ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

PROTEIN ENGINEERING APPLICATIONS OF NOVEL ESTERASE ENZYME USING RATIONAL DESIGN AND DIRECTED EVOLUTION APPROACHES

M.Sc. THESIS

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RASYONEL TASARIM VE YÖNLENDİRİLMİŞ EVRİM YAKLAŞIMLARIYLA YENİ ESTERAZ ENZİMİNİN PROTEİN MÜHENDİSLİĞİ UYGULAMALARI

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To my family and my deceased aunt Hamide KILIÇ,

FOREWORD

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February 2022

Şeyma YILMAZ KÜPOĞLU
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ABBREVIATIONS

bp	: basepair
dH_2O	: distilled water
DNA	: Deoxyribonucleicacid
dNTP	: Deoxynucleotide
E. coli	: *Escherichia coli*
EDTA	: Ethylene diamine tetra aceticacid
EP-PCR	: Error prone polymerase chain reaction
H2O	: Water
HCl	: Hydrochloric acid
His-tag	: Histidine tag
IPTG	: Isopropyl-Beta-D-Thiogalactopyranoside
Kan	: Kanamycin
KCl	: Potassium chloride
kDA	: Kilo daltons
LB	: Luria-Bertani
m	: Metre
M	: Molarity
MgCl2	: Magnesium chloride
min.	: Minute
MnCl2	: Mangan chloride
PCR	: Polymerase chain reaxion
pNP	: Para-nitrophenol
Rpm	: Revolution per minute
SDS	: Sodium deodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	: Tris-acetate-EDTA buffer
U	: Enzyme Unit
UV	: Ultraviolet

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PROTEIN ENGINEERING APPLICATIONS OF NOVEL ESTERASE ENZYME USING RATIONAL DESIGN AND DIRECTED EVOLUTION APPROACHES

SUMMARY

Enzymes mostly are protein-structured biomolecules that act as catalysts in living organisms. They regulate the rate at which chemical reactions proceed without altering themselves.

Enzymes should have properties that are suitable for the desired reaction conditions to be used as biocatalysts in industry (high activity, substrate specificity, resistance to organic solvents, etc.). However, enzymes do not always have the properties required for use in a variety of reaction conditions. So, it is necessary to develop the properties of enzymes for the desired reaction conditions. Protein engineering strategies can be used to create enzymes with unique properties for this purpose.

Esterase enzyme has great industrial importance due to its use in many different areas such as detergent, food, cosmetics and pharmaceuticals. Since esterases, which partially dissolve in water and hydrolyze ester bonds, are still able to adapt to harsh industrial conditions and do not have the required level of activity, studies are ongoing to improve the existing properties of the esterase enzyme and to bring new features.

Protein engineering methods are one of the most efficient ways to obtain enzymes that can work under harsh conditions. If these methods are applied to enzymes isolated from microorganisms living in extreme conditions it is possible to produce enzymes with superior abilities. Microorganisms living in extreme conditions can adapt to extreme conditions by the construction of certain mutations spontaneously. When protein engineering methods are applied to the adaptation tendencies of these microorganisms and their enzymes with superior properties, enzymes that can meet the needs of the industry can be obtained. Recently, our group isolated and identified microbial diversity of the samples by a sequence-based metagenomic approach using environmental samples collected from Acıgöl, which has a high salt concentration. The esterase(hAGEst) enzyme was produced recombinantly and its biochemical characterization was completed successfully. The amino acid sequence of the esterase enzyme is 91% similar to the esterase of the *Halomonas gudaonensis* organism, and the gene sequence is 75% similar to a part of the *Halolamina sediminis* genome.

This thesis aimed to determine the three-dimensional(3-D) structure and improve the substrate specificity of the newly defined esterase enzyme using two basic approaches of protein engineering, which are widely used in the literature. Firstly, Site-Directed Mutagenesis was used, which is one of the basic methods of the rational design approach. To predict the 3-D structure of the new esterase protein, when the Protein Data Bank (PDB) was examined with the SWISS-MODEL software, it was seen that the new esterase(hAGEst) enzyme had the highest similarity (34%) with the esterase ybfF protein. Thanks to this method, the importance of these amino acids in the structure were investigated by replacing the 209th serin amino acid residue, which is

estimated to be in the active region, and the 88th methionine, which is estimated to be in the oxyanion hole, with alanine amino acid. Primers were designed to obtain the Ser209Ala and Met88Ala mutations. The gene was amplified using the Q5 Site-Directed Mutagenesis Kit and was transformed into *E. Coli* C43 cells. Colonies were randomly selected from the resulting transformants sent for sanger sequencing. The DNA sequencing results indicated that the desired mutations were obtained successfully. Protein expression was initially stimulated by the addition of 1 mM IPTG, followed by a 6-hour incubation at 30 °C. The proteins from the pET-28a(+) vector containing the Ser209Ala and Met88Ala mutant esterase genes were then purified utilizing the 6xHis tag technique, and SDS-PAGE analysis was used as a control. Bands in the range of 25-35 kDa were observed after inducing gene expression with IPTG for Ser209Ala and Met88Ala. The results verified the molecular weight of esterases ranges between 27 and 54 kDa. In the next step, the protein purified by His-tag purification was removed from the unwanted proteins according to the SDS-PAGE images by ultrafiltration. The concentration of the pure protein was measured by using Bradford reagent. It was measured at 20 mg/mL for Ser209Ala mutant esterase, 20 mg/mL for Met88Ala mutant esterase and 30 mg/mL for wild-type esterase according to Bradford analysis. 1 µM of wild type enzyme, Ser209Ala and Met88Ala mutant enzymes were incubated in 1 mM para-nitrophenol (pNP) hexanoate substrate prepared in 1 mM Tris-HCl pH:8 buffer solution at 30 ºC for 20 minutes. For kinetic activity, three independent experiments were repeated and absorbance was measured at a wavelength of 410 nm. According to these results, the activity of wild type was found to be decreased by 87.1% and 17.7%, when compared to the activity of Ser209Ala and Met88Ala mutant esterases, respectively. These results show that the 88th methionine amino acid is located in the active site of the esterase enzyme due to the serious decrease in the activity and has a direct effect on the activity of the esterase enzyme. However, it was thought that the 88th methionine residue is not of great importance in the oxyanion hole, since a small decrease in the activity is observed by 17.7%.

The second part of the study aimed to create a large library with the directed evolution approach. To improve the substrate specificity of the enzyme, the library was created by using the Error-Prone PCR . It would help to develop esterase enzyme for the needs of the industry. So, it may contribute to the national economy and literature by obtaining esterases with superior properties. Firstly, the enzymes studied in the literature, especially esterases, and the Error-Prone PCR conditions applied to these enzymes were examined in detail, the most suitable conditions for the hAGEst enzyme were determined and suitable primers were designed. A suitable Error-Prone PCR reaction to generate mutant enzyme variants was achieved by decreasing adenine and guanine nucleotide concentrations and increasing thymine and cytosine concentrations. Also, commercially available Taq DNA polymerase, which does not have proofreading, was used. In addition, by increasing the concentration of 5 mM $MgCl_2$ and adding 0.25 mM $MnCl_2$, optimum mutation formation was targeted. After cleavage with EP-PCR product and pET-28a(+) *Eco*RI and *Hind*III endonuclease enzymes, the determination of purity was calculated by measuring the ratio of absorbance with 260 nm and 280 nm NanoDrop equipment. Also, the qualities of samples were assessed by calculating the ratio of A260/A280. They were transformed into *E. Coli* cells by ligation according to the ratios of 5:1 and 7:1 (insert:vector). After establishing various genetic differences as a result of mutations, a library of 159 transformants was obtained. 58 mutants showing lipolytic activity were obtained with tributyrin agar plate screening. Protein expression was ensured by IPTG induction

(incubation at 30 °C for 6 hours) of the obtained 58 mutants. Expressed proteins were extracted by using the BugBuster protein extraction reagent. The amount of protein was quantified for each of the 58 mutants by Bradford analysis and diluted to keep the equal amount of reacted protein. The activity of both wild-type and mutants was measured in triplicate with the substrates 4-nitrophenyl hexanoate (6C) and 4-nitrophenyl dodecanoate (12C). The absorbance value was measured three times for each of the 58 mutants, and these values were averaged. When the all kinetic activity comparison of mutants were examined, no mutant with significantly greater activity was found compared to wild type. After control cleavage, Mutant48, Mutant52 and Mutant53 were selected as they showed greater activity against the 12C dodecanoate substrate than the wild-type esterase enzyme. Mutant11 and Mutant36 were selected because they showed greater activity than the wild-type esterase enzyme against both 6C hexanoate and 12C dodecanoate substrates. Finally, Mutant6 and Mutant23 showed lower activity than the wild-type esterase enzyme for the two substrates; Mutant 11 was chosen randomly. DNA sequences of 8 selected mutants were analyzed. When sequencing data for these mutants using CLUSTAL OMEGA were aligned with wild type, it was seen that all the potential mutants had the same gene sequence as the wildtype esterase enzyme. As a result of the study, mutant esterase enzymes that could be used as a substrate for 6 and 12C fatty acids could not be obtained or due to the high mutation rate, mutated colonies lost their activity and were eliminated during activity screening. The library will continue to be scanned using different screening strategies. In further studies, fatty acids with a different number of C can be used as alternative substrates to observe the activity of mutants. Error-Prone PCR experiments might be set up under different conditions and the properties of the esterase enzyme might be improved by using protein engineering approaches consisting of combinations of different methods. In this way, it will be possible to contribute to the national economy and literature by obtaining esterases that can meet the needs of the industry.

RASYONEL TASARIM VE YÖNLENDİRİLMİŞ EVRİM YAKLAŞIMLARIYLA YENİ ESTERAZ ENZİMİNİN PROTEİN MÜHENDİSLİĞİ UYGULAMALARI

ÖZET

Enzimler, canlı organizmalarda katalizör görevi gören ve kimyasal reaksiyonların ilerleme hızını kendilerini değiştirmeden düzenleyen, genellikle protein yapılı biyomoleküllerdir.

Enzimlerin endüstride biyokatalizörler olarak kullanılabilmeleri için enzimlerin istenilen reaksiyon koşullarına uygun özelliklere (yüksek aktivite, substrat özgüllüğü, ekstrem koşullara dirençlilik vb.) sahip olması gerekmektedir. Buna rağmen enzimler çeşitli reaksiyon koşullarında kullanılmak için her zaman gereken özelliklere sahip değildir. Bu nedenle, doğada ihtiyaç doğrultusunda uzun zaman sürecinde meydana gelebilecek genomik değişiklikler laboratuvar ortamında meydana getirilerek enzimlerin özelliklerinin geliştirilmesi ve istenilen reaksiyon koşullarında çalışabilir hale getirilmesi gerekmektedir. Bu amaç doğrultusunda protein mühendisliği yöntemleri kullanılarak eşsiz özelliklere sahip enzimler elde edilebilmektedir.

Esteraz enzimi sanayide deterjan, gıda, kozmetik, farmasötik, biyoyoakıtlar gibi bir çok farklı alanda kullanımı nedeniyle endüstriyel açıdan büyük bir öneme sahiptir. Kısmen suda çözünerek ester bağlarını hidrolize eden bu makromoleküller hala zorlu endüstri koşullarına uyum sağlayabilecek durumda olmaması ve ihtiyaç duyulan düzeyde aktivite gösterememesi nedeniyle esteraz enziminin var olan özelliklerinin geliştirilmesi ve enzime yeni özelliklerin kazandırılmasına yönelik çalışmalar devam etmektedir.

Protein mühendisliği metodları zorlu şartlarda çalışabilen enzimler elde edebilmek için en verimli yollardan biridir. Bu metodlar ekstrem koşullarda yaşayan mikroorganizmalardan izole edilen enzimlere uygulandığında daha üstün yeteneklere sahip enzimlerin üretilebilmesi mümkündür. Ekstrem koşullarda yaşayan mikroorganizmalar belirli mutasyonların kendiliğinden gelişmesiyle ekstrem koşullara adaptasyon sağlayabilmektedirler. Bu mikroorganizmaların adaptasyon eğilimleri sayesinde sahip oldukları enzimlerine protein mühendisliği yöntemleri uygulandığında endüstrinin ihtiyaçlarını karşılayabilecek özelliklere sahip enzimler elde edilebilmektedir. yakın zamanda grubumuz tarafından yüksek tuz konsantrasyonuna sahip Acıgöl'den dizi bazlı metagenomiks çalışmalarıyla izole edilen ve tanımlanan mikroorganizmalar arasında yer alan, gen dizisi *Halolamina sediminis* genomunun bir parçasına %75 oranında; aminoasit dizisi ise *Halomonas gudaonensis* organizmasının esterazına %91 oranında benzeyen esteraz(hAGEst) enzimi rekombinant olarak üretilmiş ve biyokimyasal karakterizasyonu tamamlanmıştır.

Bu tez kapsamında ise, tanımlanmış olan yeni esteraz enziminin literatürde yaygın olarak kullanılan protein mühendisliğinin iki temel yaklaşımı kullanılarak 3 boyutlu yapının araşturılması ve substrat özgüllüğünün geliştirilmesi hedeflenmiştir. İlk olarak rasyonel dizayn yaklaşımının temel yöntemlerinden biri olan bölgeye özgü mutagenez kullanılmıştır. Yeni esteraz proteininin üç boyutlu yapısını tahmin etmek için , SWISS MODEL yazılımıyla Protein Veri Bankası (PDB) incelendiğinde yeni esteraz enziminin esteraz ybfF proteini ile en yüksek benzerliğe sahip (%34) olduğu görülmüştür. Bu yöntem sayesinde aktif bölgede olduğu tahmin edilen 209. serinin ve oksianyon deliğinde bulunduğu tahmin edilen 88. metiyoninin, alanin amino asidiyle değiştirilmesi amaçlanmış ve bu aminoasitlerin yapıdaki önemi incelenmiştir. Ser209Ala ve Met88Ala mutasyonlarını elde etmek için primerler tasarlanarak Q5 Site-Directed Mutagenesis kitiyle gen çoğaltılmıştır. *E. Coli* C43 hücrelerine transforme edilip çıkan transformantlardan rastgele seçim yapılarak sanger dizilemeye gönderilmiştir. Dizileme sonucuna göre istenilen mutasyonların elde edildiği görülmüştür. Protein ekspresyonu için 1 mM IPTG eklenmiş ve ardından 30 °C'de 6 saatlik bir inkübasyon ile uyarılmıştır. Ser209Ala ve Met88Ala mutant esteraz genlerini içeren pET-28a(+) vektöründen gelen proteinler daha sonra 6xHis etiket tekniği kullanılarak saflaştırılmış ve SDS-PAGE analizi kullanılarak kontrol edilmiştir. Ser209Ala ve Met88Ala için IPTG ile gen ekspresyonu indüklendikten sonra 25-35 kDa aralığında bantlar gözlendi. Esterazların moleküler ağırlığı 27 ile 54 kDa arasında değiştiğinden, doğru bantta olduğu doğrulanmıştır. Sonraki aşamada, His-tag purifikasyon yöntemiyle saflaştırılan protein, ultrafiltrasyon yapılarak SDS-PAGE görüntülerine göre istenmeyen proteinlerden uzaklaştırılmıştır. Bradford reagent kullanılarak saf olarak elde edilen proteinin konsantrasyon ölçümü yapılmıştır. Bradford analizine göre Ser209Ala mutant esteraz için 20 mg/ml, Met88Ala mutant esteraz için 20 mg/mL ve yabanıl tip esteraz için 30 mg/mL olarak ölçülmüştür. 1 µM yabanıl tip, Ser209Ala ve Met88Ala mutant esteraz enzimleri 50 mM Tris- HCl pH:8 tampon çözeltisinde hazırlanan 1 mM Para-nitrofenol (pNP) hexaonate substratında 30 ºC'de 20 dakika boyunca inkübe edilmiştir. Kinetik aktivite için bağımsız üç deney tekrarı yapılarak 410 nm dalga boyunda absorbansları ölçülmüştür. Bu sonuçlara göre, yabanıl türün aktivitesi, Ser209Ala ve Met88Ala mutant esterazların aktivitesiyle kıyaslandığında %87.1 ve %17.7 oranında düştüğü saptanmıştır. Bu sonuçlar, aktivitenin ciddi ölçüde azalmasına bağlı olarak 209. serin amino asidinin esteraz enziminin aktif bölgesinde yer aldığını ve esteraz enziminin aktivitesi üzerinde doğrudan etkiye sahip olduğunu göstermektedir. Aktivitede %17.7 düşüşe neden olduğu için 88. metiyonin kalıntısının aktiviteyi etkilediği, ancak oksianyon deliğinde büyük bir öneme sahip olmadığı kanısına varılmıştır.

Çalışmanın ikinci kısmında ise yönlendirilmiş evrim yaklaşımıyla geniş bir kütüphane oluşturulması amaçlanmıştır. Hata-eğilimli polimeraz zincir reaksiyonu uygulanarak elde edilen bu kütüphaneden substrat spesifitesi geliştirilmiş mutant esteraz enzimlerinin seçilmesi hedeflenmiştir. Bu kapsamda, başta esterazlar olmak üzere literatürde çalışılan enzimler ve bu enzimlere uygulanan hata eğilimli PZR koşulları detaylı bir şekilde incelenerek hAGEst enzimi için en uygun koşullar belirlenmiş ve uygun primerler tasarlanmıştır. Mutant enzim varyantları oluşturmak için uygun bir hata-eğilimli PZR reaksiyonu adenin ve guanin nükleotit konsantrasyonlarının azaltılması ve timin ve sitozin nükleotit konsantrasyonlarının arttırılmasıyla sağlanmıştır. Aynı zamanda temin edilen hatayı düzeltme özelliğine (proofreading) sahip olmayan Taq DNA polimeraz kullanılmıştır. Ek olarak, normal PCR koşullarında 1.5 mM olan $MgCl_2$ konsantrasyonun 5 mM'a arttırılmasıyla ve 0.25 mM

$MnCl_2$ eklenmesiyle optimum mutasyon oluşumu hedeflenmiştir. PCR ürünü ve pET-28a (+) *EcoR*I ve *Hind*III endonükleaz enzimleri ile kesildikten sonra 260 nm ve 280 nm "NanoDrop" ekipmanları ile absorbans oranı ölçülerek saflık değerlendirilmiştir. Ayrıca numunelerin kalitelerinin belirlenmesi amacıyla A260/A280 oranı hesaplanmıştır. 5:1 ve 7:1 (insert: vector) oranlarına göre ligasyon kurularak *E. coli* C43 kompetan hücrelerine transforme edilmiştir. Mutasyonlar sonucu çeşitli genetik farklılıklar oluşturulduktan sonra 159 transformanttan oluşan kütüphane elde edilmiştir. Tribütirin agar plate ile lipolitik aktivite gösteren 58 mutant elde edilmiştir. Elde edilen bu 58 mutantın IPTG indüksiyonuyla (6 saat boyunca 30 ^{o}C de inkübasyona bırakılarak) protein ekspresyonu sağlanmıştır. Eksprese edilen proteinler, BugBuster protein ekstraksiyon reaktifi kullanılarak ekstrakte edilmiştir. 58 mutantın her biri için protein miktarı Bradford analiziyle ölçülmüştür ve her bir örnekte reaksiyona giren protein miktarı eşit konsantrasyonda olacak şekilde dilüe edilmiştir. 4-nitrofenil hekzanoat (6C'lu) ve 4-nitrofenil dodekanoat (12C'lu) substratları ile hem yabanıl türün hem de mutantların aktivitesi üç tekrarlı olarak ölçülmüştür. 58 mutantın her biri için absorbans değeri üç kez ölçülmüş ve bu değerlerin ortalaması alınmıştır. Mutantların tüm kinetik aktivite karşılaştırması incelendiğinde, yabanıl tipe kıyasla önemli ölçüde daha yüksek aktiviteye sahip mutant bulunamamıştır. Kontrol kesiminden sonra Mutant48, Mutant52 ve Mutant53 12C'lu dodekanoat substratına karşı yabanıl esteraz enziminden daha fazla aktivite gösterdiği için; Mutant11 ve Mutant36 hem 6C'lu hekzaonat ve hem de 12C'lu dodekanoat substratlarına karşı yabanıl tip esteraz enziminden daha fazla aktivite gösterdikleri için seçilmiştir. Son olarak; iki substrat için yabanıl tip esteraz enziminden daha düşük aktivite gösteren Mutant6 ve Mutant23; rastgele olarak da Mutant 11 seçilmiştir. Seçilen 8 mutant için DNA dizi analizleri yapılmıştır. CLUSTAL OMEGA kullanılarak bu mutantlar için elde edilen DNA dizi verileri yabanıl tip ile karşılaştırıldığında, seçilen tüm mutantların vahşi tip esteraz enzimi ile aynı gen dizisine sahip olduğu görülmüştür. Elde edilen bu sonuçlara göre 6 ve 12C'lu yağ asitlerini substrat olarak kullanılabilecek mutant esteraz enzimleri elde edilememiş ya da mutasyon oranının yüksek olması nedeniyle mutasyona uğramış koloniler aktivitelerini kaybettiği için aktivite taraması sırasında elenmiştir. Bundan sonraki çalışmalarda farklı tarama stratejileri kullanılarak kütüphane taranmaya devam edilecek ve mutantların dizi analizleri tamamlanacaktır. Mutantların aktivitesini gözlemlemek için farklı sayıda C içeren alternatif yağ asitleri substrat olarak kullanılacaktır. Bunun yanısıra, hataya-eğilimli PCR deneylerinin farklı koşullarda kurulması ve farklı yöntemlerin kombinasyonlarından oluşan protein mühendisliği yaklaşımları kullanılarak esteraz enziminin özelliklerinin iyileştirilmesi sağlanacaktır. Bu sayede, endüstrinin ihtiyacını karşılayabilecek özelliklere sahip esterazların eldesiyle, ülke ekonomisine ve literatüre katkı sağlanması mümkün olabilecektir.

1. INTRODUCTION

1.1 Enzymes

Enzymes are biological catalysts which provide speed up biochemical reactions without undergoing any change. Enzymes catalyze biochemical processes at the molecular level by accelerating the transformation of substrates into products in a buried pocket inside the enzyme's active site. Several reactions would be too slow to be useful for life without enzyme catalysis, while not all reactions in nature require catalysis (Keller et al., 2015).

The substrate specificity of enzymes is one of their most important features. Enzymes have to bind their substrates before the initiation of catalyzing any chemical reaction. Binding pockets enable specificity by allowing substrates to fit with complementary shape, charge, and hydrophilic or hydrophobic properties. In 1894, Emil Fischer proposed that the enzyme and substrate have complementary three-dimensional forms that allow them to fit into one another. This is known as the "lock and key" model (Berichte & Berichte, 1898). Although the model explains the enzyme-substrate specificity, it fails as the transition state of enzymes is considered. Daniel Koshland recommended an update to the lock and key model in 1958. Koshland`s proposal was substrates consistently reshape the active site as they interact with the enzyme. According to this theory, proteins are flexible, and their stable bound conformations are "induced" by the binding of their substrate (Koshland, 1958). The conformational selection model, developed in the 1990s, is predicated on the notion that unbound enzymes exist in different conformational states and that the substrate chooses the higher energy conformation to form the enzyme-substrate complex (Yan & Zou, 2017). Binding models are shown in Figure 1.1.

Some enzymes require non-protein portions like organic or inorganic molecules. Cofactors increase the reaction rate of enzymes catalyzed by the respective enzyme and stabilize the correct three-dimensional structure of the enzyme required for its catalytic activity.

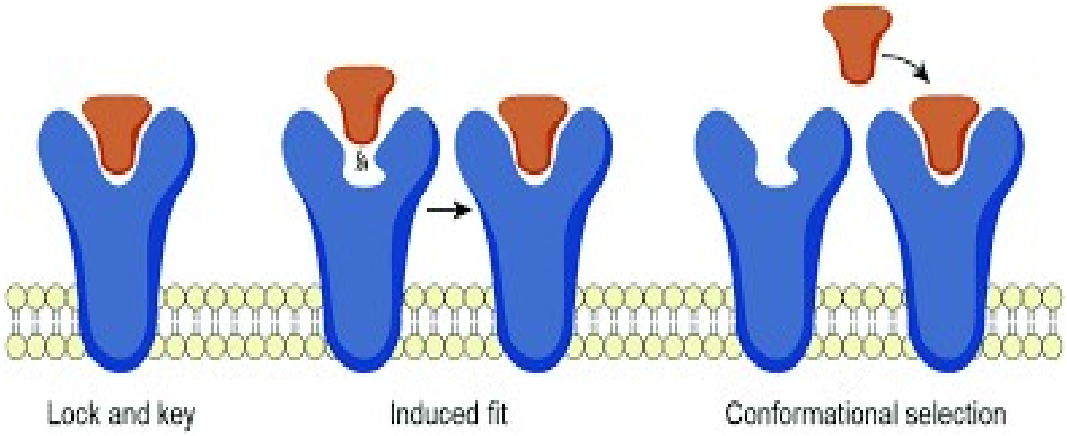

Figure 1.1 : Models of enzyme-substrat intereactions (Z. Wang et al., 2020).

There are two cofactor types; coenzyme(organic compounds) and inorganic ion group. Coenzymes and cofactors are both small, non-protein substances that play an important role in cell metabolism by helping enzymes in catalyzing biochemical reactions. (Wichmann & Vasic-Racki, 2005). Coenzymes may bind loosely and release from the active site, or they may have tight-binding (are referred, prosthetic group) and lack the ability to release easily from the enzyme. Coenzymes may bind loosely and release from the active site, or they may have tight binding (are referred prosthetic group) and lack the ability to release easily from the enzyme. There are also non-cofactor prosthetic groups, only prosthetic groups located in the active site of an enzyme are shown as cofactors (Roy Choudhury, 2019). Coenzymes act as intermediate carriers of electrons, certain atoms or functional groups to be transferred. As coenzymes undergo chemical changes as a result of enzyme action, they are also considered as a subclass of substrates or second substrates common by many various enzymes.Apoenzymes are enzymes that have not yet been connected with a required cofactor, whereas holoenzymes (active enzymes) are enzymes that have been bonded with their essential cofactors (Fischer et al., 2010).

1.2 Classification of Enzymes

An EC number is used to identify and classify enzymes in a comprehensive method; this digital number is a four-level description that is used to classify enzymes depending on the entire transformation of substrates in to the products. The first level corresponds to seven different classes based on the type of chemistry conducted.

Oxidoreductases (EC 1), catalyze oxidation/reduction reactions; transferases (EC 2) transfer a chemical group from one molecule to another.

For example, a methyl or glycosyl moiety; hydrolases (EC 3) undertake chemical bond hydrolysis by cleavage of covalent bonds with the help of water; lyases (EC 4) catalyze the breakage of C-C, C-O, C-N bonds aside from hydrolysis or oxidation; isomerases (EC 5) catalyze geometric and structural changes among isomers; ligases (EC 6) bind two molecules through the hydrolysis of a nucleoside triphosphate molecule mostly needs ATP as an energy source. A recently defined new class, translocases (EC 7) catalyze the transport of electrons or molecules across cell membranes or their separation within membranes, typically including ATP hydrolysis. These EC classes are then subdivided into subclasses and sub-sub classes (second and third level, respectively) based on a number of characteristics including the chemical bond cleaved or, the reaction centre, the transferred chemical group, and the cofactor utilized for catalysis. The substrate specificity is defined at the final level of classification (Tipton & Boyce, 2000) (Porto de Souza Vandenberghe et al., 2020).

1.3 Lipases and Esterases

Lipases and esterases are abundant in almost all organisms, from microorganisms to plants and animals; most commercially valuable esterases are obtained from microorganisms. Lipases (triacylglycerol acyl hydrolase) catalyze the hydrolysis of mono, di and triacylglycerols to glycerols and fatty acids at oil-water interfaces.

Lipolytic enzymes provide new opportunities for resolving biotechnological bottlenecks that industries are presently facing, as well as contribute tothe resolution of environmental problems. The use of enzymes in the detergent industry helps to increase detergency, minimize scaling, and shorten the time, agitation, and temperature essential for washing, therefore prolonging fabric durability. Using lipases aids to reduce the amounts of surfactants utilized (Ken Ugo et al., 2017). Lipases have been utilized to produce single isomers of chiral drugs (Gotor-Fernández et al., 2006) and optically active intermediates (Smidt et al., 1996) for pharmaceutical industries.

These can be designed to produce enantio-specific antibiotics, vitamins, alkaloids, anti-cancer, anti-arteriosclerotic, and antiallergic drugs (Aravindan et al., 2007). In the synthesis of aromatic compounds, lipases demonstrate surfactant activity.

These features have the potential to be applied in the manufacture of cosmetics. The dairy industry uses lipases to hydrolyze milk fat, and fatty acid chain lengths are altered to improve the flavours of various cheeses (Macrae & Hammond, 1985).

Lipases can also be used as supplements in shampoo compositions to enhance hair quality by increasing the clearance of excess oil from the hair and reducing dandruff (Wijaya et al., 2020).

Lipases are members of the α/β hydrolase fold superfamily and can act at both aqueous and non-aqueous interfaces which makes them different from esterases (Chandra et al., 2020).

Esterases are enzymes that have a wide substrate spectrum and cleave the ester bonds by a water molecule. As a consequence, they are members of the hydrolase family. They are described as esterases when they hydrolyze carboxylic esters, thioesterases if they target thioester bonds, and phosphoesterases when they hydrolyze phosphomonoester or phosphodiester (Peist et al., 1997).

Esterases are serine hydrolases that catalyze the cleavage of ester bonds in short-chain fatty acids to release alcohols and carboxylic acids (EC 3.1.1.1 carboxylesterases).

Despite the limited amino acid sequence similarity between lipolytic enzymes, which is frequently less than 20%, the majority of them contain a conserved α/β-hydrolase fold and a consensus canonical GXSXG pentapeptide motif around the catalytic serine residue. Subsequently, a second major structural family of lipolytic enzymes with a GDSL catalytic site consensus motif and an α/β-fold was identified. Currently, only a few lipolytic enzymes with a β-lactamase-like fold that is structurally unrelated to α/β- and α/β/α-hydrolases have been found. Black dots in Figure 1.2 represent catalytic triad residues Serin (S), Histidine (H), and Aspartic acid (D), while the white dots indicate conserved oxyanion hole residues (Glycine (G), Asparagine (N)). The canonical α/β-hydrolase fold is defined by a middle hydrophobic sheet constructed in eight β-strands and four α strands, the second of which is typically antiparallel (Kim et al., 2006).

Nucleophilic aminoacid (serine, cysteine or aspartic acid), an acidic amino acid and histidine form a highly conserved triad that is responsible for the catalytic activity. The nucleophile is located on the top of an elbow, and the aminoacid residues are Sm-X-Nu-X-Sm. While Sm indicates the small aminoacids, Nu defines the nucleophile.

This is a conserved structure in most lipases. Typically, the nucleophile is a serine, and small aminoacid residue around the nucleophile is glycine. The nucleophile can easily reach the substrate and the hydrolytic water molecule bythis conformation (Kovacic et al., 2019).

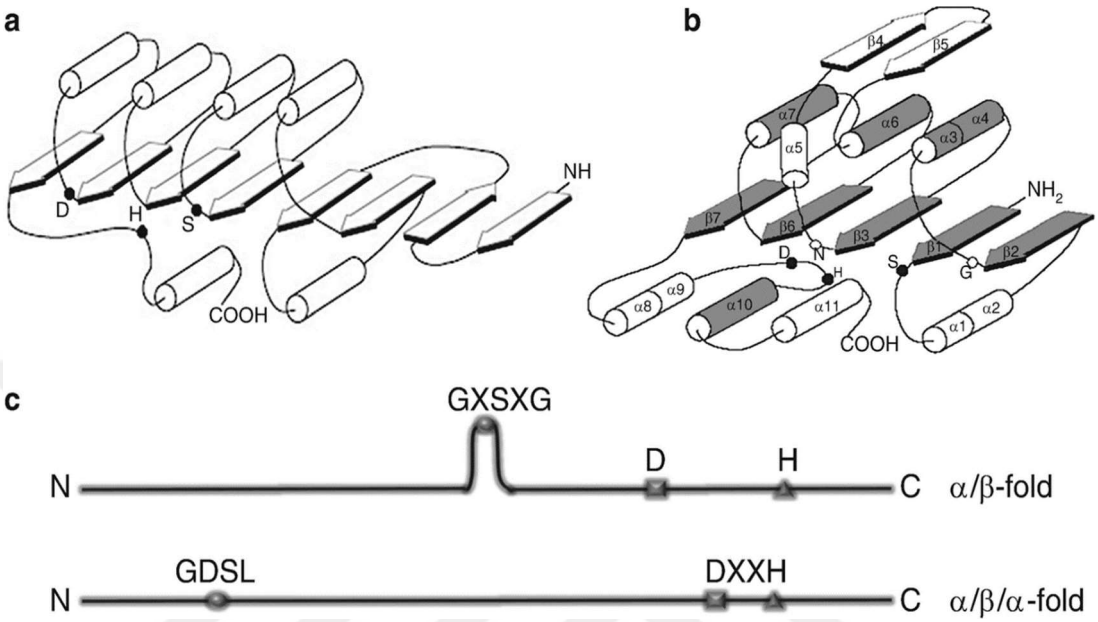

Figure 1.2 : Active site of lipases and esterases (Ramnath et al., 2017).

The mechanism by which esterases hydrolyze cleavage and formation of ester bonds basically comprises of four steps:

a) Binding of the substrate to an active serine residue which yields a tetrahedral intermediate stabilized by catalytic histidine and asparagine residues;
b) Release of the alcohol and arrangement of an acyl-enzyme complex;
c) Attack by a nucleophile and formation of a tetrahedral intermediate again;
d) Formation of the product (it can be an acid or an ester) and free enzyme by the intermediate after resolution (Gao et al., 2021).

The oxyanion hole is not a very tightly conserved region, yet helps to maintain the tetrahedral structure stable during the hydrolysis process. The oxyanion hole region consists of two residues that donate amide protons for stabilization (Castilla et al., 2017).

The activity of the protein was measured using a kinetic assay using para-nitrophenyl esters as substrate. The ester bond is hydrolyzed by the enzyme and the increased absorbance of the released pNP at 410 nm is measured. Before measuring the activity,

the path length for spectrophotometric measurements and the extinction coefficient of pNP for each reaction condition are calculated (Bardi et al., 1993).

1.4 Limitations in Industrial Enzyme Applications

For enzymes to be used as biocatalysts in industry, they must have properties suitable for the desired reaction conditions (high activity, substrate specificity, resistance to organic solvents, etc.). However, enzymes do not always have the properties necessary for use in various reaction conditions.So, it is necessary to improve the properties of enzymes and to ensure that they work under the desired reaction conditions by genomic changes that can occur in long time periods in accordance with the needs in nature. Scientists are working on enzymes that can endure severe and unfavourable circumstances widely in industrial processes, such as temperature tolerance, activity in alkaline or acidic environments, high performance in non-aqueous medium, and enhanced protease resistance (Stepankova et al., 2013).

1.5 Protein Engineering

Protein engineering is a multidisciplinary field that uses biological, computational, chemical and physical techniques such as recombinant DNA technology or X-ray chromatography, to achieve alteration or addition new functions to proteins and improved resistance of unfavourable conditions and stability. Protein engineering is employed to overcome enzyme limitations in harsh industrial processes. Protein engineering approaches to improve protein stability are targeted at some structural points, particularly surface regions, hydrophobic core, disulfide bridges, surface charges, isolated charges, binding pockets and interface of enzyme subunits. As shown in Figure 1.3, there are three types of approaches to protein engineering:

(i) directed molecular evolution,
(ii) rational design, and
(iii) semi-rational design (Bornscheuer & Höhne, 2018).

1.5.1 Rational design

Rational design is the approach of using extensive knowledge about a protein's 3D structure and function to make particular mutations to achieve the desired alterations.

However, the downside to this approach is that it cannot acquire the structural information of every desired protein. Another disadvantage of the mutation design is the difficulty in calculating long-distance interactions (Tiwari et al., 2012).

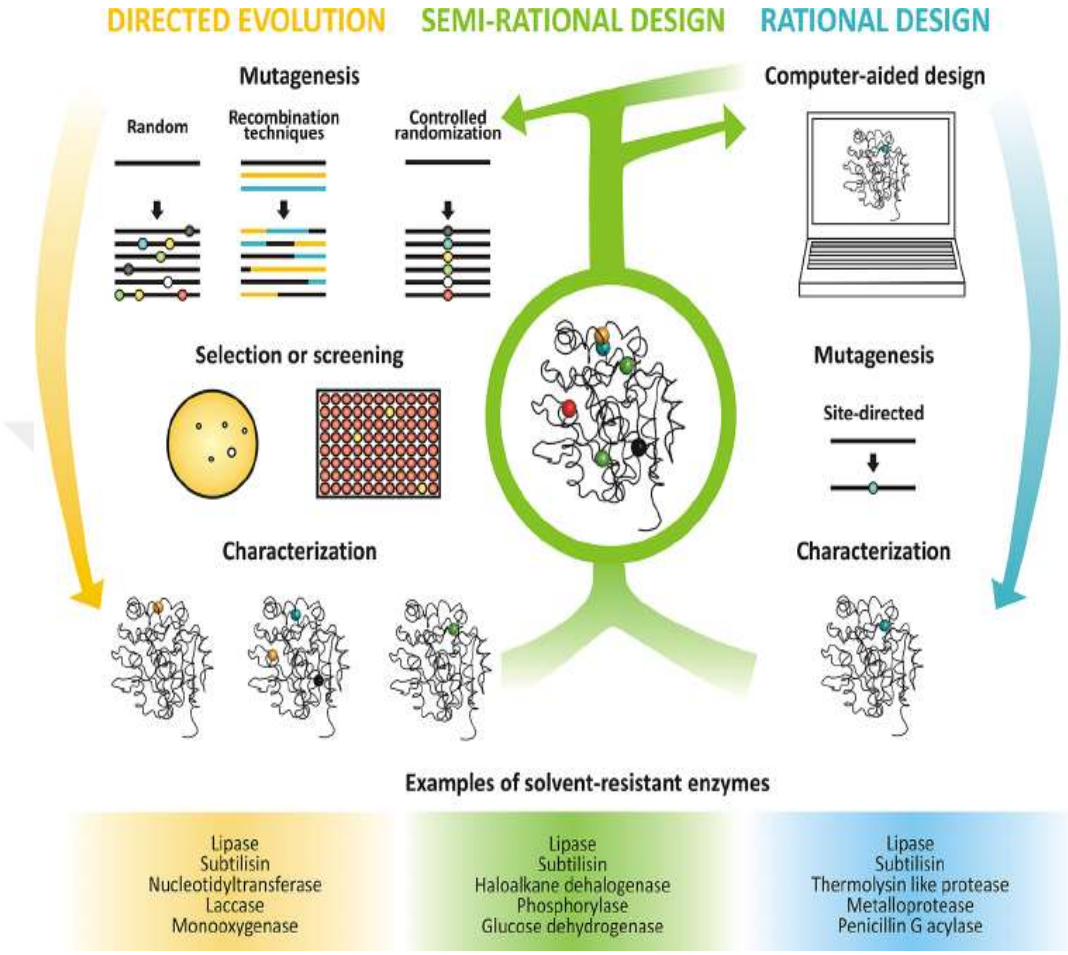

Figure 1.3 : Approaches of protein engineering (Stepankova et al., 2013).

The belief in the potential of rational design is predicated on the assumption that our present scientific knowledge is adequate to foresee function from structure. Nevertheless, the majority of enzymes lack such structural and catalytic activity mechanism information. Therefore, even if the target enzyme's structure and catalysis process are completely understood, the molecular mutation basis for the desired function may not be attained (F. H. Arnold, 2009). The potential advantages of a rational design approach include an increased likelihood of beneficial new mutations and a substantial decrease in library size, demanding less effort and time to screen the mutant library. This is especially useful if there is no high-throughput assay system available (Steiner & Schwab, 2012).

The rational design approach consists of three steps:

(1) selecting a suitable enzyme,
(2) identifying the amino acid sites to be changed, which is usually based on high-resolution crystallographic data, and
(3) characterization of the mutants (Prajapati et al., 2018).

The rational design method may allow for controllable amino acid changes such as insertion, deletion or substitution. A single amino acid can be modified or a sequence of a certain length can be modified. A single amino acid substitution or variation in a particular sequence length may also be provided (Marshall et al., 2003).

1.5.1.1 Site directed mutagenesis

Mutations are identified computationally by using enzyme structures, homology models, or sequence analyses; a desirable mutant is generated by utilizing mutant oligonucleotides that contain the target mutation at a particular location (Karagüler et al., 2007). Site-directed mutagenesis is a very useful way to determine the structure-function relationship of a protein by analyzing gene modification studies, enzyme catalytic domains, and catalytic mechanisms. Single and combined mutations evaluated by bioinformatics gene modifications are examples of site-directed mutagenesis (Hsieh & Vaisvila, 2013).

Site-directed mutagenesis permits alterations of amino acid sequences. The important residues near catalytic/substrate binding sites, which confer vital properties, are analyzed according to the properties of the protein using appropriate methods (Carter, 1986).

In this paper, tyrosine at position 245 of *Acidothermus cellulolyticus* endoglucanase Cel5A has been changed to glycine. High-resolution X-ray crystallographic structures show that the catalytic activity was increased by 40% due to the reduction of product inhibition (Baker et al., 2005).

1.5.1.2 Alanine scanning mutagenesis

The alanine scanning technique is used to determine the catalytic mechanism and functional role of a target amino acid at a specific position in addition to the energy characteristics of hotspots. This procedure includes substituting alanine amino acid for the determination of desired amino acid residues.

Alanine scanning mutagenesis is used to discover the few conserved amino acid residues in the catalytic triad and oxyanion hole, as well as the key amino acids implicated in catalytic activity in unknown proteins (Allouche, 2012). The alanine residue is the smallest, chiral and neutral amino acid because of the methyl functional group and does not interfere with side groups.

Since alanine does not cause serious electrostatic changes in the three-dimensional structure, they do not change the protein structure (Pál et al., 2006).

Alanine scanning mutagenesis was used in one research to investigate the effects of asparagine at position 371, phenylalanine at position 375, and tyrosine at position 376 on Geobacillus lipase activity, function, and structure. A single mutation has been proven to influence the primary structure and activity of lipases (Gudiukaitė et al., 2016).

1.5.2 Semi-rational design

Semi-rational design is combined with rational design and directed evolution that generates smart, small, functionally rich mutant libraries using rationally preselected target sites and limited amino acid diversity. Therefore, semi-rational design approache provides a rapid and simple method to generate novel protein variants (Stepankova et al., 2013).

In many industrial conditions, hydrogen peroxide is present in the same reaction medium of microbial lipases, so it is very important to obtain hydrogen peroxide tolerant lipases. For this purpose, a smart lipase A library consisting of 26 mutants was created and a semi-rational design approach was determined using mass spectrometry and three-dimensional structures to measure the mass changes between hydrogen peroxide and oxidized amino acids. After screening the mutant variants, 21 lipase mutant variants of LipA were observed to improve hydrogen peroxide tolerance. Compared to the wild type, the best mutant variant has two amino acid changes; Trp42Ala and Glu134Met (Jiang et al., 2020).

1.5.2.1 Site saturation mutagenesis

To generate site-saturation mutant libraries, degenerate primers are frequently used at specific locations (Adamczak & Krishna, 2004).

The site saturation mutagenesis method is used to create mutant libraries that include all possible amino acids. This is accomplished by systematic displacement of a single natural amino acid residue with the remaining 19 amino acids. As a result, the method is known as 'saturated' occurs in a specific location of the gene. PCR amplification allows the exchange of all 20 amino acids in specific regions (Iserte et al., 2013). Site saturation method is shown in Figure 1.4.

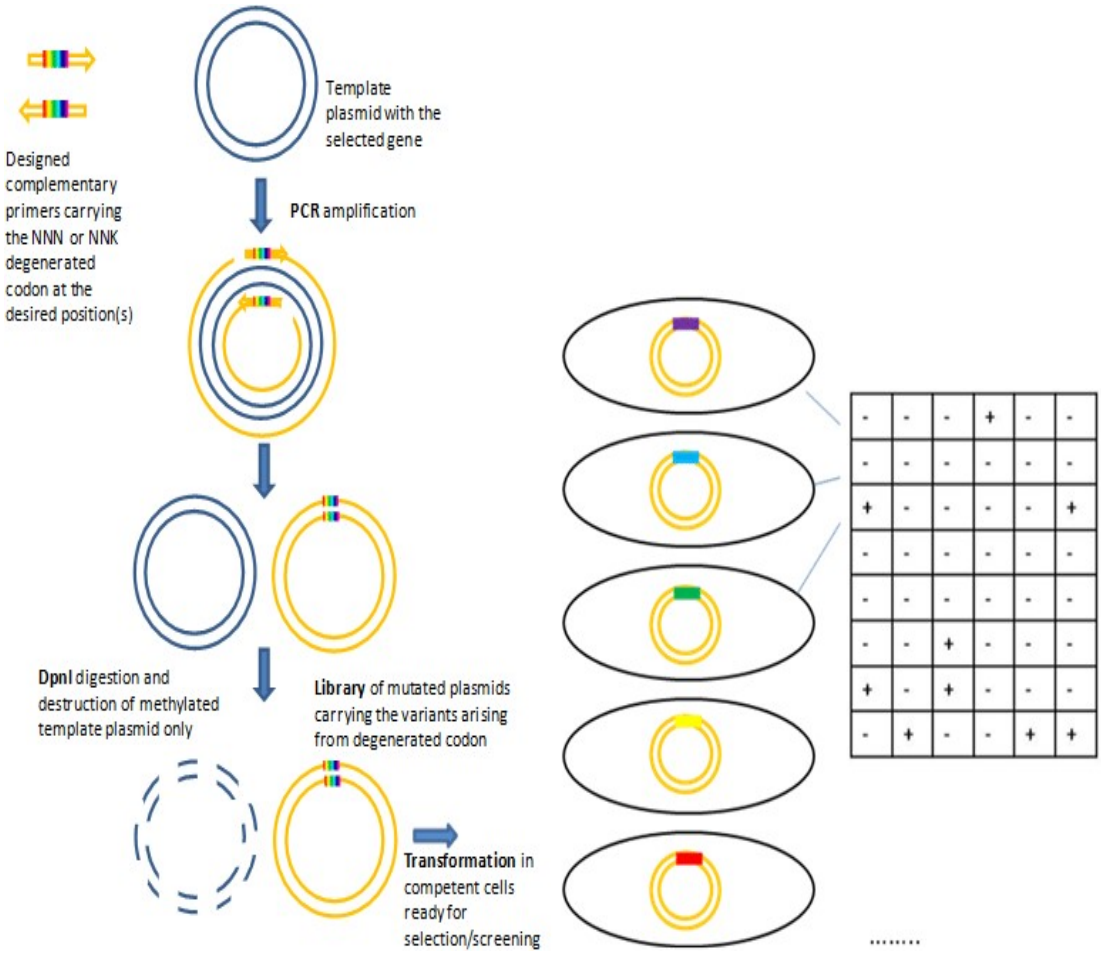

Figure 1.4 : Site saturation mutagenesis (Valetti & Gilardi, 2013).

1.5.3 Directed evolution

Directed evolution is a widespread strategy for improving the physicochemical properties of different enzymes and overcoming the limitations of native biocatalysts. Directed evolution has become a standard in protein engineering over the last two decades, forging a new era of industrial biocatalyst modification (Frances H Arnold & Geurgiou, 2014).

Because natural enzymes have limitations such as a lack of catalysis activity and resistance to extreme processes, directed evolution technologies have been developed to reshape or repurpose existing natural enzymes so that they can be effectively and significantly improved for a versatile industrial protein.

Directed evolution provides the unique advantages of adapting the biocatalyst to imposed conditions through the designing and implementation of genetic selections and function screenings without requiring considerable preexisting knowledge of gene-to-function relationships. Another significant benefit of directed evolution.

On the contrary rational design, prior knowledge of the protein's structure or mechanism is not essential. The basic disadvantage of the majority of directed evolution approaches developed for single genes is that they cannot target the multiple, isolated genetic elements that compose or regulate metabolic pathways at the same time. Another disadvantage of this approach is time-consuming and involves strenuous steps of generating gene libraries (Wilkinson et al., 2004).

The genetic diversity in directed evolution is created by random mutagenesis using a variety of techniques depending on error-prone DNA replication and chemical mutagenesis, or by homologous recombination of highly associated genes using a process is known as the family shuffling of one or more parent sequences. These modified genes are cloned back into a plasmid for expression in an optimal host organism which can be bacteria or yeast. Selection and/or screening is carried out to identify high performing clones. The process is then repeated to obtain the best variant. Screening or selecting for heterologous expression can be done in two ways: screening/selecting for the activity of a reporter protein, or screening/selecting for the protein’s own function.

Random mutagenesis does not demand knowledge of the enzyme structure, but it does require proper selection and high-throughput screening methods. The best-featured mutants are then subjected to subsequent rounds of mutations and selections (Chen, 2001).

There are some significant limitations in the approach. The most important is that it will be difficult, if not impossible, to evolve surprising new functions in nature. Apparently, there will need new methods to outcome intractable design problems in creating libraries, screening and selecting mutants(Chica et al., 2005).

1.5.3.1 DNA shuffling

DNA shuffling strategies effectively recombine mutations that consequence from point mutagenesis throughfragmentation and PCR-mediated re-assembly process. Fragments from diverse gene variants are integrated to construct a unique DNA molecule; beneficial mutations can be incorporated while undesirable mutations are removed (Druteika et al., 2020).

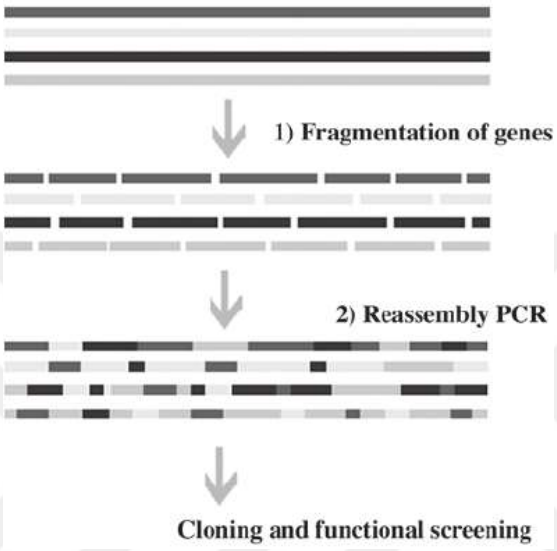

Figure 1.5 : DNA shuffling (Hoekstra & Smeekens, 2016).

As shown in Figure 1.5, DNase I produces randomly oligonucleotide fragments from selected progenitor genes and then shuffles homologous DNA sequences by PCR recombination of the resulting mixed oligonucleotides (Eton & Lepore, 2008).

1.5.3.2 Error-Prone PCR

Error-prone PCR is one of the non-recombinative methods of protein engineering. It is typically performed using conditions that decrease the fidelity of Taq DNA polymerase which permits the designing of improved enzymes by random changes within the nucleotide sequence utilizing thermostable polymerases lacking proofreading ability during DNA synthesis. It introduces a low level of point mutations randomly over a gene sequence (Pritchard et al., 2005).

That can be done in a few ways, for example increasing the concentration of magnesium chloride ($MgCl_2$) within the reaction mixture, including manganese chloride ($MnCl_2$), utilizing unequal concentrations of each nucleotide (increasing concentrations of dTTP and dCTP, and decreasing concentrations of dATP and dGTP).

In that way changing the ratios of nucleotides within the reaction, including a nucleotide analogue such as 8-oxo-dGTP or dTTP, or also by combining two or more of this options for one reaction. The modifications allow the enzyme to act in an error-prone manner, constructing mutations during the PCR cycles. The likelihood of misincorporation is proportional to the number of duplications during the PCR. One of the components for inducing randomized nucleotide sequences via PCR is additionally the utilization of mutagenic primers (Frances H. Arnold et al., 2003). Taq polymerase itself has an inherently high error rate, in addition to errors prone to changes from AT to GC. Another factor that affects error rate is template concentration, with lower template concentrations leading to larger mistake rates. Error prone pcr creation conditions are shown in the Figure 1.6.

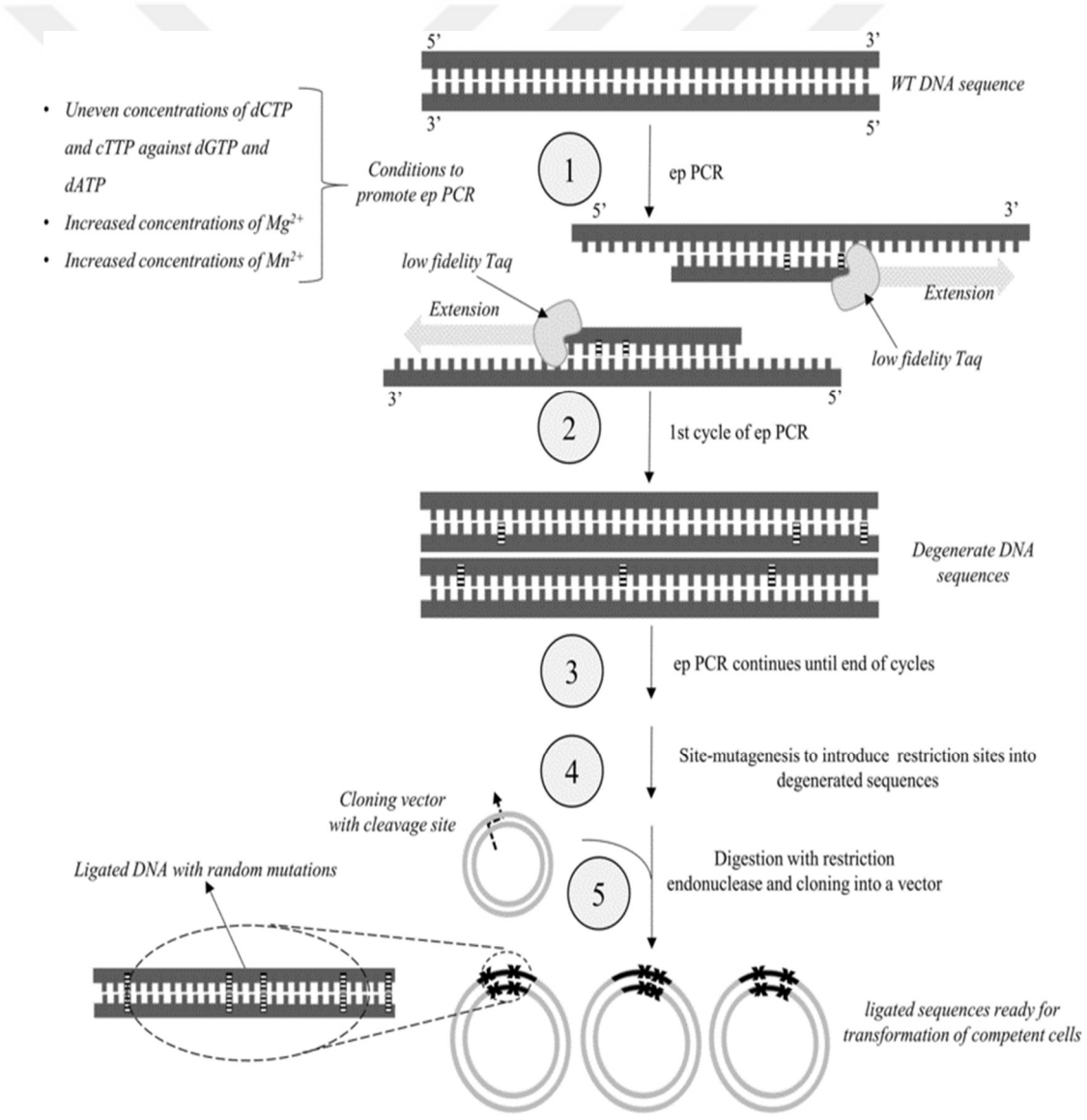

Figure 1.6 : Error-Prone PCR (Jimenez-Rosales & Flores-Merino, 2018).

Since most random mutations are neutral or harmful, it is thought that a low mutation rate enhances the likelihood of beneficial mutations happening. It is possible to introduce alterations per gene ranging from 1 to 20 (D. Wang et al., 2000).

An expression system and high-throughput assay ought to be created before a library of protein variants is generated(Kwan, 2017).

1.6 Aim of Study

Here, our main goal is to understand the three-dimensional structure and to change the substrate specificity of the novel esterase enzyme named "hAGEst" which was previously defined by our group as a result of metagenomic studies. We have used two different approaches of protein engineering strategies; rational design and directed evolution.

The first step of the study aimed to experimentally validate the predictions about the three-dimensional structure of the new esterase enzyme. When the protein database was examined, it was discovered that the novel esterase and the ybfF protein had a 34% similarity (Park et al., 2008). By using the SWISS-MODEL program, the three-dimensional structures of these two enzymes were compared. It was thought that the 209^{th} amino acid of serine could be found in the active site and the 88^{th} amino acid of methionine could be found in the oxyanion hole of the new esterase enzyme. Mutations of Ser209Ala and Met88Ala were obtained by using the alanine scanning mutagenesis method of the rational design approach.

The second part of the study involves changing the substrate specificity of hAGEst from 6C fatty esters to 12C fatty esters using the Error-Prone PCR method of directed evolution approach.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

The equipment used in this study is listed in Table 2.1 below.

Table 2.1 : Laboratory equipment that were used in this study.

Equipment	Trade Mark/Model
Analytical Balances	Precisa BT 610C
Autoclaves	Tuttnauer 2540ml (Switzerland)
Centrifuge	Allegra 25R Centrifuge Beckman
Deep freezers (-80 °C)	Ultra Low Sanyo
Electrophoresis equipments	Whatman Inc. Horizon 20-25, UV star 312nm Biometra, Biometra P25T power pack
Equipment	Trade Mark/Model
Freezer (-20 °C)	Biomedical Freezer Sanyo
Heat block	BioSan Bio TDB-100
Ice maker	IMS-50 Automatic
Magnetic stirrer	Heidolph Standard
Microfuge heater	VWR Digital Hea
Microfuge tubes	ISOLAB, Axygen
Micropipettes	Eppendorf, Gilson
Microplate Spectrophotometer	BIO-RAD Benchmark Plus
Nanodrop	Scientific
Orbital Shaker Incubator	Heidolph Duomax 1030
pH Meter	Mettler Toledo InLab Probe
Pipettes	Eppendorf
Refrigerator (+4 °C)	Electrolux SpacePlus
SDS equipment	BIO-RAD Mini Protean Tetra Cell
Sonicator	Bandelin Sonoplus 530 KE76 Probe
Thermocycler	Biometra
Thermomixer	Eppendorf
Vortex	Scientific Industries
Water Bath	Memert

2.1.2 Chemicals, enzymes and kits

The chemicals,enzymes and kits used in this study is listed in Table 2.2 below.

Table 2.2 : Laboratory equipment that were used in this study.

Chemicals	Company
4-Nitrophenyl dodecanoate	Tokyo Chemical Industry
4-Nitrophenyl hexanoate	Sigma-Aldrich
Acetic acid	Riedel de-Haen
Acrylamide	Sigma-Aldrich
Agarose	AppliChem
Ammonium persulfate (APS)	Sigma-Aldrich
Bromophenol blue	Merck
BugBuster® Protein extraction reagent	Merck
Coomassie brilliant blue R-250	Sigma-Aldrich
Ethanol	Merck
Ethylenedinitrilotetra-acetic acid (EDTA)	BDH Lab Supply
Glycerol	Carlo Erba
Glycine	Sigma-Aldrich
Gum Arabic	J. T. Baker
Hydrochloric acid (HCl)	Sigma-Aldrich
Imidazole	Merck
Isopropanol	Sigma-Aldrich
Isopropyl β-D-thiogalactoside (IPTG)	Merck
Kanamycin	Sigma-Aldrich
1 kb Gene Ruler Marker	Fermentas
Methanol	Merck
Nickel nitrilotriacetic acid (Ni-NTA Resin)	Thermo Fisher Scientific
Sodium chloride (NaCl)	AppliChem
Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$)	Sigma-Aldrich
Sodium dodecyl sulphate (SDS)	BDH Lab Supply
Sodium hydroxide (NaOH)	Sigma-Aldrich
Chemicals	**Company**
Tetramethylethylenediamine (TEMED)	Thermo Fisher Scientific
Tris (hydroxymethyl)-aminomethane	Carl Roth
Triton-X	Sigma-Aldrich
Tryptone	Bio-Life
Unstained protein MW marker	Thermo Fisher Scientific
Yeast extract powder	Bio-Life
β-mercaptoethanol	Sigma-Aldrich
Enzymes	**Company**
Taq Polymerase	Fermentas
*Hind*III	Fermentas
*Ecor*I-FD	Fermentas
*Ecor*I	Fermentas
T4 DNA Ligase	News England Biolabs
Kits	**Company**
Gel purification kit	EcoSpin
PCR product purification kit	EcoSpin
Plasmid isolation kit	EcoSpin
Q5 Site-Directed Mutagenesis Kit	Biolabs

2.1.3 Growth medium

The compositions of culture solid and liquid medium for *E. coli* C43(DE3) are given in Table 2.3 and Table 2.4. Autoclaved was applied for 15 minutes at 121 °C to sterilize the LB Agar medium.

Table 2.3 : Luria-Bertani (LB) Medium.

Luria-Bertani (LB) Medium	Amount
NaCl	1 g
Tryptone	1 g
Yeast extract	5 g
dH_2O	1000 mL

Table 2.4 : Luria-Bertani (LB) Agar Medium.

Luria-Bertani (LB) Agar Medium	Amount
Agar	15 g
NaCl	10 g
Peptone/Tryptone	10 g
Yeast extract	5 g
Distilled water (dH2O)	1000 mL

Tributyrin degradation by the microorganisms is demonstrated by clear zones surrounding the lipolytic colonies. By converting fat to water-soluble butyric acid, lipolytic organisms make the medium transparent. The components given in Table 2.5 were homogenized by sonication. A few cycles of 10 sec on/20 sec off was applied until it looks like milk.

Table 2.5 : Composition of tributyrin LB Agar medium.

Tributyrin LB Agar Medium	Amount
Peptone/Tryptone	2 g
NaCl	2 g
Yeast extract	1 g
IPTG	200 µL
dH_2O	200 mL

2.1.4 Solutions and buffers

Agarose gel (1%): 0,40 g agarose is liquefied in 40 mL 1X TAE Buffer and left to cool down and the gel is poured into the tray. The suitable comb is placed on the gel.

His-tag purification lysis buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, the pH is adjusted to 8.

His-tag purification wash buffer 1: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, the pH is adjusted to 8.

His-tag purification wash buffer 2: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, the pH is adjusted to 8.

His-tag purification wash buffer 3: 50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole, the pH is adjusted to 8.

His-tag purification elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, the pH is adjusted to 8.

IPTG (1M) : 238 mg IPTG was dissolved in 1 mL of dH_2O, passed through a filter and stored at 20 °C.

Kanamycin stock solution : 40 mg of kanamycin was dissolved in 1 mL of dH_2O, passed through a filter and stored at -20 °C.

SDS-PAGE destaining solution: 40 mL of methanol, 10 mL of acetic acid, 50 mL of dH_2O.

SDS-PAGE running buffer (10X): 30.3 g Tris, 144 g glycine and 10 g SDS were dissolved in 1L with dH2O. 10X Running buffer was diluting to 1X Running buffer with dH_2O.

SDS-PAGE sample loading buffer (5X): 90 mM TRIS-HCI, pH 6.8, 20 % Glycerol, 2% SDS, 0.02% Bromophenol blue, 0.1 M DTT.

SDS-PAGE staining solution: 0.1 g of Coomassie Brilliant Blue R-250, 40 mL of methanol, 10 mL of acetic acid, 50 mL of dH_2O.

TAE buffer (50X): 242 g Tris base (MW=121.14 g/mol), 14.6 g EDTA and 57.1 mL glacial acetic acid were dissolved in dH_2O up to 1 L. Solution was stored at room temperature.

2.1.5 Bacterial strains and vectors

pET-28a(+) bacterial expression vector (Figure 2.1) was used to produce a novel recombinant esterase enzyme in *Escherichia coli* C43(DE3) host. Length of pET-28a(+) vector is 5369 base pairs (Tutuncu et al., 2019). The vector carries a gene for the T7 RNA polymerase, which is controlled by an IPTG-inducible promoter and the T7 transcriptional promoter (the target gene, esterases, is placed downstream from the strong phage T7 transcriptional promoter on the pET expression vector). The esterase is activated by the lactose analogue isopropyl-ß-D thiogalactopyranoside (IPTG), which deactivates the lac operator and allows the production of T7 RNA polymerase, which then transcribes the target gene Since the pET expression vector includes a kanamycin resistant marker, 40 µg/mL kanamycin was introduced in all mediums to

keep the vector under selection pressure. Additionally, thrombin cleavage site and also restriction enzyme cleavage sites for SacI, *EcoR*I, *BamH*I, *Nhe*I, *Xho*I, *Not*I, *Hind*III, *Sal*I, *Nde*I, *Bmt*I, *Nco*I and *EcoR*V. pET-28a(+) vector has lac operon, N- & C-terminal Histidine tags sequences which ensure protein purification, kanamycin resistance gene (KanR). pET-28a(+) Expression Vector was used for the expression of the designed proteins. The features of the vector and multiple cloning sites are shown in Figure 2.1.

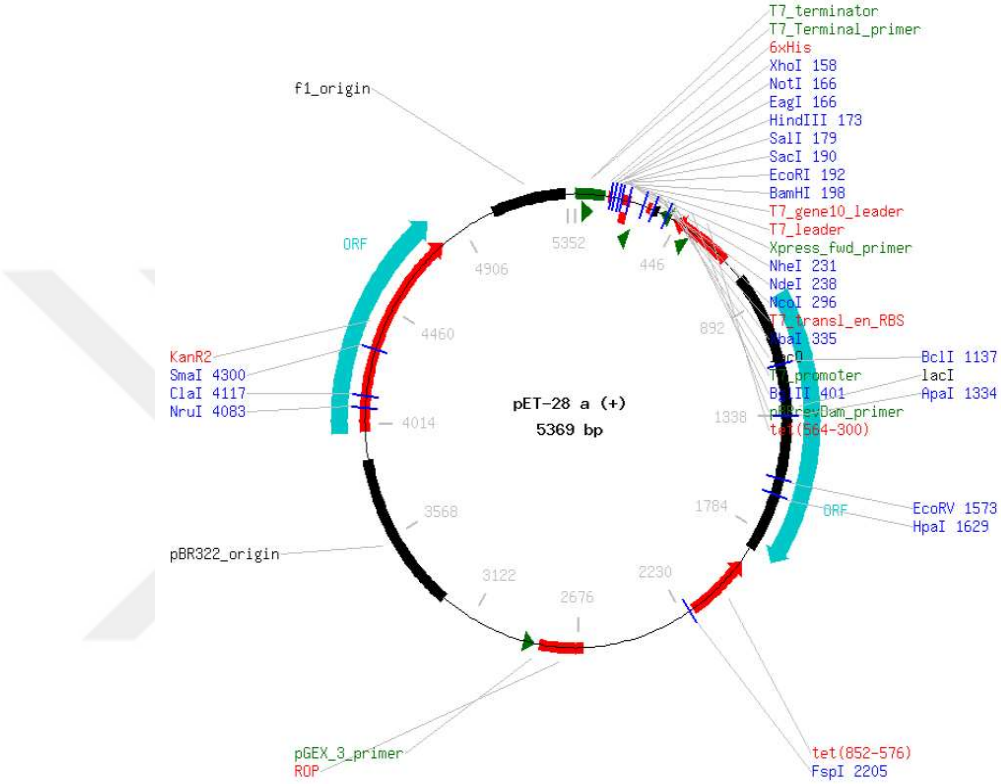

Figure 2.1 : pET-28a(+) expression vector and multiple cloning site.

2.2 Methods

2.2.1 Site-Directed Mutagenesis by Alanine Scanning

2.2.1.1 Selection of the mutation region and the mutant

The Expasy Translation Tool was used to convert the esterase gene's nucleotide sequence into a protein sequence as shown in Figure 2.2. To estimate the three-dimensional structure of the new esterase protein, the Protein Data Bank (PDB) was monitored using the SWISS-MODEL software as shown in Figure 2.3. The novel protein sequence "hAGEst" was aligned with the esterase ybfF protein having the highest similarity (34%) (Park et al., 2008) in the database.

```
3BF8_B      MKLNIRAQTAQNQHNNSPIVLVHGLFGSLDNLGVLARDLVNDHNIIQVDVRNHGLSPREP    60
hagest      --MTVDLNHLDTGGEGIPLVVIHGLLGSADNWRSHVKQWQQQCRVVAVDLRNHGRSPHAD    58
              :.:  :  :.  :. *:*::***:** **    .::  :: .:: **:**** **:

3BF8_B      VMNYPAMAQDLVDTLDAQQIDKATFIGHSMGGKAVMALTALAPDRIDKLVAIDIAPVDYH    120
hagets      GMSYAAMAADLEALLDRLGIERAHLLGHSMGGKVAISVARLHPQRVASLIVADIAPQAYG    118
             *.* *** **   **   *::* ::******..:::: * *:*: .*:. **** *

3BF8_B      VRRHDEIFAAINAVSESDAQTRQQAAAIMRQHLNEEGVIQFLLKSFVD---GEWRFN--V    175
hagest      H-DHDAVFAGLRRVEQGQPGNRREADALLAEHVEERAVRLFLATNLERDDDGILRLRIGL    177
              ** :**.:. *.:.:  .*::* *:: :*::*..*  ** ..:    *  *:.  :

3BF8_B      PVLWDQYPHIV-GWEKIPAWDHPALFIPGGNSPYVSEQYRDDLLAQFPQARAHVIAGAGH    234
hagest      DEIEGDYDAIMQAPAGEGSYDGPVLVLRGDRSPYVTDAMLPRLHQVLPQAQLVTLENAGH    237
              : .:*  *: .     ::* *.*.: *..****::     *   :***:  .: .***

3BF8_B      WVHAEKPDAVLRAIRRYLNDH--       255
hagest      WLHAEQPEAFQAAVNDFLASLEA       260
            *:***:*:*.  *:. :* .
```

Figure 2.2 : Alignment of esterase enzyme and ybfF protein using CLUSTAL OMEGA.

```
  1 M  T  V  D  L  N  H  L  D  T  G  G  E  G  I  P  L  V  V  I
  1 ATGACCGTCGATCTCAACCATTTGGACACGGGAGGGGAGGGCATCCCCCTCGTCGTGATA
 21 H  G  L  L  G  S  A  D  N  W  R  S  H  V  K  Q  W  Q  Q  Q
 61 CACGGGCTGCTGGGCAGCGCCGACAACTGGCGCTCCCACGTCAAGCAGTGGCAGCAACAG
 41 C  R  V  V  A  V  D  L  R  N  H  G  R  S  P  H  A  D  G  M
121 TGCCGTGTCGTCGCCGTCGATCTGCGCAACCACGGTCGCTCGCCCCATGCCGACGGCATG
 61 S  Y  A  A  M  A  A  D  L  E  A  L  L  D  R  L  G  I  E  R
181 AGCTATGCTGCCATGGCCGCTGATTTGGAGGCGCTCCTCGATCGGCTGGGTATTGAGCGT
 81 A  H  L  L  G  H  S  M  G  G  K  V  A  I  S  V  A  R  L  H
241 GCCCATTTGCTGGGGCACTCCATGGGCGGCAAAGTCGCGATCAGCGTGGCGCGGCTCCAC
101 P  Q  R  V  A  S  L  I  V  A  D  I  A  P  Q  A  Y  G  H  D
301 CCGCAGCGGGTTGCCTCATTGATCGTCGCCGATATCGCTCCACAAGCGTACGGACACGAC
121 H  D  A  V  F  A  G  L  R  R  V  E  Q  G  Q  P  G  N  R  R
361 CATGACGCGGTATTCGCCGGCCTGCGACGGGTCGAGCAGGGGCAGCCCGGCAATCGCCGC
141 E  A  D  A  L  L  A  E  H  V  E  E  R  A  V  R  L  F  L  A
421 GAAGCCGATGCGCTGTTGGCAGAGCACGTGGAAGAACGCGCCGTTCGGCTGTTTCTCGCC
161 T  N  L  E  R  D  D  D  G  I  L  R  L  R  I  G  L  D  E  I
481 ACCAACCTGGAGCGCGATGATGATGGCATATTGCGGCTGCGAATCGGGCTCGATGAAATC
181 E  G  D  Y  D  A  I  M  Q  A  P  A  G  E  G  S  Y  D  G  P
541 GAGGGTGACTACGACGCTATCATGCAGGCGCCGGCAGGAGAGGGCAGCTATGACGGCCCG
201 V  L  V  L  R  G  D  R  S  P  Y  V  T  D  A  M  L  P  R  L
601 GTCTTGGTGCTACGCGGCGACCGCTCTCCCTATGTGACCGACGCCATGCTGCCAAGGCTT
221 H  Q  V  L  P  Q  A  Q  L  V  T  L  E  N  A  G  H  W  L  H
661 CACCAGGTGTTACCGCAAGCGCAACTGGTGACCTTGGAAAACGCCGGTCACTGGCTGCAT
241 A  E  Q  P  E  A  F  Q  A  A  V  N  D  F  L  A  S  L  E  A
721 GCCGAGCAGCCCGAGGCATTCCAGGCTGCCGTCAATGATTTCCTCGCCTCCCTCGAGGCG
261 *
781 TGA
```

Figure 2.3 : Amino acids sequence was predicted by Translate Tool of ExPASy server. Ser209Ala and Met88Ala amino acid residues which are further subjected to alanine scanning mutagenesis shown in yellow colour.

2.2.1.2 Plasmid isolation

5 mL of *E. coli* C43(DE3) culture was grown at 37 °C until an optical density of 1.5-4 at OD_{600}. Bacterial culture ought to be inoculated using a single colony from a newly streaked selective plate to an LB medium containing the appropriate selection antibiotic as kanamycin. The bacterial culture was collected by centrifugation at 6000 rpm in a tabletop microcentrifuge for 2 minutes at room temperature. Supernatant was discarded using a micropipette. Bacterial pellet was resuspended in 250 µL of the resuspension buffer by pipetting up and down until no cell clumps remain. 20 µL of RNase A was added to resuspended the mixture. 250 µL Lysis Buffer was added and mixed delicately by inverting the tube 6-7 times. It is important to avoid vortex because of prevent to shearing of genomic DNA. The mixture was incubated at room temperature for 3 minutes. 350 µL EcoSpin Binding Buffer was added and mixed completely by inverting the tube 6-7 times then centrifuging for 5 minutes at maximum speed at room temperature. EcoSpin Column was inserted into a Collection Tube and transferred the supernatant from the EcoSpin Columns. The column was centrifuged at maximum speed in a tabletop microcentrifuge for 30 seconds at room temperature. It was discarded the flowthrough and added 400 µL of EcoSpin Wash Buffer 1 to the EcoSpin Column. It was centrifuged at maximum speed in a tabletop microcentrifuge for 30 seconds at room temperature. It was discarded the flowthrough again and added 500 µL EcoSpin Wash Buffer 2 to the EcoSpin Column. It was centrifuged at maximum speed in a tabletop microcentrifuge for 30 seconds at room temperature. It was discarded the flowthrough and added 200 µL EcoSpin Wash Buffer 2 EcoSpin Column at maximum speed for 2 minutes to thoroughly remove any residual wash buffer. It was transferred the EcoSpin Column to a sterile 1.5 mL microcentrifuge tube. 40 µL of EcoSpin Elution Buffer was added to the centre of the EcoSpin Column membrane and incubated the column at room temperature for 3 minutes. It was centrifuged at maximum speed in a tabletop microcentrifuge for 30 seconds at room temperature. It was discarded the EcoSpin Column and stored the purified DNA plasmid at -20°C.

2.2.1.3 Agarose gel electroforesis

Isolated DNA was checked on a 1% agarose gel. To prepare the gel, 0.32 g of agarose was weighed and mixed with 40 mL of TAE buffer.

The agarose was boiled in the microwave until it was completely dissolved.After cooling to about 60 ºC, the gel was poured onto the tank in order to solidification and combs were placed. After the solidification, the comb was removed gently and the gel was transferred into the tank that included 1X TAE electrophoresis buffer. 4 µL of the sample was mixed with 1 µL of DNA dye (Amresco, #N472) and loaded into gel wells. Electrophoresis was done with TAE buffer at a constant voltage 120V electric field was applied for 30-45 minutes. After the front dye reached the end of the gel, DNA was visualized utilizing UV Transilluminator.

2.2.1.4 Competent cell preparation

E. coli C43 (DE3) strain was used as competent cells for the transformation process. *E. coli* C43 cell lines from -80°C glycerol stock were spread on LB agar plates and incubated at 37°C for 16 h. After picking one colony from the LB agar plates, it was incubated in 5 mL LB broth at 37°C for 16 hours with a shaker set to 200 rpm. After incubation, 1 mL pre-culture was inoculated into a 50 mL LB broth medium and incubated at 37 °C until the OD_{600} reached 0.8. Later, the culture waited on ice for 10 minutes and it was centrifuged at 4 °C at 5000 rpm for 10 minutes. After the supernatant was discarded, the pellet was resuspended gently by pipetting in 30 mL of 80 mM $MgCl_2$ + 20 mM $CaCl_2$ solution. Then, they were kept on ice for 5 mins. After that, it was centrifuged at 5000 rpm at +4 °C for 10 minutes and the supernatant was removed. The same solution was used to dissolve the pellets. 30 mL of 80 mM $MgCl_2$ + 20 mM $CaCl_2$, and it was incubated ice at 20 minutes and then centrifuged again at 5000 rpm at +4 °C for 10 minutes. Finally, glycerol stock was prepared by adding 1 mL of 100 mM $CaCl_2$+ %15 glycerol to pellets and the mixture was aliquoted as 50 µL competent cells. They were stored at -80 °C.

2.2.1.5 Glycerol stock

300 µL of growth culture and 100 µL of 85% glycerol were mixed for future usage.

2.2.1.6 Primer design for site-directed mutagenesis

The gene was composed of 783 bp, which corresponds to 260 amino acids with a molecular weight of 28.2 kDa. The deduced amino acid sequence best matched with the esterase from *Halomonas gudaonensis* with an identity of 91%. The gene sequence of the esterase is shown below.

>hAGEst_DNA

ATGACCGTCGATCTCAACCATTTGGACACGGGAGGGGAGGGCATCCCCC
TCGTCGTGATACACGGGCTGCTGGGCAGCGCCGACAACTGGCGCTCCCAC
GTCAAGCAGTGGCAGCAACAGTGCCGTGTCGTCGCCGTCGATCTGCGCA
ACCACGGTCGCTCGCCCCATGCCGACGGCATGAGCTATGCTGCCATGGCC
GCTGATTTGGAGGCGCTCCTCGATCGGCTGGGTATTGAGCGTGCCCATTT
GCTGGGGCACTCCATGGGCGGCAAAGTCGCGATCAGCGTGGCGCGGCTC
CACCCGCAGCGGGTTGCCTCATTGATCGTCGCCGATATCGCTCCACAAGC
GTACGGACACGACCATGACGCGGTATTCGCCGGCCTGCGACGGGTCGAG
CAGGGGCAGCCCGGCAATCGCCGCGAAGCCGATGCGCTGTTGGCAGAGC
ACGTGGAAGAACGCGCCGTTCGGCTGTTTCTCGCCACCAACCTGGAGCGC
GATGATGATGGCATATTGCGGCTGCGAATCGGGCTCGATGAAATCGAGG
GTGACTACGACGCTATCATGCAGGCGCCGGCAGGAGAGGGCAGCTATGA
CGGCCCGGTCTTGGTGCTACGCGGCGACCGCTCTCCCTATGTGACCGACG
CCATGCTGCCAAGGCTTCACCAGGTGTTACCGCAAGCGCAACTGGTGACC
TTGGAAAACGCCGGTCACTGGCTGCATGCCGAGCAGCCCGAGGCATTCC
AGGCTGCCGTCAATGATTTCCTCGCCTCCCTCGAGGCGTGA

Gene-specific primers were designed to constitute of Ser209Ala and Met88Ala mutations and the OligoAnalyzer Tool was used to estimate the GC content percentage and Tm (°C) of the primers. Delta G values, as well as the creation of heterodimers, homodimers, and hairpin configurations, are all explored. Table 2.6 and Tablo 2.7 show the sequences and properties of primers.

Primer Design for Ser209Ala;

- **T**CT=>**G**CT (Serin→Alanine)

Table 2.6 : Primers for alanine scanning to obtain Ser209Ala mutation.

Name (F/R)	Primer Sequence	Len	% GC	Tm	Ta *
Q5SDM4/18/2019F	CGGCGACCGC**g**CTCCCTATGTG	22	73	76 °C	73 °C
Q5SDM4/18/2019R	CGTAGCACCAAGACCGGG	18	67	69 °C	

Primer Design for Met88Ala;

- **AT**G=>**GC**G (Methionin →Alanine)

Table 2.7 : Primers for alanine scanning to obtain Met88Ala mutation.

Name (F/R)	Primer Sequence	Len	% GC	Tm	Ta *
Q5SDM4/18/2019F	GGGGCACTCC**g****c**GGGCGGCAAA	22	77	68 °C	69 °C
Q5SDM4/18/2019R	AGCAAATGGGCACGCTCA	18	56	68 °C	

2.2.1.7 Exponential amplification (PCR)

To create insertions, deletions, and substitutions in a wide range of plasmids, the kit employs the powerful Q5 Hot Start High-Fidelity DNA Polymerase and customized mutagenic primers. The following reagents given in Table 2.8 were assembled in a thin-walled PCR tube and mixed. Then the thermocycler was run according to the conditions given in Table 2.9.

Table 2.8 : Conditions of exponential amplification.

Solutions	Volume	Final Concentrations
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µL	1X
10 µM Forward Primer	1.25 µL	0.5 µM
10 µM Reverse Primer	1.25 µL	0.5 µM
Template DNA (1-25 ng/µL)	1 µL	1-25 ng
Nuclease-free water	to 25 µL	-

To determine the annealing temperature of mutagenic primers NEBaseChanger™ was used as the online NEB primer design software. $T_a=T_m+3$ rule has been applied and necessary optimization has been made.

Table 2.9 : Thermocycling Conditions for a Routine PCR.

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 seconds	1
Denaturation	98 °C	10 seconds	25
Annealing	50-72 °C*	10-30 seconds	
Extension	72 °C	20-30 seconds/kb	
Final Extension	72 °C	2 minutes	1
Hold	4 °C	∞	1

2.2.1.8 Kinase, Ligase & DpnI (KLD) treatment

Following PCR, a KLD reaction was performed to remove non-mutant PCR products using the components listed in Table 2.10.

The following reagents are mixed by sensitively pipetting up and down. The mixture was incubated for 5 minutes at room temperature.

Table 2.10 : Components of KLD Treatment.

Solutions	Volume	Final Concentrations
10X KLD Enzyme Mix	1 µL	1X
2X KLD Reaction Buffer	5 µL	1X
PCR Product	1 µL	
Nuclease-free Water	to 10 µL	

2.2.1.9 Transformation

Competent *E. coli* cells were thawed on ice. In a tube, 5 µL of KLD mix from Step II was added to 50 µL of chemically competent cells and incubated on ice for 30 minutes. The tube was carefully shaken 4-5 times to mix without vortexing. The mixture waited on the ice for 30 minutes. For heat shock mixture was placed at 42 °C for 30 seconds and again on ice for 5 minutes. 950 µL of room temperature SOC was added into the mixture by pipette gently. It was incubated at 37 °C for 60 minutes with shaking at 250 rpm. Transformed mutants were spread on LB agar plates containing 100 µg / mL of kanamycin and incubated for 16 hours at 37 °C.

2.2.1.10 Plasmid isolation and sequencing

Agarose gel prepared as given at Session 2.2.1.2 ‘‘Agarose gel electrophoresis’’ and samples from last plasmid isolation of 7 different colonies loaded into the wells. Gel electrophoresis ran at 100 V for 30 minutes. The results were observed under UV light and captured. A few colonies were selected for sequencing to confirm that the mutation occurred. The sequence analysis of selecting isolated plasmid DNA was performed by IONTEK Company (Istanbul, Turkey) with T7 terminator and T7 promoter primers.

2.2.1.11 Protein expression

Stock cultures carrying the mutant plasmid were inoculated into 10 mL of LB medium with kanamycin. This starter culture was inoculated into 200 mL of 1 LB medium after rotational shaking for 16 hours at 150 rpm at 37 °C. Cells were induced with 0.01 mM of Isopropyl β-d-1-thiogalactopyranoside (IPTG) and incubated at 37 °C until OD_{600} reached 0.8 then incubated for 6 hours at 30 °C.

Cells were centrifuged at 5000 rpm for 20 minutes after protein expression in 1 litre of LB media. Cell pellets were stored at -20 °C for future experiments.

2.2.1.12 His-Tag purification

Affinity tags have been frequently used in the purification of recombinant proteins. In this research, an N-terminal 6 histidine tag was applied to purify the protein using the nickel-histidine affinity principle. The proteins were bound to the Ni-NTA matrix before eluting with a high amount of imidazole. First, the cell pellet from a 1L culture was resuspended in 15 mL of ice-cold lysis buffer. The suspension was then incubated

on ice for 30 minutes with lysozyme added. Sonoplus, Bancelin probe was used to sonicate the cell suspension 10 times for 20 seconds with 10 second time breaks. The debris was centrifuged for 14 minutes at 12000G at 4 °C. 1.2 mL Ni-NTA agarose was added to the lysate and incubated for 1 hour at 4 °C. After centrifuging the resin-bound proteins at 3000G for 4 minutes at 4 °C, the resin was washed three times with 4 mL of wash buffer. Finally, proteins were eluted 4 times with 1 mL of elution buffer. The collected samples of each step were controlled on SDS-PAGE.

2.2.1.13 SDS-PAGE

Purified proteins or crude lysates or purified samples were electrophoresed on a polyacrylamide gel. The stacking gel was prepared at a concentration of 5%, while the resolving gel was prepared at a concentration of 12%. Firstly, the glass plates are washed with alcohol and distilled water and then dried with blotting paper. Glass plates are carefully placed in the casting frames to prevent leakage. According to Figure 2.11, the resolving gel is prepared and put between glass plates, then isopropyl alcohol is poured over the gel to smooth it out and remove the unwanted bubbles. The isopropyl alcohol on the separation gel was removed and washed with distilled water after it had polymerized. The prepared stacking gel was poured over the resolving gel and the comb was positioned after drying the top of the gel and between the glass plates with paper. The comb was removed after polymerization and the casting frames were inserted in the electrophoresis tank. The tank was filled with 1X running buffer in the appropriate amount based on the number of used casting frames. 20 µL of samples were mixed with 5 µL of sample application buffer and heated at 95 ºC using with heater for 15 min. Without heat denaturation, 4 µl of restained Protein Ladder was loaded into the one well as a molecular weight marker.

The cables were properly connected according to the + and - current directions. The electrophoresis was started at 80 V, and after the proteins were in the resolving gel, a constant voltage of 120 V was applied until the front dye reached the end of the gel. When the procedure was completed, the glasses were opened by opening the frames from the electrophoresis tank and the gel is evacuated. Proteins were stained with Coomassie Brilliant Blue R-250 after electrophoresis on a shaker with 120 rpm overnight. Then gel was incubated with destaining buffer to remove the excess stain for roughly 3 hours.

Table 2.11 : Components of SDS-PAGE.

Chemicals	Running gel	Stacking gel
dH_2O	2000 µL	1400 µL
Acrylamide	1500 µL	250 µL
SDS solution (10%)	50 µL	20 µL
APS solution	50 µL	20 µL
TEMED	5 µL	5 µL
TRIS (pH 6.8)	-	250 µL
TRIS (pH 8.8)	1800 µL	-

To prepare the chemical composition of 10% (w/v) SDS solution, 10 g SDS was dissolved in 200 mL with dH_2O.

To prepare the chemical composition of 10% (w/v) APS solution, 10 g SDS were dissolved in 1 mL with dH_2O freshly.

2.2.1.14 Ultrafiltration

After the protein was purified, it was concentrated by using ultrafiltration. Firstly, purified proteins were collected in an ultrafiltration tube with a membrane that contains a 10 kDa molecular cut-off.

As elution buffer, 10% NaCl with pH:8 was added to a 50 mL falcon that contains 50 mL Tris-HCl. 15 mL elution buffer was added to the ultrafiltration tube and proteins were solved in it. After the mixture was centrifuged at 5000G for 20 minutes, this process addition of elution buffer and centrifuge of the mixture cycle was repeated until 1.5 mL ultra-filtered protein was obtained and its concentration was measured by Bradford analysis.

2.2.1.15 Enzyme activity

Winkler and Stuckman (1979) method was used with some modifications to kinetic analysis. Protein activity determines by measuring the release of p-nitrophenoxide from pNP-hexanoate as a result of hydrolysis of paranitrophenyl esters by esterases. 1 mM substrate and 1 µM enzyme concentrations were determined.

Firstly, 10 mg of Gum Arabic and 40 µL of Triton X-100 were added to 9 mL Glycine-NaOH (50 mM) with pH.9 and dissolved in it by heating. Secondly, pNP-hexanoate substrate mixture (11.11 mM) was produced by mixing 1 mM isopropanol and pNP-hexanoate for forming 1 mM final concentration in the reaction. Then, prepared 2 solutions mixed together and become substrate solution mixture.

After the substrate was completely dissolved, a 180 µL substrate solution mixture and 20 µL enzyme were mixed together and loaded into wells of the microplate in triplets. After the mixture was incubated at 30 ºC for 20 minutes, microplates were directly put into ice and then absorbance values were determined at 410 nm by using a microplate reader.This measurement is used to determine activity (U) which is the quantity of the enzyme that produces 1 µmol of the product per minute. The Beers Law used to calculate the activity;

$$\text{Activity (U/L)} = [(\Delta Abs/t) / (\varepsilon * d)] * 106 * V_t/V_s \quad (2.1)$$

According to equation 2.1, ΔAbs is the average absorbance value. t is used for the time (minute). Ɛ is the extinction coefficient (M^{-1} cm^{-1}), d is path length (cm). V_t is the total reaction volume (µL) and V_s is the volume of the enzyme (µL).

2.2.2 Directed evolution by Error-Prone PCR

2.2.2.1 Normal PCR and Error-Prone PCR

The gene was amplified from plasmid DNA by Normal-PCR according to the Figure 2.13. and Error-Prone PCR using a pair of degenerate oligonucleotide primers in Figure 2.12. While designing the primers, cleavage sequences for the restriction endonucleases *Hind*III and *EcoR*I were inserted. As a result, after amplification, the DNA fragment carried both restriction enzyme sites.

Table 2.12 : Primers for Normal and Error-Prone PCR.

Name	Primer Sequence	Len	% GC	Tm	Ta *
ABHF_F	GACCGAATTCATGACCGTCGATCTCAACC	29	52	63 °C	63 °C
ABHF_R	ACTGAAGCTTTCACGCCTCGAGGGAG	26	58	63 °C	

Table 2. 13 : Normal PCR Protocol.

Stock Solutions	Final Concentration
10×PCR buffer	1×
dNTPs	0.2 mM
Forward Primer	0.5 µM
Reverse Primer	0.5 µM
template DNA	10 ng
$MgCl_2$	1.5 mM
Taq DNA polymerase	U
Nanopure water	to 25 µL

Enzymes that are widely studied in the literature and commonly used EP-PCR conditions were examined. The disproportionate use of nucleotides was applied by decreasing dATP and dGTP concentrations and increasing dCTP and dTTP concentrations. Under normal conditions, 1.5 mM Mgl_2 is used in PCR. In order to increase the mutation rate for the EP-PCR, it is necessary to increase the $MgCl_2$ concentration. Accordingly, optimum EP-PCR reactions were set up and optimized using concentrations of 3 mM, 5 mM and 7 mM $MgCl_2$.

$MnCl_2$ in PCR also causes random mutations during the gene amplification reaction. While $MnCl_2$ is not added under normal PCR conditions, it is necessary to ensure mutagenic conditions. When it is found in large amounts of PCR, it inhibits the work of the enzyme. Therefore, the maximum concentration at which inhibition does not occur was determined by optimizing concentrations of components. For $MnCl_2$ optimization of EP-PCR, 0.15 mM,0.2 mM,0.25 mM,0.35 mM,0.50 mM $MnCl_2$ were used.

As a result of a series of optimizations, the most suitable results were determined as in the Table 2.14 and Table 2.15.

Table 2.14 : EP-PCR Protocol.

Stock Solutions	Final Concentration
10×PCR buffer	1×
dATP	0.2 mM
dGTP	0.2 mM
dTTP	1 mM
dCTP	1 mM
Forward Primer	0.5 µM
Reverse Primer	0.5 µM
template DNA	10-100 ng
Taq DNA polymerase	U
$MgCl_2$	5 mM
$MnCl_2$	0.25 mM
Nanopure water	to 25 µL

Table 2.15 : Thermocycling conditions N-PCR and EP-PCR.

Step	Temperature	Time	Cycles
Initial Denaturation	94 °C	3 min.	1
Denaturation	94 °C	30 sec.	35
Annealing	60 °C	30 sec.	
Extension	72 °C	1 min.	
Final Extension	72 °C	10 min.	1
Final Hold	+4 °C	∞	1

2.2.2.2 DNA recovery from agarose gel

The procedure in the following protocol was described for the extraction of DNA products from 100 mg gel. If the weight of the gel slice is larger than 100 mg, the amount of Binding Buffer is increased proportionally. PCR reaction mixture was loaded on a 0.8-2% agarose gel and the samples were run in a 1x TAE running buffer.

Electrophoresis was carried out until DNA band of interest was separated from adjacent contaminating fragments. The desired DNA band was cut from gel using an ethanol-cleaned scalpel or razor blade. It was determined the mass of a sterile 1.5 mL microcentrifuge tube and was placed excised agarose gel slice in it.It was measured the gel mass by re-weighting the tube with the excised gel slice. 300 µL EcoSpin Binding Buffer was added to each 100 mg agarose gel slice in the microcentrifuge tube. To dissolve agarose gel slice the suspension was incubated for 10 minutes at 55 °C. The tube was vortexed briefly every 2-3 minutes throughout incubation in order to release the DNA.

After the agarose gel slice was completely dissolved, added 150 µL isopropanol for every 100 mg agarose gel slice to the mixture and mix well. EcoSpin Column was inserted into a Collection Tube and transferred all of the samples. They were centrifuged at maximum speed in a tabletop microcentrifuge for 30 seconds at room temperature. The flowthrough was discarded and added 700 µL EcoSpin Wash Buffer to the solution in EcoSpin Column.

Then, they were centrifuged at maximum speed in a tabletop microcentrifuge for 30 seconds at room temperature. Again the flowthrough was discarded and the empty EcoSpin Column was centrifuged at maximum speed for an additional 2 minutes to completely remove any residual wash buffer. EcoSpin Column was transferred to a clean 1.5 mL microcentrifuge tube. 30-50 µL Elution Buffer was added to the centre of the EcoSpin Column membrane and was incubated in the column at room temperature for 3 minutes. It was centrifuged at maximum speed in a tabletop microcentrifuge for 30 seconds at room temperature. EcoSpin Column was discarded and stored the purified DNA at -20 °C.

Determination of purity were calculated by measuring the ratio of absorbance with at 260 nm and 280 nm Nanodrops equipments. Also, the qualities of samples were assessed by calculating the ratio of A260/A280.

2.2.2.3 Digestion of vector and EP-PCR product

Both pET-28a(+) vector and PCR product were had *Hind*III and *EcoR*I restriction enzyme cleavage sites. In this step, each PCR product and pET-28a (+) vector were digested using 10 U/µL *Hind*III and 10 U/µL *EcoR*I enzymes, to form sticky ends. Digestion of N-PCR and EP-PCR product components were shown in Table 2.16. Also, the digestion of the pET-28a(+) vector was shown in Table 2.17.

At 37 °C, digestion reactions were incubated for 3 hours. The reaction tubes were placed in a heat block at 80 °C for 20 minutes to stop the activity of the enzyme and finish the reactions.

Table 2.16 : Double digestion reaction component for N-PCR and EP-PCR products.

Digestion of N-PCR Products		**Digestion of EP-PCR Products**	
Stock Solutions	Final Volume	Stock Solutions	Final Volume
N-PCR Product	38 µL	EP-PCR Product	17 µL
Buffer R	4 µL	Buffer R	4 µL
*EcoR*I	1 µL	*EcoR*I	1 µL
*Hind*III	1 µL	*Hind*III	1 µL
dH_2O	6 µL	dH_2O	7 µL
Total Volume	50 µL	Total Volume	30 µL

Table 2.17 : Digestion reaction component for pET-28a(+) vector.

Digestion of pET-28a(+) vector	
Stock Solutions	Final Volume
pET-28a(+)	25 µL
Buffer R	3 µL
*EcoR*I	1 µL
*Hind*III	1 µL
dH_2O	20 µL
Total Volume	50 µL

2.2.2.4 Ligation of pET-28a(+) vector and EP-PCR product

Before setting up the ligation reaction itself, it is important to determine the amount of cut insert and vector to use for the ligation reaction. The appropriate insert volume was adjusted according to the Ligation calculator (NEB) online web tool. The ligation reaction was conducted at a ratio of 1:5 and 1:7 (vector/insert). After adding the components, the reaction tubes were incubated at 15 °C for 16 hours. The tube was incubated at 60 °C for 15 minutes to end the reaction. The components of the ligation reactions are given in the Table 2.18.

Table 2.18 : Ligation reaction components and volumes for 5:1 and 7:1 ratios.

Ligation 3:1 (insert:vector)		**Ligation 5:1 (insert:vector)**	
Stock Solutions	Volume	Stock Solutions	Volume
pET-28a(+)	3.5 µL (~100 ng)	pET-28a(+)	8.8 µL (~100 ng)
Insert	2.88 µL (~44 ng)	Insert	4.3 µL (~44 ng)
T4 DNA Ligase	2 µL	T4 DNA Ligase	2 µL
Buffer 10X	1 µL	Buffer 10X	1 µL
T4 Ligase	1 µL	T4 Ligase	1 µL
dH_2O	10.62 µL	dH_2O	3.9 µL
Total Volume	20 µL	Total Volume	20 µL

2.2.2.5 Transformation

Previously prepared frozen *E. coli* C43 (DE3) competent cells were removed from -80 °C and waited on ice until thawed completely in an ice bath for 10-15 minutes. 5 µL of ligation product was added into 200 µL of *E. coli* C43 (DE3) competent cells and gently was mixed. Cells were held on for 30 min on ice, heat-shocked at exactly 42 °C for 45 seconds and followed by incubation ice for 3 min.

Then 200 µL of LB medium was added into each tube. Tubes were incubated at 37 °C for 1 hour on the horizontal shaker at 200 rpm. After incubation, tubes were centrifuged at 10.000G for 5 minutes. After supernatants were removed, the pellet was dissolved softly by addition of 200 µL LB medium. 100 µL of each transformed cell were spread on LB agar plates containing 100 µg/mL of kanamycin and incubated for 16 hours at 37 °C.

Negative controls without any plasmids will not be able to grow on antibiotic media, providing evidence of no contamination. Each of the colonies was transferred to microplate wells containing 1 mL LB medium with 40 µg/mL of kanamycin and incubated at 37 °C on a shaker under 200 rpm for overnight. Cells growing on the microplate were precipitated by centrifuge and added the appropriate amount of glycerol for future steps. Each of the transformants was stored as glycerol stock at -80 °C.

2.2.2.6 Protein expression

Stock cultures carrying the mutant plasmid were inoculated into 6 mL of LB medium with kanamycin in falcon tubes. The cells were induced with 0.01 mM of isopropyl β-d-1-thiogalactopyranoside (IPTG) and incubated at 37 °C until OD_{600} reached 0.8 then incubated for 6 hours at 30 °C.

The cells were centrifuged at 5000 rpm for 20 minutes after protein expression. Cell pellets were stored at -20 °C for future experiments usage.

2.2.2.7 Protein extraction

Expressed proteins were extracted by using BugBuster protein extraction reagent. 700 µl BugBuster protein extraction reagent was added to the cell pellet after IPTG induction. Mixtures were stirred on a shaker at room temperature at 120 rpm for 20 minutes. Extracted proteins were centrifuged at 4000 rpm during 30 min at 4 °C. Supernatants were transferred to sterile 1.5 µL Eppendorf for using measurement of protein concentration and enzyme activity.

2.2.2.8 Bradford assay

The Bradford assay was performed to measure the protein concentration of the enzyme sample. First, 5 µL of Tris-HCl buffer and 195 µL 1X Bradford Reagent were mixed into 2 repeating 96-well plates. Seven Bovine Serum Albumin (BSA) standard solutions with concentrations ranging from 0.125 to 2 mg / mL were used. 5 µL was taken from each standard solution and put on the wells with 2 repetitive. Then 195 µL of Bradford reagent was added to each well containing standard solution. Also, 5 µL of protein samples with BugBuster Reagent and 195 µL of Bradford reagent was added on to wells with 2 repetitive like the others.

Finally, the ODs of standards and unknown proteins were measured at 595 nm with a spectrophotometer and with the absorbance value the concentration of unknown protein was measured.

2.2.2.9 Protein activity assay

For kinetic analysis, the method described by Winkler and Stuckman (1979) was used with some modifications. 90 mL of 50 mM TRIS-HCl pH 7.0 including 0.1 g of gum Arabic and 0.4 mL of Triton X-100 was mixed with 30 mg of pNP-hexanoate which was dissolved in 10 mL of isopropanol. After the substrate was completely dissolved, 150 µL of substrate solution was mixed with 50 µL crude enzyme solution and the absorbance of released pNP was measured at 410 nm at the end of 30 min incubation.

One unit of activity was represented as the amount of enzyme that converts 1 micromole of the substrate to the product in one minute.

2.2.2.10 SDS-PAGE

SDS gel electrophoresis was performed to determine the amount of the extracted proteins before further analysis according to session 2.1.1.13.

2.2.2.11 Control cleavage and sequencing

As explained previously, plasmids were isolated from transformed cells after they had grown in LB media with kanamycin (40 mg/ul).

After checking of plasmid isolation on agarose gel electrophoresis, samples were prepared to control cleavage for four samples. *EcoR*I-HF digestion enzyme was used to determine true gene cleavage. Components of control cleavage are listed in Table 2.19. Components were mixed, the mixture was incubated for 30 minutes at 37 °C.

Table 2.19 : Control cleavage protocol.

Solutions	Volume
1X FastDigest Buffer	2 µL
Plasmid	2 µL
Fast Digest *EcoR*I	1 µL
dH_2O	5 µL
TOTAL	10 µL

Following that, plasmid DNAs were examined on a 1% agarose gel. It is known that the pET-28a(+) vector containing the insert DNA is located higher up in the gel than the plasmid without the insert DNA.

According to results of control cleavage selected mutant plasmids were sequenced with universal T7 Promoter (5'-TAATACGACTCACTATAGGG-3') and T7 Terminator (5'-GCTAGTTATTGCTCAGCGG-3') primers. The sequence analysis of isolated plasmid DNA was performed by Genometri Company.

After sequencing results were obtained, reference organism similarity was checked in the BLASTX tool on the NCBI website. Also, the CLUSTAL OMEGA was used for alignment analysis between reference sequence and plasmid sequencing results.

3. RESULTS AND DISCUSSIONS

3.1 Site-Directed Mutagenesis by Alanine Scanning

3.1.1 Control of template DNA

The culture of the *E. coli* C43 cell containing the pET28-a(+) plasmid (5369 bp) carrying the esterase gene (783 bp) was previously stored at -80 °C in 85% glycerol. The plasmid DNA (6152 bp) from stock culture was isolated and was visualized on agarose gel by electrophoresis using a 1000 bp level in the DNA ladder (M). (Figure 3.1).

The bands compared with the markers, the isolated plasmid is approximately 6000 bp as expected because the pET-28a(+) vector contains 5369 bp and the esterase gene contains 783 bp.

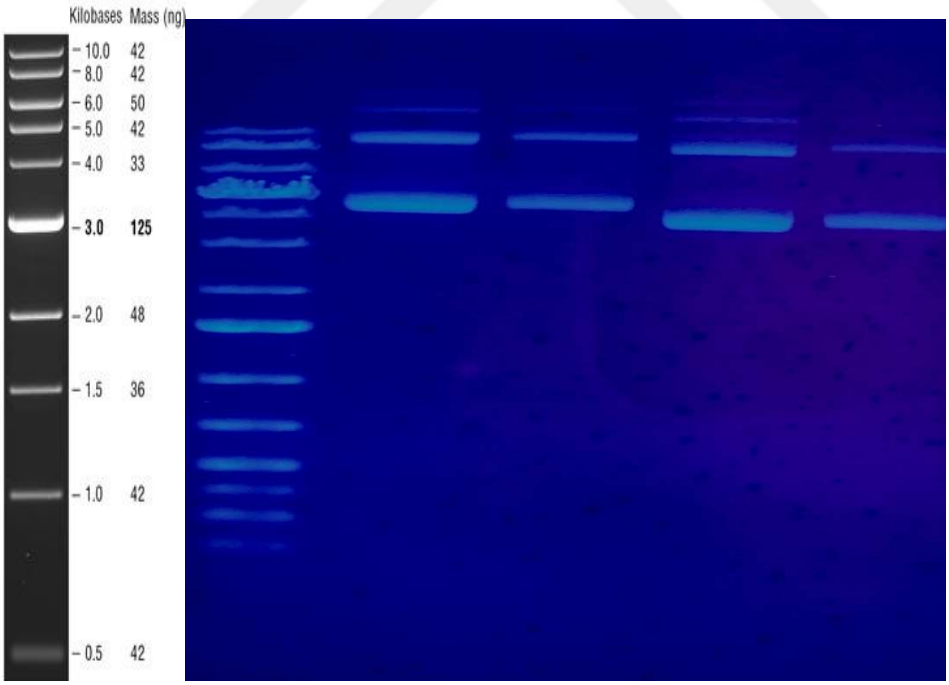

Figure 3.1 : The results of agarose gel (1%) electrophoresis of pET-28(+) vector plasmids with and without esterase gene. Line 1: 1kb Gene Ruller Marker, Line 2,3: haGEst plasmids; Line 4,5: empty pET-28a(+) plasmids.

3.1.2 Site-directed mutagenesis amplification

A PCR products of site-directed mutagenesis method were controlled by agarose gel electrophoresis in Figure 3.2.

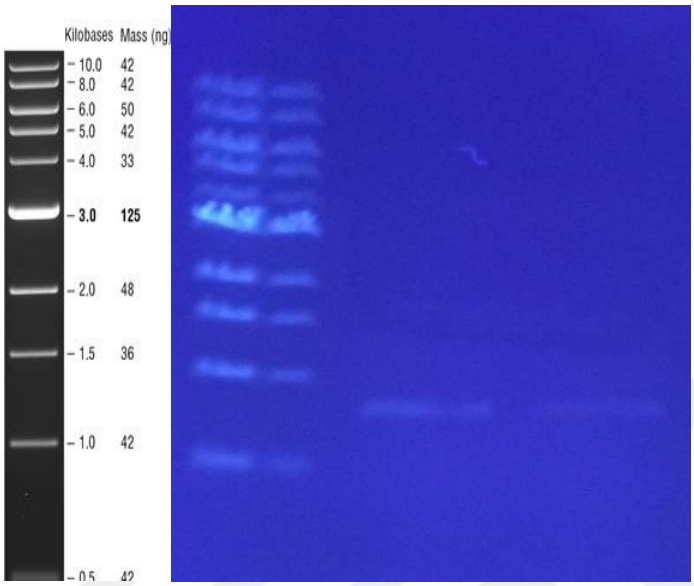

Figure 3.2 : Agarose gel (1%) electrophoresis result of PCR products. Lane 1: 1kb Gene Ruller Marker, Line 2: PCR product of Ser209Ala; Line 3: PCR product of Met88Ala.

3.1.3 Transformation and construction of libraries

Transformants obtained for the Met88Ala and Ser209Ala mutations are given in Figure 3.3 and Figure 3.4.

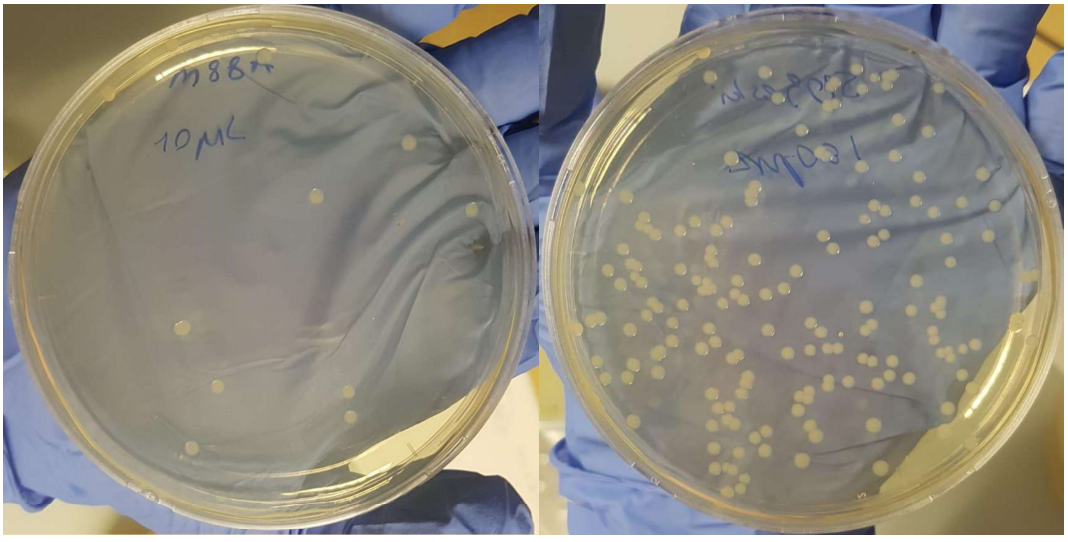

Figure 3.3 : Kanamycin screening plates containing colonies after transformation. The colonies of *E. Coli* C43 competent cells containing Met88Ala plasmid.

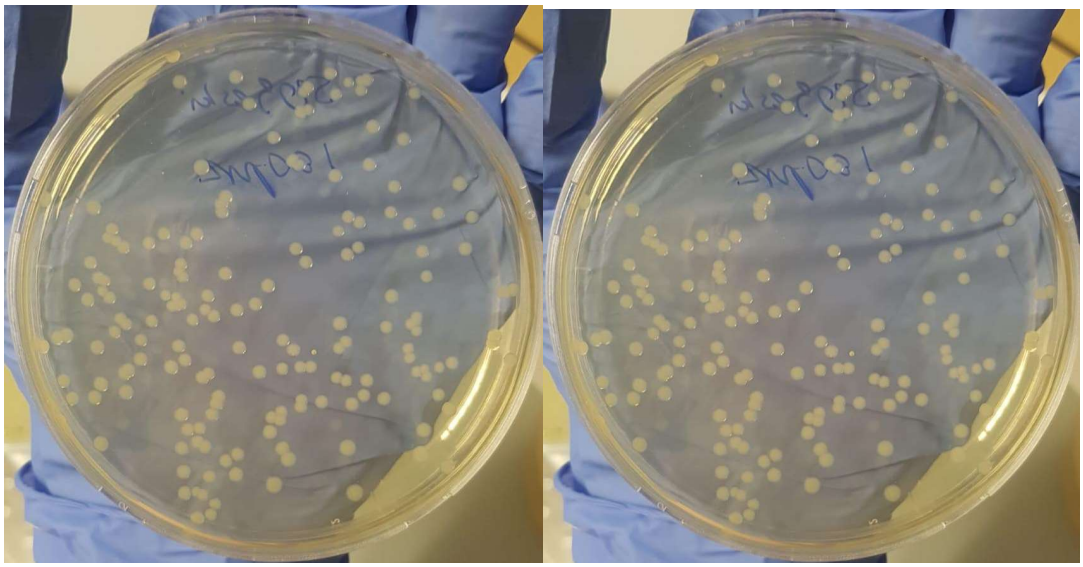

Figure 3.4 : Kanamycin screening plates containing colonies after transformation. The colonies of *E. Coli* C43 competent cells containing Ser209Ala plasmid.

3.1.4 Plasmid isolation

Colonies were selected from agar plates and placed in different falcon tubes containing LB medium with kanamycin. After the overnight growth at 37 ºC on shaker cells were harvested from each tubes. The plasmid DNAs from cultured transformants were isolated and analyzed on the agarose gel electrophoresis. According to the result of agarose gel electrophoresis shown in Figure 3.5; the 2nd and 3rd plasmids for Ser209Ala, 5th and 7th plasmids for Met88Ala were observed as the brightest ones and were subjected to sequence analysis.

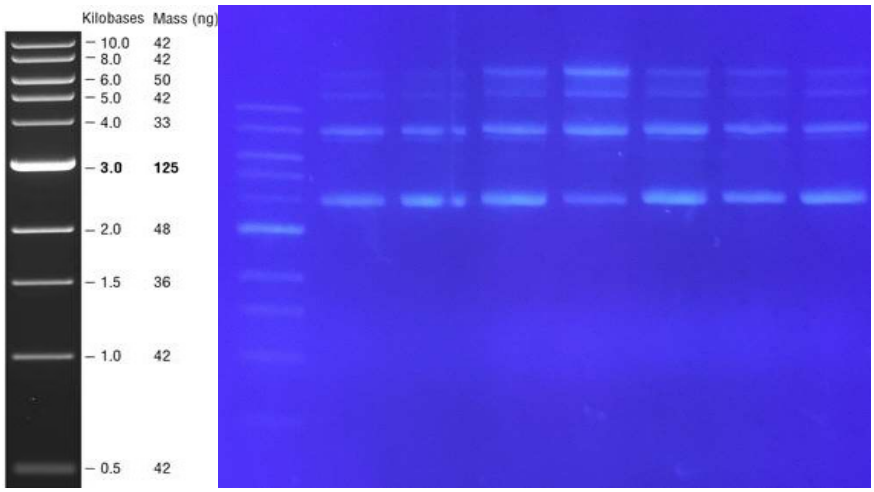

Figure 3.5 : Agarose gel (1%) electrophoresis result of isolated pET-28(+) vector including esterase gene pET-28(+). Line 1: 1kb Gene Ruller Marker, Line 2,3,4: pET-28a(+)plasmid containing Ser209Ala mutation of haGEst enzyme ; Line 6,7,8: pET-28a(+)plasmid containing Met88Ala mutation of haGEst enzyme.

3.1.5 Sequence analysis and amino acid changes

```
M88A        ATGACCGTCGATCTCAACCATTTGGACACGGGAGGGGAGGGCATCCCCCTCGTCGTGATA      60
Hagest      ATGACCGTCGATCTCAACCATTTGGACACGGGAGGGGAGGGCATCCCCCTCGTCGTGATA      60
            ************************************************************

M88A        CACGGGCTGCTGGGCAGCGCCGACAACTGGCGCTCCCACGTCAAGCAGTGGCAGCAACAG      120
Hagest      CACGGGCTGCTGGGCAGCGCCGACAACTGGCGCTCCCACGTCAAGCAGTGGCAGCAACAG      120
            ************************************************************

M88A        TGCCGTGTCGTCGCCGTCGATCTGCGCAACCACGGTCGCTCGCCCCATGCCGACGGCATG      180
Hagest      TGCCGTGTCGTCGCCGTCGATCTGCGCAACCACGGTCGCTCGCCCCATGCCGACGGCATG      180
            ************************************************************

M88A        AGCTATGCTGCCATGGCCGCTGATTTGGAGGCGCTCCTCGATCGGCTGGGTATTGAGCGT      240
Hagest      AGCTATGCTGCCATGGCCGCTGATTTGGAGGCGCTCCTCGATCGGCTGGGTATTGAGCGT      240
            ************************************************************

M88A        GCCCATTTGCTGGGGCACTCCGCGGGCGGCAAAGTCGCGATCAGCGTGGCGCGGCTCCAC      300
Hagest      GCCCATTTGCTGGGGCACTCCATGGGCGGCAAAGTCGCGATCAGCGTGGCGCGGCTCCAC      300
            ********************* ************************************

M88A        CCGCAGCGGGTTGCCTCATTGATCGTCGCCGATATCGCTCCACAAGCGTACGGACACGAC      360
Hagest      CCGCAGCGGGTTGCCTCATTGATCGTCGCCGATATCGCTCCACAAGCGTACGGACACGAC      360
            ************************************************************

M88A        CATGACGCGGTATTCGCCGGCCTGCGACGGGTCGAGCAGGGGCAGCCCGGCAATCGCCGC      420
Hagest      CATGACGCGGTATTCGCCGGCCTGCGACGGGTCGAGCAGGGGCAGCCCGGCAATCGCCGC      420
            ************************************************************

M88A        GAAGCCGATGCGCTGTTGGCAGAGCACGTGGAAGAACGCGCCGTTCGGCTGTTTCTCGCC      480
Hagest      GAAGCCGATGCGCTGTTGGCAGAGCACGTGGAAGAACGCGCCGTTCGGCTGTTTCTCGCC      480
            ************************************************************

M88A        ACCAACCTGGAGCGCGATGATGATGGCATATTGCGGCTGCGAATCGGGCTCGATGAAATC      540
Hagest      ACCAACCTGGAGCGCGATGATGATGGCATATTGCGGCTGCGAATCGGGCTCGATGAAATC      540
            ************************************************************

M88A        GAGGGTGACTACGACGCTATCATGCAGGCGCCGGCAGGAGAGGGCAGCTATGACGGCCCG      600
Hagest      GAGGGTGACTACGACGCTATCATGCAGGCGCCGGCAGGAGAGGGCAGCTATGACGGCCCG      600
            ************************************************************

M88A        GTCTTGGTGCTACGCGGCGACCGCTCTCCCTATGTGACCGACGCCATGCTGCCAAGGCTT      660
Hagest      GTCTTGGTGCTACGCGGCGACCGCTCTCCCTATGTGACCGACGCCATGCTGCCAAGGCTT      660
            ************************************************************

M88A        CACCAGGTGTTACCGCAAGCGCAACTGGTGACCTTGGAAAACGCCGGTCACTGGCTGCAT      720
Hagest      CACCAGGTGTTACCGCAAGCGCAACTGGTGACCTTGGAAAACGCCGGTCACTGGCTGCAT      720
            ************************************************************

M88A        GCCGAGCAGCCCGAGGCATTCCAGGCTGCCGTCAATGATTTCCTCGCCTCCCTCGAGGCG      780
Hagest      GCCGAGCAGCCCGAGGCATTCCAGGCTGCCGTCAATGATTTCCTCGCCTCCCTCGAGGCG      780
            ************************************************************

M88A        TGAAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAAC      840
Hagest      TGA-------------------------------------------------------      783
            ***
```

Figure 3.6 : Alignment of Met88Ala mutant and wild type esterase enzyme.

Figure 3.7 : Alignment of Met88Ala mutant and wild type esterase enzyme.

Following the sequencing of plasmids, the CLUSTAL OMEGA tool was used to align and compare the overlapping between the template sequence and the mutant gene sequencing results. Figure 3.6 and Figure 3.7 show that the serine at position 209th and the methionine at position 88th have been converted to alanine respectively.

3.1.6 Control of protein purification for Ser209Ala and Met88Ala mutants

SDS-PAGE was used to check the purity of the purified samples. According to the esterase sequence information, band formation around 28 kDa was expected for eluted proteins.

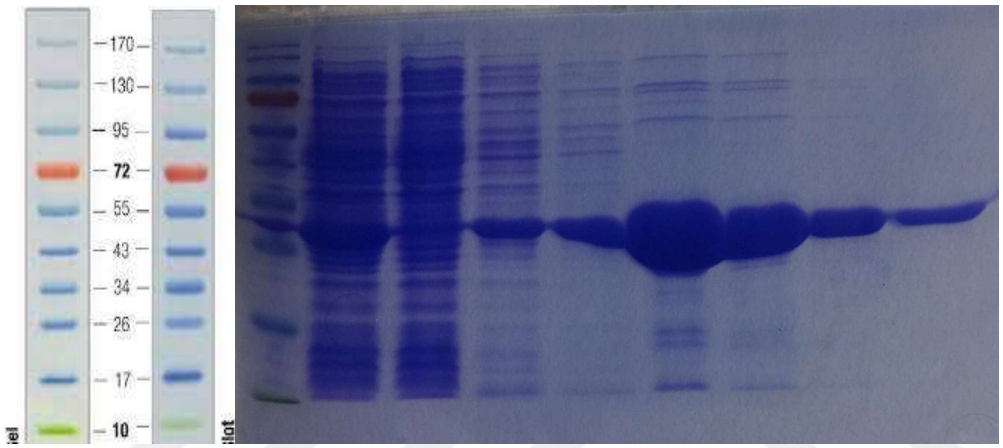

Figure 3.8 : SDS-PAGE analysis of wild type obtained by His-tag purification. 1 mM IPTG induction was performed at 30 °C for 6 hours. Lane 1: Molecular weight marker; Lane 2: clear lysate; Lane 3: unbound (flowthrough) protein fraction; Lane 4,5: 10 mM and 20 mM imidazole containing washing samples respectively; Lane 6,7,8,9 containing elution buffer.

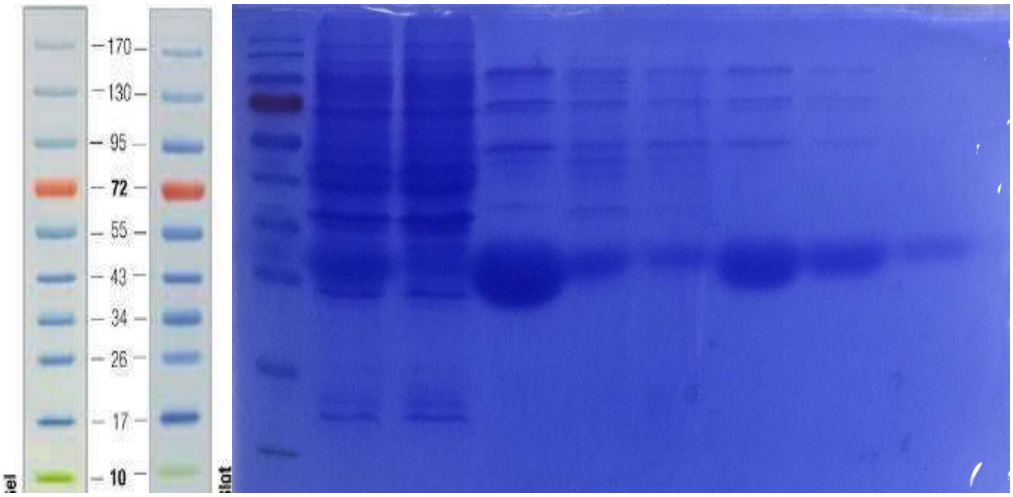

Figure 3.9 : SDS-PAGE analysis of Ser209Ala obtained by His-tag purification. 1 mM IPTG induction was performed at 30 °C for 6 hours. Lane 1: Molecular weight marker; Lane 2: clear lysate; Lane 3: unbound (flowthrough) protein fraction; Lane 4,5,6: 10 mM and 20 mM imidazole containing washing samples respectively; Lane 7,8,9 containing elution buffer.

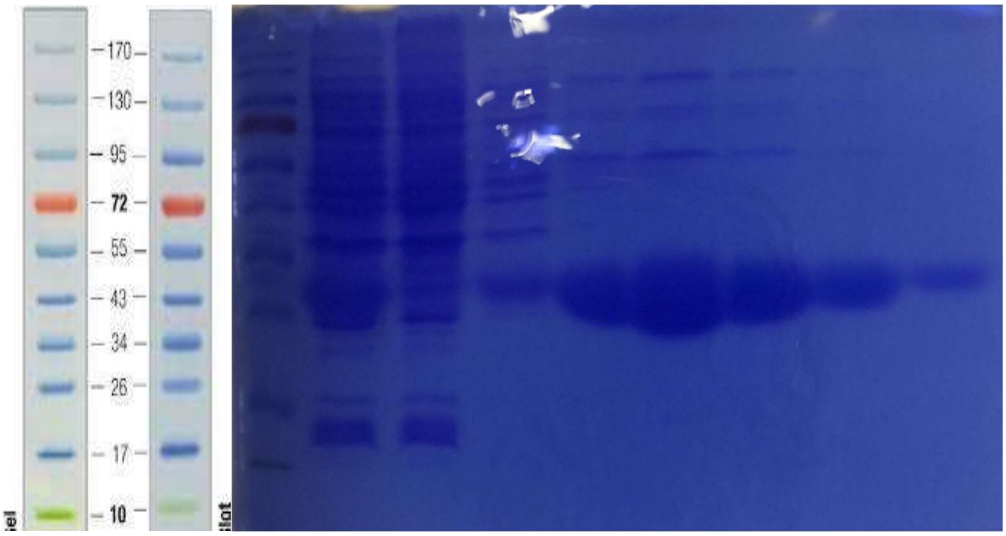

Figure 3.10 : SDS-PAGE analysis of Met88Ala obtained by His-tag purification. 1 mM IPTG induction was performed at 18 °C for 18 hours. Lane 1: Molecular weight marker; Lane 2: clear lysate; Lane 3: unbound (flowthrough) protein fraction; Lane 4,5: 10 mM and 20 mM imidazole containing washing samples respectively; Lane 6,7,8,9 containing elution buffer.

The bands around 25-35 kDa represent the molecular weight of wild type, Ser209Ala mutant and Met88Ala mutant esterases respectively which are given in Figure 3.8, Figure 3.9 and Figure 3.10. Since the literature suggests that the molecular weight of esterases is in the range of 27-54 kDa, the results are as expected. Additionally, the purification of the samples was confirmed using this method.

3.1.7 Bradford assay

Table 3.1 : Determination of concentration to Wild Type, Met88Ala and Ser209Ala.

Dilution	Abs 1	Abs 2	Abs Avg	Concentrations
WT(1/1)	0.709	0.735	0.722	6.56
WT(1/10)	0.389	0.338	0.364	2.93
WT(1/20)	0.210	0.241	0.226	1.52
Ser209Ala(1/1)	0.571	0.688	0.630	5.62
Ser209Ala(1/10)	0.33	0.423	0.377	3.01
Ser209Ala(1/20)	0.187	0.185	0.186	1.12
Met88Ala(1/1)	0.681	0.668	0.675	6.09
Met88Ala(1/10)	0.541	0.566	0.554	4.85
Met88Ala(1/20)	0.146	0.231	0.189	1.14

According to the Table 3.1; Bradford assay results are at 595 nm;

For calculation of wild type protein concentration;

30 mg/ mL * 1/ 28.400 mol/g = 1056 µM stock concentration

For using a final concentration of 10 µM of wild type enzyme, the volume was determined by the formula given below.

1056 µM* X = 1 mL* 10 µM

X= 9.5 µL

For calculation of Ser209Ala protein concentration;

20 mg/ mL * 1/ 28.400 mol/g = 704 µM stock concentration

For using a final concentration of 10 µM of wild type enzyme, the volume was determined by the formula given below.

700 µM* X = 1 µL * 10 µM

X= 14.28 µL

For the calculation of Met88Ala protein concentration;

20 mg/ mL * 1/ 28.400 mol/g = 704 µM stock concentration

For using a final concentration of 10 µM of wild type enzyme, the volume was determined by the formula given below.

700 µM* X = 1 µL * 10 µM

X= 14.28 µL

The final concentrations of 10 µM enzymes were supplemented with 1 mL of distilled water so the final concentration of the enzymes became 1 µM.

3.1.8 Enzyme activity

The enzymatic activity of the wild type and Ser209Ala and Met88Ala mutant esterases were measured for 20 minutes with 5-minute intervals at 30 ºC and 410 nm by using Microplate Reader and enzymes loaded 3 different wells as triple control as shown in Table 3.2. It was hypothesized that serin and methionine residues form part of the catalytic triad, and that substitution of these amino acids would have a negative impact on the catalytic activity of the esterase enzyme.

To demonstrate this effect using protein concentration, mathematical calculations were performed using equation 3.1, and the formula for Beers' law is presented below.

$$\text{Activity (U/L)} = [(\Delta Abs/t) / (\varepsilon * d)] * 106 * V_t/V_s \quad (3.1)$$

Table 3.2 : Observed absorbance values through 20 minutes as average of triplet, the average of absorbance values, the average of blank subtracted absorbance values of the wild type, Met88Ala and Ser209Ala mutant esterases.

	2 min	**5 min**	**8 min**	**10 min**	**12 min**	**15 min**	**18 min**	**20 min**
WT	1.212	2.474	3.032	3.034	3.674	3.776	3.871	3.952
Met88Ala	1.033	1.887	2.310	2.707	3.042	3.120	3.354	3.503
Ser209Ala	0.331	0.437	0.499	0.563	0.626	0.681	0.781	0.845

According to Beer's law, ΔAbs is the average absorbance value. t use for time and it is 20 minutes in this study. Ɛ is the extinction coefficient and equal to 15095 M^{-1} cm-1, d is path length and equal to 0.52 cm, V_t is the total reaction volume and equal to 200 µL, V_s is the volume of the enzyme and equal to 20 µL for this project. Activities of the samples were determined by using the formula given in equation 3.2 through the above-mentioned values;

d = 0.52 cm

Ɛ = 15095 M^{-1} cm^{-1}

V_t = 200 µL

$$\text{Activity (U/L)} = [(\Delta Abs/t) / (Ɛ*d)] * 106 * V_t/V_s \quad (3.2)$$

$$\text{Activity (U/L)} = [(\Delta Abs/t) / (15095\ M^{-1}\ cm^{-1}*0.52\ cm)] * 106 * (200\ \mu L / 20\ \mu L)$$

ΔAbs values taken after extraction average absorbance value of blank from average absorbance value of mutant esterases. ΔAbs values are given in Table 3.3.

Table 3.3 : Average absorbance (ΔAbs), average absorbance/time (ΔAbs/min) and calculated activities of Wild type, Met88Ala and Ser209Ala mutant esterases.

	ΔAbs	**ΔAbs-ΔAbsblank**	**ΔAbs/min**	**Activity (U/L)**
WT	3.952	3.641	0.182	0.0249
Met88Ala	3.354	3.043	0.152	0.0205
Ser209Ala	0.781	0.470	0.024	0.0032

The Activities of wild type, Met88Ala and Ser209Ala mutant esterases were found as 0.0249, 0.0205 and 0.0032 respectively. According to these results, the activity of Met88Ala and Ser209Ala mutants are decreased 17.7% and 87.1% respectively compared to the wild type. These results indicate that, these 209th serin residue take place at the active site of the esterase enzyme and have direct effect on the activity of

the esterase enzyme. However, it can be thought that 88th methionine residue is in the oxyanion hole since a small amount of activity is reduced.

3.2 Directed Evolution by Error-Prone PCR

3.2.1 Determination of EP-PCR conditions

EP-PCR provides point mutations in different gene regions. Four EP-PCR tubes were set up by providing the same conditions. As seen in Figure 3.11, the 3rd tube of the EP-PCR did not work. 1st, 2nd and 4th tubes of EP-PCR product were combined and purified as seen in Figure 3.11 and Figure 3.12.

If there is a mutation in the ancestral genes in the first cycles of EP-PCR, it is necessary to prevent the existence of a common mutation in the subsequent cycles. Therefore, PCR reactions should be set up in different tubes under the same conditions and the EP-PCR products should be combined and purified. So, it was aimed to increase the diversity of the library as a solution to the case of a mutation in ancestral genes.

Digested EP-PCR product and digested vector are shown in Figure 3.13.

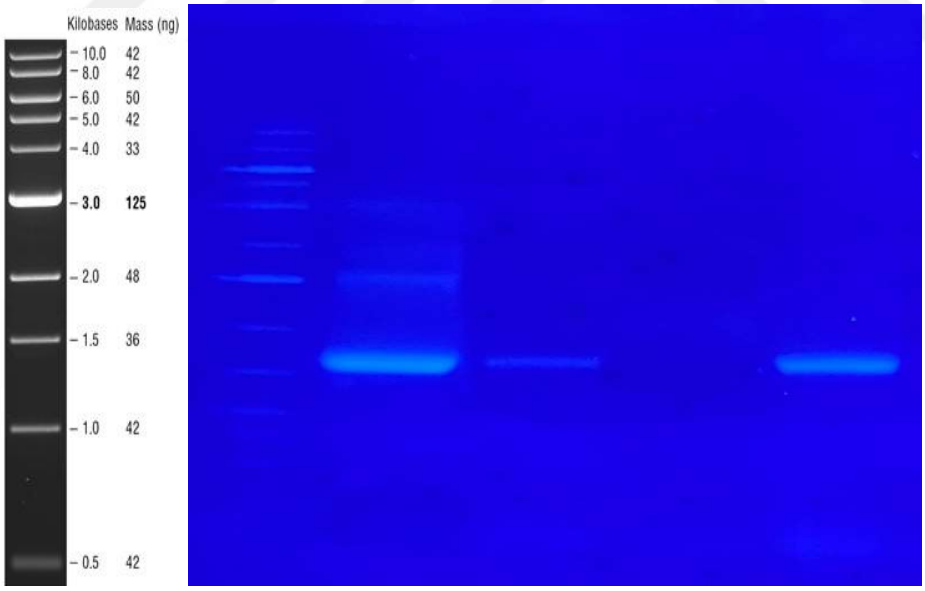

Figure 3.11 : Result of agarose Gel (1%) Electrophoresis for Error-prone PCR. Line 1:1 kb Gene Ruller Marker; Lane 2,3,4,5: EP-PCR products were obtained by using the same conditions.

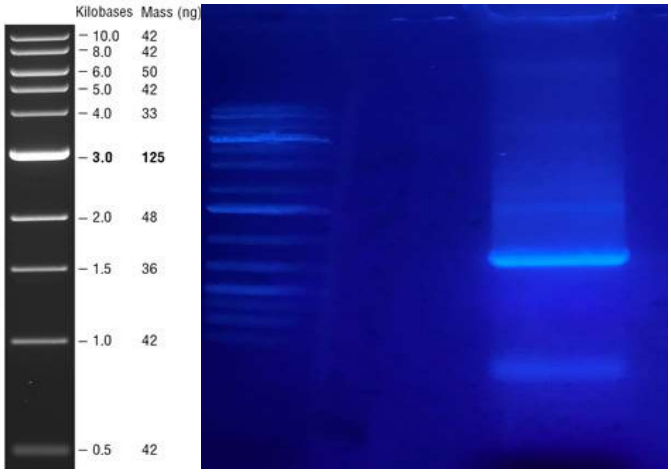

Figure 3.12 : Result of Agarose Gel (1%) Electrophoresis of pure EP-PCR product (1^{st},2^{nd} and 4^{th} tube combined).

3.2.2 Digestion of N-PCR, EP-PCR and pET-28a(+)

After clean up, concentrations and 260/280 ratio of DNA were measured with NanoDrop. The pET-28a(+) vector and insert DNA were measured as 42.1 ng/µL and 30 ng/µL respectively. 260/280 ratio of insert DNA and pET-28a(+) vector were measured as 1.70 and 1.62 respectively.

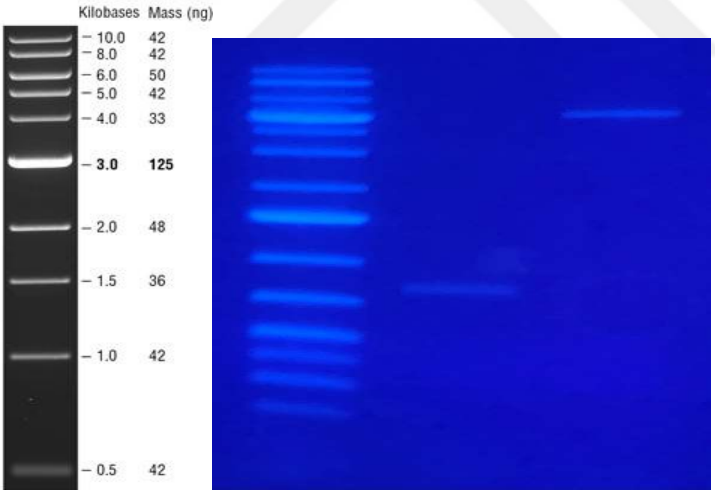

Figure 3.13 : Result of Agarose Gel (1%) Electrophoresis for digested of EP-PCR products. Line 1: 1kb Gene Ruller Marker, Line 2: digested of EP-PCR products; Line 3: digested of empty pET-28a(+).

After clean up, concentrations and 260/280 ratio of DNA samples were measured by Nano Drop. The digested pET-28a(+) vector and digested. EP-PCR Product in Figure 3.13 were measured as 28 ng/µL and 15 ng/µL respectively. 260/280 ratio measured as 1.80 and 1.85 respectively.260/280 ratio of insert DNA and pET-28a(+) vector were measured as 1.80 and 1.85 respectively.

3.2.3 Ligation

Ligation reaction was set with 5:1 and 7:1 as described above. After the ligation transformation to *E. coli* C43 (DE3) cells was performed.

3.2.4 Transformation and kanamycin screening

Transformation of PCR products into *E.coli* C43 α strain is successfully performed. Bacterial colonies on LB-Agar containing kanamycin can be seen in Figure 3.14 and Figure 3.15.

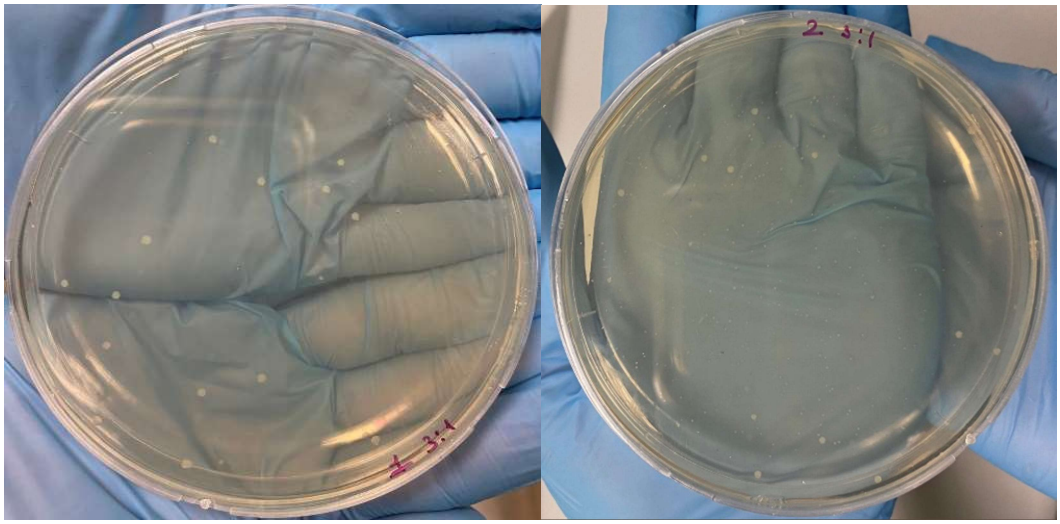

Figure 3.14 : Kanamycin screening plates containing colonies after transformation. The colonies of *E.coli* C43 competent cells containing a plasmid with 3:1 ratios (insert:vector).

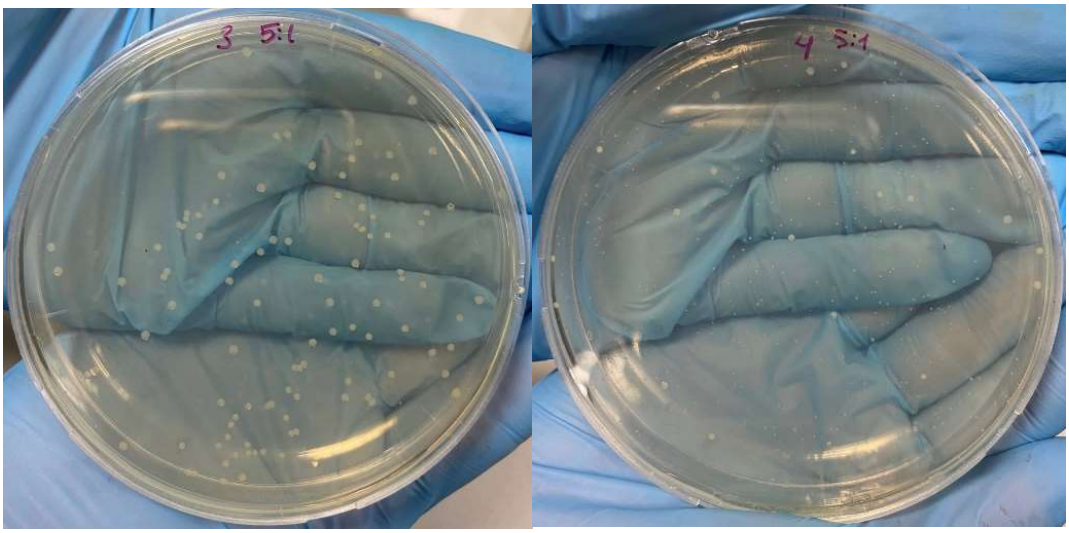

Figure 3.15 : Kanamycin screening plates containing colonies after transformation. The colonies of *E.coli* C43 competent cells containing a plasmid with 5:1 ratios (insert:vector).

3.2.5 Selection of the mutants using tributyrin agar plate

As a result of tributyrin agar screening of 159 mutants, 58 mutants were found to have lipolytic activity according to the Figure 3.16.

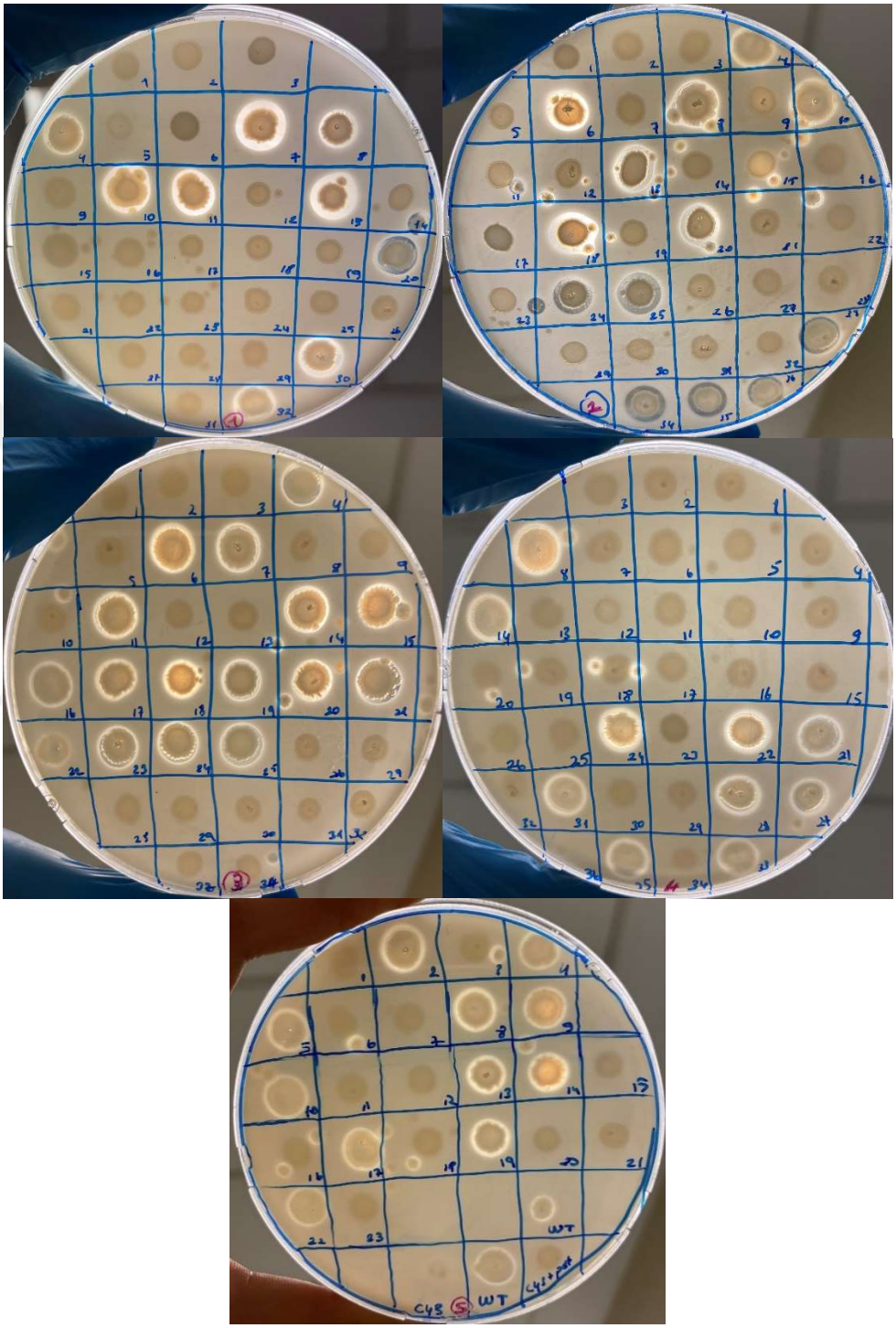

Figure 3.16 : Kanamycin screening tributyrin agar plates containing colonies after transformation. The colonies of C43 competent cells containing a plasmid with 3:1 and 5:1 ratios (insert:vector).

3.2.6 Bradford assay

The protein content of the samples was determined by using Bradford assay. 20 µL of each sample were combined with 180 l of Bradford Reagent and loaded in different wells of a 96-well plate. Seven Bovine Serum Albumin (BSA) standard solutions were used, with concentrations ranging from 0.125 to 2 mg/ml. 5 µL of each standard solution was prepared and applied to the wells repeatedly. Finally, the ODs of standards and unknown proteins were measured with a spectrophotometer at wavelenght of the 595 nm, and the concentration of unknown protein was calculated using the absorbance value. The absorbance values of the empty pET-28a(+), wild type and mutants are given in the Figure 3.17 and Figure 3.18 comparatively.

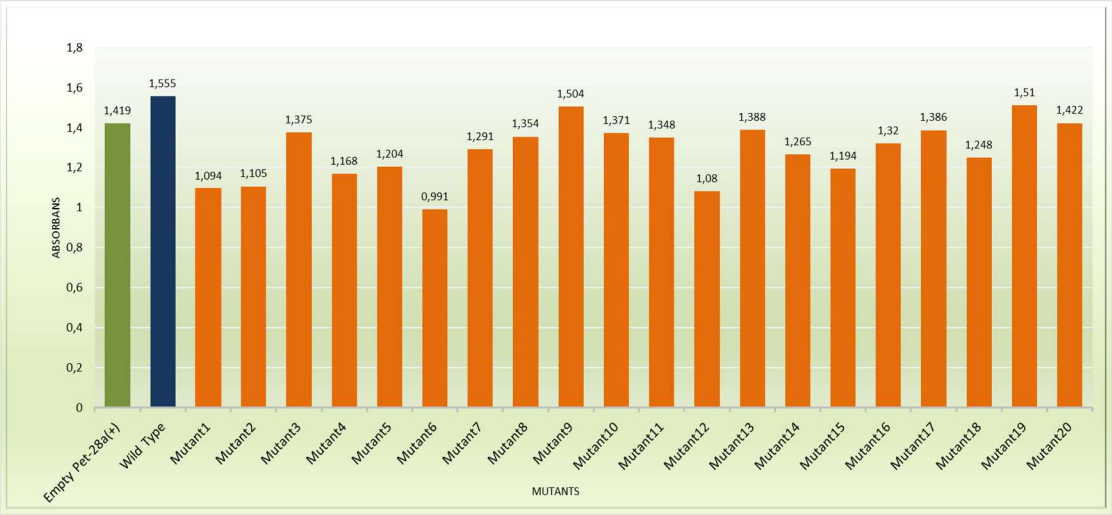

Figure 3.17 : Comparison of the concentration of mutants M1-M20.

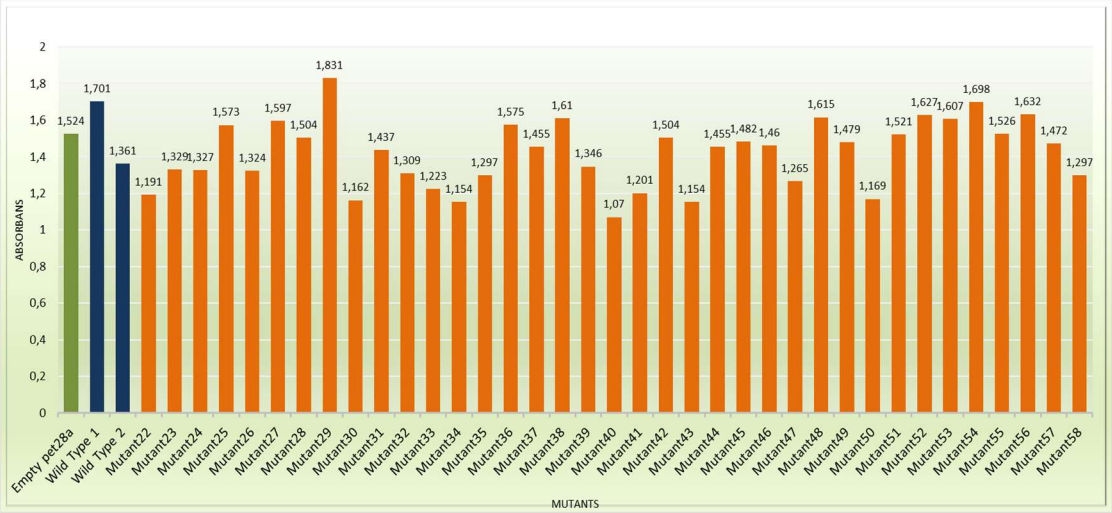

Figure 3.18 : Comparison of the concentration of mutants M22-M58.

Bradford analysis results for selected mutants with lipolytic activity are given below in Table 3.4 for 21 mutants and in Table 3.5 for 47 mutants, respectively. These tables contain the results of two replicate absorbance measurements and the concentration results calculated according to the BSA protein standards.

Table 3.4 : Bradford asssay results for M1-21.

Name	Abs 1	Abs 2	Abs Average	Concentration
Empty pET-28a(+)	0.315	0.319	0.317	1.419
Wild Type	0.352	0.353	0.353	1.555
Mutant1	0.208	0.255	0.232	1.094
Mutant2	0.209	0.259	0.234	1.105
Mutant3	0.259	0.353	0.306	1.375
Mutant4	0.239	0.264	0.252	1.168
Mutant5	0.255	0.267	0.261	1.204
Mutant6	0.193	0.217	0.205	0.991
Mutant7	0.259	0.309	0.284	1.291
Mutant8	0.269	0.330	0.300	1.354
Mutant9	0.361	0.319	0.340	1.504
Mutant10	0.294	0.315	0.305	1.371
Mutant11	0.240	0.357	0.299	1.348
Mutant12	0.191	0.264	0.228	1.080
Mutant13	0.296	0.323	0.310	1.388
Mutant14	0.279	0.273	0.276	1.265
Mutant15	0.257	0.259	0.258	1.194
Mutant16	0.253	0.329	0.291	1.320
Mutant17	0.309	0.309	0.309	1.386
Mutant18	0.273	0.272	0.273	1.248
Mutant19	0.322	0.361	0.342	1.510
Mutant20	0.319	0.318	0.319	1.422
Mutant21	0.268	0.302	0.285	1.297

Wild type and mutant cells produced different amounts of protein depending on their inducibility and protein expression conditions. The amounts of protein produced at different concentrations were diluted so that each mutant could react with the same concentration. Activity experiments were carried out with unit protein amounts. Since enzyme purification could not be done for each mutant, the reactions were established according to the fixed total protein without knowing the amount of esterase enzyme. Therefore, the amount of hagest esterase enzyme reacting with the substrate was different for each mutant. This caused fluctuations in activity experiments.

Table 3.5 : Bradford asssay results for M22-58.

Name	Abs 1	Abs 2	Abs Average	Concentration
Empty pET-28a(+)	0.302	0.307	0.305	1.524
Wild Type 1	0.314	0.347	0.331	1.701
Wild Type 2	0.279	0.263	0.271	1.361
Mutant22	0.203	0.224	0.214	1.191
Mutant23	0.244	0.245	0.245	1.329
Mutant24	0.236	0.241	0.239	1.327
Mutant25	0.318	0.297	0.308	1.573
Mutant26	0.253	0.269	0.261	1.324
Mutant27	0.290	0.344	0.317	1.597
Mutant28	0.275	0.274	0.275	1.504
Mutant29	0.349	0.390	0.370	1.831
Mutant30	0.236	0.227	0.232	1.162
Mutant31	0.296	0.263	0.280	1.437
Mutant32	0.222	0.282	0.252	1.309
Mutant33	0.231	0.248	0.240	1.223
Mutant34	0.201	0.237	0.219	1.154
Mutant35	0.266	0.255	0.261	1.297
Mutant36	0.307	0.288	0.298	1.575
Mutant37	0.280	0.252	0.266	1.455
Mutant38	0.273	0.329	0.301	1.610
Mutant39	0.259	0.259	0.259	1.346
Mutant40	0.233	0.199	0.216	1.070
Mutant41	0.230	0.228	0.229	1.201
Mutant42	0.287	0.282	0.285	1.504
Mutant43	0.215	0.218	0.217	1.154
Mutant44	0.260	0.272	0.266	1.455
Mutant45	0.301	0.284	0.293	1.482
Mutant46	0.288	0.298	0.293	1.460
Mutant47	0.239	0.264	0.252	1.265
Mutant48	0.298	0.303	0.301	1.615
Mutant49	0.279	0.286	0.283	1.479
Mutant50	0.227	0.244	0.236	1.169
Mutant51	0.304	0.281	0.293	1.521
Mutant52	0.296	0.309	0.303	1.627
Mutant53	0.323	0.290	0.307	1.607
Mutant54	0.306	0.336	0.321	1.698
Mutant55	0.290	0.280	0.285	1.526
Mutant56	0.318	0.300	0.309	1.632
Mutant57	0.285	0.309	0.297	1.472
Mutant58	0.254	0.230	0.242	1.297

3.2.7 Determination of kinetic activity for 6C and 12C substrates

Enzyme activity was determined by measuring the release of p-nitrophenoxide from pNP-hexanoate and pNP-dodecanoate as a result of hydrolysis of paranitrophenyl esters by esterases. 1 mM substrate and 1 µM enzyme concentrations were determined. The hAGEst enzyme shows higher activity against short chain pNP-esters than long chain fatty esters. The absorbance values were measured three times for each of the 58 mutants and these values were averaged. As the kinetic activity results were compared, it was found that none of the mutants had significantly greater activity than the wild type according to the Figure 3.19, Figure 3.20, Figure 3.21.

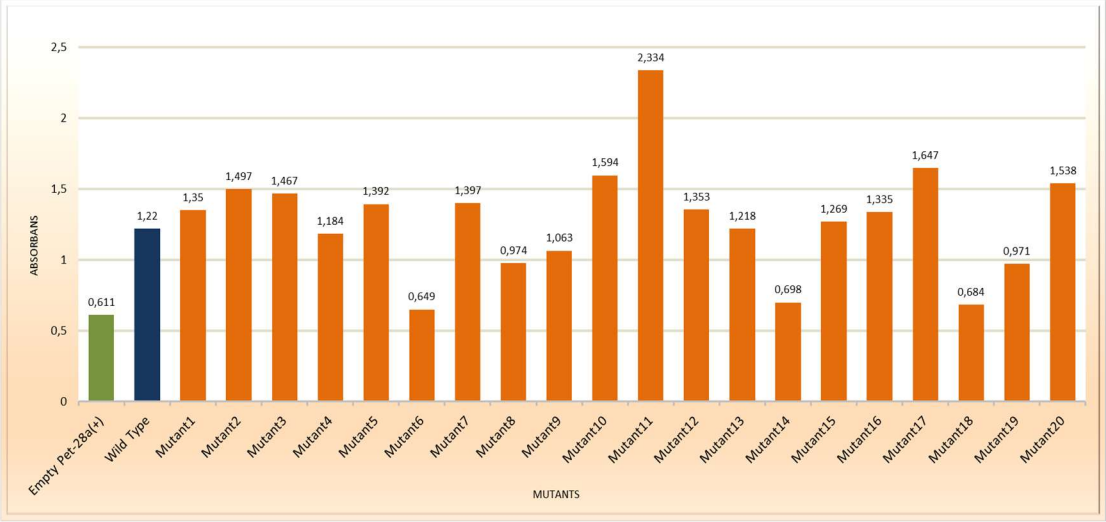

Figure 3.19 : Enzyme activity for 6C hexaonate M1-21.

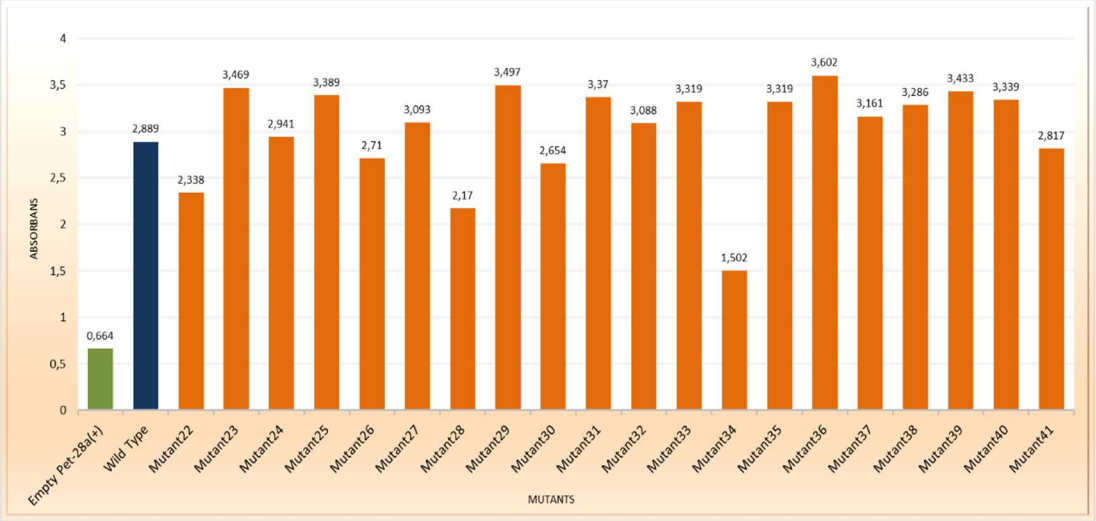

Figure 3.20 : Enzyme activity for 6C hexaonate M22-41.

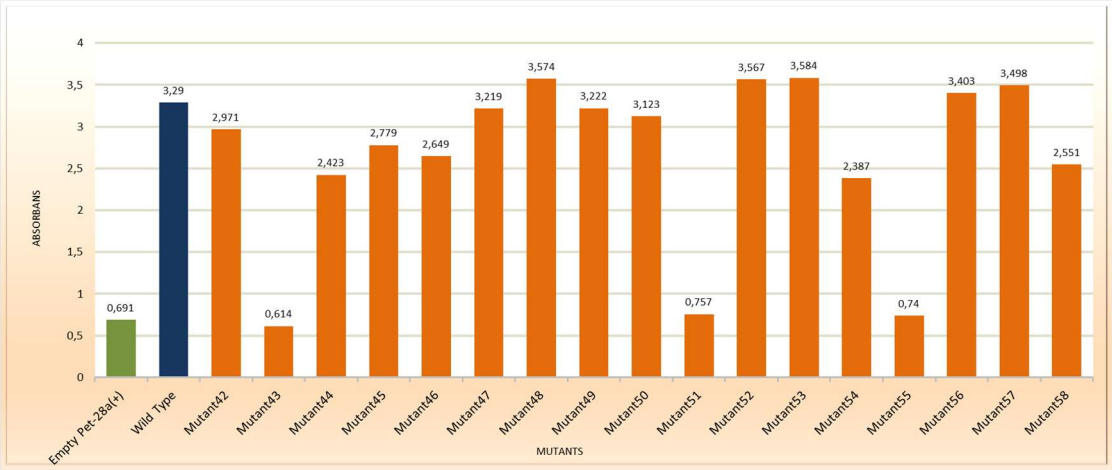

Figure 3.21 : Enzyme activity for 6C hexaonate M42-58.

For 6C hexanoate substrate; mutants 1 to 21, mutants 22 to 41, mutants 42 to 58 are given in Table 3.6, Table 3.7 and Table 3.8, respectively.

Table 3.6 : Enzyme activity for 6C hexaonate M1-21.

Name	Abs 1	Abs 2	Abs 3	Abs Average
Empty pET-28a(+)	0.595	0.613	0.625	0.611
Wild Type	1.166	1.267	1.227	1.220
Mutant1	1.352	1.319	1.379	1.350
Mutant2	1.490	1.486	1.514	1.497
Mutant3	1.439	1.463	1.499	1.467
Mutant4	1.166	1.184	1.203	1.184
Mutant5	1.359	1.424	1.394	1.392
Mutant6	0.646	0.657	0.645	0.649
Mutant7	1.398	1.394	1.398	1.397
Mutant8	0.969	0.969	0.985	0.974
Mutant9	1.031	1.076	1.082	1.063
Mutant10	1.598	1.590	1.593	1.594
Mutant11	2.305	2.363	2.333	2.334
Mutant12	1.334	1.367	1.358	1.353
Mutant13	1.202	1.240	1.213	1.218
Mutant14	0.699	0.702	0.692	0.698
Mutant15	1.265	1.285	1.258	1.269
Mutant16	1.320	1.346	1.340	1.335
Mutant17	1.560	1.727	1.654	1.647
Mutant18	0.690	0.680	0.683	0.684
Mutant19	0.938	1.000	0.974	0.971
Mutant20	1.492	1.577	1.546	1.538
Mutant21	1.332	1.337	1.346	1.338

Table 3.7 : Enzyme activity for 6C hexaonate M22-41.

Name	Abs 1	Abs 2	Abs 3	Abs Average
Empty pET-28a(+)	0.668	0.664	0.659	0.664
Wild Type	2.872	2.856	2.939	2.889
Mutant22	2.342	2.293	2.379	2.338
Mutant23	3.427	3.480	3.499	3.469
Mutant24	2.950	2.957	2.915	2.941
Mutant25	3.368	3.392	3.406	3.389
Mutant26	2.613	2.772	2.744	2.710
Mutant27	3.003	3.152	3.125	3.093
Mutant28	2.130	2.192	2.188	2.170
Mutant29	3.476	3.492	3.523	3.497
Mutant30	2.649	2.626	2.688	2.654
Mutant31	3.328	3.392	3.391	3.370
Mutant32	3.134	3.110	3.019	3.088
Mutant33	3.335	3.355	3.267	3.319
Mutant34	1.603	1.420	1.482	1.502
Mutant35	3.300	3.346	3.311	3.319
Mutant36	3.602	3.584	3.620	3.602
Mutant37	3.163	3.170	3.150	3.161
Mutant38	3.328	3.318	3.213	3.286
Mutant39	3.410	3.436	3.452	3.433
Mutant40	3.343	3.389	3.285	3.339
Mutant41	2.821	2.799	2.832	2.817

Table 3.8 : Enzyme activity for 6C hexanoate M42-58.

Name	Abs1	Abs2	Abs3	Abs Average
Empty pET-28a(+)	0.693	0.691	0.690	0.691
Wild Type	3.218	3.438	3.214	3.290
Mutant42	2.994	2.995	2.925	2.971
Mutant43	0.629	0.606	0.607	0.614
Mutant44	2.448	2.254	2.567	2.423
Mutant45	2.751	2.768	2.819	2.779
Mutant46	2.671	2.690	2.585	2.649
Mutant47	3.164	3.224	3.270	3.219
Mutant48	3.506	3.560	3.656	3.574
Mutant49	3.153	3.256	3.257	3.222
Mutant50	3.157	3.181	3.030	3.123
Mutant51	0.921	0.657	0.693	0.757
Mutant52	3.492	3.611	3.598	3.567
Mutant53	3.580	3.581	3.592	3.584
Mutant54	2.427	2.389	2.345	2.387
Mutant55	0.739	0.743	0.739	0.740
Mutant56	3.333	3.444	3.432	3.403
Mutant57	3.491	3.520	3.483	3.498
Mutant58	2.564	2.544	2.545	2.551

When the kinetic activity values were examined, it was shown that none of the mutants had substantially more activity than the wild type as shown in Figures 3.22, Figure 3.23, and Figure 3.24.

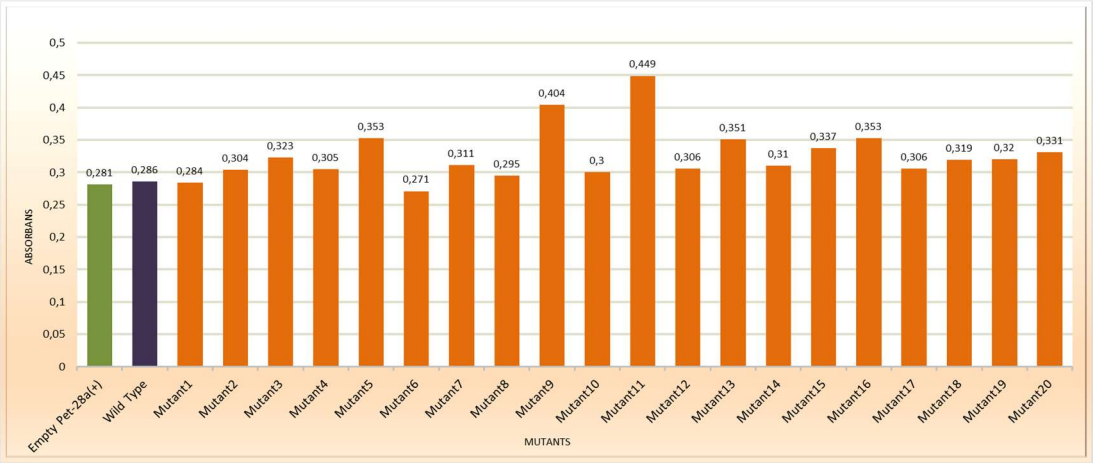

Figure 3.22 : Enzyme activity for 12C dodecanoate M1-21.

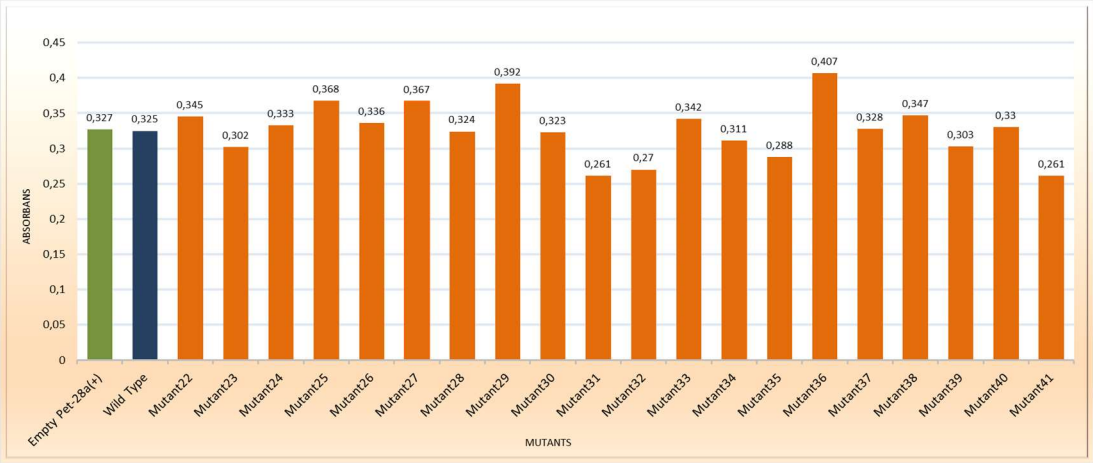

Figure 3.23 : Enzyme activity for 12C dodecanoate M22-41.

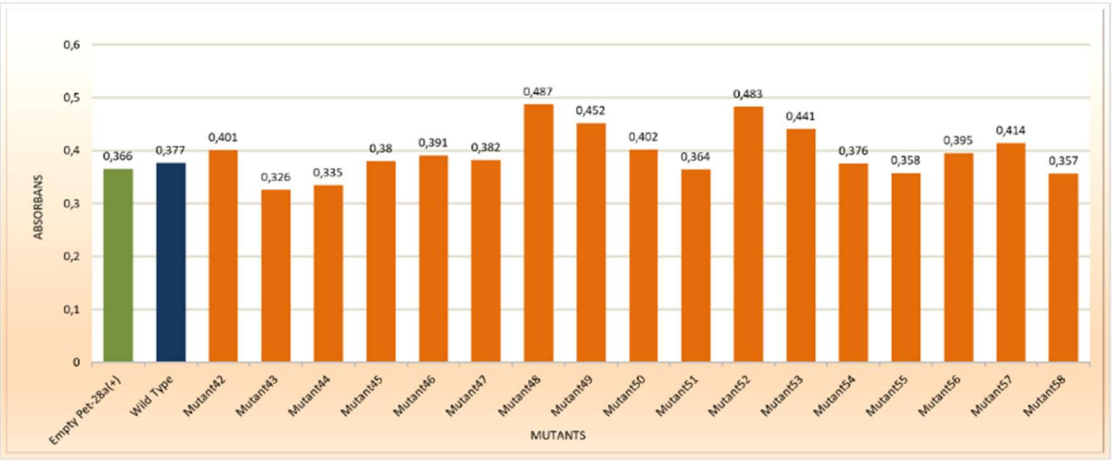

Figure 3.24 : Enzyme activity for 12C dodecanoate M42-58.

While 12C dodecanoate substrate; mutants 1 to 21, mutants 22 to 41, mutants 42 to 58 are given in Table 3.9, Table 3.10 and Table 3.11, respectively.

Table 3.9 : Enzyme activity for 12C dodecanoate M1-21.

Name	Abs 1	Abs 2	Abs 3	Abs Average
Empty pET-28a(+)	0.285	0.282	0.276	0.281
Wild Type	0.274	0.272	0.313	0.286
Mutant1	0.304	0.288	0.261	0.284
Mutant2	0.305	0.303	0.304	0.304
Mutant3	0.324	0.321	0.323	0.323
Mutant4	0.303	0.314	0.297	0.305
Mutant5	0.356	0.344	0.360	0.353
Mutant6	0.267	0.274	0.272	0.271
Mutant7	0.309	0.312	0.313	0.311
Mutant8	0.301	0.294	0.290	0.295
Mutant9	0.413	0.400	0.398	0.404
Mutant10	0.297	0.305	0.299	0.300
Mutant11	0.439	0.450	0.457	0.449
Mutant12	0.307	0.314	0.298	0.306
Mutant13	0.354	0.342	0.357	0.351
Mutant14	0.293	0.321	0.316	0.310
Mutant15	0.327	0.339	0.346	0.337
Mutant16	0.351	0.359	0.349	0.353
Mutant17	0.312	0.297	0.308	0.306
Mutant18	0.315	0.328	0.313	0.319
Mutant19	0.316	0.326	0.317	0.320
Mutant20	0.327	0.339	0.328	0.331
Mutant21	0.344	0.351	0.338	0.344
Mutant22	0.364	0.363	0.366	0.364

Table 3.10 : Enzyme activity for 12C dodecanoate M22-41.

Name	Abs 1	Abs 2	Abs 3	Abs Average
Empty pET-28a(+)	0.328	0.316	0.336	0.327
Wild Type	0.324	0.318	0.332	0.325
Mutant22	0.342	0.349	0.344	0.345
Mutant23	0.298	0.294	0.313	0.302
Mutant24	0.333	0.335	0.331	0.333
Mutant25	0.353	0.365	0.386	0.368
Mutant26	0.322	0.345	0.342	0.336
Mutant27	0.366	0.371	0.363	0.367
Mutant28	0.314	0.325	0.332	0.324
Mutant29	0.381	0.393	0.402	0.392
Mutant30	0.321	0.333	0.316	0.323
Mutant31	0.266	0.260	0.258	0.261
Mutant32	0.266	0.270	0.275	0.270
Mutant33	0.327	0.344	0.354	0.342
Mutant34	0.307	0.324	0.303	0.311
Mutant35	0.290	0.287	0.288	0.288
Mutant36	0.409	0.407	0.406	0.407
Mutant37	0.333	0.330	0.321	0.328
Mutant38	0.344	0.366	0.330	0.347
Mutant39	0.311	0.293	0.305	0.303
Mutant40	0.332	0.330	0.327	0.330
Mutant41	0.259	0.272	0.253	0.261

Table 3.11 : Enzyme activity for 12C dodecanoate M42-58.

Name	Abs1	Abs2	Abs3	Abs Average
Empty pET-28a(+)	0.357	0.374	0.366	0.366
Wild Type	0.369	0.373	0.390	0.377
Mutant42	0.413	0.399	0.390	0.401
Mutant43	0.324	0.322	0.331	0.326
Mutant44	0.318	0.348	0.339	0.335
Mutant45	0.391	0.369	0.381	0.380
Mutant46	0.393	0.385	0.395	0.391
Mutant47	0.387	0.375	0.383	0.382
Mutant48	0.463	0.504	0.495	0.487
Mutant49	0.428	0.459	0.469	0.452
Mutant50	0.411	0.399	0.396	0.402
Mutant51	0.370	0.367	0.355	0.364
Mutant52	0.472	0.492	0.485	0.483
Mutant53	0.409	0.460	0.453	0.441
Mutant54	0.381	0.376	0.371	0.376
Mutant55	0.352	0.360	0.363	0.358
Mutant56	0.393	0.397	0.394	0.395
Mutant57	0.400	0.412	0.431	0.414
Mutant58	0.357	0.352	0.362	0.357

Mutant48, Mutant52 and Mutant53 were selected because they showed more activity against the 12C dodecanoate substrate. Mutant11 and Mutant36 were selected because they showed more activity for both substrates than wild type. Mutant6 and Mutant23 were selected, which showed lower activity than wild type for the two substrates. Finally, Mutant11 was chosen randomly. As a result, eight mutants were selected and sent for sequence analysis.

3.2.8 Control cleavage of plasmids and sequencing

To find out if the plasmid carrying insert, the empty vector pET-28a(+)was also digested under the same conditions and loaded into the last well in the gel. The expected length of the pET-28a(+) vector is 5369 bp, and the insert length should be 783 bp. Therefore, the full product length should be seen at approximately 6152 bp on agarose gel after control digestion. Samples with the desired length were sent for sequencing. Looking at the Figure 3.25, the three mutant plasmids (Line 1st, 2nd and 4th, 5th and 7th) located higher up than the pET-28a(+) vector without the insert were selected for sequencing to confirm sequences of mutants.

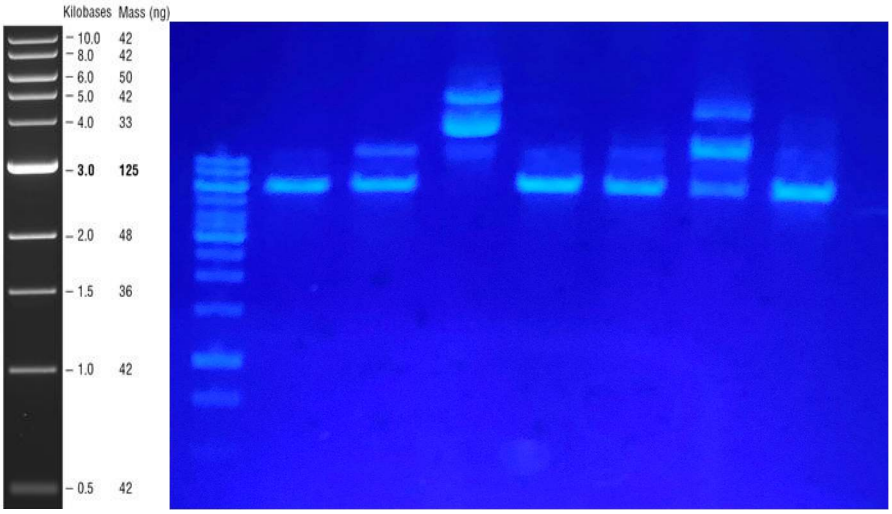

Figure 3.25 : Agarose gel electrophoresis image of control cleavage of plasmid DNAs and pET-28a (+) vector. Line 1: 1 kb Gene Ruller Marker; Line 2: plasmid of M9: Line 3:plasmid of M11 hAGest enzyme; Line 4: plasmid of M17 hAGest enzyme; Line 5: plasmid of M19 hAGest enzyme; Line 7: plasmid of M36 hAGest enzyme; Line 8: plasmid of 40 hAGest enzyme; Line 9: plasmid of M48 hAGest enzyme.

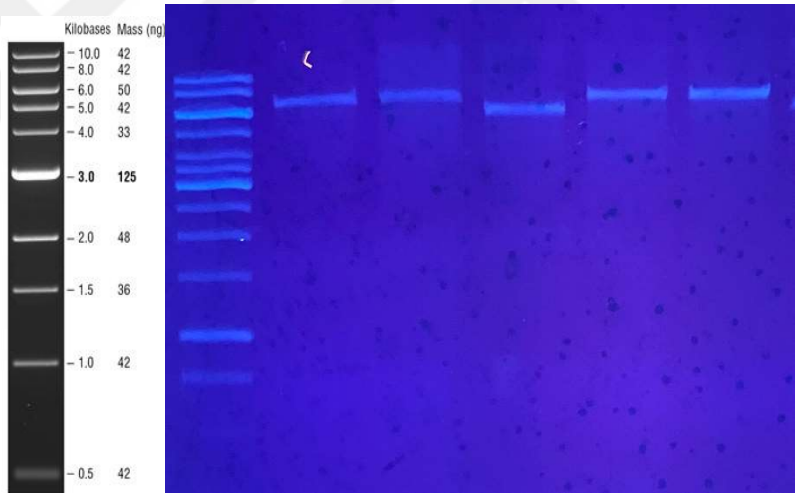

Figure 3.26 : Agarose gel electrophoresis image of control cleavage of plasmid DNAs and pET-28a (+) vector. Line 1: 1 kb Gene Ruller Marker; Line 2: plasmid of M6: Line 3:plasmid of M23 hAGest enzyme; Line 4: plasmid of M52 hAGest enzyme; Line 5: plasmid of M53 hAGest enzyme; Line 6: plasmid of WT hAGest enzyme.

When sequencing data for 8 mutants using CLUSTAL OMEGA were aligned with wild-type, it was seen that they all had the same gene sequence as the wild-type esterase enzyme. As a result of the study, mutagenic clones could not be obtained or due to the high mutation rate, the mutated clones lost their activity and were eliminated during activity screening.

It may be difficult to detect potentially rare variants of interest if the mutation rate is very low. If the mutation rate is extremely high, almost all of the molecules in the generated library will have multiple mutations and may result in loss of activity. The desired proportion of mutation depends on the type of activity wanted and the screening amount of library members. In many cases, it is a reasonable approach to construct a library containing a few unmutated molecules in the collection of mutants screened. However, obtaining an effective mutant library is often severely difficult. To achieve higher mutagenesis levels, greater dilution of the template in the first reaction will be required. A high concentration of template in the reaction can reduce or prevent mutagenicity. To reduce the occurrence of mis-priming events, the annealing temperature should be as high as possible. The optimal annealing temperature for effective amplification must be determined experimentally. Increasing the number of cycles could be another approach. When the cycles for around 50 EP-PCR replications are determined, mutations can occur in approximately 3.5% of the nucleotide positions in the DNA template. However, the precise mutation rate may vary depending on the environment and template.

4. CONCLUSIONS

Enzymes have great potential in many fields of the industry such as detergent, food, pharmaceutical, perfume, polymer and agriculture . It is very important to produce enzymes that can adapt to the harsh conditions in the industry, have a high tolerance and show high activity. Metagenomic approaches and protein engineering strategies with various combinations are used to obtain new enzymes that can be used in industrial applications. In this study, both rational design and directed evolution methods of protein engineering strategy were applied to determine the three-dimensional structure and improve the substrate specificity of the esterase enzyme which was obtained from Acıgöl, Turkey.

The alanine screening method is a rational design approach that is frequently used in studies of protein structure analysis. The structure-function relationship can be interpreted by replacing the amino acids thought to be responsible for the activity with the neutral and small alanine amino acid residue. It is also used to inhibit enzymatic activity and to direct protein-protein interactions in a targeted manner.

In the first part of the study, it was aimed to examine the roles of Ser209 and Met88 residues in the active site of the enzyme by constructing Ser209Ala and Met88Ala mutants on *E. Coli*. Compared to the wild type enzyme, the Ser209Ala mutant esterase enzyme lost its kinetic activity. The activity of the Met88Ala mutant esterase enzyme was decreased by 17.7%. It has been understood that it is not essential in the structure, but has an importance due to its complete loss of activity, but its decrease.

In the second part of the study, it was aimed to create a large mutant library by the Error-Prone PCR method of directed evolution. This library was primarily aimed at changing the substrate specificity from 6C hexaonate to 12C dodecanoate. A library containing 159 mutants was obtained by the Error-Prone PCR method and 58 mutants were found to have lipolytic activity on the tributyrin agar plate. Although kinetic activity assays were performed for these 58 mutants, sequencing was performed for only 8 selected mutants.

Regarding the results of the sequencing, mutagenic colonies could not be obtained or due to the high mutation rate, the mutated colonies lost their activity during the activity screening. The library will continue to be scanned using different screening strategies.

In further studies, fatty acids with a different number of C can be used as alternative substrates to observe the activity of mutants. Error-Prone PCR can be established in different conditions and protein engineering approaches can be applied to the esterase enzyme with combinations of different methods.

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