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# Mémoire de fin d'études

En vue de l'obtention du diplôme de Master

**Domaine :** Sciences de la matière

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**Spécialité:** Chimie Analytique

## Thème

# ACTIVITIES EVALUATION OF A LIPASE EXTRACTED FROM ACACIA SEEDS

**Présenté par:**
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Soutenu le : 14/06/2026

Devant le Jury :

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# Dedications

*This modest work is dedicated especially to my dear mother* ***BAHRI FATMA EL ZAHRA****, my reason for living as a testimony of my gratitude for her patience, love, and sacrifices.*

*To my dear father* ***BELLEIL RACHID*** *for his love and dedication. To you, my parents, I say thank you for making me the person I am today. No dedication can express my respect, my consideration, and my great admiration for you, since this work shows you my affection and deep love.*

*To my dear sisters* ***FATMA, KHAOULA****,* ***NESSRINE****,* ***NOUR*** *and to my only little brother* ***MOHAMMED ANIS***

*To my grandfather* ***TAHER*** *and his wife* ***SALIHA,***

*And all the Belleili and Bahri family .And who I know, my success is important to them. God bless you for me.*

*And for those who once gave me help, may God pay you for all your benefits*

*Finally, to all those I love and who love me, I dedicate this work.*

# Résumé

Cette étude examine l'extraction d'une lipase végétale non purifiée et à faible coût à partir de graines dormantes d'acacia (ASL). L'extrait brut a montré une activité hydrolytique spécifique de 0,0123 UI/mg (testée par hydrolyse de l'huile d'olive dans l'hexane). ASL a ensuite été utilisé comme biocatalyseur pour synthétiser des esters de sucre à partir de glucose et d'acide stéarique dans l'hexane à 60 °C. Des essais d'optimisation avec divers rapports molaires sucre: acide (1:2,5, 2,5:1, 1:1) ont montré qu'un rapport de 2,5:1 (excès de sucre) produisait une conversion maximale de 24,94 % après 24 heures. L'analyse par CCM suggère la formation d'un mono-ester.

**Mots-clés :** lipase, extrait de plante, activité hydrolytique, acide stéarique, graines d'acacia, ester de sucre.

# Abstract

This study investigates the extraction of a low-cost, unpurified plant lipase from dormant acacia seeds (ASL). The crude extract exhibited a specific hydrolytic activity of 0.0123 IU/mg (tested via olive oil hydrolysis in hexane). ASL was subsequently used as a biocatalyst to synthesize sugar esters from glucose and stearic acid in hexane at 60°C. Several trials using various molar ratios sugar:acid (1:2.5, 2.5:1, 1:1) showed that a 2.5:1 ratio (excess sugar) yielded a maximal conversion of 24.94% after 24 hours. TLC analysis suggests the formation of a monoester.

**Key Words:** lipase, plant extract, hydrolytic activity, stearic acid, acacia seeds, sugar ester

# ملخص

تبحث هذه الدراسة في استخلاص الليباز النباتي منخفض التكلفة وغير المنقى من بذور الأكاسيا .(ASL) أظهر المستخلص الخام نشاطًا مائيًا محددًا قدره 0.0123 وحدة دولية/مجم (تم اختباره عن طريق التحلل المائي لزيت الزيتون في الهكسان). تم استخدام ASLلاحقًا كمحفز حيوي لتصنيع استرات السكر من الجلوكوز وحمض دهني في الهكسان عند 60 .C°أظهرت تجارب التحسين عبر نسب مولية مختلفة (1:2.5، 1:1، 2.5:1) أن نسبة 2.5:1 (السكر الزائد) أسفرت عن تحويل ذروة بنسبة 24.94٪ بعد 24 ساعة، مع تكوين الإستر الناجح من خلال تحليل TLC.

**الكلمات المفتاحية:** الليباز، مستخلص نباتي، نشاط التحلل المائي، حمض دهني، بذور الأكاسيا، إستر السكر

# List of Abbreviations and Symbols

| | |
|---|---|
| **ASL**: | Acacia Seed Lipase |
| **ATGL:** | Adipose triglyceride lipase |
| **CALB:** | Candida antarctica Lipase B |
| **C%**: | Conversion |
| **DAG**: | Diacylglycerols |
| **DNA**: | Deoxyribonucleic Acid |
| **EA**: | Enzyme Activity |
| **EC**: | Enzyme Commission |
| **FA**: | Fatty Acid |
| **FFA**: | Free Fatty Acid |
| **g**: | Gram |
| **h**: | Hour |
| **IU/mg:** | International Unit per Milligram |
| **M**: | Molar |
| **MAG**: | Monoacylglycerols |
| **min**: | Minutes |
| **ml**: | Milliliter |
| **MM**: | Molar Mass |
| **PSVs:** | Protein Storage Vacuoles In seed |
| **Ref**: | Reference |
| **SA**: | Specific Activity |
| **T**: | Temperature |
| **t :** | Time |
| **TAG**: | Triacylglycerols |
| **TG**: | Triglyceride |
| **UIB**: | International Union of Biochemistry |
| **UV :** | ultra violet |
| **µmol**: | Micromole |
| **V**: | Volume |
| **%**: | Percentage |
| **°C**: | Degrees Celsius |

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# General Introduction

Biotechnology plays a key role in making consumer goods and industrial services more sustainable worldwide. One of the most commonly used biocatalysts is lipase, known for its use in foods, drugs, cosmetics, and detergents because of its special interfacial activation and easy biodegradability.[1] These enzymes do a great job at breaking down fats in water-rich settings, leading to the release of free fatty acids and glycerol. On the other hand, in low-water or non-aqueous media, they can carry out syntheses like esterifications and transesterifications. However, the big issue holding back their broader use is that making commercial lipases is super expensive, mostly due to costly processes for getting them from microbes and animals.[2]

Plant-derived lipases, found in oilseed reserve tissues, provide a really promising, cost-effective, and easy-to-purify alternative to pricey commercial versions. During a plant's lifecycle, these internal enzymes play a key role, kicking into action during germination to break down stored fat reserves. This process generates vital carbon and energy sources needed for the new sprout to grow. But these enzymes aren't just important inside plants. They can also handle non-aqueous conditions, making them useful in green chemistry for creating complex molecules[2]. Specifically, their ability to work in dry settings helps make special esters. Hence, plant lipases could revolutionize industrial biocatalysis, cutting out the need for costly microbial fermentation processes.[3]

The primary aim of this work is to extract a lipase from dormant Acacia seeds (ASL). This study evaluates its dual functionality: first, by assessing its classic hydrolytic activity using olive oil as substrate, and second, by investigating its synthetic potential to catalyse the synthesis of sugar esters. The manuscript is organized into the following distinct sections:

- **Chapter 1** which deals with a review of previous research on lipases, their structure, and their enzymatic activity.
- **Chapter 2** which focuses specifically on seed lipases, detailing their structural characteristics and the key factors influencing their catalytic performance.
- **Chapter 3** which presents the results and discussions concerning the evaluation of the enzymatic activity of the Acacia extract.
- **Experimental Part** that outlines the technical details and methods used**.**
- **Conclusion and perspectives**

# Chapter 1: An overview on lipases

## I. Introduction

Enzymes have been playing an essential role in our daily lives for centuries and are essential for the survival of all living organisms. As various sources explain, enzymes are characterized by their ability to accelerate biochemical reactions within cells, helping to synthesize important compounds, break down by-products, and add functional groups. This makes them indispensable in areas such as food production, cleaning, textile, and pharmaceutical industries.[4]

This chapter deals with a general overview of enzymes; it then looks at lipases in general, including their structure, mechanism of action, the reactions they catalyze and their classification and origin. The chapter also discusses the parameters affecting lipase activity and the different methods used to determine this activity.[5]

## II. Enzymes

### II.1. Definition

Enzymes can be defined as protein-based bimolecular acting as biocatalysts involved in specific metabolic reactions in living or non-living organisms. In favorable environments and conditions after having extracted them from this organisms.Without enzymatic catalysis no form of life could have existed. Moreover, without the introduction of enzymatic processes in industrial catalysis, the innovation of several products necessary for our daily life would never have seen the light of day[6]. Enzymes are the largest and most specialized class of proteins, which specifically recognizing certain molecules, accelerate the transformation reactions of these molecules sufficiently for their speed to become compatible with the functioning of the organism.

### II.2. Structure of Enzymes

Enzymes are complex macromolecular structures that exhibit a large amount of organization. The level of organization of enzymes is determined by four levels of protein folding namely primary, secondary, tertiary and quaternary structure.

## II.2.1. Primary Structure

The primary structure of an enzyme is the unique linear arrangement of amino acids in a polypeptide chain that are held together using covalent (peptide) bonds (**Figure 1**). The amino acid sequence comprises the first level of determination of the ultimate three-dimensional shape of the enzyme.[7]

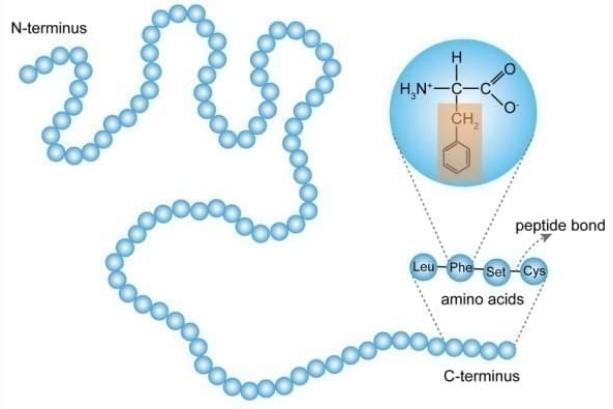



Figure 1. Primary structure of an enzyme.[7a]

## II.2.2. Secondary Structure

The secondary structure is the periodic folding of the polypeptide backbone into either a α-helix or a β-pleated sheet. Each of these secondary structure types is stabilized by the formation of hydrogen bonds between the carbonyl oxygen and the amide hydrogen atoms of the polypeptide backbone (**Figure 2**).[8]

## II.2.3. Tertiary Structure

The tertiary structure of an enzyme is the complete three-dimensional folding of a single polypeptide chain, and the tertiary structure of the enzyme is often stabilized by thinteractions between the side chains of the amino acids (**Figure 2**).[9]

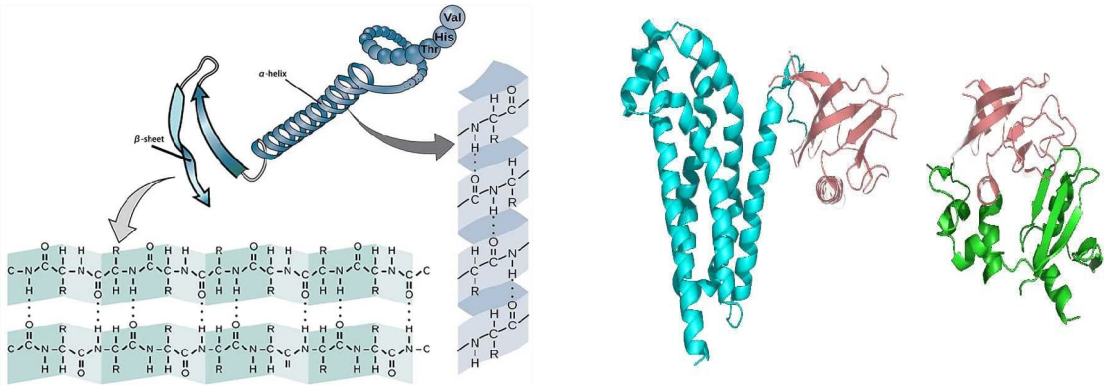



Figure 2. Secondary and tertiary structures of enzymes.[7a]

## II.2.4. Quaternary Structure

The quaternary structure of enzymes refers to how it is arranged spatially together with multiple (folded) polypeptide chains (or subunits) that combine together to create a larger functional protein complex. The individual chains (subunits) are held together by non-covalent forces, including van der Waals forces and hydrogen bonding (Figure 3).[10]

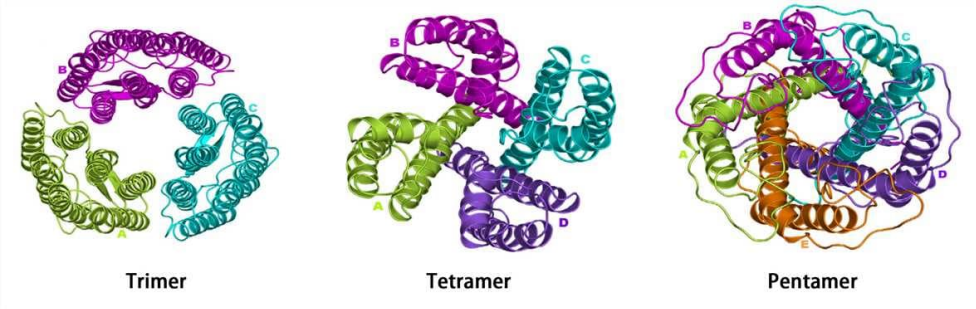



Figure 3. Quaternary structure of enzymes. [7a]

## II.3. Classification of Enzymes

Enzymes are systematically classified by the International Union of Biochemistry and Molecular Biology (IUBMB) based on the specific chemical reactions they catalyze. This system assigns each enzyme an **EC** (Enzyme Commission) number, consisting of four digits that progressively narrow down the enzyme's function [6a]

Seven enzyme classes have been recognized since the Enzyme classification and nomenclature list was reviewed by IUBMB in 2018 (Figure 4)

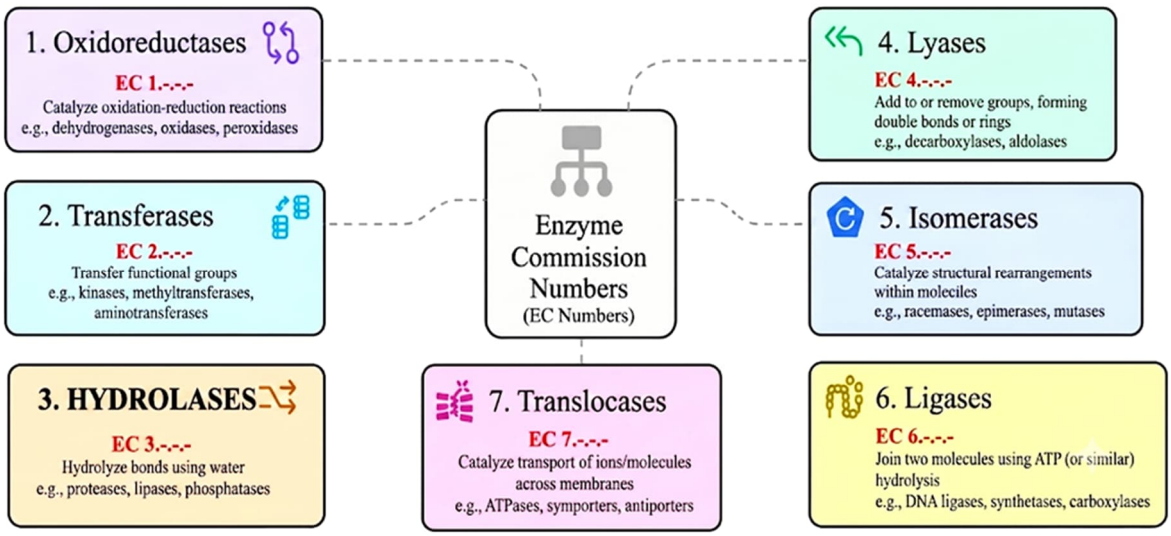



Figure 4. Seven classes of enzymes. [11]

# III. Lipases

## III.1. Definition

Lipases or triacylglycerol acylhydrolase (EC 3.1.1.3) as the official name (International Commission of Enzymes: "International Union of Biochemistry, IUB") belong to the class of hydrolases (EC3). They act specifically on the ester bonds (EC3.1) present in carboxylic esters (EC3.1.1) .The physiological role of lipase is important, they hydrolyze triacylglycerols into fatty acids, diacylglycerols, monoacylglycerols and glycerol.[12]

## III.2. Structure and mode of action

### III.2.1. Structure

Despite their low primary sequence identity, lipases exhibit a very similar fold. Other enzymes, such as esterases, proteases, dehalogenases, epoxide hydrolases and peroxidases, have similar structural characteristics and belong the α/β hydrolase family.[13] Figure 5

All lipases possess a lid domain that contains a flexible surface loop that controls access to the active site; this lid undergoes interfacial activation when the lipase adsorbs to the lipid/water interface.[14]

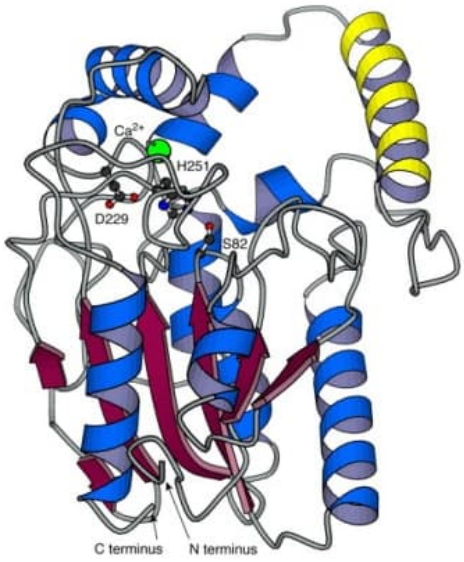
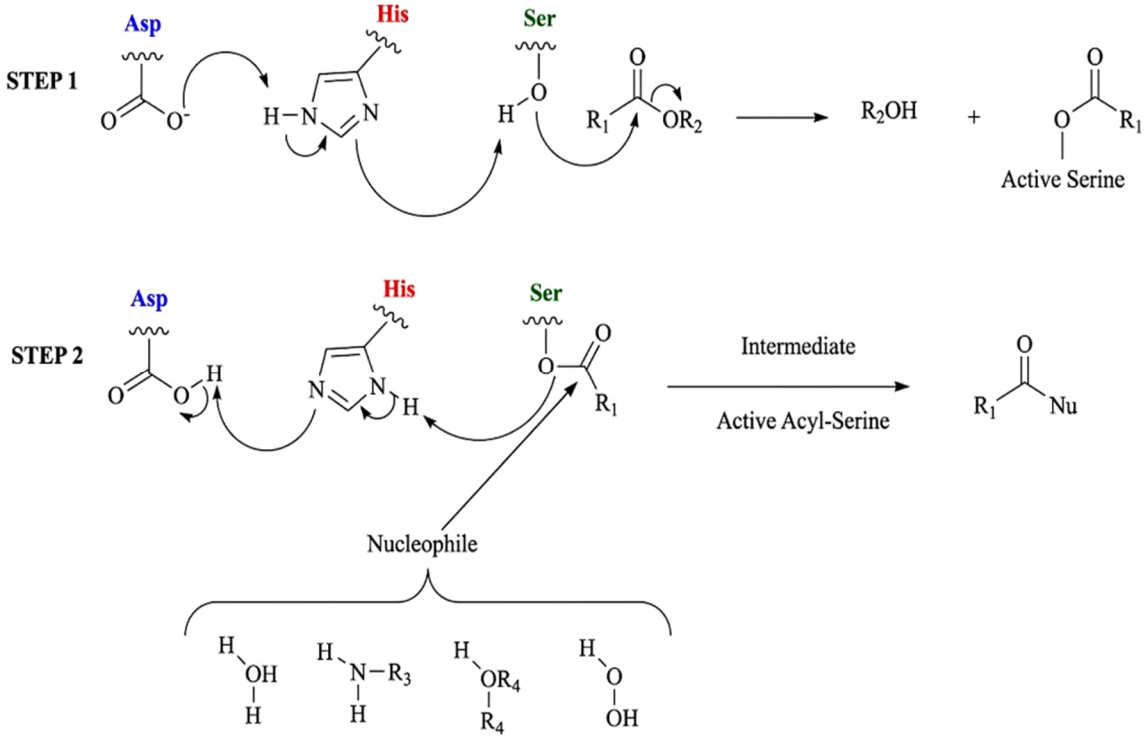



Figure 5. Lipase structure.[15]

### III.2.2. Mode of action

The active site of α/β hydrolases consists of a highly conserved **catalytic triad**: a nucleophilic residue.(serine, cysteine or aspartic acid), a catalytic acid residue (aspartic or glutamic acid), and a histidine residue (Scheme 1). In lipases, the nucleophile has always been characterized as a serine residue.[13]




Scheme 1. Action mode of lipases (catalytic triad)

## III.3. Reactions Catalyzed by Lipases

### III.3.1. Hydrolysis

Lipases naturally catalyze the hydrolysis of the ester bond of tri-, di-, and monoglycerides into fatty acids and glycerol (**Figure 6**). However, they are also active on a wide range of substrates. In all cases, the reaction takes place at the interface of a biphasic system. of an immiscible organic phase, containing the hydrophobic substrate, in water.[16]

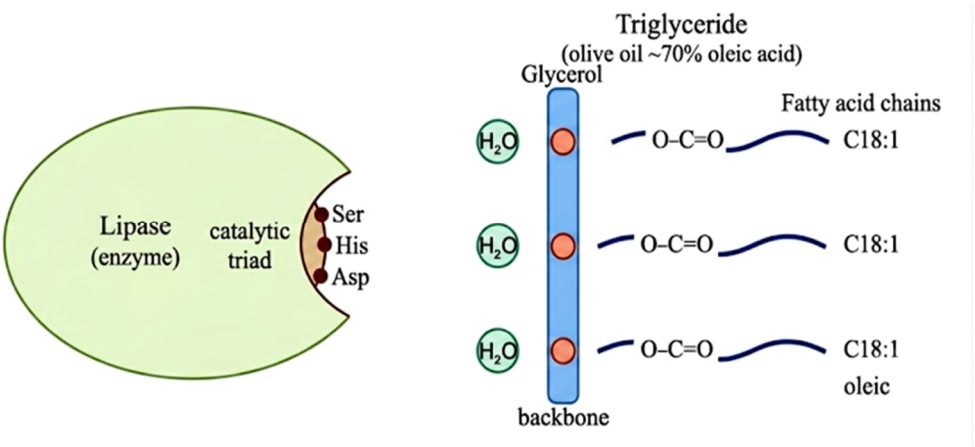


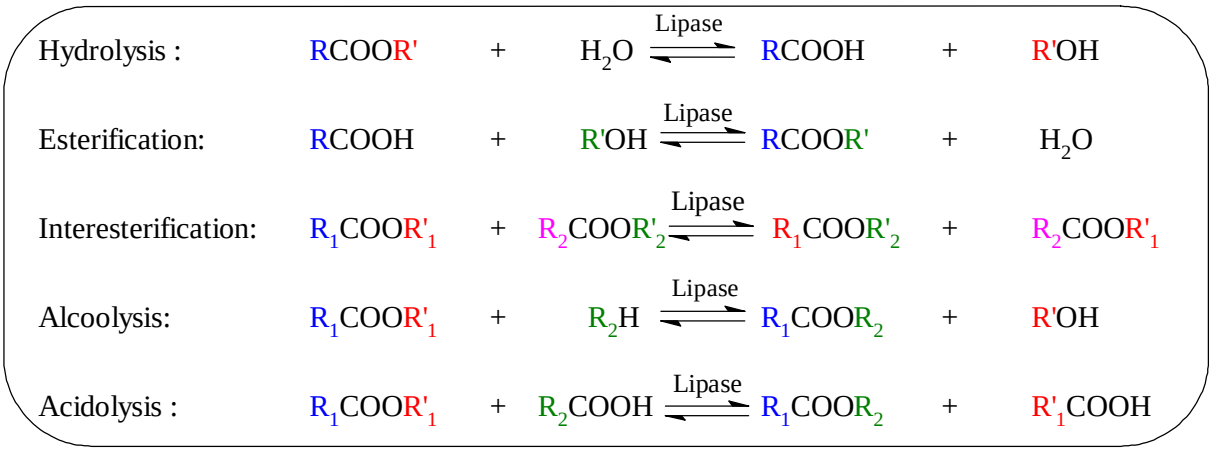

Figure 6. Hydrolysis reaction of triglyceride.[17]

### III.3.2.Synthesis reactions

Lipases under favorable thermodynamic conditions (low water activity), also catalyze a wide variety of synthesis reactions that can be classified into two main types of reactions namely esterification and transesterification.

As shown in (Scheme 2), esterification is the reaction in which one fatty acid is bonded to another fatty acid. Under the action of the enzyme, to an alcohol by an enzyme, to an alcohol by a covalent bond, producing an ester and releasing a water molecule.

Transesterification includes the reactions of alcoholysis, acidolysis, aminolysis and interesterification. In general, these synthesis reactions occur in a medium with low thermodynamic water activity. The thermodynamic activity of water is a measure of the availability of the molecule in a solvent. The medium then consists of a free solvent system (molten medium) or an organic solvent. Finally, lipases are also capable of expressing other related activities such as phospholipase, lysophospholipase, cholesterol esterase, cutinase or amidase.[16]




Scheme 2. Reactions Catalyzed by Lipases.

## III.3.3. Origin and classification of lipases

They have been classified according to two key factors: (1) the biological source of the enzyme and (2) the specific substrate or position on which they act. Together these criteria provide a solid basis for examining the biochemical properties of these substances and for establishing their evolutionary position and potential industrial uses.[18]

### III.3.3.1. Classification According to Biological Source

- ***Microbial Lipases***

Produced through fermentation on a very large scale, microbial lipases are now the most studied and commercially used as a group. They are highly diverse in their substrate specificity and demonstrate stable performance in most industrial environments. Salmon et al. (2006) stated that bacteria, fungi, and yeasts have all contributed to the production of lipases and that the individual biochemical properties of lipases are influenced by the environments of the organisms that produce them. *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Bacillus subtilis* are known for being large producers of an extracellular lipase (lipase) that has a wide range of temperature and pH stability.[19]

Fungi such as *Candida antarctica* (now known as *Moesziomyces antarcticus*) and *Rhizomucor miehei* also have widespread use as biocatalysts due to their enantioselectivity and thermal stability.[20]

- ***Animal lipases***

Lipases in vertebrates are important for the digestion of lipids, the metabolism of lipoproteins, and the regulation of energy within cells. The four major lipases are pancreatic lipase (PL), hepatic lipase (HL), lipoprotein lipase (LPL), and hormone-sensitive lipase (HSL). These types of lipases are unique to different tissues and are regulated differently within those

tissues. For instance, PL is the principal enzyme involved in the hydrolysis of TAGs in the intestine and requires colipase as a cofactor. In order for PL to work, it requires interfacial activation through the C-terminus and cleaves the fatty acids at the 1st and 3rd position of the glycerol backbone (1,3-position).[21]

HSL is activated during times of fasting to mobilise free fatty acids from adipose tissue by means of the phosphorylation of HSL by PKA. HSL is able to hydrolyse TAGs, DAGs, MAGs, and cholesterol esters. Adipose triglyceride lipase (ATGL) is acknowledged as the main lipase within adipocytes and initiates the lipolytic cascade before HSL acts on the cleaved fatty acids from TAGs.[22]

- ***Plant lipases***

Key developmental stages that express Plant lipases are: seed maturation, germination and senescence. Plant lipases play an essential role for lipid turnover; by remodeling membranes and for signalling. The main TAG (Triacylglycerol) lipase which mobilises oil body reserves during post germinative growth in Arabidopsis thaliana is the sugar-dependent 1 (SDP1) lipase also belonging to patatin-like family and uses a Ser-as-Diad for catalysis not the typical Triad. Also, the chloroplast lipase DAD1 (Defective in Anther Dehiscence 1) is involved at the start of jasmonic acid production by releasing α-linolenic acid from phospholipids in membranes; this shows some of the more diverse functions of plant lipases (e.g., important for immunity and stress signalling).[23]

### III.3.3.2. Classification According to Substrate Specificity

The main factor in categorising lipases for biotechnological and industrial purposes is the specificity of their action on the glycerol backbone, as well as their specificity towards the acyl chain of the fatty acid they catalytically hydrolyse. Based on these aspects of lipase specificity, there are three distinct areas of interest: regiospecific, fatty acids specific, and stereospecific (enantioselective).[24]

- ***Regiospecificity (Positional Specificity)***

Determining which position of the glycerol backbone is cleaved. 1,3-specific lipases (for example, pancreatic lipase and *Rhizomucor miehei*) only cleave at the sn-1 and sn-3 positions, while non-specific lipases (for example, *Candida rugosa*) cleave at all three positions. This distinction results from the geometry of the active site.[19]

- ***Fatty Acid Specificity***

Various lipases will act preferentially towards acyl chains that are of specific length or degree of unsaturation. For example, *Penicillium camemberti* will preferentially hydrolyse

short/medium chain length acyl chains (i.e., C4–C12). Similarly, *Geotrichum candidum* has a preference for hydrolysing the commonly occurring cis-Δ9 unsaturated fatty acids; such as, oleic and linoleic acids. These preferences occur due to the size/shape of the acyl-binding tunnel.[22]

- ***Stereospecificity (Enantioselectivity)***

Lipases are able to differentiate between the prochiral faces of glycerol (i.e. sn-1 versus sn-3) and also between the chiral substrate enantiomers. One of the best known lipases, *Candida antarctica* Lipase B (CALB), can resolve racemic alcohols with an enantiomeric excess of >99%. This characteristic of CALB is commonly utilized in pharmaceuticals.[22, 25]

# IV. Enzymatic Activity: Units, Specific Activity, and Calculation

## IV.1. Enzymatic Activity and Its Units

Enzyme activity measures how effectively an enzyme can catalyze the substrate to produce product(s) (or simply, measure of an enzyme ability) under defined conditions. Infact, the rate varies depending on a number of factors (e.g. temperature, pH, ionic strength of all components), etc. To ensure results are reproducible between laboratories, each of these parameters must be standardized, documented, and reported.[26]

Enzymatic activity can generally be described using the International Unit (IU or U), which represents a certain amount of substrate converted from one state to another in a given time frame. For instance, if 1 micromole (µmol) of substrate is converted within one minute by an enzyme, this is known as 1 U or 1 $\mu mol.min^{-1}$.[27] This unit continues to be used extensively by biochemists and industry alike for both physical and biological enzymes. The SI, however, has introduced a new standard of measurement called a katal (kat), where 1 kat is equal to one mole of substrate being converted to product by an enzyme in one second or 1 kat = 1 $mol.s^{-1}$; thus, IU=16.67 nkat or vice versa 1 kat = $6\times10^7$ U .Hence, many researchers tend to measure catalytic activity using the sub-multiples of katal such as millikatal (mkat), microkatal (µkat), and nanokatal (nkat) since, in most cases, katal are much larger than the amount typically prepared for laboratory work.[7b]

## IV.2. Specific Activity

One of the vital factors in characterizing and evaluating the quality of an enzyme preparation is its specific activity. This is defined by the number of enzyme units (U) per milligram of total protein in the sample, expressed as ($U.mg^{-1}$) .Total protein includes both the target enzyme and all contaminating proteins, so as you purify the target enzyme, the specific

activity increases progressively as you eliminate non-catalytic proteins from the preparation. Therefore, the higher the specific activity, the more purified the preparation is, and proportionally, the more the target enzyme comprises of the total protein in the preparation.

When the enzyme preparation is 100% pure, the specific activity has reached its maximum value, which is a property of that enzyme for the assay conditions used for the determination of that value. If the molecular weight of the enzyme is known, the specific activity can be converted into molar activity (or turnover number, kcat), indicating how many molecules of substrate are converted to product by one molecule of enzyme per time under conditions of saturating substrate.[7b]

## IV.3. Calculation of Enzymatic Activity

Enzymatic activity is determined at initial velocity conditions, meaning that the measurement is carried out when the amount of substrate consumed remains negligible compared to the total substrate present (typically below 5–20%). This ensures that the reaction rate reflects the maximal catalytic capacity of the enzyme and is not limited by substrate depletion or product inhibition.[7b]

- ***Spectrophotometric Method***

Monitors the change in absorbance (ΔA/min) at a characteristic wavelength using a UV/Vis spectrophotometer.Converted to activity via Beer–Lambert's law ($A = \varepsilon \times l \times c$). Best suited for reactions involving UV-active or chromogenic substrates and cofactors such as NADH (340 nm) or p-nitrophenol (405 nm).[28]

- ***Fluorometric Method***

It measures fluorescence emission produced or consumed during the reaction. Offers higher sensitivityth an spectrophotometry. Ideal for fluorogenic substrates or very low enzyme concentrations.[29]

- ***Calorimetric Method***

It measures heat released or absorbed (ΔH) during the reaction via microcalorimetry. Theoretically applicable to any enzyme as all biochemical reactions involve enthalpy changes. Label-free and non-destructive.[30]

- ***Electrochemical Method*** It detects changes in electrical signal (current, voltage) at an electrode associated with the enzymatic reaction, such as $H_2O_2$ production or $O_2$ consumption. Widely used in biosensors and point-of-care diagnostics.[30]
- ***Volumetric (Gasometric) Method***

It measures the volume of gas produced or consumed ($CO_2$, $O_2$, $H_2$) over time in a closed system. Applied to decarboxylases and oxidases. Gas volume is recorded manometrically and is directly proportional to substrate conversion.[31]

- ***Titrimetric Method***

The titrimetric method quantifies enzymatic activity by measuring the volume of a standardised titrant consumed to neutralise the acid or base generated during the enzymatic reaction. The assay is most commonly applied to **lipases**, **esterases**, and **proteases**, where hydrolysis of ester or peptide bonds releases protons that are titrated with a base. Two operating modes are used: pH-stat mode maintains pH constant by automatic continuous addition of titrant, while pH-drift mode determines total acid or base produced by back-titration at the end of the assay.[32]

**Total Activity (U/mL)** = $(V_{titrant} \times N_{titrant}) / (t \times V_{enzyme})$

**Specific Activity (U/mg)** =*Total Activity (U/mL)/Protein Concentration (mg/mL)*

## V. Conclusion

To conclude, this chapter has emphasized the importance of enzymes as biological catalysts and has also demonstrated that lipases (EC 3.1.1.3) are especially versatile enzymes belonging to the category of hydrolases because of their ability to catalyze reactions such as hydrolysis, esterification, and transesterification. Being diverse in origin, as well as in the substrates that they act on, and being enantioselective, lipases have been considered to be effective biocatalysts in many industrial applications [33][12b]. This chapter also explains how to measure enzyme activity and specific enzyme activity[7b, 33] which is necessary for the subsequent chapters.

# Chapter 2: Seed lipases

## I. Introduction

Apart from their biological significance, seeds are also extremely valuable to agriculture and industry. Seeds of the world's important crop plants such as soybeans, sunflower, rape (canola), peanuts, sesame, and castor oil are grown mainly for their oils, proteins, and carbohydrates. These products form the backbone of the food-processing, pharmaceutical, biofuel, and oleochemical industries.[34] Moreover, seeds also contain a wide variety of enzymes like lipases, proteases, and amylases that are increasingly being used in industry because of their cheap and environmentally friendly nature.[35]

This chapter discusses seeds in general with particular emphasis on their structural organization, chemical composition and types. Special attention is given to seed lipases, also their enzymatic activity, biological functions , and the factors influencing their catalytic performance, including pH, temperature, substrate specificity, and solvent effects.

## II. Seeds

### II.1. Definition

The seed is the basic reproductive unit of spermatophytes, carrying the genes necessary for the survival of the species from one generation to another.[36] In evolutionary history, seeds are among the most important innovations in the plant world, allowing plants to inhabit land, withstand stressful conditions, and propagate their offspring over large distances.[37]

### II.2. Structure

There is remarkable conservation in the morphology and anatomy of angiosperm seeds even as there is variation in the relative proportion of each tissue type. The adult structure of a seed is made up of three main components, namely the embryo, endosperm (or equivalent storage tissue), and testa or seed coat.[38] (Figure 7) All three components are genetically and developmentally independent entities.

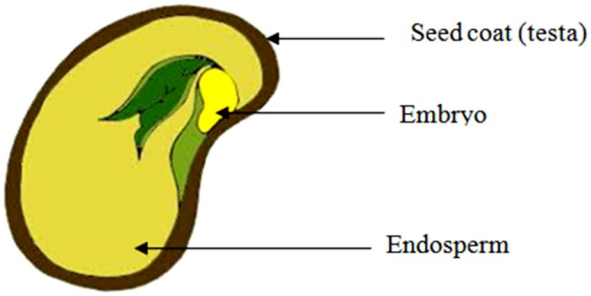



Figure 7. Seed Structure

### II.2.1. Seed Coat (Testa)

The seed coat is the outermost covering of the seed. The seed coat is formed by the integuments of the ovule of the mother plant.[39] Seed coats are maternal tissues only, and they form the physical and chemical boundary between the embryo and endosperm and the environment surrounding the seed[40] .The functions performed by the seed coat include protection from physical injury, desiccation, pathogen attacks, and herbivore attacks.

### II.2.2. Endosperm

Endosperm is a food storage tissue that is formed through the process of double fertilization that is unique to flowering plants. The union of two polar nuclei with a second sperm nucleus leads to the formation of the triploid (3n) endosperm, which stores nutritive materials that will feed the developing embryo and germinating seedling.[41]

### II.2.3. Embryo

The embryo is the diploid (2n) body that forms as a result of the union of the egg cell and the male gametophyte nucleus; it symbolizes the next generation of the plant. Architecturally, the embryo consists of the embryonic axis (which includes the radicle, hypocotyl, and shoot apical meristem) and one or two cotyledons (seed leaves).[42]

### II.2.4. Cotyledons

The cotyledons represent the seed leaves and have many roles depending upon the particular plant species. For instance, in the majority of oilseed plants belonging to dicots (sunflower, soybean, and peanut among others), the cotyledons are the main organs for storage of lipids and proteins. They are filled with oil bodies and storage vacuoles for proteins (PSVs), whose reserves get tapped after post-germinative growth.[43] The presence of lipases is high in cotyledons of germinating oil seeds, which start off in PSVs and then move to oil bodies.

### II.2.5. Oil bodies

Oil bodies or oleosomes or lipid bodies are major subcellular organellar structures for the storage of triacylglycerol (TAG). These oil bodies have an outer membrane of phospholipids containing structural proteins such as oleosins, caleosins, and steroleosins to prevent aggregation of oil bodies and interaction with other cellular organelles.[44]

## II.3. Chemical Composition of Seeds

### II.3.1. Lipids

Lipids serve as the predominant store of energy in the seeds, accounting for 20% to 60% of their dry matter content. The most common lipids present in seeds are the triacylglycerols (TAG), which occur as intracellular droplets, as mentioned earlier. Fatty acids are responsible for the variations in the composition of triacylglycerols among different species; they are regulated by the action of the enzymes involved in the process of desaturation and elongation, such as desaturases and elongases, during the development of the seed. The usual fatty acids are oleic (C18:1), linoleic (C18:2), linolenic (C18:3), palmitic (C16:0), and stearic (C18:0).[45]

### II.3.2. Proteins

Storage proteins typically constitute 15–40% of seed dry weight in leguminous oilseeds. They are classified into three main groups based on solubility: albumins (water-soluble), globulins (salt-soluble; the most abundant class, including legumins and vicilins), and prolamins (alcohol-soluble; dominant in cereal grains).[46]Storage proteins are deposited in PSVs and are mobilized during germination by protease activity. Beyond their nutritional role, storage proteins (especially soybean proteins) have significant functional properties in food processing, including emulsification, gelation, and foaming.

### II.3.3. Carbohydrates

Carbohydrates occur as starch (the primary storage form in cereal grains), structural polysaccharides (cellulose, hemicellulose, pectin), and oligosaccharides (raffinose family oligosaccharides, sucrose). In oilseeds, starch content is generally low, but structural carbohydrates constitute the major component of the seed coat.[47]

### II.3.4. Moisture, Minerals, and Bioactive Compounds

Mature and dormant seeds usually have a lower moisture content of 5-12%, thus ensuring desiccation tolerance and viability. The role of minerals such as phosphorus (present in the form of phytic acid or phytin within protein storage vacuoles), calcium, magnesium, potassium, and iron cannot be overlooked as far as enzyme activation and metabolic activities

during seed germination are concerned. Phenols, flavonoids, terpenoids, and alkaloids are bioactive compounds.[48]

Table 1 presents a comparative analysis of the chemical composition of major oilseeds, detailing the percentages of lipids, proteins, carbohydrates, moisture, and minerals on a dry weight basis (Table 1).

Table 1. Comparative Chemical Composition of Major Oilseeds (% Dry Weight). [35]

| Seed | Lipids (%) | Proteins (%) | Carbohydrates (%) | Moisture (%) | Minerals (%) |
|---|---|---|---|---|---|
| Soybean *(Glycine max)* | 17–21 | 35–40 | 30–35 | 10–12 | 4–5 |
| Sunflower *(Helianthus annuus)* | 40–55 | 15–20 | 15–25 | 5–8 | 3–4 |
| Castor bean *(Ricinus communis)* | 45–55 | 14–17 | 5–10 | 5–7 | 3–4 |
| Rapeseed/Canola *(Brassica napus)* | 38–45 | 20–25 | 18–25 | 5–8 | 4–5 |
| Peanut *(Arachis hypogaea)* | 44–56 | 22–30 | 10–18 | 5–7 | 2–3 |
| Sesame *(Sesamum indicum)* | 44–55 | 20–25 | 12–18 | 4–6 | 4–5 |
| Corn germ *(Zea mays)* | 33–40 | 15–20 | 25–30 | 8–12 | 8–10 |
| Coconut *(Cocos nucifera)* | 60–70 | 7–10 | 15–20 | 5–7 | 1–2 |

## II.4. Types of seeds

The diversity of seeds in nature reflects the breadth of ecological niches occupied by seed plants. For the purposes of biochemistry and enzyme research, the most relevant categories are described below (Table 2).

### II.4.1. Oil Seeds

Seeds with a lipid content generally exceeding 20% of dry weight. Major examples include soybean, sunflower, rapeseed, peanut, sesame, castor bean, coconut, and Jatropha. These seeds are the principal sources of plant-derived lipases and are the primary substrates for seed lipase-catalyzed reactions.[35]

### II.4.2 Cereals

Monocotyledonous grass seeds (e.g., wheat, corn, rice, barley) with a starchy endosperm as the dominant storage tissue. Lipids are concentrated in the germ (embryo plus scutellum) and constitute a relatively minor fraction (~2–8%). Lipases in cereals (notably wheat germ and

corn germ lipases) contribute to rancidity during grain storage and have industrial applications.[49]

### II.4.3. Legumes

Dicotyledonous seeds (soybean, peanut, chickpea, lentil) with high protein and moderate lipid contents. The cotyledons are the principal storage organs. Soybean is the most studied legume for seed lipase activity.[50]

### II.4.4. Industrial seeds

Seeds cultivated primarily for non-edible products. Castor bean yields ricinoleic acid for lubricants, biopolymers, and pharmaceuticals. Jatropha seeds are a feedstock for biodiesel. Linseed provides omega-3 fatty acids and drying oils for paints.[45]

### II.4.5. Edible versus non-edible seeds

Edible oilseeds (sunflower, peanut, sesame) are processed for food-grade oils and protein meals, while non-edible oilseeds (castor bean, Jatropha, tobacco) are used in industry due to the presence of toxic compounds such as ricin or phorbol esters.[45]

Table 2. Examples of seeds and their characteristics.[35]

| Seed Type | Example of Seeds | Key Characteristic |
|---|---|---|
| **Seeds** | Sunflower, soybean, rapeseed, peanut, sesame, castor bean, coconut, Jatropha, linseed, palm | Lipid content >20% DW; rich in TAGs; major lipase sources |
| **Cereals** | Wheat, corn (maize), rice, barley, oat, sorghum, rye, millet | Starchy endosperm; lipid concentrated in germ (2–8% DW) |
| **Legumes** | Soybean, peanut, chickpea, lentil, common bean, fava bean, pea, lupine,acacia | High protein (15–40% DW); cotyledons as primary storage organs |
| **Industrial Seeds** | Castor bean, Jatropha, linseed, tobacco, tung tree, niger seed, camelina | Non-edible; cultivated for biofuel, lubricants, or specialty chemicals |
| **Edible Oilseeds** | Sunflower, peanut, sesame, olive, almond, walnut, hazelnut, flaxseed | Processed for food-grade oils and protein meals; no toxic compounds |

## III. Seed Lipases:

Seed lipases refer to the lipases that are synthesized within the seed tissues, such as the endosperm, cotyledon, and embryo, at particular development periods, specifically during the processes of seed maturation and seed germination. These enzymes are unique in terms of their evolutionary history, structure, and function compared to microbial and animal lipases,

although all of them possess the alpha/beta hydrolase fold and catalytic triad comprising of serine residues.[51]

## III.1. Biological functions of seed lipases

### III.1.1. Lipid Mobilization at Germination

Lipid mobilization during seed germination serves as the main physiological role of seed lipases. Following water uptake, an array of cellular activities ensues, resulting in the activation of lipolytic enzymes. In quiescent seeds, lipases are synthesized and accumulated in PSVs (Protein Storage Vacuoles.In seed). Once the germination process commences, lipases are transported to the oil body surface, where they attach to the outer phospholipid layer and catalyze the breakdown of TAGs.[43]

The lipid mobilization activity in Arabidopsis seeds is mediated primarily by patatin-like lipase SDP1 (Sugar Dependent 1), which accounts for about 90% of the overall activity, whereas the remaining 10% is attributed to SDP1-Like (SDP1L).[52]

### III.1.2. Triacylglycerol Hydrolysis and Energy Production

The process of TAG degradation mediated by seed lipases involves a series of three steps:

(i) **Hydrolysis of a fatty acid from the sn-1 or sn-3 positions on TAGs** to produce DAG;

(ii) **Further hydrolysis of DAG** to MAG; and,

(iii) **Subsequent hydrolysis of MAG** to glycerol and free fatty acids. The released FFAs contribute in producing sucrose that contributes to hypocotyl growth, root development, and seedling establishment.[53]

## III.2. Enzymatic Activity of Seed Lipases

The natural function of lipases is to hydrolyse the ester bonds of triacylglycerols. These substrates are poorly soluble in water, and the reaction thus normally takes place at an organic–aqueous interface at which lipases work very well.[54]

### III.2.1. Hydrolytic Activity

The first step in the catalytic process carried out by seed lipases is the hydrolysis of triacylglycerides to diacylglycerides, monoacylglycerides, glycerol, and three molecules of free fatty acids[35] .This process involves the creation of an acyl-enzyme intermediate that undergoes further deacylation by water to yield the fatty acid and regenerates the free enzyme. Apart from the hydrolysis of TAGs, other potential substrates for the lipase enzymes include diacylglycerides, monoacylglycerides, fatty acid esters, and wax esters; however, triacylglycerides are the main physiological substrates.

The degree of hydrolysis depends on factors such as enzyme concentration, substrate specificity, pH, temperature, water activity, and reaction time.[55]

### III.2.2. Esterification

On the contrary, when subjected to water-restricted or anhydrous conditions, the lipases' catalytic pathway can be redirected towards ester synthesis via direct esterification between free fatty acids and alcohols to form fatty acid alkyl esters and water.[35] In this case, the mechanism is thermodynamically reversed, involving the formation of a covalent acyl-enzyme intermediate, in which deacylation occurs via nucleophilic attack of the alcohol substrate on the bound fatty acid rather than water, resulting in the production of esters and the regeneration of the free enzyme molecule.[54] They are particularly useful in creating diverse waxes, biodiesels, and food flavoring agents.

A comparative overview of the reaction types and activities of various seed lipases is presented in Table 3.

Table 3. Comparaison of reaction types and activities of various seed lipases.

| Seed Source | Studied Reaction | Methodology and activity | Ref |
|---|---|---|---|
| Lupin | Hydrolysis of triglycerides | Titrimetric; specificity for primary (sn-1,3) positions; more active on saturated than unsaturated FA; induction period 10 min; 70% activity loss after 24 h | [56] |
| Rice Bran | Esterification (Oleic acid + EtOH → OAEE) | GC quantification of OAEE; hydrolytic activity 10.57 U/g; esterification activity 324.03 U/g; optimum OAEE yield 810.77 µmol | [57] |
| Barbados Nut | Transesterification | Michaelis-Menten ( $V_{max}$) $V_{max}$ of 0.24–0.26 \□mol /mg/min | [58] |
| Bambara Groundnut | Hydrolysis of TAG (triglycerides and cholesteryl esters) | Titrimetric / spectrophotometric. Km = 6 mM; Vmax = 1120 U/L. Partially purified by 60% ammonium sulfate precipitation, dialysis and CM-cellulose chromatography | [59] |
| Black-Cumin | Transesterification | Titration method with( 0.1 N) NaOH 48.72 % conversion | [60] |
| Hazelnut | Hydrolysis of triacylglycerols (triolein / tributyrin / olive oil) | Titrimetric & spectrophotometric (NaOH). Km = 4.545 mM; Vmax = 80 U/min•mg. Purified 1255-fold; MW = 20 kDa by SDS-PAGE. 83% activity retained after 9 months at −20 °C | [61] |
| Citrullus lanatus | Hydrolysis of triacylglycerols | specific activity of 211.81 U/mg | [62] |

## IV. Factors Affecting Seed Lipase Activity

### IV.1. Effect of pH

The pH is one of the most important factors determining the activity of lipase enzymes, including their ionization status and stability. Seed lipases have been found to vary in their pH optima (Table 4). For example, the castor bean lipase has an optimum pH of 4.5–5.0 [55] while the optimum for the alkaline lipase from the same organism is 8.0–9.0.[63] The sunflower lipase on the other hand has an optimum of 7.5.[64] In general, plant lipases have high stability within a wide pH range of 4.0–9.0.[35] Any deviation from the optimum will lead to decreased lipase activity due to changes in the ionization status of the His residue in the catalytic triad.

Table 4. pH optima of different seed lipases.

| Seed Source | Optimal pH | Ref |
|---|---|---|
| Castor bean (acid) | 4.5–5.0 | [55] |
| Castor bean (alkaline) | 8.0–9.0 | [63] |
| Sunflower (germinated) | 7.0–7.5 | [64] |
| Soybean (GmSDP1) | 6.5–8.0 | [3] |
| Cocoa bean | 9.0 | [50] |
| Linseed (Flaxseed) | 7.0–8.0 | [65] |

### IV.2. Effect of Temperature

Lipases in seeds exhibit mesophilic behavior, operating optimally within the temperature range of 30 to 55 degrees Celsius. The castor bean lipase has an optimum activity range of 37 to 50 degrees Celsius,[55] while the sunflower seed lipase shows stability between 30 and 50 degrees Celsius.[64] Any temperature exceeding this optimum will cause the unfolding of the protein, rendering it inactive (Table 5).

Table 5. Temperature optima of different seed lipases.

| Seed source | Optimal temp °C | Ref |
|---|---|---|
| Peanut | 30–45 | [66] |
| Rapeseed | 35–50 | [67] |
| Corn germ | 35–45 | [68] |
| Jojoba | 60 | [69] |
| Sesame | 38 | [70] |
| Luffa | 50-80 | [62] |

### IV.3. Effect of Substrate Concentration

Kinetics at lower concentrations of substrates and their activities increase until they reach substrate saturation (Table 6). In case of emulsified TAG substrates, concentration is related

to interfacial area in such a way that more substrates mean higher interfacial area and interfacial activation; too much substrate can inhibit the enzyme.

Table 6. How different seed lipases respond to substrate amount.

| Seed Source | Substrate trend with activity | Notes | Ref |
|---|---|---|---|
| Prouted melon (C. manni) | Increases up to 5 g, then activity drops | Km 6.25 g, Vmax 13.33 %FFA/h | [71] |
| African oil bean | Optimum at 5 g; inhibition above | Dormant seeds | [72] |
| Fermented oil seeds | Michaelis–Menten dependence | Km ~0.066–0.076 mM | [73] |
| Soybean germinating seeds | Km 7.67 mg (olive oil emulsion) | Classic MM kinetics | [74] |
| Castor bean powder | Activity depends on oil:aqueous ratio | Hydrolysis of high-oleic sunflower oil | [75] |

## IV.4. Effect of Organic Solvents

Many seed lipases tolerate the presence of organic solvents, a property that is valuable for catalysis in non-aqueous media (Table 7). Tolerance varies among solvents and enzymes: hydrophobic solvents (hexane, heptane) generally have minimal effect on activity, while polar solvents (acetone, methanol at high concentrations) may cause denaturation.[35] The inhibitory effect of DMSO on cocoa bean lipase activity has been reported.[64]

Table 7. Preferred solvents for lipase activity across various seed lipases.

| Seed Source | Prefered solvent | Ref |
|---|---|---|
| Maize (Corn) | Aqueous buffer / 30% Ethanol | [35] [76] |
| Wheat germ | n-hexane, isooctane | [35] [77] |
| Pumpkin | Tris-HCl buffer (50 mM, pH 8.0); Triton X-100 phase | [78] |
| Barbados Nut | Ethanol (for precipitation) | [58] |
| Sunflower | Aqueous buffer (pH 7.5–8.0) | [35] |

## IV.5. Effect of Metal Ions

Metal ions have different impacts on seed lipase activity (Table 8). The presence of calcium ions ($Ca^{2+}$) and magnesium ions ($Mg^{2+}$) can act as an activator when present in small quantities because of protein stabilization or substrate binding. On the other hand, heavy

metal ions like $Hg^{2+}$, $Cu^{2+}$, $Fe^{3+}$ and chelating agents like EDTA usually act as inhibitors due to their interaction with key residues of the enzyme. Sodium ions ($Na^{+}$) have no impact at all.[63-64]

Table 8. Activators and inhibitors of various seed lipases

| Seed source | Activator | Inhibitor | Ref |
|---|---|---|---|
| Africa bean seed | $Ca^{2+}$ | EDTA | [35] |
| French bean seed | Tween-20 | $Ca^{2+}$ | [35] |
| Lupin seed | $Ca^{2+}$ ,$Mg^{2+}$ $K^{2+}$ | – | [35] |
| Almond seed | $Ca^{2+,}$ $Fe^{2+}$, $Mn^{2+,}$ $Co^{2+}$ and $Ba^{2+}$ | $Mg^{2+}$, $Cu^{2+}$ and $Ni^{2+}$ | [35] |
| Laurel seed | $Ca^{2+}$ , $Mg^{2+}$, $Co^{2+}$, $Cu^{2+}$ and $Fe^{2+.}$ | – | [35] |

### IV.6. Effect of Incubation Time

The reaction kinetics of lipase depends on time, which means that there is an increase in the activity of lipase up to a particular point (enzyme saturation) followed by a decrease as a result of product inhibition (Table 9), denaturation of enzymes, or depletion of substrate. The optimum incubation time for sunflower lipase ranges from 10-40 minutes.[64] Long incubation time can lead to esterification.

Table 9. Comparison of optimal incubation times across seed lipase systems.

| Seed source | Optimal incubation time | Ref |
|---|---|---|
| Sprouted melon | 2 h | [71] |
| Pachira aquatica | 90 min | [79] |
| African oil bean | ~60 min | [72] |
| Sunflower (germinated) | 20 min (stable 10–40 min) | [80] |
| Sunflower (purified) | 5 min | [81] |

## V. Conclusion

To summarize, this chapter emphasized the significance of seeds as living organisms consisting of many storage compounds and enzymes.[38] Special emphasis was placed on seed lipases, which have a very important function of breaking down lipids during seed

germination by cleaving triacylglycerols to yield free fatty acids and glycerol.[43] Lipases are known for their efficient catalysis, substrate specificity, and susceptibility to physicochemical factors like pH, temperature, substrate concentration, organic solvents, metals, and incubation time. By controlling these factors, the thermodynamic balance can be driven away from hydrolysis to esterification to synthesize high-value products through green chemistry.

An improved understanding of seed structure, composition and enzymes location is necessary for optimum extraction and maximal lipase activity.

# Chapter 3: Activity Estimation of an Acacia Lipase Extract

## I. Introduction

The plant seeds constitute a much diversified source of lipases due to their high efficiency and stability as catalysts. Out of the various botanical origins, seeds from Acacia plants offer an especially interesting platform for biocatalytic reactions.[35] The objective of this work is to extract lipases from the Acacia seeds and analyze their hydrolysis capability. In addition, the efficacy of this enzyme as an esterifying agent shall be tested. Prior to presenting the detailed findings of this study, a summary on Acacia tree, seed structure and uses will be given.

## II. General Acacia Geographic Distribution

Acacia is found everywhere warm and tropical, spreading farther than any other woody plant group on the planet. With over 1350 species, almost half are native to Australia about 1,000 in total there. Other spots around the world have the rest: 144 types in Africa and roughly 89 in Asia.[82] Many parts of Australia see species like Acacia *aneura*, or *mulga*, controlling how these regions look. They make big woodlands that stretch for miles. In Africa, what folks now call *Vachellia* and *Senegalia*, after recent updates, grow in wide-open savannas, sub-desert steppes, and by rivers.[83] These plants really matter too, 'cause they build key pieces of their ecosystems and help out tons of animal buddies living there.

## III. Acacias in Algeria

The distribution of *Acacia* species in Algeria varies significantly across geographic and climatic gradients, extending from the hyperarid southern desert regions up to the northern zones of the country.

### III.1. Local Species of Acacia

In Algeria, native Acacia species are really important for Saharan and pre-Saharan ecosystems. *A. laeta, A. nilotica, and A. seyal* only grow in the driest spots that get under 100 mm of rain each year. One standout species is the Acacia *tortilis* subsp. *Raddiana* (now

known as *Vachellia tortilis subsp. raddiana*), which is perfect for desert life.[84] This tree covers the whole Algerian Sahara and is especially common in the northwestern part, forming big communities along with *Panicum turgidum* and *Foleyola billotii* in river beds and alluvial plains.

### III.2. Introduced Species of Acacia

They were introduced and acclimatised mostly from Australia at the end of the 19th century. There are about 17 species of acacia that have been introduced to Algeria. 13 of them are native to Australia and the other four species are native to South and Central America. For example, species like *Acacia saligna* and *Acacia farnesiana* were introduced in the north.[85]

## IV. Applications and Uses

### IV.1. Bark

Like Fabaceae family members, Acacia species have bark packed with tannins. People use these tannins a lot in industries because they're eco-friendly. They help in tanning leather, making stuff water resistant, and creating strong wood adhesives. But that's not all – traditional medicine has long used Acacia bark too. Research shows it's full of helpful compounds that act as antioxidants, fight infections, reduce inflammation, and even help with diabetes.[86]

### IV.2. Leaves and Foliage

The leaves and foliage of acacia serve important ecological functions. As a nitrogen-fixing legume,[87] the plant forms symbiotic associations with rhizobia bacteria in root nodules, converting atmospheric nitrogen into plant-available forms .Leaf litter decomposition therefore enriches soil nitrogen, benefiting surrounding vegetation. This property makes the species valuable in agroforestry, land reclamation on degraded soils, and erosion control .The feathery foliage is also used ornamentally. However, allelopathic effects have been noted in related Acacia species, with leaf extracts reported to inhibit germination of competing plants.[82]

### IV.3. Wood

Acacia wood is well-known both locally and commercially as top-notch fuel, often chosen for firewood and making high-energy charcoal.[88] Many Acacia species grow super fast, making them perfect for short-rotation biomass fuel crops.[88-89] Beyond fuel, Acacia's dense, hard

wood makes it great for more than just heating up the fireplace; folks use it for crafting, mine support, pulp, and even building stuff.[88]

## IV.4. Uses of seeds

### IV.4.1. Pods and seeds description

After pollination, the flat elongated legume pods of acacia are formed, with the potential to reach a maximum length of approximately 10 cm.[90] The immature pod is green and changes to dark brown or black when mature, while the seeds have a linear arrangement within the pod, being dark-coloured to oval-shaped and relatively hard-shelled (Figure 8). Hard-shelled seeds are characteristic to many leguminous species, which contributes to the long-term viability of seeds in soil.

**A**

**B**

Figure 8. Pods (A) and seeds (B) of the used acacia

### IV.4.2. General Uses

Acacia species seeds, collectively called wattle seeds, pack a big nutritional punch and are seen as a secure food source .These tough and adaptable seeds are full of protein, fiber, and essential fatty acids .During germination, the seeds own lipase enzymes break down triacylglycerides using their fat content as a main ingredient. In industry, plant seed lipases have become go-to options for creating eco-friendly detergents and green chemicals because they are cheap and sustainable.[91] In addition, the seeds durable coats mean they keep well, making Acacia seeds a dependable choice for both biochemical research and adding nutrition to diets worldwide.[92]

# V. Results and discussion

## V.1. Extraction of Lipase from dormant Acacia seeds

### V.1.1. Grinding

Using a coffee grinder, the Acacia seeds were ground into a uniform powder. The goal of grinding is to disrupt the cellular matrix in order to release lipases and achieve greater uniformity. This type of preparation improves extraction efficiency and allows to achieve high-quality crude lipases.

### V.1.2. Delipidation

In order to increase both lipase productivity and thermal resistance, the natural oils that are found in vegetable material have to be separated from the protein using solvents, to create a lipid-free extract of the vegetable material. The most common solvents that are utilized for this process are acetone and n-hexane. In this study, cold acetone, as described in the experimental procedures, was used for delipidation. After filtration, the resulting solid extract was dried at ambient temperature and then stored at 4°C.

### V.1.3. Sieving

After storage, the lipase extract was sieved using a vibratory sieve shaker (see experimental part). To make a homogeneous enzyme preparation, impurities and cotolydon part of the seed had to be eliminated. The most effective catalytic powder is the fraction in which the particles diameter is between the **0.125mm** and **0.063mm** sieves (Figure 9). This crude lipase extract (ASL) will be tested for both hydrolytic and synthesis activities.

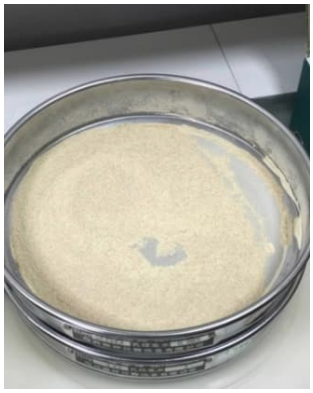


Figure 9. Recovered powder (0.125mm and 0.063mm).

Figure 10 summarizes the complete extraction process of the enzymatic powder ASL extract from dormant Acacia seeds.

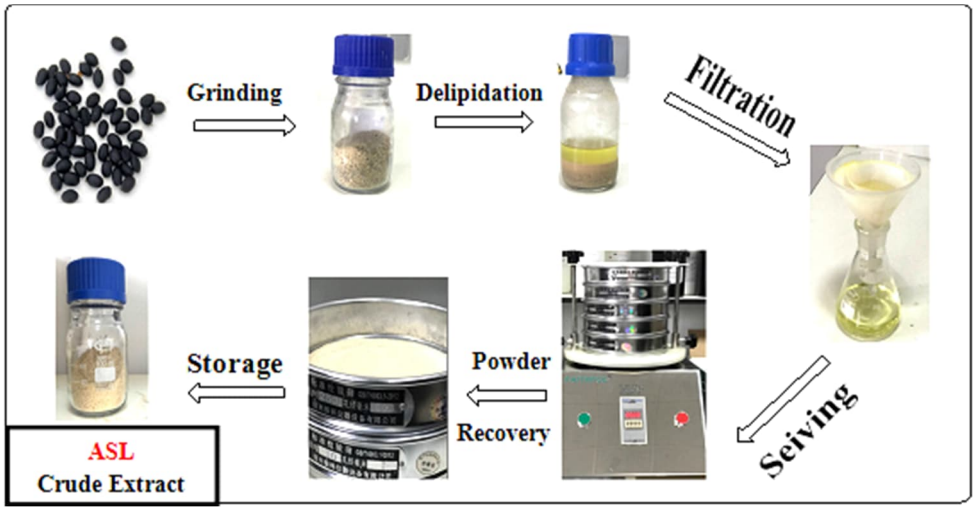
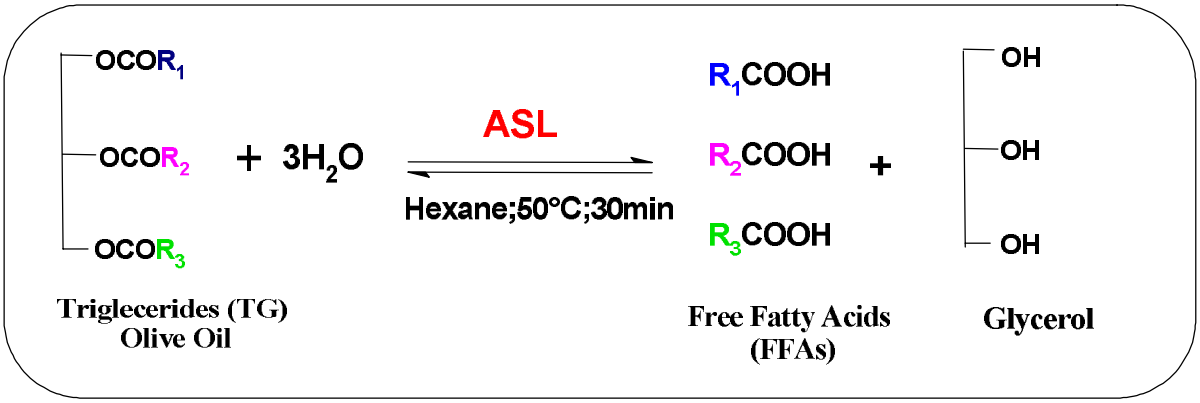



Figure 10. Complete extraction process of lipase powder (**ASL**) from dormant Acacia seeds

## VI. Measuring Lipase Activity

An hydrolysis test was performed to identify seed extract lipase activity. Olive oil was used as substrate for the different hydrolysis tests (Scheme 3). In addition, volumetric assay was choosen to measure lipase activity because it is simple and easy, and enables us to quickly determine the amount of FFAs formed after hydrolysis, and thus the enzymatic activity.




Scheme 3. Hydrolysis reaction of olive oil in the presence of enzyme extracts.

### VI.1. Powder extract

Two assays were conducted: a blank test (without enzyme) and a test using ASL powder extract. The blank test was first performed to confirm that no hydrolysis occurred in the absence of enzymatic activity. For this, a reaction was carried out using olive oil as substrate in hexane, with stirring at 50 °C for 30 min. The reaction was then stopped by adding a mixture of acetone and 0.1% phenolphthalein in ethanol, followed by titration with 0.1 M sodium hydroxide (NaOH) solution.

In parallel, a separate mixture of olive oil and hexane was directly titrated with NaOH solution to evaluate any initial FFA content. These two control tests were used to quantify the

amount of free fatty acids (FFAs) present in the reaction mixtures. The results of the tests performed without the-ASL are presented in the following table (Table 10).

Table 10. Results of volumetric measurement of olive oil hydrolysis in the absence of ASL

| | Test at (t=0) | Blank Test |
|---|---|---|
| t (Min) | 0 | 30 |
| $V_{NaOH}$ (µL) | 500 | 500 |

According to the presented data (Table 10), there was no change in the amount of NaOH used after a half-hour in the blank assay, which indicates that free fatty acids (FFAs) were not produced during this time and confirms that hydrolysis does not occur without enzymes. However, with the acacia seed extract and olive oil as substrate in hexane, the reaction was carried out at 50 °C and stirred continuously in a reaction mixture using 0.5 g of enzymatic extract. After 30-minutes, the reaction was terminated by adding a mixture of acetone and phenolphthalein (0.1%), and the resulting solution was immediately titrated with 0.1M NaOH. The specific activity **SA** and free faty acids percentage released (**FFAs**) were then calculated using the following equations (see experimental part).

$$SA = \frac{n\ (\mu mol)}{x(mg) \times t(mn)} \qquad \%FFAs = \frac{100 \times C_{NaOH} \times V_{NaOH} \times MM_{Olive\ oiL}}{m_{Olive\ oil}}$$

Table 11. Evaluation of ASL hydrolytic activity (powder extract).

| **Blank $V_{NaOH}$ µl (t=0)** | **$V_{NaOH}$ µl (t=30min)** | **Specific activity SA (IU/mg)** | **Free fatty acids FFAs %** |
|---|---|---|---|
| 500 | 2350 | 0.0123 | 2.09 |

According to this table (Table 11), it can be observed that the crude extract of Acacia seed possesses hydrolytic activity with olive oil as substrate. This confirms the existence of one or several lipases that have the ability to break down ester linkages in TAGs of olive oil. The specific activity of the ASL enzyme extract from Acacia seeds is 0.0123 IU/mg. In order to better estimate the hydrolysis activity of our crude extract, the percentage of FFAs obtained was compared with those of literature. The following table (Table 12) reviews FFAs% released when different seed extracts are used as source of lipases.

Table 12. Enzymatic Activity of Lipases from Different Sources in the Hydrolysis of Various Oils.

| Input | Lipase source | Substrate | FFAs (%) | Ref. |
|---|---|---|---|---|
| 1 | Acacia seeds (ASL) | Olive oil | 2.09 | This work |
| 2 | Yellow maize seeds | Olive oil | 2.34 | [93] |
| 3 | Brown mustard seeds | Olive oil | 2,95 | [94] |
| 4 | African bean seeds | Palm oil | 1,21 | [95] |
| 5 | Canephor nuts | Peanut oil | 1,25 | [96] |
| 6 | Flax seeds | Olive oil | 7.27 | [96] |

Analysis of Table 12 shows that high FFAs percentages can be obtained using different sources of lipase, with these percentages varying based on both the lipase source and the type of oil used as a substrate. The best result (FFAs% =7.27) was obtained during the hydrolysis of olive oil catalyzed by lipases extracted from flax seeds (input 6). Using ASL, extracted from dormant Acacia seeds, the result (FFAs %= 2.09) is comparable to those found in the literature, demonstrating the efficacy of this lipase as a potential source for efficient FFAs production.

## VI.2. Solution extract

Despite the existence of many biochemical techniques used to extract active enzyme solutions from acetone powder extracts, including various buffer media,[97] detergent solubilization,[98] and ultrasound treatment[99], cold saline extraction technique was chosen for this experiment due to its operational simplicity and the immediate availability of all necessary reagents within our laboratory. The first step in the extraction and preservation of the ASL enzyme involves dissolving 0.9g of sodium chloride in 100mL of distilled water in order to make a 0.9% saline solution.[100] This ensures that a chemically stable environment is created which does not allow the enzyme to be degraded. After the preparation of the saline solution, 10g of ASL enzyme powder are added to the saline solution. It is important to note that the saline solution is kept in a cool environment in order to prevent the degradation of the sensitive proteins. The solution is then agitated for five minutes in order to disperse the enzyme powder. The next process involves the use of a centrifuge machine where the solution is centrifuged at 4200 rpm for ten minutes in order to separate the useful enzyme from other unwanted particles. The liquid that remains on the upper surface of the solution is extracted and placed in a container at 4°C. This following figure (Figure 11) summarizes the complete process used to obtain the crude solution of the extracted lipase.

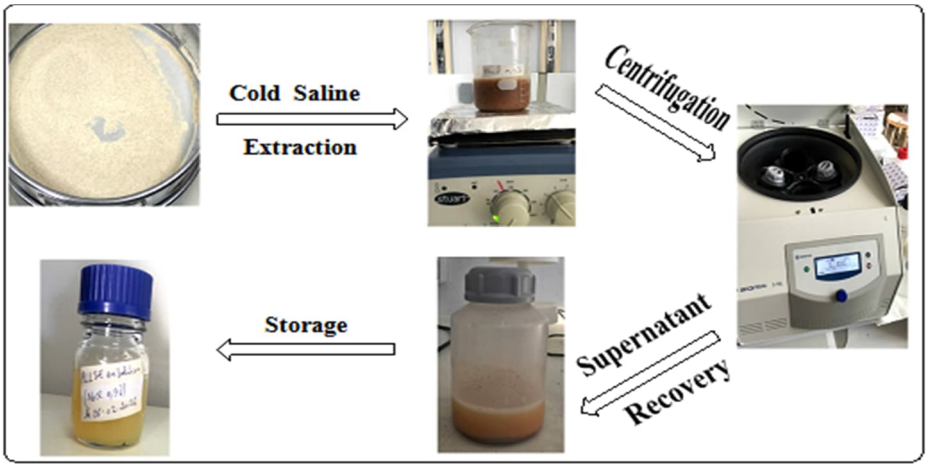



Figure 11. Complete process for the obtention of solution extract from dormant Acacia seeds

The resulting enzyme solution will be subjected to hydrolysis to compare its activity with that of the crude powder (Table 13).

Table 13. Evaluation of ASL activity (solution extract).

| **Blank $V_{NaOH}$ µl (t=0)** | **$V_{NaOH}$ µl (t=30min)** | **Specific activity SA(ui/mg)** | **Free fatty acids FFAs%** |
|---|---|---|---|
| 500 | 800 | 0.002 | 0.34% |

The comparison of the experimental values, table 12 and table 13, reveals a considerable gap in activity level between the two extracted enzymes (powder extract and solution extract). The enzyme activity value and the percentage of free fatty acids released after hydrolysis in the presence of crude powder extract are roughly six times greater than the values obtained for the solution extract (Figure 12).

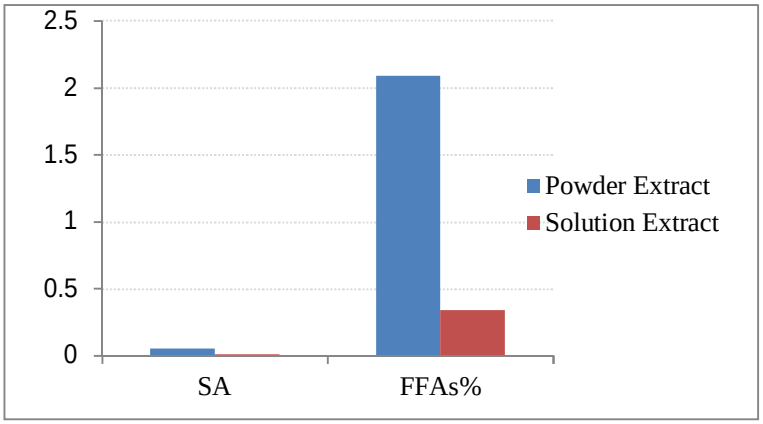


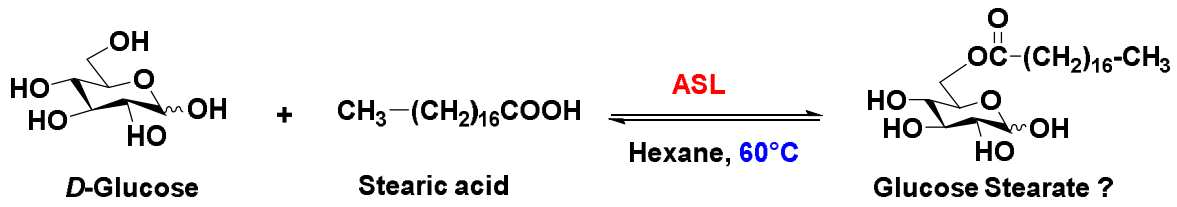

Figure 12. Comparative Analysis of Crude Powder and Solution Enzyme Extracts.

It can be assumed that the loss of enzyme activity was caused by placing the enzyme in a saline solution and extracting it through the process of centrifugation in a solution form. These results suggest that this method is not suitable in our case. It also suggests that the hydrolytic activity obtained with the crude powder extract reflect the real enzyme activity

level. Therefore, the crude powder extract will be used to test the synthesis capacity in the following study.

## VII. Esterification reactions

Enzymatic esterification is a type of condensation synthesis reaction where the lipase enzyme acts on an organic solvent such as hexane,[101] which is utilized as a hydrophobic solvent because its nonpolar nature preserves the essential hydration shell around the lipase, thereby maintaining enzymatic stability and shifting the chemical equilibrium toward ester synthesis. A reaction temperature of 60°C is applied to maximize catalytic kinetics and enhance substrate solubility while remaining safely below the boiling point of hexane (69°C) to minimize solvent evaporation.[102] To work against the usual breakdown process, brings about the linkage between a carbohydrate (glucose) and a fatty acid (stearic acid) for the formation of a sugar ester, as well as water (Scheme 4).




Scheme 4. Synthesis of glucose stearate (effect of reactants ratio).

To determine the best results for the particular synthesis reaction within certain periods of time (24 hours, 48 hours), the amount of starting materials that remained unreacted is determined through titration with sodium hydroxide (NaOH); Conversion of reactants was measured through titration using 0.1 N NaOH. All experiments performed are detailed in the following table (Table 14).

Table 14. Effect of molar ratio of Sugar (**S**)/Stearic Acid (**A**) on conversion.

| Substrate | Ratio (S:A) | Time | Blank $V_{NaOH}$ | Final $V_{NaOH}$ | Conversion (C%) |
|---|---|---|---|---|---|
| a | 2.5 : 1 | 24 h | 13190 µl | 9900 µl | 24.94 |
| Glucose | 2.5 : 1 | 48 h | 13190 µl | 10900 µl | 17.36 |
| b | 1 : 2,5 | 24 h | 12100 µl | 9700 µl | 19.83% |
| Glucose | 1 : 2,5 | 48 h | 12100 µl | 1100 µl | 10.56% |
| c | 1 : 1 | 24 h | 13000 µl | 12000 µl | 7.7% |
| Glucose | 1. : 1 | 48h | 13000 µl | 12900 µl | 0.9% |

The following formula was used to calculate conversion: $C\% = \frac{V0-Vt}{V0} \times 100$

Esterification of glucose using ASL extract proved to be very efficient even if modest conversions were obtained, provided that there was an adequate molar ratio of reactants and sufficient reaction time. The kinetic curves presented in Figure 13 demonstrate how this correlation works and how the efficiency of conversions starts decreasing at 24 hours.

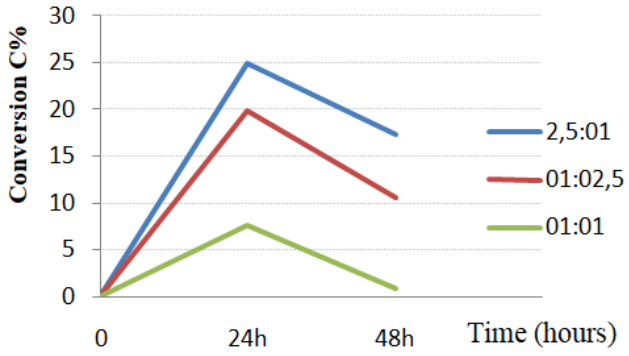



Figure 13. Kinetics of glucose conversion over 48 hours under varying reactants molar ratios.

Over all the molar ratios of the substrate tested, the reaction shows a distinct parabolic kinetic profile with the same allure. The maximum conversion was recorded after 24-hours of reaction, with the maximum amount of converted glucose (24.94%) being noted at 2.5:1 molar ratio (Figure 14).This excess seems to make a greater contribution towards shifting the thermodynamics of the process than 1:2.5 and 1:1 molar ratios (19.83% and 7.7% conversions, respectively). On the other hand, the kinetics of the process showed a significant drop in the amount of converted glucose after 48 hours; particularly, it fell to nearly zero (0.9%) (Figure 14).The rapid decrease in conversion suggests the dominance of the reverse reaction caused by the presence of water products or decreased enzyme activity in the crude lipase powder of ASL. Thus, for amelioring the synthesis of these sugar esters, it is essential to limit reaction time to 24 hours or conduct the reaction in an environment where water is continuously removed.

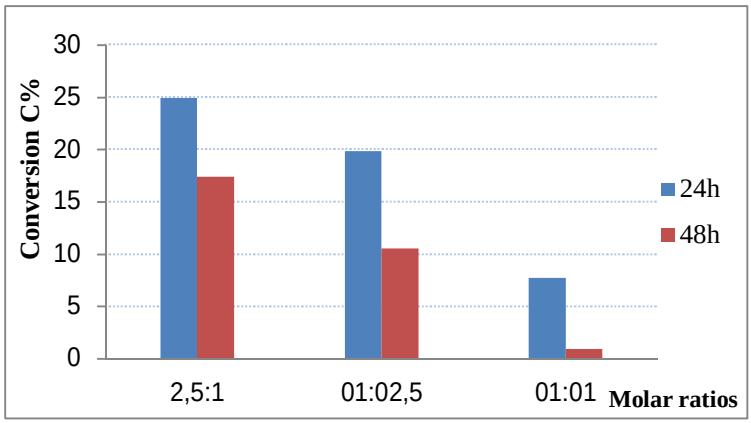



Figure 14. Effect of reactants molar ratios (a = 2.5:1, b = 1:2.5, b = 1:1) and reaction time on the glucose conversion (**C %)** on enzymatic esterification.

Maximum conversion of 24.94% is observed in the presence of crude extract ASL powder without the use of any drying agents. Literature shows that when physical water removal is done using molecular sieves, vacuum evaporation, and azeotropic distillation, glucose esterification yields can be increased from 65% to 90%.[103]

Three essays were explored in order to optimize conversion rate at 2.5:1 molar ratio: solubilization of sugar for 24 h in order to achieve homogeneity, and reaction time variations such as 6, 18 and 72 hours. However, since no significant increase in yield was observed after these attempts, it is clear that the optimal condition remains that of 24-hour reaction time at 2.5:1 molar ratio.

## VIII. TLC Analysis

To check the progression of the enzymatic esterification reaction and to verify that a new species had been formed, TLC (Thin Layer Chromatography) technique was used to compare the products of the ongoing reaction with three main control samples. The elution was conducted using a solvent system of chloroform and hexane (50:50, v/v). The different spots identification was achieved through oxidative visualization using a solution of basic potassium permanganate (see experimentale part).

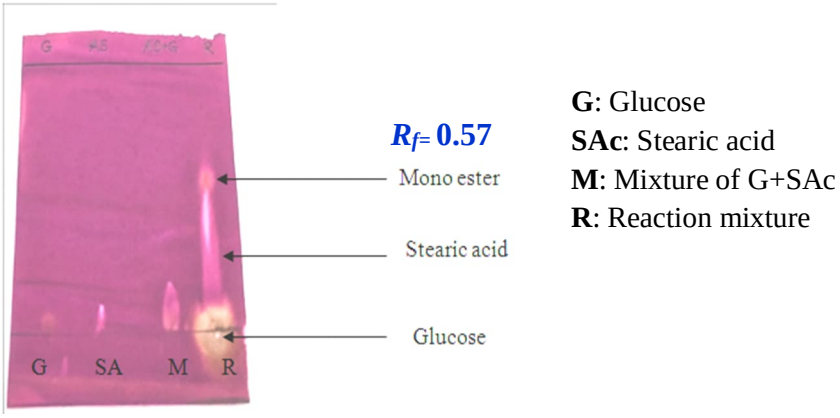



Figure 15. TLC analysis of the esterification t

TLC chromatogram (Figure 15) suggests the successful synthesis of the desired sugar ester through enzymatic synthesis after 24 hours of reaction time. With the use of the eluent system Chloroform/Hexane (50:50 v/v), it is consistent to observe the product spots , with tightly clustered $R_f$=0.57, compared with literature, the TLC plate analyses indicate the formation of a new product which may be the expected mono-ester with an $R_f$ of 0.50-0.59,[104] which shows the consistency and reliability of the condensation reaction with glucose. In addition, this production indicates the reliability of the pre-solubilization stage, in which the solubility problem of hydrophilic carbohydrates in normal hexane was overcome. According to the

literature, lipase-catalyzed synthesis of sugar esters in organic solvents showed high regioselectivity toward the primary hydroxyl group, leading to the preferential formation of monoesters at the C-5 position[105].

Normally, after TLC analysis, the separation of esters must be carried out in order to separate the formed product and identify it by different analytical methods such as (NMR, IR, MS...) to compare them with standards and those in literature.[101] [106] [107] Unfortunately, the separation carried out according to the protocol described in the literature did not succeed.

## X. Conclusion:

In this chapter, ASL was successfully extracted and its specific activity was determined SA=0.0123 IU/mg in the presence of olive oil as substrate in hexane at 50°C. Furthermore, this extract was used as a catalyst for enzymatic esterification, in different ratios of glucose to stearic acid, in the presence of ASL in hexane at 60 °C. The best results (C%= 24.94%) were obtained after 24h of reaction with a molar ratio of 2,5/1, The TLC analysis suggests the formation of a new product which is probably a mono-ester.

# Experimental Part

## I. Plant Biomass and Chemicals

### I.1. Plant Biomass

In our work, a species of Acacia seeds, (Figure 16) is harvested from a local forest (in El Kala). It was used as a source of lipases to catalyze the hydrolysis of olive oil in order to determine the enzymatic activity of the extracts and esterification reactions.

A  B 

Figure 16. Acacia pods (A) and seeds (B) used in this work

### I.2. Chemicals

**Substrate**: The olive oil used in this work is from local source (Figure 17) .Its fatty acid composition is given in the following table (Table 15)

**Solvents**: acetone, n_hexane, methanol, propanol, butanol, ethanol.

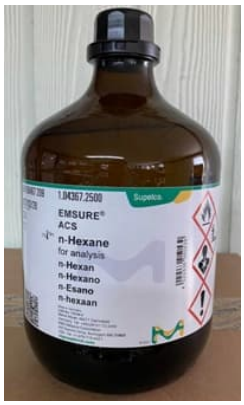
Table 15. Fatty Acid Composition of Olive Oil Used in this Work

| Fatty acid | Composition (%) |
|---|---|
| Palmitic (C16:0) | 11.4 |
| Palmitoleic (C16:1) | 0.65 |
| Stearic (C18:0) | 2.60 |
| Oleic (C18:1) | 80.6 |
| Linoleic (C18:2) | 4.20 |
| Linolenic (C18:3) | 0.60 |
| Arachidic (C20:0) | 0.20 |
| Average molar mass | 279.6 |

Figure 17. Olive Oil Used in this Work

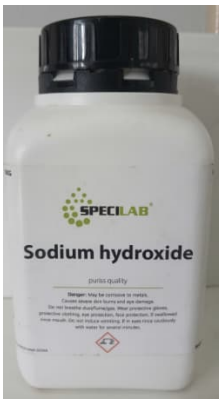
- **Chemicals**: Sodium hydroxide, Phenolphthalein, stearic acid, glucose, sodium carbonate, potassium permanganate.
- **Solvents**: acetone, n-hexane, ethanol (Figure 18)


Acetone




n-hexane

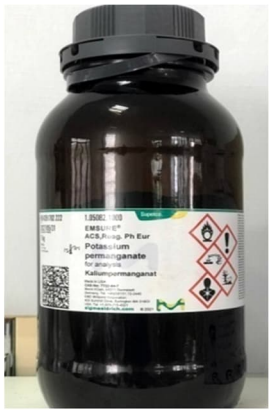

ethanol




Sodium hydroxide

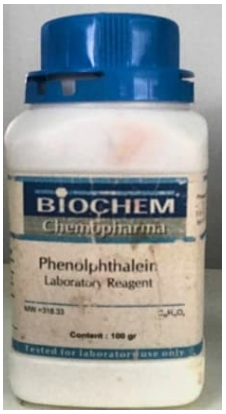

Stearic acid


Glucose




Potassium permanganate


Sodium carbonate




Phenolphtalein

Figure 18. Chemicals and solvents used in this work.

# II. Materials

## II.1. Precision Balance

The OAHUS precision balance (Figure 19) was used for all weighing operations with an accuracy of 0.0001 g.

## II.2. Magnetic Stirrer

To carry out the enzymatic hydrolysis, a Stuart brand heated magnetic stirrer and a bar magnet were used (Figure 19).

## II.3. Mechanical Micropipettes

In order to improve precision during the determination of lipase activity, two micropipettes were used one from **Accumax** with a precision of 20-200 µl, and a **Socorex** micropipette with a fixed volume of 1000 $\mu$ l (Figure 19).

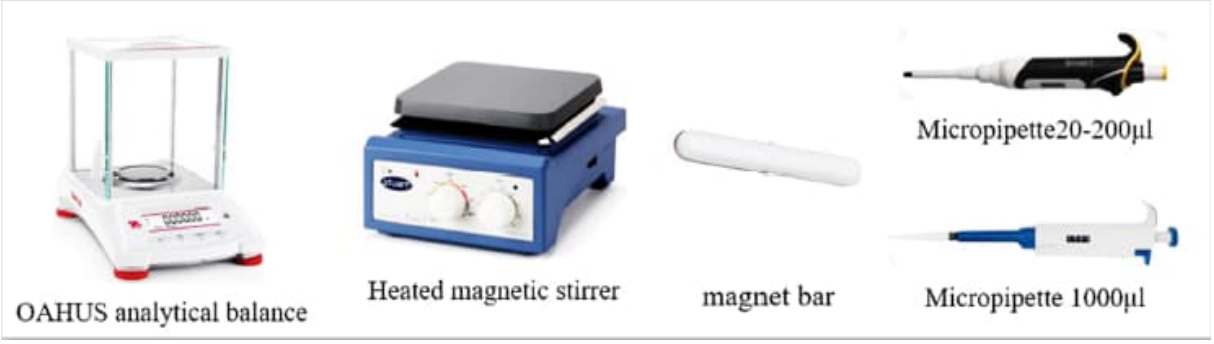



Figure 19. Analytical balance, Stirrer, magnet bar and micropipettes used in this work.

## II.4. Vibratory sieve shaker

To optimize the physical state of the biocatalyst, the crude **ASL** powder was subjected to a mechanical fractionation process using an electric vibratory sieve shaker from **FAITHFUL** (Figure 20)

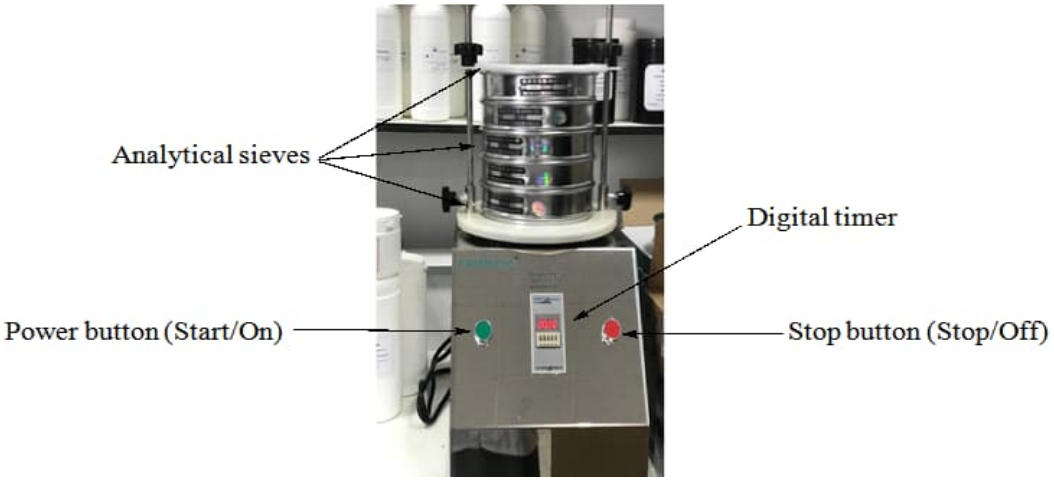



Figure 20. Vibratory sieve shaker from FAITHFUL .

# III. Methods

## III.1. Procedure for Extracting Lipases from Dormant Acacia Seeds

### III.1.1. Cleaning

The Acacia seeds were purified by cleaning them with a disinfectant and sterilizer.

### III.1.2. Drying and Grinding

Dehulled dormant acacia seeds are dried at room temperature for 48 hours, then in an oven for 24 hours at 40°C. The dried seeds are ground using a coffee grinder.

### III.1.3. Sieving

A vertical stack of standard analytical sieves, including mesh sizes for **0.125 mm (120 mesh)** and **0.063 mm (250 mesh)** (Figure 21) , was employed to separate the extract particles based on their diameter. The vibration parameters were set 2 minutes of vibrations to ensure a complete and homogeneous distribution of the powder across the different levels of the sieve stack.

| Analytical sieve (0.45 mm /40 mesh) | Analytical sieve (0.3 mm /60 mesh) | Analytical sieve (0.125 mm /120mesh) | Analytical sieve **(0.063mm/250mesh)** |
|---|---|---|---|
|  |  |  |  |

Figure 21. Different analytical sieves used in this analysis.

### III.1.4. Delipidation

20 g of the obtained powder was placed in 40 ml of cold acetone and left to infuse for 2 hours at (-20°C). After filtration through filter paper (150 $\mu$ m), the acacia seed powder was washed 4 to 7 times with cold acetone until the solvent became clear and transparent. The extracted powder was dried on filter paper at room temperature and then stored at 4°C for use in the following reactions.

### III.1.5. Hydrolysis Procedure in the Presence of Plant Extract

2.5 g of olive oil and 1.25 ml of n hexane in a flask are heated to 50°C and stirred for 10 minutes, then 0.5 g of the lipase extract is added and the mixture is incubated for 30 minutes with continuous stirring (Figure 22). The addition of 12.5 ml of a mixture of acetone-phenolphthalein 0.1% (50/50) interrupts the incubation and simplifies the measurement of enzyme activity by volumetric assay.

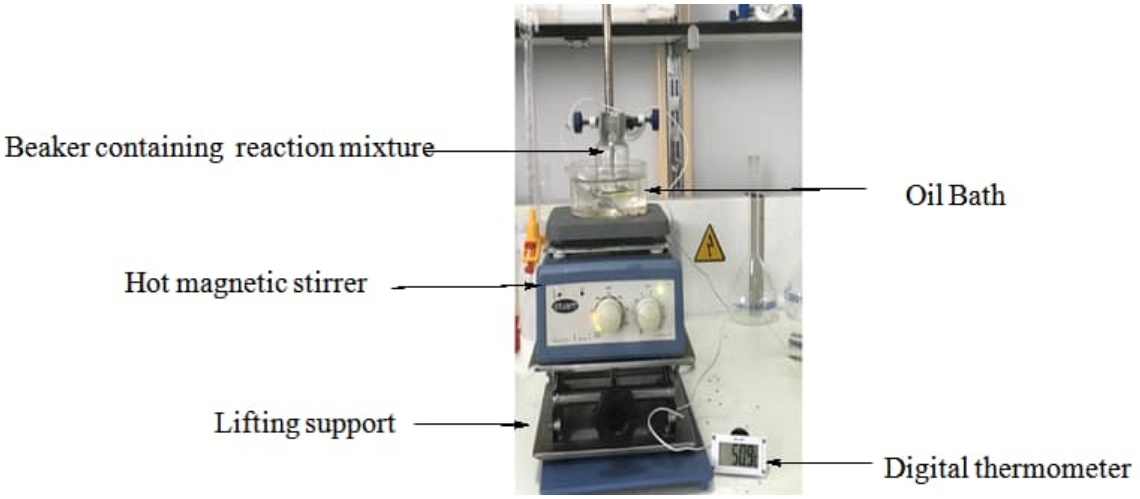
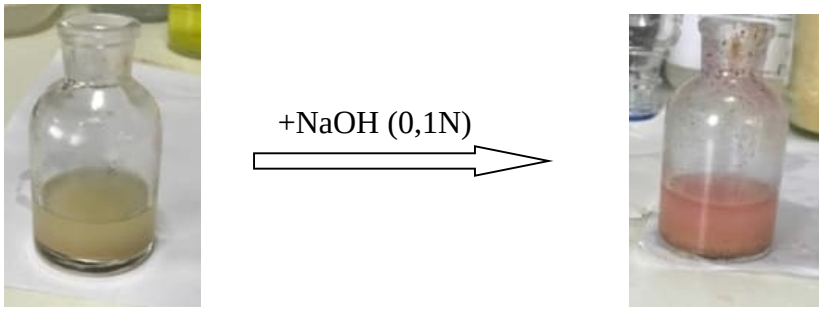



Figure 22. Experimental Setup for Olive Oil Hydrolysis in the Absence or Presence of ASL extract

## IV. Measurement of Lipase Activity by Volumetric Assay

The amount of fatty acid released (FFAs) was determined by direct determination using a sodium hydroxide solution (0.1 M). This solution was added until the solution turned a persistent pink (Figure 23).




Before addition of NaOH

After the addition of NaOH at the equivalence point (pink color)

Figure 23. Titration of the Mixture after 30 minutes of Reaction.

Formula used to calculate specific activity **SA** and free fatty acids **FFAs** are:

$$SA = \frac{n\ (\mu mol)}{x(mg) \times t(mn)}$$

$$\%FFAs = \frac{100 \times C_{NaOH} \times V_{NaOH} \times MM_{Olive\ oiL}}{m_{Olive\ oil}}$$

n (µmol): Number of moles of free fatty acid.
x (mg) : Mass of plant extract in mg.
t (mn) : Reaction time in minutes

$MM_{Olive\ oil}$: Average molar mass of olive oil (=279.6(g/mol).
$m_{Olive\ oil}$ : Mass of olive oil in (g)
$C_{NaOH}$ and $V_{NaOH}$ : Concentration and volume of used NaOH

## V. Esterification reactions:

The esterification reaction began by placing a carbohydrate substrate glucose and stearic acid in a reaction flask containing 4 mL of n hexane. The mixture was stirred and heated to 60 °C for 15 minutes as a pre-incubation period to ensure substrate dissolution and thermal equilibrium. The reaction was then catalyzed by adding ASL and allowed to proceed with

continuous stirring for 24, 48, and 72 hours (Figure 24). Incubation was interrupted by adding 12.5 mL of an acetone mixture (50/50 v/v) with 0.1% phenolphthalein. The amount of fatty acid released (FFAs) was determined by direct determination using a sodium hydroxide solution (0.1 M). This solution was added until the solution turned a persistent pink the molar equivalence between the sugar and the acid was varied to achieve optimal conversion.

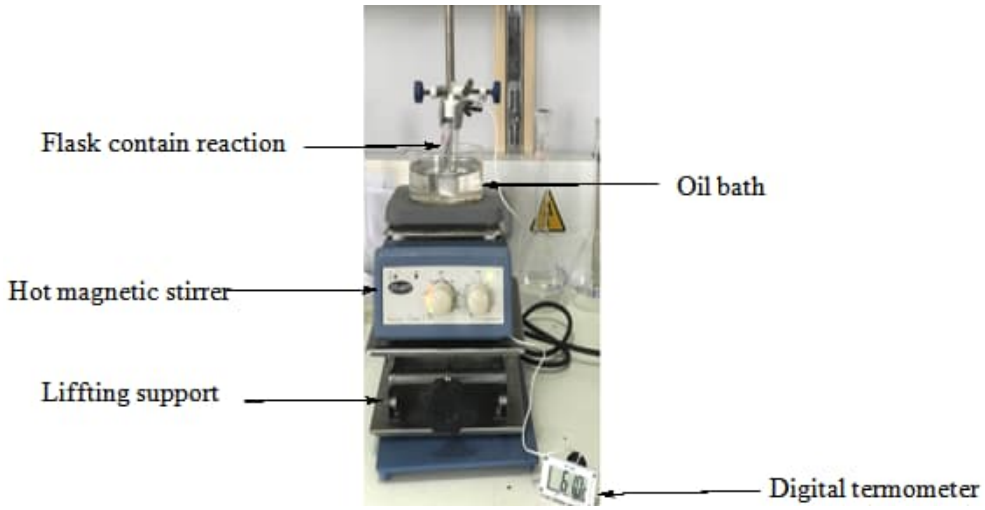



Figure 24. Experimental Setup for esterification reaction in the Absence or Presence of Lipase Extract.

The following formula was used to calculate conversion :

$$C\% = \frac{V_0 - V_t}{V_0} \times 100$$

$V_0$ : NaOH volume before adding ASL extract.

$V_t$:NaOH volume after adding ASL extract

## V.1. TLC Analysis:

### V.1.1. TLC plates used in this analysis:

Thin-layer chromatography (TLC) was performed using Merck silica gel 60 $F_{254}$ plastic backed plates, with 20*20 cm dimensions coated with 0.2 mm layer of silica gel. (Figure 25)


Figure 25. TLC plates on silica gel 60 $F_{254}$ used in this work.

Thin Layer Chromatography (TLC) was employed to monitor the progress of the enzymatic esterification and confirm the synthesis of new chemical species. The analysis compared the reaction products against three primary control references to validate the conversion.

### V.1.2.Elution and visualisation

The elution was performed using a Chloroform/Hexane (50:50 v/v) eluent system. The plate consisted of 4 primary application zones. The plates were visualized using a staining solution of 2% $KMnO_4$ and 4% $Na_2CO_3$, revealing the spots through oxidative revelation.[108]

The frontal report $\boldsymbol{R_f}$ : represents the ratio of the distance traveled by the product (h) to the distance travelled by the solvent (H), is specific to each substance.

$$\boldsymbol{R_f = h/H}$$

# General Conclusion

Plant biomass represents a potential source of cheap, abundant and easily exploitable biocatalysts without prior purification. Among these biocatalysts, lipases are extracted from different parts of the plant. The seeds are the main parts that contain more concentrations of lipases that play an important role in the growth of the embryo during germination by providing it with the energy necessary for its development. These lipases can be extracted easily and used in hydrolysis and synthesis. Among synthetic products, sugar esters are natural surfactants that are used as inexpensive, renewable and biodegradable raw materials.[35]

This project was carried out with the aim of extracting a lipase from acacia seeds (ASL) to measure enzymatic activity and test the catalysis of the esterification of stearic acid by glucose. ASL was successfully extracted and its specific activity was determined **SA=0.0123** IU/mg in the presence of olive oil as substrate in hexane at 50°C. Furthermore, this extract was used as a catalyst for enzymatic esterification, in different ratios of glucose to stearic acid, in the presence of ASL in hexane at 60 °C. After many attempts, the best result (**C%= 24.94%**) was obtained after 24h of reaction with a molar ratio of 2,5/1. In addition, TLC analysis confirmed the formation of a new product which is probably a mono-ester.

The findings of this work open up broad perspectives for studying advanced strategies in lipase utilization, focusing on several critical aspects:

- The production of ASL under optimized conditions.
- The optimization of esterification conditions taking into account all the parameters influencing this reaction.
- The separation and identification of the reaction product.

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