IDENTIFICATION OF A LIPOLYTIC *Trichoderma* sp. AND CHARACTERIZATION OF ITS CRUDE EXTRACELLULAR LIPASE

By

N.K. ATHUKORALA

A REPORT
In Partial Fulfillment of the Requirement of the Degree of Bachelor of Science Honors in Molecular Biology and Biotechnology

of

UNIVERSITY OF PERADENIYA
SRI LANKA

2020

IDENTIFICATION OF A LIPOLYTIC *Trichoderma* sp. AND CHARACTERIZATION OF ITS CRUDE EXTRACELLULAR LIPASE

By

NADEESHA KUMARI ATHUKORALA

A REPORT
In Partial Fulfillment of the Requirement of the Degree of Bachelor of Science Honors in Molecular Biology and Biotechnology

of

UNIVERSITY OF PERADENIYA
SRI LANKA

2020

DECLARATION

I do hereby declare that the work reported in this project report was exclusively carried out by me under the supervision of Prof. Preminda Samaraweera, Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya. It describes the results of my own independent research project where due references have been made in the text. No part of this research has been submitted earlier or concurrently for the same or any other degree.

Name of the Candidate: Nadeesha Kumari Athukorala

31/5/2021 …… Nadeesha ……..

Date Signature of the Candidate

***Certified by*:**

Supervisor,

Name: Prof. Preminda Samaraweera

Signature: …… Date: …….

Head,

Department of Molecular Biology and Biotechnology,

Faculty of Science,

University of Peradeniya.

Name: Prof. R.G.S.C. Rajapakse

Signature: …… Date: …….

Department Stamp

IDENTIFICATION OF A LIPOLYTIC *Trichoderma* sp. AND CHARACTERIZATION OF ITS CRUDE EXTRACELLULAR LIPASE

N.K. Athukorala

Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya, Sri Lanka

Fungal lipases are abundantly used in a wide range of industries due to their low cost of production, catalytic activity, ability to tolerate polar solvents, and stability at high temperatures. The demand for lipases has kept researchers exploring new lipolytic fungi. However, the success of such studies lies in the optimization of the growth conditions to maximize lipase secretion and characterization of the enzyme. Therefore, the current research has been undertaken to characterize the crude enzyme extracted from a lipolytic *Trichoderma* species and to accomplish species-level identification of the fungus. The lipolytic activity of the fungus was determined using Tween 20 and phenol red plate assays and *para*-nitrophenyl palmitate assay. The growth medium of the fungus was optimized, followed by the characterization of crude enzyme activity. Since species-level identification is vital in reporting a lipolytic fungus, DNA fragments amplified using *Internal Transcribe Spacer 1* and *Internal Transcribe Spacer 4* primers were sequenced. The study results showed the effect of growth conditions on extracellular lipase production. Maximum lipase secretion was examined with olive oil as the carbon source and; ammonium sulfate as the nitrogen source at pH of 7.0. The crude enzyme activity was highest at a pH of 6.0 and 40 °C. The enzyme activity was significant at high temperatures and enhanced with Ca^{2+} and NO_3^-. Database analysis of the sequenced DNA fragment revealed the fungus as *Trichoderma longibrachiatum*. The results of the study uncovered that this lipolytic fungus could be improved for industrial applications.

Keywords: Enzyme optimization, fungal lipase, *ITS*, pNPP assay.

DEDICATION

To all the teachers and my family, whose encouragement and advice guided me throughout my education.

ACKNOWLEDGEMENT

Firstly, I express my sincere gratitude to my research supervisor Prof. Preminda Samaraweera, Senior lecturer of the Department of Molecular Biology and Biotechnology, University of Peradeniya, for his continuous guidance throughout the research project. His encouragement to think deeply and rationally helped me to accomplish this task and intensified my curiosity and interest in the field. It was a great fortune for me to work under his guidance.

Secondly, I would like to express my deep gratitude to Prof. S.D.S.S. Sooriyapathirana, Senior lecturer of Department of Molecular Biology and Biotechnology, University of Peradeniya, for teaching us the methodology to carry research successfully and present it clearly through the Scientific Writing and Research Methodology course. Moreover, his encouragement greatly motivated me to engage in the research field. I would also like to express my deep sense of thanks to Prof. R.G.S.C. Rajapakse, the Head of the Department of Molecular Biology and Biotechnology, University of Peradeniya, Dr. C. Dhanapala, lecturer of the Department of Molecular Biology, and Dr. D. Beligala, lecturer of the department of Molecular biology and Biotechnology for making me knowledgeable in the subject.

I thank profusely all the members of the temporary staff for their immense support. I specially thank Miss M.T.H. Tharika for her ideas and guidance; and Mr. Rasika Rathnayake for helping us to successfully complete the research. I express my gratitude to Mrs. Manoja Dayananda (Technical officer), Mr. Gamini Chandrarathne, and Mr. Priyantha Abesekara (Non-academic staff) for their willingness to help me doing the research. I also thank Prof. Inoka Karunaratne, the Head of the Department of Zoology, and Mr. Niroshan Samarasingha (Technical officer), Department of Zoology, the University of Peradeniya, for helping us to complete microscopic observations. Finally, I express my gratitude to all my friends for their support and friendship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Asp	Aspartic acid
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CALA	*Candida antarctica* lipase A
CALB	*Candida antarctica* lipase B
CTAB	Cetyl Trimethyl Ammonium Bromide
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
dTTp	Deoxythymidine Triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Asssay
FTIR	Fourier Transform Infrared Spectroscopy
His	Histidine
hr	hour
ITS	Internal Transcribed Spacer
LED	Lipase Engineering Database
LPCB	Lacto Phenol in Cotton Blue
M	Molar
mM	milli molar
mins	minutes
mRNA	messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
nm	nano meter
OD	Optical Density
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Protein Data Bank
PHAs	Polyhydroxyalkanoates
pNP	*para*-Nitrophenol
pNPP	*para*-Nitrophenyl Palmitate
Rh. B	Rhodamine B

rpm	revolutions per minute
rRNA	ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
secs	seconds
Ser	Serine
UPGMA	Unweighted Pair Group Method with Arithmetic mean

1. INTRODUCTION

Lipases are constituting the most significant group of biocatalysts. They are triacylglycerol acyl hydrolases hydrolyzing carboxylic ester bonds to release carboxylic acids and alcohols (Daiha *et al*., 2015). Besides, lipases can catalyze other chemical reactions such as esterification, trans-esterification, aminolysis, and acidolysis (Mehta *et al*., 2017). True lipases attack triacylglycerol constitutes of fatty acid chain lengths of more than C10. This property of lipases distinguishes lipases from esterases that hydrolyze glycerol esters with fatty acid chains of C10 or less (Piscitelli *et al*., 2017). Lipases belong to the α/β-hydrolase fold superfamily where the core of the enzyme is composed of eight β-sheets connected by α-helices (Ollis *et al*., 1992). Their conserved catalytic triad consists of Serine (Ser), Aspartic acid (Asp), and Histidine (His) residues (Piscitelli *et al*., 2017). Several factors such as increased substrate concentration, conformation changes, the better orientation of ester bond, and reduction of water shell around the ester trigger lipase activation at a lipid-water interface (Brzozowski *et al*., 1991). Lipases are an attractive and versatile group of enzymes due to their chemoselectivity, stereoselectivity, availability, independence on cofactors (Jaeger and Eggert, 2002), and active in organic solvents (Daiha *et al*., 2015). The potential for a wide field of applications has encouraged the studies to search for novel lipases. Extensive studies have been conducted to enhance production and yield, understand structural basis accompanied with enantioselectivity, and engineer enzyme specificity (Gupta *et al*., 2015). Numerous research on lipases have revealed the significance of these enzymes and the importance of further studies.

Lipases are ubiquitous. They are being found in microorganisms such as bacteria and fungi, plants, and animals. However, commercially available lipases are derived from microorganisms (Schmid and Verger, 1998). Microbial lipases are utilized in various industries such as food, biodegradable polymers, pharmaceutical, textile, detergent (Hasan *et al*., 2006), paper, biodiesel, ester synthesis, and bioremediation. Fungal lipases standing out from other lipases from different sources have gained attention from several industries. This is due to their chemoselectivity, enantioselectivity, thermostability, stability under extreme pH, and stability in organic solvents (Mehta *et al*., 2017). Lipases exhibiting such advantageous properties are already identified from several fungi genus such as *Trichoderma*, *Aspergillus*, *Fusarium*, *Penicillium*, *Mucor*, *Geotrichum*, *Rhizomucor*, etc. (Hasan *et al*., 2006; Deshmukh *et al*., 2016). Fungal lipases are extracellular. Several factors

such as nitrogen source, carbon source, inorganic salts, temperature, pH, agitation rate, and dissolved oxygen concentration influence the production (Mehta *et al*., 2017). Therefore, determining sources of lipases and the proper conditions for enzyme production is crucial.

Many years of studies on lipases have not lessened the researchers' attention on this area. A study aimed to answer the question "Are lipases still attractive?" has revealed the increasing demand for these enzymes in kinetic resolution, detergent industry, food and feed, and biodiesel production (Daiha *et al*., 2015). Many fungi exhibiting lipolytic activity are already discovered and are still being explored to discover unidentified lipolytic fungi. It is not only the identification of lipolytic activity, characterization of the production and activity of the enzyme is also equally essential. For instance, lipases' temperature stability is the most significant characteristic for their use in several industries. Psychrophilic lipases are utilized to produce frail compounds such as chiral intermediates due to their high activity at low temperatures (Joseph *et al*., 2008). Uncovering such abilities of lipases require further characterization studies.

Trichoderma is a genus of fungi where its first description dates back to 1794 and 1865. Distinguishing different species of *Trichoderma* by observing morphological characteristics alone was difficult and often led to misidentifications of species until the development of oligonucleotide barcode and similarity search tools (Schuster and Schmoll, 2010). Currently, lipolytic activity was observed for *Trichoderma harzianum* (Toscano *et al*., 2013), *T. lentiforme* (Wang *et al*., 2018), *T. longibrachiatum* (Gochev *et al*., 2012), *T. reesei* (Silano *et al*., 2019), etc. Characterization studies on such *Trichoderma* lipases have revealed several significant properties of the enzymes. An alkaline and surfactant tolerant lipase from *T. lentiforme* has exhibited its application potential in the detergent industry (Wang *et al*., 2018). Observation of elevated lipase activity by *T. harzianum* with inexpensive agro-industrial medium has made it a good candidate for biodiesel production (Toscano *et al*., 2013). It is now evident that optimization of the lipase production medium and enzyme activity conditions are used to disclose lipases with industrially demanding properties.

Well-tested and simple laboratory techniques are available to identify and characterize the lipolytic activity of fungi. Simple plate assays employing the pH drop due to fatty acids released upon lipolysis are available in scientific literature. Indicators such as phenol red have offered more sensitive and reproducible screening methods (Singh *et al*., 2006).

Lipolytic activity can be detected using agar media incorporated with various lipid substrates such as tributyrin, Tween 20, and Tween 80. In these methods, lipolytic microorganisms break down the lipid substrate to form a clear zone (Lanka and Latha, 2015). Colorimetric estimation of *para*-nitrophenol (pNP) released by lipase-catalyzed hydrolysis of *para*-nitrophenyl palmitate (pNPP) allows the estimation of the esterolytic activity of lipases. This method provides a quantitative analysis of lipolytic activity (Gupta *et al*., 2002). The availability of well-tested procedures to optimize the growth medium and lipase activity make the research realistic and achievable. Moreover, the need to understanding the factors that affect enzyme production and activity ensures the necessity of characterization experiments (Sharma *et al*., 2001; Shahid *et al*., 2011). With these understandings and demand, this study aims to optimize the growth medium of a previously isolated lipolytic *Trichoderma* species to obtain a high lipase yield and identify the conditions that result in high lipase activity. The research intends to identify the properties that could make it a suitable candidate as an industrially demanding biocatalyst. Moreover, species-level identification is essential in specifically reporting the lipolytic fungi. So, the current study has been undertaken to identify the species of the lipolytic *Trichoderma* by molecular identification techniques.

2. REVIEW OF LITERATURE

2.1 Lipases (E.C 3.1.1.3)

Lipases are carboxylesterases that can catalyze the hydrolysis and synthesis of long-chain acylglycerols. These nature's catalysts form the most significant biocatalysts for biotechnological applications (Jaeger and Eggert, 2002). Lipases are also esterases that hydrolyze triglycerides at the water-oil boundary (Schmid and Verger, 1998). In addition, lipases catalyze chemical reactions such as aminolysis, acidolysis, esterification, and trans-esterification (Mehta *et al*., 2017). Kinetics studies show the interfacial activation of the majority of lipases. The lipase-catalyzed reactions' rates increase after a particular substrate concentration known as critical micellar concentration (Demera *et al*., 2019). Enhanced catalytic activity at high temperature, changes in substrate specificity (Zaks and Klibanov, 1984), ability to tolerate polar organic solvents (Liu *et al*., 2015), available in large quantities, and facilitating rational engineering (Jaeger and Eggert, 2002), make lipases an attractive candidate to catalyze specific industrially demanding reactions. These remarkable properties make lipase an environmentally friendly alternative to chemical catalysts (Schmid *et al*., 2001).

2.1.1 Historical background and current trends related to lipases

As evidenced by published literature, research on lipase date back to the 1930s. Even after more than 85 years of studies, lipases are still important biocatalysts (Daiha *et al*., 2015). By 1981, extreme thermostability and flexibility in substrate specificity of specific lipases are reported. The studies aimed at improving enzymes' catalytic properties have been conducted for several decades (Zaks and Klibanov, 1984). By 1992, the characteristic folding pattern known as alpha-beta (α/β)-hydrolase fold was experimentally explained by the researchers. As reported, the α/β-hydrolase fold pattern has been preserved as a standard feature, providing the scaffolding for the catalytic triad involved in enzyme activity (Ollis *et al*., 1992). Lipases were traditionally extracted from the animal pancreas as a digestive aid for human consumption. Due to the pancreas shortage and difficulties in the extraction process, the initial interests in microbial lipases were provoked (Hasan *et al*., 2006). Up till now, the existence of lipolytic microorganisms has been reported by several authors. Some of the lipolytic fungi species are *Penicillium citrinum*, *Trichoderma viride*, *Geotrichum candidum*, etc. (Gopinath *et al*., 2005). In 2015, a study investigating the technological development in industries employing lipases reported the significance and growing demand for lipases. They

uncovered lipases as a preferred type of catalyst in the detergent industry, food and feed products, and biodiesel production (Daiha *et al*., 2015). These facts uncover the undiminishing demand for lipases and their importance in related research in the past and present.

2.1.2 Structure of lipase related to the mechanism

Lipases show a folding pattern known as the α/β hydrolase fold. The α/β-hydrolase fold gives the scaffolding for the lipase's catalytic triad involve in enzymatic activity (Ollis *et al*., 1992). The catalytic triad possesses a well-preserved arrangement. Serine-Aspartic Acid-Histidine is identified as the catalytic triad residues (Suplatov *et al*., 2012). The α/β-hydrolase fold is common to several hydrolytic enzymes showing different phylogenetic origins (Figure 2.1). All those enzymes show a standard arrangement of the catalytic triad but diversity in the binding site (Ollis *et al*., 1992). The diversity in the binding site allows enzymes of the α/β-hydrolase superfamily to show diverse catalytic activities (Suplatov *et al*., 2012).

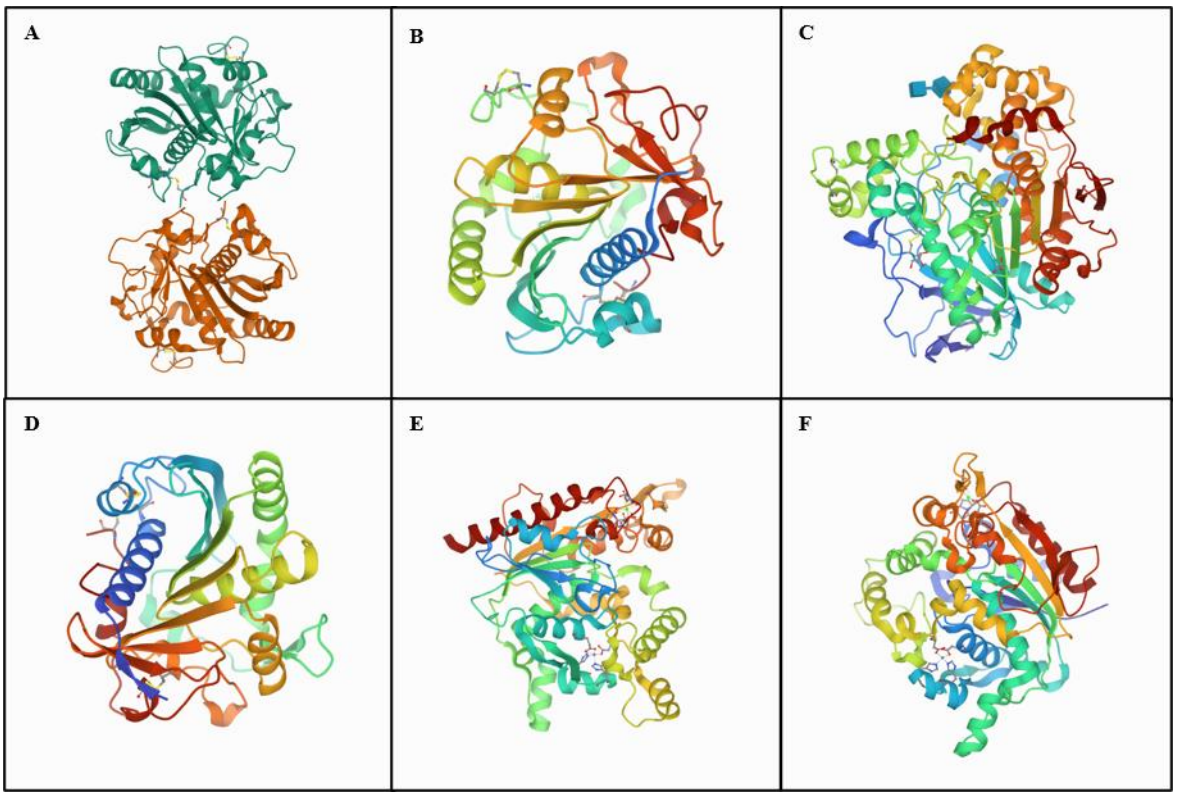

Figure 2.1 Structure of lipases with different microbial origins. A: Crystal structure of Fungal lipase from *Thermomyces lanuginose* (4EA6); B: Crystal structure of lipase/esterase from the fungus *Rasamsonia emersonii* (6UNV); C: Crystal structure of the closed state of lipase from *Candida rugosa* (1GZ7); D: Lipase ii from *Rhizopus niveus* (1LGY); E: Crystal structure of *Staphylococcus hyicus* lipase (2HIH); F: Crystal structure of thermostable, organic solvent tolerant lipase from *Geobacillus* sp. (4FMP). Source: Protein Data Bank (PDB).

Key units of the catalytic mechanism are the nucleophile, the catalytic acid, the catalytic histidine, and the residues of the oxyanion hole. These critical units are coordinated by conserved structural elements that comprise the α/β-hydrolase fold enzymes conserved catalytic core (Denesyuk *et al*., 2020). A central β sheet comprising up to eight β (β_1-β_8) strands joined by up to six α helices (A-F) makes the lipase core. The nucleophilic serine in the β_5 strand is in a highly conserved pentapeptide forming the characteristic β-turn-α motif called nucleophilic elbow (Jaeger and Reetz, 1998). The nucleophilic elbow is a sharp turn. Placing the nucleophilic serine with a short side chain on this sharp turn allows the nucleophilic serine to stand proud of the rest of the active site surface. The sharp turn also optimally positions the serine residue at the end of the α helix. Thus, the helix dipole will stabilize the tetrahedral intermediate formed in the catalytic reaction and nucleophile ionized form (Ollis *et al*., 1992). A group of conserved structural elements coordinating the catalytic histidine adjacent to the catalytic nucleophile and catalytic acid leads to an excellent tuning of three catalytic residues (Denesyuk *et al*., 2020). Oxyanion hole influences the catalytic efficiency of the enzyme. The substrate gains access to the buried active site, and the oxyanion hole is crucial for stabilizing tetrahedral intermediate during the catalytic reaction (Derewenda *et al*., 1994). A mobile lid enables the ability of lipase to function at the lipid-water interface. Like a flap, the lid domain is located over the active site, modulated access to the active site, and contributes to the substrate-binding surface. It is reported that the lid domain is crucial in substrate recognition and that the lid's open conformation is vital to position the oxyanion hole correctly (Brocca *et al*., 2003). In this way, the enzyme structure is well arranged to perform its functions during catalytic reactions.

2.1.3 Interfacial activation of lipase

Most of the lipases get activated by a water-lipid interface. This phenomenon is known as interfacial activation (Brocca *et al*., 2003). It is observed that the interfacial activation is connected to the amphiphilic lid's ability to undergo conformational changes. This phenomenon helps the substrate to enter the active site. The lid covers the active site of the enzyme. When in contact with a water-lipid interface, the lid opens, making the substrate's active site accessible. A conformational rearrangement achieves the open conformation in the enzyme's structure (Verger, 1997). The hydrophobic side of the lid becomes exposed in the open conformation and expands the nonpolar surface around the active site. The exposure of the enzyme's catalytic residues is linked with a marked increase in the non-polarity of the surrounding surface. The lipid environment stabilizes the nonpolar surface and creates a

catalytically competent enzyme (Brzozowski *et al*., 1991). A study on *Pseudomonas glumae* shows that the α5 helix serves as the lid (Noble *et al*., 1993). Another research has revealed two clearly defined hinge regions, one on each side of the lid. These hinges help the movement of the lid (Brzozowski *et al*., 1991). Many studies have focused on elucidating the structure of lipase. Most studies show the link between the lid structure and control of substrate access (Noble *et al*., 1993). Study results have revealed interfacial activation as a significant aspect of lipolytic enzymes.

2.1.4 Ways to analyze the lipase structure

The structural elucidations of lipases are crucial in designing and engineering lipases for specific industrial reactions. X-ray crystallography technique is frequently employed in determining proteins' three-dimensional structures necessary to understand reaction mechanisms at the atomic level. For successful structure determination, X-ray crystallography, sample preparation, and crystallization steps are vital (Feiten *et al*., 2017). X-ray analysis is used to understand the lipase's interfacial activation by analyzing *Rhizomucor michei* lipase structure (Brzozowski *et al*., 1991). Fluorescence spectroscopy is also used in lipase structure analyses. Conformations of *Humicola lanuginose* lipase in buffer and substrate solutions were reported to compare using fluorescence spectroscopy (Jutila *et al*., 2004). Fourier transform infrared spectroscopy (FTIR) analyzing molecular vibrations allows structural characterization of proteins in aqueous and non-aqueous media. As water absorbs infrared radiation, short path length cells are used to record proteins' spectra in water. In this technique, it is always essential to maintain the purity of the tested protein sample (Haris and Severcan, 1999). Mapping electrostatic potential distribution provides insights into the distribution of polar and nonpolar residues in the active site. Such studies reveal the active site's negative charge when in open conformation than in closed conformation, at their most active pH (Petersen *et al*., 2001). Moreover, bioinformatics is a novel approach to protein structure prediction based on sequence analysis. Protein visualization programs such as PROMOTF, Chime, TOPS, and RasMol are significant in lipase structure predictions. Lipase Engineering Database (LED) designed in 2000 is exclusively on lipases and related proteins sharing α/β-hydrolase fold. LED helps understand the sequence-structure relationships and protein engineering (Mala and Takeuchi, 2008). Instrumental, chemical, and bioinformatics studies on lipase structure are currently employed for understanding lipase structure and are of great importance in shaping lipase as an efficient biocatalyst.

2.1.5 Lipases as biocatalysts

Lipases can catalyze industrially demanding reactions such as hydrolysis, esterification, transesterification, acidolysis, and aminolysis (Figure 2.2). Hydrolysis is the breaking of fat into its constituent acid and glycerol in the presence of water. Hydrolysis of lipids to form fatty acids and glycerol is known as lipolysis (Gandhi, 1997). Fungal strains such as *Aspergillus flavus*, *Trichoderma viride*, *Fusarium oxysporum*, and *Geotrichum candida* are few lipolytic fungal stains (Gopinath *et al*., 2005). The applications of lipase-catalyzed reactions are enormous. For instance, lipase as a feed enzyme to hydrolyze lipids for increasing energy supply is a novel lipase application. Lipase-mediated hydrolysis of lipid in fish diet can support fish growth through energy supply and storage (Yan *et al*., 2018). Hydrolysis reaction starts upon the nucleophile attack by the serine oxygen of the catalytic site triad on the ester bond's carbonyl carbon in the substrate. A tetrahedral intermediate is formed with the nucleophilic attack, followed by the liberation of alcohol, leaving behind an acyl-lipase complex. The acyl-lipase complex is finally hydrolyzed to form fatty acid and the free enzyme (Jaeger and Reetz, 1998).

During esterification, lipase forms water and esters. The optimal substrate and enzyme concentrations determine the optimum yield during an esterification reaction. Lipase-mediated ester synthesis can be carried out at room temperature, neutral pH in vessels operated batch-wise or continuously (Rajendran *et al*., 2009). Alcoholysis and acidolysis are two transesterification reactions catalyzed by lipases. In transesterification reactions, alcohol, acid, or ester is formed instead of water (Gandhi, 1997). Transesterification reactions are affected by the type of catalyst, temperature, and the presence of impurities. A study on the transesterification of soybean oil with methanol has uncovered the occurrence of three stepwise and reversible reactions (Rajendran *et al*., 2009). Lipases secreted by *Candida antarctica*, *Pseudomonas cepacian*, and *Mucor miehe*i are involved in transesterification reactions. Enzymatic transesterification can overcome the problems associated with chemical transesterification employing alkali-catalysis. Some of the overcame drawbacks are energy-intensive nature, difficulty in recovering glycerol, interference from free fatty acids and water with the reaction, and the requirement to remove the acid or alkaline catalyst from the product (Fukuda *et al*., 2001). Lipase-catalyzed acidolysis, and aminolysis reactions are also equally important. Lipase-catalyzed acidolysis results in triacylglycerols and the amount of water needed in the reaction is low. A low amount of water requirement is vital in industries (Xu *et al*., 2000). The possibility of using

lipase to catalyze aminolysis reactions using several amines is reported. The selective aminolysis of various amino alcohols can be achieved using immobilized *Candida antarctica* lipase B (CALB). Currently, this reaction is used to synthesis cosmetic ingredients (Couturier *et al*., 2009). Varied lipase-catalyzed reactions are already utilized in numerous industries.

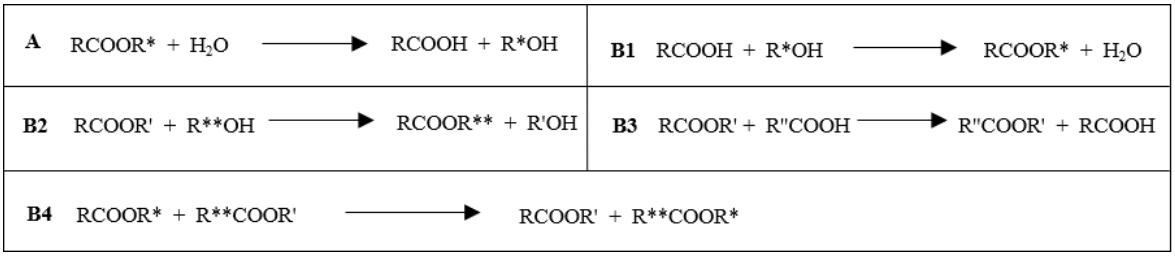

Figure 2.2 Lipase-catalyzed reactions. A: Hydrolysis reaction involves breaking fat into its constituent acid and glycerol in the presence of water. B1, B2, B3, and B4 are lipase-catalyzed synthesis reactions. Where B1: Esterification reaction forming esters and water. B2: Alcoholysis reaction involving alcohol and ester synthesis. B3: Acidolysis reaction synthesizing triacylglycerols. B4: Interesterification reaction involving modification of structure and functionality of fats and oils.

2.1.6 Factors affecting lipase production and activity

Several factors affect the lipase production in lipolytic fungi and the activity of secreted lipases. Factors such as temperature, incubation period, pH of the medium, Carbon source, and Nitrogen source can influence lipase secretion from lipolytic fungi. Affect from the amount of dissolved oxygen for the growth of lipolytic fungi (e.g., *Aspergillus wentii*) is studied. High lipase production in shake cultures, compared to stationary cultures, proves the requirement of large quantities of dissolved oxygen for aerobic fungi such as *Aspergillus* species. It is clear that some modifications are required to enhance lipase production (Chander *et al*., 1980; Akyil and Cihangir, 2018). Moisture content in the culture medium also influences lipase production. Excessively low moisture content can reduce the solubility of solid media and generate higher water tension. However, excessively high moisture content can decrease porosity, minimize oxygen transfer and enhance the risk of contamination (Boratyński *et al*., 2018). The optimum values of each factor affecting lipase activity can vary among strains, and the determination of optimum levels is vital for enhanced lipase production (Boratyński *et al*., 2018). Similarly, several factors affect the activity of secreted lipases. Factors such as pH, temperature, substrate concentration, and metal ions in the medium can affect lipase activity. The highest lipase activity is usually observed at the optimized level of each affecting factor. Optimizing temperature can also provide insights into enzymes' thermostability (Ülker *et al*., 2011). Influence from solvents

and additives on lipase activity is also reported. Replacing volatile and toxic organic solvents with ionic liquids is of great interest. Ionic liquids can enhance enantioselectivities in kinetic resolutions and reuse the enzyme (Reetz, 2002). Lipases are used in processes that employ detergents that can inhibit lipase activity. Therefore, studying the effect of detergents is also equally important (Boratyński *et al*., 2018). Conditions for different types of reactions catalyzed by lipases can be slightly different. For instance, the lipase-catalyzed transesterification reaction of 3-hydroxy esters in hydrophobic organic solvents does not require water addition. However, the reaction is affected by the acylating agent and the solvent (Bornscheuer *et al*., 1993). Therefore, determining the factors that affect lipase secretion and activity is essential.

2.1.7 Classification of lipases

Lipases are ubiquitous and catalyze several reactions. Lipases can be classified based on the structure and source. Through classification, a deep understanding of the applicability, functionality, and availability of lipases can be achieved (Sarmah *et al*., 2017). Two main classification systems are discussed under the 2.1.7.1 and 2.1.7.2 sections.

2.1.7.1 Lipase Engineering Database classification

α/β-hydrolase class includes lipases, esterases, cutinases, carboxylases, and several other groups. LED intends to function as a navigation tool to systematically analyze the highly diverse α/β-hydrolase class (Fischer and Pleiss, 2003). LED concerns microbial lipases and provides information relevant to the sequence-structure-function relationship. Proteins in the database are assigned into categories based on the sequence and structure of the oxyanion hole. Lipase's oxyanion hole comprises two residues stabilizing the substrate in the transition state. One residue is located in a structurally conserved nucleophile elbow and the other residue in a loop on top of the β-strand 3. Based on the sequence and structure of the oxyanion hole, two groups, GX and GGGX, are identified (Pleiss *et al*., 2000). Class GX includes bacterial, fungal, and eukaryotic lipase and several other enzymes. The class GGGX consists of bacterial esterases, bile-salt activated lipases, hormone-sensitive lipases, eukaryotic carboxylesterases, etc. (Fischer and Pleiss, 2003). Y-class with a positionally variable oxyanion hole tyrosine residue is also later reported (Borrelli and Trono, 2015). *Candida antarctica* lipase A (CALA) is classified as a Y-class lipase (Widmann *et al*., 2010). The classification has aided in understanding the structure and functionality of different lipase groups.

2.1.7.2 Classification based on the source

Up to date, various sources of lipases are reported (Table 2.1). Several plant parts, including seeds, fruits, leaves, and latex of specific plants, act as lipase sources. Plant seeds are an attractive source due to the higher lipase activity than in any other plant part with a high concentration of triacylglycerols, serving as energy sources for plant growth. Animal lipases are mainly used in clinical diagnosis other than commercial products due to the difficulties in culture handling and product separation (Sarmah *et al*., 2017). Microbial lipases are the most attractive due to the availability, solubility in organic solvents, broad substrate specificity, high enantioselectivity (Jaeger and Reetz, 1998), and ability to remain active under extreme pH and temperature (Mehta *et al*., 2017). The significant properties of fungal lipases have made them industrially demanding enzymes.

Table 2.1 Different lipase sources and their properties and/or applications.

Lipase Source	Examples	Properties and/or applications of the extracted lipases	References
Plant sources	*Vernonia galamensis* (ironweed) seeds	Hydrolyzing triglycerides that are present in the seeds with high selectivity.	Adlercreutz *et al*., (1997)
	Oats	Splitting off only one fatty acid radicle and not complete hydrolysis of triglyceride to glycerol and fatty acid.	Peers, (1953)
	Castor beans	Acid lipase associated with the membrane of the lipid bodies.	Huang and Moreau, (1978)
	Peanuts	Maximum lipase activity at alkaline pH and some cannot hydrolyze diglycerides and triglycerides.	Huang and Moreau, (1978)
Animal sources	Human pancreatic lipase	A single-chain glycoprotein fulfills key functions in dietary fat absorption.	Winkler *et al*., (1990)
	Liver of sea bass (*Lates calcarifer*)	To remove fat in fish skin.	Sae-leaw and Benjakul, (2018)
	Rainbow trout (*Oncorhynchus mykiss*)	Contain two hormone-sensitive lipase-encoding mRNAs that code for lipases serve in the lipid metabolism.	Kittilson *et al*., (2011)
	Bovine pancreatic gland	Traditional cheese flavor enhancement.	Aravindan *et al*., (2007)
	Porcine pancreatic gland	Traditional cheese flavor enhancement.	Aravindan *et al*., (2007)
Fungal source	*Aspergillus niger*	High thermostability and polar organic solvent-tolerance lipase with potential applications in pharmaceutical and food industries.	Liu *et al*., (2015)
	Trichoderma harzianum	Active in a wide range of pH and high thermostability. Possible applications in detergent industry.	Ülker *et al*., (2011)
	Trichoderma citrinoviride	Considerable lipase production using cost-effective molasses.	Akyil and Cihangir, (2018)

	Penicillium camemberti	Byproducts from oil production can be used as cost-effective medium for lipase biosynthesis. Used in food industry.	Boratyński *et al*., (2018)
	Penicillium simplicissimum	Acidic and thermostable lipase showing high yield at a low production cost.	Gutarra *et al*., (2009)
	Rhizopus oryzae	Thermostable recombinant lipase expressed in *Pichia pastoris* using a simple purification procedure.	Minning *et al*., (1998)
	Geotrichum candidum	Lipase with a specific affinity to long-chain fatty acids produced in less time and cost effectively.	Burkert *et al*., (2005)
	Fusarium globulosum	An alkaline, low-temperature, and stability in the presence of detergents, proteases, and bleaching agents.	Gulati *et al*., (2005)
Bacterial sources	*Staphylococcus aureus*	Can be used in cosmetic, medical, detergent, and food industries.	Xie *et al*., (2012)
	Pseudomonas fluorescence	Transesterification capacity allows it use in organic synthesis (biodiesel production).	Liu *et al*., (2017)

2.2 Fungi as a promising source for lipases

Fungi are ubiquitous and can survive in inhospitable habitats because of their efficient enzymes (Gopinath *et al*., 2005). Lipase is a critical enzyme for fungi in infection processes and degrading different substrates (Ali *et al*., 2009). Fungal lipase can catalyze a wide range of reactions including, hydrolysis, transesterification, esterification, alcoholysis, saponification, and ethanolysis (Sarmah *et al*., 2017). Out of fungal sources, filamentous fungi are the preferred sources of lipases due to their extracellular nature and the possibility of extraction from fermentation media (de Oliveira Carvalho *et al*., 2005). Fungal genera such as *Aspergillus niger* (Liu *et al*., 2015), *Rhizopus oryzae* (Minning *et al*., 1998), *Humicola lanuginosa* (Jutila *et al*., 2004), *Trichoderma harzianum* (Ülker *et al*., 2011), *Penicillium camemberti* (Boratyński *et al*., 2018), and *Candida rugosa* (Brocca *et al*., 2003) are significant as lipolytic fungi. Fungi stand out as an excellent source of lipases mainly due to their low cost of production, catalytic activity, and ease in genetic manipulation (Mehta *et al*., 2017). Some fungal lipases are significant due to their ability to tolerate polar solvents, thermostability, and stability at acidic pH (Liu *et al*., 2015). Acidic and thermostable fungal lipases are also of great importance. Lipase from *Penicillium simplicissium* is studied to show a high activity at 35-60 °C and in pH of 4.0-6.0 (Gutarra *et al*., 2009). The presence of lipolytic fungi on a wide range of substrates enables the isolation of lipolytic fungi producing lipases with unique properties. Moreover, the availability of well-tested isolation, screening, and characterization methods make the lipolytic fungi isolation steps easier (Gopinath *et al*., 2005).

Optimized process parameters can maximize lipase secretion from lipolytic fungi. Optimization of temperature, Carbon and Nitrogen source, metal ions, initial pH, and moisture content enhance lipase secretion. Substrate optimization studies reveal agro-industrial byproducts as a cost-effective alternative for lipolytic fungi such as *Penicillium camemberti* (Boratyński *et al*., 2018). The ability to employ recombinant DNA techniques and ease in genetic manipulations intensifies the importance of fungi as lipase sources (Minning *et al*., 1998). In 1994, an extracellular lipase from the fungus *Humicola lanuginosa* S-38, expressed in the fungus *Aspergillus oryzae* was reported. The trade name for the fungal lipase was Lipolase and its applicability in fabric washing detergents are uncovered. Studies have reported water-soluble Lipolase's ability to catalyze the hydrolysis of water-insoluble triacylglycerol substrate (Duinhoven *et al*., 1995).

Similarly, efficient expression of the mature lipase of the fungus *Rhizopus oryzae* in the methylotrophic yeast *Pichia pastoris* is reported to produce the recombinant enzyme in a functional form. Recombination has also allowed the extraction of pure lipase by a simple purification method (Minning *et al*., 1998). The versatility of properties and ease of mass production make fungal lipases biotechnologically essential enzymes. Currently, fungal lipases are extensively used in detergent, food, textile, paper, pharmaceutical, and cosmetic industries. Fungal lipases have also spread their roots in waste treatment, biodiesel production, tea processing, and biosensors (Singh and Mukhopadhyay, 2012). In addition, the efficiency of filamentous fungi to degrade lignified plants is significant in Earth's Carbon cycle (Arntzen *et al*., 2020). Thus, studies have shown the significance of fungal lipases in numerous industries.

2.2.1 Lipolytic *Trichoderma* species

The genus *Trichoderma* was well known for its ability to biocontrol plant pathogens. They attack other fungi and involve in producing antibiotics that affect other microbes. *Trichoderma* species are very efficient in making a wide range of extracellular enzymes (Harman, 2006). Studies have identified *Trichoderma* species with lipolytic activity. *Trichoderma harzianum* produces extracellular lipase with high thermostability and stability across a wide range of pH (Ülker *et al*., 2011). Another study reporting on *Trichoderma lentiforme* has revealed its high tolerance to both anionic and non-ionic surfactants such as Sodium Dodecyl Sulfate (SDS), Tween 20, Tween 80, and triton X-100. Such *Trichoderma* lipases have potential applications in the detergent industry (Wang *et al*., 2018). Applications

of *Trichoderma* lipase are not restricted to the detergent industry. Its applications in the food industry have also been reported. For example, the food enzyme triacylglycerol lipase synthesized using genetically modified *Trichoderma reesei* is approved for baking and cereal-based processes. In such instances, it proves that genetic modifications do not give rise to safety concerns. Furthermore, the modified enzyme was free from recombinant DNA or viable cells of the production organism (Silano *et al*., 2019). *Trichoderma* species, such as *Trichoderma reesei* are suitable as model organisms for heterologous expression due to their high secretory capacity, excellent post-translational modifications, and simplified downstream purification. Due to these characteristics, *Trichoderma reesei* has been successfully transformed with the lipase gene from *Talaromyces thermophilus* (Zhang *et al*., 2015). *Trichoderma reesei* is widely exploited as an efficient and low-cost industrial enzyme production platform (Rantasalo *et al*., 2019). The above information reveals the importance of studying lipolytic *Trichoderma* species for various fields.

Studies have reported the possibility of engineering nutritive medium to enhance extracellular lipase production by *Trichoderma* species. For instance, an optimized medium for *Trichoderma longibrachiatum* has resulted in a 2.5 times increase in lipase production than the basal medium. Therefore, nutritive medium optimization is an excellent approach for enhancing lipase secretion from *Trichoderma* species (Gochev *et al*., 2012). Lipase production by solid-state fermentation using agro-industrial residues to increase cost-effectiveness has gained the attention of several industries. *Trichoderma* species, such as *Trichoderma harzianum* have shown elevated enzyme activity in solid-state fermentation using wheat bran. The secreted lipase has exhibited favorable properties for its use in industries and environmental applications (Toscano *et al*., 2013). Finally, the isolation of novel *Trichoderma* species is essential to extract lipases with industrially demanding characteristics. Similarly, characterizing the lipase from already identified lipolytic *Trichoderma* can enhance enzyme secretion and activity efficiencies, giving rise to industrially important enzymes.

2.3 Macroscopic and microscopic identification of fungi

Fungi are morphologically, phylogenetically, and ecologically diverse eukaryotes (Raja *et al*., 2017). As estimated, there are 1.5 million fungi on the Earth. Culturing fungi after collecting them from the environment is vital because of the need to identify them (Blackwell, 2011). Fungal identification is essential in research and can be done

macroscopically by observing the color, shape, size, pigment formation, and hyphae (Figure 2.3). According to Watanabe, (2010), growing habitats, physiological properties, and morphology of different structures in cultures and nature are crucial in the identification process. Also, the morphology can be studied microscopically using a mycelium specimen under a compound microscope (Alsohaili and Bani-Hasan, 2018). Fungal mycelium is a vegetative part of fungi and has a filamentous and porous network structure. Mycelium is composed of filaments called hypha. Also, microscopic imaging of the mycelium using a scanning electron microscope helps identify the fungus (Islam *et al*., 2017). Various spores help classify fungi into Zygomycota, Ascomycota, Oomycetes, Basidiomycota, and Deuteromycetes (Watanabe, 2010). Morphology of spore-forming structures is also widely used to identify fungal species (Raja *et al*., 2017). A frequently used microscopic identification method is the slide culture technique using lactophenol cotton blue for staining for such microscopic observation. In this, lactophenol in cotton blue (LPCB) acts as a mounting medium (Tankeshwar, 2015). Specific problems associated with the slide culture technique encouraged the use of cavity slide cultures, in which sabouraud agar is used to fill the cavity of the cavity slide, on which the fungal sample is inoculated (Wijedasa and Liyanapathirana, 2012). However, the slide culture technique is widely used in fungal microscopic studies due to its simplicity and clear microscopic images.

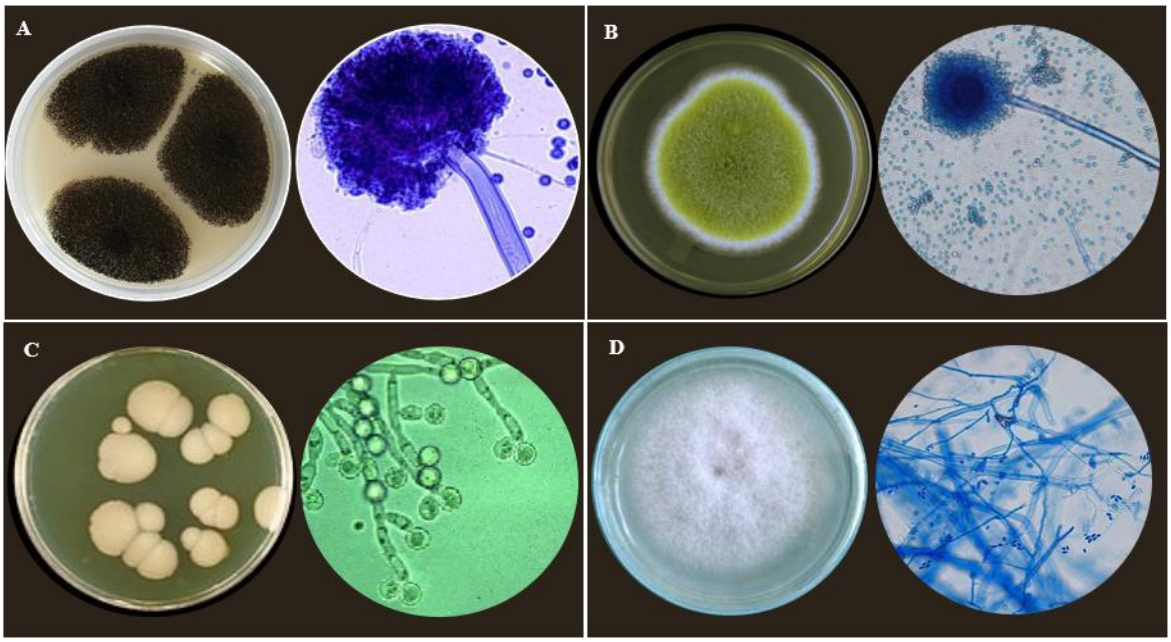

Figure 2.3 Morphology of fungal colonies on PDA medium and microscopic view by slide culture technique, stained with LPCB. Macroscopic and microscopic characteristics of the fungus allow distinguishing different fungus and thereby genus identification. A: *Aspergillus niger*, B: *Aspergillus flavus*, C: *Candida albicans*, D: *Fusarium oxysporum*.

2.3.1 Identification of *Trichoderma* species

Fungi of the genus *Trichoderma* are soilborne. They are usually green-spored ascomycetes. *Trichoderma* species show rapid growth, repetitively branching conidiophores, and mostly bright green conidia (Schuster and Schmoll, 2010). Most of the *Trichoderma* colony colors vary from white to green, forming concentric rings on potato dextrose agar (PDA) (Bissett, 1991). For instance, *T. harzianum* produces green to yellow conidia found densely at the center and undulates concentric rings towards the edge. *T. virens* form dark green conidia that uniformly cover the plate (Savitha and Sriram, 2015). Identification of *Trichoderma* species is difficult with the few morphological characteristics available, where most features overlap among species (Samuels, 2006). However, the color and pattern of appearance of conidia help to identify different *Trichoderma* species under the microscope (Figure 2.4). For this, slide culture stained with LPCB can be used. Under the microscope, conidiophores' branching pattern and structure can be observed. These microscopic features help distinguish *Trichoderma* species (Savitha and Sriram, 2015). For example, *T. flavofuscum* has conidiophores aggregated into flat pustules while *T. harzianum* has conidiophores aggregated in loose, flat pustules. Different species have conidiophores arranged in compact, hemispherical pustules or sparingly branched conidiophores with principal branches arising singly or paired (Bissett, 1991). Therefore, both macroscopic and microscopic features are required to identify *Trichoderma* species.

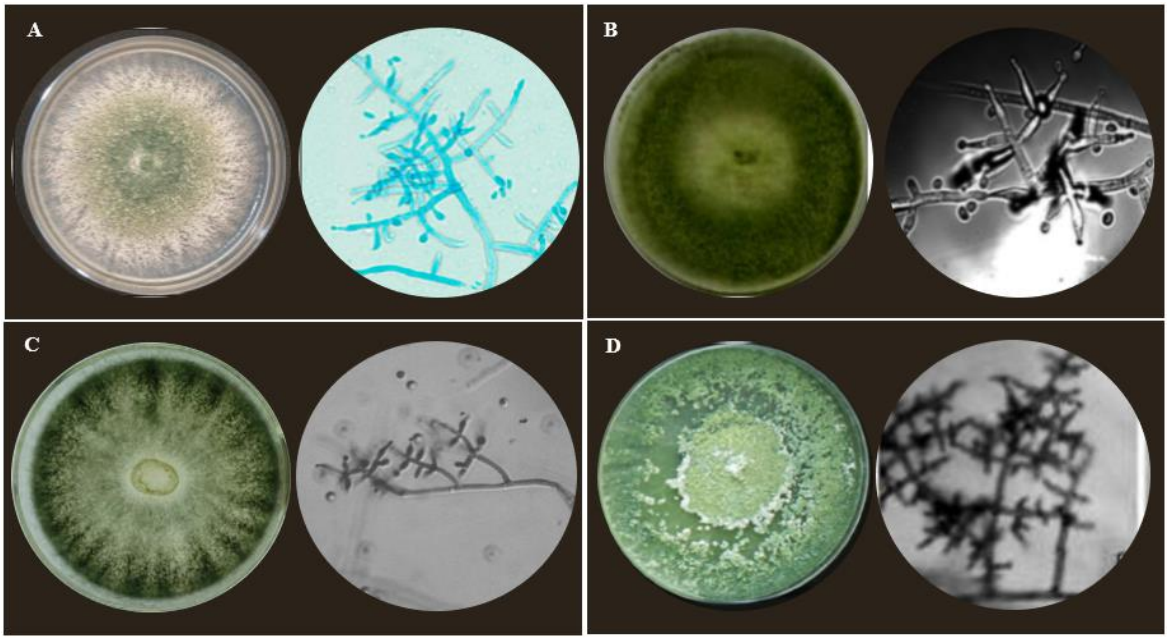

Figure 2.4 Morphology of *Trichoderma* colonies and their microscopic views. The pattern of conidia on culture plates and microscopic view together aids *Trichoderma* species identification. A: *T. longibrachiatum*, B: *T. aureoviride*, C: *T. atrovirde*, and D: *T. citrinoviride*.

2.3.2 Molecular identification of fungi

Several drawbacks of using morphology alone in the fungal species identification have encouraged molecular techniques in the species identification. Morphological characters are misleading due to hybridization, convergent evolution, and cryptic speciation (Raja *et al*., 2017). Molecular techniques of identifying fungi include polymerase chain reaction (PCR) technology, nucleic acid-based probe technology, and immunological methods (Aslam *et al*., 2017). Potential advantages of amplifying internal transcribed spacer (*ITS*) regions in the ribosomal RNA (rRNA) in species-level identification of fungi are reported. *ITS* regions evolve faster and exhibit high variation, allowing species-level identification (Raja *et al*., 2017). Initial genomic DNA extraction, followed by PCR amplification using *ITS* primers, has to be done. Sequencing the PCR product and comparing the obtained sequences with the other related sequences using basic local alignment search tool (BLAST) in GenBank of National Center for Biotechnology Information (NCBI) allows the fungi identification (Sadati *et al*., 2015). Frequently used primers for sequence-based fungal identification at the species level are *ITS1*, *ITS2*, *ITS3*, and *ITS4*. The primer pair *ITS4* amplifying the *ITS2* region of the rRNA operon is identified as the most suitable primer pair for fungal communities' characterization with metabarcoding (De Beeck *et al*., 2014). In nucleic acid-based probe technology, a probe is employed for nucleic acid analysis without its amplification. The probe can be labeled with a reporter molecule or with radio-labeled isotopes. DNA probes are better alternatives to PCR for fungal identification studies (Aslam *et al*., 2017). Immunological techniques like enzyme-linked immunosorbent assay (ELISA) for the quantitative detection of an antigen using a conjugated antibody can also be used. In this technique, monoclonal antibodies result in high specificity but a low background, unlike polyclonal antibodies that increase the breadth of the assay and allows the detection of multiple isolates of the same species of fungus (Peruski and Peruski, 2003).

2.4 Screening methods to detect lipase activity

Sensitive and rapid methods to screen lipase activity are essential for discovering novel lipolytic fungi and their enzymes with various industrial applications. Reactions catalyzed by lipolytic enzymes take place at a lipid-water interface, and monitoring changes in the physical properties of the lipid-water interface permits measuring lipolysis. These methods are technically sophisticated and require specialized instruments (Gilham and Lehner, 2005). The detection of microbial lipases is currently based on the estimation of free fatty acids synthesized from triglycerides (Samad *et al*., 1989). Evolving from intensive and laborious

titrimetric methods, currently, various lipase screening methods, such as colorimetric methods, spectrophotometric methods, fluorescence assays, and gel diffusion assays are reported. Gel diffusion assays using various lipid sources or indicator dyes are already available in the scientific literature (Lanka and Latha, 2015). Techniques such as agar plate tests allow prescreening of the lipase activity. These facilitate the initial identification of the lipolytic fungi with effective lipase activities (Brandt *et al*., 2018). Lipase activity screening methods can be broadly discussed as quantitative and qualitative screening methods. In addition, recently introduced molecular methods are becoming attractive as these methods overcome the problem of the cultivation of lipolytic fungi (Lanka and Latha, 2015). Simple and well-tested screening methods allow efficient identification of the lipolytic fungus.

2.4.1 Quantitative methods to detect lipase activity

Initial use of the silica gel technique and a modified Majonnier extraction method were reported to determine lipase activity in milk. In these methods, the fatty acids released by the enzyme activity were extracted and measured using a non-aqueous titration. Later, titration methods involving incubating the enzyme with the lipid substrate followed by an alkali titration to quantify the released fatty acids were introduced (Parry *et al*., 1966). By 1988, a turbidimetric esterase assay was designed using Tween 20, $CaCl_2$, and the enzyme source. In 1989, the enzyme activity was determined by measuring the increase in the optical density (OD) at 500 nm due to the precipitation of released fatty acids from Tween 20 as calcium salts. As reported, this turbidimetric assay was at least 36 times more sensitive than the Tween 20 based titrimetric assay (Tigerstrom and Stelmaschuk, 1989).

Fluorescence methods for screening lipase activity use fluorescent compounds, which allow the measurement of the fluorescence emitted from the released fluorescent fatty acids by the lipase activity (Demera *et al*., 2019). In 1991, a continuous fluorescence-displacement assay for quantifying long-chain fatty acids released by triacylglycerol lipase was reported. In this assay, the enzyme activity results in fatty acids that displace the highly fluorescent fatty acid probe 11-(dansyl amino) undecanoic acid from the proteins that bind rat liver fatty acids (Wilton, 1991). Furthermore, the enzymatic reactions' colorimetric determination of free fatty acids allows quantitative screening of lipase activity with high sensitivity. Cupper soap method is one such colorimetric method. The free fatty acids released by the enzymatic action form blue color soaps of cupric complexes. Spectrophotometry then allows the evaluation of the formed cupric complexes extracted into an organic solvent (Lowry and

Tinsley, 1976; Lanka and Latha, 2015). Frequently used spectrophotometric procedures determine lipolytic activity using *para*-nitrophenyl esters and measure released pNP by lipolytic activity at 410 nm (Gilham and Lehner, 2005). Spectrophotometric methods employing pNPP as a substrate are effectively used to detect lipase activity (Demera *et al*., 2019). These quantitative screening methods to detect lipase activity are essential to isolate potential lipolytic fungi to serve industrial needs and quantitatively determine lipase activity.

2.4.1.1 pNPP assay

In 1979, U. K. Winkler and M. Stuckmann assayed exolipase using pNPP as the substrate (Winkler and Stuckmann, 1979). The assay is applicable for estimating the esterolytic activity of lipases and esterases (Gupta *et al*., 2002). pNPP is a well-known lipase substrate (Pencreac'h and Baratti, 1996). The hydrolysis of pNPP to pNP is a standard reaction to determined lipase activity. The optical absorption of pNP is very high in alkaline solutions. According to the Beer-Lambert Law, absorbance is related to the concentration of the absorbing species (Swinehart, 1962). These understandings are used to determine the lipase activity in the pNPP assay. The method involved the estimation of pNP released by the enzymatic hydrolysis of pNPP (Figure 2.5). In the assay, the lipase activity is quantified by measuring the increase of pNP released at 410 nm. The substrate solution is comprised of pNPP, isopropanol, and gum Arabic, to which the enzyme extract is added (Demera *et al*., 2019). The assay is simple and rapid. However, the turbidity due to the immiscibility of released fatty acids by the reaction and pNPP can interfere with the spectrophotometric measurements. This problem is controlled by the addition of Triton X-100 into the substrate solution. Triton X-100 acting as a surfactant disperses fatty acids released by enzymatic hydrolysis of pNPP (Gupta *et al*., 2002).

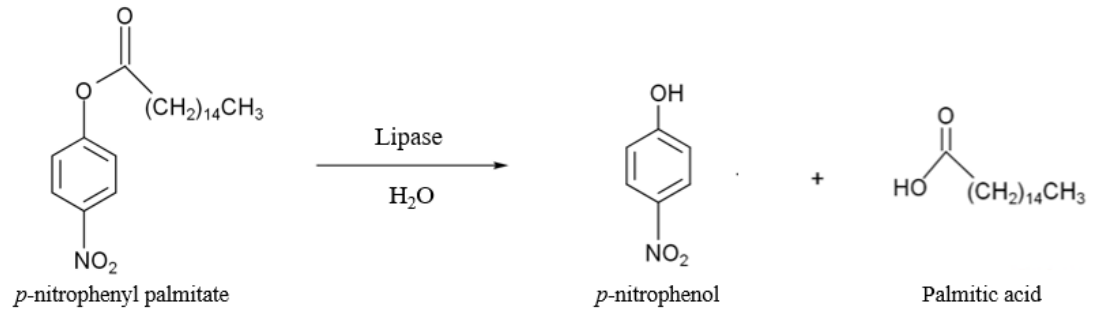

Figure 2.5 Lipase-mediated hydrolysis of pNPP forming pNP and palmitic acid. The released pNP can be estimated by absorbance measurements at 410 nm and thereby evaluate lipase activity.

2.4.2 Qualitative screening of lipase activity: Gel diffusion assays

Primary plate assays employing lipid substrate with an indicator determine lipolytic activity by forming a zone of intensification. Lipid substrates such as Tween 20, Tween 60, and Tween 80 with Victoria Blue B, rhodamine B (Rh. B), or methyl red allow qualitative screening of lipolytic activity (Samad *et al*., 1989). Later, simple and rapid detection of lipase activity was developed based on pH reduction due to the released fatty acids during lipolysis. Chromogenic plates incorporated with phenol red indicator and a lipidic substrate, kept at pH 7.3, provide a fast and sensitive lipolytic activity staining method. Phenol red with an endpoint at pH 7.3-7.4, pink, turns yellow with a slight pH reduction to 7.0-7.1 (Figure 2.6). In this method, the incorporation of $CaCl_2$ to the medium quenches the fatty acids preventing fatty acids' spread during prolonged incubations (Singh *et al*., 2006). Tween 20, Tween 60, and Tween 80 esters differ in the fatty acid chain lengths. Therefore, they are potential substrates for the detection of lipolytic activity. Results have indicated esters with medium-chain lengths are attacked rapidly with high affinity, making Tween 20 (C_{12}) a better substrate for lipase (Sakai *et al*., 2002). In this method, lipase cleaves Tween esters to produce fatty acids and alcohols. In the presence of calcium, free fatty acids precipitate, forming insoluble fatty acid salt and indicate the presence of lipolytic activity in the medium (Lonon *et al*., 1988). The quantitative screening methods are therefore widely used in lipase characterization studies.

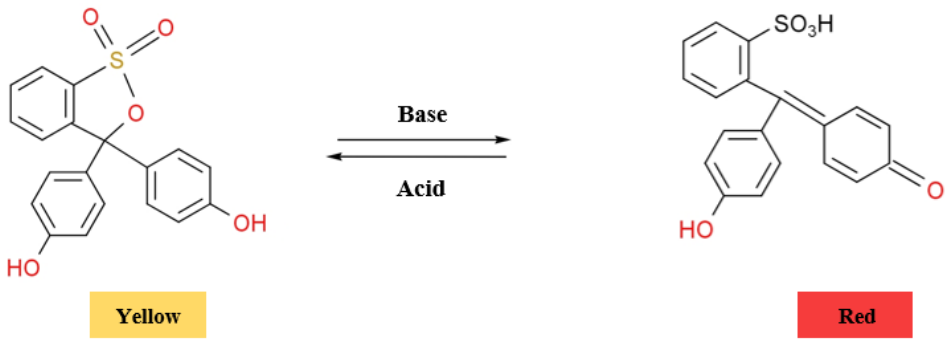

Figure 2.6 Phenol red acting as an indicator. The color change from red to yellow takes place with the acidification of the medium due to the release of fatty acids by lipase activity on olive oil in the medium.

2.5 Applications of fungal lipases

Lipases are ubiquitous enzymes that constitute the most attractive biocatalysts for applications in several fields due to their diversified enzymatic properties and substrate specificities (Mehta *et al*., 2017). Lipases are employed in food, pharmaceutical, detergent, agrochemicals, cosmetics, biodiesel, paper, bioremediation, and many other fields (Hasan *et*

al., 2006). Due to the increasing demand, lipolytic fungi in several habitats, including oil-contaminated soils, seeds, and deteriorated food, are isolated and used to extract lipases (Singh and Mukhopadhyay, 2012). Ultimately, the rising demand has kept the researchers exploring new lipolytic fungi and characterization of their lipase.

2.5.1 Medical industry

Since lipase is the primary enzyme responsible for fat metabolism, its deficiency directly influences health. Lipase deficiency requires external administration of lipase as a corrective measure (Mehta *et al*., 2017). In addition, epidemiological studies show the role of serum triglyceride levels in carcinogenesis (Takasu *et al*., 2012). Lipases are applied in curing hair loss and skin scalp diseases (Chandra *et al*., 2020). These studies have ultimately focused on the industrial-scale production of lipase for medical applications. Lovastatin drug manufactured from *Candida rugosa* lipase is administrated to reduce serum cholesterol levels. Lovastatin provides evidence for the importance of fungi as a source of lipase. Methods to assess gene expression provide opportunities to direct rational strain improvements and develop industrial strains producing lovastatin (Askenazi *et al*., 2003). Lipases from endophytic fungi such as *Emericella nidulans*, *Dichotomophtora boerhaaviae*, and *Dichotomophtora portulacae* show biological activity against *Leishmania sp*, *Microsporum sp*, and *Malassezia sp* causing zoonotic diseases (Alves *et al*., 2018).

2.5.2 Food industry

Lipases are attractive enzymes in food processing. Lipases are used for the modification and breakdown of biomaterials. Commercial lipases are currently used for flavor improvement in the dairy industry and processing food such as vegetables, fruits, meat, milk, beer, and bakery (Singh and Mukhopadhyay, 2012). The specificity and selectivity of lipases enabled many food products (Sarmah *et al*., 2017). Lipase catalyzed transesterification and esterification reactions are widely used in the food industry. Lipases acting as additives modify flavor by producing esters of short-chain fatty acids and alcohols. In addition, the use of enzymes during food processing is advantageous due to their ability to operate under a broad range of temperatures and pH, retention the desired structure of the food, and reduce the energy requirements during processing (Saxena *et al*., 2001).

Lipases from *Penicillium*, *Aspergillus*, *Mucor*, and *Candida* are currently widespread in the food industry. For instance, *Aspergillus niger*, *Rhizopus oryzae*, and *Candida cylindracea*

lipases are used in bakery products. Meanwhile, *Mucor meihei*, and *Aspergillus oryzae* lipases are used in cheese manufacturing (Mehta *et al*., 2017) (Table 2.2). The involvement of *Trichoderma* species in the food industry is also significant. The food enzyme triacylglycerol acyl hydrolase synthesized using genetically modified *Trichoderma reesei* has been reported. The enzyme has been approved for its intended uses due to its safety (Silano *et al*., 2019). In the lipid industry, lipases are used to retail vegetable oil with nutritionally important structural triacylglycerols and improved physicochemical properties (Ferreira-Dias *et al*., 2013). The production of trans-fatty acid is another attractive application of lipases in the food industry (Mehta *et al*., 2017).

Table 2.2 Applications of lipase in the food industry.

Source of lipase	Application in the food industry	Reference
Candida rugosa	Catalyzes esterification and transesterification reactions used in flavor synthesis.	Linko and Yan Wu, (1996)
	In production of carbohydrate esters, amino acid derivatives, and ice cream.	Raveendran *et al*., (2018)
Candida parapsilosis	The lipase-acyltransesterase catalyzes fatty hydroxamic acids synthesis, which are constituents of food additives.	Vaysse *et al*., (1997)
Candida antarctica	Synthesis of polyphenolic antioxidants applied in sunflower oil production by esterification of functionalized phenols.	Raveendran *et al*., (2018)
Geotrichum sp (*LIP2* gene)	To produce bread with a uniform crumb structure and higher loaf volume.	Monfort *et al*., (1999)
Immobilized lipase from *Mucor miehei* (Lipozyme ™)	Synthesis of short-chain flavor thioester derivatives.	Cavaille-Lefebvre and Combes, (1997)
Aspergillus niger and *Aspergillus oryzae*	In the production of cheddar cheese	Raveendran *et al*., (2018)
Penicillium camemberti	In the production of camembert cheese	Raveendran *et al*., (2018)

2.5.3 Detergent industry

Lipases are significant additives used in detergents of industrial laundry and household dishwashing. Moreover, heavy-duty powder detergents and automatic dishwasher detergents are incorporated with lipases to improve detergency (Hasan *et al*., 2006). The use of lipases as additives in the detergent industry can save energy by enabling a low wash temperature (Mehta *et al*., 2017). In addition to the efficient removal of fat stains, lipases can prevent the redeposition of fat on washed textiles. However, detergent formulations are incorporated with chemical surfactants, while washing liquids contain both anionic and nonionic surfactants with complexing agents at a high alkaline pH. Such conditions do not favor enzymatic reactions. These challenges accelerated the screening programs for lipases stable under such conditions (Schmid and Verger, 1998). Extensive studies have shown that fungal

lipases have properties that support their usage in the detergent industry and cleaning. The recombinant fungal lipase (Lipolase) from *Humicola lanuginosa*, manufactured by Novo-Nordisk company was the first commercial lipase (Hasan *et al*., 2006). Lipase from *Talaromyces thermophilus* has shown high resistance to alkaline pH and various surfactants and commercial wash agents. Lipase from *Humicola lanuginosa* showing thermostability, stability towards anionic surfactants, and activity at alkaline pH is an excellent candidate as a detergent additive (Mehta *et al*., 2017). Moreover, *Trichoderma lentiforme* secreting an alkaline and surfactant-tolerant lipase has uncovered its potential in the detergent industry (Wang *et al*., 2018).

2.5.4 Biodiesel production

Environment-friendly and renewable biodiesel can be synthesized by transesterifying various feedstocks (Hwang *et al*., 2014). The transformation of byproducts during food processing into revenue streams such as biofuel via lipase-mediated transesterification is recently documented (Figure 2.7). Lipase transesterification prevents soap formation, reduces byproduct formation, enhanced activity in solvent-free conditions (e.g., waste cooking oil), and promotes the use of greener processing conditions (Andler and Goddard, 2018). Moreover, lipase-catalyzed biodiesel production involves lower energy consumption than the chemical catalyzed process (Hwang *et al*., 2014). From different lipase sources, microorganisms are preferred as a lipase source in lipase-catalyzed biodiesel production due to the shortest generation time for lipase production, improved substrate to product conversion, ability to conduct genetic operations, simplicity in harvesting (Chandra *et al*., 2020), and ability to optimize lipase production systems (Hwang *et al*., 2014). *Aspergillus niger*, *Rhizopus oryzae*, *Candida rugosa*, and *Streptomyces* species are some effective lipase-producing microbes for biodiesel production (Chandra *et al*., 2020). Interestingly, *Trichoderma reesei* ability to degrade lignified plants has gained attention from the biofuel production sector (Arntzen *et al*., 2020). Some other fungal lipase, such as CALB showing high esterification activity, is heterologously produced by *Aspergillus oryzae* and used for enzymatic biodiesel production (Hwang *et al*., 2014). Solvent tolerant lipases produced by *Fusarium* species are also crucial in biodiesel production (Fukuda *et al*., 2001). Immobilization of *Thermomyces lanuginosus* lipase onto microporous polymer and its usage to synthesize biodiesel using soybean, sunflower, and waste cooking oils is also reported (Dizge *et al*., 2009).

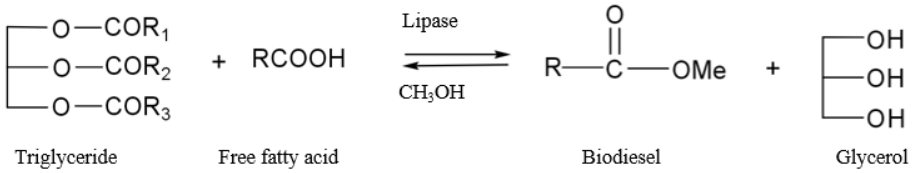

Figure 2.7 Production of biodiesel by enzymatic transesterification. Methanolysis of soybean oil and methanol utilizing lipase in a solvent-free reaction system can produce biodiesel.

2.5.5 Bioremediation

Bioremediation of toxic compounds from industrial effluents is vital before releasing such effluents into the environment due to their carcinogenicity, mutagenicity, and persistence in the environment (Deshmukh *et al*., 2016). Bioremediation technologies have offered safe and economical alternatives in place of physical and chemical treatments to remove such contaminants (Karigar and Rao, 2011). Fungal lipases degrade oil spills on the coasts, ensuring eco-restoration and degrading polyvinyl alcohol films in polluted soils. Lipases have offered new solutions in bioremediation for waste disposal (Mehta *et al*., 2017). Lipases enhance the efficiency and rates of composting and bio methanation by anaerobic digestion. These two processes are used to manage municipal solid wastes (Deshmukh *et al*., 2016). In most instances, lipases catalyze the hydrolysis of triacylglycerols to form glycerol and fatty acids, thereby degrading lipids derived from a wide range of animals, plants, and microorganisms (Karigar and Rao, 2011). Due to these reasons, screening potential lipolytic fungi was encouraged, and lipases from *Trichoderma*, *Aspergillus*, *Fusarium*, *Penicillium*, *Mucor*, and *Rhizopus* isolated from contaminated soils are now available for bioremediation. These enzymes have opened up new avenues to efficiently bioremediate toxic pollutants such as polyaromatic hydrocarbons (Deshmukh *et al*., 2016).

2.5.6 Paper industry

Pulp supplemented with lipase before the incubation can decrease pitch deposits on paper machines (Fischer and Messner, 1992). Pitch is the triglycerides and waxes of wood causing severe problems in the paper industry (Sharma *et al*., 2001). In this, lipases hydrolyze triglycerides in the resins, and sodium hydroxide washes away the released fatty acids. This process is termed biological pitch control (Fischer and Messner, 1992). Nippon Paper Industries in Japan has developed a method to control pitch using *Candida rugosa* lipase that hydrolyzes wood triglycerides up to 90% (Sharma *et al*., 2001). Furthermore, *Candida cylindrica* and *Candida antarctica* lipases are used in pitch control. Besides pitch control, lipases aid lipid stain removal during paper recycling (Mehta *et al*., 2017).

2.5.7 Cosmetics

Active lipases are found in surficial cleansers, cosmetics in anti-cellulite treatment, and overall body slimming cosmetics. In these products, lipases are responsible for removing dirt and small flakes of dead skins and breaking down fat deposits (Ansorge-Schumacher and Thum, 2013). Lipases promote the penetration of preparation in hair-waving (Gandhi, 1997). In addition, lipases are used for the controlled in situ release of an active ingredient from an inactive precursor. This application is significant in functional perfumery to maintain an even development of odor over time (Ansorge-Schumacher and Thum, 2013). Lipase from *Candida antarctica* used to synthesize isopropyl myristate, *Rhizomucor meihei* lipase acting as a biocatalyst in sun-tan creams, and amphiphilic compounds produced by CALB have already won the attention of cosmetic industries (Mehta *et al*., 2017). Enzymatically prepared wax esters using *Candida cylindracea* lipase utilized in personal care products is reported. In this process, the overall production cost is higher compared to conventional methods. But the improved product quality justifies the price (Hasan *et al*., 2006).

2.5.8 Biodegradable polymer production

Biopolymers such as polyphenols, polyesters, and polysaccharides are significant due to their biodegradability and ability to synthesize from renewable natural resources. Lipases are used as catalysts during polymeric synthesis due to their high selectivity under mild reaction conditions (Jaeger and Eggert, 2002). Naturally produced biopolymers such as polyhydroxyalkanoates (PHAs) by various microbes have several applications in drug delivery and packing. Frequently used PHAs are poly(3-hydroxybutyrate) P(3HB). But the applications are restricted due to the brittle nature and high melting temperature. To overcome the problem, monomers are incorporated into P(3HB) to produce co-polymers, and lipases catalyze the process (Bhatia *et al*., 2019). For instance, CALB is used to functionalize medium chain length PHAs with sucrose. Such functional polymers show high biodegradability, and the functionalization process commonly utilizes lipases to catalyze ester bond synthesis in micro-aqueous media (Gumel *et al*., 2013). Lipase-catalyzed reactions can polymerize monomers with structural complexity and multifunctional reactive groups. Besides, diverse lipase-catalyzed polymer libraries can be achieved by different combinations of diesters and diols using lipases (Jaeger and Eggert, 2002).

3. MATERIALS AND METHODS

3.1 Fungus sample and initial culturing

Previously isolated, the uncharacterized lipolytic fungus was used to conduct media optimization and enzyme characterization. Fresh and pure cultures were obtained by subculturing the fungus on a PDA medium. The PDA medium was supplemented with 0.005% (w/v) ampicillin and 0.005% (w/v) spectinomycin antibiotics after autoclave. The medium contained 5 g of dextrose and 5 g of agar dissolved in 125 mL of potato infusion and 125 mL of distilled water, adjusted to a pH of 5.8 ± 0.2. Point inoculation of the fungal mycelium onto the PDA medium was done using a sterilized inoculating loop. The inoculated medium was incubated for 3 days at 32 °C. The complete procedure was repeated thrice to obtain pure cultures.

3.2 Culture maintenance

PDA Petri plates were point inoculated with actively growing fungal colonies and sealed with parafilm to avoid contamination and drying. Pure visible colonies were established at 32 °C followed by storage at 4 °C. The cultures were revived every 15 days to avoid contamination.

3.3 Identification of the fungus

Initially, morphological characteristics such as colony growth, top, and bottom views, form, color, elevation, growth rate, and pigmentation were evaluated. For this, 3-day old fungus cultures on PDA plates were used.

Slide culture technique with lactophenol in cotton blue (LPCB) as the stain was used in microscopic identification. The bottom of an empty Petri plate was covered by a circular filter paper, on which a glass slide and a coverslip were placed. The whole setup was autoclaved to prevent contamination. A block of PDA (1 cm x 1 cm) was placed on the glass slide; four corners of the agar block were inoculated with small colonies of fungus using sterilized toothpicks and covered with the coverslip. The filter paper was wetted daily with distilled water to prevent being dry. The inoculums were allowed to grow under 32 °C for 4 days. Then, the coverslip was carefully removed, stained with LPCB, and examined under a microscope. Morphology of spores and patterns of mycelium branching were observed to identify the genus of the lipolytic fungus.

3.4 Determination of lipase activity

The lipase activity was determined qualitatively and quantitatively. The plate-based screening of lipolytic activity of the fungus was based on the Tween 20 and phenol red plate methods. The quantification of lipolytic activity of the crude enzyme extract was analyzed using pNPP assay utilizing pNPP as the substrate under standard assay conditions.

3.4.1 Qualitative screening

The lipolytic activity of the fungus was detected by using Tween 20 plate method. The medium was composed by: 1% peptone, 0.5% NaCl, 0.01% $CaCl_2$, 2% agar, and 1% Tween 20 as the lipid source. The final pH was adjusted to 5.8 ± 0.2. The medium was supplemented with 0.005% (w/v) ampicillin and 0.005% (w/v) spectinomycin antibiotics after autoclave. After solidification, the Tween 20 medium was inoculated with a small inoculum, incubated for 3 days at 32 °C, and examined to develop a white halo around the inoculum. The controls were conducted in the absence of Tween 20 in the medium.

The lipolytic activity of the isolated fungus was further confirmed by growing the fungus mycelia on plates with phenol red medium. The medium was supplemented with 0.01% phenol red as the indicator, 1% olive oil as the lipid substrate, 0.1% $CaCl_2$, and 2% agar. The pH was maintained at 7.8 ± 0.2. The medium was supplemented with 0.005% (w/v) ampicillin and 0.005% (w/v) spectinomycin antibiotics after autoclave. After solidification, the fungal mycelium was point inoculated in the middle of the plate, incubated in the dark for 3 days at 32 °C, and examined to develop a yellowish halo around the fungus inoculum, indicating the presence of lipolytic activity. The controls were conducted without fungus inoculation.

3.4.2 Quantitative screening

Crude lipase activity was determined spectrophotometrically using pNPP as the substrate. Crude lipase for the assay was extracted by culturing the lipolytic fungus in a basal mineral medium.

3.4.2.1 Lipase production medium

The basal mineral medium contained 10 gL^{-1} $(NH_4)_2SO_4$, 12 gL^{-1} NaH_2PO_4, 2 gL^{-1} KH_2PO_4, 0.33 gL^{-1} $CaCl_2$, 0.03 gL^{-1} $ZnSO_4$, 0.03 gL^{-1} $MgSO_4$, and 1% olive oil as the carbon source. The pH was adjusted to 6.0. After autoclaving, 20 mL of the medium was inoculated with 1

mL of fungal suspension. The fungal suspension was prepared by dissolving 10 loops of fungal culture in 10 mL of distilled water. The inoculated medium was incubated at 32 ℃ in a shaking incubator.

3.4.2.2 Extraction of crude lipase

The crude enzyme was extracted by filtering the inoculated liquid medium after incubation through a Whatman No.1 filter paper (Akyil and Cihangir, 2018), followed by the centrifugation of the filtrate at 10,000 rpm at 4 ℃ for 15 mins. The extracted crude enzyme was stored at 4 ℃ until used.

3.4.2.3 pNPP assay

Lipase activity was determined by adding 0.06 mL of crude lipase to 1.44 mL of the substrate solution. The substrate solution was prepared by dissolving 6 mg of pNPP in 2 mL of isopropanol with 0.32 mL of TritonX-100 followed by mixing with 38 mL of 0.1 M Tris-HCl buffer (pH 8) with 100 mg of gum Arabic dissolved in it. The mixture was left for 15 mins at room temperature to stabilize. Then, the mixture of substrate solution and crude lipase was incubated at 40 ° C for 15 mins. The lipase activity was quantified daily for 5 days by detecting the released product, pNP at 410 nm. To detect other influences on the absorbance readings, 0.06 mL of distilled water was added instead of the enzyme, and the absorbance was recorded.

3.5 Optimization of lipase production medium

The effect of the incubation period, carbon source, nitrogen source, pH, and temperature were estimated concerning the crude lipase yield. The basal mineral medium mentioned under 3.4.2.1 was modified to test the effect of different factors on lipase production, and pNPP assay mentioned under 3.4.2.3 was used to determine lipase activity after each test. For all the tests, the fungal suspension was prepared by dissolving 10 loops of fungus inoculum in 10 mL of distilled water. Each test was repeated to assure the variation of the results, and the results of one trial were used to represent data using charts.

3.5.1 Effect of the incubation period

Initially, the best incubation period for the highest lipase secretion was determined as mentioned under 3.4.2 by quantifying the lipase activity of lipolytic fungus in the liquid

medium for 5 consecutive days. The procedure was repeated to ensure the variation of the observations.

3.5.2 Effect of carbon source

The optimization of the carbon source was done using the following carbon sources: olive oil, coconut oil, vegetable oil, cellulose, margarine, and gingerly oil. Each of the above carbon sources was separately used with the basal mineral medium explained under 3.4.2.1. Separate Erlenmeyer flasks with 20 mL of basal mineral medium were supplemented with 1% of the above carbon sources. In addition, a separate flask was maintained without any carbon sources. The autoclaved liquid media with different carbon sources were inoculated with 1 mL of fungal suspensions. A separate set of flasks with the above carbon sources in the liquid medium but without the fungus were maintained to detect the influences on the absorbance readings other than lipase activity. The inoculated media were incubated for 3 days at 32 °C in a shaking incubator at 120 rpm. The lipase activity was quantified by using pNPP assay.

3.5.3 Effect of nitrogen sources

Basal mineral medium described under 3.4.2.1 with 1% olive oil was supplemented with the following nitrogen sources (0.5% w/v): ammonium sulfate, peptone, ammonium nitrate, sodium nitrate, and urea. In addition, a separate flask was maintained without any nitrogen source in the medium. The media were autoclaved, and 20 mL of each medium was inoculated with 1 mL of fungal suspension. A separate set of flasks with media supplemented with the above nitrogen sources but without the fungus were maintained to detect the influences on the absorbance readings other than from lipase activity. The inoculated media were incubated for 3 days at 32 °C in a shaking incubator at 120 rpm. The lipase activity was quantified by using pNPP assay.

3.5.4 Effect of pH

The effect of pH on the lipase secretion was evaluated by adjusting the pH of the basal mineral medium across the range of 3.0 to 10.0. The autoclaved media supplemented with olive oil and ammonium sulfate at different pH values were inoculated with 1 mL of fungal suspension. A separate set of flasks with media at different pH but without the fungus were maintained as controls to detect the influences on the absorbance readings other than from

lipase activity. The inoculated media were incubated for 3 days at 32 °C in a shaking incubator at 120 rpm. The lipase activity was quantified by using pNPP assay.

3.5.5 Effect of temperature

The incubation temperature of the inoculated basal mineral medium was adjusted to determine the optimized growth temperature. The composition of all the media was kept constant, and 20 mL of the medium was inoculated with 1 mL of fungal suspension. The inoculated media were incubated for 3 days in a shaking incubator at 120 rpm. Controls were conducted at each temperature without the fungus.

3.6 Characterization of crude enzyme

The effect of pH, temperature, cations, and anions on the activity of the crude enzyme was analyzed using the enzyme recovered by the procedure mentioned under 3.4.2.2. The lipolytic activity of the extracted crude enzyme under different conditions was determined by changing the conditions of the pNPP assay given in section 3.4.2.3. Each test was repeated appropriately to ensure the consistency of results, and the results of one trial were used to represent data.

3.6.1 Effect of pH on lipase activity

The optimum pH of lipase activity was determined from 3.0 to 11.0 using appropriate buffer solutions. The lipase activity was measured at 40 °C by using 50 mM citrate phosphate buffer (pH 3.0-7.0), 50 mM Tris HCl (pH 8.0-9.0), and 50 mM sodium glycine (pH 10.0-11.0) respectively in the pNPP assay. The reaction was carried out for 15 mins at a specific pH value. The pH of all the samples was adjusted to 8.0 using a constant volume of 1 M Tris HCl at pH 8.0 before measuring the absorbance at 410 nm. The controls were conducted at each pH value by adding an equal volume of distilled water in place of crude enzyme in the pNPP assay.

3.6.2 Effect of temperature on lipase activity

The effect of temperature was investigated across the range of 20-70 °C under optimized assay conditions. The pH of the pNPP substrate solution was maintained at 6.0. After the 15 mins incubation period at different temperatures, the pH of all the samples was adjusted to 8.0 using 1 M Tris HCl (pH 8.0) before measuring the absorbance at 410 nm. The controls

were conducted by adding an equal volume of distilled water in place of the enzyme to the reaction mixture.

3.6.3 Effect of cations on unchelated lipase activity

The effect of cations on the crude enzyme activity was examined by incubating 0.1 mL of the crude enzyme with 0.1 mL of the following metal chlorides: $MgCl_2$, NaCl, KCl, $MnCl_2$, and $CaCl_2$ at 50 mM concentrations. The incubation was conducted for 60 mins at room temperature. The remaining lipase activity was measured under optimized assay conditions. The controls were conducted for each cation with an equal volume of distilled water in place of crude enzyme.

3.6.4 Effect of anions on unchelated lipase activity

The effect of anions was examined by incubating 0.06 mL of the crude enzyme with 0.06 mL of the following sodium salts: NaCl, $NaNO_3$, NaI, and Na_2SO_4 at 50 mM concentrations. The crude enzyme with each anion was incubated for 60 mins at room temperature. The remaining lipase activity was measured under optimized assay conditions. The controls were conducted for each anion with an equal volume of distilled water instead of the crude enzyme.

3.7 Molecular identification of the lipolytic fungus

The molecular identification of the lipolytic fungus was made by initial genomic DNA extraction, PCR amplified to target the specific *ITS* regions, followed by sequencing reactions to establish a draft nucleotide sequence of the fungus.

3.7.1 DNA isolation

The DNA of the lipolytic fungus was isolated with the following Cetyl Trimethyl Ammonium Bromide (CTAB) method. The fungal mycelia (~100 mg) were scrapped from a fresh culture plate using a sterilized scalpel into a 1.5 mL Eppendorf tube. The fungal mycelia were treated with 500 µL of Tris-EDTA buffer (1 mM Tris HCl, pH 8, 0.1 mM EDTA, 2% SDS w/v), 140 µL of 5 M NaCl, and 70 µL of 10% CTAB at 60 °C. The mixture was vortexed for 2-3 secs followed by incubation for 60 mins at 65 °C. To the incubated mixture, 600 µL of chloroform: isoamyl alcohol (24: 1 v/v) was added, mixed for 1 min, and centrifuged at 5000 rpm for 5 mins at room temperature. The supernatant was extracted into a new Eppendorf tube, and the extraction step involving chloroform: isoamyl was repeated.

The supernatant after centrifugation was extracted into a new Eppendorf tube while measuring the volume, and 0.2 volumes of 5 M NaCl and 2.5 volumes of 100% ethanol were added. The tube was gently mixed, kept at room temperature for 5 mins, and centrifuged for 5 mins at 13,000 rpm. After discarding the supernatant, the pellet was washed with 80% ice-cold ethanol. The pellet was dried for 30 mins at room temperature and re-suspended in 50 µL of Tris-EDTA buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0). The DNA was visualized in a 1% agarose gel and stored at -20 °C.

3.7.2 PCR amplification and sequencing of *ITS* region

The *ITS*-1/5.8S rRNA/*ITS*-2 region of the genome was amplified with *ITS1*/ forward primer: 5'-TCCGTAGGTGAACCTGCGG-3' and *ITS4*/ reverse primer: 5'-TCCTCCGCTTATTGATATGC-3' (De Beeck *et al*., 2014). 15 µL of the reaction mixture was comprised of 5 µL of GoTaq® Green Master Mix [2X Green GoTaq® reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM $MgCl_2$], 3 µL of nuclease-free water, 0.5 µL of forward primer, 0.5 µL of reverse primer, and 1 µL of template DNA. The PCR profile comprised of an initial denaturation step at 94 °C for 30 secs followed by 45 cycles of double-stranded DNA denaturation for 30 secs at 94 °C, annealing for 40 secs at 55 °C, and 1 min extension at 72 °C. Lastly, a final extension for 5 mins at 72 °C was done. The amplified DNA sample was visualized in a 2% agarose gel with Ethidium Bromide as the stain. A 100 bp ladder was used to quantify the DNA band under a UV transilluminator. The PCR product was sequenced and compared with other sequences using the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed to show the relationship of the lipolytic fungus with several other fungi. The sequences for the phylogenetic tree were obtained from the Gen Bank Database. Unweighted pair group method with arithmetic mean (UPGMA) statistical method with 1000 bootstrap replications and Tamura-Nei model was used to infer the phylogenetic tree in MEGA 7.0 tool.

4. RESULTS

4.1 Initial culturing of the fungus

A pure fungus culture was established on PDA (Figure 4.1) by repeated subculturing, and colony characteristics were recorded (Table 4.1). The fungus was cultured by point inoculation and allowed to grow at room temperature for 3 days. The fungus was identified as a *Trichoderma* species based on basic morphological characteristics.

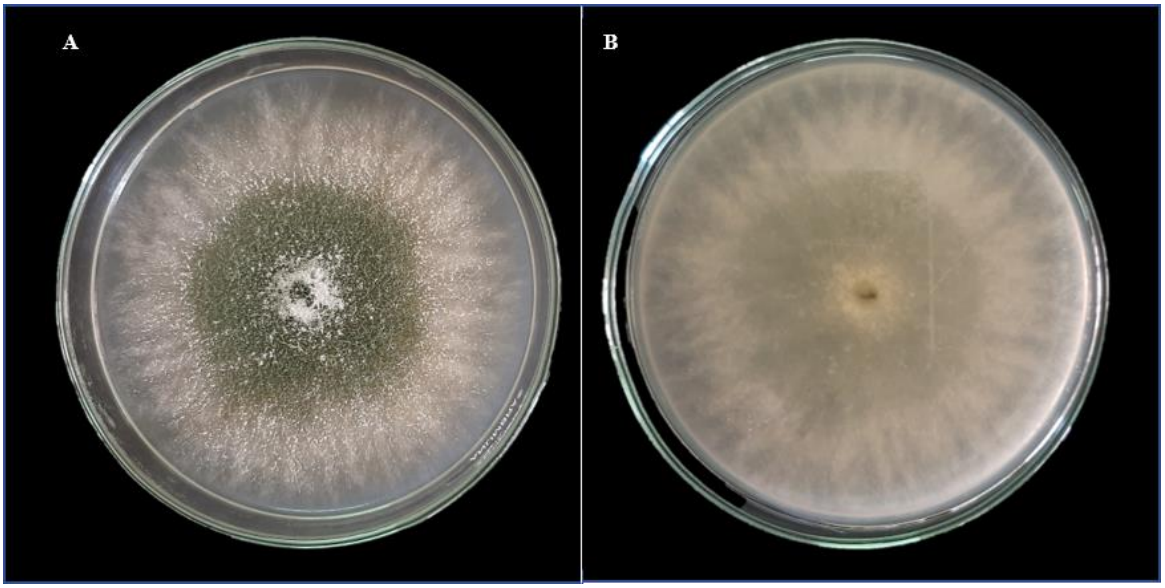

Figure 4.1 Morphology of the pure fungus culture on a PDA plate cultured by point inoculation. A: Top view of the culture plate. B: Bottom view of the culture plate.

Table 4.1 Morphological characteristics of the fungus.

Morphological Character	Observation
Top view	White and green circular growth
Bottom view	Circular growth pattern with a yellow pigmentation
Elevation	Flat
Form	Circular
Color	White mycelium with green colored conidia
Margin	Filamentous
Colony growth	Fast

4.2 Microscopic identification of the fungus

Microscopic observation of slide culture stained with LPCB provided the details of the structure of fungal mycelium and spore shape (Figure 4.2). Fungus slide culture allowed to grow for 3 days was used. The slide was observed under mid-power (×10 ×10). As observed, mycelium branching was bidirectional, and spores were spherical. The hyphae were not properly stained and observed as hyaline structures. Spores were observed in both green and hyaline and found clustered at some positions. The microscopic features observed agreed with the characteristic of *Trichoderma* species.

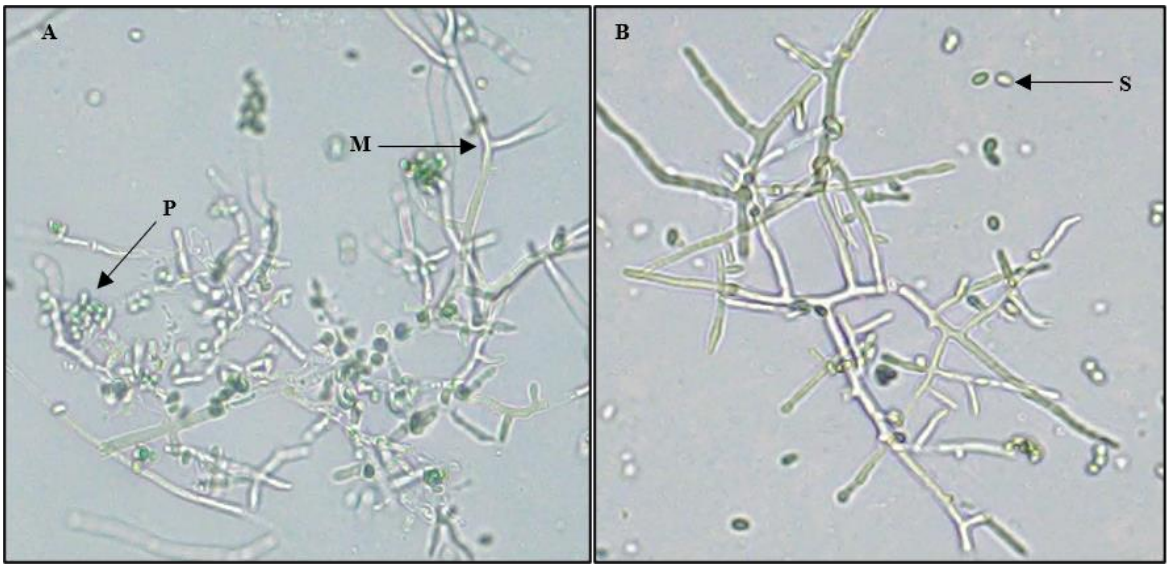

Figure 4.2 Microscopic view of the fungus. A: Represents the mycelium structure with bidirectional branching (M) and clusters of spores (P). B: Indicates the spherical shape of the spores clearly (S).

4.3 Qualitative determination of lipolytic activity

In the Tween 20 plate assay, the formation of a visible precipitate of white due to the calcium salt formed by the fatty acid released by the hydrolysis of Tween 20 was observed around the inoculum. This indicated the lipolytic activity of the fungus in Tween 20 medium (Figure 4.3 A1). The control conducted without Tween 20 showed no visible precipitation around the fungus inoculum (Figure 4.3 A2). However, growth of the fungus was observed in the control.

The presence of lipase activity was further indicated by the color change of phenol red indicator from red to yellow around the inoculum in the phenol red plate assay due to the formation of fatty acids by the lipolytic fungus (Figure 4.3 B1). The control conducted without fungus inoculation showed no color change into yellow (Figure 4.3 B2).

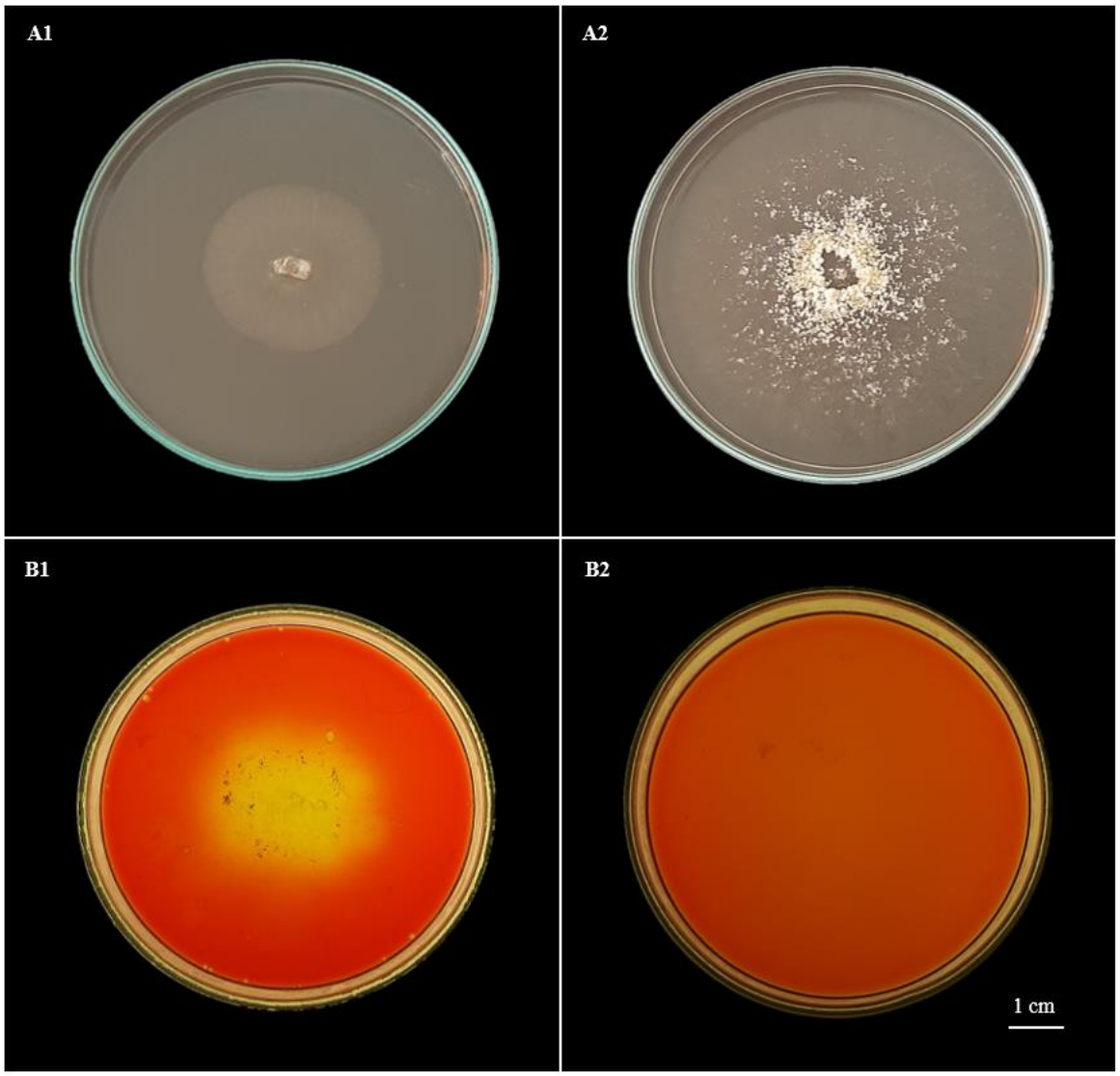

Figure 4.3 Primary screening of lipolytic fungus by plate assays. A1: Tween 20 plate with precipitation around the inoculum. A2: The control of the Tween 20 test showed no visible precipitate formation. B1: Phenol red plate indicating yellow coloration around the inoculum. B2: The control for phenol red test exhibited no yellow coloration.

4.4 Quantitative determination of lipolytic activity: pNPP assay

The crude enzyme's lipase activity evaluation was based on the enzyme's ability to induce hydrolysis of pNPP to form pNP that can be spectrophotometrically determined at 410 nm. The optimized growth period was analyzed by obtaining absorbance from the same inoculated liquid culture for 5 consecutive days. The lipase secretion was highest on day 3, as indicated by the highest absorbance value, and the secretion was almost absent on day 1 (Figure 4.4). After day 3, the absorbance measurements gradually reduced, indicating the decreasing lipase production from the lipolytic *Trichoderma* species. Therefore, a 3-day incubation period was selected for culturing the fungus in the liquid medium.

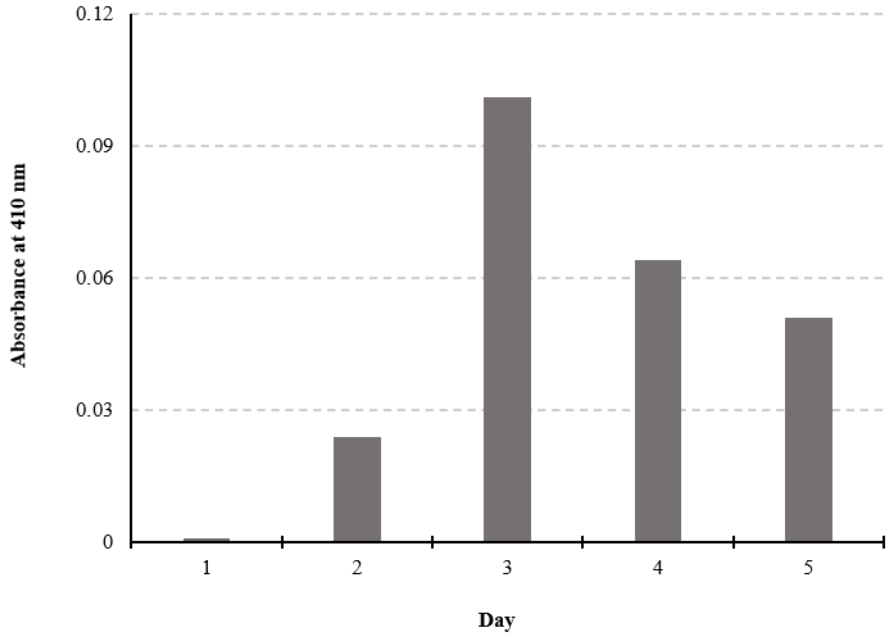

Figure 4.4 Quantitative determination of the lipase activity of the fungus. The graph depicts the absorbance variation at 410 nm (y-axis) for 5 consecutive days (x-axis). The highest absorbance on day 3 indicated the most elevated lipase secretion from the lipolytic fungus.

4.5 Optimization of lipase production medium

4.5.1 Carbon and nitrogen source optimization

An optimized carbon source for lipase secretion was tested using different carbon sources in the basal mineral medium. The highest lipase secretion was observed with olive oil as the carbon source. A significant lipase secretion was recorded with coconut oil in the medium. The lowest lipase secretion was observed in the absence of a carbon source. Cellulose as a carbon source reported the least absorbance value indicating a very low lipase secretion (Figure 4.5 Graph A).

The nitrogen source's effect on lipase production from the fungus was determined using different nitrogen sources in the liquid mineral medium. The lipase secretion was highest with ammonium sulfate as the nitrogen source, and the lipase secretion was least with sodium nitrate as the nitrogen source. The absorbance observed in the absence of a nitrogen source was higher than the absorbance recorded with sodium nitrate as the nitrogen source (Figure 4.5 Graph B). Ammonium nitrate, urea, and peptone did not show a significant lipase secretion compared to ammonium sulfate as the nitrogen source.

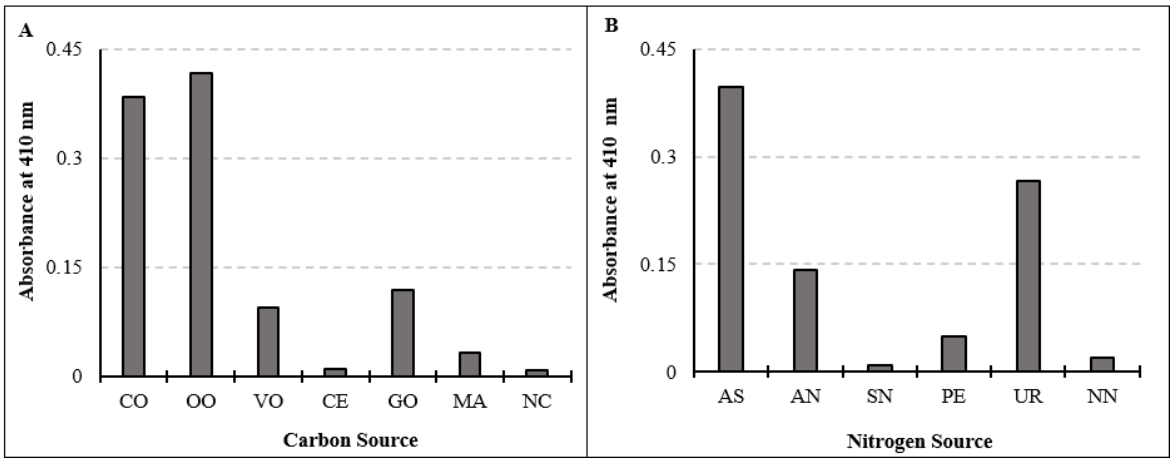

Figure 4.5 Media optimization for lipase secretion. A: Effect of different carbon sources on lipase secretion. CO: Coconut oil, OO: Olive oil, VO: Vegetable oil, CE: Cellulose, GO: Gingerly oil, MA: Margarine, and NO: No carbon source. B: Effect of different nitrogen sources on lipase secretion. AS: Ammonium sulfate, AN: Ammonium nitrate, SN: Sodium nitrate, PE: Peptone, UR: Urea, and NN: No nitrogen source.

4.5.2 pH optimization

An optimum pH for lipase secretion was analyzed by adjusting the pH of the basal mineral medium across the range of 3.0 to 10.0. The absorbance was highest at pH 7.0, followed by a drastic reduction of the absorbance at pH 8.0. In addition, pH 6.0 also reported a significant absorbance indicating a considerable lipase activity. Lipase activity in an alkaline medium was negligible (Figure 4.6).

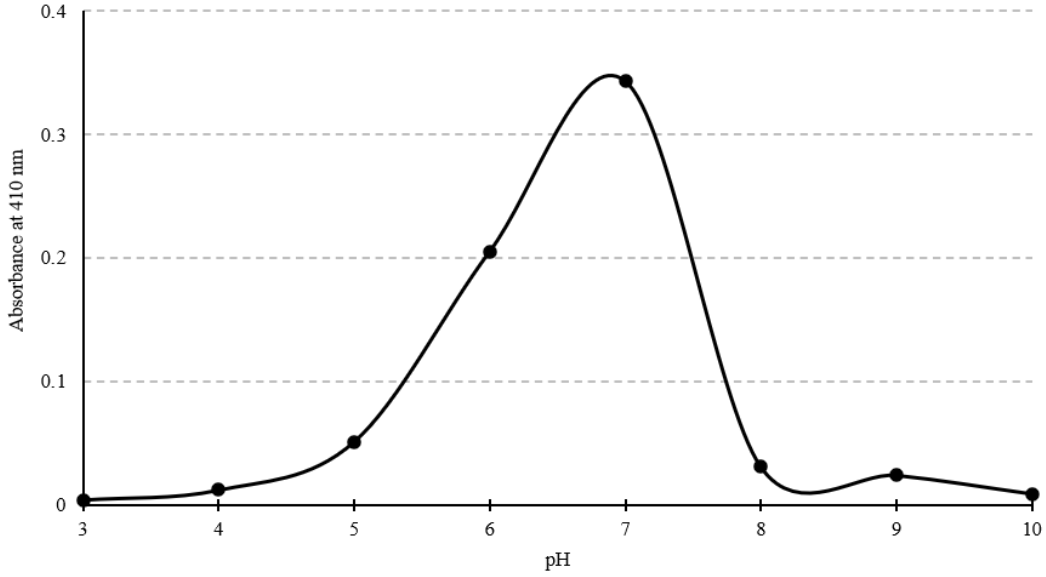

Figure 4.6 Effect of pH on lipase secretion by the fungus. The variation of absorbance measurements (y-axis) with the medium at pH across the range 3.0 to 10.0 (x-axis).

4.5.3 Temperature optimization

The absorbance recorded at 35 °C was significant than the absorbance at 30 °C, indicating a high lipase secretion at 35 °C during the single trial. However, the lipase secretion at 30 ° C was substantial, showing a considerable growth of the lipolytic fungus at the temperature. Temperature optimization requires further trials to ensure the variation of the results.

4.6 Characterization of crude enzyme

4.6.1 Effect of pH and temperature on the crude enzyme activity

The effect of the pH on the crude enzyme activity was determined across the range of 3.0 to 11.0. The maximum lipolytic activity was at pH 6.0. A drastic reduction in crude lipase activity was observed when moving from pH 6.0 to 7.0 (Figure 4.7 A). Low lipolytic activity was observed at alkaline pH values. The optimum temperature for lipase activity was determined as 40 °C and the lipase activity at 50 °C was also significant (Figure 4.7 B).

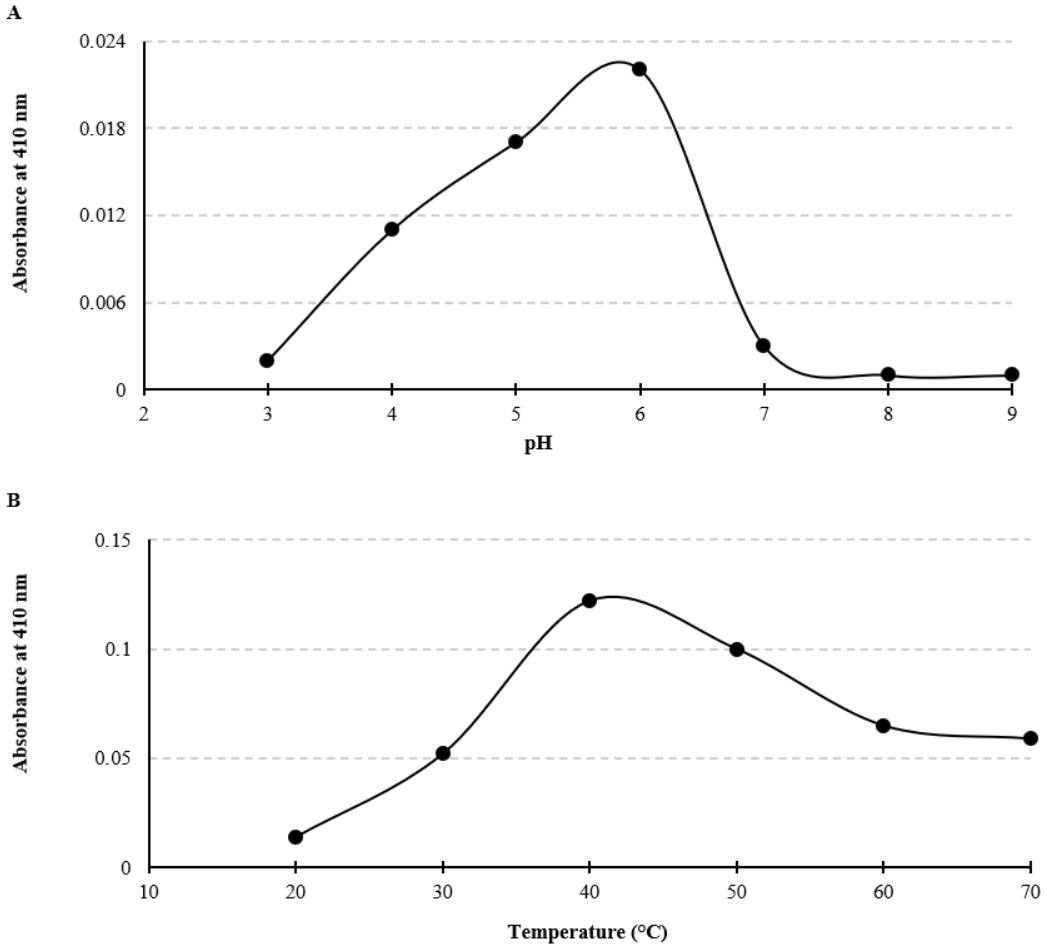

Figure 4.7 Impact of pH and temperature on lipase activity. A: Lipase activity across the pH range 3.0 to 9.0 (x-axis) was analyzed by obtaining absorbance measurements at 410nm (y-axis). B: Dependence of lipase activity on temperature range 20 to 70 °C (x-axis), monitored by absorbance measurements at 410 nm (y-axis).

4.6.2 Effect of different cations and anions on unchelated enzyme

The effect of several cations at a concentration of 50 mM on the activity of the crude enzyme was examined. The crude enzyme activity was highest in the presence of Ca^{2+}, while Mg^{2+} and Mn^{2+} moderately activated the enzyme. Na^{+} showed an inhibitory effect on the crude enzyme, while K^{+} showed no considerable modification in lipase activity (Figure 4.8 Graph A). Characterization of anions (50 mM) indicated the highest lipase activity with NO_3^{-}. In addition, SO_4^{2-}, I^{-}, and Cl^{-} showed moderate enzyme activation (Figure 4.8 Graph B).

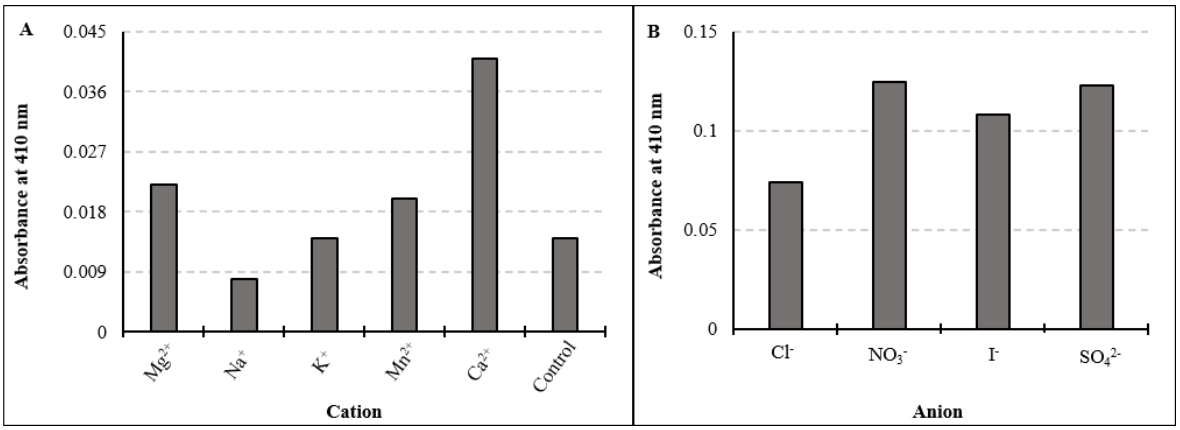

Figure 4.8 Effect of cations and anions at 50 mM concentration on lipase activity. A: Variation of unchelated lipase activity with different cations (x-axis) analyzed by absorbance measurements at 410 nm (y-axis). The control was conducted without any cation. B: Change in unchelated lipase activity with different anions (x-axis) analyzed by absorbance measurements at 410 nm (y-axis).

4.7 Molecular identification of the fungus

The genus was identified as *Trichoderma* based on colony characteristics and microscopic observations. To further ensure that the fungus belongs to the identified genus and predict the species, the DNA fragment amplified using *ITS1* and *ITS4* primers were sequenced. The amplified sample to be sequenced was quantified in a 2% agarose gel, and it resulted in a band of 600 bp. The obtained sequence showed 98% query cover, 100% identity, and 0.0 E value with *Trichoderma longibrachiatum* compared with the sequences available in online databases using the BLAST program. The phylogenetic tree constructed using the unknown sequence and several other sequences from Gen Bank Database showed the relationship of the unknown sequence to the selected Gen Bank Database sequences. The unknown sequence clusters with *Trichoderma longibrachiatum* as indicated in the phylogenetic tree.

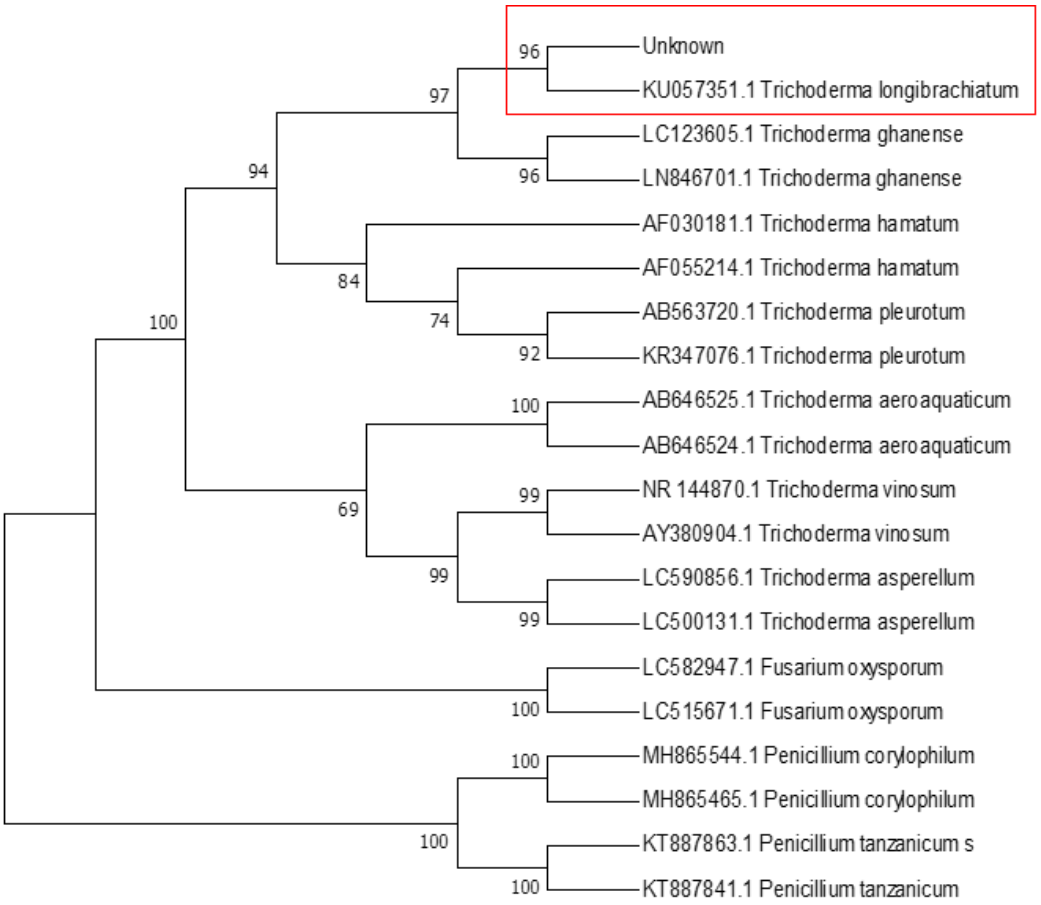

Figure 4.9 The phylogenetic relationship of the unknown sequence to the selected sequences from the Gen Bank Database. For the phylogenetic tree, 1000 bootstrap replications were used. The red colored box indicates the position of the unknown sequence in the phylogenetic tree.

5. DISCUSSION

Obtaining pure cultures of the interested fungus was an initial and essential step of the research. PDA medium was used for this purpose as it allows isolation and cultivation of fungi. PDA medium comprised agar, dextrose, with nitrogen, phosphorous, micronutrients, and vitamins derived from potato infusion. The increased carbon: nutrient ratio of the medium permits growth, sporulation, and pigmentation of many fungi taxa (Griffith *et al*., 2007). The medium was supplemented with ampicillin and spectinomycin antibiotics to avoid bacterial contamination. Ampicillin act by inhibiting the formation of cross-links in the peptidoglycan layer while spectinomycin binds 30S ribosomal subunits of bacteria to interfere with protein synthesis. The fungus was point-inoculated since the streak plate method was unsuccessful with *Trichoderma* species due to the high growth rate. The morphological characteristics of fungal colonies on PDA confirmed the genus of the lipolytic fungus as *Trichoderma*. The most prominent feature of the colony was the green and white concentric circular growth pattern, observed by day 3 (Figure 4.1). Overgrown colonies were green in color, and the circular pattern of growth was less prominent. Supported by the microscopic images, the green color can be due to spore formation. Microscopic observation of fungal specimens stained with LPCB revealed spherical-shaped spores and bidirectionally branched mycelium (Figure 4.2). The three components of LPCB have different functions in the technique. Phenol kills the living microbes, cotton blue stains the chitin structures of the fungus, while lactic acid preserves the structure of the fungus (Tankeshwar, 2015).

Separate assays to identify the lipolytic activity were used. They were Tween 20 and phenol red plate assays. In Tween 20 plate assay, the lipolytic activity was indicated by a zone of precipitate around the inoculum (Lanka and Latha, 2015). The precipitation was due to the insoluble fatty acid salt formed by the released fatty acids by hydrolysis of Tween 20 and calcium provided by the $CaCl_2$ supplied in the medium (Lonon *et al*., 1988). The controls without Tween 20 showed no visible precipitate. Instead, the fungus growth without considerable pigmentation was observed (Figure 4.3 A1 and A2). The presence of lipase cannot be determined with the observations from Tween 20 plate method since esterases hydrolyze Tween 20 and can give rise to similar observations (Tomioka, 1983). Another method using phenol red as an indicator to detect lipolysis as a function of pH drop due to the hydrolysis of olive oil into fatty acids was conducted. In the phenol red plate method, the lipase activity was indicated by a color change from red to yellow around the inoculum. In

this assay, the yellow zone can increase due to the spread of fatty acids with prolonged incubation. The incorporation of $CaCl_2$ overcame the problem and quenched fatty acids (Singh *et al*., 2006). The control without any inoculations showed no color changes due to the absence of lipolytic activity to release fatty acids (Figure 4.3 B1 and B2).

Lipase activity was quantitatively determined using pNPP assay employing pNPP as the substrate for lipase. Following the lipase reaction, the amount of released pNP by the hydrolysis of pNPP was estimated spectrophotometrically at 410 nm. Besides the fact that the method is simple and rapid, forming a turbid solution due to the immiscibility in pNPP or released fatty acids by lipase activity has to be overcome. The turbidity can interfere with spectrophotometric measurements. Hence triton X-100 was incorporated into the pNPP dissolved isopropanol solution (Gupta *et al*., 2002). This enhanced the dissolution of pNPP in isopropanol and resulted in a clear solution after agitating for several minutes. Without Triton X-100 in the medium, it was almost impossible to get a clear solution of pNPP. The preparation of pNPP solution by dissolving 0.006 g of pNPP and 320 µL of triton X-100 in 2 mL of isopropanol was done immediately before the assay. Immediate preparation of the solution minimizes the hydrolysis of pNPP with time and resulting in a yellow-colored solution. It was also examined that the color change was accelerated at high temperatures. Appropriate controls for each test were conducted to determine the influence of such background effects on absorbance readings. The controls could be used as references during spectrophotometric measurements. But, the absorbance readings for the controls and tests were separately obtained against distilled water for easy comparison and analysis of the readings. The pNPP assay was used in both media optimization and enzyme activity characterization studies.

Tests were conducted to optimize growth conditions such as the incubation period, carbon source, nitrogen source, pH, and temperature to enhance lipase secretion from lipolytic fungus. As determined by the results of the pNPP assay, a 3-day incubation period was selected as the best incubation period for a high enzyme secretion (Figure 4.4). Trials were conducted appropriately to ensure the variation of test parameters. The results for each factor were presented using the readings from a single trial since the amount of inoculum used to inoculate the medium can vary between the trials. Obtaining similar inoculums is challenging and could be achieved by preparing fungal suspensions with a similar OD at 600

nm. Each test was conducted together with controls to identify the influence of background effects on the absorbance readings.

The effectiveness of the following carbon sources on lipase production was examined: coconut oil, olive oil, vegetable oil, cellulose, gingerly oil, and margarine. Olive oil exhibited the highest lipase secretion. The lipase production was also significant with coconut oil as the carbon source. Lipase activity was less effective with gingerly oil, vegetable oil, and margarine but greater than cellulose that exhibited the lowest lipase production (Figure 4.5 A). The lipase production was deficient in the medium without any carbon source and indicated the need for a carbon source for lipolytic fungus growth and subsequent lipase secretion. The results clearly showed that lipase production is highly dependent on the carbon source of the growth medium. The effect of nitrogen source was studied using ammonium sulfate, ammonium nitrate, sodium nitrate, peptone, and urea as the nitrogen source of the growth medium. Among the studied nitrogen sources, the highest lipase production was observed when ammonium sulfate was utilized as the nitrogen source, followed by urea, ammonium nitrate, peptone, and sodium nitrate. The absorbance reading with sodium nitrate was lower than the reading recorded without any nitrogen source in the medium (Figure 4.5 B) and indicated an inhibitory effect of sodium nitrate on lipase production. Most of the previous studies have reported yeast extract as a good nitrogen source. But in this study, yeast extract was not used as a nitrogen source since it is a complex hydrolysate of yeasts providing carbon, sulfur, growth factors, trace nutrients alone with nitrogenous compounds. These observations for optimized carbon and nitrogen source agreed with the results of a previous study involving *Trichoderma longibrachiatum* where olive oil has shown the highest lipase production among the studied plant oils and ammonium sulfate as the optimized nitrogen source (Gochev *et al*., 2012).

The pH of the basal mineral medium was adjusted across the range of 3.0-10.0 to examine the effect of pH on lipase production from the fungus. The highest absorbance reading was reported at pH 7.0, followed by a drastic drop at pH 8.0. Meanwhile, pH 6.0 also exhibited a considerable lipase production (Figure 4.6). The results explained that the alkaline pH inhibits lipase secretion from the tested *Trichoderma* species. The hypothesis that lipase has been secreted but inactivated in an alkaline pH medium is disproved as the lipases are active over the range 4.0-11.0 (Mehta *et al*., 2017). The incubation temperature can also affect lipase secretion (Akyil and Cihangir, 2018). Fungi cannot control the internal temperature;

instead, the ambient climate governs it. The growth rate and metabolism of fungi are dependent on temperature when all other conditions are kept constant (Li *et al*., 2009). The inoculated liquid media were incubated for 3 days at specific temperatures, and 35 °C was observed as a favourable temperature for considerable lipase secretion. The need for a moderate temperature avoids high costs to maintain high temperatures on an industrial scale.

The extracted crude enzyme's activity was optimized by changing the pH and temperature of the pNPP assay medium in which the lipase-catalyzed reaction would take place. The pH of the pNPP assay was adjusted over the range 3.0-9.0 using several buffers with suitable buffering ranges. Changing pH affects the charges on amino acids forming the enzyme, affecting the active site's shape. For the studied lipase, the highest activity was examined around pH 6.0. The enzyme activity was drastically decreased at pH 7.0. Considerable lipase activity was observed at pH 4.0 and 5.0 (Figure 4.7 A). The results indicated that the crude lipase extracted from the *Trichoderma* species is active under acidic conditions. During pH optimization studies, previous studies have determined the specific lipase activities at each pH by obtaining absorbance at 348 nm without adjusting the pH to 8.0, which is the standard pH of the pNPP assay. The 348 nm is the pH-independent isosbestic wavelength of pNP (Prive *et al*., 2013). In this study, the final pH of the reaction mixture was adjusted to pH 8.0 using 1 M Tris-HCl (pH 8.0), and the absorbance was measured at 410 nm since the 410 nm wavelength gives high sensitivity. The temperature optimization for lipase activity was done by changing the temperature of 15 mins incubation of pNPP assay in the range of 20 to 70 °C. The highest lipase activity was observed at 40 °C. The enzyme activity between 50 to 70 °C was also significant, while the activity at 20 and 30 °C was less significant (Figure 4.7 B). High temperatures lead to protein denaturation, disrupting the shape of the active site and reduces enzyme activity. However, for the tested crude lipase, considerable enzyme activity was observed at high temperatures. The crude lipase activity at high temperatures makes the enzyme an attractive candidate for industrial applications employing high temperatures. However, most reported lipases are characterized by rapid inactivation at high temperatures. High absorbance values in the study can be due to other background reactions at high temperatures, which requires further analysis.

The effect of cations and anions on unchelated crude enzyme was examined by preincubating the enzyme with each cation and anion at 50 mM concentration for 60 mins at room temperature. The highest enzyme activity was observed with Ca^{2+}, and this can be due to a

stabilizing effect on enzyme conformation. Meanwhile, Mg^{2+} and Mn^{2+} moderately activated the enzyme (Figure 4.8 A). K^{+} showed no considerable enzyme activation while Na^{+} exhibited an inhibitory effect on the crude enzyme, compared to the absorbance reading recorded without any cation incorporation (control). These results indicated the possible activation of crude enzyme activity by preincubating with Ca^{2+}, which could be important during industrial applications. Lipases are documented as a metalloenzyme. Metalloenzyme usually requires metal ions in their active site for catalytic activity (Lestari *et al*., 2016), and for the tested crude enzyme, Ca^{2+} can be stabilizing the confirmation. Among tested anions, NO_3^{-} exhibited the highest crude enzyme activity. However, significant enzyme activation was achieved with I^{-} and SO_4^{2-}. Meanwhile, Cl^{-} recorded the lowest crude enzyme activity (Figure 4.8 B). A substantial variation in crude enzyme activity was not observed with tested anions as observed with different cations.

Sequencing techniques have provided researchers with quick and affordable methods to access genomic data (Prive *et al*., 2013). Sequencing has facilitated species-level identification that could be vital in reporting specific fungi with lipolytic activity. Especially with the *Trichoderma* genus, species identification based on morphological characteristics is difficult. For this, DNA was extracted as the first step. A clear band for genomic DNA was not observed in 1% agarose gel during the first trial with a one-week-old culture and when the final pellet dissolved in distilled water. But a clear band was observed when the fungal sample was obtained from growing edges of a 3-day old culture, prolonged grinding, and when the final pellet was dissolved in TE buffer. This suggests that cell lysis has not occurred efficiently with old cultures and that prolonged grinding is required to extract DNA. Molecular identification of fungi usually employs *ITS1* and *ITS4* primers that amplify ITS-1/5.8SrRNA/ITS-2 region (Li *et al*., 2009). The amplified DNA fragment using the above primers resulted in a band around 600 bp. The obtained sequence showed 98% query cover, 100% identity, and 0.0 E value with *Trichoderma longibrachiatum* compared with similar sequences available online using the BLAST program. Inferred phylogenetic tree indicated the clustering of the obtained sequence with *Trichoderma longibrachiatum* sequence from Gen Bank Database. A previous study involving *Trichoderma longibrachiatum* has also reported its lipase activity (Gochev *et al*., 2012). However, this study has accomplished media optimization, crude enzyme characterization, and molecular identification of the fungus to provide a complete report about the lipolytic activity of the *Trichoderma* species.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Tween 20 and phenol red plate assays verified the lipolytic activity of the *Trichoderma* species. The current study results revealed that the crude lipase secretion from the lipolytic *Trichoderma* species could be increased by media optimization. As observed, olive and ammonium sulfate, as the carbon and nitrogen sources, respectively, showed an optimized crude lipase secretion. The enzyme production was prominent at a pH of 7.0. Furthermore, significant lipase production was observed at pH 6.0, but the production was drastically inhibited at pH 8.0. The lipase secretion was maximum at the temperature of 35 °C. The enzyme activity characterization studies revealed that the crude lipase activity could be enhanced under optimized conditions. The crude enzyme activity was significant at a pH of 6.0 and a temperature of 40 °C. Retention of activity at high temperatures makes the enzyme a potential candidate in industrial applications. Moreover, the enzyme activity could be enhanced using Ca^{2+} and NO_3^-. The lipase activity was also prominent with SO_4^{2-}. Finally, the molecular identification indicated the lipolytic fungus as *Trichoderma longibrachiatum*.

More studies are needed to test the enzyme's thermostability with more precision. Characterizing the enzyme activity with frequently used chemicals in the industries can expose more industrially favourable enzyme properties. The stability and activity of the enzyme with chemicals make the enzyme more attractive as an industrially demanding biocatalyst. Testing the effect of frequently used reagents in activity assays such as pNPP assay will also indicate any inhibition or activation of the enzyme. After enzyme purification, analysis of the extracted lipase using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) can provide more details regarding the enzyme. SDS-PAGE will allow the estimation of the relative molecular mass and abundance of the enzyme. Furthermore, improving the strain by modifying the fungus' genetic makeup will allow higher enzyme secretion and activity.

REFERENCES

Adlercreutz, P., Gitlesen, T., Ncube, I. and Read, J.S. (1997). Vernonia lipase: A plant lipase with strong fatty acid selectivity. *Methods in enzymology* **284:** 220-232. DOI: https://doi.org/10.1016/S0076-6879(97)84014-1.

Akyil, M.H. and Cihangir, N. (2018). Detection of lipase production from newly isolated *Trichoderma Citrinoviride*. *Hacettepe Journal of Biology and Chemistry* **46**(2): 229-235. DOI: 10.15671/HJBC.2018.231.

Ali, S., Huang, Z. and Ren, S.X. (2009). Production and extraction of extracellular lipase from the entomopathogenic fungus *Isaria fumosoroseus* (Cordycipitaceae; Hypocreales). *Biocontrol Science and Technology* **19**(1): 81-89. DOI: 10.1080/09583150802588524.

Alsohaili, S.A. and Bani-Hasan, B.M. (2018). Morphological and Molecular Identification of Fungi Isolated from Different Environmental Sources in the Northern Eastern Desert of Jordan. *Jordan Journal of Biological Sciences* **11**(3): 229-337.

Alves, D.R., Morais, S.M.D., Tomiotto-Pellissier, F., Vasconcelos, F.R., Freire, F.D.C.O., Silva, I.N.G.D., Cataneo, A.H.D., Miranda-Sapla, M.M., Pinto, G.A.S., Conchon-Costa, I., Noronha, A.D.A.A. and Pavanelli, W.R. (2018). Leishmanicidal and fungicidal activity of lipases obtained from endophytic fungi extracts. *PloS one* **13**(6): e0196796. DOI: https://doi.org/10.1371/journal.pone.0196796.

Andler, S.M. and Goddard, J.M. (2018). Transforming food waste: how immobilized enzymes can valorize waste streams into revenue streams. *npj Science of Food* **2**(1): 1-11. DOI:10.1038/s41538-018-0028-2.

Ansorge-Schumacher, M.B. and Thum, O. (2013). Immobilised lipases in the cosmetics industry. *Chemical Society Reviews* **42**(15): 6475-6490. DOI: 10.1039/c3cs35484a.

Aravindan, R., Anbumathi, P. and Viruthagiri, T. (2007). Lipase applications in food industry. *Indian Journal of Biotechnology* **6**(2):141-158.

Arntzen, M.Ø., Bengtsson, O., Várnai, A., Delogu, F., Mathiesen, G. and Eijsink, V.G. (2020). Quantitative comparison of the biomass-degrading enzyme repertoires of five filamentous fungi. *Scientific reports* **10**(1): 1-17. DOI: https://doi.org/10.1038/s41598-020-75217-z.

Askenazi, M., Driggers, E.M., Holtzman, D.A., Norman, T.C., Iverson, S., Zimmer, D.P., Boers, M.E., Blomquist, P.R., Martinez, E.J., Monreal, A.W., Feibelman, T.P., Mayorga, M.E., Maxon, M.E., Sykes, K., Tobin, J.V., Cordero, E., Salama, S.R., Trueheart, J., Royer, J.C. and Madden, K.T. (2003). Integrating transcriptional and metabolite profiles to direct the engineering of lovastatin-producing fungal strains. *Nature biotechnology* **21**(2): 150-156. DOI:10.1038/nbt781.

Aslam, S., Tahir, A., Aslam, M.F., Alam, M.W., Shedayi, A.A. and Sadia, S. (2017). Recent advances in molecular techniques for the identification of phytopathogenic fungi–a mini review. *Journal of Plant Interactions* **12**(1): 493-504. DOI: 10.1080/17429145.2017.1397205.

Bhatia, S.K., Wadhwa, P., Hong, J.W., Hong, Y.G., Jeon, J.M., Lee, E.S. and Yang, Y.H. (2019). Lipase mediated functionalization of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) with ascorbic acid into an antioxidant active biomaterial. *International journal of biological macromolecules* **123:** 117-123. DOI: 10.1016/j.ijbiomac.2018.11.052.

Bissett, J. (1991). A revision of the genus *Trichoderma*. III. Section Pachybasium. *Canadian journal of botany* **69**(11): 2373-2417. DOI: https://doi.org/10.1139/b91-298.

Blackwell, M. (2011). The Fungi: 1, 2, 3… 5.1 million species? *American journal of botany* **98**(3): 426-438. DOI: 10.3732/ajb.1000298.

Boratyński, F., Szczepańska, E., Grudniewska, A., Gniłka, R. and Olejniczak, T. (2018). Improving of hydrolases biosythesis by solid-state fermentation of *Penicillium camemberti* on rapeseed cake. *Scientific reports* **8**(1): 1-9. DOI: 10.1038/s41598-018-28412-y.

Bornscheuer, U., Herar, A., Kreye, L., Wendel, V., Capewell, A., Meyer, H.H., Scheper, T. and Kolisis, F.N. (1993). Factors affecting the lipase catalyzed transesterification reactions of 3-hydroxy esters in organic solvents. *Tetrahedron: asymmetry* **4**(5): 1007-1016. DOI: https://doi.org/10.1016/S0957-4166(00)80145-7.

Borrelli, G.M. and Trono, D. (2015). Recombinant lipases and phospholipases and their use as biocatalysts for industrial applications. *International journal of molecular sciences* **16**(9): 20774-20840. DOI:10.3390/ijms160920774.

Brandt, S.C., Ellinger, B., van Nguyen, T., Thi, Q.D., Van Nguyen, G., Baschien, C., Yurkov, A., Hahnke, R.L., Schäfer, W. and Gand, M. (2018). A unique fungal strain collection from Vietnam characterized for high performance degraders of bioecological important biopolymers and lipids. *PloS one* **13**(8): e0202695. DOI: https://doi.org/10.1371/journal.pone.0202695.

Brocca, S., Secundo, F., Ossola, M., Alberghina, L., Carrea, G. and Lotti, M. (2003). Sequence of the lid affects activity and specificity of *Candida rugosa* lipase isoenzymes. *Protein science* **12**(10): 2312-2319. DOI: 10.1110/ps.0304003.

Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.P., Bjorkling, F., Huge-Jensen, B., Patkar, S.A. and Thim, L. (1991). A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature* **351**(6326): 491-494. DOI: 10.1038/351491a0.

Burkert, J.F.D.M., Maldonado, R.R., Filho, F.M. and Rodrigues, M.I. (2005). Comparison of lipase production by *Geotrichum candidum* in stirring and airlift fermenters. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology* **80**(1): 61-67. DOI: 10.1002/jctb.1157.

Cavaille-Lefebvre, D. and Combes, D. (1997). Lipase synthesis of short-chain flavour thioesters in solvent-free medium. *Biocatalysis and Biotransformation* **15**(4): 265-279. DOI: https://doi.org/10.3109/10242429709003194.

Chander, H., Batish, V.K., Sannabhadti, S.S. and Srinivasan, R.A. (1980). Factors affecting lipase production in *Aspergillus wentii. Journal of food Science* **45**(3): 598-600. DOI: https://doi.org/10.1111/j.1365-2621.1980.tb04109.x.

Chandra, P., Singh, R. and Arora, P.K. (2020). Microbial lipases and their industrial applications: a comprehensive review. *Microbial Cell Factories* **19**(1): 1-42. DOI: https://doi.org/10.1186/s12934-020-01428-8.

Couturier, L., Taupin, D. and Yvergnaux, F. (2009). Lipase-catalyzed chemoselective aminolysis of various aminoalcohols with fatty acids. *Journal of Molecular Catalysis B: Enzymatic* **56**(1): 29-33.

Daiha, K., Angeli, R., de Oliveira, S. D., & Almeida, R. V. (2015). Are lipases still important biocatalysts? A study of scientific publications and patents for technological forecasting. *PloS one* **10**(6): e0131624. DOI: https://doi.org/10.1371/journal.

De Beeck, M.O., Lievens, B., Busschaert, P., Declerck, S., Vangronsveld, J. and Colpaert, J.V. (2014). Comparison and validation of some *ITS* primer pairs useful for fungal metabarcoding studies. *PloS one* **9**(6): e97629. DOI: 10.1371/journal.pone.0097629.

de Oliveira Carvalho, P., Contesini, F.J., Bizaco, R. and Alves Macedo, G. (2005). Kinetic properties and enantioselectivity of the lipases produced by four *Aspergillus* species. *Food Biotechnology* **19**(3): 183-192. DOI: 10.1080/08905430500316342.

Demera, L.L., Barahona, P.P. and Barriga, E.J.C. (2019). Production, extraction and characterization of lipases from the Antarctic yeast *Guehomyces pullulans*. *American Journal of Biochemistry and Biotechnology* **15**: 75-82. DOI: 10.3844/ajbbsp.2019.75.82.

Denesyuk, A., Dimitriou, P.S., Johnson, M.S., Nakayama, T. and Denessiouk, K. (2020). The acid-base-nucleophile catalytic triad in ABH-fold enzymes is coordinated by a set of structural elements. *PloS one* **15**(2): e0229376. DOI: https://doi.org/10.1371/journal.pone.0229376.

Derewenda, U., Swenson, L., Wei, Y.U.N.Y.I., Green, R., Kobos, P.M., Joerger, R., Haas, M.J. and Derewenda, Z.S. (1994). Conformational lability of lipases observed in the absence of an oil-water interface: crystallographic studies of enzymes from the fungi *Humicola lanuginosa* and *Rhizopus delemar*. *Journal of lipid research* **35**(3): 524-534.

Deshmukh, R., Khardenavis, A.A. and Purohit, H.J. (2016). Diverse metabolic capacities of fungi for bioremediation. *Indian Journal of Microbiology* **56**: 247–264). DOI: https://doi.org/10.1007/s12088-016-0584-6.

Dizge, N., Aydiner, C., Imer, D.Y., Bayramoglu, M., Tanriseven, A. and Keskinler, B. (2009). Biodiesel production from sunflower, soybean, and waste cooking oils by transesterification using lipase immobilized onto a novel microporous polymer. *Bioresource technology* **100**(6): 1983-1991. DOI: 10.1016/j.biortech.2008.10.008.

Duinhoven, S., Poort, R., Van der Voet, G., Agterof, W.G.M., Norde, W. and Lyklema, J. (1995). Driving forces for enzyme adsorption at solid-liquid interfaces: The serine protease savinase. *Journal of colloid and interface science* **170**(2): 340-350. DOI: https://doi.org/10.1006/jcis.1995.1111.

Feiten, M.C., Di Luccio, M., Santos, K.F., de Oliveira, D. and Oliveira, J.V. (2017). X-ray crystallography as a tool to determine three-dimensional structures of commercial enzymes subjected to treatment in pressurized fluids. *Applied biochemistry and biotechnology* **182**(2): 429-451. DOI 10.1007/s12010-016-2336-9.

Ferreira-Dias, S., Sandoval, G., Plou, F. and Valero, F. (2013). The potential use of lipases in the production of fatty acid derivatives for the food and nutraceutical industries. *Electronic Journal of Biotechnology* **16**(3): 12-12. DOI: 10.2225/vol16-issue3-fulltext-5.

Fischer, K. and Messner, K. (1992). Adsorption of lipase on pulp fibers during biological pitch control in paper industry. *Enzyme and microbial technology* **14**(6): 470-473. DOI: https://doi.org/10.1016/0141-0229(92)90139-F.

Fischer, M. and Pleiss, J. (2003). The lipase engineering database: a navigation and analysis tool for protein families. *Nucleic acids research* **31**(1): 319-321. DOI: 10.1093/nar/gkg015.

Fukuda, H., Kondo, A. and Noda, H. (2001). Biodiesel fuel production by transesterification of oils. *Journal of bioscience and bioengineering* **92**(5): 405-416. DOI: https://doi.org/10.1016/S1389-1723(01)80288-7.

Gandhi, N.N. (1997). Applications of lipase. *Journal of the American Oil Chemists' Society* **74**(6): 621-634. DOI: https://doi.org/10.1007/s11746-997-0194-x.

Gilham, D. and Lehner, R. (2005). Techniques to measure lipase and esterase activity in vitro. *Methods* **36**(2): 139-147. DOI: 10.1016/j.ymeth.2004.11.003.

Gochev, V., Montero, G., Kostov, G., Toscano, L., Stoytcheva, M., Krastanov, A. and Georgieva, A. (2012). Nutritive medium engineering enhanced production of extracellular lipase by *Trichoderma longibrachiatum*. *Biotechnology and Biotechnological Equipment* **26**(2): 2875-2882. DOI: 10.5504/BBEQ.2011.0138.

Gopinath, S.C., Hilda, A. and Anbu, P. (2005). Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments. *Mycoscience* **46**(2): 119-126. DOI 10.1007/s10267-004-0221-9.

Griffith, G.W., Easton, G.L., Detheridge, A., Roderick, K., Edwards, A., Worgan, H.J., Nicholson, J. and Perkins, W.T. (2007). Copper deficiency in potato dextrose agar causes reduced pigmentation in cultures of various fungi. *FEMS microbiology letters* **276**(2): 165-171. DOI:10.1111/j.1574-6968.2007.00923.x.

Gulati, R., Isar, J., Kumar, V., Prasad, A.K., Parmar, V.S. and Saxena, R.K. (2005). Production of a novel alkaline lipase by *Fusarium globulosum* using neem oil, and its applications. *Pure and Applied Chemistry* **77**(1): 251-262. DOI: 10.1351/pac200577010251.

Gumel, A.M., Annuar, S.M. and Heidelberg, T. (2013). Single-step lipase-catalyzed functionalization of medium-chain-length polyhydroxyalkanoates. *Journal of Chemical Technology & Biotechnology* **88**(7): 1328-1335. DOI 10.1002/jctb.3980.

Gupta, N., Rathi, P. and Gupta, R. (2002). Simplified para-nitrophenyl palmitate assay for lipases and esterases. *Analytical Biochemistry* **311**(1): 98-99. DOI: 10.1016/s0003-2697(02)00379-2.

Gupta, R., Kumari, A., Syal, P. and Singh, Y. (2015). Molecular and functional diversity of yeast and fungal lipases: their role in biotechnology and cellular physiology. *Progress in Lipid Research* **57**:40-54. DOI: 10.1016/j.plipres.2014.12.001.

Gutarra, M.L., Godoy, M.G., Maugeri, F., Rodrigues, M.I., Freire, D.M. and Castilho, L.R. (2009). Production of an acidic and thermostable lipase of the mesophilic fungus *Penicillium simplicissimum* by solid-state fermentation. *Bioresource technology* **100**(21): 5249-5254. DOI: 10.1016/j.biortech.2008.08.050.

Haris, P.I. and Severcan, F. (1999). FTIR spectroscopic characterization of protein structure in aqueous and non-aqueous media. *Journal of Molecular Catalysis B: Enzymatic* **7**(1-4): 207-221.DOI: https://doi.org/10.1016/S1381-1177(99)00030-2.

Harman, G.E. (2006). Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* **96**(2): 190-194. DOI: 10.1094/PHYTO-96-0190.

Hasan, F., Shah, A.A. and Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial technology* **39**(2): 235-251. DOI: https://doi.org/10.1016/j.enzmictec.2005.10.016.

Huang, A.H. and Moreau, R.A. (1978). Lipases in the storage tissues of peanut and other oil seeds during germination. *Planta* **141**(1): 111-116. DOI: 10.1007/BF00387752.

Hwang, H.T., Qi, F., Yuan, C., Zhao, X., Ramkrishna, D., Liu, D. and Varma, A. (2014). Lipase-catalyzed process for biodiesel production: Protein engineering and lipase production. *Biotechnology and bioengineering* **111**(4): 639-653. DOI 10.1002/bit.25162.

Islam, M.R., Tudryn, G., Bucinell, R., Schadler, L. and Picu, R.C. (2017). Morphology and mechanics of fungal mycelium. *Scientific reports* **7**(1): 1-12. DOI:10.1038/s41598-017-13295-2.

Jaeger, K.E. and Reetz, M.T. (1998). Microbial lipases form versatile tools for biotechnology. *Trends in biotechnology* **16**(9): 396-403. DOI: 10.1016/s0167-7799(98)01195-0.

Jaeger, K.E. and Eggert, T. (2002). Lipases for biotechnology. *Current Opinion in Biotechnology* **13**(4):390-397. DOI: 10.1016/s0958-1669(02)00341-5.

Joseph, B., Ramteke, P.W. and Thomas, G. (2008). Cold active microbial lipases: some hot issues and recent developments. *Biotechnology advances* **26**(5): 457-470. DOI: https://doi.org/10.1016/j.biotechadv.2008.05.003.

Jutila, A., Zhu, K., Tuominen, E.K. and Kinnunen, P.K. (2004). Fluorescence spectroscopic characterization of *Humicola lanuginosa* lipase dissolved in its substrate. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* **1702**(2): 181-189. DOI: 10.1016/j.bbapap.2004.08.011.

Karigar, C.S. and Rao, S.S. (2011). Role of microbial enzymes in the bioremediation of pollutants: a review. *Enzyme research* **2011**: 1-11. DOI:10.4061/2011/805187.

Kittilson, J.D., Reindl, K.M. and Sheridan, M.A. (2011). Rainbow trout (*Oncorhynchus mykiss*) possess two hormone-sensitive lipase-encoding mRNAs that are differentially expressed and independently regulated by nutritional state. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **158**(1): 52-60. DOI: 10.1016/j.cbpa.2010.09.010.

Lanka, S. and Latha, J.N.L. (2015). A short review on various screening methods to isolate potential lipase producers: lipases-the present and future enzymes of biotech industry. *International Journal of Biological Chemistry* **9**(4): 207-219. DOI: https://dx.doi.org/10.3923/ijbc.2015.207.219.

Lestari, P., Raharjo, T.J., Matsjeh, S. and Haryadi, W. (2016). Partial purification and biochemical characterization of extracellular lipase from *Azospirillum* sp. JG3 bacteria. In *AIP Conference Proceedings* **1755**(1): 080003. DOI: 10.1063/1.4958511.

Li, Y., Wadsö, L. and Larsson, L. (2009). Impact of temperature on growth and metabolic efficiency of Penicillium roqueforti–correlations between produced heat, ergosterol content and biomass. *Journal of applied microbiology* **106**(5): 1494-1501. DOI: 10.1111/j.1365-2672.2008.04110.x.

Linko, Y.Y. and Yan Wu, X. (1996). Biocatalytic production of useful esters by two forms of lipase from *Candida rugosa*. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental AND Clean Technology* **65**(2): 163-170.

Liu, G., Hu, S., Li, L. and Hou, Y. (2015). Purification and characterization of a lipase with high thermostability and polar organic solvent-tolerance from *Aspergillus niger* AN0512. *Lipids* **50**(11): 1155-1163. DOI 10.1007/s11745-015-4052-6.

Liu, W., Li, M. and Yan, Y. (2017). Heterologous expression and characterization of a new lipase from *Pseudomonas fluorescens* Pf0–1 and used for biodiesel production. *Scientific reports* **7**(1): 1-11. DOI:10.1038/s41598-017-16036-7.

Lonon, M.K., Woods, D.E. and Straus, D.C. (1988). Production of lipase by clinical isolates of *Pseudomonas cepacia*. *Journal of clinical microbiology* **26**(5): 979-984. DOI: 10.1128/JCM.26.5.979-984.1988.

Lowry, R.R. and Tinsley, I.J. (1976). Rapid colorimetric determination of free fatty acids. *Journal of the American Oil Chemists' Society* **53**(7): 470-472. DOI: https://doi.org/10.1007/BF02636814.

Mala, J.G. and Takeuchi, S. (2008). Understanding structural features of microbial lipases--an overview. *Analytical Chemistry Insights* **27(**3):9-19. DOI: 10.4137/aci.s551.

Mehta, A., Bodh, U. and Gupta, R. (2017). Fungal lipases: a review. *Journal of Biotech Research* **8**(1)**:** 58-77.

Minning, S., Schmidt-Dannert, C. and Schmid, R.D. (1998). Functional expression of *Rhizopus oryzae* lipase in *Pichia pastoris*: high-level production and some properties. *Journal of biotechnology* **66**(2-3): 147-156. DOI: 10.1016/s0168-1656(98)00142-4.

Monfort, A., Blasco, A., Sanz, P. and Prieto, J.A. (1999). Expression of LIP1 and LIP2 genes from *Geotrichum* species in Baker's yeast strains and their application to the bread-making process. *Journal of agricultural and food chemistry* **47**(2): 803-808.

Noble, M.E.M., Cleasby, A., Johnson, L.N., Egmond, M.R. and Frenken, L.G.J. (1993). The crystal structure of triacylglycerol lipase from *Pseudomonas glumae* reveals a partially redundant catalytic aspartate. *FEBS letters* **331**(2): 123-128. DOI: https://doi.org/10.1016/0014-5793(93)80310-Q.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J. and Sussman, J.L. (1992). The α/β hydrolase fold. *Protein Engineering, Design and Selection* **5**(3): 197-211. DOI: 10.1093/protein/5.3.197.

Parry Jr, R.M., Chandan, R.C. and Shahani, K.M. (1966). Rapid and sensitive assay for milk lipase. *Journal of dairy science* **49**(4): 356-360. DOI: https://doi.org/10.3168/jds.S0022-0302(66)87874-8.

Peers, F.G. (1953). Oat lipase and tributyrin. *Nature* **171**(4361): 981-982.

Pencreac'h, G. and Baratti, J.C. (1996). Hydrolysis of p-nitrophenyl palmitate in n-heptane by the *Pseudomonas cepacia* lipase: a simple test for the determination of lipase activity in organic media. *Enzyme and Microbial Technology* **18**(6): 417-422.

Peruski, A.H. and Peruski, L.F. (2003). Immunological methods for detection and identification of infectious disease and biological warfare agents. *Clinical and diagnostic laboratory immunology* **10**(4): 506-513. DOI: 10.1128/CDLI.10.4.506–513.2003.

Petersen, M.T.N., Fojan, P. and Petersen, S.B. (2001). How do lipases and esterases work: the electrostatic contribution. *Journal of biotechnology* **85**(2): 115-147. DOI: 10.1016/s0168-1656(00)00360-6.

Piscitelli, A., Tarallo, V., Guarino, L., Sannia, G., Birolo, L. and Pezzella, C. (2017). New lipases by mining of *Pleurotus ostreatus* genome. *PloS one* **12**(9): e0185377. DOI: https://doi.org/10.1371/journal.

Pleiss, J., Fischer, M., Peiker, M., Thiele, C. and Schmid, R.D. (2000). Lipase engineering database: understanding and exploiting sequence–structure–function relationships. *Journal of Molecular Catalysis B: Enzymatic* **10**(5): 491-508. DOI: 10.1093/nar/gkg015.

Prive, F., Kaderbhai, N.N., Girdwood, S., Worgan, H.J., Pinloche, E., Scollan, N.D., Huws, S.A. and Newbold, C.J. (2013). Identification and characterization of three novel lipases belonging to families II and V from *Anaerovibrio lipolyticus* 5ST. *PLoS One* **8**(8): e69076. DOI: 10.1371/journal.pone.0069076.

Raja, H.A., Miller, A.N., Pearce, C.J. and Oberlies, N.H. (2017). Fungal identification using molecular tools: a primer for the natural products research community. *Journal of natural products* **80**(3): 756-770. DOI: 10.1021/acs.jnatprod.6b01085.

Rajendran, A., Palanisamy, A. and Thangavelu, V. (2009). Lipase catalyzed ester synthesis for food processing industries. *Brazilian archives of biology and technology* **52**(1): 207-219. DOI: https://doi.org/10.1590/S1516-89132009000100026.

Rantasalo, A., Vitikainen, M., Paasikallio, T., Jäntti, J., Landowski, C.P. and Mojzita, D. (2019). Novel genetic tools that enable highly pure protein production in *Trichoderma reesei*. *Scientific reports* **9**(1): 1-12. DOI: https://doi.org/10.1038/s41598-019-41573-8.

Raveendran, S., Parameswaran, B., Beevi Ummalyma, S., Abraham, A., Kuruvilla Mathew, A., Madhavan, A., Rebello, S. and Pandey, A. (2018). Applications of microbial enzymes in food industry. *Food technology and biotechnology* **56**(1): 16-30. DOI: 10.17113/ftb.56.01.18.5491.

Reetz, M.T. (2002). Lipases as practical biocatalysts. *Current opinion in chemical biology* **6**(2): 145-150. DOI: 10.1016/s1367-5931(02)00297-1.

Sadati, R., Barghi, A. and Larki, R.A. (2015). Isolation and screening of lipolytic fungi from coastal waters of the Southern Caspian Sea (North of Iran). *Jundishapur journal of microbiology* **8**(4): 1-7. DOI: 10.5812/jjm.8(4)2015.16426.

Sae-Leaw, T. and Benjakul, S. (2018). Lipase from liver of seabass (*Lates calcarifer*): Characteristics and the use for defatting of fish skin. *Food chemistry* **240:** 9-15. DOI: 10.1016/j.foodchem.2017.07.089.

Sakai, Y., Hayatsu, M. and Hayano, K. (2002). Use of Tween 20 as a substrate for assay of lipase activity in soils. *Soil Science and Plant Nutrition* **48**(5): 729-734. DOI: 10.1080/00380768.2002.10409263.

Samad, M.Y.A., Razak, C.N.A., Salleh, A.B., Yunus, W.Z.W., Ampon, K. and Basri, M. (1989). A plate assay for primary screening of lipase activity. *Journal of microbiological methods* **9**(1): 51-56.

Samuels, G.J. (2006). *Trichoderma*: systematics, the sexual state, and ecology. *Phytopathology* **96**(2): 195-206. DOI: 10.1094/PHYTO-96-0195.

Sarmah, N., Revathi, D., Sheelu, G., Yamuna Rani, K., Sridhar, S., Mehtab, V. and Sumana, C. (2017). Recent advances on sources and industrial applications of lipases. *Biotechnology progress* **34**(1): 5-28. DOI: 10.1002/btpr.2581.

Savitha, M.J. and Sriram, S. (2015). Morphological and molecular identification of *Trichoderma* isolates with biocontrol potential against Phytophthora blight in red pepper. *Pest Management in Horticultural Ecosystems* **21**(2): 194-202.

Saxena, R.K., Gupta, R., Saxena, S. and Gulati, R. (2001). Role of fungal enzymes in food processing. In *Applied Mycology and Biotechnology* **1**: 353-386. DOI: https://doi.org/10.1016/S1874-5334(01)80015-0.

Schmid, A., Dordick, J.S., Hauer, B., Kiener, A., Wubbolts, M. and Witholt, B. (2001). Industrial biocatalysis today and tomorrow. N*ature* **409**(6817): 258-268. DOI: 10.1038/35051736.

Schmid, R.D. and Verger, R. (1998). Lipases: Interfacial enzymes with attractive applications. *Angewandte Chemie International Edition* **37**(12): 1608-1633. DOI: 10.1002/(SICI)1521-3773(19980703)37:12<1608: AID-ANIE1608>3.0.CO;2-V.

Schuster, A. and Schmoll M. (2010). Biology and biotechnology of *Trichoderma*. *Applied microbiology and biotechnology* **87**(3):787-799. DOI: 10.1007/s00253-010-2632-1.

Shahid, M., Singh, A., Srivastava, M., Mishra, R.P. and Biswas, S.K. (2011). Effect of temperature, pH and media for growth and sporulation of *Trichoderma longibrachiatum* and self -life study in carrier-based formulations. *Annals of Plant Protection Science* **19**(1): 147-147. DOI: https://www.researchgate.net/publication/270339292.

Sharma, R., Chisti, Y. and Banerjee, U.C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnology advances* **19**(8): 627-662. DOI: 10.1016/s0734-9750(01)00086-6.

Silano, V., Barat Baviera, J.M., Bolognesi, C., Cocconcelli, P.S., Crebelli, R., Gott, D.M., Grob, K., Lampi, E., Mortensen, A. and Rivière, G., Steffensen, I., Tlustos, C., Loveren, H.V., Vernis, L., Zorn, H., Glandorf, B., Herman, L., Mala, J., Liu, Y., Rainieri, S. and Chesson, A. (2019). Safety evaluation of the food enzyme triacylglycerol lipase from *Trichoderma reesei* (strain RF10625). *EFSA Journal* **17**(10): p.e05837. DOI: 10.2903/j.efsa.2019.5837.

Singh, A.K. and Mukhopadhyay, M. (2012). Overview of fungal lipase: a review. *Applied biochemistry and biotechnology* **166**(2): 486-520. DOI 10.1007/s12010-011-9444-3.

Singh, R., Gupta, N., Goswami, V.K. and Gupta, R. (2006). A simple activity staining protocol for lipases and esterases. *Applied microbiology and biotechnology* **70**(6): 679-682. DOI: https://www.researchgate.net/publication/7592331.

Suplatov, D.A., Besenmatter, W., Švedas, V.K. and Svendsen, A. (2012). Bioinformatic analysis of alpha/beta-hydrolase fold enzymes reveals subfamily-specific positions responsible for discrimination of amidase and lipase activities. *Protein Engineering, Design & Selection* **25**(11): 689-697. DOI:10.1093/protein/gzs068.

Swinehart, D.F. (1962). The beer-lambert law. *Journal of chemical education* **39**(7): 333. DOI: https://doi.org/10.1021/ed039p333.

Takasu, S., Mutoh, M., Takahashi, M. and Nakagama, H. (2012). Lipoprotein lipase as a candidate target for cancer prevention/therapy. *Biochemistry research international* **2012**: 1-9. DOI:10.1155/2012/398697.

Tankeshwar, A. (2015). Lactophenol Cotton Blue (LPCB) mounts: Principle and staining protocol. Microbe Online. Available on-line at: https://microbeonline.com/lactophenol-cotton-blue-lpcb-mounts-principle-staining-protocol/.

Tigerstrom, R.G.V. and Stelmaschuk, S. (1989). The use of Tween 20 in a sensitive turbidimetric assay of lipolytic enzymes. *Canadian journal of microbiology* **35**(4): 511-514. DOI: 10.1139/m89-079.

Tomioka, H.A.R.U.A.K.I. (1983). Purification and characterization of the tween-hydrolyzing esterase of Mycobacterium smegmatis. *Journal of bacteriology* **155**(3): 1249-1259.

Toscano, L., Montero, G., Cervantes, L., Stoytcheva, M., Gochev, V. and Beltrán, M. (2013). Production and partial characterization of extracellular lipase from *Trichoderma harzianum* by solid-state fermentation. *Biotechnology & Biotechnological Equipment* **27**(3): 3776-3781. DOI: http://dx.doi.org/10.5504/BBEQ.2012.0140.

Ülker, S., Özel, A., Çolak, A. and Karaoğlu, Ş.A. (2011). Isolation, production, and characterization of an extracellular lipase from *Trichoderma harzianum* isolated from soil. *Turkish Journal of Biology* **35**(5): 543-550. DOI:10.3906/biy-1004-107.

Vaysse, L., Dubreucq, E., Pirat, J.L. and Galzy, P. (1997). Fatty hydroxamic acid biosynthesis in aqueous medium in the presence of the lipase-acyltransferase from *Candida parapsilosis*. *Journal of biotechnology* **53**(1): 41-46. DOI: 10.1016/s0168-1656(96)01660-4.

Verger, R. (1997). 'Interfacial activation'of lipases: facts and artifacts. *Trends in Biotechnology* **15**(1): 32-38. DOI: https://doi.org/10.1016/S0167-7799(96)10064-0.

Wang, Y., Ma, R., Li, S., Gong, M., Yao, B., Bai, Y. and Gu, J. (2018). An alkaline and surfactant-tolerant lipase from *Trichoderma lentiforme* ACCC30425 with high application potential in the detergent industry. *AMB Express* **8**(1): 1-11. DOI: https://doi.org/10.1186/s13568-018-0618-z.

Watanabe, T. (2010). Pictorial atlas of soil and seed fungi: morphologies of cultured fungi and key to species. CRC press. DOI: https://www.routledgehandbooks.com/doi/10.1201/EBK1439804193-c3.

Widmann, M., Juhl, P.B. and Pleiss, J. (2010). Structural classification by the Lipase Engineering Database: a case study of *Candida antarctica* lipase A. *BMC genomics* **11**(1): 1-8. DOI: https://doi.org/10.1186/1471-2164-11-123.

Wijedasa, M.H. and Liyanapathirana, L.V.C. (2012). Evaluation of an alternative slide culture technique for the morphological identification of fungal species. *Sri Lankan Journal of Infectious Diseases* **2**(2): 47-52. DOI: http://dx.doi.org/10.4038/sljid.v2i2.4070.

Wilton, D.C. (1991). A continuous fluorescence-displacement assay for triacylglycerol lipase and phospholipase C that also allows the measurement of acylglycerols. *Biochemical Journal* **276**(1): 129-133. DOI: 10.1042/bj2760129.

Winkler, F.K., d'Arcy, A. and Hunziker, W. (1990). Structure of human pancreatic lipase. *Nature* **343**(6260): 771-774. DOI: 10.1038/343771a0.

Winkler, U.K. and Stuckmann, M. (1979). Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *Journal of bacteriology* **138**(3): 663-670. DOI: 10.1128/jb.138.3.663-670.1979.

Xie, W., Khosasih, V., Suwanto, A. and Kim, H.K. (2012). Characterization of lipases from *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from human facial sebaceous skin. *Journal of microbiology and biotechnology* **22**(1): 84-91. DOI: 10.4014/jmb.1107.07060.

Xu, X., Fomuso, L.B. and Akoh, C.C. (2000). Synthesis of structured triacylglycerols by lipase-catalyzed acidolysis in a packed bed bioreactor. *Journal of agricultural and food chemistry* **48**(1): 3-10. DOI: 10.1021/jf990836p.

Yan, J., Han, B., Gui, X., Wang, G., Xu, L., Yan, Y., Madzak, C., Pan, D., Wang, Y., Zha, G. and Jiao, L. (2018). Engineering *Yarrowia lipolytica* to simultaneously produce lipase and single cell protein from agro-industrial wastes for feed. *Scientific reports* **8**(1): 1-10. DOI: 10.1038/s41598-018-19238-9.

Zaks, A. and Klibanov, A.M. (1984). Enzymatic catalysis in organic media at 100 degrees C. *Science* **224**(4654): 1249-1251. DOI: 10.1126/science.6729453.

Zhang, X., Li, X. and Xia, L. (2015). Heterologous expression of an alkali and thermotolerant lipase from *Talaromyces thermophilus* in *Trichoderma reesei*. *Applied biochemistry and biotechnology* **176**(6): 1722-1735. DOI 10.1007/s12010-015-1673-4.