Comparative *In-silico* Study of Pathogenic *Candida* and Edible Mushroom Lipases as Green Catalysts for Sustainable Biodiesel Production

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Thesis Acceptance Certificate

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This thesis is dedicated to our beloved parents who have supported us and our mentors and friends whose guidance and assistance were unparalleled.

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List of Abbreviations

1CRL 1	Candida Rugosa Lipase 1
AA	Amino Acids
Asp187	Aspartic Acid
Asp/Glu	Aspartate/Glutamine
BLASTP	Basic Local Alignment Search Tool for Proteins
C6i2tc	Human 2 Butyrylcholinesterase
ChEMBL	Chemical Database of Bioactive Molecules with Drug like Properties
CRL	Candida Rugosa Lipase
Dscore	Druggability Score
GASAG	Active Serine Hydrolase Motif
His224	Histidine 224
kDa	Kilo Dalton
kcal/mol	Kilo Calorie per mole
Lip2 Gene	Lipase 2 Gene
MEGA X	Molecular Evolutionary Genetics Analysis
Mol2	Molecular File Format
Nnscore	Neutral Network Based Scoring Function.
PC15	*Pleurotus ostreatus*
PDB Code	Protein Data Bank

Phyre2	Protein Homology/Analogue Recognition Engine 2
Pleolip241	*Pleurotus ostreatus* lipase 241
Pleolip369	*Pleurotus ostreatus* lipase 369
RMSD(Lb)	Root Mean Square Deviation (Lower Bound)
RMSD(Ub)	Root Mean Square Deviation (Upper Bound)
Scoop-Prediction	Stability Change Prediction
SDF	Spatial Data File
Ser-Glu-His	Serine-Glutamine-Histidine
SSF	Solid State Fermentation
UCSF Chimera	University of California, San Francisco Chimera

Abstract

The scarcity of the fossil fuels and their use as conventional energy source has inflicted drastic effects on the environment, stimulating the research to explore renewable and sustainable energy resources. Biodiesel produced *via* enzymatic catalysis is committed to serve as an alternative green energy fuel. This signifies the role of lipase to be integrated as a biocatalyst in the transesterification process behind biodiesel. Lipase being a ubiquitous enzyme is naturally produced by a plethora of organisms including animals, plants and microorganisms. Lipases from *Candida rugosa* are currently utilized in biodiesel synthesis, however, due to the specie's pathogenicity and containment during the fermentation process the overall production cost is elevated. The current study is a comparative investigation of a novel lipase of edible mushroom; *Pleurotus ostreatus* (Pleolip241) with *Candida rugosa* (1GZ7 and 1CRL). To distinguish the optimum biocatalyst activities of these lipases, comparative assessment was performed to study the protein structure, amino acid assemblies, catalytic triads, active sites and its residues. Based on the molecular docking against different substrate and binding energy scores the best enzyme-substrate model was identified. Edible mushroom lipase (Pleolip241) showed better affinity towards the substrate along with more stability at higher temperatures as compared lipases of *Candida rugosa*. In conclusion, Pleolip241 proved be a promising candidate and can be considered as green catalyst for sustainable biodiesel production.

Keywords: Biodiesel, biocatalyst, lipase, *Candida rugosa,* IGZ7, 1CRL, *Pleurotus ostreatus*, Pleolip24, molecular docking

Chapter 1

1.0 Introduction

The world is faced with the twin crisis of fossil fuel depletion and climate change. Excessive use of fossil fuels has led to disastrous impacts in the form of global warming, natural habitat destruction, more carbon emissions, disturbed ecosystem and harm to biodiversity. In such situations, the importance of alternative energy sources has increased many folds. It is the need of the hour to switch to sustainable and organic alternatives that do not harm the lives of living beings and the environment. Biodiesel is one such alternative fuel that is similar to conventional fossil fuel but is made from green substrates and is not harmful to the environment due to almost negligible carbon emissions.

Biodiesel is made by the process of transesterification in the presence of either chemical or enzymatic catalysts [1]. Acid and base-catalysed transesterification is done commonly due to high yields while if we look at the quality of the product, enzymatic catalysis is more favourable. This study focuses on the utilization of enzymes obtained from different fungal sources in the process of transesterification to produce methyl esters. Lipase is one of the most important industrial enzymes that can be used as a catalyst in the process of transesterification to produce sustainable biodiesel. Lipases (triacylglycerol acyl hydrolases) are class of enzymes which catalyse the hydrolysis of long-chain triglycerides. They also display alcoholysis, aminolysis, interesterification, and esterification activity, with strict regioselectivity, stereoselectivity, and chemoselectivity [2].

Lipases are classified according to the sources from which they are obtained, such as microorganism, animal and plant and can easily be produced in high yields, by fermentation processes and following few basic purification steps, from microorganisms such as fungi (e.g., *Candida antarctica, Candida rugosa*) which are of high industrial potential. The lipase enzyme has some properties; it is an octamer with a molecular mass of 30-40 kDa, an isoelectric point of 4.5, and works best at pH 5.8 and 40 ˚C. Its active site contains the well-known Ser-Glu-His catalytic triad. A 23-aa signal peptide precedes the N-terminus of the enzyme [3]. Out of all the enzymes, lipases are most significant industrially as they have a wide range of applications in food, detergent, cosmetics, sewage waste treatment plants, pharmaceutical, cheese, leather, textile

and paper industry. It is a very important component of food and agro-industries [4] due to its application in the production of beverages, dairy products and in baking etc. lipase is also used as a substitute for cocoa butter and human milk fat. Lipases are also used widely in the production of biofuels [5].

Lipases are obtained from animal sources, plant sources and microorganisms such as bacteria, filamentous fungi and yeast. Microbial lipases are the most commonly employed enzymes due to the higher yields in microorganisms as compared to plants and animals. The most common source of microbial lipases is fungi [6].

The following combination of six characteristics, is used to define organisms classified in the Kingdom Fungi: Eukaryotes, reproduce by spores, the fungus body is either in the form of hypha (filamentous structure) or yeast, fungi are heterotrophs and absorb their food through cell wall by secreting digestive enzymes, and their cell wall is typically composed of chitin [7].

Fungi can be classified on the base of the mode of nutrition into saprophytic, parasitic and symbiotic fungi and based on spore formation into four major classes that are Ascomycetes, Zygomycetes, Basidiomycetes and Deuteromycetes [8].

The focus of our project is the comparative analysis of lipases from different fungal sources to determine their potential to be utilized as a catalyst in biodiesel production. We are mainly focusing on the comparative analysis of the yeast *Candida rugosa* and the edible mushrooms, oyster mushroom or *Pleurotus ostreatus* based on computational analysis of the structure and function of different lipases present in them.

Candida rugosa is a pathogenic yeast species in the genus Candida. It belongs to the phylum Ascomycota of the kingdom fungi. It produces two distinct types of lipases named as lipase A and lipase B. Both of these lipases have a huge potential of industrial applications. Most common industrial application of lipases obtained from *C. rugosa* is in biotransformation. The analysis of the structure of the lipase molecules in *Candida rugosa* will help in assessing more uses of *C. rugosa* associated lipases [9].

The other fungus we chose for analysis is edible mushrooms out of which we chose oyster mushrooms. Oyster mushroom or *Pleurotus ostreatus*, is a common edible known for its oyster-shaped cap. *Pleurotus* sp. belong to phylum Basidiomycota that produce oyster shaped mushrooms (basidiocarps), and accordingly, they have been called as oyster mushrooms. While they look like a similar mollusc, three different types of oyster are found: *Pleurotus ostreatus, Pleurotus*

pulmonarius and *Pleurotus populinus.* The only true difference is the season they grow in. We have specifically chosen *Pleurotus ostreatus* due to high lipase content in it [10].

Through the comparative analysis of lipases from *Candida rugosa* and *Pleurotus ostreatus*, we will observe the structure of the proteins, assemblies of amino acids, the catalytic triads, active sites followed by its residues. We will also determine the catalytic activity of the lipases from both these sources. The study of the structure of active sites will help is better interpret the molecular docking of lipases against the substrates and the binding energy of each model. Thus, this analysis will lead us to distinguish the best enzyme-substrate suitable for its application in biodiesel production. As *Candida rugosa* is pathogenic and has containment issue during fermentation which compromises the industrial process by elevating the cost. Hence, we are focused on elucidating whether the lipase from oyster mushroom is efficient enough to successfully replace the candida-based lipase for the green catalysis of sustainable biodiesel production.

1.1 Problem Statement

C. rugosa is an important source for lipase production. However, due to its pathogenicity, biosafety and containment expenses during fermentation leads to increase the cost of production

1.2 Research Hypothesis

Lipases from *Candida rugosa* and *Pleurotus ostreatus* are equally good candidates to be used as biocatalysts for sustainable biodiesel production.

1.3 Research Objectives

Considering the above hypothesis, the research objectives of this research are mentioned below:

- To decipher the interaction of suitable substrates with lipases from the pathogenic, *Candidia rugosa* and edible mushroom*; Pleorutus ostreatus* by molecular docking.
- To identify the best biocatalyst by comparing enzymatic parameters of fungal lipases against desired substrates using computational assessment.

Chapter 2

2.0 Literature Review

2.1 Lipase Enzyme-An Overview

The lipase enzyme was first discovered by its property of emulsification and saphonation of fatty substances in the pancreatic juice by Claude Bernard in 1856 [11]. From its discovery till this date, lipase has been a considerable contributor of the bio-industry as a leading biocatalyst. Previously, animal pancreatic extracts were the major source of lipase for commercial use. However, due to its vast variety of industrial applications and a gap between the supply and demand; the enzyme has created opportunities for exploration and exploitation of new sources for its production. Over time, the focus has been diverted from animals and plants to microorganisms as a large-scale source of diverse form of lipases and their potential use in the industry. 'Lipolase' was the first commercial recombinant form of lipase extracted from the fungal strain of *Thermomyces lanuginosus* in 1994 by Novo Nordisk [12]. This enzyme was used by the detergent industry because of its ability for removing oil and grease stains from fabrics [13].

2.2 Structural Features of Lipases

Lipases are all α/β hydrolase proteins. They have an in-center core containing parallel β-pleated sheets configuration and a conserved sequence of catalytic triad comprising of serine, histidine, and an acidic amino acid either glutamate or aspartate and a lid domain covering the active site [78].

2.2.1 Active Site of Lipase Enzyme

The active site comprising of a catalytic triad consisting of Ser, His, and Asp/Glu (figure 2.1) within a secondary alcohol binding pocket and a binding pocket for acyl [79].

On the carboxy-terminal edge of the parallel β -sheet, the serine triad is located. In the tight turn between *the* β*-sheet* and the next α helix, the catalytic serine residue is present. The sequence surrounding the serine is generally the only conserved region existing in lipases. A consensus sequence GxSxG is found in lipases, and other α / β -hydrolases. The active site histidine, His224, is positioned at the start of the α helix such that the side chain projects into

the active site. The active site aspartic acid, Asp187, is located in a turn preceding the sixth strand [79]. All these characteristics make lipases an efficient enzyme for catalyzing reactions of a wide range of lipid molecules [80].

2.2.2 The Lid Domain of Lipases

The important feature of many lipases is the presence of a movable subdomain lid or flap located over the active site as shown in figure 2.1 below. The key characteristic of many lipases is the presence of a movable subdomain lid or flap found on the active site [85]. If closed conformation, the lipase is inactive because the active site is not accessible to the substrate. If the lid is open, substrates can enter the lipases' active sites and can be converted io products [86].

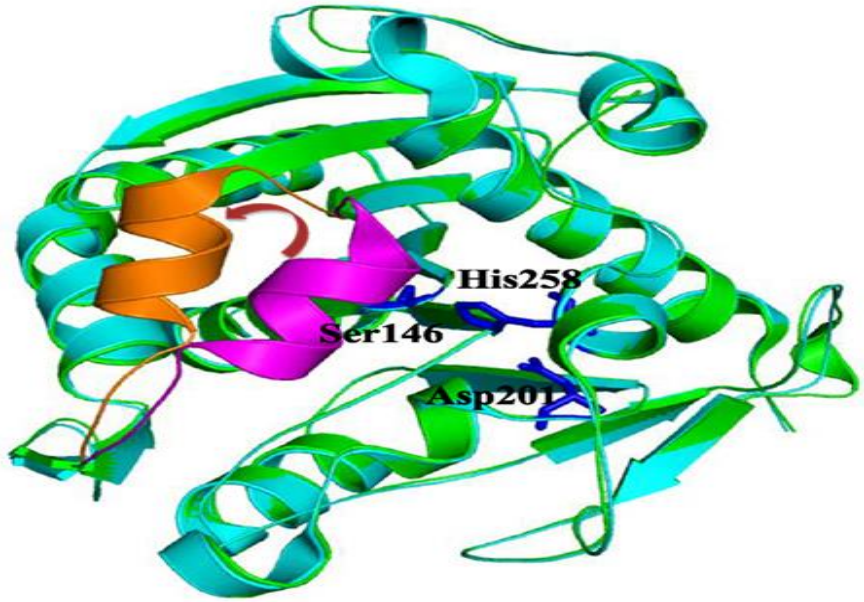


Figure 2.1 Superimposition of close (green, PDB code: 1DT3) and open (cyan, PDB code: 1EIN) conformations of Thermomyces lanuginosus lipase. The close lid, open lid, and catalytic triads were highlighted by magenta, orange, and blue colors, respectively[78]

The lid is responsible for substrate recognition and binding. It is amphipathic structure; if the lid is closed, its hydrophilic side is directed towards the solvent, whereas the hydrophobic one faces the protein core. As the lipase shifts to the open enzyme conformation, uncovering of the hydrophobic face occurs contributing to the substrate-binding region. Therefore, the amphipathic nature of the flap as well as the presence of specific amino acid is important for the activity and specificity of the enzyme [85]. The same is also reported in fungal lipases [87].

2.2.3 Catalytic Mechanism of Lipase Enzyme

The Michaelis-Menten kinetics are not followed by lipase-catalysed hydrolysis reactions. Instead, they are characterized by a two-step catalytic mechanism: Firstly, interfacial activation occurs by the enzyme being adsorbed to a heterogeneous interface, followed by an increase in the lipolytic activity afterward [81]. The covalent catalysis is performed by the serine present in the catalytic triad and this serine is activated by histidine to undergo this nucleophilic attack [82].

Serine nucleophilic attack on the carbonyl carbon generates acyl-enzyme intermediate, promoted by a histidine and an aspartate residue. This intermediate reacts with a nucleophile in a second nucleophilic attack, for example water, resulting in the formation of product that is methyl ester and returning the functional hydroxyl group to serine [83] and completing the transesterification reaction as shown in the figure 2.2. below.

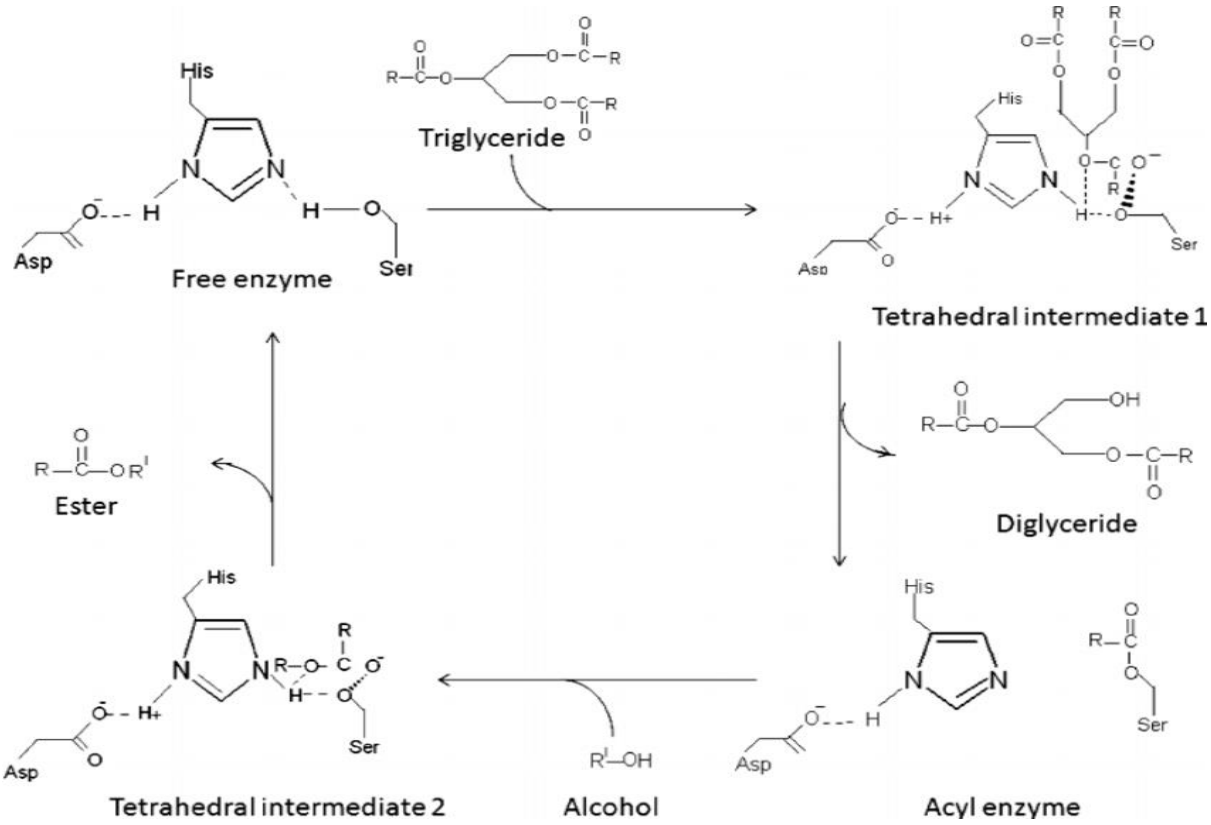


Figure 2.2 Mechanism of lipase enzyme in transesterification process [114]

2.3 Industrial Implication of Lipases

Lipases are generally integrated in bioprocessing due to their high specificity – chemo, regio, enantio – economical sources and low environmental impact [14]. Furthermore, they can catalyse diverse bio-transformations which leads to their highly increased demand on a commercial scale [15]. Use of lipase has marked its position in industries like food, detergent, oil and fat, pulp and paper, leather, textile, cosmetics, animals feed and biodiesel production. The table below (Table 2.1) highlights some major industries that incorporates microbial lipases as biocatalysts for industrial products.

List of Important Industries	Application	Microbial Source of Lipases	Reference
Detergent Industry	Decomposition of fatty material. Removal of fatty stains such as fats, butter, salad oil, sauces and the tough stains on collars and cuffs.	*P. alcaligenes* M-1	[16]
Pharmaceutical Industry	Asymmetric hydrolysis of esters like phenylglycidic acid, for manufacturing diltiazem hydrochloride	*Serratia marcescens*	[17]
Cosmetic Industry	Production of emulsifiers and moisturizers.	*Pseudomonas fluorescens*	[1]
Food Industry	Ester synthesis to produce trans-fat free margarines and shortening.	*Candida cylindracea* (AY3)	[5]
	Flavor development.	*Staphylococcus xylosus*	[19]
Dairy Products	Increase in fat content of cheese.	*Aspergillus niger*	[15]

	Cheese production.	*Candida rugosa*	[20]
Bakery Items	Bread making.	*Geotrichum* sp.	[21]

Table 2.1 Industries that use microbial lipases as biocatalysts for industrial products.

2.3.1 Application of Lipase in Biodiesel Production

Biodiesel is a type of biofuel consisting of long-chain fatty acids and short-chain alcohol, hence also called methyl esters. Transesterification reaction of oil feedstocks catalysed by immobilized lipase yields biodiesel [22]. Among various types of lipases, immobilized lipase is a promising source for yielding biodiesel due to its enhanced tolerance to organic solvents, resistance to heat and shear and most importantly its recovery [23]. Methanol and ethanol are widely used in short chain alcohols, particularly due to their low cost and physicochemical benefits. This is a method commonly employed to reduce the viscosity of triglycerides, improving the physical characteristics and efficiency of renewable fuels [24]. By using lipase for biocatalysts, both triglycerides and fatty acids are esterified, and a cleaner glycerol is obtained [25]. This approach demonstrates strong potential for the manufacture of sustainable biodiesel on a large scale because of substantial advantages over chemical methods [23]. This study will focus will further highlight the potential sources of effective lipase enzyme that can significantly catalyse the process of biofuel production.

2.4 Important Sources of Lipase

Lipases are ubiquitous enzymes produced by a plethora of organisms like plants and animals as well as numerous microorganisms such as bacteria, fungi, archaea and eukarya [26]. Lipases are present in the pancreas and on the surface of the gastric mucosa of animals. Additionally, they are also found in the salivary glands, fat bodies, muscles and plasma of certain insects like *Rhynchophorus palmarum* [27] *Drosophila melanogaster* and *Anopheles gambiae* [28]. Plants also exhibits lipase activity, but a high concentration is present in the seeds [29]. For the newly emerging plants, triglycerides are one of the importance feeds sources hence the quantity of lipases are usually high in the seeds. The activity of these enzymes is considerably high during the germination stages, as they act as rate controllers for the germination process in many seeds of the plants [30]. *Heliantusannuus* [31], *Brassica napus* [32], *Jatropha curcas* [33], *Lupinus luteus* [34] and Linum usitatissimumare [35] are some major sources of plant-based lipases. A significant amount of work has been contributed to plant-based lipases in cereal seeds, rice and wheat. However, lipases from animal or plant sources are rarely used in

industry due to the high cost of extraction and complicated downstream signaling processes [36]

2.4.1 Microbial Sources

Microbial lipases own the ability to catalyse the hydrolysis and synthesis of fatty acids. Their specific characteristics render them a great place in the enzyme technology and industry. Attributable to their high yields in the microorganisms as compared to animals and plants and their utilization in bioprocess technologies, lipases are the most commonly employed type of enzymes in different industries [37]. A vast diversity in bacteria, yeast, and fungi [38] are producers of extracellular lipase. Based on the source of the enzyme, different techniques are utilized to extract it, for example, fungal lipases are cultivated in solid-state fermentation (SSF) while for yeast and bacterial lipases submerged fermentation is considered [39]. Moreover, microbial lipases can catalyse diverse reactions, yielding high quality and quantity of products [40]. Along with low production costs and enhanced stability, these microbial lipase sources can be genetically manipulated according to the need [41]. Thus, the number of available lipases has surged high majorly because of the extensive research and achievements made in the cloning and expression of enzymes from microorganisms [42].

2.4.2 Bacterial Lipases

In 1901, *Serratia marescens* and *Pseudomonas aeruginosa* were the first reporters of bacterial lipases [43]. Since then, lipases have been widely researched and documented by several different bacterial organisms. Numerous publications on the development of bacterial lipases, particularly of *Bacillus sp., P. aeruginosa* and *Pseudomonas*, are available [44]. The extracellular lipases are essential commercially because they are much simpler to produce. While there are a variety of bacterial sources containing lipase, only a handful are commonly used as wild or recombinant strains. Several strains of *Pseudomonas* and *Bacillus* bacteria are known for producing biotechnologically important lipases. For instance, Lumafast and Lipomax from *Psuedomanas sp*. are widely used enzymes in detergents [45]. Similarly, lipases from *Bacillus spp* have tremendous potential in bioremediation of oil-contaminated wastewater [46]. A few other bacterial species that account for the production of lipases *are Acinetobacter, Achromobacter, Alcaligenes, Arthrobacter,* [47], *Staphylococcus, Burkholderia and Chromobacterium* [48].

2.4.3 Fungal Lipases

Fungal based lipases are better investigated among all microbial lipases. Consequently, fungi are rendered as the best source for the production of industrially important lipases due to extracellular lipase production. They are also considered economical in contrast to bacterial due to cost-effective extraction *via* batch fermentation for fungal lipases [49]. The SSF further incorporates different wastes with low amount of water. Fungal production of lipases differs with strain, growth medium, structure, cultivation conditions, pH, temperature and the form of sources of carbon and nitrogen Investigation of lipase production by a new isolated of *Aspergillus sp.* World Journal of Microbiology & Biotechnology. Several fungal species belonging to different classes are reported to be the producers of lipase. Different phycomycetes like *Rhizopus oligosporous* [50] and *Rhizopus deleme* [51] along with some aquatic species like *Allomycesanomalus, Allomyces arbuscular* and *Brevilegnia diclina* [52] are mass-producers of a variety of lipases. Similarly, different classes of ascomycetes–*Aspergillus niger* [53], *Penicillium restrictum* [54] *Sarcomycetes, – Geotrichum candidum* [55] *Candida antartica* [56]and *basidiomycetes–Schizophyllum commune* [57] *Pleurotus ostreatus* [58] are also contributing to the production of biotechnologically important lipase. There is a significant diversity in the fungal sources of lipase enzyme ranging from pathogenic species of yeast to non-pathogenic mushrooms. The variation in the structure and properties of lipases in these organisms is considerable with regard to the diversity in the source. Many pathogenic fungi which causes serve infections are the leading producers of lipase for bio-catalysis of industrial products. For instance, *Rhizomuco miehei* causes fatal mycotic diseases but is additionally producing lipases with high reactivity and stability. The lipase is widely used in the food industry as a potent biocatalyst in the transesterification and modification of fats and oils [59].

2.4.3.1 Lipases in Pathogenic Fungi-Candida

Candida, a genus of yeast is responsible for inflicting a plethora of fungal infections worldwide. Many species of this genus like *Candida albicans, Candida rugosa* and *Candida antartica* lives as endosymbionts with humans, however, they can inflict many opportunistic mycotic infections like systemic illness causing abscesses, thrombophlebitis, endocarditis, or infections of the eyes if immune barriers are invaded [60]. In-contrast the lipase extracted from these species serves in many biotechnological processes such as lipid hydrolysis and esterification reactions [61]. *Candida rugosa* is one of 154 Candida species [62]. *Candida*

rugosa lipases were initially described in the sixties when the yeast was extracted from natural soils because of its high development potential for lipase [63]. Although it is recently being cited as an emerging cause of Fungemia [64], [65] but due to miscellaneous lipase applications and the growing interest, much work has been dedicated to the production of *Candida rugosa* based lipases for biocatalysts. Hence, two isozymes – Lipase A and B – were isolated, processed and optimized [66]. At least seven genes are now well known to be involved in *C. rugosa* lipase-producing machinery among which five of them are characterized biochemically for commercial use [67], [68].

Lipase from *Candida rugosa* has been reported to exhibit significant hydrolytic activity. The esterification reaction of palmitic acid and lauryl alcohol catalysed by lipase from *Candida rugosa* yielded 90% pure lauryl palmitate. Similarly, lipase B from the same species is widely used in the cosmetic industry for producing beneficial skin products [69]. Studies also suggest that this lipase has been acting as an amperometry sensor [70] and for detection of triglycerides [71]. Due to its ability to catalyse the hydrolysis of triacylglycerol to glycerol and fatty acids, lipase is also used in the biosensing of β-hydroxy acid esters and triglycerides in blood serum [72]. Biodiesel is an ester group manufactured via fatty acids transesterification reaction with alcohols in the presence of biocatalysts. Immobilized *Candida rugosa* lipase has been reported as the most promising biocatalyst for the synthesis of biodiesel via enzyme catalysis [73]. Moreover, *Candida rugosa* lipase (CRL) has also been experimented and reported in a recent study for the production of biodiesel from Chinese Tallow kernel oil [74].

2.4.3.2 Non-Pathogenic Fungal Sources of Lipase Enzyme

Apart from pathogenic fungal species of yeast, many edible mushrooms from the class; basidiomycetes are also known for contributing to efficient lipase production in large quantities. However, little work have been done for developing potential lipase enzymes from these species except a few like *Agaricus bisporus* [75] and *Lentinus edodes* [75] and *Pleurotus florida* [76]. Additionally, the heterologous expression of the extracellular lipase [77] and functional expression of the lip2 gene producing lipase was studied in *Pleurotus sapidus.* The genome mining of the *Pleurotus ostreatus* shows that 53 lipases were annotated automatically among which Pleolip241 and Pleolip369 were further characterized. The study also revealed that Pleolip241 could be substantially used on a large scale for removing hydrophobic layer from wool surface in order to improve its dyeability. There are many non-pathogenic fungal sources of the lipase enzymes, yet to be explored. The lipases extracted from mushrooms like

Pleurotus florida and *Pleurotus ostreatus* are economically beneficial and eco-friendly. These enzymes can be optimized for large-scale industrial applications such as biodiesel production. Due to less scientific attention given to extraction of industrially important lipases from mushrooms followed by the limited amount of data present on their biochemical and physiological analysis, this study will open windows for further research on *Pleurotus ostreatus* based lipase to be administered in biotechnologically important applications. Besides, it will provide a comparative analysis of lipase producing, pathogenic fungi; *Candida rugosa* with non-pathogenic, edible mushroom; *Pleurotus ostreatus* to determine and identify more effective lipase among the two species based on structural and functional relationship [58].

2.5 Comparative Computational and Experimental Analysis of Fungal Lipases

Various microbial lipases of bacterial and fungal origins have been investigated and compared for analysis of their structures to identify lipases with high stability and enantioselectivity and their subsequent utilization in the industries [84].

In a comparative study of commercially available lipases, nineteen commercial lipases obtained from different sources were analyzed to compare their activity. Based on the result of the study it was concluded that particular attention should be paid to the source organism in selecting lipases for use in the industrial enzymatic processes [113].

As lipases coming from different sources have different properties therefore, a comparison must be done to select the tailor-made lipases for their specific use in green chemistry. The determination of the best suitable substrates for the lipases from microbial sources is an area of growing research. Therefore, the prediction and iterative synthesis of the best lipase model according to the industrial need is the essential goal of lipase engineering [88].

There is insufficient data on computational comparative analysis of fungal lipases specially between pathogenic and edible lipases. Present study will bridge the gap by computationally comparing the lipases from two industrially important fungal sources namely an ascomycete pathogenic *Candida rugosa* and a basidiomycete edible *Pleurotus ostreatus.*

Chapter 3

3.0 Methodology

3.1 Ligand Retrieval and Preparation

T 105 different ligands that are an important substrate for lipase enzyme were selected based on their potential use in biodiesel production. The ligands were downloaded in SDF (Spatial data file) format from a database of bioactive molecules "ChEMBL" [94, 95] and were visualized using PyMOL.

These ligands were prepared for molecular docking by converting SDF files into mol2 format using a chemical toolbox open babel [96, 97]. Furthermore, the ligands were docked as mol 2 since it is standard format for input and output of dock that modelling program can read.

3.2 Protein Sequence Retrieval

Structural data of Lipases of *Candida rugosa* was retrieved using the PDB database. Two distinct Lipases from *Candida* were selected for further analysis. This includes PDB accession codes: 1CRL [98, 99] and 1GZ7 [100, 101]. The PDB file containing structural coordinates and information of the two lipases (1CRL, 1GZ7) along with FASTA file containing protein sequence was downloaded. The protein sequence of lipase from *Pleurotus ostreatus* Pleolip241[58] was kindly provided by Dr. Alessendra Piscitelli, from the University of Naples Federico II, Italy.

3.3 3-D Structure Prediction through Phyre2

The protein sequence of Pleolip241 was used to predict the 3-D structure of the enzyme using phyre2 [105] using intensive mode profile-profile alignment algorithm. After prediction Phyre2 investigator was also run. The PDB file was downloaded.

3.4 Protein Preparation for Molecular Docking Analysis

All three proteins were prepared for molecular docking by predicting the active site pocket using DogSiteScorer [103, 104]. DogSiteScorer was used to search for ligand binding pockets

and calculate their geometrical as well as their physicochemical properties. In general, pockets with a Dscore higher than 0.7 were considered good candidates for the active site, thus pocket P_0 of each lipase was chosen. The changes with active site pockets were applied to PDB of the enzyme structures.

After active site prediction, the protein was modelled using UCSF chimera [105]. Changes were made to adjust the pH by Addition of H^+, Removal of H_20 was done, appropriate charges were also applied, energy minimization was done and PDB files of enzymes were converted to pdbqt file format, a useable file format for docking afterward.

3.5 Molecular Docking of Ligands with Lipases using PyRx

Molecular docking between residues of the active site of the lipase enzyme and ligands was performed by Autodock Vina PyRx version 0.8 [106] for Windows [107]. Table 3.1 lists the parameters used for docking in Vina Search Space. In short, the lipase macromolecule was loaded into the software first, and then molecular docking was carried out with ligand files. The grid size was set up to cover all possible interactions between active sites of enzyme and each ligand. Also, the analysis was extended to ensure that each ligand was in its own position with reference to the structure of the active site. A total of eight runs were performed for each docking by setting the exhaustiveness at 8. The docking was performed in two batches 52 and 53 ligands docked in each batch. The same process was repeated for all three (Pleolip241, 1CRL, 1GZ7) lipases and corresponding 105 substrates.

Center (Å)		**Dimensions (Å)**	
X	**–27.5501**	X	65.5753
Y	**–0.2162**	Y	62.4444
Z	**50.3675**	Z	57.9704

Table 3.1 Parameters used in Vina search space for docking.

After docking interactions, the conformation was chosen with the lowest docking energy, since the higher the negative value of the binding energy, the stronger is the affinity between the enzyme and the ligand.

3.5.1 Verification of Docking Data

The verification of docking scores of docked poses of ligands and enzymes was done by using the python implementation of NNScore 1.0 [108] along with a consensus of 24 best scoring networks.

3.5.2 Analysis of Docking Data

The 3-D interactions between best ligands and respective enzymes after docking was visualized using PyMOL. The data obtained from the docking of three enzymes with ligands were further analyzed statistically to identify the best substrate for each enzyme. The statistical analysis involving frequency of the highest number of maximum binding energy with ligands under consideration with each enzyme was calculated using statistical tool R version 4.0.1 [109].

3.6 Phylogenetic Analysis of Fungal Lipases

Multiple sequence alignment of three lipases was done using CLUSTAL W [110]. Conserved residues in active sites of 3 lipases were identified by analysis of lipase coding sequences of *Candida rugosa* and *Pleurotus ostreatus.*

The BLASTP homology search was done for each lipase to identify closely related sources of fungal lipases. The total of 19 protein sequences of fungal lipases from varied sources were chosen for further phylogenetic analysis. The multiple sequence alignment of these protein sequences was done using T-coffee [111]. The Phylogenetic tree of 19 chosen protein sequences of fungal lipases by the Maximum likelihood method was constructed using MEGA X software.

3.7 Effect of Temperature on Enzyme Stability

The comparative stability of enzymes as a function of changing temperature and thermodynamics was predicted using SCooP-Prediction of the stability curve of protein [112]. Comparative analysis for the three enzymes was performed based on the thermal stability of each, defined by the melting temperature, and the thermodynamic stability, specified by the folding free energy at room temperature.

3.8 Overview of Methodology

Complete methodology followed in this computational comparative study of 3 different fungal lipases from pathogenic (1CRL and 1GZ7) and edible (Pleolip241) origin along with the bioinformatic tools used is presented below in the schematic flowchart figure 3.1.

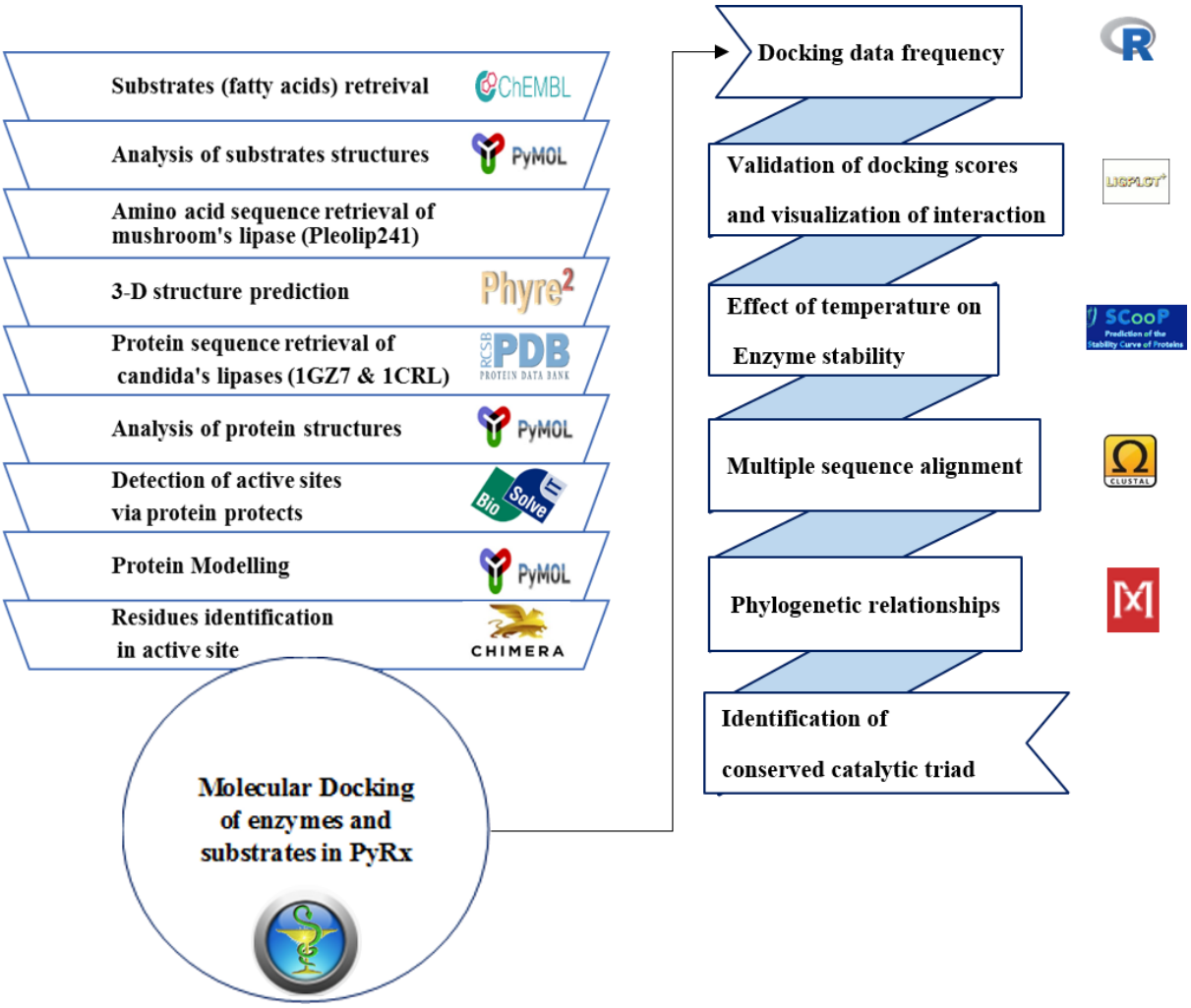


Figure 3.1 Summary of complete methodology of the study along with bioinformatic tools used.

Chapter 4

4.0 Results

4.1 Retrieval of Ligands and Proteins

Almost 105 suitable ligands consisting mainly of fatty acids and triglycerides were chosen. The prominent fatty acids included stearic acid, nervonic acid, palmitic acid, oleic acid, myristic acid, heptadecanoic acid, erucic acid, behenic acid, linoleic acid, elaidic acid and so on. The lipases from *Candida rugosa* with PDB accession number 1CRL and 1GZ7 were chosen based on their commercial use in biodiesel production. Lipase enzyme selected from *Pleurotus ostreatus* was Pleolip241 and amino acid sequence was used to construct 3-D model.

4.2 Predicted Three-Dimensional (3-D) Structure Model of Novel Pleolip241

A comparative result was obtained from Phyre2 software which showed the presence of helices and coil in the 3-D space. From a total of 120 templates aligned to the query sequence, 32 templates showed 100% confidence with the query input and gave a 3-D model. The human2 butyrylcholinesterase (c6i2tC) tetramer which showed maximum per cent identity of 30% and confidence 100% with the query sequence and was used as a template. The most probable structure predicted by Phyre2 software has been presented in Figure 4.1 below. The result was summarized by 86% of residues modelled at >90% confidence. Six templates were selected to model protein based on heuristics to maximize confidence, percentage identity and alignment coverage. The 86 residues were modelled by ab initio.

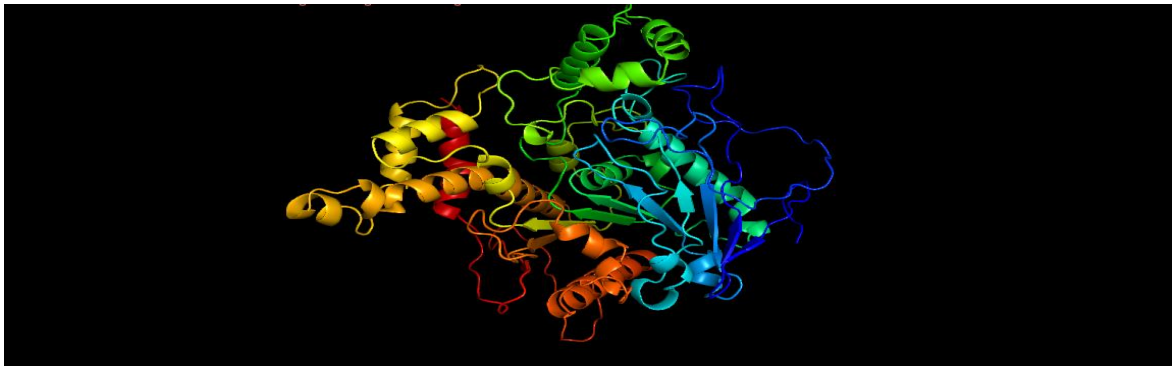
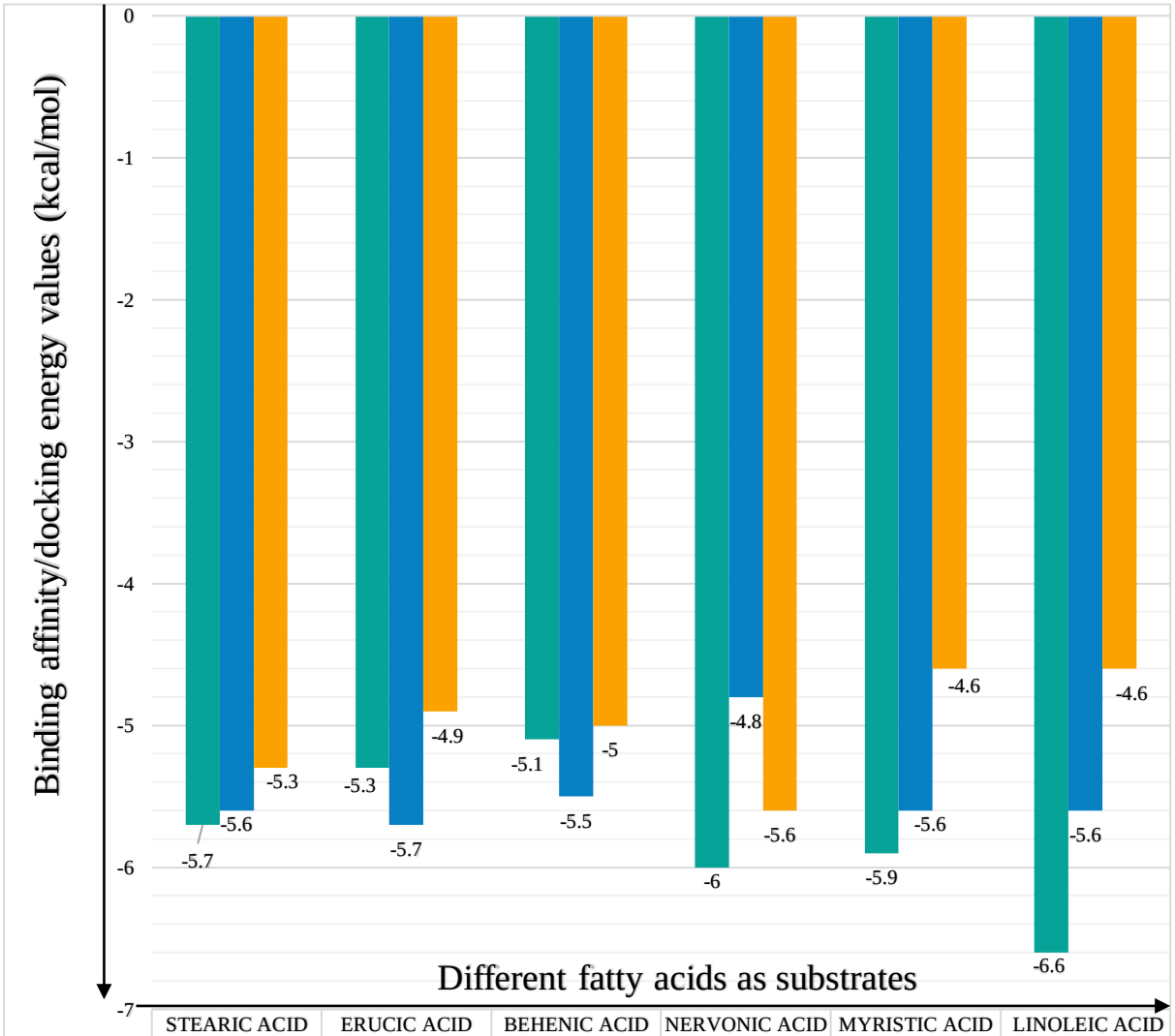


Figure 4.1 Phyre2 predicted 3-D model of Lipase of P. ostreatus-Pleolip241. (VIBGYOR color pattern from N→C terminals) Model dimensions (Å): X:59.935 Y:71.574 Z:56.978

4.3 Results of Docking by Pyrx and Autodock Vina

The results of the data of molecular docking by Autodock vina was obtained in form of excel data files containing values of binding affinity and RMSD(lb) as well as RMSD(ub) for each enzyme with each ligand molecule and its 8 different poses. Using this data, the best substrates for each of the three enzymes were chosen based on least binding affinity value. Threshold for good binding is below zero. All 3 lipases showed binding affinity in ranges of –4 to -7 kcal/mol to these fatty acids. Data of these top 6 substrates in shown in figure 4.2 below.



	STEARIC ACID	ERUCIC ACID	BEHENIC ACID	NERVONIC ACID	MYRISTIC ACID	LINOLEIC ACID
pleolip241	-5.7	-5.3	-5.1	-6	-5.9	-6.6
1CRL	-5.6	-5.7	-5.5	-4.8	-5.6	-5.6
1GZ7	-5.3	-4.9	-5	-5.6	-4.6	-4.6

Figure 4.2 Important fatty acids for biodiesel production and their binding efficiency with Fungal Lipases calculated by docking through Autodock Vina.

4.3.1 Molecular Docking of Best Substrates Against the Lipases for Potential Biodiesel Production

The best substrate for each enzyme was identified based on docking affinity and less difference between RMSD values of each docking pose as indicated in table 4.1 below.

Lipase Enzyme	Binding affinity(Kcal/mol)	Best Substrate	Source/feedstock for biodiesel
Pleolip241 (*Pleurotus ostreatus*)	-6.6	Linoleic acid	Spent vegetable oil, waste industrial oil Jatropha plant oil
1CRL (*Candida rugosa*)	-5.7	Erucic acid	Mustard oil, Jatropha plant oil
1GZ7 (*Candida rugosa*)	-5.6	Nervonic acid	*Lunaria annua*, Marine oils mainly algal sources

Table 4.1 Best performing Substrate for each enzyme based on molecular docking

The best substrate for 1GZ7, the four chained lipase from *C. rugosa* showed best binding affinity with nervonic acid (figure 4.3). Nervonic acid is found in highest amount in oil of *Lunaria annua*, a plant of the family of Brassicaceae as well as other seed oils important for biodiesel production.

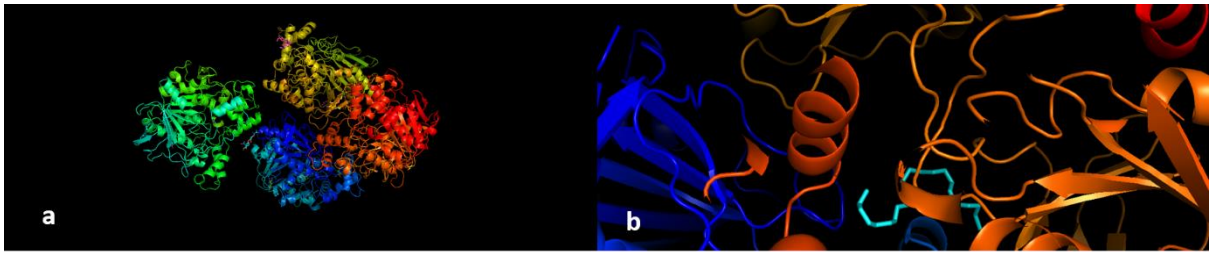


Figure 4.3 a. 1GZ7(Rainbow N->C Terminus) enzyme docked with best substrate nervonic acid (Light blue), b. Zoomed in figure of interaction with ligand on right

The best substrate for 1CRL enzyme was identified to be erucic acid (figure 4.4) It is also an important fatty acid found in abundance in mustard oil and Jatropha plant oil-an important

feedstock for biodiesel production.

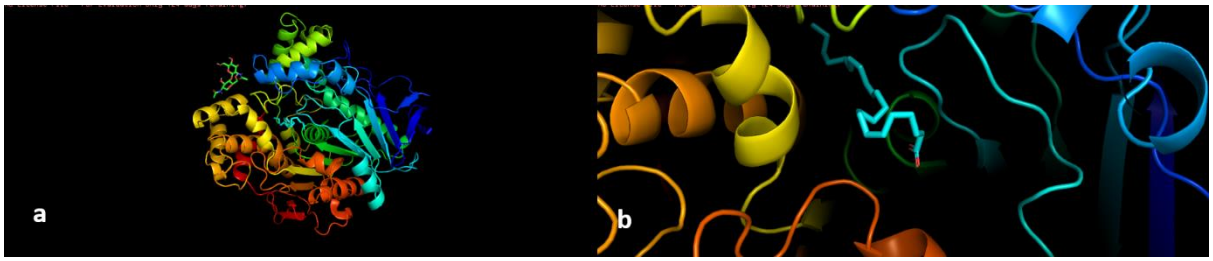


Figure 4.4 a. 1CRL (Rainbow N->C Terminus) enzyme docked with best suited ligand-Erucic acid (Light blue), b. Zoomed in figure of interaction on right

The best substrate for Pleolip241 was found to be linoleic acid (figure 4.5). It is one of the most important fatty acid with high content in many oil sources including cotton, coconut, palm kernel, castor beans, rapeseed, soybeans, and sunflowers. It is also found in spent vegetable oil and jatropha plant.

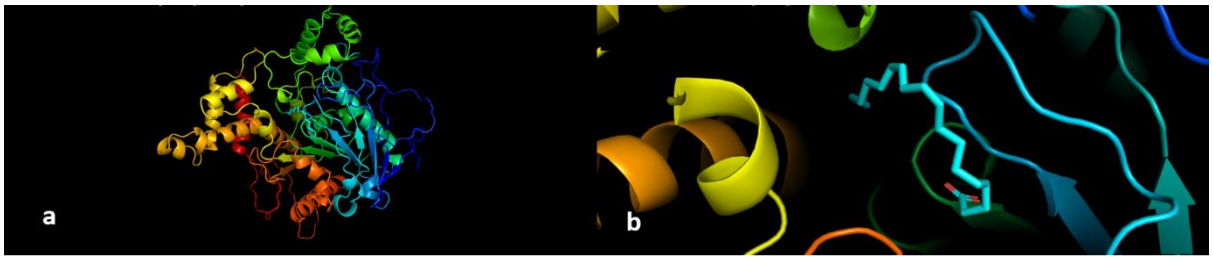


Figure 4.5 a. Pleolip241 (Rainbow N->C Terminus) enzyme docked with better performing substrate-Linoleic acid (Light blue colour) b. Zoomed in figure of interaction on right.

4.3.2 Statistical Analysis of Docking Data

Comparative affinities of 3 fungal (1CRL, 1GZ7 and Pleolip241) lipases under consideration with substrates was calculated by statistical analysis of docking data by calculating frequency. The algorithm on R was set to find the lipase with highest number of maximum binding affinities for the substrates considered. The result indicated that for 45% of the substrates, Pleolip241 was the enzyme that showed best binding affinity. 1CRL enzyme showed best binding efficiency with 30% of the ligands under study.

Lipase Enzyme	**Maximum docking energy with substrates molecules (105 total)**
Pleolip241 (*Pleurotus ostreatus*)	48 (45%)
1CRL (*Candida rugosa*)	31 (30 %)
1GZ7 (*Candida rugosa*)	26 (25 %)

Table 4.2 Frequency of best binding affinity of enzymes with their substrates by Statistical analysis using R

4.4 Multiple Sequence Alignment of Lipases

The multiple sequence alignment of 2 lipases from Candida rugosa (1CRL and 1GZ7) and Pleolip241 indicated the presence of conserved pentapeptide (GASAG). The presence of conserved catalytic triad comprising serine, histidine and aspartate indicated confirming the sequence similarity between lipases that allow them to have similar substrate specificity.

Figure 4.6 Multiple alignment among lipase coding sequences from P. ostreatus and sequences of lipases from Candida Rugosa. In the black box the conserved pentapeptide is displayed. The red arrows indicate the three amino acids (Serine,Aspartate,Histidine) of catalytic triad.

4.5 Phylogenetic Analysis of Fungal Lipases by Maximum Likelihood Method

Using Maximum likelihood method, the condensed tree is made, and the test of phylogeny has performed is bootstrap method with the number of bootstrap replications as 100. The model used is Jones Taylor Thonton (JTT) with frequencies (+F) model. The tree interference options considering ML Heuristic Method is Nearest Neighbor Interchange (NNI). The cut off value is 50%. The results indicate that Lipases of *Candida sp.* are more closely related to each other while the lipase from *Pleurotus ostreatus* (PC15) resemble more closely to other fungal lipases like *Penicillium sp.* and *Aspergillus sp.* As indicated in phylogenetic tree below.

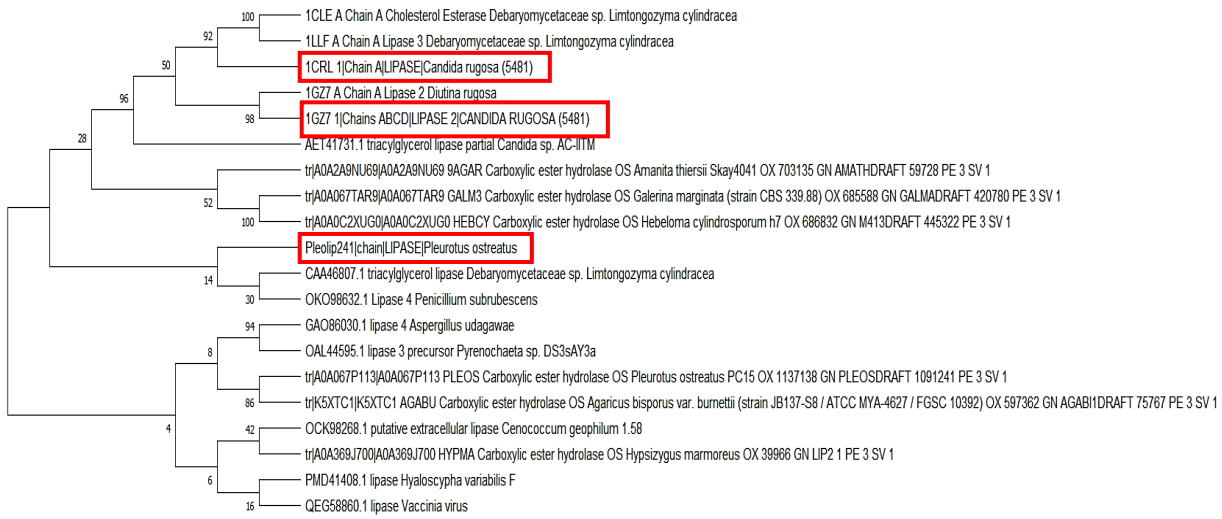


Figure 4.7 Maximum likelihood tree of lipase amino-acid sequences. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pair wise deletion option). This analysis involved 20 amino acid sequences. There was a total of 621 positions in the final dataset. Phylogenetic analyses were conducted in MEGA X. P. ostreatus lipase along with lipases from Candida rugosa are highlighted

4.6 Impact of Temperature on Enzyme Stability

Stability of all three enzymes using temperature as function was calculated. The stability curve of all three enzyme were generated that showed that the stability of Pleolip241 is more as compared to the other two lipases from *Candida rugosa.* The temperature range at which enzyme remain stable is also widest for putative Pleolip241 from oyster mushroom followed by 1CRL from *Candida rugosa*. The thermostability indicate the Tm, the temperature at which

half the protein is folded, and the other half is unfolded and deltaG is =0, the delta free energy of transition from the unfolded to the native state at room temperature, the heat capacity of the folding transition as shown in table 4.3 below.

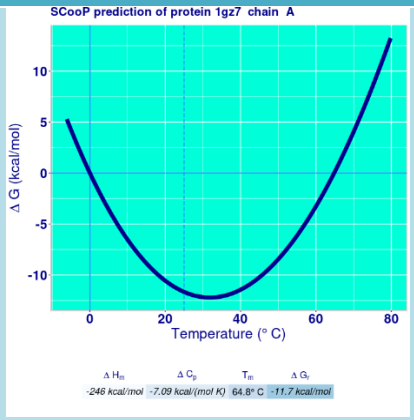
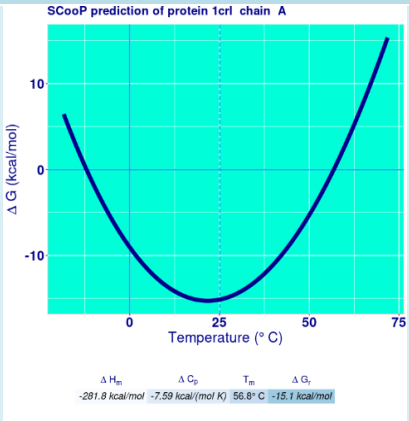
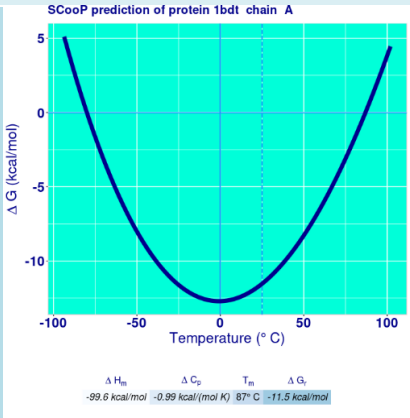
Enzyme	Delta H_m	Delta C_p	T_m	Delta G_r	Temperature range	Stability curve
1GZ7	-246 kcal/mol	-7.09 kcal/(mol K)	64.8 °C	-11.7 kcal/mol	-6.15; 79.83 °C	
1CRL	-281.8 kcal/mol	-7.59 kcal/(mol K)	56.8 °C	-15.1 kcal/mol	-18.38; 71.84°C	
Pleolip241	-99.6 kcal/mol	-0.99 kcal/(mol K)	87°C	-11.5 kcal/mol	-93.58; 101.99 °C	

Table 4.3 Comparison of thermodynamic stability of lipase enzymes by Scoop protein stability Prediction curves.

Chapter 5

5.0 Discussion

There are many commercial lipases available to use as a biocatalyst in biodiesel production but none of them is potent enough to replace chemical catalysis. The Pleolip241 from edible mushroom *Pleurotus ostreatus* proved to be a really good candidate for substrate catalysis for biodiesel production. It's higher binding efficiency and ability to retain its structure even at high temperatures was found indicating its better efficacy. All these properties are conferred to it because of its evolutionary advantage of having one lipase chain with residues of catalytic triad present in close proximity to each other as compared to other fungal lipases. Between 1CRL and 1GZ7 from *Candida rugosa*, 1CRL proved to the better catalyst as it had higher binding affinity as well as more temperature tolerance as compared to 1GZ7. The reason behind this can be the four-chain conformation of the lipase. That makes it less suitable for interaction with the substrate and contributes to its less fold stability.

The substrates that proved to be best for each enzyme also reveal interesting information. The difference in the binding affinities of different substrates with each enzyme is due to their varied 3-D configuration. The active site of lipases is highly selective, but it is oriented in such a way to accommodate every type of fatty acid. Although the interaction is not uniform among similar fatty acids. Pleolip241 is a putative enzyme and the growth of its source oyster mushroom is comparatively very easy as compared to pathogenic *Candida rugosa.* Thus, pleolio241 from *Pleurotus ostreatus* can serve as a potential catalyst for biodiesel production.

As the multiple sequence alignment indicates, the conserved pentapeptide and the residues of the catalytic triad that play a very important role in catalysis are conserved in all three lipases under consideration. This justifies the efficient binding of all the chosen substrates with all three lipases. The molecular configuration of the active site might not be the same but similar substrate specificity is due to the similar function of catalysing the reaction of the formation of methyl esters for biodiesel production.

The best substrates identified for each enzyme also give very significant information for preferred feedstock for the reactor while using each of the lipases as biocatalyst. The linoleic acid-rich feedstocks like spent vegetable oil and industrial waste oil can be a better feedstock for biodiesel production using Pleolip241. Thus, reutilizing the waste streams into useful compound-biodiesel by greener process using pleolip241 as an enzymatic catalyst.

Chapter 6

6.0 Conclusion

Sustainability, renewable energy and carbon neutrality in the future. Presently, the world is concerned over the non-renewable nature of the fossil fuels and their devastating impact on the environment. This significantly directs the attention of the scientific community towards the advancement in sustainable and alternate energy fuels. Biodiesel being an eco-friendly diesel fuel and a promising green energy source gives the impetus to integrate biotechnology with cost-effective industrialization processes for its production.

Biodiesel is synthesized from renewable resources like vegetable oil and animal fats via transesterification process. The enzyme derived catalytic transesterification is known to produce cleaner and more economical biodiesel. An effective enzyme and a suitable substrate are the important factors that will influence the synthesis of biodiesel in terms of considerable yield and environmental and economic feasibility. Consequently, the focus of this study was on fungal lipases from two distinct species – pathogenic, *Candida rugosa* and an edible mushroom, *Pleurotus ostreatus* – and the exploration for triglycerides as their potential substrates.

The focal point of the comparative approach was to assess the molecular pattern of docking of the lipases derived from candida (1GZ7 & 1CRL) and mushroom (Pleolip241) against various substrates. Resultantly, the best enzyme source followed by a stable enzyme-substrate model were identified. Pleolip241 demonstrated better affinity with substrates of feedstock in biodiesel based on the docking scores and residue efficiency. The feedstock originating from algal sources confirmed preferable affinity towards 1GZ7 and 1CRL of the pathogenic fungi, but the containment issue remains a major hindrance. Furthermore, the vegetable oil and the industrial biowaste proved to be an ideal feedstock for Pleolip241lipase. Additionally, its protein structure exhibited considerable stability at higher temperatures in-contrast to the structures of 1GZ7 and 1CRL.

As proved, the lipase from the edible mushroom; *Pleurotus ostreatus* is epitomized as an effective biocatalyst for the synthesis of biodiesel via transesterification. Thus, Pleolip241 has the potential to be integrated into the green biotechnological process for generating a sustainable, alternative fuel for the future.

Chapter 7

7.0 Future Prospects

Our project is uniquely positioned to grow and continue to have a trace record of success. The roadmap of utilization of lipases enzyme from edible mushroom in industry first require the confirmation of activity and testing efficiency in production of biodiesel production. Any shortcomings, if observed while lipase is in action can be covered by protein engineering. Then after the enzyme is ready for commercialization, a integrated system based on circular bioeconomy consisting of self-sustaining three units which operates in environment friendly way is presented below.

7.1 *In-vitro* Confirmation of Lipase Activity

The first step that is *in-vitro* confirmation which can be done by first collecting the sample followed by identification of specie and confirming it to be *Pleurotus ostreatus*. Then, the specie can be grown in bioreactor and media can be provided for maximum secretion of extracellular lipase. Then lipase can be purified, converted into nanoparticles and then be utilized as biocatalyst in production of biodiesel in lab scale equipment using waste vegetable oil as a feedstock to confirm the potential of the enzyme. Ensuring that methyl ester of green origin can be obtained efficiently.

7.2 Protein Engineering to Improve Native Lipases

The native lipases can then be subjected to protein engineering to improve efficacy of enzyme if any shortcomings are observed. This can be done via site directed mutagenesis, focused mutagenesis, random mutagenesis, substrate engineering, medium engineering, structure gadded engineering, directed evolution. and other computational design methods.

As a result of the steady growth of the biocatalyst market, there is a desire for enzymes that can either catalyse the production of new organic compounds through novel reaction pathways or work more efficiently in established processes. To accomplish these goals and obtain better biocatalysts known enzymes can be modified via random or targeted mutation.

Over the past decade, enzyme properties have been tailored both through evolutionary approaches as well as by applying computer-aided rational strategies [89].

The industrially relevant properties of lipase can also be optimized by directed evolution [90].

There are two types of methods for diversity generation in lipase protein engineering including random and focused mutagenesis. By using focused mutagenesis, rationally well addressable properties like activity, specificity, enantioselectivity, substrate profile, and thermal stability can be targeted. While in the case of random mutagenesis almost all the properties including organic solvent resistance, ionic liquid resistance, pH stability, molar substrate concentration, and protein concentration can be altered [91].

Random mutagenesis can be achieved using directed enzyme evolution which includes steps of random mutagenesis at DNA level then screening for improved properties in enzymes at protein level followed by gene isolation and sequencing and at the end the iterative cycle for mass production of the enzyme having superior qualities [92].

So, essentially directed evolution increases the efficiency of natural enzymes including lipase many folds to suit the dynamic needs in sustainable bioeconomy including biodiesel production [93].

7.3 Lipase as Biocatalyst in Circular Bioeconomy

The catastrophic conditions, fossil fuels are causing massive destruction to environment due to large carbon emissions. This is also causing harm to biodiversity, global warming and harm to public health. Sustainable and renewable energy sources are the need of the hour, but due to high production cost, the production of biodiesel is not common.

The project is designed into cost effective manner keeping in view the current situation. It will protect our environment in organic way. The industrial adaptation is the essence of our idea. There are three parts of self-sufficiency model as indicated in schematic diagram in figure7.1

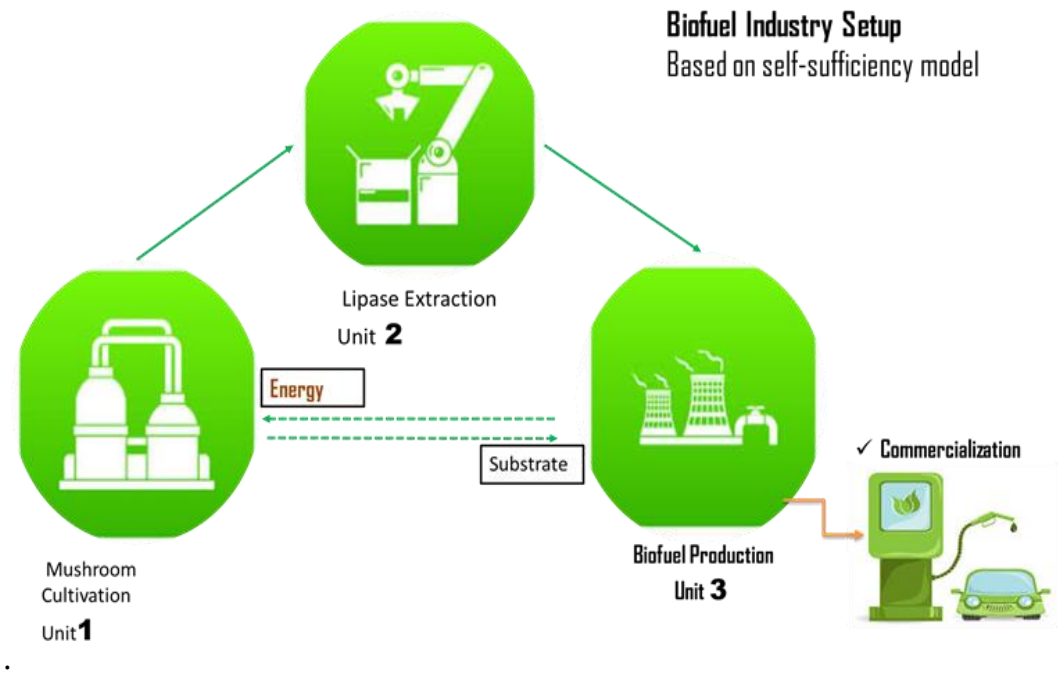


Figure 7.1 Integrated 3Unit model for biodiesel production

First unit is mushroom cultivation unit. It requires minimal power supply, optimal conditions and substrate (Spent vegetable oil and waste industrial oil) to grow the *Pleurotus ostreatus* in bioreactor and make it release maximum extracellular lipase enzyme. Then comes the next part of the model, which includes lipase purification and its conversion into nanoparticles. Finally, the last unit is where the Biodiesel will be produced using lipase nanoparticles as biocatalyst.

Two types of biofuel will be produced by working of this unit. It includes biodiesel and biogas. Biogas will be used to Bioreactor and lipase extraction. The biodiesel will be sent for commercialization and will serve as alternative for fossil fuels. The project has potential thrive across the business cycle and will be successful in long term but more importantly will provide cleaner greener energy. The vision is to provide eco -friendly, economically viable, organic and sustainable alternatives through our project to improve our economy and climate.

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