Random Mutagenesis on a De-novo enzyme : increasing the hydrolase activity of a Polycarbonate degrading enzyme.

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Random Mutagenesis on a de-novo enzyme : increasing the hydrolase activity of a polycarbonate degrading enzyme.

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Abstract:

The aim is to experimentally increase the hydrolase activity of a given polycarbonate degrading enzyme by directed evolution. 206 candidates were picked for screening from the provided library. During the course of this project 4 screening were done and the expressed proteins are screened for their activity on long chain and short chain fatty acid esters, using tributyrin and olive oil plate assays at 25°C and 37°C for 10 days. The variants were evaluated based on three criteria: absence of background activity and the order of appearance of hydrolase activity compared to the controls. Restriction enzyme analysis was performed to ensure the presence of translation start codon in the plasmid DNA of variants showing activity. Out of 206, there are 5 interesting candidates 6A,11B,11C, 20I, and 18E, having hydrolase activity at 25°C.

Preface

This report is written by Hira Abdullah as the short master thesis of the Master's degree in Nanobiotechnology at the Department of Materials and Production at Aalborg University. This project was written over a period from February 2^{nd} to November 21^{st} 2023, with supervision from Eva Maria Petersen.

This project is focused on the method of directed evolution by random mutagenesis in-order to experimentally increase the hydrolase activity of a given polycarbonate degrading enzyme based on a de-novo designed armadillo repeat protein (dArmRP). The candidates were evaluated based on three criteria: absence of background expression, the order of appearance of hydrolysis activity, and the strength of hydrolysis activity.

The report is composed of six chapters. Chapter one is the state of the art, chapter two is an introduction to the experiment and includes the relevant theory of the de-novo enzyme and random mutagenesis library and the theory of some of the methods used in the report. Chapter three is about the material and methods used in the expression and screening of the mutants. The results are reported in chapter four while the fifth chapter is the discussion and explains and reflects on the results obtained. The conclusion is drawn in chapter six.

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I want to thank my family and friends for consistently believing in me and for being my anchor during my lowest moments, and for continuously making the journey easier.

Contents

List of Abbreviations

3D	Three-dimensional
Ala (A)	Alanine
Escherichia Coli	*E. coli*
Ethidium Bromide	EtBr
DNA	Deoxyribonucleic acid
H-bonding	Hydrogen-bonding
H-bonds	Hydrogen-bonds
Isopropyl-β-D-thiogalactopyranoside	IPTG
Kilo base pair	kbp
Lower Molecular Weight	LMW
Lysogeny Broth	LB
Lysogeny Broth-Ampicillin	LB-Amp
Optical Density	OD
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis	SDS-PAGE
Tetramethylenediamine	TEMED
Tris-Acetate-EDTA	TAE
Trisaminomethane	Tris
Ultraviolet	UV
wt.%	Mass fraction

1 State of the art

Plastic is a synthetic compound derived from crude oil, natural gas, plants, salts, and coal [1]. With a global output of approximately 368 million metric tons in 2019, plastic is one of the most commonly used materials on the planet. Due to its high demand and low degradation rate plastic has become a significant threat to the environment. Upon degrading, plastic breaks down into smaller components which are difficult to analyze. Microplastic which is plastic of length 1-5000 µm is one of the rising concerns for environmental scientists [2]. Microplastics emerges as a new source of persistent environmental pollution which is widely distributed to different ecological territories and food chain. These concerns have led to numerous studies on plastic degradation processes, including physical, chemical, and biological approaches. With the advancement in technology, scientists are able to gather strong data related to microplastic nature, reactivity, structure, and chemical composition [3]–[5].

Microbial plastic degradation is a promising method that has attracted attention over a few decades due to its eco-friendliness and commercial application possibilities. Microbes such as bacteria and fungi produce enzymes capable of degrading polymers such as polystyrene, polyethylene, and polycarbonate [6]. Microbial enzymes break down plastics into small compounds or monomers that bacteria may subsequently consume as a source of energy or carbon. Yoshida et al. reported bacteria *Ideonella sakaiensis* uses plastic polyethylene terephthalate (PET) as a sole carbon and energy source by breaking it down into into terephthalic acid and ethylene glycol [7], [8].

While microbial enzymatic degradation holds significant promise for the treatment of waste, it also presents several constraints that need to be acknowledged. Enzyme selectivity is an important limitation, as enzymes may only degrade specific polymer types, which constrains their broad applicability for diverse waste forms [9]. The relatively slow pace of microbial degradation compared to enzymatic processes may impede reaction rates [10]. Environmental factors, including the applied pH, thermo stability, substrate molecular weight, and complexity, can significantly influence the enzymatic degradation of biodegradable plastics [11].

Polymers like petro-plastics are highly resistant to natural biodegradation processes, including microbial and enzymatic degradation, thus posing another hurdle [12]. Microbial enzymatic degradation, from a bioremediation perspective, is viable only when microbial nutrients are readily accessible [13]. Lastly, the financial implications of employing enzymes on a large industrial scale may be prohibitive, thus adding to the challenges of implementing microbial enzymatic degradation [9].

Modern biotechnological methods combining protein engineering, genetic engineering, and bioinformatics present an excellent field of research for microplastic degradation by creating novel pathways for bioremediation. De-novo enzyme design presents a potent strategy capable of overcoming the limitations of microbial enzymatic degradation. This approach designs enzymes with a higher degree of selectivity towards specific polymers [14], thereby can be designed to target recalcitrant polymers, such as petro-plastics, that naturally resist biodegradation processes [11], [14]. These de-novo enzymes, are constructed to be more efficient in polymer breakdown[14] and can withstand a diverse range of environmental conditions [15], thereby increasing their efficacy in plastic degradation. Furthermore, these enzymes can be engineered to more efficiently utilize available nutrients, thus bolstering their effectiveness in degrading plastics [16]. Finally, cost-efficiency and scalability considerations can be incorporated into the design of de-novo enzymes, making them a potentially viable choice for large-scale industrial applications [14].

Although de-novo enzyme design (rational design) is a promising approach, it also has some limitations. The enzyme design can be complex and computationally intensive, which can limit the number of possible designs that can be tested [17].De-novo enzyme tends to be less predictable as the relationship between enzyme sequence and function is not fully understood [17]. Though de-novo enzymes are specifically designed for substrates, they may have low activity or specificity, which can limit their effectiveness in degrading plastics [17]. They may also be unstable under certain environmental conditions, which can limit their effectiveness in industrial applications[17].

Directed evolution is a strategy that can overcome some of the limitations of de-novo enzyme design. In contrast to de-novo enzyme design, which involves designing new enzymes from scratch, directed evolution involves modifying existing enzymes to improve their activity, specificity, and stability. Directed evolution can be less complex and more straightforward than de-novo enzyme design, as it involves modifying existing enzymes rather than designing new ones from scratch [18]. However, a significant limitation of directed evolution is its inability to thoroughly explore the extensive sequence space of a protein. Unlike rational design methods, directed evolution induces random mutations in the target gene without necessitating information about the protein's structure. This approach often results in a majority of mutants showing

no enhancement or, in some cases, exhibiting reduced activity [18].

To circumvent this limitation and facilitate a more effective search, an efficient strategy involves integrating directed evolution with computational enzyme design [19].

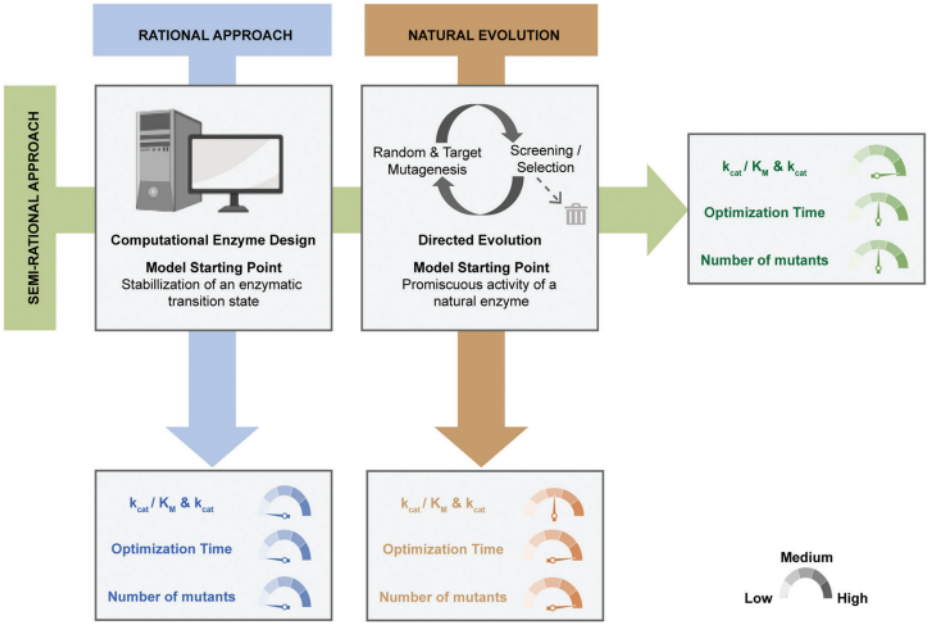


Figure 1.1: *The figure shows the various approaches to artificial enzyme development. The semi-rational approach gives the highest turnover number and catalytic efficiency, compared to the rational approach and natural evolution. It takes an adequate amount of time to generate a library with diverse genes. The rational design approach though takes less optimization time and produces fewer mutants with very little catalytic efficiency. The natural evolution process takes a long time to optimize and produces a huge number of mutants with average catalytic functions, making it difficult to screen and highly laborious. The semi-rational approach that combines the good of both is a better way for enzyme development. The figure used by Beatrize et.al 2021 [19].*

1.1 Problem statement

Can the hydrolase activity of a given polycarbonate degrading enzyme based on a de novo designed armadillo repeat protein (dArmRP) be increased by directed evolution?

1.2 Project approach

In this project, the given polycarbonate degrading enzyme based on a de novo designed armadillo repeat protein (dArmRP) is subjected to directed evolution by random mutagenesis. 206 candidates from a library are screened for hydrolase activity

towards long and short-chain fatty acid substrates. The assays are not specific to carbonate esters, the enzyme in context. Direct evolution is a method that takes advantage of substrate promiscuity to develop enzymes that can degrade alternative substrates. The aim of this thesis is to find out will direct evolution through random mutagenesis produces mutants that express enzymes with activity toward tributyrin and olive oil as substrates.

2 Introduction

2.1 Enzyme design

In this project, a de-novo polycarbonate enzyme developed by Holst et.al by redesigning an armadillo repeat protein is used[20]. Armadillo repeat proteins offer several advantages as scaffolds for enzyme redesign due to their repetitive structural motif, which provides a stable and modular framework for modifications [21]. Additionally, natural armadillo repeat proteins have a high affinity for binding peptides along grooves on their surface[21]. The mutated designed armadillo repeat protein will be referred to as mutated dArmRP, while the original armadillo repeat protein serving as the starting point will be called the native dArmRP[20]. Through directed evolution, the mutated dArmRP is being optimized in this project to enhance its hydrolase activity.

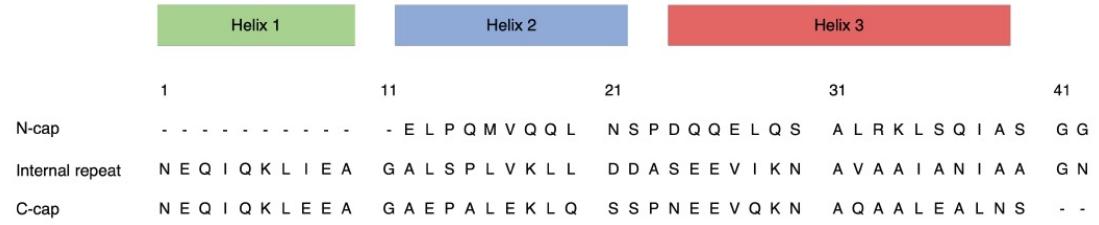


Figure 2.1: The components of armadillo repeat protein. The sequence components of armadillo repeat protein are represented on separate rows. Helix 1, helix 2, and helix 3 are designated regions indicating the portions of the sequence that form each helical structure within the protein. Figure taken from- Recombinant production and characterization of a designed polycarbonate degrading enzyme - semester project by Group 5.319 [20].

Figure 2.1 illustrates the components of the armadillo repeat protein. The N-cap and C-cap sequences are located at the N-terminal and C-terminal ends, respectively. They play a crucial role in stabilizing the protein's structure and shielding the hydrophobic core from solvent exposure[21]. The cap sequences differ from the internal repeat sequence. The internal repeat sequence, which consists of approximately 42 amino acid residues repeated multiple times forms the foundation of the armadillo

repeat structure. This repetitive motif contributes to the overall stability and folding of the protein[21]. Each component contributes to a three-helix motif, with the helices arranged in a triangular manner and repeating to form a spiral staircase. This highly symmetric and repetitive structure is characteristic of armadillo repeat proteins[21]. It optimizes dipole alignment of the α-helical secondary structure, salt-bridges between helices, and has a high proportion of intra-molecular hydrogen bonds.[20], [21]

The polycarbonate degrading enzyme was designed based on the structure of a native armadillo repeat protein (5MFBY)[20]. As shown in the figure Figure 2.2, the mutated protein structure closely resembles the native structure, with four specific mutations introduced. To enable the enzyme to break down polycarbonate, a catalytic triad consisting of Serine, Histidine, and Glutamic acid was incorporated[20].The design process focused on two key aspects: ensuring the enzyme's ability to bind to its target molecules and accurately placing the amino acids involved in catalysis[20]. A loop region and an α-helix were utilized to position the catalytic triad in the desired location within the protein structure. Notably, the active site responsible for catalytic activity differs from the region where the native protein typically binds peptides[20].

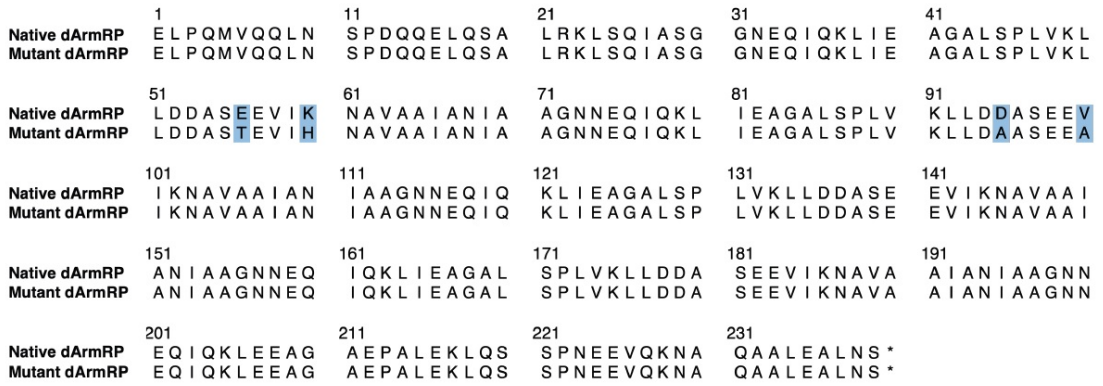


Figure 2.2: The aligned amino acid sequences of the native dArmRP and the mutant dArmRP. The sequence alignment highlights the mutations in blue. One-letter amino acid abbreviations were employed in representing the sequences. Figure taken from- Recombinant production and characterization of a designed polycarbonate degrading enzyme - semester project by Group 5.319 [20].

The pMutantdArmRP plasmids was designed by the supervisor. [20]. The design employs a T7/lac system to regulate gene expression and includes a polyhistidine tag for affinity purification. His tag can be removed by TEV protease, which is site specific to a ENLYFQG/S sequence that follows the polyhistidine tag. The gene include many restriction sites to allow for quick mutation of amino acids by using short primers. Mutations can also be introduced in the gene by flanking the restriction sites on both sides. The restriction sites can be cleaved by NdeI and BamHI restriction enzymes. The design was synthesized, sequenced, and cloned into a pET11a vector. The construct

is illustrated in Figure 2.3.The pET11a plasmid is 5675 bp and the insert (mutant dArmRP codon sequence) is 800 bp which makes the entire construct 6475 bp.[20]

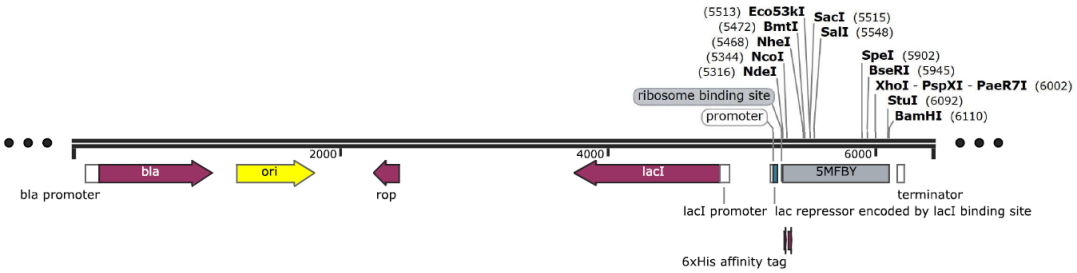


Figure 2.3: A linearized representation of the designed pMutantdArmRP plasmid construct. The numbers associated with the restriction site enzymes indicate the specific base pair positions.A pET11a plasmid with T7/lac system is used[20].

2.1.1 Enzyme Characteristic

The mutated designed armadillo repeat protein (dArmRP) is cytosolic enzyme weighing 27kDa. It has a midpoint denaturation temperatures (TM) of 102°C [20]. The mutant and native enzyme fold in similar way. Enzyme has a high secondary structure stability at high temprature with fast refolding rate. The highly symmetric triangular staircase repeat of the armadillo repeat protein contributes to its exceptionally stable secondary structure at high pH and in the presence of organic solvents (15% acetonitrile)[20]. The enzyme was also found to be stable at high pH (5-10).The negative electrostatic surface potential increased with increase in pH. The histidine component of the catalytic triad possesses a negative surface charge, which is essential for its role in the catalytic process, as it must be deprotonated to function effectively [20]. The surface potential of this catalytic histidine undergoes a shift from positive to negative when the environmental pH changes from 7 to 9. This transition occurs because histidine becomes deprotonated at pH levels above 8, thus making conditions above this pH more conducive to catalysis. Additionally, the presence of Glu/Lys salt bridges, which are abundantly distributed across the surface of the dArmRP, significantly contributes to the protein's high stability.The pKa shifts by lowering the pKa of Glu and increasing the pKa of Lys [20]. It can degrade polycarbonate and also has activity towards *p*-nitrophenyl butyrate (1.2 µM min^{-1}) and bis-(4-nitrophenyl) carbonate (1.3 $\cdot$ 10 s^{-3} s^{-1})[20].

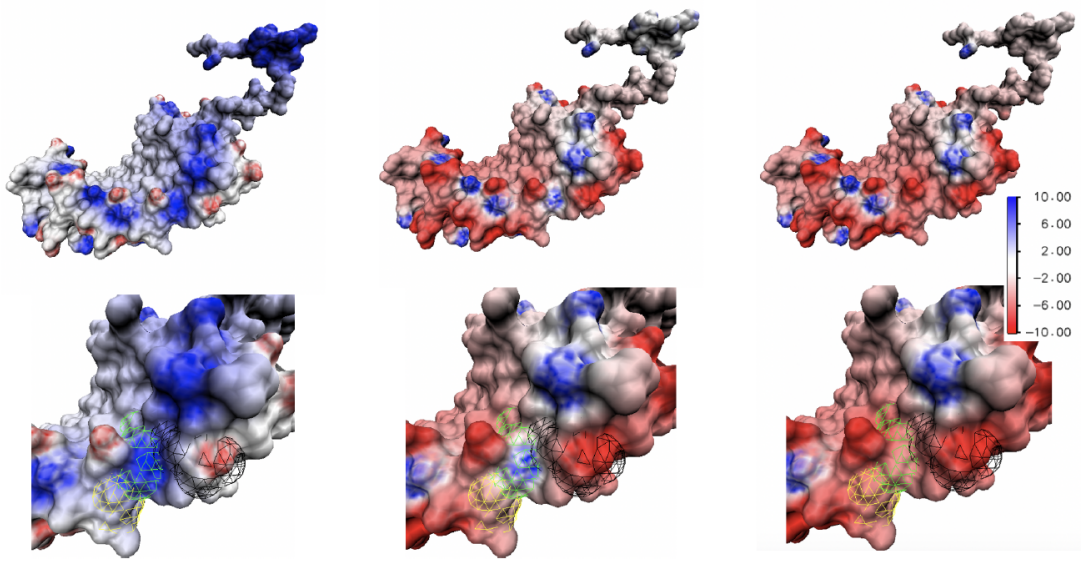


Figure 2.4: *The presented images depict the varying electrostatic surface potential of the modified dArmRP enzyme, inclusive of a polyhistidine tag, across different pH conditions: pH 4, pH 7, and pH 9. In each set, the top images demonstrate the global electrostatic surface potential across the full protein. This offers a broad view of charge dispersal over the protein's surface, which can provide insights into the protein's interactions within a solution or with other proteins or ligands.The bottom images in the sets give a focused view of the active site of the enzyme, with catalytic residues distinctively marked. In this view, Serine (Ser) is indicated in yellow, Histidine (His) is in green, and Glutamic acid (Glu) is in black. These depictions allow a detailed examination of the active site's electrostatic environment under the given pH conditions, which can influence its catalytic activity. Figure taken from- RECOMBINANT PRODUCTION AND CHARACTERIZATION OF A DESIGNED POLYCARBONATE DEGRADING ENZYME- semster project by Group 5.319 [20].*

2.1.2 Hydrolase activity

Hydrolase activity refers to the ability of enzymes called hydrolases to catalyze the hydrolysis of chemical bonds in biomolecules[22], [23]. Hydrolases facilitate the breaking of chemical bonds by utilizing water, typically leading to the division of larger molecules into smaller ones. Although the hydrolysis of these bonds is an energetically favorable (downhill) process, it occurs slowly under physiological conditions. Hydrolases enhance the rate of these reactions by reducing the activation energy necessary for the reactions to proceed.[23]. The active site of the enzyme provides a favorable environment for the reaction to occur, and the enzyme-substrate complex stabilizes the transition state of the reaction, making it easier for the bond to be broken[22], [23]. The mechanism of hydrolase catalysis is similar for different types of hydrolases, but the specific details of the mechanism can vary depending on the enzyme and the substrate involved[22], [24].Hydrolases are the most diverse class of enzymes and are involved in many biological processes, including digestion, metabolism, and signaling. Hydrolase activity is essential for many biological processes and is also important in industrial applications, such as the production of detergents and phar-

maceuticals[22]. Hydrolases can be classified in many different ways, including by sequence or structure-based methods, and by the type of bond they hydrolyze[23].

The catalytic residues in the enzyme mutant dArmRP, play distinct roles in the hydrolysis mechanism. Serine, already present in the native dArmRP, binds to the the carbon of the carbonyl of the substrate, forming the enzyme-substrate complex. The addition of histidine enhances the reactivity of the catalytic serine by abstracting and donating protons, making it more effective in breaking down polycarbonate. Aspartic acid or glutamic acid helps orientate the histidine, facilitating proton transfer to or from serine. The formation of a tetrahedral intermediate during substrate binding generates charge, which can be stabilized by an oxyanion hole. In the mutant dArmRP enzyme, threonine is positioned opposite the serine to form the oxyanion hole. This stabilization is crucial for the enzymatic reaction. The introduced mutations involve replacing certain amino acids with smaller residues, reducing the overall number of positive or negative charges in the modified enzyme compared to the native dArmRP[20].

The catalysis mechanism of Ser-His-Asp/Glu consists of two steps: acylation and deacylation. During acylation, the enzyme binds to the substrate, facilitating the release of the alcohol component. However, a portion of the substrate remains bound to the enzyme. In deacylation, hydrolysis occurs, leading to the release of the carbonate portion. After deacylation, the enzyme is ready to catalyze a new reaction[20].

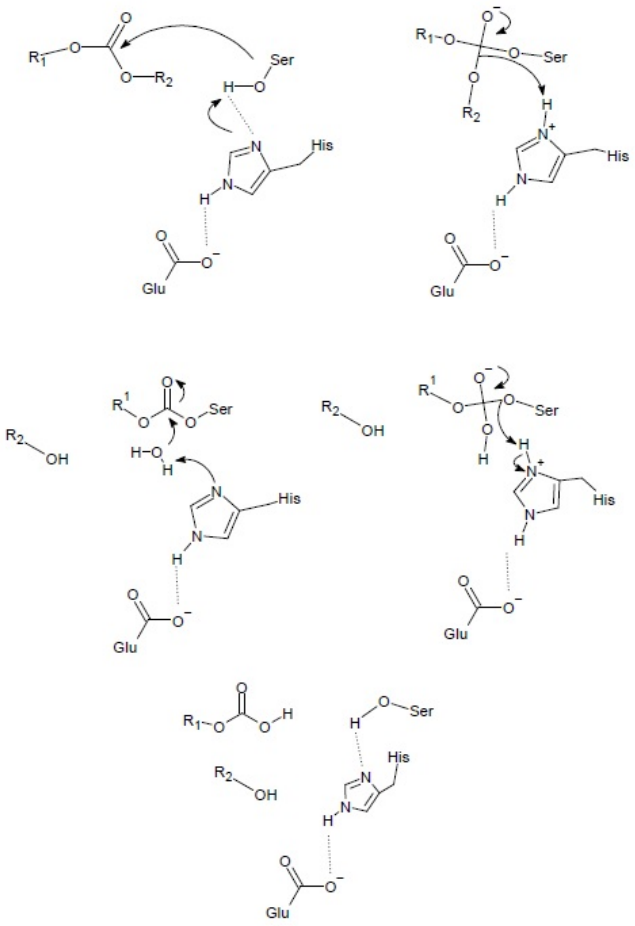


Figure 2.5: *The enzymatic cleavage mechanism of a carbonate group proceeds as follows: To initiate the process, a proton is transferred from serine (Ser) to histidine (His). This proton abstraction makes Ser negatively charged and capable of interacting with the carbon atom of the substrate's carbonyl group. The interaction between the negatively charged Ser and the carbonyl carbon forms an unstable tetrahedral intermediate. The carbonyl double bond is then reestablished, pushing the better leaving group (OR2 in this scenario) away, which concurrently gains a proton from His. In the next step, His pulls a proton from a water molecule, generating a hydroxide ion. This hydroxide ion acts as a nucleophile, attacking the carbonyl carbon and forming another unstable tetrahedral intermediate. Again, the instability of this intermediate causes the reformation of the carbonyl, facilitating the departure of Ser (now a more competent leaving group) upon receiving a proton from His. The enzymatic process is completed with the release of a carbonate (R1OCOOH) from the enzyme active site. Figure taken from- RECOMBINANT PRODUCTION AND CHARACTERIZATION OF A DESIGNED POLYCARBONATE DEGRADING ENZYME- semster project by Group 5.319[20].*

2.1.3 Enzyme promiscuity

The de-novo enzyme is designed as a carbonate ester hydrolase and has been demonstrated to possess hydrolytic activity towards polycarbonate [20].

Enzymes are capable of catalyzing substrate reactions that are structurally homologous to the previously known designated substrate. Some have even demonstrated to react with fundamentally different compounds altogether[25], [26].Enzyme promiscuity has been defined as the phenomenon of an enzyme catalyzing any other substrate that is not part of the native reaction [27]. Figure 2.8 illustrates the concept of promiscuity in enzymes simply.

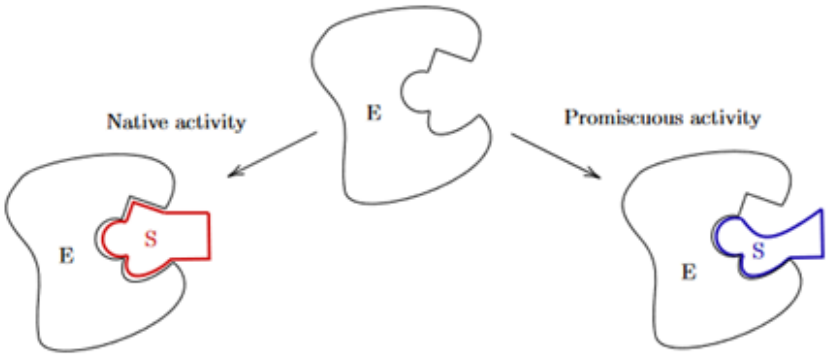


Figure 2.6: This image illustrates an enzyme displaying native behavior and promiscuous behavior. The native behavior is characterized by the complementary binding of substrate and enzyme, while the promiscuous activity has been characterized by an incomplete usage of the active site as the substrate continues to be successfully catalyzed by the enzyme[20].

Directed evoloution can be used to induce promiscuous activity in enzyme. Directed evolution is a protein engineering technique that draws inspiration from the "survival of the fittest" idea – a core principle of Darwinian evolution. A functional protein is first selected and placed in a test tube. Then, several generations of mutations is induced to create different iterations of the same protein. These iterations are then subjected to the thermodynamic conditions that the protein is expected to perform in, and the fittest of those are then taken through more generations of mutations[28].There is no one single characteristic in an enzyme that can define promiscuity. The traditional concept of "lock and key" has been proven inadequate. Factors like accommodation, conformational selection, subsites, cofactors, and protonation play much more active roles[27]. Accommodation arises from the numerous reactions that employ catalytic geometries, with the differentiation of the enzyme being determined by its binding motif. To demonstrate promiscuousness, enzymes may bind to substrates with hydrophobic interactions due to the nonspecific nature of these reactions. Thus, the catalytic geometries of these enzymes may accommodate for promiscuous activity. Conformational selection may contribute by exploiting the less favorable conformers of the enzyme to interact with non-native substrates. Research has confirmed that the subsites make further, more specified use of catalytic geometries. For example, if a catalytic triad is known to induce the enzyme's native catalysis reaction,

then a subdivision of the amino acids in the sequence can be utilized for another catalysis reaction[28]. In the case of metalloenzymes, cofactors can induce conformational changes in the protein to interact with different substrates based on the ions the catalytic center is exposed to. Protonation states can also aid the promiscuity of an enzyme, with different titratable residues acting as bases or acids to catalyze a wider variety of catalyzing reactions [27]. Even though all these factors contribute to an enzyme's promiscuity, they also conversely contribute to the substrate specificity of the enzyme as well. Either KM or kcat can be individually affected, however both variables are linked to each other. One cannot be considered without taking the other into consideration as well[29].

In this project, the mutated dArmP is subjected to directed evolution, random mutations in the gene have been introduced through random mutagenesis. It is to increase the hydrolase activity of the polycarbonate degrading enzyme by inducing promiscuous activity towards long-chain and short chain hydrolase substrates. If an enzyme can degrade polycarbonate, it is not necessarily a polyesterase. Polycarbonate is a thermoplastic polymer that contains carbonate groups, while polyester is a category of polymers that contain ester groups[30], [31]. *Rhizopus d.* can degrade aliphatic polycarbonates prepared from epoxides and carbon dioxide with a single enzyme and *Amycolatopsis sp.* can degrade polycarbonate by a polyester-degrading strain strain HT-6 [26], [32].

2.2 Directed Evolution

Directed evolution is a method inspired by natural evolution. It has been adapted for laboratory applications in order to develop biological entities with desirable attributes[33], [34]. It helps in speeding the evolutionary processes of biological molecules and systems within an in vitro setup [35]. Directed evolution allows for the enhancement of protein stability and biochemical functionality. This is achieved through repeated rounds of mutation and selection [36].

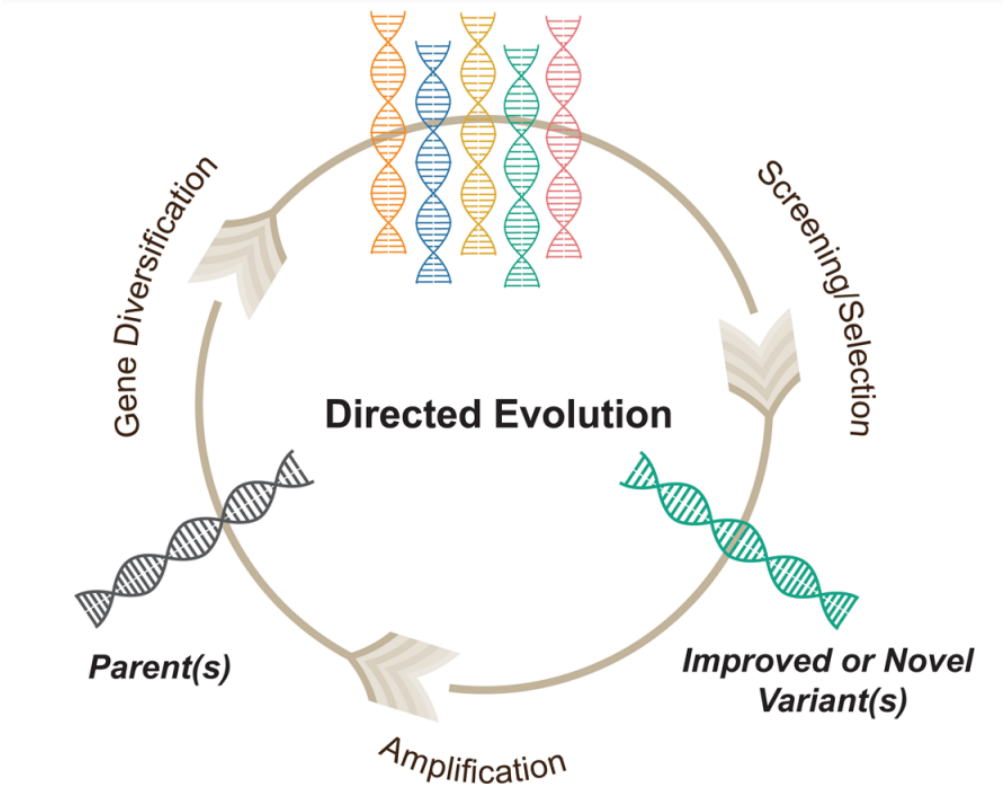


Figure 2.7: Principle of directed evolution. A traget gene is chosen, mutations are introduced through in-vitro and in-vivo methods. After repeated rounds of screening the improved variants with desirable qualities are obtained. The image is from [35].

Directed evolution has been widely used to improve the efficiency of enzymes in various applications, such as biotechnology, pharmaceuticals, and plant research[18], [37], [38]. To improve enzyme efficiency, directed evolution involves subjecting a gene to iterative rounds of mutagenesis and screening or selection for variants with the desired characteristics [36], [39].The principle of directed evolution is illustrated in Figure 2.7. The methods of gene diversification can be divided into two fundamental types, in-vivo and in-vitro[35]. The in-vitro approach involves random mutagenesis, focussed mutagenesis and recombination based DNA shuffling. Random mutagenesis enables rapid engineering of a target protein without requiring knowledge of the protein structure-function relationships[35]. When structure-function links are ambiguous random mutagenesis is a better option for reaching functional library members. than focusing library diversity on wrongly chosen residues, which may not give the appropriate activities upon mutation[35], [40].

Directed evolution can be used to improve enzyme efficiency by enhancing enzyme activity through altering their active sites or increasing their affinity for substrates[36], [38], [39].It can also help in increasing the specificity of enzymes for a particular sub-

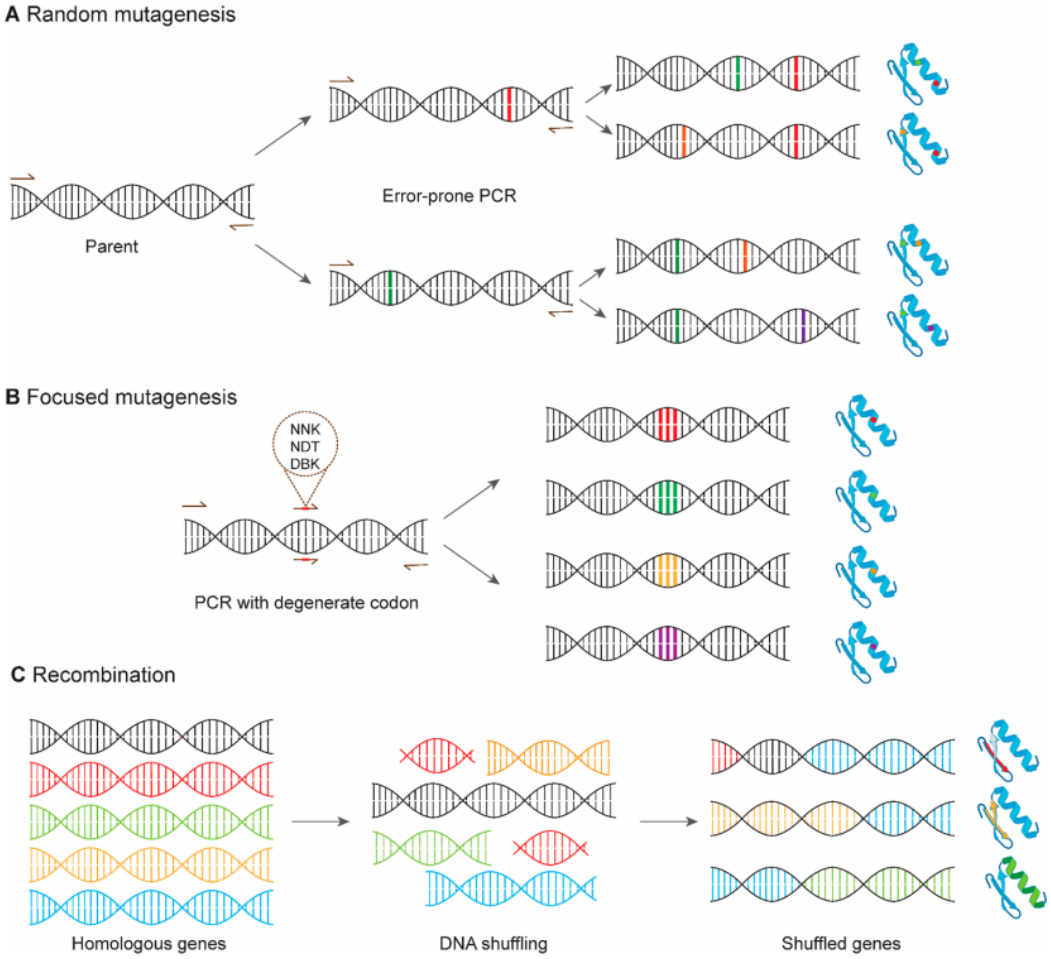


Figure 2.8: The figure shows various directed evolution methods. The image is taken from [35].

strate or product by altering their substrate-binding pockets[38], [39].Enzymes produced through directed evolution have improved stability. The process increases their resistance to heat, pH, or other environmental factors[36], [38], [39]. Directed evolution can modify the kinetics of enzymes by altering their rate constants or changing their reaction mechanisms[38].

For the purpose of this project an intresting application of directed evolution, Protein breeding is used for the optimization of enzymes. In this mutagenesis is used for creating gene diversification between enzymes. The targeted DNA is first isolated, placed in the parental pool, it is then crossed, cloned and then expressed to produce recombinant product to be screened for desired characteristic. The selected variants are then subjected to another cycle of mutagenesis and selection till the desired level of activity is achieved[41].

2.3 Recombinant protein expression

Organisms serve as biologically active factories capable of producing a vast array of proteins. Every organism synthesizes proteins, each with a distinct role vital to the organism's functionality and survival[42] . The timescale for protein production varies widely among organisms. For example, bacteria can produce proteins within a matter of days, while mammalian systems might require several months for the same process.The advent of recombinant protein production has radically transformed the field of biochemistry. In this realm, a significant number of proteins are produced and investigated for their structural and functional attributes. Key determinants of success in the protein industry revolve around the speed of production and yield[43]. Advancements in genetic engineering and molecular biology have enabled scientists to surmount these challenges. It is now feasible to achieve substantial quantities of virtually any protein type by leveraging the power of recombinant expression systems[42].

Recombinant proteins are acquired by integrating a protein gene from an organism typically characterized by slow production (e.g., mammals) into a host organism generally known for its faster production rates (e.g., bacteria)[44]. This approach enables the high-yield production of proteins once expression is triggered. The extraction of the desired protein necessitates the use of protein purification and identification systems. Recombinant systems express proteins in different types of cultures, which may be either prokaryotic cells - often used for smaller proteins - or eukaryotic cells, such as yeast or mammalian cells, which are utilized for the expression of larger and more complex proteins[45]. Key factors like protein quality, functionality, production speed, and yield must be taken into account when choosing an appropriate expression system for recombinant protein production. The selection of a system is highly contingent upon the characteristics of the protein to be produced[44], [46]. For instance, if

the protein necessitates glycosylation or phosphorylation, the use of a eukaryotic expression system is warranted. Despite these variations, the *E. coli* host system is often the preferred choice for protein expression, given its cost-effectiveness, quick culture times, high biomass, and potential for high protein yields.Following the selection of the host system, an appropriate plasmid must be chosen for the transformation and subsequent expression[47].

A plasmid is a small, extrachromosomal DNA molecule, usually circular, that is capable of autonomous replication (contains its own origin of replication). Plasmids naturally occur in many prokaryotes and often contain critical genes necessary for antibiotic resistance, but they are also used as vectors[44]. There are two types of vectors: cloning vectors, used to generate large amounts of copies of the same plasmid, and expression vectors, used to express the desired protein in high amounts[44]. All plasmid expression vectors share some common features: a selection marker (e.g., antibiotic resistance), origin of replication, effective transcriptional promoter, 5' untranslated region (5'UTR), and a translation initiation site. An effective promoter should allow a protein accumulation of 10-30% of the total cellular proteins, have a low basal activity, enable inexpensive induction, and have activity that can be precisely tuned[44].

The vector used in this project is the pET-11a vector with an AMP^R resistance marker and a T7/lacZ expression system. With this vector and promoter, the expressed recombinant protein can accumulate up to 50% of the total cell proteins, and it can be induced using isopropyl-β-D-thiogalactopyranoside (IPTG)[43]. Although *E. coli* is commonly used for expressing recombinant proteins, this system has some major drawbacks. One of the major drawbacks is the correct disulfide bond formation of more complex proteins (e.g., mammalian proteins), which leads to the formation of inclusion bodies[46]. Inclusion bodies (IB) are the aggregation of proteins caused by their instability in the *E. coli* microenvironment, which may differ from the original environment (pH, redox potential, cofactors, etc.). Different approaches have been developed to obtain the correct disulfide bond formation and avoid the formation of IB. One of them is adding a signal peptide to the N-terminus of the recombinant protein to direct the protein into the periplasm for proper folding[46].

Escherichia coli

Escherichia coli (*E. coli*), a Gram-negative, rod-shaped bacterium, is widely recognized as the most commonly used organism for the expression of recombinant proteins[43]. This facultative anaerobe efficiently generates ATP through aerobic respiration but can adapt to fermentation in the absence of oxygen. Predominantly, *E. coli* forms a part of the normal gut flora in humans and other animals. Although generally non-pathogenic, certain strains do possess pathogenic qualities, often acquired via plasmids, transposons, or bacteriophages[48].

E. coli offers several advantages for recombinant protein expression. Key benefits include its rapid generation time of approximately 20 minutes under optimal conditions, ease of maintaining these conditions, and the simplicity of transformation using plasmids. Standard cultivation involves growing *E. coli* in LB medium at 37°C, typically yielding a cell density of less than 1×10^{10} cells/mL. However, using more complex media can enhance cell density, even during recombinant protein production, which is beneficial given that metabolic demands from protein production can impact generation time. Among the numerous *E. coli* strains available, the BL21(DE3) and certain K-12 strains are notably significant for initial expression screens[43].

Cloning with the DH5α Strain

The DH5α strain is a popular *E. coli* cloning strain, renowned for its ability to maintain the stability of inserted DNA. This characteristic makes DH5α an ideal choice for storing recombinant DNA for later use in expression strains[49].

Expression in the BL21 (DE3) Strain

The BL21 (DE3) *E. coli* strain, distinguished by its absence of Lon and OmpT proteases, is specifically designed for recombinant gene expression. It contains the λDE3 lysogen with the T7 RNA polymerase gene, regulated by the lacUV5 promoter. Inducing this strain with IPTG in low-glucose conditions triggers T7 RNA polymerase production, which selectively transcribes genes under T7 promoter control. This makes BL21 (DE3) adept at expressing genes regulated by a range of promoters such as T7, T7-lac, tac, lac, trc, PrhaBAD, ParaBAD, and T5[50].

Utilizing the pET-11a Vector

The chosen vector for this study is pET-11a, an expression vector utilizing the T7 expression system. This system, governed by the T7 promoter, specifically recruits T7 RNA polymerase for the transcription and subsequent expression of the recombinant gene[51]. The vector features an N-terminal epitope T7 tag for gene product localization[52] and is regulated by the lac promoter, with expression induced by lactose or IPTG. The pET-11a vector is particularly well-suited for the Origami 2 (DE3) strain[53].

Figure 2.9 shows a picture of pET-11a vector.

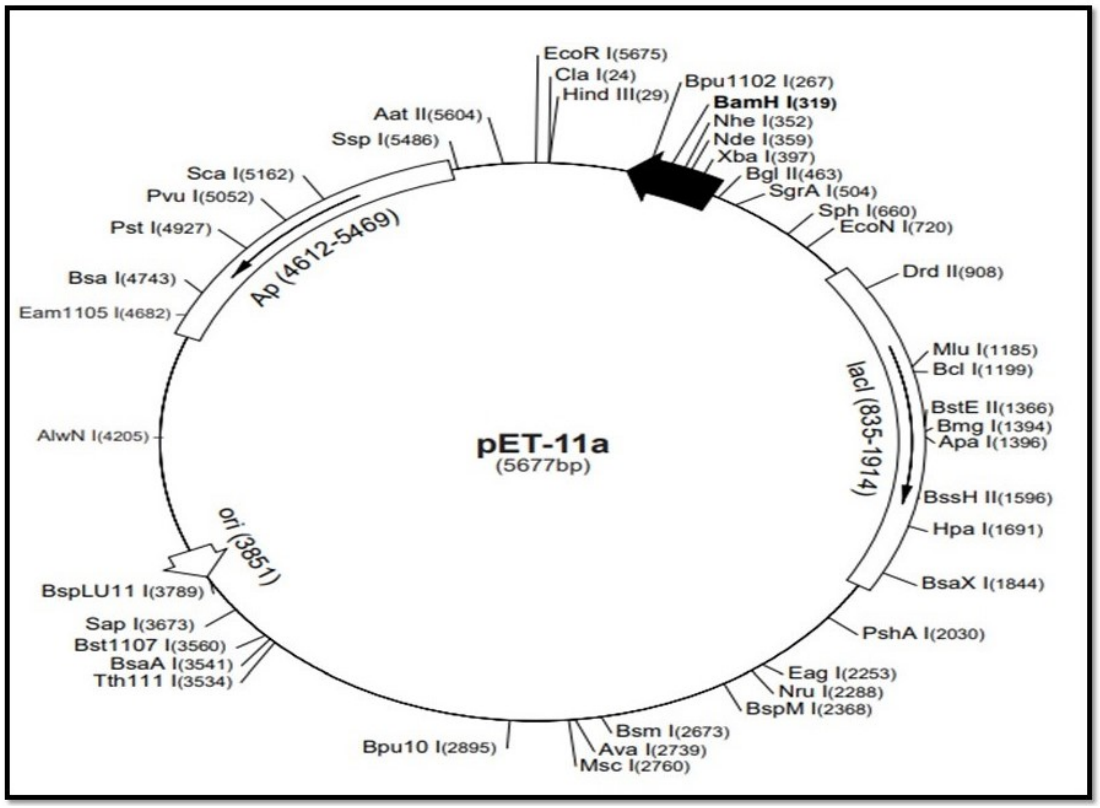


Figure 2.9: Plasmid map of the vector pET-11a with various restriction enzyme sites.These sites, marked by abbreviations such as EcoRI, BamHI, and XbaI, correspond to specific sequences in the DNA where each enzyme will cut.The arrow indicates the direction of transcription for an inserted gene, and the overall size of the plasmid is given as 5677 base pairs[53].

2.4 Random mutagenesis

Genetic diversity in DNA occurs naturally as a result of errors generated during genome duplication or DNA damage from UV radiation or exposure to reactive substances. Modern polymerase chain reaction (PCR) techniques are used to produce random genetic diversity in DNA in the lab. By using error-prone PCR, genetic variation is most frequently produced.

The mutation rate and mutational spectrum are two critical factors to consider when conducting a random mutagenesis experiment[35]. Mutation rate refers to the frequency at which mutations occur in a given organism or DNA sequence over a defined period of time. It is usually expressed as the number of mutations per unit of DNA or per generation[35]. Mutational spectrum refers to the distribution and types of genetic mutations that occur within a genome[35]. Conventional genetic diversi-

fication procedures, using chemical and physical agents, cause random DNA damage. These include base analogues like 2-aminopurine, alkylating chemicals like ethyl methanesulfonate (EMS), deaminating drugs like nitrous acid, and UV light. Chemical mutagenesis can be used to randomly deactivate genes for genome-wide screening, however due to mutational spectrum biases, it is less frequently utilized in directed evolution. The error rate during DNA replication is usually increased by non-chemical means of random gene mutation.

Because of the low mutation rates and lack of control inherent in most in vivo random mutagenesis approaches, in vitro random mutagenesis strategies have gained popularity. This method entails amplification of the gene in the presence of manganese ions and altered magnesium concentration. It uses mutagenic deoxyribonucleotide triphosphate (dNTP) nucleotide analogs that encourage the DNA polymerase to incorporate nucleotides incorrectly. [54]. It also uses DNA polymerases' poor fidelity under particular conditions to create point mutations during PCR amplification of a target gene.[35].

In this project the Mn-DiTP method for error-prone PCR is used because normal error prone PCR has transition;transversion ratio bias because they favor transitional point mutation over transversional, making it difficult to introduce all 20 AA at each position[41]. In transversional point mutation a pyrimidine is exchanged with a purine or vice versa. In translation point mutation a pyrimidine is exhachanged with a pyrimidine or a purine is exchanged with a purine[55]. Mn-DiTP error prone PCR consist of two PCR. In the initial PCR, manganese is introduced into the PCR reaction buffer. This addition causes the less stringent *Taq* DNA polymerase to sporadically incorporate point mutations. Deocyinosine triphosphate is added in the second PCR is added to compensate for the transition transversional bias of the first PCR[56], [57].The final library has error rates of 1 nt/kb due to the presence of Mn2+ and an excess of dGTP and dTTP in the amplification process. The amount of Mn^{2+} in the reaction or the number of amplification cycles can both, within certain bounds, affect the extent of mutagenesis.[57]

2.5 Activity Assay

For selecting an appropriate substrate for the hydrolysis test several factors were considered. The substrate was selected to be a lipid that can detect the activities of esterases, true lipases, and polyesterases. It is water-insoluble to form an opaque medium, inexpensive and readily available[58].

The role of a polycarbonate degrading enzyme is to degrade polycarbonates by acting on the ester bonds present in the polycarbonate structure. Triglycerides consisting

of glycerol bound to three fatty acids via ester bonds. An enzyme that can break down the ester bonds in polycarbonate can also theoretically break down the ester bonds in triglycerides, although the surrounding chemical environment is different. Enzymes often don't just act on one specific substrate but on a range of similar substrates, enzyme promiscuity. By testing the enzyme on lipids, it is tested for its specificity and an incresaded hydrolase activity towards polycarbonates. A polycarbonate degrading enzyme may not show activity against lipids as a substrate. The exact three-dimensional shape and electronic environment of the substrate can greatly influence enzymatic activity.But variants from random mutagenesis library may have evolved to have higher hydrolase activity towards polycarbonate and thus may show activity towards lipids.

Tributyrin plate assay is a method used to detect lipolytic enzymes which hydrolyzes tributyrin oil. It is a triglyceride consisting of glycerol and three short chain fatty acids. Upon breakdown, these components may be converted into diverse end-products, which the cell can then use for energy production or in various other metabolic processes. If the microorganism produces polyesterases, it will hydrolyze the tributyrin oil in the agar medium, resulting in the formation of a clear zone around the colony. The size of the clear zone around the colony is proportional to the amount of lipase produced by the microorganism. A larger clear zone indicates a higher level of lipase production, while a smaller clear zone indicates a lower level of lipase production.[59]

The principle behind the olive oil plate assay is that lipase hydrolyzes the ester bonds in olive oil, producing glycerol and fatty acids[60]. The hydrolysis of olive oil by lipase reduces the pH and a green zone or halo around the bacterial growth on the LB-agar plate is observed, indicating the presence of lipolytic activity[61].

For the purpose of this project, tributyrin and olive oil plate assays are used to test the hydrolase activity. These assays are not specific to carbonate esters. Carbonate ester hydrolase (CEH) and carboxylic ester hydrolase catalyze the hydrolysis of ester bonds but they have different substrates[62]. Carboxylic ester hydrolase can hydrolyze tributyrin as well as olive oil. [63], [64].Mutant candidates demonstrating hydrolysis activity for these substrates will be selected for sequencing to identify mutations and study the relationship between enzyme sequence and function .

2.6 Designing a high-throughput screening for Plastic degradation

Designing a high-throughput assay for plastic degradation presentsis difficult because of heterogeneity of Plastics. Plastics can vary greatly in terms of chemical composition, physical properties, and additives. Therefore, an assay effective for one type of plastic may not work for another[65]. Plastics are also typically insoluble and have a high melting point, which makes them difficult to handle in liquid-based high-throughput assays.Detecting and quantifying plastic degradation is difficult because it often requires sensitive, indirect methods of measurement[65]. For instance, monitoring the decrease in plastic weight, the release of CO2, or changes in water turbidity due to microplastic formation can be complex to set up in a high-throughput manner.If the assay relies on microbial action (like using bacteria to break down the plastic), it may be difficult to ensure consistent and rapid growth of the microbes. Additionally, the activity of microbes can be influenced by a wide variety of factors (like temperature, pH, and nutrients), making it hard to standardize conditions for high-throughput screening.Despite these challenges, the development of high-throughput assays for plastic degradation remains an active area of research. Improved techniques could significantly accelerate the discovery of new methods or organisms capable of breaking down plastics[65].

2.7 Construction of the library

This section describes the construction of the library by the professor. The pET-11a plasmid vector with GFP gene was cleaved with BamH1 and NdeI from the construct. The DNA from the error prone PCR was ligated into the cleaved plasmid. 20µL of ligated plasmid DNA was acquired. 5µL of ligated DNA was used to transform 100 µL of E.coli BL21(DE3) cells. The product was poured on a LB-ampicillin plates containing IPTG and was labelled as transformation 1. 15µL of the remaining ligated DNA was used to transform 100 µL of E.coli BL21(DE3) cells. The product was poured on a LB-ampicillin plate without IPTG and was labelled as transformation 2. The plates were incubated at 37°C overnight to grow the bac- teria. A second ligation of the random PCR product with cleaved plasmid vector was performed and the whole volume 20µL was used to transform 100 µL of E.coli BL21(DE3) cells. They were grown on LB-ampicillin plates without IPTG and was labelled as transformation 3. The non fluroscent colonies from the theses transformation were picked for the screening. Figure 2.10 describes the procedure as a flow chart. The obtained colonies were further divided into 4 batches based on the transformation they were picked from. In order to confirm that the GFP gene is completely cleaved from the pET-11a plasmid and the DNA from error prone PCR is ligated into the plasmid and to identify background expression controls were used. In order to test the variants for these background ex-

pression the *E.coli BL21 DE3* cells with pET-11a plasmid vector with GFP gene were used as a control on each plate for comparison of GFP expression. It is labelled as **G** when used in plates. The *E.coli BL21 DE3* cell colonies transformed with plasmid vector with original 5MFBY gene insert (before directed evolution on the gene) is also used as a negative control for testing hydrolase activity. It is labelled as **C** when used in plates. It has less background expression. It expresses the original version of the dArmP enzyme, a polycarbonate hydrolase, which in theory can not degrade the triglycerides or express GFP.

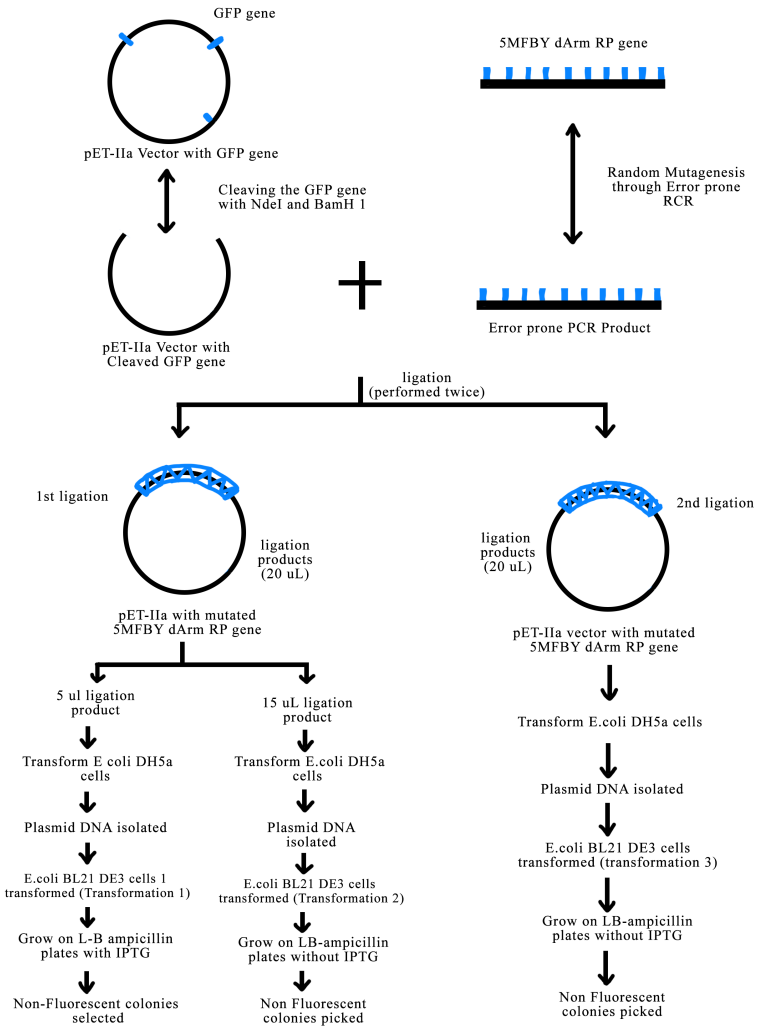


Figure 2.10: Description of the procedure for the constrcution of library by the professor [53].

3 Materials and Methods

3.1 Materials

This section lists the materials used in the experiments. All chemicals used are listed in Table 3.1, respectively.

Table 3.1: Chemicals used.

Chemical	CAS no	Lot no	Supplier
Agar	9012-36-6	080M1575	Sigma Aldrich
Agarose	9012-36-6	19G0256361	VWR Life Sciences
Ampicilin	69-53-4		In house stock
EDTA	60-00-4	BCBN7602V	Sigma Aldrich
Ethanol 96%	200-578-6	1680643	Kemetyl
Ethidium Bromide	1239-45-8	SLBF7132V	Sigma Aldrich
GeneJET plasmid miniprep kit		00714520	Thermofisher Scientific
IPTG	367-93-1		In house stock
DNA Loading Dye	2650-17-1	00071285	Fermentas
LMW Ladder	16858-02-09	N3232S	New England Biolab
Olive oil			
Sodium Chloride	7647-14-5	17L184138	VWR Life Sciences
TAE Buffer			In house stock
Tributyrin			
Tryptone	91079-40-2	02235-2023-05	Piove Di Sacco Italy
Yeast Extract		M0186W	VWR Chemicals

Table 3.2: Materials used for cutting of DNA and bacterial strains used for transformation.

Enzymes & Cloning Materials	Cas & Lot no	Supplier
AccuGENE Molecular Biology Water	8MB248	Lonza
BamHI	10020678	New England Biolabs
E.coli BL21(DE3)		New England Biolabs
CutSmart 10x Buffer	10043914	New England Biolabs
E.coli DH5α	1321707	New England Biolabs
NdeI	10020970	New England Biolabs
Sac II	——	New England Biolabs

3.2 Methods

3.3 Lysogeny Broth-Medium

The 1 Litre LB medium was prepared by adding 10g/L tryptone, 5g/L of yeast extract, 5 g/L sodium chloride and the solution was autoclaved. In order to make agar plates 15 g of agar was added in the solution and it was stirred till a homogenous solution was obtained and the solution was autoclaved. The solution was bought to 55ºC and 1 ml ampicillin was added and the medium was poured in plates. The plates were left to solidify and stored for further use.

3.4 Bacterial growth in plates

In order to grow the *Escherichia coli* (*E. coli*) strains (DH5α and BL21(DE3)) containing the protein of interest and later express it. *E. coli* liquid precultures were made. Precultures for BL21(DE3) and DH5α *E. Coli* strains were made following the same procedure. *E. Coli* cells from the LB-ampicillin (LB-amp) agar plates cultures were transferred into a 10mL culture tube containing 5mL of LB medium and 20 μL of stock ampicillin (100mg/mL). The solution was mixed until the culture was completely dissolved. Then, the liquid preculture was incubated at 37ºC, and 275 rpm overnight.

The procedure of inoculating the LB-amp agar plates with liquid precultures of *E. coli* is as follows: The transformed competent *E. coli* cells were inoculated into LB-ampicillin plates, which were then incubated at 37ºC overnight. An LB-ampicillin plate without ampicillin resistance plasmid was incubated as well as a reference plate. After the incubation, the plates were stored in the fridge at 4ºC for later use.

3.5 Isolation of Plasmid DNA from *Escherichia Coli*

The transformed competent E. coli cells from the culture plates prepared as explained in (section 3.4) were used to prepare a liquid culture as explained in (section 3.4) and incubated overnight at 37ºC. The DNA isolation procedure is the same for all cultures as described below using GeneJET plasmid miniprep kit.

The culture was centrifuged at 7,000 rpm for 10 minutes at 10ºC. The supernatant, consisting of the LB medium, was discarded by decantation, which left a pellet made up of cells. The pellet was then dryed out as much as possible, pouring the remaining LB medium over a tissue to avoid the interference of LB with the lysis solution. Once dryed out, the pellet was resuspended in 250 μL of resuspension solution. The solution was vortexed until the pellet was completely dissolved. The solution was then transferred into a new Eppendorf tube. Then, 250 μL of the lysis solution was added and the solution was mixed gently by flipping the tube. 350 μL of the neutralising buffer was added to the Eppendorf tube, and the solution was mixed once again. The solution was centrifuged, in a microcentrifuge, which ran at 12,000g for 10 minutes. The supernatant, which contains plasmid DNA, was carefully pipetted and transferred into a spin column. The pellet, composed of cell debris and unwanted chromosomal DNA, was discarded. The spin column was then centrifuged for a minute under the aforementioned conditions. The flowthrough in the tube, composed of various debris of similar size to the plasmid DNA, was decanted away. The material left in the spin column was washed with 500 μL of the wash solution. The column was once again centrifuged under the same conditions for a minute and the resulting flowthrough was decanted away. This step was then repeated once more, before the columns were centrifuged again without the wash solution for a minute to remove the remaining washing solution. The filter part of the spin column was moved to a new Eppendorf tube and 50 μL of the elution buffer were carefully added, ensuring the tip of the pipette was close to the membrane without touching it. The tube was incubated for two minutes at room temperature, so that the elution buffer reached the entire membrane, then it was centrifuged for another two minutes at 12,000 rpm. The supernatant was collected and transferred into a new Eppendorf tube and stored at 4ºC for later use.

3.6 Agarose Gel Electrophoresis

For quick analysis of DNA fragments throughout the work, agarose gel electrophoresis was used to determine fragment sizes and ensuring the cutting of GFP and insert fragments with restriction site.

3.6.1 Tributyrin and Olive oil Plates

To test the variants activity on short chain substrates LB-agar plates infused with tributyrin is used and for activity on long chain substrates Olive oil infused plates are prepared. The substrate differ in their most abundant fatty acid.

For the preparation of the LB-agar plates infused with olive oil 5 g/L of the oil was added LB agar medium cooled down to 55C containing 100 µ L ampicillin (100 mg/L) per 100 ml agar medium and 100 µ L IPTG (100 mg/L) per 100 ml agar medium. 125µL/ L of the dye Brilliant Green (72 mg/L in 96 % ethanol) was used as a pH-indicator. The solution is immediately mixed using an ULTRA-TURRAX T 25 from IKA with a S 25 N-8 G ST mixing drill from IKA at 20,000 rpm.

For the preparation of the LB-agar plates infused with tributyrin plates 7 g/L of the oil was added LB agar medium cooled down to 55C containing 100 µ L ampicillin (100 mg/L) per 100 ml agar medium and 100 µ L IPTG (100 mg/L) per 100 ml agar medium. The solution is immediately mixed using an ULTRA-TURRAX T 25 from IKA with a S 25 N-8 G ST mixing drill from IKA at 20,000 rpm.

The oil-infused agar solution is poured into petri dishes, allowed to solidify, and subsequently stored at 5 °C. The mixer is cleaned before, in between, and after use by using multiple rounds of milli-Q water at 20,000 rpm followed by 70 % ethanol at 20,000 rpm.

3.7 Screening of library

Tributyrin and olive oil plates infused LB-ampicillin plates with IPTG to induce activity were used for screening the candidates chosen from library. Four rounds of screening were conducted to identify candidates exhibiting enhanced hydrolase activity. The variants were evaluated based on three criteria. First, they were screened at 25°C for background expression of GFP, as this is the optimal temperature for GFP expression in *E. coli*. Second, their activity at 37°C was assessed, as this is the optimal temperature for protein expression in E. coli, thereby facilitating the study of enzyme activity. Lastly, the candidates were compared against one another to identify the variant demonstrating the highest increase in hydrolase activity. The culmination of these evaluations will allow for the selection of the most promising candidate for further studies and potential applications as hydrolases.

For the tributyrin screening, the presence of a halo around the colonies was expected, while for the olive oil screening, a dark green color around the colony indi-

cated activity. Streaking method with the help of toothpick was used to transfer the variants on the plates and the area was labelled.

The condition for the screening are explained below:

Screening 1 at 25°C

To investigate the background expression and hydrolase activity, both the variants and controls were meticulously transferred to LB-ampicillin agar plates infused with tributyrin and olive oil using toothpick and incubated at 25 degrees Celsius for a total of 10 days. The plates were first incubated at a 25°C overnight. This initial incubation period was for the preliminary growth of the variant colonies. To further assess the background expression—which is optimally expressed at this ambient temperature—the plates were kept at 25°C for an extended period of 96 hours. This step was pivotal for enabling the variants to exhibit GFP expression. Subsequently, any variant colonies that demonstrated background activity were carefully screened out to refine the selection process. Subsequently, the variants were examined daily for hydrolase activity against both short-chain and long-chain fatty acids at room temperature over the next six days. To determine whether the activity was attributable to the organism or to mutations in the variants, transformed *E. coli* BL21(DE3) cells carrying the original 5MFBY gene plasmid were used as a negative control.

Screening 2 at 37°C

The variants showing no background expression were taken for further screening of their activity at 37°C. Variants and both controls were transferred on olive oil and tributyrin infused LB- ampicillin plates with the help of tooth pick and labelled respectively. The plates were first incubated overnight at 37°C to grow the variant and control colonies. The plates were further incubated at 25°C till any sign of activity was observed on the plates. Variants that showed activity at the same time or after the controls were eliminated. Variants were not tested for their activity on Olive oil plates after this stage.

Screening 3

The variants that expressed activity first and before the controls in screening 2 were chosen for third screening on plates with and without IPTG. Fresh tributyrin infused plates with chosen variant colonies were first incubated overnight at 37°C to grow. The plates was further incubated at 25°C for the variants to express and show activity. Here the variants were compared for their activity among each other, the one having most activity were chosen for further screening.

Screening 4

This was the final screening. The variants that have not expressed background activity and have shown strong activity before the controls were screened for final activity against each other and the controls. The variants with good activity were chosen and a master plate was made.

3.7.1 DNA isolation of selected candidates

With the observed hydrolysis activity in the screening results, plasmid extraction and restriction enzyme analysis was performed. The insert at the beginning has a restriction site for NdeI, therefore the plasmid should linearize when cut with NdeI, confirming the ligation of the insert and transnational start codon. There is also a site for Sac II on the insert, if the DNA is not cut by Sac II it maybe due to mutations in the insert at the site. Variants from screening 4 were chosen for DNA isolation and digestion with restriction enzyme.

For this testing, the precultures of the selected variants were made as described in section 3.4 and plasmid isolation was performed as described in section 3.5. The DNA was cut with procedure as described below:

Cutting plasmid DNA with restriction enzyme

In order to cut the plasmid with NdeI and BamH1 10 µl of plasmid DNA containing the GFP gene was mixed with 2 µl of 10x cut smart buffer, 1 µl of each restriction enzyme and 6 µl of DNA water to make the total volume of 20 µl. The procedure was carried out at room temprature. In order to cut the variant plasmid DNA using NdeI and SacII restriction enzyme 5 µl of variant plasmid DNA was mixed with 2 µl of 10x cut smart buffer, 1 µl of either NdeI or SacII restriction enzyme and 12 µl of DNA water to make the total volume of 20 µl. The procedure was carried out at room temprature. The obtained product was incubated at 37°C for 15 minutes. The acquired cut DNA was strored in fridge till further use.

An agarose gel elctrophoresis was performed with uncut variant plasmid DNA, cut variant plasmid DNA and cut original plasmid with GFP gene to visualize and analyse it.The procedure was performed as described in section 3.6.

4 Results

This chapter serves to introduce the results obtained for this project.

4.1 Bacterial Transformation

After transformation in section 2.7, fluroscent colonies were observed. These colonies were eliminated due to background expression of GFP. The non-fluroscent colonies were picked for screening. The non-fluroscent colonies from transformation 1 are called batch 1. The plates were labelled 1-12 each containing 3 colonies labelled A-C. Since there were more green colonies in batch 1, more library DNA(15µL) was used for second transformation. After second transformation many green colonies were observed but they were less intense in color and white color colonies were picked for screening. The non-fluroscent colonies picked from transformation 2 are called batch 2. The plates were labelled 13-17 each containing 10 colonies labelled A-J. It was later observed that there were some slow growing non-fluroscent colonies in the plates containing cells from both transformation 1 and 2. Slow growing colonies from transformation 1 were transferred to LB-ampicillin plates labelled as 18 and 19 each containing 10 colonies labelled A-J. Slow growing colonies from transformation 2 were transferred to a LB-ampicillin plates plate labelled 20 containing 10 colonies labelled A-J. These slow growing colonies from both transformation makes batch 3.The non-green colonies from transformation 3 were picked and transferred to new LB-ampicillin plate. They were labelled from 21-29 each containing 10 colonies labelled as A-J.They make batch 4. Table 4.1, shows a description of all the batches. In total 206 colonies were selected from thelibrary for screening.

Table 4.1: Description,labelling and nomenclature of variants in different batches.

	Description	Labelling	No. of colonies
Batch 1	The non-fluroscent colonies from the *E.coli BL21(DE3)* cells transformed with 5µL of the library DNA.	Plates labelled = 1-12 Colonies on each plate = A-C	36
Batch 2	The non-fluroscent colonies from the *E.coli BL21(DE3)* cells transformed with 15µL of the first ligation product.	Plates labelled = 13-17. Colonies on each plate = A-J	50
Batch 3	The slow-growing non-fluroscent colonies from plates with *E.coli BL21(DE3)* cells transformed with both 5µL and 15µL of the first ligation product.	Plate 18 & 19 = original plate 1 Plate 20 = original plate 2 Colonies on each plate = A-J	30
Batch 4	The non-fluroscent colonies from the transformed *E.coli BL21(DE3)* cells with 20µL of library DNA.	Plates labelled = 21-29 Colonies on each plate = A-J	90

To estimate the concentration of library DNA from batch 1 and batch 2 plasmid DNA was isolated and run on a gel elctrophorosis.Very faint bands are visible between 6000 bp and 3000 bp representing less concentration of plasmid DNA present in the transformed cells.

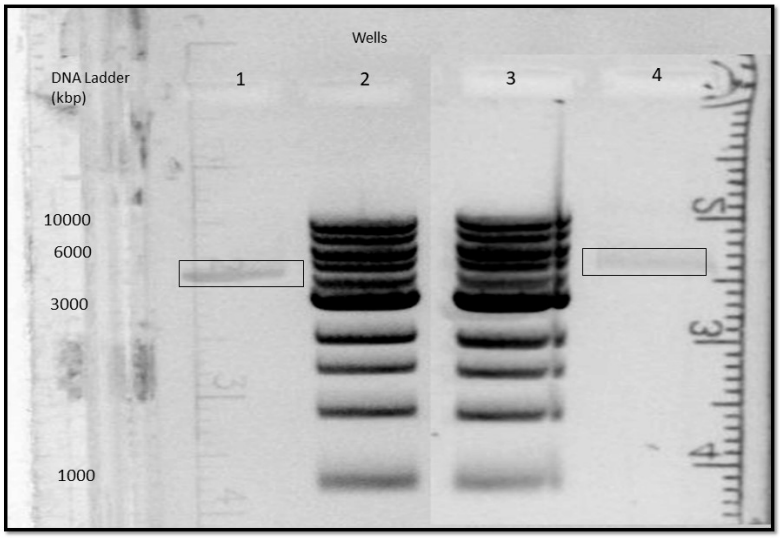


Figure 4.1: *The 1% agarose gel of miniprep from plasmid isolation of library DNA from batch 1 and batch 2. The wells are loaded as follows: 1: 9 µL of plasmid DNA from batch 1, 2: 1 kb DNA Ladder, 3: 1 kb DNA Ladder, 4: 9 µL of plasmid DNA from batch 2. Very faint bands around 6000 bp are visible in the gel, that is the size of the plasmid construct.*

4.2 Screening of the Library

In this section the results of the screening using tributyrin and olive oil assay at 25°C and 37°C are explained. The plates were incubated at both temprature for a total of 10 dayssection 3.7.
During the incubation the plates were examined for activity and compared with the negative control **C** to ensure the activity is not a result of cell's normal metabolic processes. A marking system was developed to compare the activity based on the size of the colony growth and the halo area around it, it is described in Table 4.3. Table 4.2 describes the control used in these screening. Since the optimal temperature for the expression of original 5MFBY polycarbonate degrading enzyme is 37°C, variants having good activity with no or little observable activity by controls on the same plate are considered interesting at 25°C. The original 5MFBY polycarbonate degrading enzyme is known to have no hydrolase activity against tributyrin and olive oil. The variants that had activity after incubation at 37°C with no or little sign of activity from controls from the same plates are considered interesting.

Table 4.2: Controls used for screening.

Control	
C	The E.coli BL21 DE3 cell cells with plasmid vector with original 5MFBY gene insert (before directed evolution on the gene
G	The E.coli BL21 DE3 cells with pET-11a plasmid vector with GFP gene

Table 4.3: System for marking the hydrolase activity of the variants during screening.

Mark	
+++	Higher Activity: This was used to when a well defined activity area, comparatively around the same size of the colony, was observed around the grown variant colonies on the plates.
++	Good Activity: This was used to when a well defined activity area but less compared to the size of the grown variant colonies was observed on the plates.
+	Low activity: This was used to define very little and not well defines activity area close to the peripheries of the grown variant colonies on the plates.
-	No activity: This was used to define no activity around the grown variant colonies on the plates.

4.2.1 Screening 1

Hydrolase activity at 25°C

On the seventh day of incubation, some hydrolase activity was observed around both the variants and the control on tributyrin infused plates, indicated by halo regions around the peripheries of the colony growth. The plates continued to be incubated at this temperature to allow for the expansion of these halo regions, to check increased activity with time. Notably, several variants demonstrated significantly higher activity compared to the controls and other variants from the same plate.

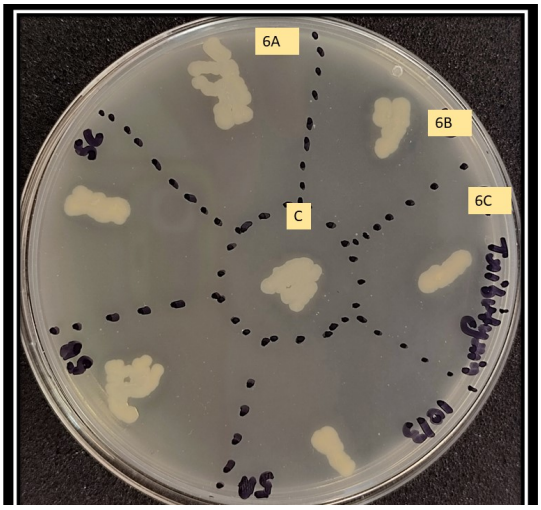
From **batch 1**, only 3 out of 36 candidates had good activity as compared to no activity from the control. The variants and their activity was noted as shown in Table 4.9. After 10 days of incubation, activity in controls were seen in plates 1,4, 5 and 6. Whereas no or very little activity was observed around controls in plate 3and 2. Variants 6B, 11B and 11C with good activity were considred interesting and are highlighted in the Table 4.9. Plates 3 and 6 are shown in Figure 4.2

Table 4.4: Record of activity of batch 1 variant after 10 days of incubation on tributyrin and olive oil plates at 25°C as compared with the negative control. In table **C** represents the activity of transformed *E.coli* BL21 (DE3) carrying plasmid vector with original 5MFBY gene which was used as a negative control. The activity is marked as, +++; high activity, ++; good activity, +; low activity, -; no activity. 3 out of 36 candidates had good activity as compared to no activity around the control.Those variants are highlighted in the table. No activity was observed on olive oil plate assays.

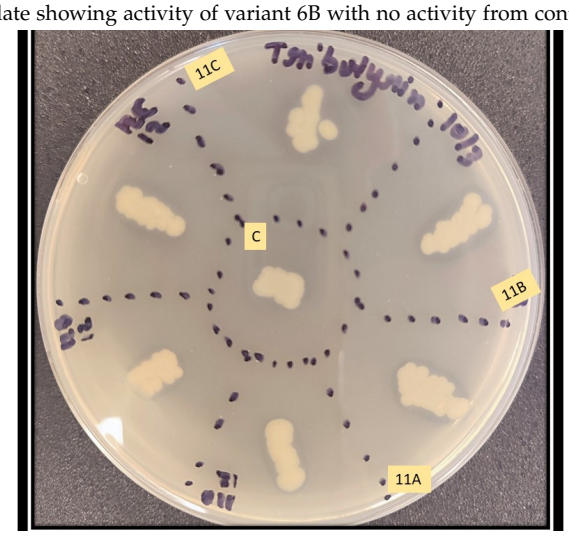
Batch1								
Variant	Tribytyrin	Olive oil	Variant	Tribytyrin	Olive oil	Variant	Tribytyrin	Olive oil
Plate 1			**Plate 3**			**Plate 5**		
C	+	-	C	-	-	C	+	-
1A	+	-	5A	+	-	9A	+	-
1B	+	-	5B	++	-	9B	+	-
1C	+	-	5C	+	-	9C	+	-
2A	+	-	6A	+	-	10A	+	-
2B	+	-	6B	++	-	10B	+	-
2C	-	-	6C	+	-	10C	+	-
Plate 2			**Plate 4**			**Plate 6**		
C	-	-	C	+	-	C	+	-
3A	+	-	7A	+	-	11A	+	-
3B	+	-	7B	+	-	11B	++	-
3C	+	-	7C	++	-	11C	++	-
4A	+	-	8A	-	-	12A	+	-
4B	+	-	8B	+	-	12B	+	-
4C	+	-	8C	-	-	12C	+	-

In **batch 2,3 and 4** after 10 days of incubation, approximately same degree of activity around grown control and variant colonies was observed on all the plates. Indicating this activity could be from the hosts metabolic processes itself and not due to mutations introduced through random mutagenesis. Variant 20I and 18E, had more activity compared to other variants as well as control from the same plate.

After screening all 206 variants for background activity at 25°C for 96 hrs. The candidates that expressed background activity ie, showed presence of GFP, similar to the control **G** on the plate were eliminated from further screening. It was observed that none of the candidates from batch 1 and 4 had any background activity. Two candidates 15E and 17D expressed GFP after 96 hrs of incubation at 25°C. Variant 20J from batch 3 had expressed small amount of GFP after long incubation, indicated by the greenish appearance of the colony on the plate. As explained in the Figure 4.3 these candidates are from batch 2 and 3 were eliminated from further screening.



(a) Plate showing activity of variant 6B with no activity from control (C)



(b) Plate showing activity of variant 11B and C with little activity from control (C)

Figure 4.2: The image above is the tributyrin infused LB plate assay with variants. Here **C** is transformed *E.coli* BL21 (DE3) colonies carrying plasmid vector with original 5MFBY gene which was used as a negative control. **(a)** 6A,B and C on the top plate. It was observed that 6B had a more clear halo around it indicating good activity compared to no activity from control.**(b)** 11B and 11C had more activity marked by clear halo around the grown colonies compared to little activity from control and other variants on the plate.

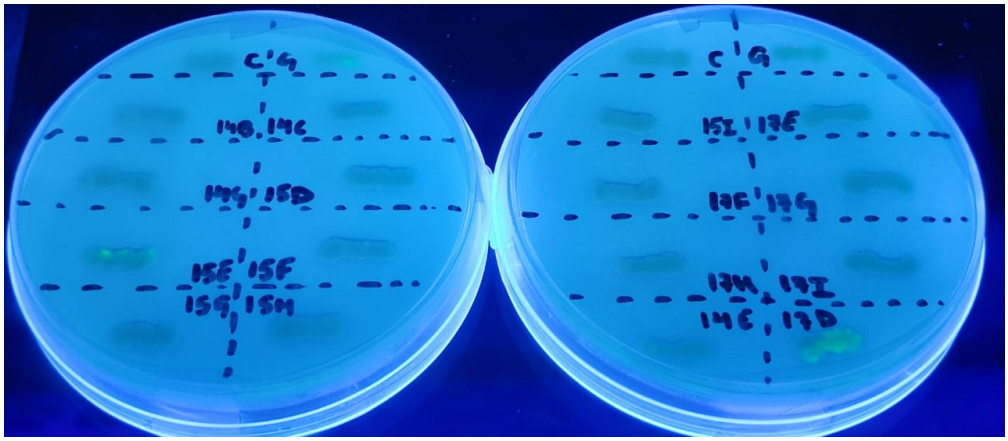


Figure 4.3: ***Tributyrin plates for GFP activity*** *The above shown image here is from tributyrin infused LB-ampicillin plates with transferred colonies of batch 2 variants with the purpose of screening for background activity. Candidate 15E and 17D shows background expression, marked by presence of GFP after 96hrs. The picture taken under UV-light and green flurosence can be observed on the both plates at the areas marked with 15E and 17D representing the variant colony respectively. Similiar flurosence can also be seen on the top right of both the plates, labelled as* ***G****. Here* ***G*** *represents E. coli BL21(DE3) cells carrying plasmid with GFP gene were used as a negative control on each plate for the confirmation of comparison of background expression. Transformed E.coli BL21 (DE3) carrying plasmid vector with original 5MFBY gene are labelled as* ***C*** *and is used as a positive control. Notably, the expressed GFP can be observed in the left plate in* ***G*** *and 15E colonies. There is very small amount of GFP produced in the negative control of right plate but 17D had evident amount of GFP.*

Throughout the incubation period, no lipolytic activity was detected in any batch using the olive oil plate assays, hence the absence of corresponding images or tables in the results section. Additionally, technical difficulties with lighting and camera quality precluded the effective photographic documentation of the plates. Consequently, a qualitative activity marking system was established to characterize and record the observed activity.

4.2.2 Screening 2

After 7 days of incubation some signs of activity at the peripheries of the grown colonies was visible in few variants. The variants were incubated 3 more days for the activity to increase.

Table 4.5: Record of activity of batch 1 variant after 10 days of incubation on tributyrin and olive oil plates at 37°C as compared with the negative control. In table **C** represents the activity of transformed *E.coli* BL21 (DE3) carrying plasmid vector with original 5MFBY gene which was used as a negative control. The activity is marked as, +++; high activity, ++; good activity, +; low activity, -; no activity. No activity was observed on olive oil plate assays. Only 4 variants had very little activity of interest compared to no activity in control, they are highlighted in the table.

Batch1								
Variant	Tribytyrin	Olive oil	Variant	Tribytyrin	Olive oil	Variant	Tribytyrin	Olive oil
Plate 1			**Plate 3**			**Plate 5**		
C	+	-	C	+	-	C	+	-
1A	-	-	5A	-	-	9A	-	-
1B	+	-	5B	-	-	9B	-	-
1C	+	-	5C	-	-	9C	-	-
2A	+	-	6A	+	-	10A	-	-
2B	+	-	6B	-	-	10B	-	-
2C	+	-	6C	-	-	10C	+	-
Plate 2			**Plate 4**			**Plate 6**		
C	-	-	C	+	-	C	+	-
3A	+	-	7A	-	-	11A	-	-
3B	+	-	7B	+	-	11B	+	-
3C	+	-	7C	+	-	11C	+	-
4A	+	-	8A	+	-	12A	+	-
4B	-	-	8B	+	-	12B	+	-
4C	-	-	8C	-	-	12C	+	-

Table 4.6: Record of activity of batch 2 variants after 10 days of incubation on tributyrin and olive oil plates at 37°C as compared with the positive and negative controls.**C**- Transformed *E.coli* BL21 (DE3) carrying plasmid vector with original 5MFBY gene is used as a negative control. The activity is marked as, +++; high activity, ++; good activity, +; low activity, -; no activity. There was activity in controls in all the plates.

Batch2								
Variant	Tribytyrin	Olive oil	Variant	Tribytyrin	Olive oil	Variant	Tribytyrin	Olive oil
Plate 1			**Plate 2**			**Plate 3**		
C	++	-	C	++	-	C	++	-
13A	+	-	14A	+	-	15A	+	-
13B	+	-	14B	++	-	15B	+	-
13C	+	-	14C	++	-	15C	+	-
13D	+	-	14D	++	-	15D	+	-
13E	-	-	**14E**	-	-	**15E**	+	-
13F	+	-	14F	+	-	15F	+	-
13G	++	-	14G	+	-	15G	+	-
13H	+	-	14H	+	-	15H	+	-
13I	+	-	14I	++	-	15I	+	-
13J	+	-	14J	++	-	15J	+	-
Plate 4			**Plate 5**					
C	+	-	C	++	-			
16A	+	-	17A	+	-			
16B	+	-	17B	+	-			
16C	-	-	17C	+	-			
16D	-	-	**17D**	+	-			
16E	+	-	17E	+	-			
16F	+	-	17F	+	-			
16G	+	-	17G	++	-			
16H	+	-	17H	++	-			
16I	+	-	17I	++	-			
16J	+	-	17J	++	-			

Table 4.7: Record of activity of batch 3 variants at tributyrin plates after 10 days of incubation on tributyrin and olive oil plates at 37°C as compared with the positive control.**C**- Transformed *E.coli* BL21 (DE3) carrying plasmid vector with original 5MFBY gene is used as a negative control. The activity is marked as +++; high activity, ++; good activity, +; low activity, -; no activity. 8 out of 30 candidates showed high activity. The intrested candidates are highlighted in table.

Batch 3								
Variant	Tribytyrin	Olive oil	Variant	Tribytyrin	Olive oil	Variant	Tribytyrin	Olive oil
Plate 1			**Plate 2**			**Plate 3**		
C	+	-	C	+	-	C	-	-
18A	+	-	19A	+	-	20A	+	-
18B	+	-	19B	-	-	20B	+	-
18C	+	-	19C	-	-	20C	+	-
18D	+	-	19D	+	-	20D	+	-
18E	++	-	19E	+	-	20E	-	-
18F	++	-	19F	-	-	20F	+	-
18G	++	-	19G	+	-	20G	++	-
18H	+	-	19H	++	-	20H	++	-
18I	-	-	19I	++	-	20I	++	-
18J	+	-	19J	+	-	**20J**	-	-

Table 4.8: Record of activity of batch 4 variants at tributyrin plates after 10 days of incubation on tributyrin and olive oil plates at 37°C as compared with the positive and negative controls. **C**- Transformed *E.coli* BL21 (DE3) carrying plasmid vector with original 5MFBY gene is used as a negative control.**G**-*E.coli* BL21 (DE3) cells carrying plasmid with GFP gene is also used as a negative control. The activity is marked as +++; high activity, ++; good activity, +; low activity, -; no activity.The variants with interesting activity are highlighted.

Batch4								
Variant	Tributyrin	Olive oil	Variant	Tributyrin	Olive oil	Variant	Tributyrin	Olive oil
Plate 1			**Plate 2**			**Plate 3**		
C	-	-	C	++	-	C	+	-
G	-	-	G	++	-	G	+	-
21A	+	-	22A	++	-	23A	-	-
21B	-	-	22B	++	-	23B	++	-
21C	+	-	22C	++	-	23C	-	-
21D	+	-	22D	++	-	23D	+	-
21E	-	-	22E	++	-	23E	-	-
21F	+	-	22F	++	-	23F	-	-
21G	-	-	22G	++	-	23G	-	-
21H	-	-	22H	++	-	23H	-	-
21I	-	-	22I	++	-	23I	-	-
21J	+	-	22J	++	-	23J	-	-
Plate 4			**Plate 5**			**Plate 6**		
C	+	-	C	+	-	C	+	-
G	+	-	G	+	-	G	+	-
24A	++	-	25A	++	-	26A	+	-
24B	++	-	25B	+	-	26B	+	-
24C	++	-	25C	+	-	26C	+	-
24D	++	-	25D	++	-	26D	+	-
24E	++	-	25E	+	-	26E	+	-
24F	++	-	25F	+	-	26F	+	-
24G	++	-	25G	++	-	26G	+	-
24H	+	-	25H	+	-	26H	+	-
24I	+	-	25I	+	-	26I	+	-
24J	++	-	25J	++	-	26J	+	-

Table 4.9: Record of activity of batch 4 variants at tributyrin plates after 10 days of incubation on tributyrin and olive oil plates at 37°C as compared with the positive and negative controls. **C**- Transformed *E.coli* BL21 (DE3) carrying plasmid vector with original 5MFBY gene is used as a negative control.**G**-*E.coli* BL21 (DE3) cells carrying plasmid with GFP gene is also used as a negative control. The activity is marked as +++; high activity, ++; good activity, +; low activity, -; no activity.The variants with interesting activity are highlighted.

Batch4								
Variant	Tribytyrin	Olive oil	Variant	Tribytyrin	Olive oil	Variant	Tribytyrin	Olive oil
Plate 7			**Plate 8**			**Plate 9**		
C	++	-	C	-	-	C	+	-
G	+	-	G	-	-	G	+	-
27A	+	-	28A	+	-	29A	++	
27B	+	-	28B	+	-	29B	+	-
27C	+	-	28C	-	-	29C	++	-
27D	+	-	28D	-	-	29D	++	-
27E	+	-	28E	-	-	29E	+	-
27F	+	-	28F	-	-	29F	++	-
27G	+	-	28G	-	-	29G	++	-
27H	+	-	28H	-	-	29H	+	-
27I	+	-	28I	-	-	29I	+	-
27J	+	-	28J	-	-	29J	+	-

The variants were incubated at 37°C to grow overnight and then incubated at room temperature for 9 days to analyze their activity. Very less activity was produced by the variants in batch 1 and 2. Only eight variants were able to express activity a little more than the control.There was activity in negative control before the variants. In Batch 1, variants 3A, 3B, 3C, and 4A demonstrated comparably more activity than the control. Batch 3, derived from slow-growing colonies, variants 18E, 18F, 18G, 19H, 19I ,20G, 20H and 20I showed better activity compared to the control. Batch 4, 24 variants demonstrated considerably higher activity compared to the control. There was no sign of activity by variants or control on olive oil plates. Therefore this assay was not used in further screening.

4.2.3 Screening 3

Variants that had shown promising activity were selected for the third round of screening. Some of the variants with less activity was also included because of their promising activity at 25°C. This screening was conducted to identify the variants exhibiting activity before the control.The experiments were conducted both with and without

Isopropyl β-D-1-thiogalactopyranoside (IPTG) in order to test the impact of IPTG, on inducing the activity of the variants. All the previous screening was conducted on tributyrin infused plates with IPTG. The activity was recorded and is presentedin Table 4.10 A significant increase in activity was observed in the plates with IPTG within 5 days. 11 variants (24A ,24B ,24E ,24F, 15I ,19H, 23B, 20G, 23B, 18E , and 19I) that had good activity in plates with IPTG were selected for master plate and final screening. 3 variants (6B, 11B, 11C) were also selected based on their initial high activity at 25°C.

Table 4.10: Record of comparative activity of variants on tributyrin plates without and with (WO/w) IPTG after 10 days and 5 days of incubation at 37°C as compared with the negative controls. **WO/w**-without IPTG/with IPTG. The plates with IPTG were incubated 5 days. The plates without IPTG were incubated for 10 days. **C**- Transformed *E.coli* BL21 (DE3) carrying plasmid vector with original 5MFBY gene is used as a negative control.**G**- *E.coli* BL21 (DE3) cells carrying plasmid with GFP gene is also used as a negative control. The activity is marked as +++; high activity, ++; good activity, +; low activity, -; no activity. The variants that show increase in activity are highlighted.

Variant	Tribytyrin	Variant	Tribytyrin	Variant	Tribytyrin	Variant	Tributyrin
	WO/W		**WO/w**		**WO/w**		**WO/w**
C	-/+	C	+/-	C	-/+	C	+/-
G	+	G	++/-	G	-/++	G	+/+
1B	-/-	11B	+/+	17J	-/-	20J	-/-
1C	-/-	11C	+/+	18B	+/-	20I	+/-
2A	-/-	14B	++/-	18G	+/-	21A	-/-
2B	+/-	14C	++/-	18F	+/-	21C	-/+
2C	-/-	14D	-/-	18E	+/++	21D	-/+
3A	-/-	14I	-/-	20D	-/-	21F	-/+
3B	-/-	14J	-/-	20E	+/+	21G	-/-
3C	+/-	17G	-/-	20F	-/-	21I	-/+
6B	+/+	17H	-/-	20G	+/++	21J	-/+
5B	+/+	17I	-/-	20H	-/-	22J	+/+
	WO/w		**WO/w**		**WO/w**		
C	-/+	C	-/+	C	-/+		
G	+/+	G	+/+	G	+/+		
24C	-/+	29A	-/-	25A	-/-		
24A	-/++	29C	-/-	25D	-/-		
24B	-/++	29D	+/-	25G	-/-		
24G	-	29F	-/-	23B	+/+++		
2E	+/+	29G	+/+	28B	-/+		
2E	+/++	15I	+/++	26J	-/-		
24F	-/++	19I	-/++				
25J	-/+	19H	+/++				
28A	-/+	24D	+/-				

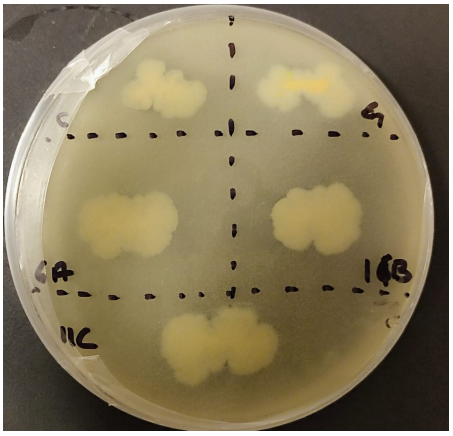
4.2.4 Screening 4

The variants that had good activity in screening 3 and the ones with good activity at 25°C were selected for final screening to reciprocate the previous activity result and proceed with DNA isolation. The screening was performed 3 times with inconsistent

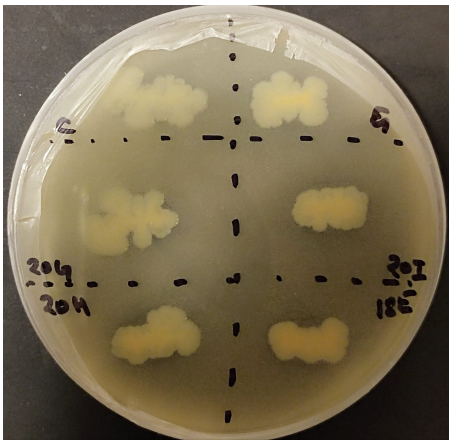
results. The final observations are presented in Table 4.11. Vaiants from batch 1, that had good activity at 25°C had no activity at 37 °C after 5 days of incubation. Variant 15 I had an infection growth, the high activity is suspected from the infection. It is shown in figure Figure 4.4. The figure shows the master plate.

Table 4.11: Record of activity of variants on tributyrin plates with IPTG after 5 days of incubation at 37°C as compared with the negative controls.**C**- Transformed *E.coli* BL21 (DE3) carrying plasmid vector with original 5MFBY gene is used as a negative control.**G**- *E.coli* BL21 (DE3) cells carrying plasmid with GFP gene is also used as a negative control. The activity is marked as +++; high activity, ++; good activity, +; low activity, -; no activity. I- represents infection.

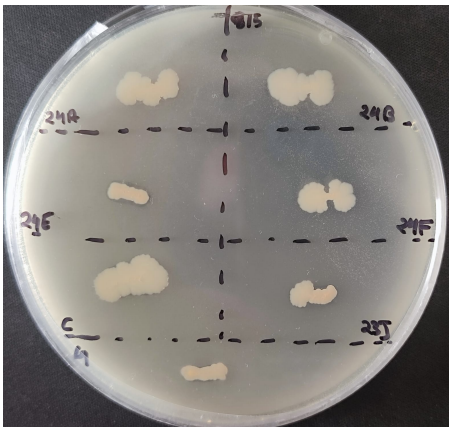
Variant	Tribytyrin	Variant	Tribytyrin	Variant	Tribytyrin	Variant	Tributyrin
Plate 1		**Plate 2**		**Plate 3**		**Plate 4**	
C	+	C	+	C	+	C	+
G	+	G	+	G	+	G	+
6A	-	20G	+	24A	-	15I	I
11B	-	20I	+	24B	-	25E	-
11C	-	20H	+	24E	+	19H	+
		18E	+	24F	+	23B	+
				22J	+	19I	+



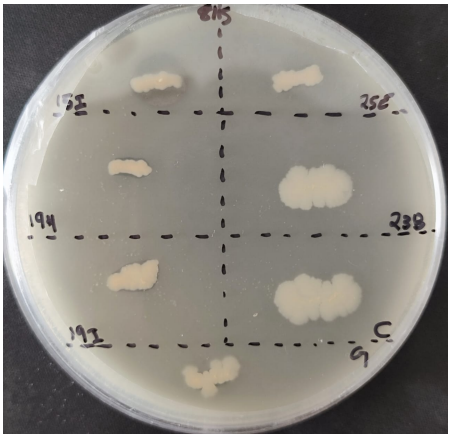
(a) Variants from batch1. 6A,11B,11C



(b) Variants from batch 3, 18E,20G,20H,20I



(c) Variants from batch 4, 24A,24B,24E,24F,23J



(d) Variants 15I,25E,19H,23B,19I

Figure 4.4: These are the pictures of the plates from final screening. The variants were screened for their activity on tributyrin infused plates with IPTG for 96hrs (incubated 37°C growth overnight and 96hrs at 25°C for expression)**C**- Transformed *E.coli* BL21 (DE3) carrying plasmid vector with original 5MFBY gene is used as a negative control.**G**- *E.coli* BL21 (DE3) cells carrying plasmid with GFP gene is also used as a negative control.Variants 20I,20H,18E,19H and 19I had some activity.

4.3 DNA isolation

With the observed inconsistencies in the screening results, plasmid extraction and restriction enzyme analysis was performed. The obtained 1% gel electrophoresis results are presented and explained here for selecting the candidates with potential increased hydrolase activity.

In Figure 4.5 lane 8, the double restriction digestion of GFP results in two band, one bright at around 6000bp and the second very fade at 800bp which is the insert. These results are in accordance with expected results[20]. A single restriction digest linearizes the plasmid, and a double restriction digest should result in two fragments with the insert being around 800 bp. Also in Figure 4.5 well 3 containing 6A plasmid DNA with NdeI, has a single strong band around 6000 bp, which is the size of the construct. This indicates a successful digestion and NdeI was able to linearize the plasmid DNA compared to Uncut super-coiled DNA of mutant 6A, shown in well 1,which has a higher mobility than the digested linearized in well 3. Therefore, variant **6A**, does have the translation start codon ligated on the plasmid construct confirming the expression of enzyme. Whereas, in well 4 containing plasmid DNA Sac II, the bands are at the same position as the uncut plasmid DNA in lane 1, this could indicate that the digestion did not go to completion, or that there might be unexpected alterations in the plasmid that are affecting the recognition and cutting by SacII. Therefore variant 6A presents as an interesting candidate for further study. Variant 7C DNA an example of the poor ligation of the insert on the plasmid. It was not successfully digested by both the restriction enzymes NdeI, indicating absence of translation start codon,and also Sac II. The bands pattern in well 6 and 7 containing plasmid DNA with restriction enzyme is to well 5 having uncut super coiled plasmid DNA. It was not successfully digested by both the restriction enzymes, indicating absence of translation start codon.

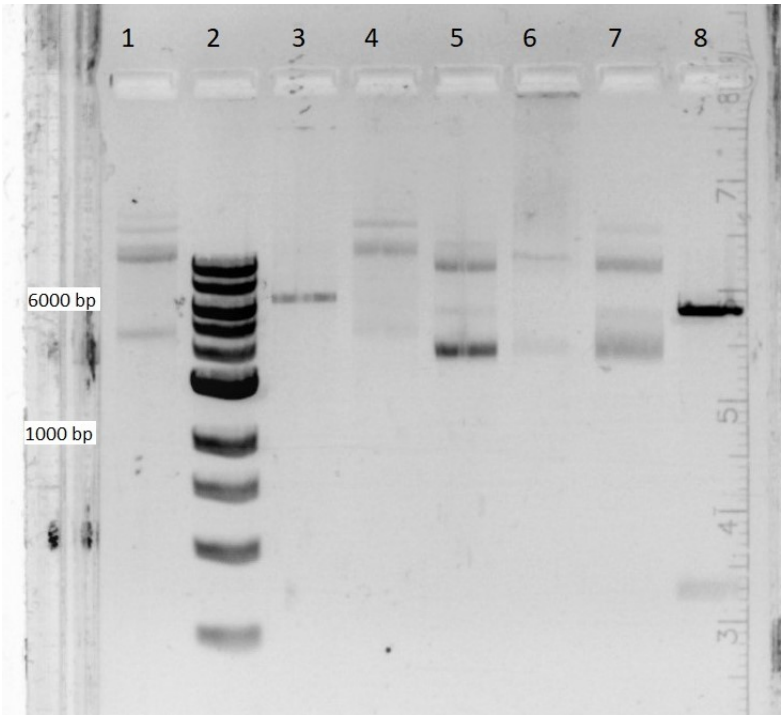


Figure 4.5: The 1% agarose gel with the results from DNA digestion with restriction enzymes. 1; uncut supercoiled plasmid DNA of variant 6A, 2; 1 kb standard DNA ladder, 3; 6A plasmid DNA cut with NdeI, 4; 6A plasmid DNA cut with SacII, 5; 7C uncut plasmid DNA, 6; 7C plasmid DNA cut with NdeI, 7; 7C plasmid DNA cut with SacII, 8; pGFP cut with NdeI and BamHI.

As seen in Figure 4.6, compared to uncut super-coiled DNA of variant 11B and 11C, in lane 1 and 5, plasmid DNA with NdeI in lane 2 and 6 which has a single band at 6000 bp. This band is around the same size as the plasmid construct of 6475 bp. Indiacating that the variants 11B and 11C, plasmid DNA was successfully linearized with NdeI restriction enzyme, therefore the insert was successfully ligated. Since, enzyme SacII despite having a restriction site on insert could not linearize the 11B and 11C DNA hints there might be unexpected alterations in the plasmid that are affecting the recognition and cutting. Therefore 11B and 11C also considered interesting candidate for further study.

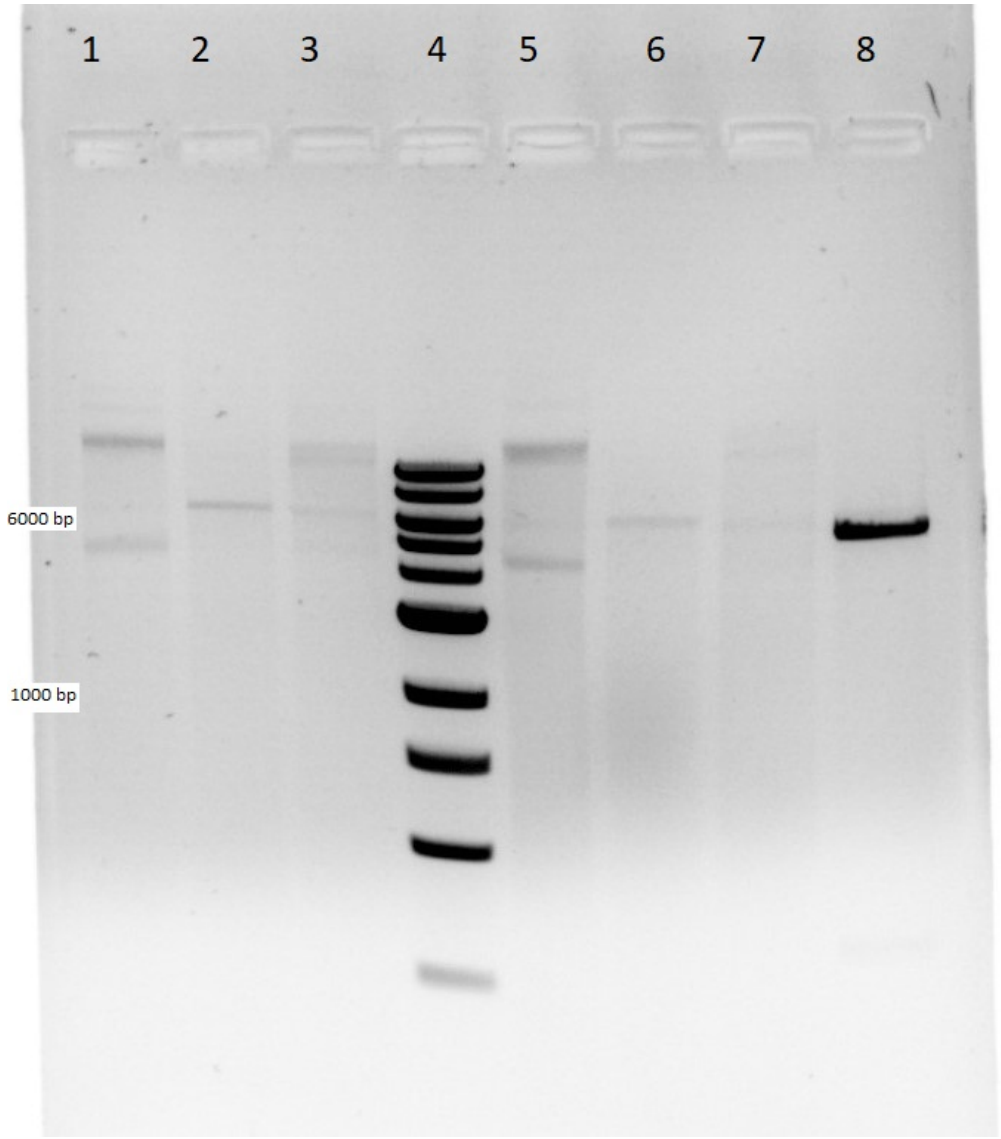


Figure 4.6: The 1% agarose gel with the results from DNA digestion with restriction enzymes. 1; 11B uncut plasmid DNA, 2; 11B plamsid DNA cut with NdeI, 3; 11B plasmid DNA cut with SacII, 4; 1 kb standard DNA ladder 5; 11C uncut plasmid DNA, 6; 11C plasmid DNA cut with NdeI, 7; 11C plasmid DNA cut with SacII, 8; pGFP cut with NdeI and BamHI.

??, shows results of the restriction enzyme digest of variants 20G and 20I. Plasmid DNA with NdeI in lane 2 and 6 has a single band around 6000 bp. Indicating that the variants 20G and 20I, plasmid DNA was successfully linearized with NdeI restriction enzyme, therefore the insert was successfully ligated. In well 3, containing plasmid DNA of 20G with NdeI, a strong band around 6000 BP indicating the linearization of plasmid DNA. Whereas in well 7, containing variant 20I plasmid DNA with SacII, the

band are at the same position as the supercoiled plasmid in lane 5, this could indicate that the digestion did not go to completion, or that there might be unexpected alterations in the plasmid that are affecting the recognition and cutting by SacII. Variant 20 I seems to be an interesting candidate.

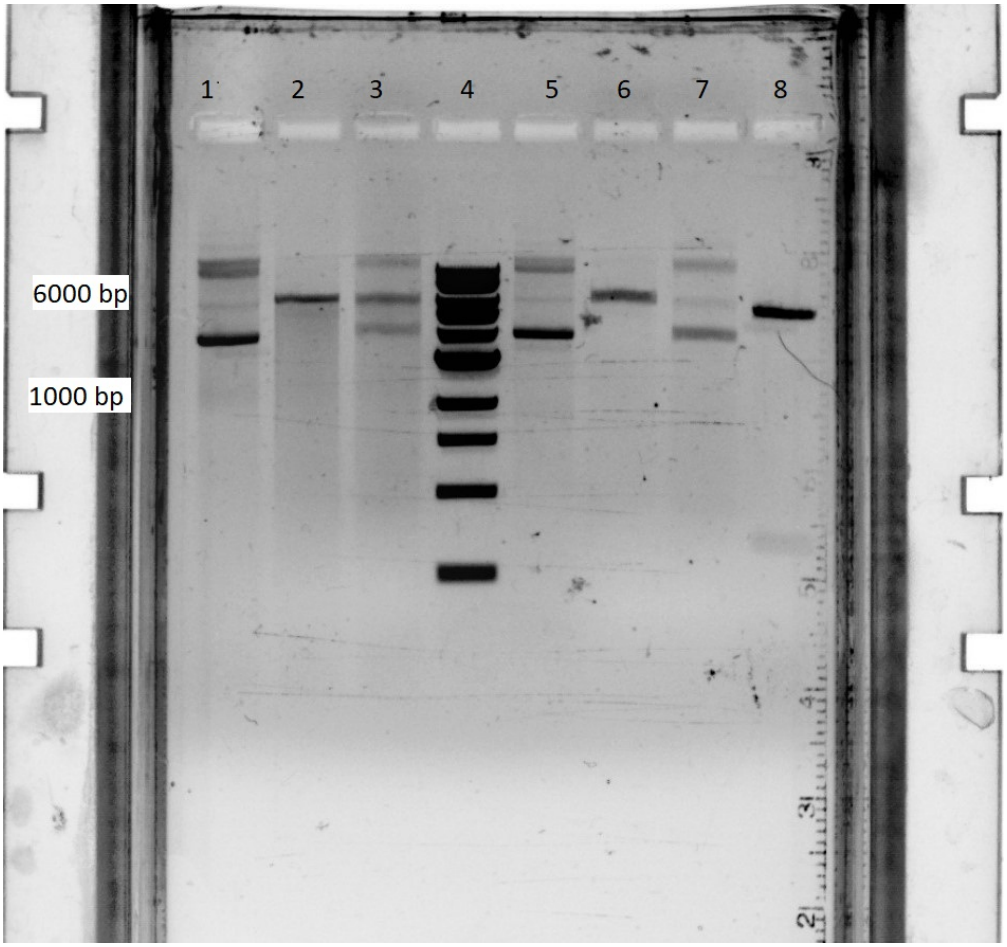


Figure 4.7: The 1% agarose gel with the results from DNA digestion with restriction enzymes. 1; 20G uncut plasmid DNA, 2; 20G cut with NdeI, 3; 20G cut with SacII, 4; 1 kb standard DNA ladder, 5; 20I uncut plasmid DNA, 6; 20I cut with NdeI, 7; 20I cut with SacII, 8; GFP cut with NdeI and BamHI.

The results of the restriction enzyme digest of 18E and 20H mutant are shown on a 1% agarose gel in **??**. Uncut supercoiled DNA of variant 18E and 20H, shown in lane 1 and 4, has a higher mobility than the plasmid DNA with NdeI in lane 2 and 5 which has a single band at around 6000 bp. It can also be seen that in lane 8, containing the double restriction enzyme digestion of PGFP results in two band, one bright at around 6000bp and the second very fade at 800bp which is the insert. Therefore, the plasmid DNA is linearized as this aligns with expected result. As seen in well 6 there are faint bands around 6000bp, indicating SacII was able to linearize variant

20H plasmid DNA.Therefore there might not be any possible mutations in the variant insert gene and the activity mostly be from the organism itself. Whereas in well 3, containing variant 18E plasmid DNA with SacII, the band are at the same position as the supercoiled plasmid in lane 1, this could indicate that the digestion did not go to completion, or that there might be unexpected alterations in the plasmid that are affecting the recognition and cutting by SacII. Variant 18E seems to be an interesting candidate.

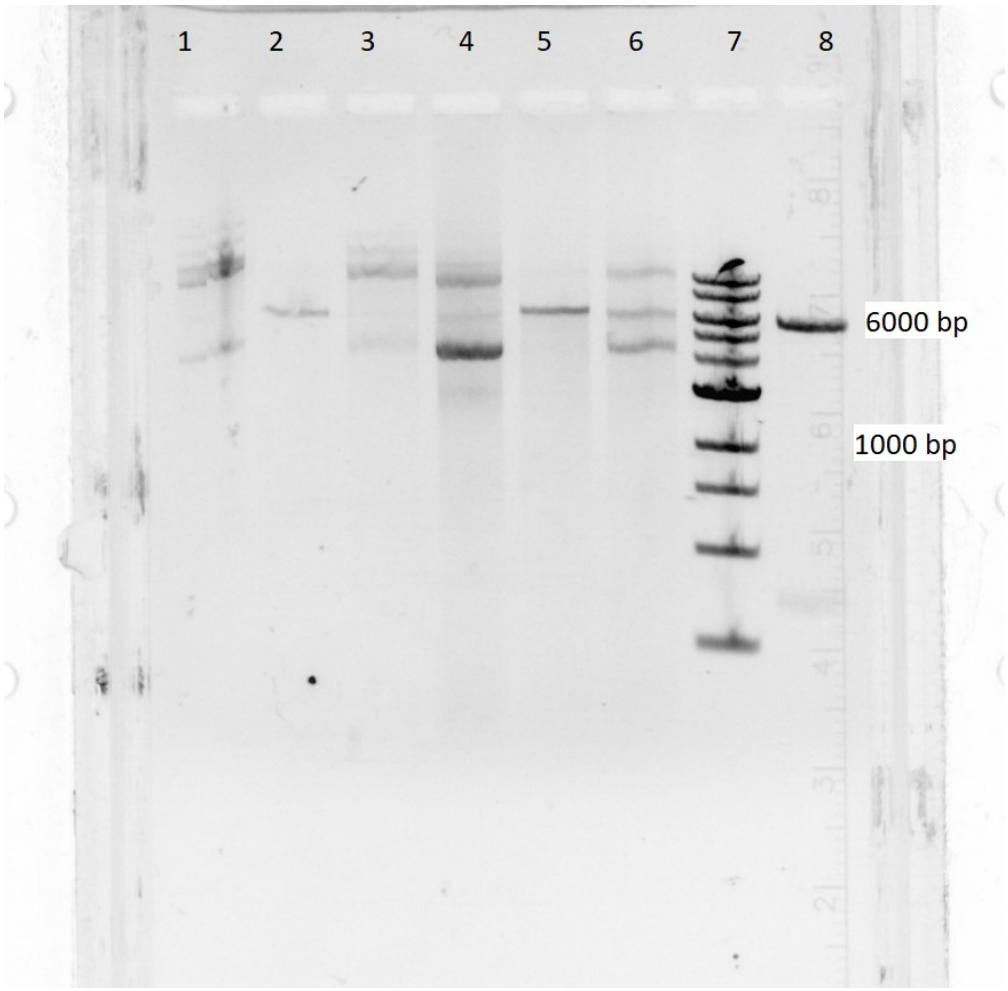


Figure 4.8: The 1% agarose gel with the results from DNA digestion with restriction enzymes. 1; 18E uncut plasmid DNA, 2; 18E cut with NdeI, 3; 18E cut with SacII, 4; 20H uncut plasmid DNA, 5; 20H cut with NdeI, 6; 20H cut with SacII, 7; 1 kb standard DNA ladder, 8; GFP cut with NdeI and BamHI.

It was found that variant 15I,18G and 19H did not have the translation start codon, therefore the insert was not ligated properly. As seen in **??** well 3,5 and 7, containing plasmid DNA with NdeI plasmid DNA, no sinlge band at 6000 bp can be seen therefore the plasmid DNA was not successfully digested with the restriction enzyme. The

variants are eliminated from having potential increased enzyme promiscuity. So the activity showed by the variants is most like from the host organism.

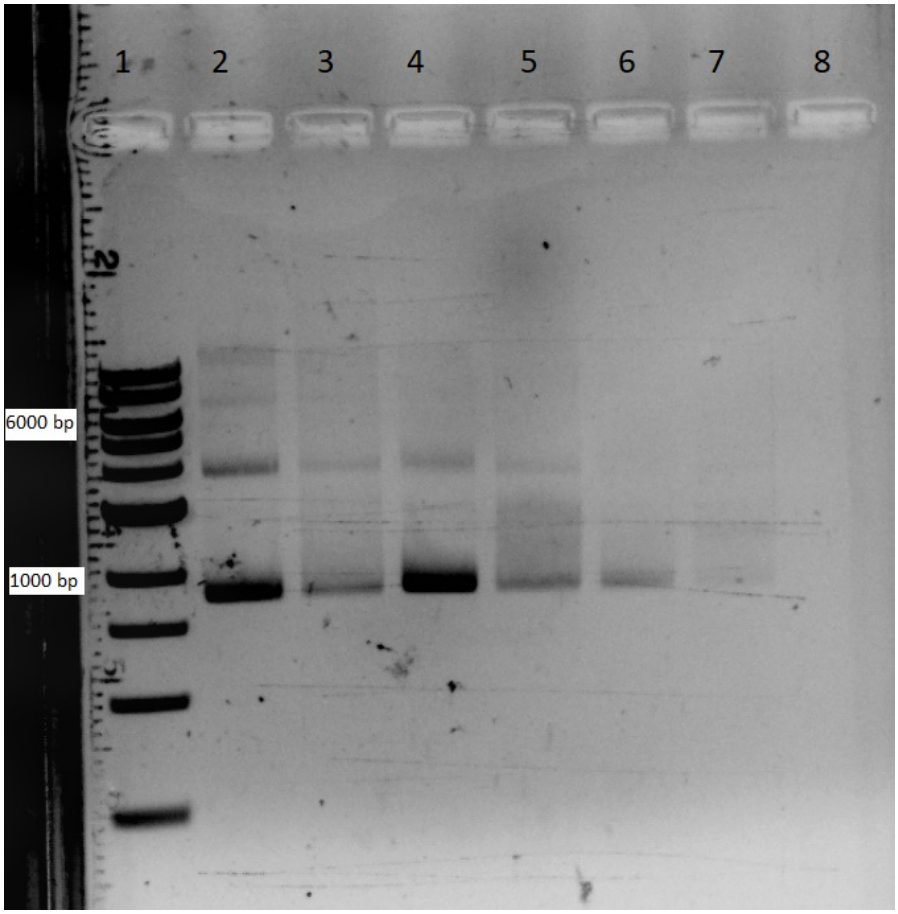


Figure 4.9: The 1% agarose gel with the results from DNA digestion with restriction enzymes.1; 1 kb standard DNA ladder, 2; 15I uncut plasmid DNA, 3; 15I cut with NdeI, 4; 18G uncut plasmid DNA, 5; 18G cut DNA with NdeI, 6; 19H uncut plasmid DNA, 7; 19H cut NdeI.

For variant 24E it was found that the insert was not successfully ligated in the plasmid marked by the absence of translation start codon as NdeI was not able to linearize the plasmid DNA. This can be seen in **??** well 5, containing plasmid DNA with NdeI. Whereas, ,a strong band at 6000bp was seen in well 3, contaning plasmid DNA of variant 23B with NdeI. This indicates linearization of plasmid DNA and presence of start codon. Further analysis using SacII is required to confirm if the activity is due to some alteration in the insert from random mutagenisis.

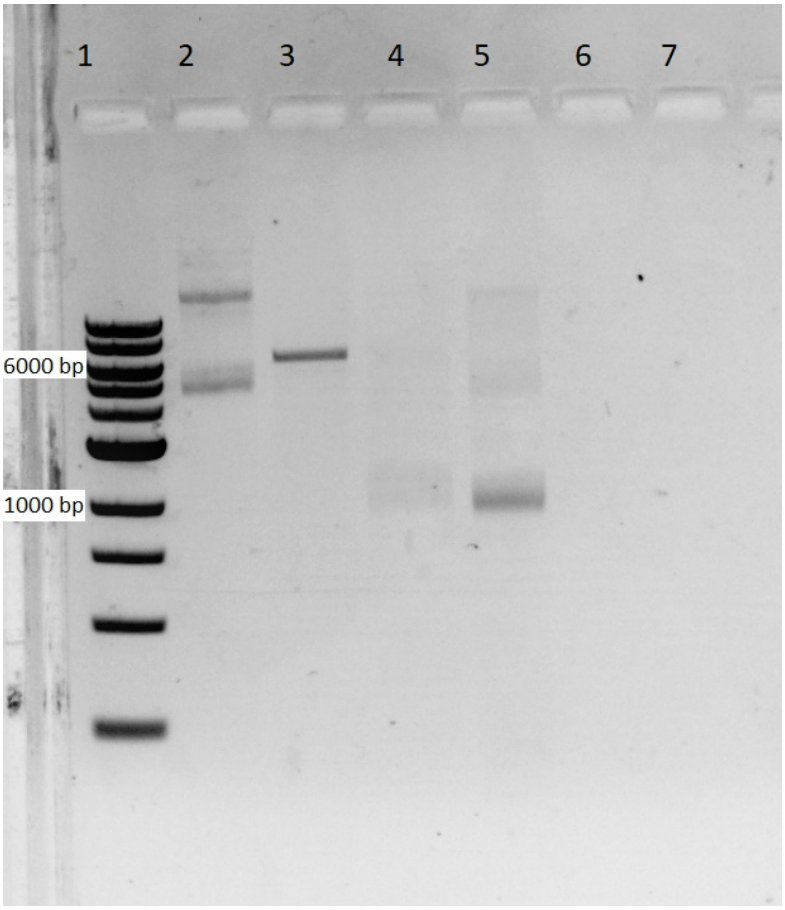


Figure 4.10: The 1% agarose gel with the results from DNA digestion with restriction enzymes.1; 1 kb standard DNA ladder, 2; 23B uncutplamsi DNA, 3; 23B cut with NdeI, 4; 24E uncut plasmid DNA, 5; 24E cut DNA with NdeI.

Upon digestion of the plasmid DNA by restriction enzyme, variants 6A,11B,11C, 20I, and 18E that had activity at 25 °C, showed a linearized DNA cut with NdeI.The plasmid DNA was not successfully digested with Sac II ,this could indicate that there might be unexpected alterations in the plasmid that are affecting the recognition and cutting by SacII. The activity substantially decreased when the variants were incubated at 37°C. The variants 15I, 18G, 19H, 20G, 23B and 24E had shown hydrolase activity after 7 days at 37°C incubation temprature with no significant activity at 25°C. Only 23B among these had linearized DNA upon digestion. Therefore there activity was part of host cells metabolic path. For 23B, when incubated for 24 hrs, 48 hrs, 96hrs at 37°C to reciprocate the activity, no hydrolase activity was observed. The plates were then kept at 25°C to check for activity for a total of 10 days but no activity was observed.

5 Discussion

The purpose of this thesis is to improve the hydrolase activity of a polycarbonate-degrading enzyme by directed evolution. This section discuss the findings of the performed experiment, the possibility of directed evolution in enzyme optimization, and to provide a broader reflection on enzyme activity and specificity.

For this thesis, directed evolution by random mutagenesis is used to increase the enzyme promiscuity of a 5MFBY hydrolase. In order to test the increased enzyme promiscuity, tributyrin and olive oil plate assays were employed section 4.2. The 5MFBY original enzyme has a Ser-His-Asp/Glu active site specific for cleaving carbonate groups in polycarbonate (R-O-C(=O)-O-R') [20]. This is distinct from the ester bond found in triglycerides, like tributyrin and the fats in olive oil (R-C(=O)-O-R') [66]. Therefore, these ester bonds in these plate assays have a different spatial and electronic configuration, which may not be adequately recognized or acted upon by the original 5MFBY enzyme . With the help of directed evolution, the goal was to introduce mutations that can increase promiscuity. These assays provided a perspective to observe and quantify enzyme activity beyond its known specificity.

The incubation period for the variants to exhibit activity was observed to be around 10 days. There could be many reasons that the variants to take longer to show activity. After translation, proteins must fold into their active form. The time frame for protein folding can vary substantially from microseconds to hours based on the specific protein size [67]. The original 5MFBY polycarbonate-degrading enzyme is of 27 kDa, having high secondary structure stability, especially at high temperatures. Properties suggest that it may not necessarily require an exceptionally long time to fold, especially given its fast refolding rate [20]. It was found that random mutagenesis can impact the protein stability and its folding time [68]. Therefore, induced mutations might have caused change in the structure and folding time of the enzyme, thus impacting its expression levels.

This long incubation period could also be attributed to the lower expression levels of the variant enzyme, necessitating more time for sufficient protein expression and

accumulation to observe activity[69]. There is a direct yet complex relationship between enzyme expression and accumulation which depends on various factors such as the efficiency of translation, protein stability, and cellular mechanisms for protein degradation [69]. The rate of formation of the product may not increase linearly with the increase in enzyme expression levels, and the measured activity of the enzyme can be falsely low if the incubation time is too long [69], [70]. Therefore, over extended periods can lead to enzyme degradation, loss of enzyme activity, or changes in cell condition can occur, which can cause an underestimation of the enzyme's true activity [69]. That is why the variants were not incubated for more than 10 days.

In the initial screening at 25°C Figure 4.3, variants 17D and 15E showed background activity with the presence of GFP within 96 hours, suggesting that the 5MFBY gene may not have been successfully ligated into the plasmid, leading to leaky expression of GFP gene. This GFP expression could be ascribed to improper regulation or interactions with the host cell machinery caused by surrounding genetic elements such as promoters or regulatory regions [71]. Furthermore, after a week of incubation, variant 20J exhibited GFP expression, indicating a potential problem with the excision of the GFP gene from the plasmid prior to the ligation of the error-prone PCR DNA [72]. This delayed expression of GFP could potentially be caused by mutations or changes within the GFP gene, which would influence its fluorescence intensity and behavior in the *E. coli* host [73]. The translational efficiency of foreign proteins can be influenced by factors such as the sequence context around the enzyme gene, including the ribosome binding site and translation initiation site [74]. A poor sequence context may have resulted in ineffective translation of the 5MFBY gene, resulting in the observed GFP expression instead. No GFP was expressed by the variants at 37°C maybe because the optimal temperature for the expression of GFP by *E.coli* is at 25°C.

Variants 15I, 18G, 19H, 20G and 24E showed hydrolase activity after 7 days at 37°C Table 4.7. As revealed by agarose gel analysis of the plasmid DNA digested with restriction enzymes section 4.3, the absence of the translation start codon, indicates that the intended polycarbonate enzyme (encoded by the plasmid) was not expressed. *E.coli* cells can produce various types of hydrolases, including peptidoglycan hydrolases, serine hydrolases [75], signal peptide hydrolases [76], and (p)ppGpp hydrolases [77]. These enzymes are crucial for cellular functions, such as, cell wall remodeling and maintaining cell integrity [75]. The hydrolase activity observed in these variants could be attributed to the background activity of *E. coli's* native hydrolases. In the absence of the translation start codon for the intended enzyme, these native hydrolases might be responsible for the observed activity. This also explains why little activity was observed in variants 17D and 15E despite expressing GFP.

It should also be noted that variants 6A, 11B, 11C, 20I, and 18E exhibited hydrolase

activity at 25°C, a temperature lower than the optimal 37°C for the expression of the original 5MFBY polycarbonate degrading enzyme, this suggests that these variants may have acquired beneficial mutations that enhance their enzymatic function at this lower temperature. In a study conducted by the University of Texas, enzyme variants that could break down plastics at low temperatures were designed [78]. Additionally, other studies have focused on mutations of the hydrolases, such as cutinase, PETase, and MHETase, to obtain mutants with improved thermostability and catalytic activity in the hydrolysis of polyethylene terephthalate (PET) and other polymers [79]–[81]. These findings collectively support the idea that mutations can enhance enzymatic function at lower temperatures, as observed in the above mentioned variants.

The observed activity at lower temperatures can be attributed to the slower rates of protein production, which afford newly transcribed recombinant proteins ample time for folding may be leading to increased promiscuity [43]. Protein synthesis at higher temperature may lead to the formation of inclusion bodies or problems with folding of the protein. A prevalent method to decelerate protein synthesis is to decrease the incubation temperature[43]. Especially, when the formation of inclusion bodies (IBs) poses an issue, it is advisable to conduct recombinant protein synthesis at temperatures between 15°C and 25°C [43], [82]. Such adaptability in enzymatic function is not only pertinent for various industrial applications, where processes often operate across a spectrum of temperatures, but also hints at an evolutionary advantage [83]. It suggests that these enzymes can maintain functionality under diverse environmental conditions, a trait that could be critically important in natural ecosystems characterized by frequent temperature fluctuations [81].

A significant decrease in the activity of the variants was observed when screened on plates without IPTG. Variants displayed high activity within just five days of incubation with IPTG, compared to ten days of incubation without it. This could be because enzyme expression of the variant is under the control of the T7/lac system, an IPTG-inducible promoter [84], [85]. Given that the bacteria were incubated at 37 °C and transferred to 25 °C for enzyme expression, this imposed stress on the bacteria [86]. When the variants are grown in the presence of IPTG, it results in the production of the target enzyme with enhanced activity [84], [85].

The catalytic site of the original 5MFBY enzyme is characterized by its broad nature, resulting in a relatively loose interaction with its substrate. This structural feature could account for the moderate hydrolase activity observed in enzyme variants. To enhance substrate affinity and increase catalytic efficiency, targeted mutations are necessary to refine the architecture of the active site. Such modifications aim to promote a more precise and stronger interaction with the substrate, thereby potentially improving the enzyme's hydrolase activity.[20]. During the screening, it was found

that variants that had good activity till screening three did not reproduce the same result in screening four. The inconsistency in the screening result could be that bacteria may start to dry out or die during the transfer for testing, leading to reduced enzyme activity due to a lower number of viable cells [87]. Also over time, especially in prolonged incubations, agar plates can dry out, which may concentrate substrates and reagents or alter the enzyme-substrate interaction, affecting the activity [87].

In summary, this study aims to utilize the potential of directed evolution as a tool for creating enzymes with novel and enhanced functionalities. However, it is essential to acknowledge the limitations and challenges inherent in this approach. The vastness of the potential sequence space in directed evolution means that while we have identified promising candidates, they represent only a fraction of the possible configurations. The solution space to be explored for any given protein is large enough to feasibly allow sufficient library coverage[54]. Therefore, there is a high likelihood that the vast majority of the generated variants will exhibit no improvement or even inferior performance compared to the parent protein. As suggested by Miyazaki et.al[55], if a desired level of modified activity is not achieved, other techniques for creating the gene diversity for directed evolution can be employed. Future work should focus on further refining these variants, possibly through more targeted mutagenesis or by employing alternative screening strategies to isolate enzymes with even greater specificity and efficiency for plastic degradation.

6 Conclusion

This thesis has embarked on a quest to augment the hydrolase activity of a polycarbonate-degrading enzyme through the methodical application of directed evolution.The library is divided into 4 batches depending upon the concentration of the mutagenized plasmid expression construct (5µL, 15µL and 20µL) used for transformation of the *E.coli BL21(DE3)* cells. The transformative potential of these variants was rigorously assessed through their ability to catalyze long chain and short chain fatty acid esters, employing tributyrin and olive oil plate assays at the temperatures of 25°C and 37°C. The variants were evaluated based on three criteria: no background activity, the order of appearance of hydrolysis activity compared to the controls, and the strength of hydrolysis activity. During the course of this project 4 screening were done to choose the potential candidate. Variants 6A,11B,11C, 20I, and 18E that had high activity at 25 °C, showed a linearized DNA upon digestion with NdeI. These variants are from batch 1 and 3 (the slow growing colonies of transformation one). From the screening observation, the activity is comparatively very less, more cycles of directed evolution can be used to increase targeted hydrolysis activity.

In order to confirm that the activity is from to induced mutation high-throughput screening and sequencing is suggested.

Bibliography

[1] P. Baheti, "How is plastic made? a simple step-by-step explanation," *British Plastic Federation. https://www. bpf. co. uk/plastipedia/how-is-plastic-made. aspx*, 2021.

[2] L. Anagnosti, A. Varvaresou, P. Pavlou, E. Protopapa, and V. Carayanni, "Worldwide actions against plastic pollution from microbeads and microplastics in cosmetics focusing on european policies. has the issue been handled effectively?" *Marine Pollution Bulletin*, vol. 162, p. 111 883, 2021.

[3] K. Kik, B. Bukowska, and P. Sicińska, "Polystyrene nanoparticles: Sources, occurrence in the environment, distribution in tissues, accumulation and toxicity to various organisms," *Environmental Pollution*, vol. 262, p. 114 297, 2020.

[4] C. Ripken, D. G. Kotsifaki, and S. N. Chormaic, "Analysis of small microplastics in coastal surface water samples of the subtropical island of okinawa, japan," *Science of the total Environment*, vol. 760, p. 143 927, 2021.

[5] R. Ambrosini, R. S. Azzoni, F. Pittino, G. Diolaiuti, A. Franzetti, and M. Parolini, "First evidence of microplastic contamination in the supraglacial debris of an alpine glacier," *Environmental pollution*, vol. 253, pp. 297–301, 2019.

[6] M. Karamanlioglu, R. Preziosi, and G. D. Robson, "Abiotic and biotic environmental degradation of the bioplastic polymer poly (lactic acid): A review," *Polymer Degradation and stability*, vol. 137, pp. 122–130, 2017.

[7] S. Yoshida, K. Hiraga, T. Takehana, *et al.*, "A bacterium that degrades and assimilates poly (ethylene terephthalate)," *Science*, vol. 351, no. 6278, pp. 1196–1199, 2016.

[8] U. T. Bornscheuer, "Feeding on plastic," *Science*, vol. 351, no. 6278, pp. 1154–1155, 2016.

[9] D. Danso, J. Chow, and W. R. Streit, "Plastics: Environmental and biotechnological perspectives on microbial degradation," *Applied and environmental microbiology*, vol. 85, no. 19, e01095–19, 2019.

[10] C. Vargas-de la Cruz and D. Landa-Acuña, "Microbial bioremediation of azo dye through microbiological approach," in *Emerging Technologies in Environmental Bioremediation*, Elsevier, 2020, pp. 425–441.

[11] K. Bano, M. Kuddus, M. R Zaheer, *et al.*, "Microbial enzymatic degradation of biodegradable plastics," *Current pharmaceutical biotechnology*, vol. 18, no. 5, pp. 429–440, 2017.

[12] N. Mohanan, Z. Montazer, P. K. Sharma, and D. B. Levin, "Microbial and enzymatic degradation of synthetic plastics," *Frontiers in Microbiology*, vol. 11, p. 580 709, 2020.

[13] L. P. Wackett and S. L. Robinson, "The ever-expanding limits of enzyme catalysis and biodegradation: Polyaromatic, polychlorinated, polyfluorinated, and polymeric compounds," *Biochemical Journal*, vol. 477, no. 15, pp. 2875–2891, 2020.

[14] J. Herbert, A. H. Beckett, and S. C. Robson, "A review of cross-disciplinary approaches for the identification of novel industrially relevant plastic-degrading enzymes," *Sustainability*, vol. 14, no. 23, p. 15 898, 2022.

[15] J. Kaushal, M. Khatri, and S. K. Arya, "Recent insight into enzymatic degradation of plastics prevalent in the environment: A mini-review," *Cleaner Engineering and Technology*, vol. 2, p. 100 083, 2021.

[16] J. Zrimec, M. Kokina, S. Jonasson, F. Zorrilla, and A. Zelezniak, "Plastic-degrading potential across the global microbiome correlates with recent pollution trends," *MBio*, vol. 12, no. 5, e02155–21, 2021.

[17] W. Xiong, B. Liu, Y. Shen, K. Jing, and T. R. Savage, "Protein engineering design from directed evolution to de novo synthesis," *Biochemical Engineering Journal*, vol. 174, p. 108 096, 2021.

[18] F. Wen, M. McLachlan, and H. Zhao, "Directed evolution: Novel and improved enzymes," *Wiley Encyclopedia of Chemical Biology. John Wiley & Sons, Inc*, 2008.

[19] B. de Pina Mariz, S. Carvalho, I. L. Batalha, and A. S. Pina, "Artificial enzymes bringing together computational design and directed evolution," *Organic & Biomolecular Chemistry*, vol. 19, no. 9, pp. 1915–1925, 2021.

[20] L. H. Holst, N. G. Madsen, F. T. Toftgaard, *et al.*, "De novo design of a polycarbonate hydrolase," *bioRxiv*, pp. 2023–03, 2023.

[21] S. Hansen, P. Ernst, S. L. König, *et al.*, "Curvature of designed armadillo repeat proteins allows modular peptide binding," *Journal of structural biology*, vol. 201, no. 2, pp. 108–117, 2018.

[22] E. Shukla, A. D. Bendre, and S. M. Gaikwad, "Hydrolases: The most diverse class of enzymes," in *Hydrolases*, IntechOpen, 2022.

[23] D. L. Nelson, A. L. Lehninger, and M. M. Cox, *Lehninger principles of biochemistry*. Macmillan, 2008.

[24] J. Z. Long and B. F. Cravatt, "The metabolic serine hydrolases and their functions in mammalian physiology and disease," *Chemical reviews*, vol. 111, no. 10, pp. 6022–6063, 2011.

[25] K. Hult and P. Berglund, "Enzyme promiscuity: Mechanism and applications," *Trends in biotechnology*, vol. 25, no. 5, pp. 231–238, 2007.

[26] H. Pranamuda, R. Chollakup, and Y. Tokiwa, "Degradation of polycarbonate by a polyester-degrading strain, amycolatopsis sp. strain ht-6," *Applied and environmental microbiology*, vol. 65, no. 9, pp. 4220–4222, 1999.

[27] O. K. Tawfik and D. S, "Enzyme promiscuity: A mechanistic and evolutionary perspective," *Annual review of biochemistry*, vol. 79, pp. 471–505, 2010.

[28] P. A. Romero and F. H. Arnold, "Exploring protein fitness landscapes by directed evolution," *Nature reviews Molecular cell biology*, vol. 10, no. 12, pp. 866–876, 2009.

[29] D. S. Tawfik, "Accuracy-rate tradeoffs: How do enzymes meet demands of selectivity and catalytic efficiency?" *Current opinion in chemical biology*, vol. 21, pp. 73–80, 2014.

[30] V. Serini, "Polycarbonates," *Ullmann's encyclopedia of industrial chemistry*, 2000.

[31] G. Sudha and V. Ganesh, "Enzymatic degradation of polycarbonates: Response surface methodology (rsm) based approach.," in *Journal of Physics: Conference Series*, IOP Publishing, vol. 1979, 2021, p. 012 006.

[32] M. Zhou, M. Takayanagi, Y. Yoshida, S. Ishii, and H. Noguchi, "Enzyme-catalyzed degradation of aliphatic polycarbonates prepared from epoxides and carbon dioxide," *Polymer Bulletin*, vol. 42, pp. 419–424, 1999.

[33] M. S. Packer and D. R. Liu, "Methods for the directed evolution of proteins," *Nature Reviews Genetics*, vol. 16, no. 7, pp. 379–394, 2015.

[34] R. E. Cobb, R. Chao, and H. Zhao, "Directed evolution: Past, present, and future," *AIChE Journal*, vol. 59, no. 5, pp. 1432–1440, 2013.

[35] Y. Wang, P. Xue, M. Cao, T. Yu, S. T. Lane, and H. Zhao, "Directed evolution: Methodologies and applications," *Chemical reviews*, vol. 121, no. 20, pp. 12 384–12 444, 2021.

[36] J. C. Avise, F. J. Ayala, *et al.*, "In the light of directed evolution: Pathways of adaptive protein evolution," in *In the Light of Evolution: Volume III: Two Centuries of Darwin*, National Academies Press (US), 2009.

[37] J. D. Garcıa-Garcıa, K. Van Gelder, J. Joshi, *et al.*, "Using continuous directed evolution to improve enzymes for plant applications," *Plant Physiology*, vol. 188, no. 2, pp. 971–983, 2022.

[38] E. G. Hibbert and P. A. Dalby, "Directed evolution strategies for improved enzymatic performance," *Microbial Cell Factories*, vol. 4, pp. 1–6, 2005.

[39] H. Xiao, Z. Bao, and H. Zhao, "High throughput screening and selection methods for directed enzyme evolution," *Industrial & engineering chemistry research*, vol. 54, no. 16, pp. 4011–4020, 2015.

[40] D. Lenug, "A method for random mutagenesis of a defined dna segment using a modified polymerase chain reaction," *Technique JMCMB*, vol. 1, pp. 11–15, 1989.

[41] C. Fenton, H. Xu, E. I. Petersen, S. B. Petersen, and M. R. El-Gewely, "Random mutagenesis for protein breeding," *In Vitro Mutagenesis Protocols*, pp. 231–241, 2002.

[42] S. Sanchez and A. Demain, "Special issue on the production of recombinant proteins," *Biotechnology Advances*, vol. 5, no. 30, pp. 1100–1101, 2012.

[43] G. L. Rosano and E. A. Ceccarelli, "Recombinant protein expression in escherichia coli: Advances and challenges," *Frontiers in microbiology*, vol. 5, p. 172, 2014.

[44] B. Jia and C. O. Jeon, "High-throughput recombinant protein expression in escherichia coli: Current status and future perspectives," *Open biology*, vol. 6, no. 8, p. 160 196, 2016.

[45] P. T. Wingfield, "Overview of the purification of recombinant proteins," *Current protocols in protein science*, vol. 80, no. 1, pp. 6–1, 2015.

[46] R. Assenberg, P. T. Wan, S. Geisse, and L. M. Mayr, "Advances in recombinant protein expression for use in pharmaceutical research," *Current opinion in structural biology*, vol. 23, no. 3, pp. 393–402, 2013.

[47] R. Chen, "Bacterial expression systems for recombinant protein production: E. coli and beyond," *Biotechnology advances*, vol. 30, no. 5, pp. 1102–1107, 2012.

[48] J. Y. Lim, J. W. Yoon, and C. J. Hovde, "A brief overview of escherichia coli o157: H7 and its plasmid o157," *Journal of microbiology and biotechnology*, vol. 20, no. 1, p. 5, 2010.

[49] M. Kostylev, A. E. Otwell, R. E. Richardson, and Y. Suzuki, "Cloning should be simple: Escherichia coli dh5α-mediated assembly of multiple dna fragments with short end homologies," *PLoS One*, vol. 10, no. 9, 2015.

[50] N. E. Biolabs, *FAQ: What is the difference between BL21 and BL21(DE3) competent E.coli cells?* `https://international.neb.com/faqs/2016/01/15/what-is-the-difference-between-bl21-and-bl21-de3-competent-e-coli-cells1`, Online; accessed 12 November 2020.

[51] N. E. Biolabs, *E. coli Expression Strains*, `https://international.neb.com/products/competent-cells/e-coli-expression-strains/e-coli-expression-strains`, Online; accessed 16 September 2020.

[52] T. Scientific, *OT7 Tag Polyclonal Antibody*, `https://www.merckmillipore.com/DK/en/product/Origami-2DE3-Singles-Competent-Cells-Novagen,EMD_BIO-71408?ReferrerURL=https%3A%2F%2Fwww.google.com%2F`, Online; accessed 16 September 2020.

[53] SnapGene, *pET-11a Vector Map*, https://www.snapgene.com/resources/plasmid-files/?set=pet_and_duet_vectors_(novagen)&plasmid=pET-11a, Online; accessed 16 September 2020.

[54] H. Leemhuis, R. M. Kelly, and L. Dijkhuizen, "Directed evolution of enzymes: Library screening strategies," *IUBMB life*, vol. 61, no. 3, pp. 222–228, 2009.

[55] K. Miyazaki and F. H. Arnold, "Exploring nonnatural evolutionary pathways by saturation mutagenesis: Rapid improvement of protein function," *Journal of molecular evolution*, vol. 49, pp. 716–720, 1999.

[56] H. Xu, E. I. Petersen, S. B. Petersen, and M. R. El-Gewely, "Random mutagenesis libraries: Optimization and simplification by pcr," *Biotechniques*, vol. 27, no. 6, pp. 1102–1108, 1999.

[57] C. Neylon, "Chemical and biochemical strategies for the randomization of protein encoding dna sequences: Library construction methods for directed evolution," *Nucleic acids research*, vol. 32, no. 4, pp. 1448–1459, 2004.

[58] D. X. Sahonero-Canavesi, M. Zavaleta-Pastor, L. Martınez-Aguilar, I. M. López-Lara, and O. Geiger, "Defining substrate specificities for lipase and phospholipase candidates," *JoVE (Journal of Visualized Experiments)*, no. 117, e54613, 2016.

[59] C. Avendaño and J. Menéndez, "Chapter 10-other approaches to targeted therapy," *Avendaño, C. and Menéndez, JCBT-MC of AD (eds). Amsterdam: Elsevier*, pp. 307–349, 2008.

[60] A. Mustafa, A. Karmali, and W. Abdelmoez, "A sensitive microplate assay for lipase activity measurement using olive oil emulsion substrate: Modification of the copper soap colorimetric method," *Journal of oleo science*, vol. 65, no. 9, pp. 775–784, 2016.

[61] L. Ramnath, B. Sithole, and R. Govinden, "Identification of lipolytic enzymes isolated from bacteria indigenous to eucalyptus wood species for application in the pulping industry," *Biotechnology Reports*, vol. 15, pp. 114–124, 2017.

[62] M. Levisson, J. van der Oost, and S. W. Kengen, "Carboxylic ester hydrolases from hyperthermophiles," *Extremophiles*, vol. 13, pp. 567–581, 2009.

[63] A. Placido, T. Hai, M. Ferrer, *et al.*, "Diversity of hydrolases from hydrothermal vent sediments of the levante bay, vulcano island (aeolian archipelago) identified by activity-based metagenomics and biochemical characterization of new esterases and an arabinopyranosidase," *Applied microbiology and biotechnology*, vol. 99, pp. 10 031–10 046, 2015.

[64] V. Gupta, I. Singh, P. Kumar, S. Rasool, and V. Verma, "A hydrolase with esterase activity expressed from a fosmid gene bank prepared from dna of a north west himalayan glacier frozen soil sample," *3 Biotech*, vol. 9, pp. 1–7, 2019.

[65] A. A. de Souza Machado, W. Kloas, C. Zarfl, S. Hempel, and M. C. Rillig, "Microplastics as an emerging threat to terrestrial ecosystems," *Global change biology*, vol. 24, no. 4, pp. 1405–1416, 2018.

[66] D. Y. Colin, P. Deprez-Beauclair, N. Silva, L. Infantes, and B. Kerfelec, "Modification of pancreatic lipase properties by directed molecular evolution," *Protein Engineering, Design & Selection*, vol. 23, no. 5, pp. 365–373, 2010.

[67] A. N. Naganathan and V. Muñoz, "Scaling of folding times with protein size," *Journal of the American Chemical Society*, vol. 127, no. 2, pp. 480–481, 2005.

[68] A. Dror, E. Shemesh, N. Dayan, and A. Fishman, "Protein engineering by random mutagenesis and structure-guided consensus of geobacillus stearothermophilus lipase t6 for enhanced stability in methanol," *Applied and environmental microbiology*, vol. 80, no. 4, pp. 1515–1527, 2014.

[69] M. Arvas, T. Pakula, B. Smit, *et al.*, "Correlation of gene expression and protein production rate-a system wide study," *BMC genomics*, vol. 12, no. 1, pp. 1–25, 2011.

[70] R. de Sousa Abreu, L. O. Penalva, E. M. Marcotte, and C. Vogel, "Global signatures of protein and mrna expression levels," *Molecular BioSystems*, vol. 5, no. 12, pp. 1512–1526, 2009.

[71] E. Wons, D. Koscielniak, M. Szadkowska, and M. Sektas, "Evaluation of gfp reporter utility for analysis of transcriptional slippage during gene expression," *Microbial Cell Factories*, vol. 17, pp. 1–10, 2018.

[72] H. J. Cha, R. Srivastava, V. N. Vakharia, G. Rao, and W. E. Bentley, "Green fluorescent protein as a noninvasive stress probe in resting escherichia coli cells," *Applied and Environmental Microbiology*, vol. 65, no. 2, pp. 409–414, 1999.

[73] K. R. Siemering, R. Golbik, R. Sever, and J. Haseloff, "Mutations that suppress the thermosensitivity of green fluorescent protein," *Current Biology*, vol. 6, no. 12, pp. 1653–1663, 1996.

[74] M. R. Hanson and R. H. Köhler, "Gfp imaging: Methodology and application to investigate cellular compartmentation in plants," *Journal of Experimental Botany*, vol. 52, no. 356, pp. 529–539, 2001.

[75] J. van Heijenoort, "Peptidoglycan hydrolases of escherichia coli," *Microbiology and Molecular Biology Reviews*, vol. 75, no. 4, pp. 636–663, 2011.

[76] P. Novak, P. H. Ray, and I. Dev, "Localization and purification of two enzymes from escherichia coli capable of hydrolyzing a signal peptide.," *Journal of Biological Chemistry*, vol. 261, no. 1, pp. 420–427, 1986.

[77] J.-W. Lee, Y.-H. Park, and Y.-J. Seok, "Rsd balances (p) ppgpp level by stimulating the hydrolase activity of spot during carbon source downshift in escherichia coli," *Proceedings of the National Academy of Sciences*, vol. 115, no. 29, E6845–E6854, 2018.

[78] S. Gault, P. M. Higgins, C. S. Cockell, and K. Gillies, "A meta-analysis of the activity, stability, and mutational characteristics of temperature-adapted enzymes," *Bioscience Reports*, vol. 41, no. 4, BSR20210336, 2021.

[79] Z. Song, Q. Zhang, W. Wu, Z. Pu, and H. Yu, "Rational design of enzyme activity and enantioselectivity," *Frontiers in Bioengineering and Biotechnology*, vol. 11, p. 1 129 149, 2023.

[80] K. Charupanit, V. Tipmanee, T. Sutthibutpong, and P. Limsakul, "In silico identification of potential sites for a plastic-degrading enzyme by a reverse screening through the protein sequence space and molecular dynamics simulations," *Molecules*, vol. 27, no. 10, p. 3353, 2022.

[81] R. P. Magalhães, J. M. Cunha, and S. F. Sousa, "Perspectives on the role of enzymatic biocatalysis for the degradation of plastic pet," *International Journal of Molecular Sciences*, vol. 22, no. 20, p. 11 257, 2021.

[82] C. H. Schein and M. H. Noteborn, "Formation of soluble recombinant proteins in escherichia coli is favored by lower growth temperature," *Bio/technology*, vol. 6, no. 3, pp. 291–294, 1988.

[83] A. K. Urbanek, K. E. Kosiorowska, and A. M. Mirończuk, "Current knowledge on polyethylene terephthalate degradation by genetically modified microorganisms," *Frontiers in bioengineering and biotechnology*, vol. 9, p. 771 133, 2021.

[84] P. Dvorak, L. Chrast, P. I. Nikel, *et al.*, "Exacerbation of substrate toxicity by iptg in escherichia coli bl21 (de3) carrying a synthetic metabolic pathway," *Microbial cell factories*, vol. 14, no. 1, pp. 1–15, 2015.

[85] L. Gomes, G. Monteiro, and F. Mergulhão, "The impact of iptg induction on plasmid stability and heterologous protein expression by escherichia coli biofilms," *International journal of molecular sciences*, vol. 21, no. 2, p. 576, 2020.

[86] J.-M. Xu, Z.-S. Wu, K.-J. Zhao, *et al.*, "Iptg-induced high protein expression for whole-cell biosynthesis of l-phosphinothricin," *Biotechnology Journal*, vol. 18, no. 9, p. 2 300 027, 2023.

[87] R. D. Heselpoth and D. C. Nelson, "A new screening method for the directed evolution of thermostable bacteriolytic enzymes," *JoVE (Journal of Visualized Experiments)*, no. 69, e4216, 2012.