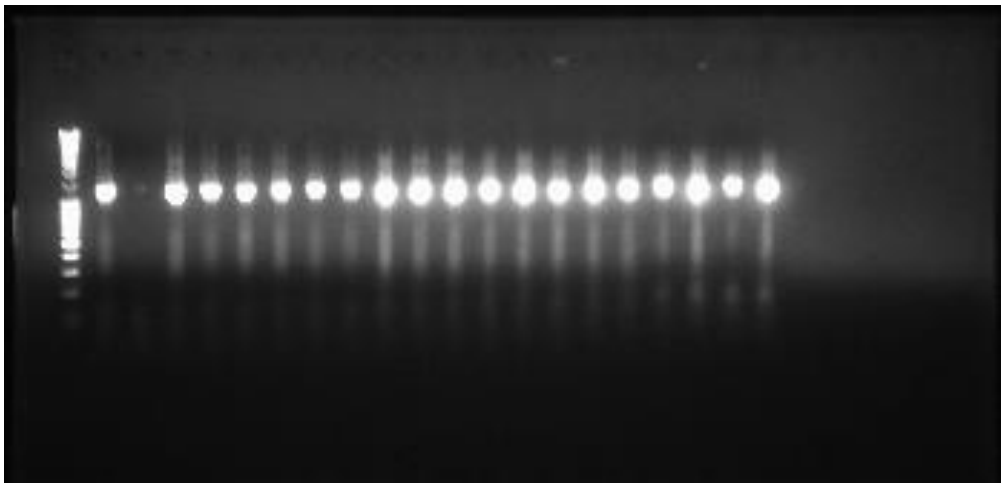


**Figure 3.3:** Cloning Confirmations of BTL2_cys Mutant by Colony PCR.

Colony PCR was performed using BTL2_LIC primers to twenty single colonies obtained from the transformation plates. Analysis was made in agarose gel under 100V for 20 minutes.

Twenty single colonies of BTL2_cys were selected from the transformation plates and colony PCR was performed. Nineteen colonies of these twenty single colonies indicated the presence of the insert and confirmed to be as positive colonies (Figure 3.3).

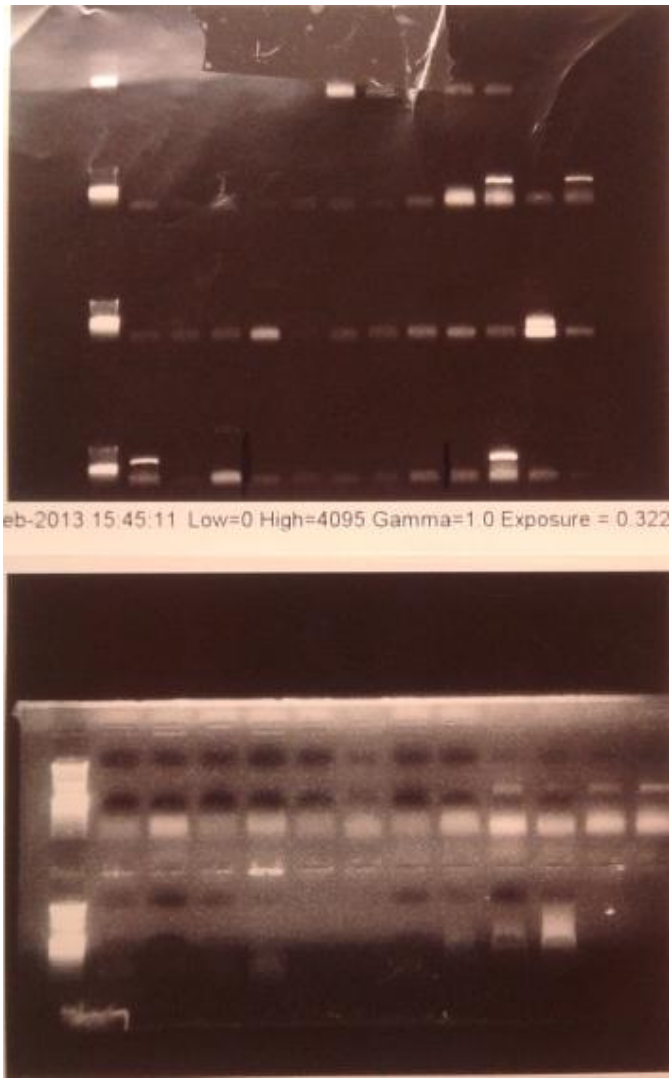



**Figure 3.4:** Cloning Confirmations of N7G and N7Q Mutants by Colony PCR.

Colony PCRwas applied to single colonies from N7G and N7Q transformation plates. First gel image indicates N7G and second gel image indicates N7Q colonies.Colony PCR was performed to adequate number of single colonies obtained from the transformation plates. Analyses were made in agarose gel under 100V for 20 minutes.

N7G and N7Q mutants were generated via Overlap-Extension PCR and colony PCR was performed with the BTL2_LIC primer pair and confirmed the presence of the positive colonies. Three colonies for N7G and three colonies for N7Q were chosen for the second confirmation step which is performed by *Bam*HI and *Kpn*I double digestion to the isolated plasmids of the selected colonies (Figure 3.5).

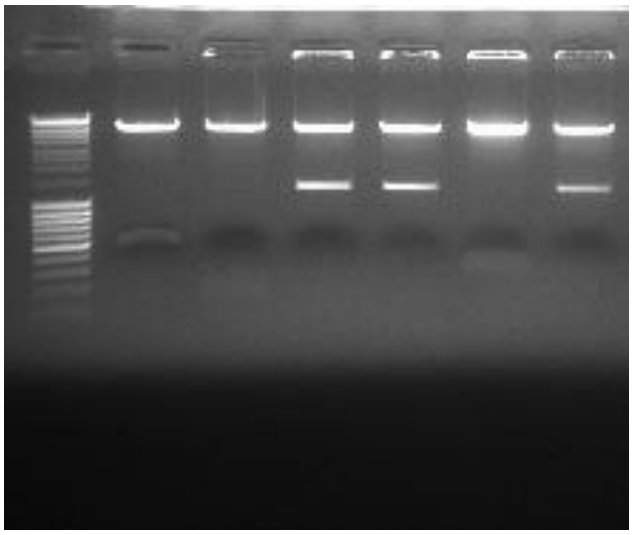
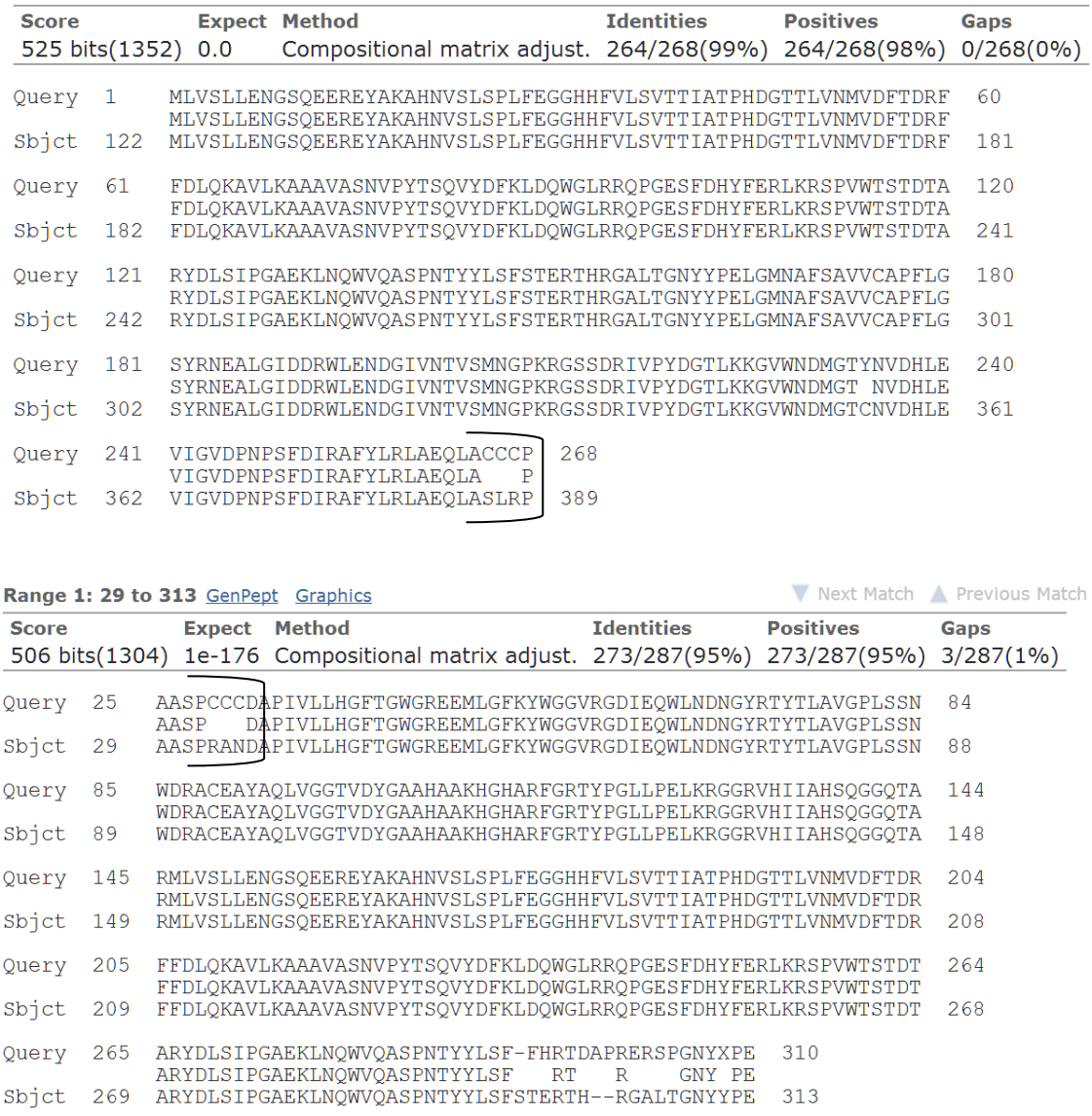


**Figure 3.5:** Cloning Confirmations of N7G and N7Q Mutants by Double-Digest.

Confirmation of the pure plasmids of N7G and N7Q mutations are performed using *Bam*HI and *Kpn*I digestion enzymes and the analysis were made in agarose gel under 100V for 20 minutes. The order of the wells: Gene-Ruler Marker / N7G-1 / N7G-2 / N7G-3 / N7Q-1 / N7Q-2 / N7Q-3

Double-digest digestion of three pure plasmids from both mutations for cloning confirmation indicated that the third plasmid of N7G mutation and both first and third plasmids of N7Q mutations were cut by both *Bam*HI and *Kpn*I restriction enzymes confirming that these plasmids carry the desired nucleotide fragments.



| Score | Expect | Method | Identities | Positives | Gaps |
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           SYRNEALGIDDRWLENDGIVNTVSMNGPKRGSSDRIVPYDGTLKKGVWNDMGT NVDHLE
Sbjct  302  SYRNEALGIDDRWLENDGIVNTVSMNGPKRGSSDRIVPYDGTLKKGVWNDMGTCNVDHLE  361

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Sbjct  362  VIGVDPNPSFDIRAFYLRLAEQLASLRP  389
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**Range 1: 29 to 313** GenPept Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Method | Identities | Positives | Gaps |
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| 506 bits(1304) | 1e-176 | Compositional matrix adjust. | 273/287(95%) | 273/287(95%) | 3/287(1%) |

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Sbjct  209  FFDLQKAVLKAAAVASNVPYTSQVYDFKLDQWGLRRQPGESFDHYFERLKRSPVWTSTDT  268

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Sbjct  269  ARYDLSIPGAEKLNQWVQASPNTYYLSFSTERTH--RGALTGNYYPE  313
```

**Figure 3.6:** Cloning Confirmations of BTL2_cys Mutant by Sequencing

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

Sequencing result for BTL2_cys mutant confirmed the cloning by site directed mutagenesis. Cysteine amino acids are introduced to the desired sites of the DNA fragment which is seen in Figure 3.6. . The BTL2_cys mutant contained 6 mutations that are R5C, A6C, N7C, S386C, L387C and R388C.

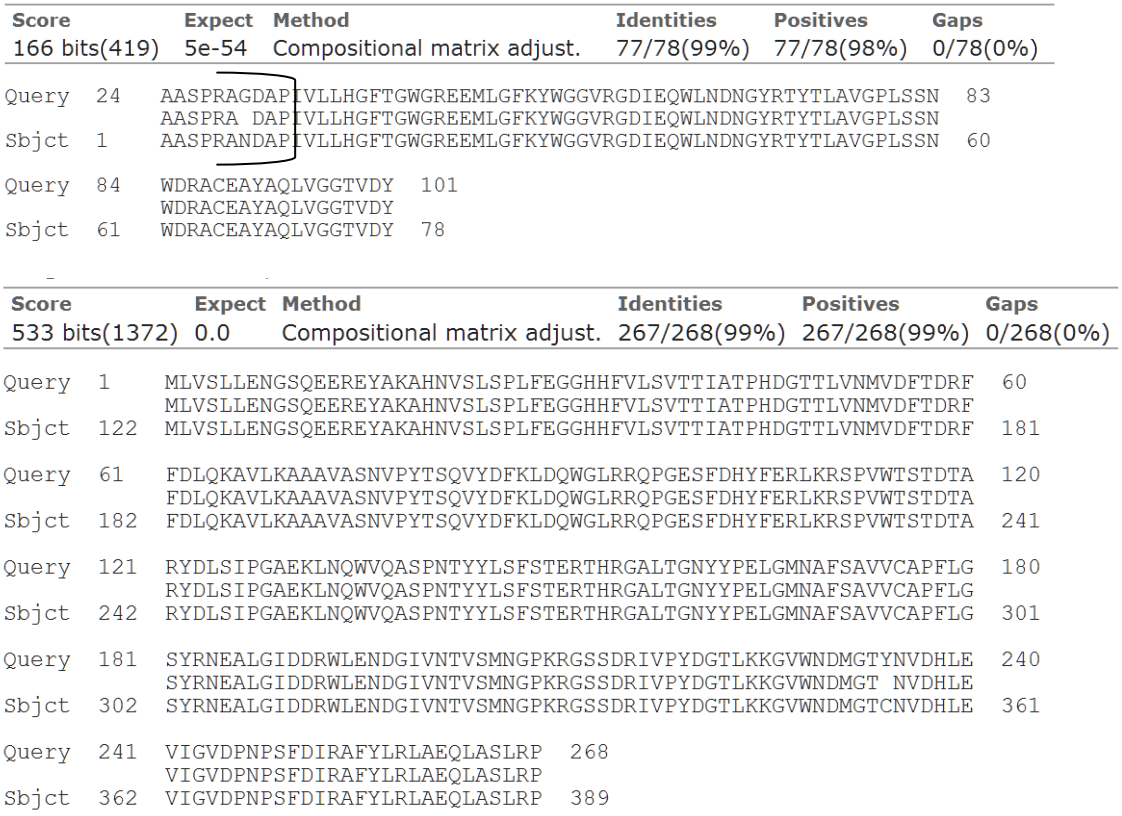


**Figure 3.7:** Cloning Confirmations of N7G Mutant by Sequencing

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

Sequencing result for N7G mutant confirmed the cloning by site directed mutagenesis. Asparagine amino acid at residue 7 was converted to glycine amino acid which is indicated in Figure 3.7 with black bracket.

| Score | Expect | Method | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| 511 bits(1315) | 0.0 | Compositional matrix adjust. | 279/302(92%) | 281/302(93%) | 5/302(1%) |

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Sbjct  61   WDRACEAYAQLVGGTVDYGAAHAAKHGHARFGRTYPGLLPELKRGGRVHIIAHSQGGQTA  120

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Sbjct  181  FFDLQKAVLKAAAVASNVPYTSQVYDFKLDQWGLRRQPGESFDHYFERLKRSPVWTSTDT  240

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Sbjct  241  ARYDLSIPGAEKLNQWVQASPNTYYLSFSTERTHR--GALTGNYY-PEL-GMNAFSAVVC  296
```

| Score | Expect | Method | Identities | Positives | Gaps |
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| 533 bits(1372) | 0.0 | Compositional matrix adjust. | 267/268(99%) | 267/268(99%) | 0/268(0%) |

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Sbjct  362  VIGVDPNPSFDIRAFYLRLAEQLASLRP  389
```

**Figure 3.8:** Cloning Confirmations of N7Q Mutant by Sequencing

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

Sequencing result for N7G mutant confirmed the cloning by site directed mutagenesis. Asparagine amino acid at residue 7 was converted to glutamine amino acid which is indicated in Figure 3.8 with black bracket.

## 3.2 Expressions and Purifications of BTL2_cys, N7G and N7Q Mutant Lipases

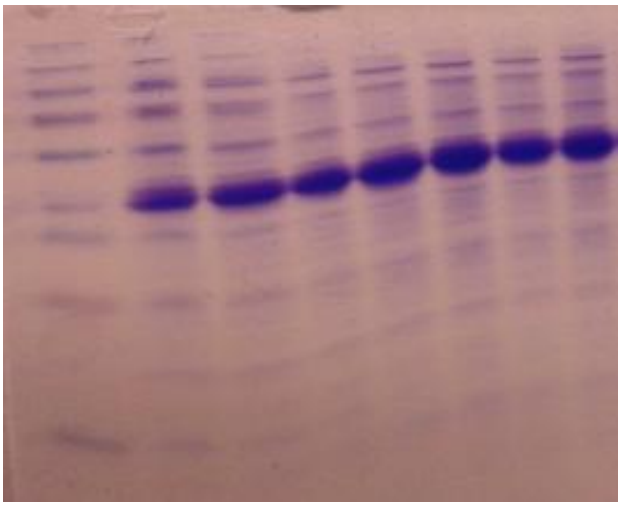

**Figure 3.9:***E.coli* SHuffle Expressions of BTL2_cys. Whole cell-fraction from the mutant collected for eight hours in one-hour intervals, analyzed in 12% SDS-PAGE which was stained with coomassie dye. The gel indicates: Marker, $t_0$, $t_1$, $t_2$, $t_3$, $t_4$, $t_5$, $t_6$, $t_7$, $t_8$ samples of the BTL2_cys.

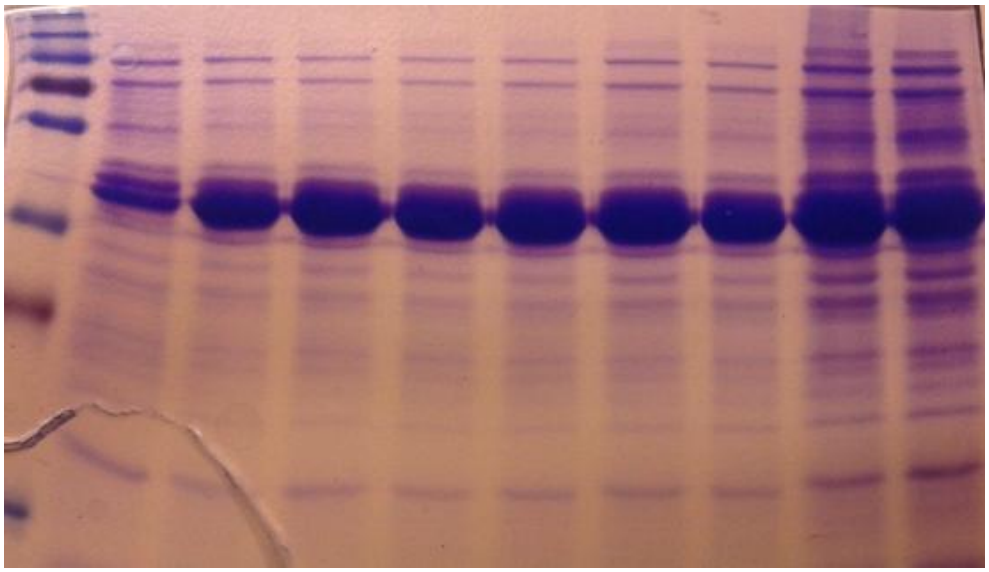

**Figure 3.10:***E.coli* SHuffle Expressions of N7G Mutation. Whole cell-fraction from the mutant collected for eight hours in one-hour intervals, analyzed in 12% SDS-PAGE which was stained with coomassie dye. The gel indicates: Marker, $t_0$, $t_1$, $t_2$, $t_3$, $t_4$, $t_5$, $t_6$, $t_7$, $t_8$ samples of N7G.

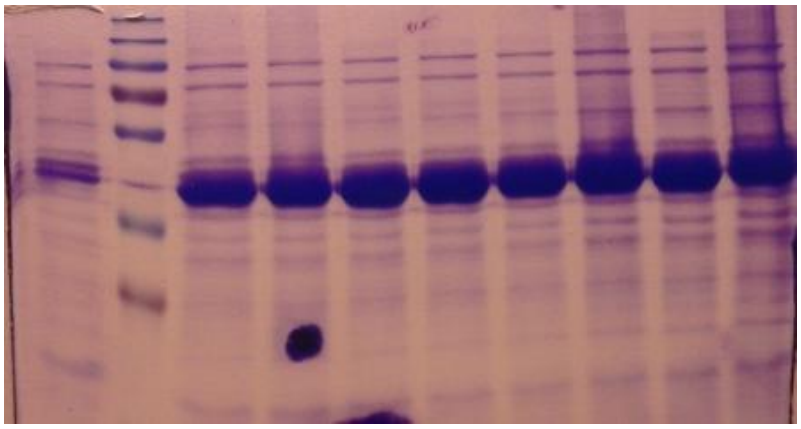

**Figure 3.11:***E.coli* SHuffle Expressions of N7Q Mutation. Whole cell-fraction from the mutant collected for eight hours in one-hour intervals, analyzed in 12% SDS-PAGE which was stained with coomassie dye. The gel indicates: Marker, $t_0$, $t_1$, $t_2$, $t_3$, $t_4$, $t_5$, $t_6$, $t_7$, $t_8$ samples of N7Q.

*Bacillus thermocatenulatus* lipase is 43 kDa protein and BTL2_cys, N7G and N7Q expressions were confirmed through the presence of high amount of proteins which were 43 kDa on the SDS_PAGE gels (Figure 3.6, Figure 3.7, and Figure 3.8). There was no significant change in the expression levels of the proteins in each time interval. Therefore, $t_5$ was selected as expression level of the high-scale cell culture which was used for the purification of the lipases.

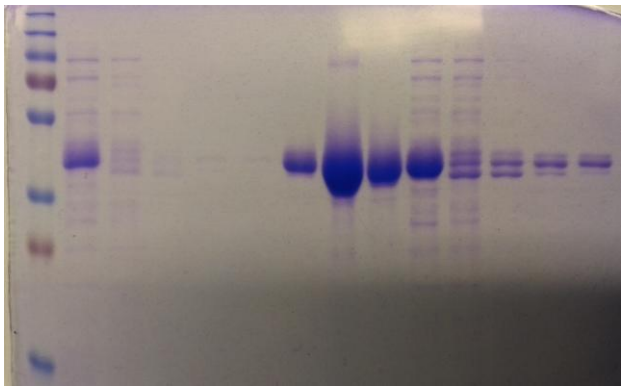

**Figure 3.12:** Column Purification for the BTL2_cys Mutation.

Column was washed with 50 mM imidazole for three consecutive times as Wash I (W1), WashII (W2), and Wash III (W3) and the protein was eluted through the column using 500 mM imidazole as three elution steps; Elution I (E1), Elution II (E2) and Elution III (E3). Flow-through (FT) and the inputs (I) of the proteins were analyzed for comparison. The wells consecutively indicate: Marker / BTL2_cys_I / BTL2_cys_FT / BTL2_cys_W1 / BTL2_cys_W2 / BTL2_cys_W3 / BTL2_cys_E1 / BTL2_cys_E2 / BTL2_cys_E3 / N7G_I / N7G_FT / N7G_W1 / N7G_W2 / N7G_W3

The purification gel for the BTL2_cys mutation confirmed the protein expression, which was shown in the input protein and purely, eluted proteins (43 kDa) in the elution steps. Moreover, input of the N7G protein indicated the protein expression and small amount of N7G protein was observed in the washing steps (Figure 3.9).

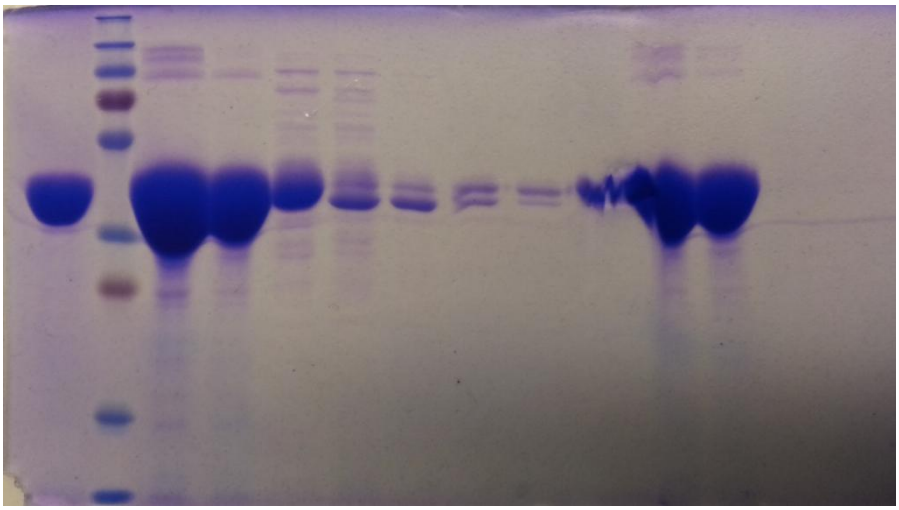

**Figure 3.13:** Column Purification for N7G and N7Q Mutation.

Column was washed with 50 mM imidazole for three consecutive times as Wash I (W1), WashII (W2), and Wash III (W3) and the protein was eluted through the column using 500 mM imidazole as three elution steps; Elution I (E1), Elution II (E2) and Elution III (E3). Flow-through (FT) and the inputs (I) of the proteins were analyzed for comparison. The wells consecutively indicate: N7G_E1 / Marker / N7G_E2 / N7G_E3 / N7Q_I / N7Q_FT / N7Q_W1 / N7Q_W2 / N7Q_W3 / N7Q_E1 / N7Q_E2 / N7Q_E3

The purification gel for N7G and N7Q proteins confirmed the protein expression which was shown in the input protein and purely eluted proteins (43 kDa) in the elution steps (Figure 3.10).

## 3.3 Characterization of BTL2_cys, N7G and N7Q Mutant Lipases

### 3.3.1 Determination of the Linear Range for Fluorescent Lipase Assays

BTL2 is 43 kDa and its molar concentration is equal to 23.26 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 23.26 nM and 2.32 nM, respectively. Therefore, 10 nM, which corresponds to 430 ng/ml, was used in the fluorescent lipase assays. 20 µl of enzyme solutions were used in all assays.

Bradford protein assays were applied to determine the concentration of the wild type BTL2, and mutations of BTL2_cys, N7G and N7Q proteins, which were measured with spectrophotomer (ELISA reader) at 595 nm. The concentration of wild type BTL2 protein was measured as 25 mg/ml; BTL2_cys protein was 56 mg/ml; N7G was 81 mg/ml; and N7Q protein was 63 mg/ml. All of the proteins were diluted to 0.43 µg/ml to reach the linear range in fluorescent lipase assays.

### 3.3.2 Thermostability Assay

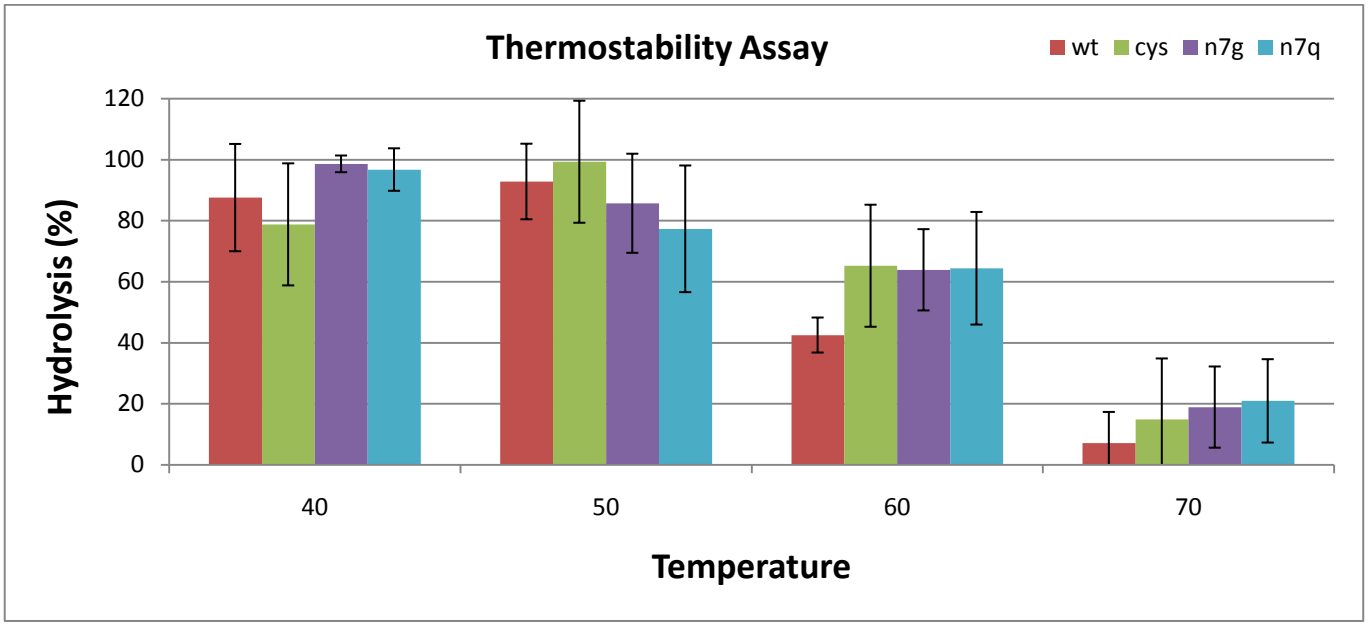


**Figure 3.14:** Thermostability Assay for WT, BTL2_cys, N7G and N7Q from Three Independent Measurements.

Each lipase was incubated at the given temperature for 30 minutes using 4MU-caprylate as substrate. Assay was performed in 0.1 mM Tris-Cl, pH 7.25 at room temperature using 250 µM substrate. Percent activity was demonstrated with error bars representing standard deviations from three independent experiments. The highest activity for each mutant was set to 100%.

Results of the thermostability assay indicated that wild type BTL2 shows the highest activity between 40ºC and 50ºC. As the temperature increases to 60ºC and 70ºC, stability of the wild type decreases and percent activity of the wild type becomes almost zero at 70ºC. BTL2_cys, N7G and N7Q mutations also showed the similar pattern compared to wild type BTL2. Mutants showed slightly higher activity at 60ºC. However, when the error bars take into account, no significant change in thermostability of wild types and mutants were seen (Figure 3.14).

### 3.3.3 Thermoactivity Assay

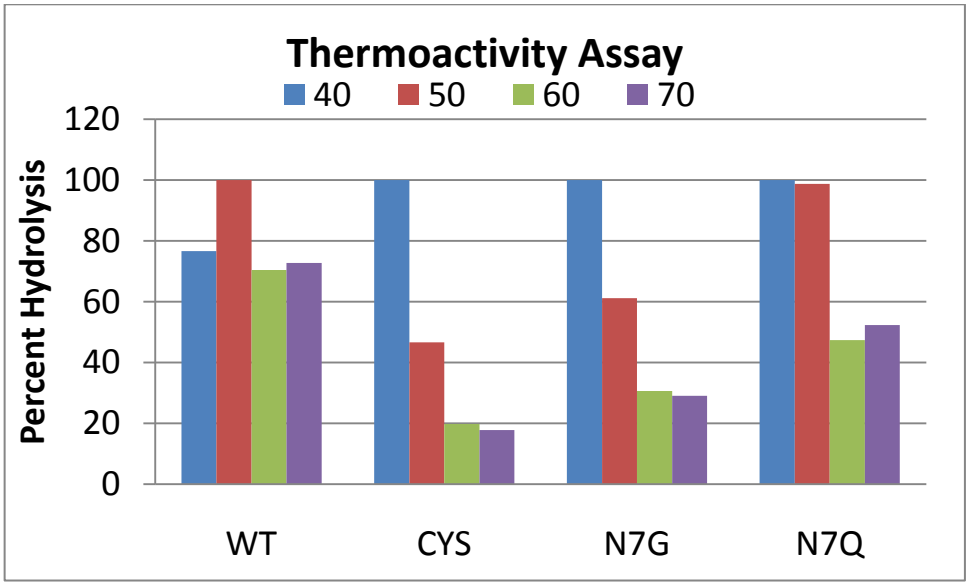


**Figure 3.15:** Thermoactivity Assay for WT, BTL2_cys, N7G and N7Q.

Thermoactivity assay was performed in 0.1 mM Tris-Cl, pH 7.25 at given temperatures (40ºC-70ºC) using 250 µM substrate (4MU-caprylate). Percent activity was demonstrated and the highest activity for each mutant was set to 100%.

Results of the thermoactivity assay indicated that wild type BTL2 shows the highest activity at 50ºC and the percent hydrolysis is between 60-80 % at 40ºC, 60ºC and 70ºC. However, mutants of BTL2_cys, N7G show lower thermoactivity at 50ºC, 60ºC and 70ºC compared to wild type BTL2. Mutant N7Q has higher thermoactivity compared to BTL2_cys and N7G mutants but lower thermoactivity at 60ºC and 70ºC compared to wild type BTL2 (Figure 3.15).

### 3.3.4 Substrate Selectivity Assay

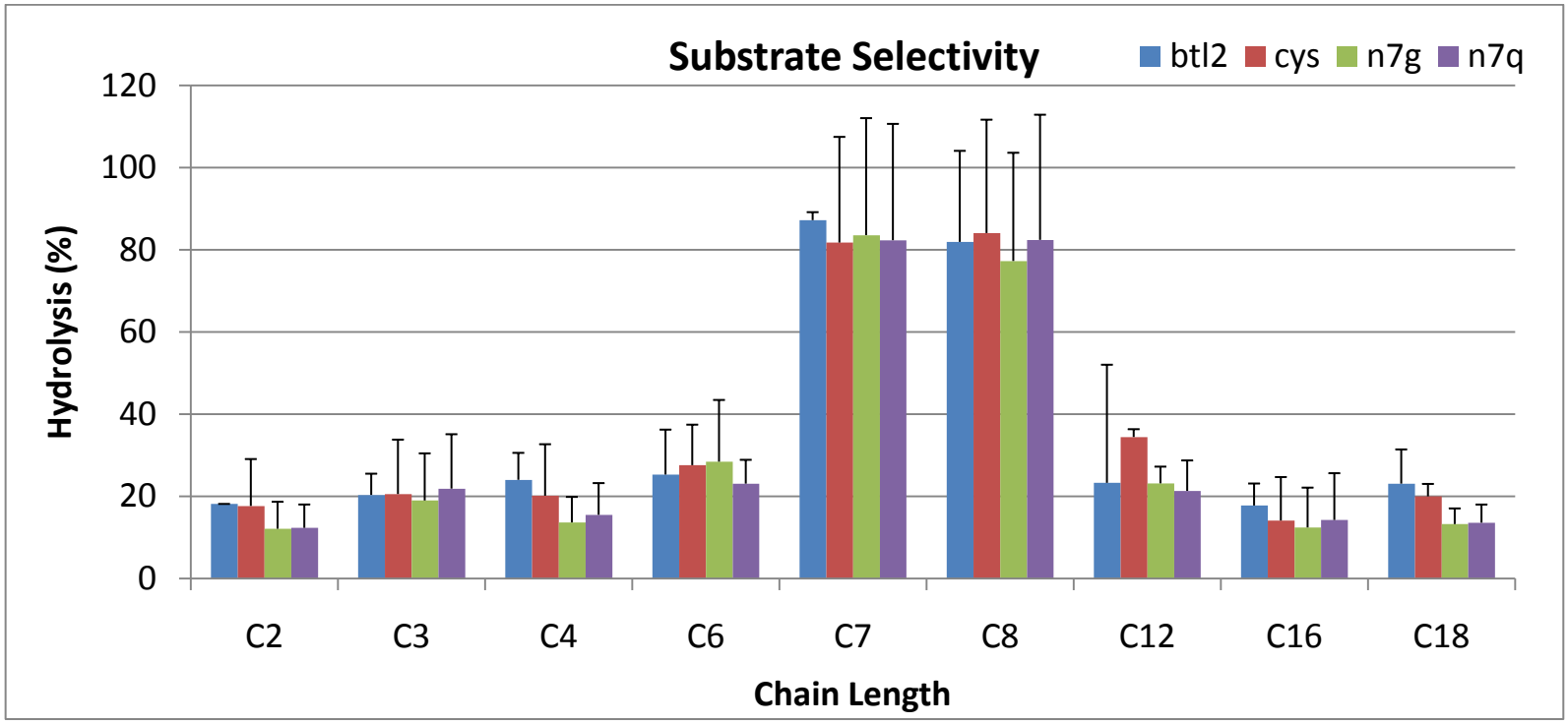


**Figure 3.16:** Substrate Selectivity Assay for WT, BTL2_cys, N7G and N7Q.

Substrate selectivity assay was performed using 4MU-based substrates; 4MU-acetate (C2), 4MU-propionate (C3), 4MU-butyrate (C4), 4MU-caproate (C6), 4MU-enanthate (C7), 4MU-caprylate (C8), 4MU-laurate (C12), 4MU-palmitate (C16) and 4MU-elaidate (C18). The assays were performed in 0.1 mM Tris-Cl pH 7.25 at room temperature using 250 µM from each substrate. Percent activity was demonstrated with error bars representing standard deviations from three independent experiments. The highest activity for each mutant was set to 100%.

Results of the substrate selectivity assay indicated that wild type BTL2 shows the highest activity with substrates 4MU-enanthate (C7) and 4MU-caprylate (C8). Similar to BTL2, the mutants of BTL2_cys, N7G and N7Q showed selectivity towards substrates; C7 and C8 (Figure 3.16).

### 3.3.5 Circular Dichorism Spectroscopy

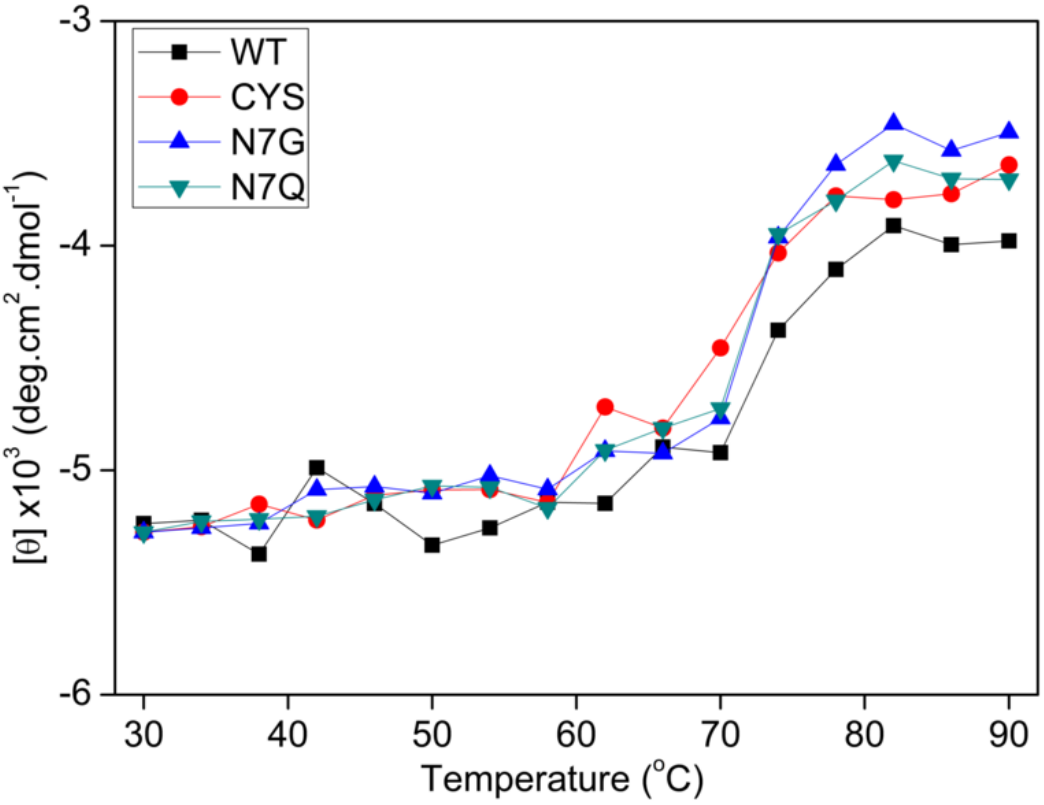


**Figure 3.17:** Far UV-CD Spectra Results for WT (black), BTL2_cys (red), N7G (blue) and N7Q (green).

CD spectroscopy was performed to monitor thermal denaturation of wild type BTL2 and the mutants of BTL2_cys, N7G and N7Q. Figure 3.17 shows the far-UV CD spectra collected at different temperatures. The spectra obtained for wild type BTL2 and mutant lipases were not significantly different from each other such that all lipases showed fairly stable conformation in the temperature range of 30-60ºC. Wild type experienced a temperature induced change at 80ºC. However, mutant lipases experienced a temperature induced change at 70ºC (Figure 3.18). For the temperature range of 70-80ºC, all lipases displayed dramatic structural changes (Figure 3.17).

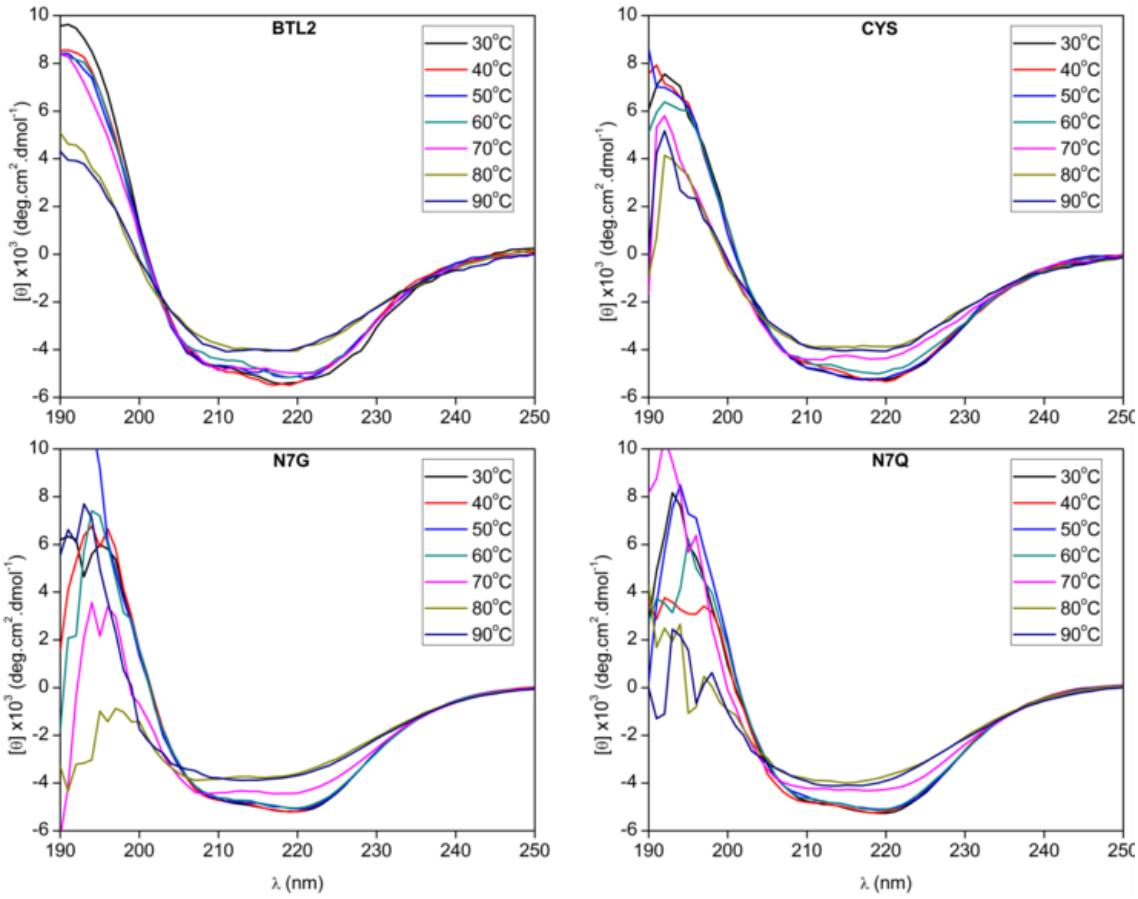


**Figure 3.18:** Far UV-CD Spectra Results for WT, BTL2_cys, N7G and N7Q.

Different colors correspond to different temperatures and the graph shows the mean residue ellipticity as a function of wavelength. The spectra of each lipase are subtracted from blank spectra collected for the buffer system. Data steps for each measurement are 1nm.

For wild type BTL2 and the mutants of BTL2_cys, N7G and N7Q, ellipticity at 222 nm was shown as a function of temperature in Figure 3.18. Table 3.1 demonstrates that the melting temperature for wild type BTL2 was found to be 69.48ºC which was almost not changed for mutants N7G (70.96ºC) and N7Q (70.86ºC) and significantly lower for mutant of BTL2_cys (66.14ºC). Therefore, mutant of BTL2_cys caused more drastic destabilization of lipase structure at high temperatures compared to wild type BTL2.

**Table 3.1:** Melting Temperatures for WT, BTL2_cys, N7G and N7Q

| **Lipases** | **Tm (°C)** |
|---|---|
| WT_BTL2 | 69.48±0.31 |
| BTL2_cys | 66.14±0.15 |
| N7G | 70.96±0.41 |
| N7Q | 70.86±0.41 |

# 4 DISCUSSION

*Bacillus thermocatenulatus* lipase (BTL2) is a thermostable enzyme that shows resistance towards inactivation agents such as high temperature, organic solvents, alkaline pH. These properties of make BTL2 a prominent target in industry [68]. In addition to that, the termini of BTL2 backbone seated close to each other (less than 5 Å). Owing to the fact that circular proteins possess higher resistance to both proteolytic cleavage and thermal conditions [64], the close proximity of BTL2 termini makes it a candidate structure to mimic circular backbone and to investigate the impacts of the termini proximity on thermotolerance. Also, modification on the N- and/or the C-terminus of BTL2 will enable us to understand the effects of the termini interactions with the overall protein structure and function.

In this thesis, rational design of BTL2 was performed using the insights revealed by the crystallographic evidence [27]. The design is realized via site-directed mutagenesis to modify protein termini of BTL2 under investigation of the impacts on the structure, activity and stability. Since, previous mutagenesis strategies to obtain higher thermostable lipase variants have included forming extra disulfide bonds via cysteine knot motifs [63], R5C, A6C, N7C, S386C, L387C and R388C mutations were carried out to induce formation of cysteine bonds between N- and C- terminus of the BTL2. Moreover, N7G and N7Q mutation were performed to eliminate hydrogen bonding between N- and C- termini since the side chain of asparagine amino acid at residue 7 makes hydrogen bond with the 387th leucine at the C terminus. Apart from the carbon-carbon interactions, the hydrogen bond at the protein termini is considered to be an effective factor for the structure of the termini. Since converting asparagine to glycine would eliminate the formation of the hydrogen bond in the native structure, N7G mutations can provide information about the function and the impact of hydrogen bond to overall protein structure, thermostability and thermoactivity. Additionally, converting asparagine to glutamine would not have such drastic effects on the hydrogen bond as the N7Q mutation does not necessarily eliminate the hydrogen bond owing to the very similar side chain of glutamine with asparagine.

Ligation independent cloning (LIC) via overlap-extension PCR was used to generate N7G and N7Q mutants. BTL2_cys mutant contains R5C, A6C, N7C, S386C, L387C and R388C amino acid substitutions and generated via polymerase chain reaction. All three mutations were confirmed by colony PCR using BTL2_LIC primers. Since BTL2_LIC primers would amplify the recombinant clones, cloning was confirmed by the presence of the bands corresponding to BTL2 gene on the agarose gel electrophoresis. Moreover, further confirmation was performed by sequencing that proved the alteration of the DNA in the desired region. Overlap-extension PCR provided successful cloning since the altered site is close to the terminus and the method ligation-independent cloning was the appropriate chose to perform the procedure.

*E.coli* expression system worked for lipases of BTL2_cys, N7Q and N7G mutations due to the fact that *Bacillus thermocatenulatus* lipase has a bacterial origin. SDS-PAGE results indicated efficient expressions from $t_1$ to $t_8$ for all mutant lipases. Lipase purifications were performed by using nickel-coated beads of poly-histidine tagged proteins and batch purifications were resulted in high-purity lipases which enabled sensitive measurements such as CD spectropolarimetry.

Thermostability assay were performed to measure the stability of each mutants in 40ºC, 50ºC, 60ºC and 70ºC to measure the resistance of lipases to thermal conditions. Fluorescent assay results indicated no significant change between BTL2_cys, N7Q and N7G mutants and wild type BTL2. Although, it was assumed that the introducing cysteine residues to the N and C termini contributes to disulfide bridge (cysteine bond) formation and enhance stability, in this case mutagenesis resulted in no effect. The reason of that might be not coupling of thiol groups of the cysteine amino acids to form disulfide bridges due to not being close enough to each other or different orientations of the side chains in the backbone. Moreover, the relation between enthalpy and entropy affects the thermostability of the proteins. More negative Gibbs free energy (dG= dH-TdS) contributes to increased thermostability. Introducing cysteine bonds to the termini of BTL2 induces a decrease in entropy (dS) thus expected to have a negative impact on the stability but its effect to enthalpy may be so strong that it may overcome the negative impact of loss of flexibility due to disulfide bonds thus the entropy. If the entropy loss is larger than the enthalpy gain then this would destabilize the protein. One should consider both affects when trying to enhance the stability.

Either increase flexibility (entropy) without too much loss in the enthalpy or increase enthalpy without too much loss of entropy. In our case, despite the cysteine bonds are presumed to be formed, if total change in Gibbs free energy is not more negative, then BTL2 may not become more thermostable. This maybe the case in our study. But one should confirm the establishment of disulfide bonds via introduced mutations.

Thermoactivity assays were performed to measure the activity of each mutant in 40ºC, 50ºC, 60ºC and 70ºC. All three mutant lipases showed lower activity compared to wild type BTL2. However, among the mutants N7Q had the higher thermoactivity than N7G which might stem from the similar biochemical properties of asparagine and glutamine, both of which are polar amino acids. Eliminating the hydrogen bond between N- and C-terminus decreased the thermoactivity as expected that emphasize the effect of that particular hydrogen bond. However, the decrease of thermoactivity in BTL2_cys mutant indicated a more dramatic structural change in the termini such that the mutant also denatured at lower temperatures than the BTL2. CD results which showed the melting temperature of the wild type as 69.48ºC, N7G as 70.96ºC, N7Q as 70.86ºC and BTL2_cys as 66.14ºC were consistent with the data obtained from thermoactivity assays. Parallel to the melting temperature of BTL2_cys mutant as the lowest one, the optimal temperature of BTL2_cys mutant was measured as the lowest. Moreover, substrate selectivity assay results indicated that all of the lipases including the mutants BTL2_cys, N7G and N7Q showed selectivity towards substrates 4MU-enanthate (C7) and 4MU-caprylate (C8), showing that the mutations did not change the selectivity at all. Despite the changes in the thermoactivity profiles of the mutants, the same mutations did not have any significant impacts on the substrate selectivity emphasizing that the mutations does not alter the catalytic properties of the native BTL2. Considering the locations of these mutations, as they are located at the protein termini, it has been already anticipated that such mutations would less likely to affect the characteristics of the active site which was located in the core domain.

# 5 CONCLUSIONS

In this thesis, backbone modifications in the termini of BTL2 was applied to generate the disulfide bridge by introducing cysteine residues and to eliminate the hydrogen bonds and the impacts of these changes on activity and stability are investigated. Elimination of the hydrogen bonding did not affect the thermostability significantly but decreased the thermoactivity slightly while introducing cysteine residues to the termini of the BTL2 resulted in decreased thermoactivity and thermal denaturation. Overall, these results suggested that the hydrogen backbone at the protein termini is a less likely important interaction regarding thermostability and thermoactivity. On the other hand, the presence of consecutive cysteines disrupted the thermal stability of BTL2 proposing that the cysteine substitutions have a dramatic effect on the thermal stability of the lipase without affecting the function. In conclusions such modifications performed at the protein termini might be advantageous in such a way that without altering the catalytic machinery the protein stability might be tuned via changing the composition of the N- and/or C- terminus.

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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

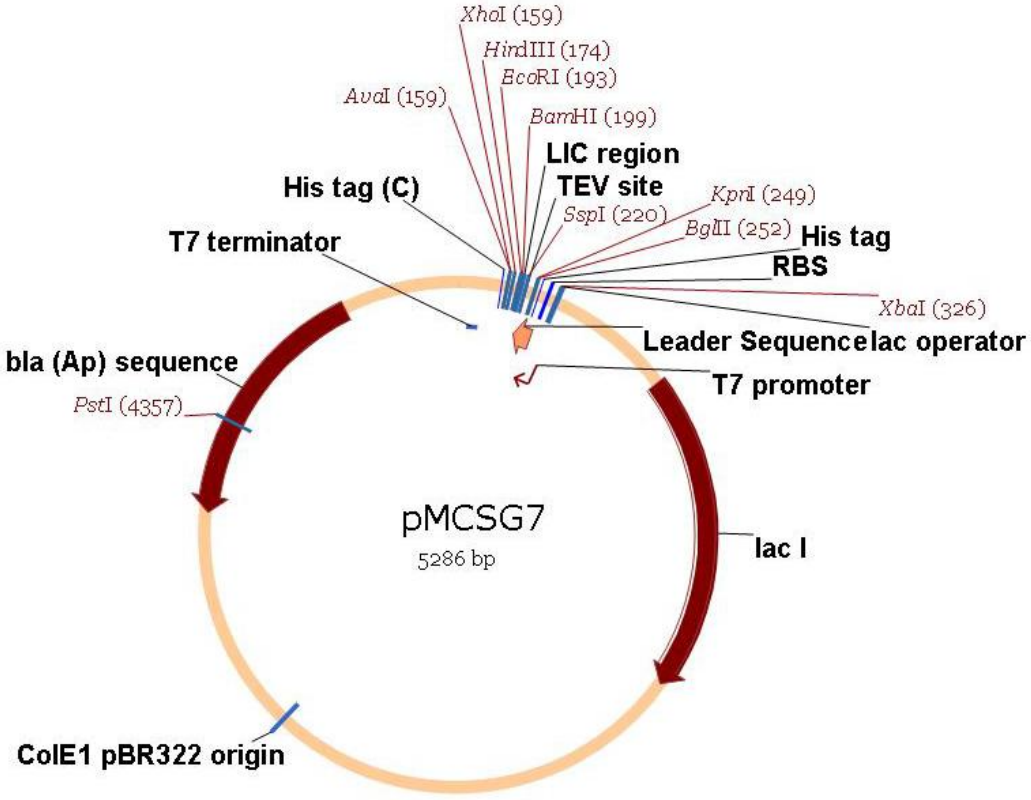


**Figure A.1:** Expression Vector Map

## 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

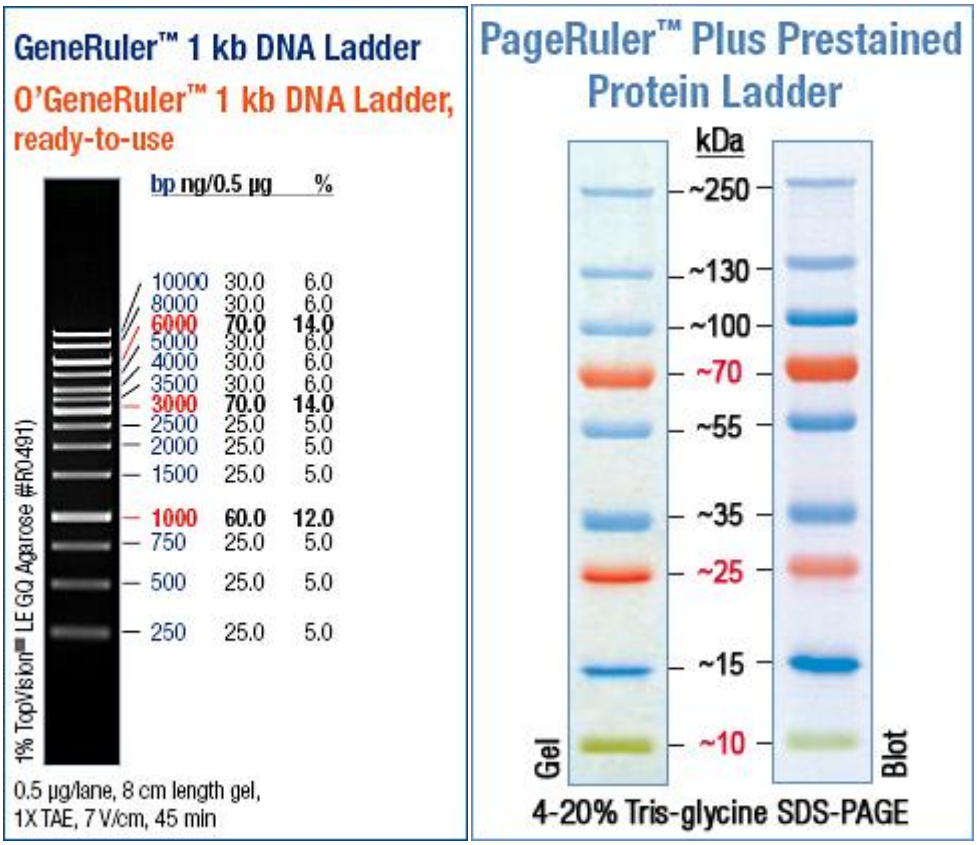


**Figure A.3:** Electrophoresis Marker Legends

## 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.