IN VITRO AND *IN SILICO* ANALYSIS OF *Sphingobacterium* sp. LIPASE CO-EXPRESSION WITH LEA K PEPTIDE

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by

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LIST OF SYMBOLS

%	Percentage
±	Plus/minus
Å	Angstrom
ºC	Degree Celsius
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µg/mL	Microgram per milliliter
µmol	Micromole
ϕ	Phi
ψ	Psi
pI	Isoelectric point

LIST OF ABBREVIATIONS

BLAST	Basic Local Search Alignment Tool
CASTp	Computer Atlas of Surface Topography of Protein
Co^{2+}-CMA	Copper (II) carboxylmethylaspartate
DNA	Deoxyribonucleic acid
dH_2O	Distilled water
3D	Three dimensional
EC	Enzyme code
H_2O	Water
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
K_m	Michaelis-Menten constant
K_{cat}	Reaction Turnover
kDa	Kilo Dalton
LB	Luria Bertani
LEA	Late embryogenesis abundant
Na_2HPO_4	Disodium phosphate
NaH_2PO_4	Monosodium Phosphate
NCBI	National Center for Biotechnology Information
OD	Optical density
p-NPP	Para-Nitrophenyl Phosphate
PDB	Protein Data Base
PDBQT	Protein Data Bank, Partial Charge (Q), & Atom Type (T)
RMSD	Root-mean-square deviations

rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
UV	Ultraviolet
V_o	Initial velocity
V_{max}	Maximum velocity

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***ANALISIS IN VITRO* DAN *IN SILICO* LIPASE *Sphingobacterium* sp. YANG DIEKSPRESS BERSAMA PEPTIDA LEA K**

ABSTRAK

Sejenis bakteria Gram-negatif bernama *Sphingobacterium* sp. selalunya dapat dibezakan berdasarkan jumlah sphingophospholipids yang banyak dalam kandungan lipidnya. Disebabkan hanya beberapa kajian saja yang telah dibuat terhadap lipase *Sphingobacterium* sp., maka objektif kajian ini adalah untuk menyediakan ilmu penting mengenai aktiviti lipase *Sphingobacterium* sp. dengan menggunakan LEA-K sebagai ekspresibersama melalui kaedah siliko. Suatu perkara yang penting untuk mengkaji aktiviti pemangkinan lipase *Sphingobacterium* sp. tanpa peptida LEA-K kerana ia mungkin bersifat unik atau berbeza daripada rakan sejenisnya. Melalui penjelasan mengenai struktur lipase tiga dimensi, maka tidak mustahil untuk memahami dengan lebih baik tentang cara enzim berfungsi dan memanfaatkan interaksi (saling tindak) molekulnya apabila dirangsang oleh peptida LEA-K. Bagi memahami interaksi molekul dan kualiti lipase *Sphingobacterium* sp. yang tersendiri, maka kajian secara *in vitro* dilakukan terhadap aktiviti lipase dengan ekspresibersama peptida LEA-K. Oleh itu, keputusan kajian ini akan lebih membantu dalam memberikan pemahaman asas nilai aplikasi yang sesuai untuk lipase *Sphingobacterium* sp. Rekombinan lipase *Sphingobacterium* sp. telah diklon oleh pengkaji sebelum ini di dalam Lab406 USM, ia diekstrak dan diikuti dengan transformasi melalui kejutan haba, dan kemudian diekspresikan dengan peptida LEA-K. Minyak zaitun digunakan sebagai substrat di dalam ujian kolorimetri untuk mengukur aktiviti lipase. Struktur lipase diramalkan menggunakan tapak sesawang SWISS-MODEL, dan dok molekul dilakukan menggunakan program AutoDock

Vina untuk menyiasat interaksi molekul. Keadaan ekspresi optimum ditentukan pada 25ºC selama 24 jam pengeraman dengan IPTG 0.5mM. Lipase Ab3 yang telah dibuat His-Tag telah ditulenkan dan mencapai 40.2 kali ganda dengan hasil 7.91% dan aktiviti khusus 480.53 U/mg melalui teknik penulenan afiniti selangkah. Aktiviti enzimatik dijalankan pada suhu optimum 15ºC dan di bawah pH 7. Kajian kekhususan substrat menunjukkan bahawa Ab3 lipase mempunyai afiniti tertinggi terhadap minyak zaitun dengan K_m 0.2353 mM, V_{max} ialah 98.04 µmol min^{-1} mg^{1}, dan K_{cat} adalah dikira sebagai 3,624.13 min^{-1}. Jujukan lipase asid amino Ab3 menunjukkan identiti 100% dengan lipase *Sphingobacterium* sp. dan identiti jujukan 32.03% dengan struktur terlarut esterase Est8. Struktur tertier yang menggunakan lipatan α/β-hydrolase telah diramalkan dengan keyakinan yang tinggi. Peptida LEA-K juga diramalkan dengan heliks-α di tengah struktur. Sisa lipase Ab3 berinteraksi dengan sisa peptida LEA-K melalui elektrostatik, ikatan hidrogen dan interaksi hidrofobik. Hasil dok substrat yang dilekatkan ke dalam kompleks Ab3-LEA K menunjukkan elektrostatik, ikatan hidrogen dan interaksi hidrofobik yang kuat. Walau bagaimanapun, hasil dok substrat ke dalam lipase Ab3 tanpa LEA K adalah lemah berbanding dengan yang didok dengan kompleks Ab3-LEA K. Kajian ini menunjukkan bahawa LEA dalam sistem ekspresi bersama meningkatkan ekspresi protein dan aktiviti enzim dan bahawa peningkatan ini bergantung kepada kehadiran asid amino hidrofobik dalam jujukan. Analisis lanjut perlu dijalankan untuk memahami batasan aktiviti enzimatik untuk aplikasi industri yang lebih baik.

IN VITRO AND *IN SILICO* ANALYSIS OF *Sphingobacterium* sp. LIPASE CO-EXPRESSION WITH LEA K PEPTIDE

ABSTRACT

A yellowish Gram-negative bacterium called *Sphingobacterium* sp. is frequently distinguished by large quantities of sphingophospholipids in its lipid composition. Since few studies have been done on the *Sphingobacterium* sp. lipase, which has been the subject of limited research, this study aims to provide essential knowledge on the lipase activities of *Sphingobacterium* sp. with LEA-K as co-expression and *in silico* studies. To characterize Ab3 lipase from *Sphingobacterium* sp. that has been produce with LEA K. To predict the 3-dimensional structure of *Sphingobacterium* sp. lipase and LEA-K peptide using an *in-silico* approach. To investigate the interaction of Ab3 lipase-LEA K peptide complex that enhanced catalytic activity using in silico studies. Therefore, this study's results will be more helpful in providing a basic grasp of favourable application values for *Sphingobacterium* sp. lipase. Olive oil was used as the substrate in a colourimetric test technique to measure lipase activity. The lipase structure was predicted using the SWISS-MODEL website, and molecular docking was done using the AutoDock Vina program to investigate molecular interactions. The optimum expression conditions were determined at 25ºC for 24 hours of incubation with 0.5mM IPTG. The His-tagged Ab3 lipase was purified and achieved 40.2 folds with a 7.91% yield and 480.53 U/mg specific activity via a one-step affinity purification technique. The enzymatic activity was carried out at an optimized temperature of 15ºC and under pH 7. Substrate specificity study indicated that Ab3 lipase has the highest affinity

towards olive oil with the K_m of 0.2353 mM, V_{max} was 98.04 µmol min^{-1} mg^1, and K_{cat} was calculated as 3,624.13 min^{-1}. The Ab3 lipase's amino acid sequence exhibits 100% identity with *Sphingobacterium* sp. lipase and 32.03% sequence identity with the esterase Est8 solved structure. The tertiary structure adopted α/β-hydrolase fold was predicted with high confidence. The LEA K peptide was also predicted with an α-helix at the centre of the structure. The Ab3 lipase residues interact with the LEA K peptide residues via electrostatic, hydrogen bonding, and hydrophobic interactions. The docking result of the substrate docked into the Ab3-LEA K complex shows good binding energy, strong electrostatic, hydrogen bonds, and hydrophobic interactions. However, the docking result of the substrate into Ab3 lipase without LEA K is poor compared to the one docked with the Ab3-LEA K complex. This study demonstrates that LEA in the co-expression system enhances the expression of proteins and enzyme activity, and that this enhancement depends on the presence of hydrophobic amino acids in the sequence. Further analysis should be conducted to understand the limitations of the enzymatic activities for better industrial applications.

CHAPTER 1
INTRODUCTION

1.1 Introduction

The Enzymes Industry Data Book provides an extensive overview encompassing the industrial enzymes and specialty enzymes markets. Valued at USD 12.28 billion in 2022, the global enzymes market is poised for robust expansion, with an anticipated compound annual growth rate (CAGR) of 6.5% projected from 2023 through 2030 (Share *et al*., 2023). Enzymes catalyse reactions faster and more precisely than chemical compounds, resulting in fewer side products and reduced waste treatment time and cost. Enzymes also lower energy consumption in commercial bioprocesses because they only need mild conditions to work. Furthermore, a single enzyme can catalyse various reactions using different substrates and catalysing states, leading to various desired products. It is also environmentally friendly because it is biodegradable and non-toxic (Choi *et al.*, 2015; Li *et al.*, 2012).

Lipase protein (triacylglycerol hydrolases EC 3.1.1.3:) is listed among the essential enzyme types after carbohydrase and protease. Lipases have a subjective function in the formation and hydrolysis of fatty acid ester in saturated and unsaturated environments. In contrast with the true esterase (carboxyl ester hydrolases), Long-chain fatty acid esters can also be hydrolyzed by lipase (Guncheva & Zhiryakova, 2011). Lipases serve as a bridge between hydrophilic aqueous media and substrate of hydrophobic lipid in order to establish esters bond hydrolysis in triglyceride molecules to produce glycerol, diglycerol, monoglycerides, and free fatty acids (Feller & Gerday, 1997).

The lipase enzyme has many biotechnological applications, bio-refining, and industrial settings. In industrial settings, faster reaction speeds occur when the higher

temperature stability of these protein lipase catalysts allows for reactions (Vieille & Zeikus, 2001). Lipases are employed in a wide range of industries, including paper, leather, detergent, textile, cosmetic, and pharmaceutical (Sharma *et al.,* 2001). Most industrial processes and operations might be more cost-effective, convenient, and environmentally friendly with a well-rounded microbial enzymes uses (Hasan *et al.*, 2006; Nigam, 2013). Despite these benefits, common enzymes were useless in numerous industrial processes when exposed to high pH, temperature, and salt environments (Karan *et al.*, 2012; Sarmiento *et al.*, 2015). Under these harsh conditions, common enzymes would clump together, precipitate, and denature, drastically reducing their ability to perform their intended functions (Karan *et al*., 2012; Yadav *et al*., 2021).

Sphingobacterium sp. represents a genus within the phylum Bacteroidetes and is recognized as a Gram-negative, rod-shaped bacterium (Fu *et al*., 2017). This genus encompasses various species of bacteria that are commonly found in diverse environments, including soil, water, and clinical settings. *Sphingobacterium* species are known for their distinct lipid composition, notably characterized by the presence of sphingophospholipids in their cell membranes (Fu *et al*., 2017; Tóth *et al*., 2021).

A few years ago, a few researchers claimed that *Sphingobacterium* sp. showed high lipolytic activity; however, their claims were backed up by only a few studies (Bharathi *et al.*, 2022). Recently, there have been several new investigations on *Sphingobacterium* sp. phospholipase and mesophilic lipase (Yadav *et al.*, 2018). Despite this, there was insufficient information on finding the cold-adapted lipase from *Sphingobacterium sp*. (Li *et al.*, 2021; Zhu *et al*., 2020). This study was proposed to analyse the *Sphingobacterium sp*. lipase that was isolated from an Arctic soil sample (Kuo *et al.*, 1999; Li *et al*., 2020; Satti *et al.*, 2019). *Sphingobacterium*

sp. lipases are sensitive to changes in temperature that cause the enzyme to denature. At 25°C, these enzymes lose most of their lipase activity (Anitori, 2012; Horikoshi *et al.*, 2010).

Studies have proved that an LEA-like peptides co-expression system can improve protein production in the *E. coli* host (Ikeno & Haruyama, 2013). Additionally, LEA-like peptides enhance cellular resistance to UV, pH fluctuation, temperature, and salinity (Huwaidi et al., 2018; Metwally & Ikeno, 2020a; Pathak & Ikeno, 2017). But no research has been done and sees if LEA-like peptides can aides in improving lipase's heat and cold resistance. As a result, a simple and efficient procedure was chosen as a new way to enhance lipase expression and heat or cold resistance. In this work, peptides obtained from the Group III Late Embryogenesis Abundant (LEA) protein motif were modified and co-expressed with *Sphingobacterium* sp. lipase to investigate the peptide's potential function in boosting their expression, heat resistance, and cold resistance (Ikeno & Haruyama, 2013).

1.2 The rationale of the research

In recent times, psychrophiles' cold-adapted lipase has attracted much interest as a possible enzyme for use in cold industrial processes such as skin hair removal and cold washing (Kourist *et al.*, 2010). The interactions between common enzymes and more ordered water molecules at low temperatures pushed these enzymes toward an unfolded and degraded state. Cold-adapted lipases were shown to have flexible structures that allowed them to hang onto water molecules firmly while adapting to such an environment, maintaining their lipolytic activity with low energy consumption. Since these enzymes provided an equivalent enzymatic activity to their non-extreme equivalents while providing superior product quality preservation by

minimizing the high-temperature requirement, these cold-adapted lipases' distinctive properties became the main attention. In the previous study by Ng *et al.*, (2022), The characterization of the Ab3 lipase involved evaluating its activity across varying conditions such as temperature, pH, exposure to organic solvents, detergents, and different metal ions. Interestingly, the expression of Ab3 lipase was notably absent in the absence of the LEA peptide. However, the presence of the LEA K peptide significantly enhanced the expression of Ab3 lipase, resulting in markedly improved expression levels.

To explore additional mechanisms associated with the Ab3 lipase when co-expressed with the LEA K peptide, further in silico investigations will be conducted. This includes delving into protein structure determination through homology modeling and conducting molecular docking studies to gain deeper insights into their interactions and functional dynamics. This will enable the determination of the Ab3 lipase and LEA K peptide interaction mechanisms through amino acid residues (Sarrouh *et al.*, 2012). The simple overexpression in *E. coli* and compact size of this lipase, Ab3 lipase (37kDa), make them attractive candidates for structure-function research and genetic modification to produce desired outcomes (Goomber *et al.*, 2016; Madan & Mishra, 2014). Therefore, this research was proposed to determine the 3-dimensional structure of the Ab3 lipase and LEA K peptide and investigate the mechanisms involved in the expression and better activity through *in silico* studies.

1.3 Problem Statement

The challenge is to fully characterize the Ab3 lipase from *Sphingobacterium* sp. produced with the LEA K peptide. Although this variant shows promise for diverse applications, its detailed properties, including enzymatic behaviour, stability,

substrate preferences, and possible structural changes caused by LEA K, remain poorly understood. This lack of understanding limits the tailored use of this modified lipase in various industries like biocatalysis and pharmaceuticals. Closing this knowledge gap is crucial to optimize the utility of this unique enzyme in different fields.

The challenge is to precisely predict the 3D structures of both the Ab3 lipase and the LEA-K peptide using computational methods. Despite advancements, their intricate structures pose complexity, hindering accurate predictions. This lack of reliable structural models limits our understanding of their interactions, hampering exploration of their roles in biotechnology and therapeutics. Addressing this challenge is vital for uncovering their structure-function relationships and maximizing their potential across scientific and applied fields.

The challenge lies in comprehensively understanding the interaction dynamics between the Ab3 lipase-LEA K peptide complex, which reportedly enhances catalytic activity, through *in silico* studies. Despite indications of augmented enzymatic performance resulting from this complex formation, the precise molecular mechanisms underlying this enhancement remain unclear. Elucidating the detailed interactions, including binding sites, conformational changes, and the stabilizing effect of LEA K on the Ab3 lipase, presents a significant hurdle due to the intricate nature of such macromolecular interactions. The lack of detailed insights into this complex impedes the rational design and optimization of enzyme modifications for improved catalytic efficiency. Addressing this knowledge gap is critical to unlocking the full potential of this enhanced catalytic system for various industrial, biomedical, or biotechnological applications.

1.4 Research Objective

Substrate specificity and *in-silico* methods was used to examine *Sphingobacterium* sp. lipase activity with LEA-K peptide as co-expression. This investigation would yield further fundamental understanding and useful applications of *Sphingobacterium* sp. lipase. Thus, the following are the study's objectives:

1. To characterize Ab3 lipase from *Sphingobacterium* sp. that has been produce with LEA K.

2. To predict the 3-dimensional structure of *Sphingobacterium* sp. lipase and LEA-K peptide using an *in-silico* approach.

3. To investigate the interaction of Ab3 lipase-LEA K peptide complex that enhanced catalytic activity using *in silico* studies.

1.5 Scope of the Study

This study focuses on the following: -

1. Investigating the combined effect of co-expressing Ab3 lipase from *Sphingobacterium* sp. with the LEA K peptide. This involves studying the interaction dynamics between these molecules to understand potential alterations in enzymatic behaviors, substrate specificity, and structural changes induced by their co-expression.
2. Utilizing computational simulations and modeling techniques to predict the behaviors, binding sites, and potential structural modifications of the Ab3 lipase and LEA K peptide when co-expressed. This includes exploring how this interaction might impact the functional properties of the Ab3 lipase.

3. Exploring potential biotechnological applications stemming from the interaction between the lipase and Lea K peptide. This may involve assessing the potential enhancement of enzymatic activity, stability.

CHAPTER 2
LITERATURE REVIEW

2.1 Lipase from *Sphingobacterium* sp.

In nature, lipases are widely distributed and can be found in both microorganisms and multicellular creatures like plants and animals. Microbes, which include bacteria and fungi, account for 45% and 21% of lipases, respectively, according to a study on lipase biodiversity. Animals, plants, and algae come in second, third, and fourth, with 18%, 11%, and 3% of the vote, respectively (Patil *et al.*, 2011).

Yabuuchi *et al.*, (1983) were the first to define the genus *Sphingobacterium*, which they placed in the family *Sphingobacteriacaea* of the phylum Bacteroidetes. *Sphingobacterium* members are Gram-negative rods that are positive for catalase and oxidase, negative for heparinase and gelatinase activities, and variable in indole synthesis (Kakumanu *et al*., 2021).

Since the early days of research, the genus *Sphingobacterium* sp. has exhibited heterogeneity, encompassing Gram-negative species characterized by colonies ranging in color from yellow to orange. These organisms possess the capability to produce acid from carbohydrates and vary in motility, displaying either peritrichous flagella for motility or lacking motility entirely. Notably, their DNA compositions exhibit diverse G+C contents, spanning from high (63 to 70 mol%) to low (26 to 34 mol%) (Holmes *et al.*, 1988; Shivaji *et al.*, 1992; Takeuchi & Yokota, 1992). Some recommended classifying bacteria based on their G+C content in their DNA, their motility, the sort of respiratory quinone system they have, and their susceptibility to antimicrobial agents (Holmes *et al*., 1988; Takeuchi & Yokota, 1992). Following the reclassification, the genus was reduced to only consisting of

non-motile, yellow, rod-shaped Gram-negative, bacteria with a low G+C content (30 percent to 42 percent) (Shivaji *et al.*, 1992).

There are now 24 species of *Sphingobacterium*, most of which are Gram-negative, rod-shaped and non-fermenting bacteria (Farfan *et al.*, 2014). Only a small number of *Sphingobacterium*, including *Sphingobacterium multivorum* and *Sphingobacterium spiritivorum* isolated from cellulitis, end-stage kidney illness, lung infections, and bloodstream infections, are connected to human problems (Seemann *et al.*, 2017). Nonetheless, several new *Sphingobacterium sp*. strains, such as *Sphingobacterium composti*, have recently been proposed.

The initial discovery of the lipase enzyme in pancreatic juice was credited to Clade Bernard in 1856, as it demonstrated the transformation of insoluble oil droplets into soluble ones by dissolving them. Over time, advancements in lipase development led to the isolation of lipases from various sources. Notably, lipases were subsequently derived from the fungus *Thermomyces anugiwnosos*, marking a significant milestone. The emergence of the first commercial recombinant lipase occurred in *Aspergillus oryzae* in 1994, as documented by Chandra *et al.*, (2020). Further discoveries expanded the understanding of lipase enzymes, revealing their presence in various bacterial species such as *Bacillus pyocyaneus*, *Bacillus prodigious*, and *Bacillus fluorescence* in 1901. Additionally, bacteria including *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* have been identified as lipase producers, as reported by Hasan *et al.*, (2009)**.** This comprehensive progression of discoveries illustrates the diverse sources and origins of lipase enzymes, contributing significantly to our knowledge of their existence and functionalities. Lipases are digestible for human nutrition, either alone or in a crude mixture with other hydrolases derived from the pancreas of animals. Temperature,

pressure, pH, high conversion rates, and highly natural catalysts are among them (Huang *et al.*, 2019). Triglyceride hydrolysing enzymes have been studied for over 300 years, and the capacity to hydrolyse lipid and synthesise esters was discovered around 90 years ago (Hasan *et al.*, 2006).

The timeline of lipase structural discoveries highlights significant milestones in the understanding of these enzymes. The first lipase structure isolated from a bacterium, specifically *Pseudomonas glumae,* was unveiled in 1993. Before this, in 1990, the structure of the first lipase isolated from a fungus, *Rhizomucor miehei,* was characterized (Anobom *et al.*, 2014). Presently, various methodologies are employed to further advance the understanding and utility of lipases. These approaches encompass a spectrum of techniques, including protein engineering and strategic modifications based on insights derived from structural data. Leveraging these structural insights enables scientists to make rational modifications to lipase enzymes, enhancing their functional properties or tailoring them for specific industrial or biotechnological applications (Anobom *et al.*, 2014). This multidisciplinary approach involving structural data plays a crucial role in the continual refinement and optimization of lipase enzymes, expanding their potential across diverse fields such as food processing, pharmaceuticals, and biofuels.

The Enzyme Commission (EC) number structure serves as a code that provides detailed information about the characteristics and functions of an enzyme. In the case of the enzyme discussed, its EC number, 3.1.1.3, serves as a descriptor of its specific properties. The first digit, '3', within the EC number categorizes the enzyme as a hydrolase. This classification denotes enzymes that catalyze hydrolysis reactions, breaking down chemical bonds through the addition of water molecules (Cristalli et al., 2001). The second digit, '1', indicates the specific type of bond the

enzyme works on, in this case, an ester bond. Ester bonds are prevalent in various biological molecules and are often associated with fats, oils, and other lipid compounds. The third digit, again '1', refers to the substrates upon which the enzyme primarily acts. Specifically, it signifies carboxyl esters as the core substrates for this enzyme. Carboxyl esters are compounds with a particular chemical structure that includes a carboxyl group and an ester linkage. Lastly, the final figure, '3', denotes the enzyme type, specifically identifying it as a lipase. Lipases are a specific subgroup of hydrolases that specialize in breaking down lipid molecules, such as fats and oils, into smaller components through hydrolysis (Cristalli *et al.*, 2001). In summary, the EC number 3.1.1.3 provides a systematic breakdown of the enzyme's classification, the type of bond it acts upon (ester bond), the core substrates it targets (carboxyl esters), and its categorization as a lipase, offering a comprehensive insight into its enzymatic function.

2.2 Structure of Lipase

Microorganism lipases are members of the family of α/β-hydrolase fold proteins, which also includes proteins with molecular weights of 20 to 60 kDa (Nardini & Dijkstra, 1999; Singh & Mukhopadhyay, 2012). Ser, His, and Asp control the activity of the enzymes known as hydrolases (Farrokh *et al.*, 2014; Laachari *et al.*, 2015). The Ser residues are all located in the α/β-hydrolase in the order Ser-Asp-His, and they are all part of a consistent catalytic Gly-X-Ser-X-Gly sequence as shown in Figure 2.1. All serine hydrolases have this conserved pentapeptide motif, which is crucial for this family of lipases' catalytic activity (Nardini & Dijkstra, 1999b; Schreck & Grunden, 2014).

The quantity and configuration of α-helices, as well as the number and angle of folds in the β-strands, are the primary variances seen in the secondary structures of all lipases (Schrag & Cygler, 1997). Figure 2.2 depicts a common α/β fold.

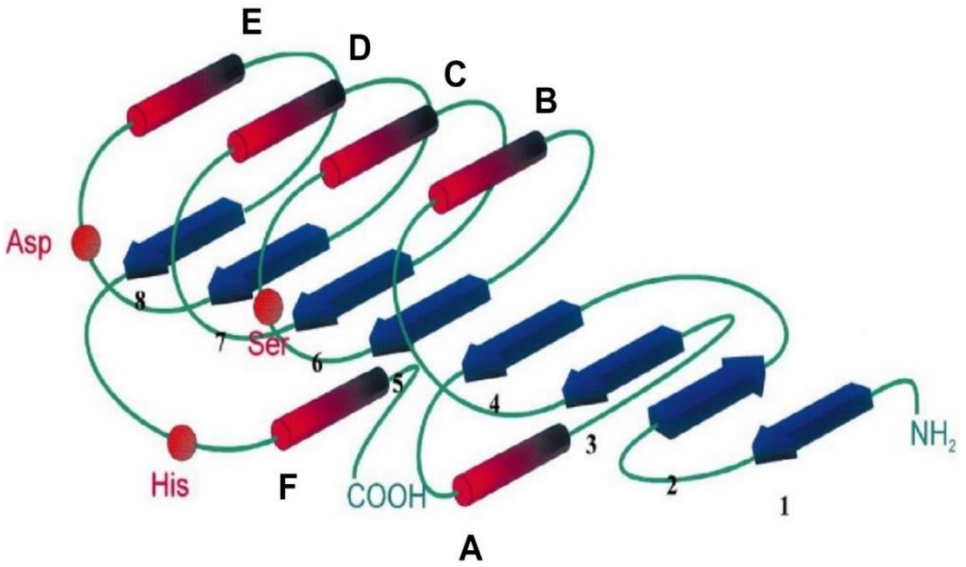

Figure 2.1 Illustration depicting the α/β-hydrolase fold (Bornscheuer, 2002)

Due to the relative proximity of the residues, alanine, serine, threonine, or valine may be substituted for glycine in the conserved region (Jaeger et al., 1999). The nucleophile elbow is created by the steep turn that this pentapeptide region creates between helix E and strands β5 (Sarmah *et al.*, 2018). Meanwhile, the substrate and the histidine residue of the catalytic triad can readily access the serine residue on the active site surface (Sarmah *et al.*, 2018). As a result of serine's strategic placement at the helix E N-terminal end, the sharp bend stabilizes the tetrahedral intermediate and amino acid ionized form (Khan *et al.*, 2017; Monteiro *et al.*, 2021). The acidic residue, however, can sometimes be seen following the β6 strand in lipases. After the β7 strand, the acidic residue of the catalytic triad is located and hydrogen-bonded to the catalytic histidine (Khan *et al.*, 2017). Finally, the catalytic triad's histidine residue is positioned after the β8 strand in a loop of various lengths and conformations (Pouderoyen *et al.*, 2001).

A "lid structure" is an unstable domain found in most lipases (Sarmah *et al.*, 2018). The form of the lid covers the active site when it is far from the oil-water interface; however, when the active site is near the oil-water interaction, the lid opens to allow interactions between the catalytic site and the substrate, hence boosting the number of solvent catalyzed processes (Khan *et al.*, 2017; Monteiro *et al.*, 2021). For instance, the *T. lanuginosus* lipase structure exhibited a variety of behaviors both closed and open. Figure 2.3 compares the two conformations (Khan *et al.*, 2017). The "oxygen anion hole" is another significant property of lipases. Based on their preference for the "oxygen anion hole" while catalyzing various substrates, Arpigny and Jaeger categorized lipases into three types: GGGX, GX, and g (Albayati *et al.*, 2020). Moreover, some interfacial recognition sites in the lipase structure have an effect on the lipase conformation when amphiphilic molecules and lipid are present, as well as the lipase stability at the interface (Albayati *et al.*, 2020).

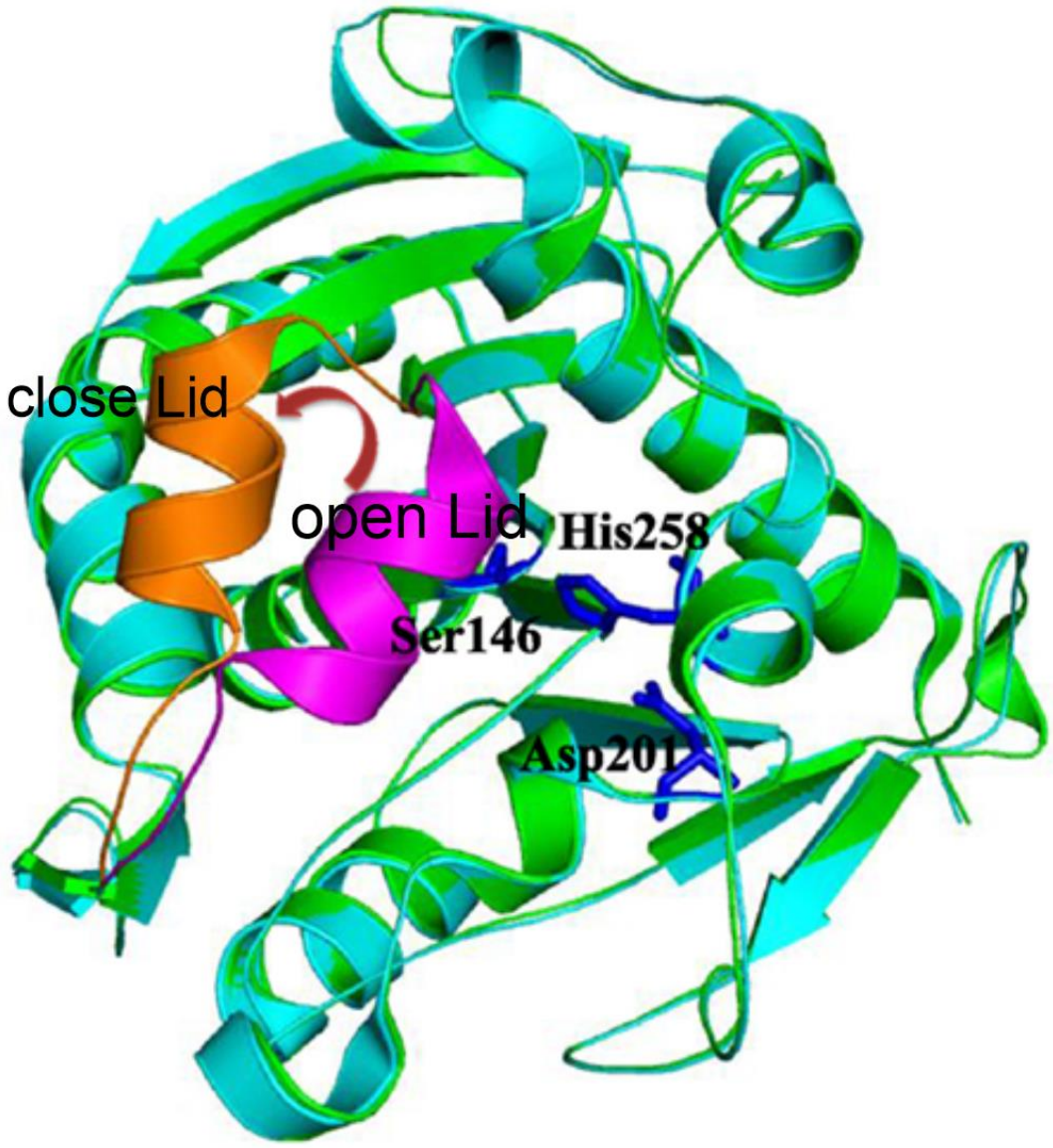

Figure 2.2 *Thermomyces lanuginosus's* lipase lid's opening and closing structure is depicted in a superimposition, indicted the colors magenta, orange, and blue to denote the catalytic triads and the close lid, respectively (Khan *et al.*, 2017)

2.3 Types of reactions catalysed by lipase

The capacity of the enzyme to catalyse various reactions is one of the reasons causing lipases to be used extensively in industries, as shown in Figure 2.2. In the presence of water, lipase is used to hydrolyse triglycerides into monoglycerides, diglycerides, glycerols, and fatty acids. It can, however, be utilised to make ester in a low-water medium by esterification or transesterification. Esterification is the reaction that forms when an acid reacts with alcohol to form an ester and water. The phenomenon of interesterification occurs when esterification and hydrolysis coincide (Sharma *et al.*, 2001). In the presence of organic solvents, a transesterification reaction hydrolyses triglycerides to produce methyl esters and glycerols. Acidolysis, aminolysis, alcoholysis, and thiolysis are the most common transesterification processes, as follows (Patil *et al.*, 2011). Acidolysis occurs when an ester and an acid combine in an organic solvent. For instance, from the fungus lipase Caprylic acid and certain oils are acidolyzed by *Rhizomucor miehei* to produce structured lipid (Fomuso & Akoh, 2002).

Aminolysis occurs when an amine interacts with alcohol to form an amide and an ester. Under solvent-free enzymatic conditions, lipase isolated from *Candida antarctica* catalysed the aminolysis of linoleyl ethyl ester with amino alcohols to create fatty alkanolamides (Couturier *et al.*, 2009). Alcoholysis occurs when a triglyceride interacts with alcohol, creating an ester. For instant, lysozyme (lipase from immobilised *Rhizomucor miehei*) and Novozyme (lipase B from immobilised *Candida antarctica*) catalyse the reaction of triolein with oleyl alcohol to create oleyl oleate, a wax ester (Couturier *et al.*, 2009). Thiolysis is a process in which a thiol group splits a chemical into two. For example, with the help of lipase from the pig

pancreas, thiophenol undergoes thiolysis with styrene oxide to produce -hydroxysulfides (Saima *et al.*, 2019).

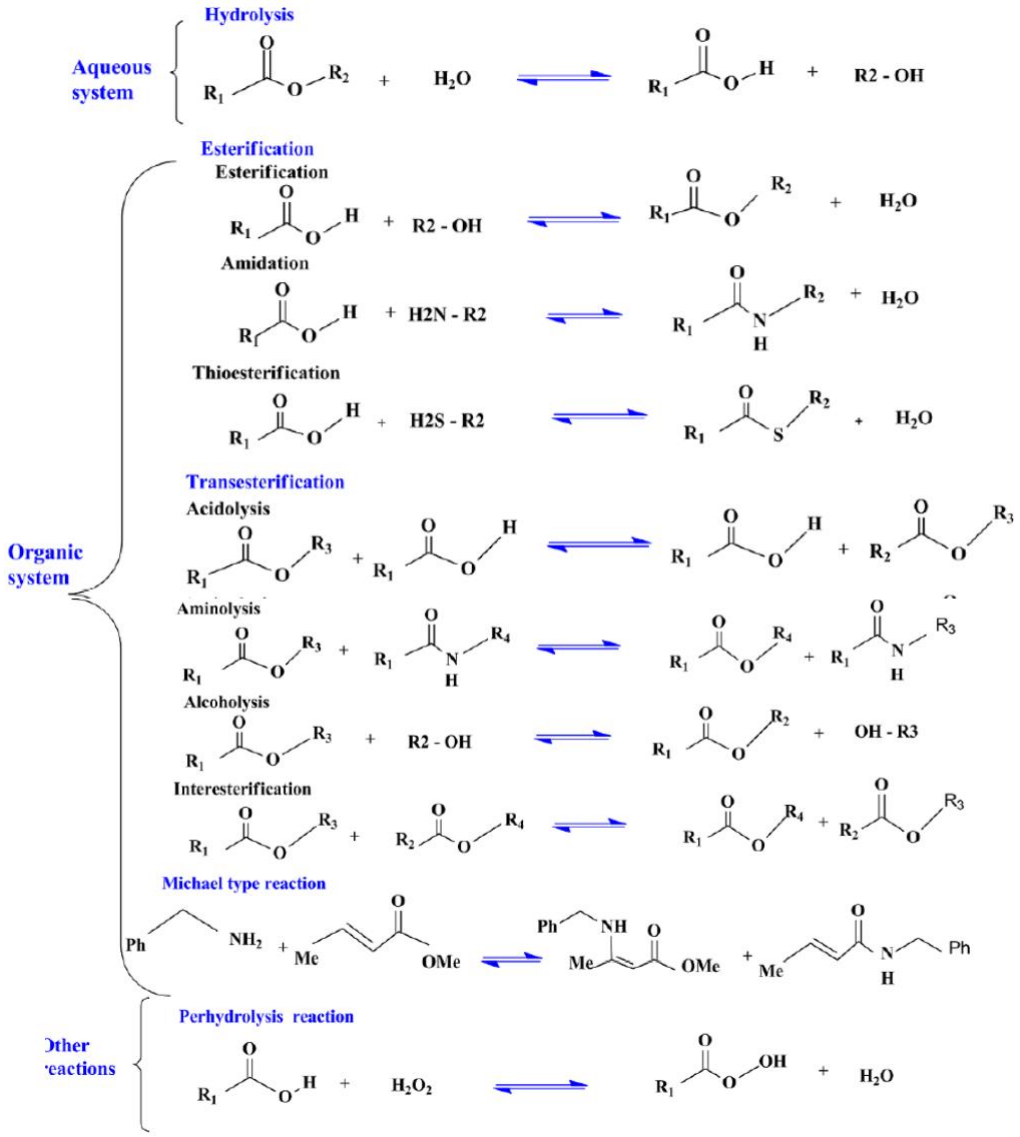

Figure 2.3 Reactions catalysed by lipase (Castillo *et al.*, 2016).

2.4 Lipase Catalytic Mechanisms

Microbial lipases use a catalytic triad like that seen in serine hydrolases for their catalytic processes (Schreck & Grunden, 2014). The four steps that make up lipase's catalytic mechanism are depicted in Figure 2.4: (a) A negatively charged oxygen molecule is created when the histidine residue at the catalytic triad absorbs

hydrogen from the serine hydroxyl group and combines it with a positively charged carbonyl carbon to form covalent bonds. (b) After a steady enzyme-substrate tetrahedral transition state forms, the lipase electrophilic area develops (i.e., the oxygen anion hole). (c) After the ester link is broken, the fatty-acid alcohol is liberated and acyl covalent intermediate complexes are produced. (d) The release of the acyl substrate occurs after the temporary bonds between the serine and the substrate are broken.

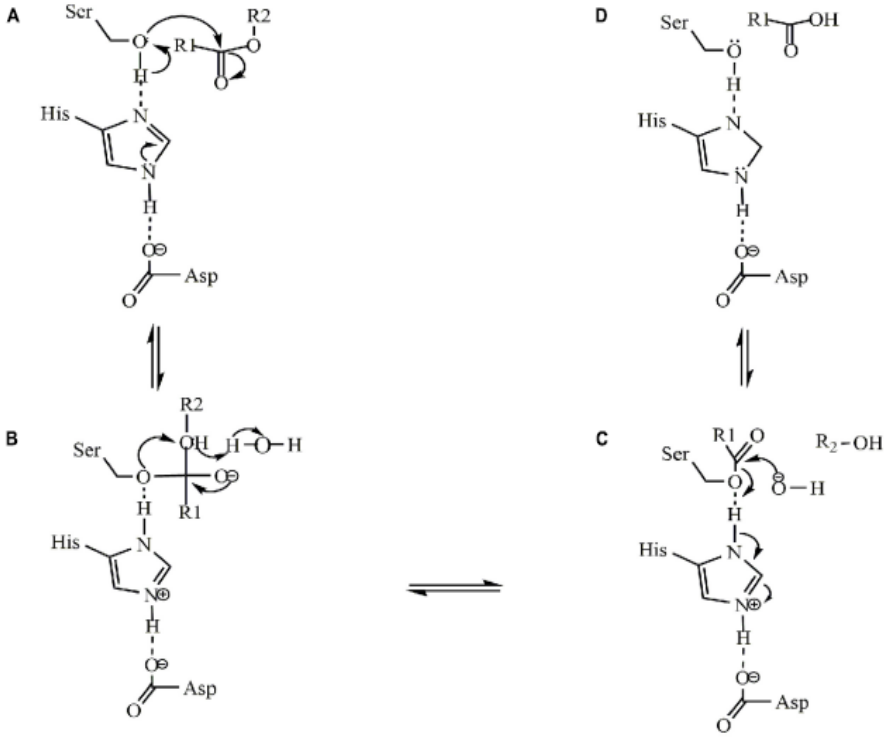

Figure 2.4 Lipase catalysis mechanisms

2.5 Psychrophiles *Sphingobacterium* sp.

Psychrophiles are a type of extremophile that can survive in icy conditions, such as the oceans and the polar regions. Bacteria, archaea, algae, yeast, plants, and animals make up most species, with polar fish flourishing beneath the ice pack being the most psychrophilic. Consequently, psychrophiles dominate in terms of diversity, biomass, and dispersion among microorganisms (Parvizpour *et al.*, 2017). In

previous decades, distinct unfavourable environmental variables were assumed to limit microbial life (Kumar *et al.,* 2011; Rothschild & Mancinelli, 2001; Van Den Burg, 2003). But over time, various earlier researchers discovered that microscopic life could thrive in extreme settings, which led to the labeling of such animals as "extremophiles" (Moyer & Morita, 2007).

The genus *Sphingobacterium* belongs to the family *Sphingobacteriacaea* within the phylum Bacteroidetes. These bacteria are Gram-negative and rod-shaped. Psychrophilic *Sphingobacterium species* are typically found in cold environments such as polar regions, alpine glaciers, permafrost, cold ocean waters, and snowfields (Farfan *et al.,* 2014). They thrive in temperatures ranging from 0°C to 20°C, with optimal growth occurring at lower temperatures. Psychrophilic *Sphingobacterium species* have evolved various adaptations to survive and thrive in cold environments. These adaptations may include (Tamboli *et al.,* 2011).

Production of antifreeze proteins to prevent ice crystal formation within cells. Modification of membrane composition to maintain fluidity at low temperatures. Enhanced expression of cold-shock proteins to facilitate cellular functions in cold conditions. Efficient nutrient uptake mechanisms to compensate for slower metabolic rates at low temperatures (Satti *et al.,* 2019; Shivaji *et al.,* 1992). Psychrophilic *Sphingobacterium species* play important roles in cold ecosystems, contributing to nutrient cycling, organic matter degradation, and microbial community dynamics. They are involved in the decomposition of organic material, including plant debris and animal carcasses, in cold environments (Tamboli *et al.,* 2011). Psychrophilic *Sphingobacterium species* have potential biotechnological applications due to their ability to produce cold-active enzymes and metabolites. These enzymes, such as lipases, proteases, and amylases, have industrial applications in processes requiring

low temperatures, such as food processing, detergent formulations, and bioremediation of cold environments (Tamboli *et al.*, 2011).

Psychrophilic *Sphingobacterium species* are of interest to researchers studying extremophiles, microbial ecology, cold adaptation mechanisms, and bioprospecting for cold-active enzymes. Understanding their physiology and molecular adaptations provides insights into microbial life in extreme environments and may lead to the discovery of novel biotechnological resources. While psychrophilic *Sphingobacterium species* thrive in cold environments, they may face challenges such as competition for resources, fluctuations in environmental conditions, and vulnerability to climate change-induced alterations in cold habitats (Farfan *et al.*, 2014).

The terms psychros and philus, which in ancient Greek meant "cold" and "love," are the scientific names for organisms known as psychrophiles, which are acclimated to frigid temperatures (Horikoshi *et al.,* 2010; Nevalainen *et al.*, 2012). According to prior studies, psychrophiles can be found in areas with a consistent low temperature, such as polar and deep marine conditions. Any creature that can only grow at a specific range of temperatures, such as 15 to 20 °C or less, but not at 32 °C, is said to be an obligatory psychrophile, according to Morita's prior disagreements. In contrast, a facultative psychrophile can thrive at temperatures ranging from 0 to 32°C (Horikoshi *et al.*, 2010; Nevalainen *et al.*, 2012; Paul & Jiang, 2001). Many studies concluded that real psychrophiles were uncommon because most bacteria in cold habitats were psychrotrophic, meaning they had a temperature optimum of 20°C or above yet could grow at 0°C (Horikoshi *et al.*, 2010; Nevalainen *et al.*, 2012; Paul & Jiang, 2001). At low temperatures, psychrotolerant bacteria have growth rates

comparable to or better than psychrophiles (Horikoshi *et al.*, 2010; Nevalainen *et al.*, 2012; Paul & Jiang, 2001).

2.6 Application of lipase enzymes

Microbial lipase is currently used extensively in biotechnology and industrial usage, and the need for these enzymes is anticipated to increase over the next five years. New technologies that will eventually aid in extending the usefulness of lipases in numerous crucial industries have taken a lot of time and effort to create. Numerous products, including food, pharmaceuticals, detergents, paper, biodiesel fuel, textiles, and cosmetics, are produced using microbial lipases (Market, 2018). Table 2.1 summarises the major industrial applications of lipase.

Table 2.1 Microbial lipases are used in industry

Industry	Action	Product or application	References
Fats and oils	Transesterification; hydrolysis	Margarine, fatty acids, glycerol, mono-, and diglycerides, cocoa butter	Konkit & Kim, 2016
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavouring agents in milk, cheese, and butter	Esteban-Torres *et al.*, 2015; Konkit & Kim, 2016
Bakery foods	Flavour improvement	Shelf-life prolongation	Lamsal & Faubion, 2009
Beverages	Improved aroma	Beverages	Chandra *et al.*, 2020
Food dressings	Quality improvement	Spices and seasonings	Gricajeva *et al.*, 2018
Pharmaceuticals and Medical equipment	Transesterification, hydrolysis	Treatment of tumors, chiral drug the resolution, antioxidants, digestive aids and Blood sensor	Bancerz, 2017; Dai & Xia, 2005; Herrera-López, 2012; Horchani *et al.*, 2012; R. Kumar *et al.*, 2017; Memarpoor-Yazdi *et al.*, 2018
Pulp and paper	Hydrolysis	Paper with improved quality	Almeida *et al.*, 2018; Demuner et al., 2011; Horchani *et al.*, 2012; Liu *et al.*, 2012; Patel *et al.*, 2019
Washing	Hydrolysis	Removal of fats	Abol-Fotouh et al., 2021; Holland *et al.*, 2020; A. Kumar *et al.*, 2016; Mehta *et al.*, 2017; Niyonzima & More, 2015; Phuah *et al.*, 2015; Saraswat *et al.*, 2017; Unni *et al.*, 2016
Biodiesel	Transesterification, hydrolysis, synthesis	Biodiesel production	Karmee *et al.*, 2015; Khosla *et al.*, 2017; Li *et al.*, 2020; Sorte *et al.*, 2020; Talavari *et al.*, 2021; Tian *et al.*, 2017; Tran *et al.*, 2016

Table 2.1 (Continued)

Industry	**Action**	**Product or application**	**References**
Leather	Hydrolysis	Leather products	Kavitha, 2019; Rashid *et al.*, 2018
Cosmetics	synthesis	Emulsifiers, moisturizers	Agobo *et al.*, 2017; Gupta *et al.*, 2015; Kim *et al.*, 2015; Tufiño *et* **al**., 2019; Uppada *et al.*, 2017
Environmental protection	Transesterification, hydrolysis	Environmental monitoring and maintenance	Bucur *et al.*, 2018; Hassan *et al.*, 2018; A. Kumar et al., 2020; Lauprasert *et al.*, 2017; Ma *et al.*, 2018

Microbial lipases in the food industry: Since the hydrolysis or esterification of oil requires highly harsh reaction conditions and specialized equipment, inorganic acids or metal oxidation have long been utilized in food preparation (Chandra *et al.*, 2020). The reaction cycle is also time-consuming, expensive, energy-intensive, and potentially polluted (Esteban-Torres *et al.*, 2015; Konkit & Kim, 2016). On the other hand, because lipase-mediated reactions are extremely efficient and selective, lipase can get over these problems when used as a biocatalyst (Gricajeva *et al.*, 2018). As a result, lipase-catalyzed processes are gradually replacing conventional methods.

Lipase's use in the paper and pulp industry: In the paper and pulp processing sector, resin is frequently found; this could be because of raw materials in the reservoir. Paper degradation, downtime, and unstable operations can be brought on by resin build-up on the paper stock, oils from the rolling resin binder, and other factors. Oils and resins are difficult to maintain and clean in machinery, which is quite uncomfortable. The effectiveness of pulp washing, screening, and purification will decrease because of resin deposition. The removal of esters by lipase from pulp could improve its quality and capacity (Horchani *et al.*, 2012).

Lipase can be used in the papermaking industries to remove just the contaminated resins and oils from the paper, reducing breakage and ensuring paper quality and output (Almeida *et al.*, 2018). In order to avoid detrimental effects on the functionality of the equipment, lipase can also be employed to remove anion residual and bitumen deposition during the papermaking process (Demuner *et al.*, 2011; Liu *et al.*, 2012). By hydrolyzing wood pitch (by 90%) with C. rugosa fungal lipase, Nippon, one of Japan's leading paper companies, has identified a means to prevent wood pitch contamination (Patel *et al.*, 2019).

Applications of lipase in the detergent industry: One of lipase's most significant industrial uses is as a component in detergents. According to estimates, over thirteen billion tonnes of detergent incorporate approximately one thousand tonnes of lipases, constituting 32% of all lipase sales and making them the second most widely used detergent additive after proteases (Mehta *et al.*, 2017). Without harming the environment, adding lipase to the detergent can increase purification and make the fabric more flexible (Kumar *et al.*, 2016). Lipases added to detergents must function under conditions of alkalinity, elevated temperatures, and various surfactants commonly found in detergent formulations.

Lipase from *Geobacillus spp*., *B. flexus* XJU-1, *B. licheniformis*, *Bacillus pumilus* SG2, and *Serratia marcescens* DEPTK21 (Niyonzima & More, 2015), *Bacillus cepacia, Candida spp*., *Staphylococcus arlettae*, and *Pseudomonas fluorescens* are often employed in (Phuah *et al.*, 2015; Su *et al.*, 2015). According to specific research, lipase produced by *A. niger* has been observed to function efficiently in typical temperature wash cycles, making it suitable for incorporation as an ingredient in detergents (Unni *et al*., 2016). *B. subtilis* lipase is suitable for use in the production of detergent due to its resistance to conventional detergents, oxidizing agents, and surfactants (Saraswat *et al.*, 2017). Others discovered that *G. stearothermophilus* FMR12 had high lipolytic activity at pH9 and temperature 70ºC, and they hypothesized that it could be used as a detergent (Abol-Fotouh *et al.*, 2021). In 2017, a patent application was made for the use of lipase in powdered laundry detergent for cleaning in cold water (Sato, 2020).

Lipase uses in the production of biodiesel: Biofuels, such as biodiesel, has emerged as a sustainable alternative to fossil fuels (Bilal *et al*., 2021; Shuba & Kifle, 2018). A contemporary source of sustainable energy, biodiesel is typically produced