International Journal of Environment, Agriculture and Biotechnology
Vol-8, Issue-6; Nov-Dec, 2023
Peer-Reviewed International Journal
Journal Home Page Available: https://ijeab.com/
Journal DOI: 10.22161/ijeab

Lipases: Sources, immobilization techniques, and applications

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Received: 30 Oct 2023; Received in revised form: 01 Dec 2023; Accepted: 08 Dec 2023; Available online: 16 Dec 2023

Abstract**—** *Enzymes serve as natural catalysts that exhibit high specificity to their respective substrates and function effectively under mild temperature, pressure, and pH conditions, resulting in superior conversion rates compared to traditional chemical catalysts. These catalysts, sourced from animals, plants, and microorganisms, offer versatility, with lipases standing out for their broad applicability, capturing the interest of various industries. However, the widespread adoption of soluble lipases is hindered by challenges such as high acquisition costs, limited operational stability, and difficulties in recovery and reuse. To address these limitations, enzymatic immobilization has emerged as a viable alternative, aiming to enhance the stability of soluble enzymes while simplifying their recovery and reuse processes. This approach significantly mitigates the overall cost associated with enzyme-dependent processes. This review examines the diverse sources of enzymes, explores various immobilization methods for lipases, and discusses their wide-ranging applications.*

Keywords**—** ***Lipase, Classification, Sources, Immobilization, Application.***

I. INTRODUCTION

Enzymes stand out as efficient biocatalysts with significant potential, particularly in the food industry, where they offer a range of benefits such as safety, efficiency, specificity, controlled reactions, and minimal energy and chemical consumption. Their widespread application in the manufacturing of fats and oils, guiding the transformation of raw materials into final products, has become increasingly prevalent (Cieh et al., 2023; Dijkstra, 2009). The use of various enzymes in lipid processing and modification, though met with varied success within the inherent limitations of bio-catalytic reactions, has gained considerable attention in the field of enzymology over the past few decades (Pliego et al., 2015). Enzyme catalysis represents a notable initiative facilitating reaction processes under near-ambient conditions, showcasing enhanced specificity and velocity. This approach has played a key role in numerous industrial scenarios, contributing significantly to process intensification (Wohlgemuth et al., 2015).

Lipases (E.C.3.1.1.3) are enzymes that possess specific properties concerning their substrates. These properties include chemo-, region-, and stereo-specificity. They can facilitate heterogeneous reactions in systems that are both water-soluble and water-insoluble. Due to their broad catalytic properties, lipases are widely utilized as biocatalysts in numerous industries, including agrochemicals, pharmaceuticals, detergents, tanning, food, and surfactant production (Ananthi et al., 2014; Iftikhar et al., 2012; Kumar et al., 2012; Thakur et al., 2014).

Additionally, lipases are positioned as the third most prevalent enzymes in terms of usage, after amylases (carbohydrases) and proteases, owing to their diverse utility (Ülker et al., 2011). Lipases, also known as fat-splitting enzymes, triacylglycerol acylhydrolases, or glycerol ester hydrolases, are a class of enzymes that catalyze hydrolysis processes. They catalyze the hydrolysis of triglycerides in these processes, transforming them into fatty acids and glycerol at the oil-water interface. Notably, lipases can also reverse this reaction in aqueous and non-aqueous conditions. (Laachari, El Bergad, et al., 2015; Lee et al., 2015; Nadeem et al., 2015; Priji et al., 2015; Ramos-Sánchez et al., 2015). Parts of the α/β-hydrolase fold family, lipases, possess an active site containing a catalytic triad of Ser-His-Asp/Glu located beneath a brief amphiphilic helical segment that obstructs substrate access. Interacting with hydrophobic substrates prompts a structural rearrangement, leading the enzyme to shift from a closed to an open conformation, allowing accessibility to the active site (Miled et al., 2003; Verger, 1997). Specific lipases exhibit enantioselective properties and catalyze processes such as esterification, interesterification, transesterification, acidolysis, and aminolysis (Hasan et al., 2009). The substrates of lipases, which consist of triacylglycerols with lengthy chain lengths, are water-insoluble. A two-phase system is produced by dissolving these substrates in organic solvents before combining them with a buffer. Lipases, which can catalyze reactions in both aqueous and organic media, are water-soluble. However, applying organic solvents presents difficulties due to the potential for denatured and conformationally altered lipases, which could impact their functional and catalytic capabilities (Guo et al., 2015). In particular, several lipases have a movable lid structure that, depending on displacement, either permits or prohibits access to their active site. The lipase lid usually remains closed in an aqueous media, hiding the active site. But the lid can open at a contact between water and an organic solvent. Based on molecular dynamics simulations, the lipase lid can move in nonpolar conditions, providing access to the active site and making the lipase active while the lid is open. In contrast, the lipase is inactive when the lid is closed (Barbe et al., 2009). The benefits of using lipases as biocatalysts include excellent specificity and selectivity and the capacity to sustain catalytic activity in both aqueous and non-aqueous conditions (Sánchez et al., 2018; Vanleeuw et al., 2019; Zou et al., 2023). As the manufacturing industry increasingly focuses on climate change and environmental concerns, there is a gradual shift towards developing alternative, greener, safer, and sustainable processes (Wohlgemuth et al., 2015).

"Immobilized enzymes" are enzymes that are restricted or localized physically; however, they still can catalyze reactions and be recycled (Brena et al., 2013). Enzyme immobilization technology is acknowledged as a potent tool for adjusting and customizing a range of catalytic characteristics, such as enzyme activity, selectivity, specificity, stability at varying pH and temperature ranges, inhibitor resistance, and recyclability over multiple catalytic cycles (Bilal, Asgher, et al., 2019; Bilal, Zhao, et al., 2019). Additionally, the products produced have a higher purity because the biocatalyst may be easily removed from the reaction medium when enzymes are attached to solid supports (Homaei et al., 2013; Singh et al., 2013; Zdarta et al., 2018; Zhang et al., 2015). Choosing the suitable support materials and the immobilization techniques used is an important challenge to obtaining the desired immobilization results. This is important because it significantly affects the catalytic system's and the enzyme's characteristics. Several parameters define suitable immobilization support, including pore width, specific surface area, mechanical resistance, internal geometry, and support activation degree (Bilal, Asgher, et al., 2019). This contribution offers a thorough overview and discussion of the most recent advancements in the application of immobilized enzymes in the food industry, taking into account the body of knowledge currently available on enzyme immobilization, which includes a variety of strategies, supports, and applications (Bilal, Asgher, et al., 2019; Datta et al., 2013; Jesionowski et al., 2014; Mohamad et al., 2015; Zdarta et al., 2018). They are used in the fruit juice, dairy, baking, and brewing industries and provide fresh perspectives, especially when considering the food industry.

This review aims to provide a comprehensive and up-to-date overview of lipase research by combining recent studies and developments in classification, sources, immobilization techniques, and various industrial applications.

II. CLASSIFICATION OF LIPASES

This section discusses the categorization of lipases based on their specificity, while the subsequent part is focused on exploring the sources of lipases. Basically, lipases can be categorized according to specificity and origin, as depicted in **Fig. 1**. **Table 1** summarizes the functional characteristics of distinct lipase classes, highlighting examples from previous year's research developments.

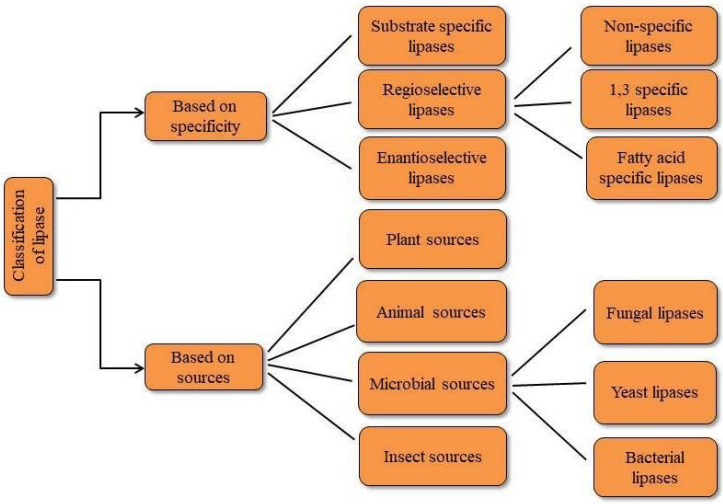

Fig.1: Classification of lipases

2.1. Based on specificity

This section provides an overview of the classification of lipases according to their specificity. The subsequent section focuses on the sources of lipases. Their specificity significantly influences the characterization of the industrial applications of lipases; thus, these uses can be classified into three broad categories: (i) substrate-specific, (ii) regioselective, and (iii) enantioselective.

2.1.1. Substrate specific lipases

Lipases exhibiting substrate specificity play an important role in reactions that precisely target particular substrates within a mixture of raw materials. This precision is instrumental in achieving desired outcomes, as exemplified by the effective utilization of lipases in processes such as biodiesel production (Ribeiro et al., 2011) and the production of high-purity diacylglycerols (Borza et al., 2015). Typically, these specialized lipases demonstrate proficiency with substrates like fatty acids and alcohols (Kapoor & Gupta, 2012). Recent research underscores the paramount importance of considering both substrate specificity and enzyme stability, highlighting their fundamental roles in optimizing the application of lipases across various industrially significant processes (Brígida et al., 2014).

2.1.2. Regioselective lipases

Regioselective lipases are essential for maximizing desirable side effects and guiding reactions in the right direction. This characteristic is very important, especially in the chemical and pharmaceutical industries, where it is crucial to produce isomeric molecules that perform best in specific configurations. The acylation of quercetin with ferulic acid, using *Rhizopus oryzae* lipase to synthesize flavonoid derivatives (Kumar et al., 2016), the deprotection of per-O-acetylated thymidine, producing 3'-OH-5'-OAc-thymidine using lipase from *Candida rugosa* (Rivero & Palomo, 2016), and the synthesis of acacetin and resveratrol 3,5-di-O-beta-glucopyranoside using *Candida antarctica* lipase B (Novozym 435) and *Burkholderia cepacia* lipase (Amano PS-IM) are examples of recent discoveries in the field of regioselective lipase (Hanamura et al., 2016). Lipases are classified into three classes based on positional specificity or regiospecificity.

2.1.2.1. Non-specific lipases

These particular types of lipases are exceptionally versatile, acting on a wide range of substrates. *Mucor meihei* lipases are a prime example of this, as they are versatile enough to catalyze a wide range of reactions, from the manufacture of biodiesel to uses in the cosmetics sector. These lipases generally catalyze the hydrolysis of triacylglycerols into glycerol and free fatty acids, using mono- and diacylglycerols as intermediates. The method's ability to function under ambient circumstances with little thermo-degradation makes it very beneficial (Kapoor & Gupta, 2012; Ribeiro et al., 2011). High-purity structured lipids have been produced through the acidolysis of canola oil with caprylic acid using the non-specific *Candida Antarctica* lipase, according to a study by Savaghebi et al. (2012).

2.1.2.2. 1,3 specific lipases

These lipases help hydrolyze triacylglycerols at the C_1 and C_3 locations, which produces fatty acids 1, 3 or 2, 3 diacylglycerols and 2-monoacylglycerols. The latter two molecules become unstable, which causes acyl migration and the synthesis of 1- or 3-monoacylglycerols and 1,3-

diacylglycerol (Barros et al., 2010). It is noted that the synthesis of diacylglycerols happens considerably more quickly than the conversion of triacylglycerols into monoacylglycerols (Ribeiro et al., 2011). A specific conformation is produced by the 1,3 specificity, as demonstrated in the example of *Carica papaya* latex lipase (Rivera et al., 2017). Recently, long-chain fatty acids have been synthesized by acidolyzing walnut oil with caprylic acid using 1,3-specific immobilized lipases from *Rhizomucor miehei* (Lipozyme) and *Rhizopus delemar* (PP-RhDL) (Todorova et al., 2015). Moreover, biodiesel production has used 1,3-specific immobilized *Rhizopus oryzae* lipase produced in Pichia pastoris (Clementz et al., 2016).

2.1.2.3. Fatty acid-specific lipases

These lipases work well to degrade esters that contain double bonds on C-9 of long-chain fatty acids (Ribeiro et al., 2011). A detailed study into lipases with fatty acid selectivity showed several enzymes that target various substrates with various carbon chain lengths, saturation levels, and unique side chains. Some lipases showed strong selectivity for medium- or long-chain and branched esters. In contrast, lipases sourced from S9 *Geotrichum candidum*, S11 *Candida lypolytica*, YM *Bacillus coughing*, MJ1 *Aspergillus niger*, MJ2 *Aspergillus oryzae*, and S3 *Penicillium citrinum* showed unique specificity for short-chain esters (Song et al., 2008). Selected microbial strains that could produce lipases suited for medium- and long-chain saturated fatty acids were found through screening in another study (Miettinen et al., 2013).

2.1.3. Enantioselective lipases

In a racemate, enantioselective lipases hydrolyze one isomer more than the other, especially when starting with prochiral precursors. These lipases are very good at separating enantiomers from a racemic mixture (Barros et al., 2010). Enatiospecific lipases catalyze various processes, such as the transesterification of secondary alcohols into drugs (Borza et al., 2015), the hydrolysis of menthol benzoate for the production of cosmetic and food items (Dhake et al., 2013), and the hydrolysis of glycidic acid methyl ester for the synthesis of medical and health care products (Su et al., 2014).

Table 1: Classification of lipases based on specificities.

Classification basis	Reaction details			Application	References
	Substrate-enzyme system	Type	Mode		
Substrate specific	Camellia oil + *Penicillium camembertii* lipase	Esterification	Batch	Production of high purity di-acylglycerols	(P. Zheng et al., 2014)
Regio-selective	Novozym 435, nonspecific *Candida antarctica* lipase + refined, bleached and deodorized (RBD) canola oil	Acidolysis (nonspecific)	Batch-shaker flask systems	Production of structured lipids (SLs)	(Savaghebi et al., 2012)
	1,3-specific immobilized lipases from *Rhizomucor miehei* +Walnut oil + caprylic acid	Acidolysis (1,3 specific)	Batch-immobilized enzyme system	Production of MLM-type structured lipids	(Todorova et al., 2015)
	1,3 specific immobilized lipase from *Rhizopus oryzae*	Transesterification (1,3 specific)	Fed-batch shake flask system	Biodiesel	(Clementz et al., 2016)

	+ olive oil + methanol + hexane				
	MJ2 *Aspergillus oryzae* lipase is highly specific to methyl butyrate	Hydrolysis (fatty acid specific)	Batch-silica gel GF_{254} plates	Transportation fuel, wastewater denitrification, fuel cell hydrogen carrier production, biodiesel transesterification, electricity generation	(Song et al., 2008)
Enantio-specific	*C. antarctica* lipase A entrapped in sol-gel + Secondary alcohols	Transesterification	Batch-shaker flask systems	Large-scale processes in pharmaceutical industry	(Borza et al., 2015)

2.2. Based on sources

Lipases are found extensively in plants, animals, insects, and microbial organisms (Maldonado et al., 2014; Patil et al., 2011; Ray, 2012). Microbes are particularly important among the various sources of lipase due to their substantial industrial potential, easy culture handling, wide availability, and potential for scalable production (Patil et al., 2011).

2.2.1. Plant sources

Plant materials that include lipases include latex, leaves, bran, seeds, beans, and fruits. Plant lipases are particularly abundant in seed sources such as castor bean, African bean, elm, sunflower, physic nut, lupin, linseed, coconut, almond, black cumin, wheat grain, rice, corn, oat, barley, sesame, sorghum, etc. Since seeds are the primary energy source for plant growth and contain a high concentration of triacylglycerols, they have higher lipase activity than other plant components. Due to increased lipase activity, triacylglycerols in plant seeds are converted into soluble sugars during germination (Patil et al., 2011). As detailed in the provided **Table 2**, these lipases find diverse applications, including the hydrolysis of vegetable oils (Salaberría et al., 2017), processing of seeds and oils (Lampi et al., 2015; Mohd Zin et al., 2017), production of structured lipids (da Silva Serres et al., 2017), beverage production (Moreau et al., 2016), and synthesis of pharmaceutical and therapeutically significant compounds (Hamden et al., 2017). Lipases derived from palm fruit mesocarp have proven effective in manufacturing pharmaceuticals, detergents, and cosmetic products (Suwanno et al., 2017). Recent studies highlight the efficiency of lipases from the drumstick tree in treating obesity (Kadouf et al., 2015).

Plant lipases are attractive because of their direct use as biocatalysts, accessible acceptability, particular uses, and inexpensive cost. However, their investigation has been restricted due to poor abundance, instability, and activity loss during conventional purifying procedures (Seth et al., 2014). Addressing these challenges, a novel technique based on immuno-purification has been developed for rapeseed lipase preparation (Belguith et al., 2013). Research is ongoing to get lipases from inedible plant sources so that different fatty acids can be hydrolyzed to produce biodiesel (Banković-Ilić et al., 2012).

Table 2: Lipases from plant sources.

Lipase source	Applications	References
Castor bean	Hydrolysis of vegetable oils.	(Salaberría et al., 2017)
Barley	Hydrolysis of lower molecular weight water-soluble substrates and long-chain insoluble triglycerides.	(Schneider et al., 2016)
Rice/rice bran	Preferential hydrolysis of *sn*-2 position of phosphatidylcholine.	(Qi et al., 2015)
Almond	Hydrolysis of oil.	(Huang et al., 2017)

Carica papaya latex	In reactions involving fats and oils demanding *sn*-3 selectivity, interesterification, transesterification and acidolysis using homogenous triacylglycerol and various other acyl donors industrially and also a crucial component in cure for dengue.	(Rivera et al., 2017)
Elm	Synthesis of emulsifiers or oiling agents for foods, spin finishes and textiles, antifoaming and antistatic agents for plastics and lubricants, water treatment, metal working fluids, personal care products and dispersing agents from tricaprin.	(Barros et al., 2010)
Fenugreek	Synthesis of pharmaceutical and therapeutically significant products.	(Hamden et al., 2017)
African bean	Production of fermented condiments such as Iru, Soumbala, daddawa, sonsu, afitin, ugba	(Seth et al., 2014)
Sunflower seed	Production of structured lipids by action on other oils.	(da Silva Serres et al., 2017)
Physic nut	Production of biodiesel within a hybrid system of chemical and enzymatic process.	(Sousa et al., 2015)
Sorghum	Production of alcoholic beverages.	(Moreau et al., 2016)
Palm fruit mesocarp	Pharmaceuticals, industrial detergent, food and cosmetics.	(Suwanno et al., 2017)
Coconut	Processing coconut oil.	(Mohd Zin et al., 2017)
Oat	Processing oats.	(Lampi et al., 2015)
Vermonia sp	Specific towards trivernolei.	(Barros et al., 2010)
Lupin	Specific towards lupin oil to release fatty acids from them.	(Stephany et al., 2016)
Sesame	Non-specific lipases.	(Oliveira et al., 2017)
Linseed	Treatment of triacylglycerols in acidic environment.	(Qiu et al., 2017)
Moringa olifera (Drum stick tree)	Treatment of obesity.	(Kadouf et al., 2015)
Black cumin	For enrichment of Γ- Linolenic acid in the unhydrolyzed acylglycerol fractions of Γ-Linolenic acid containing oils.	(Siow et al., 2016)
Wheat grain	Determination of the storage quality of wheat and wheat bran.	(Ahmad et al., 2015)
Corn	To act on oleyl ester than stearyl ester to release fatty acids.	(Eze et al., 2007)

2.2.2. Animal and insect sources

Lipases are produced by animal cells and are necessary for digesting fats and lipids (Patil et al., 2011). However, because of the difficulties in managing cultures and separating products, these animal lipases are used more frequently in clinical diagnosis than in commercial production. Animal lipases have not been explored as much as plant and microbial lipases. **Table 3** below lists several animal tissue-derived lipases used in different research projects. Among the variety of animal lipases, pancreatic lipase has been widely used as a tool for lipid

chemistry and biochemistry research, exhibiting efficient catalysis in the hydrolysis of primary alcohol esters (Pahoja & Sethar, 2002).

Lipases derived from the porcine pancreas play a significant role in preparing Monoacylates of 2-Substituted (Z)-But-2-ene-1,4-diols (Kawashima et al., 2016), asymmetric aldol reactions (J. Zheng et al., 2014), and the synthesis of bis (indolyl) alkanes (Xiang et al., 2013). Furthermore, starfish lipases are a viable substitute for porcine pancreatic lipases and have significant uses in the food processing sector, as shown in **Table 3**. It is important that insect tissues also yield lipases, primarily contributing to the development of the insect's larval stage (Sakate & Salunkhe, 2013) or residing in their gut (Delkash-Roudsari et al., 2014; Rong et al., 2014). Research shows that lipases active against diacylglycerols are found in various insect flight muscles and bodily tissues, except for the alimentary canal (Pahoja & Sethar, 2002). Research on gut lipase activity to create bio-control agents is becoming increasingly popular (Khan et al., 2012; Sandhu et al., 2012).

Table 3: Lipases from animal and insect sources.

Lipase sources	References
Human pancreas	(Borrelli & Trono, 2015)
Human gastric cells	(Patil et al., 2011)
Porcine pancreas	(Borrelli & Trono, 2015)
Guinea pig pancreas	(Borrelli & Trono, 2015)
Cyprinion macrostomus	(Patil et al., 2011)
Chicken adipose cells	(Patil et al., 2011)
Scorpion	(Patil et al., 2011)
Rainbow trout	(Kittilson et al., 2011)
Dasyatis pastinaca	(Borrelli & Trono, 2015)
Seabass liver	(Sae-Leaw & Benjakul, 2018)

2.2.3. Microbial sources

Microbial lipases, derived from bacteria, yeast, and fungi, are desirable for industrial use because of their ease of production and adaptability (Ray, 2012). The following lists the several biotechnological uses for which microbial lipases are helpful because of their remarkable selectivity. These lipases are predominantly extracellular and mainly produced from bacterial and fungal species. Physical and chemical parameters like temperature, pH, and dissolved oxygen significantly impact their creation, as does the makeup of the medium (Thakur, 2012). Further insights into microbial lipases can be gained from recent review papers covering topics like the general overview of microbial lipases (P Kanmani et al., 2015), microbial alkaline lipases (Niyonzima & More, 2015), and the purification of microbial lipases (Show et al., 2015). Recent findings indicate that microbial lipases can be obtained through the fermentation of agricultural waste (Zubiolo et al., 2015) and dairy waste (Marques et al., 2014), contributing to environmental protection. This opens up possibilities for potential future bio-catalytic applications.

2.2.3.1. Fungal lipases

Among the several types of microbial lipases, fungal lipases are widely used due to their unique characteristics, which include substrate specificity, stability at both pH and temperature, affordability during extraction, and effective activity in organic solvents (Patil et al., 2011). Because of the carbon and nitrogen content of the medium, these lipases can be found either intracellularly or extracellularly (Sharma et al., 2010). Fungal lipases are interesting for their exceptional adaptability, as they may catalyze a variety of processes such as alcoholysis, acidolysis, saponification, ethanolysis, hydrolysis, esterification, transesterification, deacetylation, and hydrolytic kinetic resolution.

Solid-state fermentation technology holds great potential for producing fungal lipases, as evidenced by the latest developments in this field (Ramos-Sánchez et al., 2015). A further review focuses on the molecular and functional variety of fungal lipases, examining transdisciplinary strategies that lead to enhanced substrate selectivity and thermostability (Gupta et al., 2015). A special review also covers the use of fungal lipases to produce biodiesel, outlining developments and important

variables that impact lipase stability and activity, like the type of biocatalyst used, the water content, solvent usage, and raw material selection (Aguieiras et al., 2015).

Recent research on fungal lipases includes isolating several fungal strains from Divle Cave cheese, which identified *Mucor racemosus* as a strong lipase producer with possible uses in the cheese industry (Ozturkoglu-Budak et al., 2016). Several fungal strains were screened to assess their lipase-producing capabilities using various substrates, such as sugar cane bagasse, soybean bran, and wheat bran (Fleuri et al., 2014). *Aspergillus niger*, *Nectria haematococca*, and *Trichoderma reesei* are a few examples of fungal sources whose lipases were cloned, expressed, and characterized, and their catalytic activity was compared by Vaquero et al. (2015). A study by Khasanov et al. (2015) examined the catalytic activity of fungal lipases isolated from *Rhizopus microsporus*, *Penicillium sp*., and *Oospora lactis*. In addition, the immobilization of fungal lipases from *Rhizomucor miehei* and *Thermomyces lanuginosus* on nanozeolite supports was studied by de Vasconcellos et al. (2015).

2.2.3.2. Yeast lipases

Because of their unique properties and ease of cultivation, yeast lipases are an essential source of enzymes in great demand across various industries, including biodiesel, chemicals, and pharmaceuticals. The primary yeast producers of lipases are *Candida antarctica*, *Candida rugosa*, *Candida utilis*, and *Saccharomyces* species. These enzymes catalyze a wide range of processes and find applications in various industrial sectors. A literature review indicates that lipases derived from *Candida rugosa* are important examples of biocatalysts that are effective in various reactions. These reactions include the hydrolysis of conjugated linoleic acid methyl ester by adding an organic solvent (Kobayashi et al., 2012), transesterification of palm oil with methanol and ethanol (Moreno-Pirajan & Giraldo, 2011), and enantioselective hydrolysis of menthol benzoate (Dhake et al., 2013), etc. Another significant yeast source is *Candida antarctica*, effectively employed in the synthesis of valuable fatty acids (L. D. Santos et al., 2015), biodiesel production through the transesterification of *Simarouba glauca* oil (Garlapati et al., 2013), and the production of pharmaceutical products through acylation and alcoholysis (Baldessari & Iglesias, 2012). Additionally, *Candida antarctica* lipases are utilized in the synthesis of cosmetic and detergent products by acidolysis of butter oil with conjugated linoleic acid (Garcia et al., 2001) and acidolysis of acyl glycerols (Senanayake & Shahidi, 2002).

Yeast lipases, enzymes from different yeast species involved in diverse processes, are essential to biodiesel synthesis. *Thermomyces lanuginosus* lipases, for example, are used in the hydro esterification of soybean oil (Cavalcanti-Oliveira et al., 2011), *Rhodotorula mucilaginosa* in the esterification of palm oil (Nuylert & Hongpattarakere, 2013), *Thermomyces lanuginosus* in the hydrolysis of soybean oil (Cavalcanti-Oliveira et al., 2011), and *Thermomyces lanuginosus* in the transesterification of rapeseed oil (Price et al., 2014). An overview of the production and use of *Candida lipolytica/Yarrowia lipolytica* lipases in the bio-surfactant manufacturing process is given in a recent review by Brígida et al. (2014).

2.2.3.3. Bacterial lipases

Bacterial lipases can be found in several parts of cells, such as extracellular, intracellular, or membrane-bound. Some extracellular bacterial lipases are lipoproteins, while the majorities are glycoproteins. Bacterial lipases are remarkably numerous and frequently described as thermo-stable and substrate non-specific (Dhake et al., 2013; Vaquero et al., 2016). Recent studies highlight the thermal stability and industrial potential of bacterial lipases, such as those from *Thermophilic Bacterium*, *Bacillus licheniformis* lipases (Rashid et al., 2013), *Bacillus pumilus* lipase isolated from tannery waters (Laachari, El Bergadi, et al., 2015), and extracellular lipase of *Bacillus licheniformis* (Rashid et al., 2013). For instance, the lipase *Serratia marcescens* N3 is used to hydrolyze several types of edible oils, and it is most active when applied to Gingily oil (Zaki & Saeed, 2012). Serratia effectively carries out the kinetic resolution of racemic alcohols in organic solvents *marcescens* H30 lipase (Su et al., 2014). *Staphylococcal* lipases isolated from facial sebaceous skin are used as biocatalysts in the food, cosmetic, pharmaceutical, and detergent sectors (Xie et al., 2012).

Several bacterial lipases exhibit promising applications in biodiesel production, such as *Geobacillus thermodenitrificans* AV-5 (Christopher et al., 2015), cross-linked *Staphylococcus haemolyticus* lipase immobilized on solid polymeric carriers (Kim et al., 2013), *Pseudomonas fluorescens* lipase (Salis et al., 2009), *Staphylococcus haemolyticus* L62 lipase expressed in *Escherichia coli* cells (Kim et al., 2013), and *Staphylococcus haemolyticus* L62 lipase (Jo et al., 2014). Furthermore, the use of elite-immobilized crude lipase produced from *Staphylococcus pasteuri* for the pretreatment of coconut mill effluent shows the effectiveness of bacterial lipases in waste management and treatment (Palanisamy Kanmani et al., 2015) and by the lipase from *Pseudomonas aeruginosa*

AAU2 in treating lipid-rich industrial effluents (Bose & Keharia, 2013).

III. IMMOBILIZATION TECHNIQUES

Enzymes function as biological catalysts in enzymatic reactions, enhancing reaction rates without undergoing depletion. This property allows for the repeated utilization of enzymes as long as they remain active. Various methods for immobilizing enzymes on solid surfaces have been developed, and these methods are classified as follows and shown in **Fig. 2**.

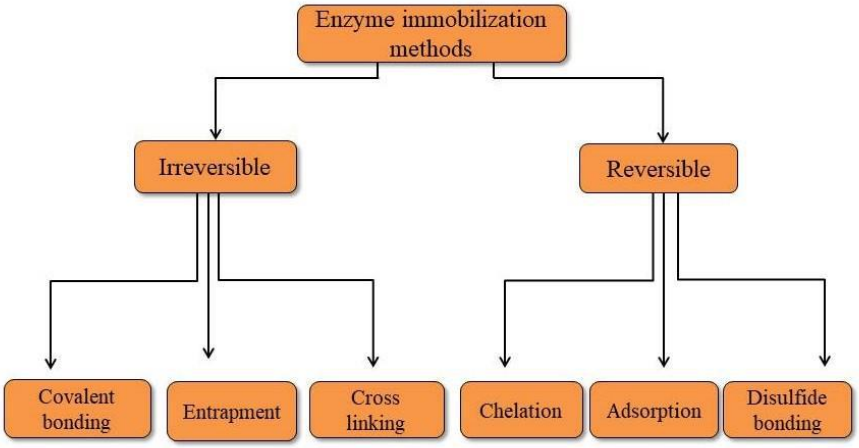

Fig.2: Schemes for major enzyme immobilization methods

3.1. Irreversible mechanism

In irreversible immobilization, the only way to separate a biocatalyst from a support is to undermine its biological activity. The most popular approaches for permanently immobilizing enzymes include coupling and cross-linking procedures and covalent trapping like microencapsulation.

3.1.1. Covalent bonding

Making covalent connections between proteins and a support matrix is often used for permanently immobilizing enzymes. Because of the stronger interactions created between the matrix and the enzyme, this method has the benefit of keeping the enzyme from escaping into the solution while it is being used. In some cases, keeping amino acid residues essential for catalytic action from being involved in the covalent attachment to the support can be challenging, which makes achieving high binding activity difficult. During the coupling reaction, substrate analogues can be added to the reaction media as a simple way to improve this activity (Brena et al., 2013). Covalent techniques are usually used for immobilization when the enzyme does not need to remain in the finished product. Researchers have developed various reactions, considering the functional groups present in the matrix (Jasti et al., 2014). In a broad classification, coupling methods are divided into two main groups: (1) activating the matrix by adding a reactive function to a polymer and (2) modifying the polymer backbone to generate an activated group. Activation approaches are typically recommended to provide electrophilic groups on a set of supports that react with potent nucleophiles on proteins during the coupling step. The covalent binding of covalents to matrices is governed by parameters comparable to those used in protein chemical modification. Various commercially available supports are available for immobilization; the best one to employ will rely on the properties of the catalyst and its intended usage. To find the best method depending on specific circumstances, it is often essential to experiment with a number of different approaches (J. C. S. d. Santos et al., 2015). When covalent processes are used, ether, amide, carbamate, and thioether bonding are frequently used by enzymes to attach to the support. As a result, a strong link known for its high stability is established between the matrix and the enzyme. Because the linkage is covalent, throwing out the matrix and the enzyme becomes important when the enzymatic activity decreases.

A leak-proof link between the enzyme and the matrix can be formed using the covalent immobilization technique, but there are also disadvantages, including high cost, limited immobilization activity yield, and irreversibility. However, modifications to the immobilization method can alleviate these constraints to

differing degrees. A number of variables, including the carrier material's size, shape, and composition, as well as the particulars of the coupling reactions, affect the activity of covalently immobilized enzymes (Bilal, Asgher, et al., 2019). For covalent bond-based immobilization, hydrophobic or hydrophilic supports can be used; silica/chitosan-based supports have also been shown to be effective carriers for covalent immobilization in recent research (Cazaban et al., 2018; Manzo et al., 2018; Singh et al., 2017; Van Den Biggelaar et al., 2017). Due to its high surface area, low diffusional limitations, good mechanical properties, chemical resistance, and abundance of hydroxyl groups, silica is well-suited for surface functionalization with agents such as glutaraldehyde and 3-aminopropyltriethoxysilane (APTES) and enzyme attachment. Because of its advantageous structural characteristics, chitosan, a common natural biopolymer, is a hydrophilic, biodegradable, and biocompatible polysaccharide frequently employed for enzyme immobilization. Many covalent attachments of the amino groups on the enzyme's surface onto cross-linked agarose beads are an effective covalent technique for immobilizing enzymes. This procedure increases the enzyme structure's stiffness, giving it more stability against conformational changes by denaturing chemicals (Romero-Fernández & Paradisi, 2020). Covalent immobilization can also be site-directed to obtain immobilized enzymes with enhanced stability and reactivity. Using the Huisgen 1,3-dipolar cycloaddition "click" reaction and non-canonical amino acid incorporation, Wu et al. (2015) developed the PRECISE (protein residue-explicit covalent immobilization for stability enhancement) system, which allows for directed enzyme immobilization at specifically selected residues throughout an enzyme. This system facilitates the evaluation of the impact on activity and stability under severe conditions by enabling immobilization at both close and remote locations from the active site.

3.1.2. Entrapment and cross-linking

The entrapment approach works by encasing enzymes in a network of polymers, which allows products and substrates to pass through while keeping the enzyme in place. In contrast, this strategy does not limit the enzyme to a matrix or membrane-like coupling system. Numerous methods for entangling enzymes, including fiber entrapment, gel and microencapsulation, have been suggested by Livage and Coradin (2018). These approaches change the encapsulating material for ideal pH, polarity, or amphiphilicity to improve enzyme stability, minimize leaching and denaturation, and optimize the microenvironment (Nguyen & Kim, 2017). However, mass transfer limits through gels or membranes limit the practical applicability of these techniques. It's important to remember that more recent approaches, including cross-linked enzyme aggregates (CLEAs) and crystals (CLECs), have been proposed to immobilize enzymes (Galliani et al., 2018; Tran & Balkus Jr, 2011) and differ from conventional immobilization approaches. Though these techniques developed at the turn of the century, they are still the go-to option for enzymatic applications in biorefineries and the food sector. With their highly active immobilized enzymes and adjustable particle sizes, high catalytic and volumetric productivities, ease of recycling, and operational stability, CLECs are perfect for industrial biotransformations. However, a major drawback of this technique is the thorough protein purification needed to generate CLECs. With the addition of salts or nonionic polymers, enzymes from an aqueous solution precipitate to form physical aggregates of protein molecules, which is how the more recently produced CLEAs are an upgraded version of CLECs (Homaei et al., 2013; Nguyen & Kim, 2017). Because of their increased catalytic activity, improved operational and storage stabilities, ease of use, superior reusability, and multi-point attachment through intermolecular cross-linking between enzyme molecules, cleavage-free amnesias CLEAs have become a promising carrier-free immobilization system. Enzyme preparation from lipases, horseradish peroxidase, penicillin acylases, and laccases is the subject of many ongoing investigations to produce CLEAs of high yield (Šulek et al., 2011).

The development of multipurpose cross-linked enzyme aggregates (multi-CLEAs) and combined cross-linked enzyme aggregates (combi-CLEAs), which cross-link multiple enzymes together, is a development in the immobilization process. As a result, CLEAs have an increased ability to catalyze a range of biotransformation reactions, either one at a time or in succession as catalytic cascade processes (Bilal, Zhao, et al., 2019). Recent examples have been reported for these cross-linked immobilization approaches. Periyasamy et al. (2016) successfully combined b-1, 3-glucanase, cellulase, and xylanase for one-pot cascade saccharification of sugarcane bagasse (SCB). After six consecutive applications, the combi-CLEAs produced improved temperature and storage qualities, maintaining 90% of their activity (Periyasamy et al., 2016). Another example is the production of three distinct, independent catalytic reactions using multi-CLEAs of pectinase, xylanase, and cellulase. The produced multi-CLEAs showed excellent recyclability and thermostability (Dalal et al., 2007). Combining CLEAs with magnetic nanoparticles (M-CLEAs) has also increased their stability and reusability in the food industry. The benefit of this integration is that it is simple to separate from the reaction mixture, making it

easier to use again and increasing the possibility of developing continuous biocatalytic processes (Martins et al., 2018; Nadar & Rathod, 2016). In a recent advancement, magnetic combi-CLEAs based on glucose dehydrogenase and ketoreductase have been developed. Compared to the original CLEAs, they demonstrated better catalytic activity and stability in both aqueous and biphasic media (Su et al., 2018).

Furthermore, using CLEAs combined with the bio-imprinting process has been extensively investigated over the past years to improve the stability and catalytic efficacy of various enzymes. Imprinted CLEAs are a revolutionary combinatorial cross-linked imprinting technique (iCLEAs). Bio-imprinting technology is valuable for adjusting enzymes' stability, enantioselectivity, reusability, and catalytic characteristics. (Bilal, Asgher, et al., 2019; Cui & Jia, 2015; De Winter et al., 2012).

3.2. Reversible mechanism

Regarding the binding between the support and the enzyme, reversibly immobilized enzymes can separate from the support in mild circumstances. In response to this issue, reversible techniques have attracted much interest in enzyme immobilization, primarily because of their sound financial basis. The principal factor contributing to these enzymes' cost-effectiveness is their declining activity, which triggers regeneration and reloading of the support with new enzymes. The support cost primarily determines the catalyst's total immobilization cost. Reversible enzyme immobilization is very important for bioanalytical systems and the immobilization of labile enzymes (Bilal, Zhao, et al., 2019).

3.2.1. Adsorption (Non-covalent interactions)

Nonspecific adsorption: Nonspecific adsorption is a relatively simple technique for immobilization that operates predominantly through physical or ionic means of binding (Mohamad et al., 2015). Enzymes bind to the matrix through salt bonds in ionic bonding. On the other hand, in physical adsorption, the enzymes use hydrophobic interactions, hydrogen bonds, and van der Waals forces to bind to the matrix. Changes in ionic strength, pH, solvent polarity, and temperature, among other factors that regulate interaction strength, can reverse the process caused by the forces involved in non-covalent immobilization. The procedure of adsorption immobilization is mild and straightforward, usually maintaining the enzyme's catalytic activity. Despite their economic appeal, these methods have certain disadvantages, such as the possibility of enzyme leakage from the matrix in the event of weak connections.

Ionic binding: Utilizing protein-ligand interactions via chromatography is commonly used for reversibly immobilizing enzymes. The use of ion exchangers for enzyme reversible immobilization is an example of an early application of this method (Vaz & Filho, 2019). This method is reversible and convenient, but it can be challenging to identify the exact circumstances that ensure strong bonding and high enzyme activity. Lately, immobilized polymeric ionic ligands have been used to manipulate the protein-matrix interaction, allowing for the optimization of derivative characteristics. Despite the advantages, problems could occur, especially if highly charged supports are used, especially if the goods or substrates are charged. Diffusion and partition, for example, might upset kinetics in certain situations and change the enzyme's ideal pH and pH stability range (Ward et al., 2016). However, recent research over the last few years has shown that pH values can be adjusted to achieve ideal circumstances for a particular enzyme. (Benítez-Mateos et al., 2017; Furuya et al., 2017; Wang et al., 2017).

Hydrophobic adsorption: Hydrophobic interactions driven by entropy instead of chemical bond formation can also be achieved by the reversible immobilization of enzymes. This technique has been a chromatographic concept for over thirty years, relying on conventional experimental parameters; including temperature, pH, and salt content (Mohamad et al., 2015). Both the hydrophobicity of the protein and the adsorbent influence the intensity of the interaction. The size of hydrophobic ligand molecules and the support substitution level can be changed to modify the adsorbent's hydrophobicity. It has been possible to immobilize reversible β-amylase and amyloglucosidase to hexylagarose carriers (Das & Kayastha, 2019). The use of different supports, such as silica, magnetic nanoparticles, and synthetic beads made of polymethacrylate matrices, as support materials for the hydrophobic absorption method has been reported in research in recent years (Abreu Silveira et al., 2017; Gao et al., 2019; Hüttner et al., 2017; Koutinas et al., 2018; Srivastava et al., 2012; Vescovi et al., 2017).

Affinity binding: Determining the immobilization of enzymes has been attributed to the fundamental principle of utilizing the attraction between complementary biomolecules. The approach shows a significant benefit through a highly selective interaction. The requirement for covalent attachment of a costly affinity ligand to the matrix, such as an antibody or lectin, is a drawback (Brena et al., 2013). Strong connections between the protein structure and a surface functionalized with a complementary affinity ligand can be established using affinity tags, which can be intrinsic or injected at a

specified place away from the active site in the original enzyme structure. In contrast to random immobilization, which can result in the immobilized enzyme in numerous orientations, directed immobilization results from greater specificity in enzyme adsorption to support materials (Bolivar & Nidetzky, 2013).

One of the two methods for achieving enzyme immobilization based on affinity binding between enzymes and support materials is the ionic exchange or covalent bonding. A recent advancement in ionic immobilization is affinity binding, in which enzymes are fixed to substrates containing metal ions such as Ni^{2+}, Cu^{2+}, Co^{2+}, Fe^{3+}, and Zn^{2+} by use of a fused peptide tag containing a polyhistidine chain (His-tag) (Böhmer et al., 2018; Liu et al., 2017; Vahidi et al., 2018). (Böhmer et al., 2018) showed how ionic affinity binding allowed co-immobilizing chimeric amine dehydrogenase (AmDH) and alcohol dehydrogenase (ADH) on regulated porosity glass Fe^{3+} ion-affinity beads. Over five cycles, the immobilized dual-enzyme system demonstrated recyclability, with total turnover numbers for ADH and AmDH surpassing 4000 and 1000, respectively. Immobilization of enzymes with a fused discrete protein domain is a further form. Enzymes, for instance, fused to the modified Z_{basic2} binding domain are bonded to immobilization matrices with anionic surface groups by ionic interactions. Compared with smaller tags like the His-tag, this method provides a substantial benefit for oriented immobilization. Oriented immobilization is highly preferred because it uses the Z_{basic2} module more efficiently to achieve spatial separation between the immobilization matrix and the enzyme catalytic activity. This method reduces the possibility that surface binding will negatively affect the enzyme's activity (Bolivar et al., 2017; Romero-Fernández & Paradisi, 2020). The formation of covalent bonds between the enzyme and the immobilization support is another method for achieving affinity-based enzyme immobilization. This approach integrates a tiny peptide tag, a distinct protein domain, or a genetically fused protein into the target enzyme. It is comparable to affinity binding via ionic exchange. Attaching horse liver alcohol dehydrogenase, fused to a polyhistidine tag, onto metal-activated polymethacrylate support using epoxy groups is a recent development in covalent affinity binding (Contente & Paradisi, 2018). Using a second protein as a spacer to block the direct covalent connection between the enzyme and the immobilization matrix, this novel method of covalent immobilization via affinity binding increases the catalytic activity of the target enzyme. His-tag and different target enzymes are fused to form T4L lysozyme, the second protein rich in lysine amino acid, increasing immobilized enzymes' recovered activity (Planchestainer et al., 2017). A recent development is affinity binding to immobilize enzymes on magnetic beads, which improves the functionality of immobilized enzymes in analytical tests. Faster assay kinetics are achieved by the abundance of binding sites that magnetic beads provide for biological reactions (Sassolas et al., 2020).

3.2.2. Chelation or metal binding

Salts of transition metals or metal hydroxides, such as zirconium or titanium, applied to the surface of an organic carrier can create bonds by coordinating with nucleophilic groups on the matrix. The term "metal link immobilization" describes this method well (Singh et al., 2013). Usually, heating or neutralization causes the hydroxide or metal salt (such as chitin, cellulose, alginic acid, and silica-based carriers) to precipitate onto the support. Some coordination places in the metal still need to be occupied for coordination with groups in enzymes because steric constraints prevent the matrix from occupying all the coordination positions in the metal. Even though the process is relatively simple, the immobilized specific activity of enzymes ranges from 30% to 80%. However, the operational stabilities that are attained are frequently irregular and difficult to replicate, potentially because of irregular adsorption sites and metal ion leakage from the support. A way to overcome these difficulties is to use stable covalent bonds to immobilize chelator ligands on solid supports, enabling metal ions to be bound via coordination. As a result, stable complexes are formed, which can be used to retain proteins. Elution of the bound proteins can be accomplished by lowering the pH of the process or by competing with soluble ligands. The support is renewed by rewashing it with a potent chelator (such as ethylenediaminetetraacetic acid or EDTA). Immobilized Metal-Ion Affinity (IMA) adsorbents, which are these supports, are widely used in protein chromatography (Kagedal, 2011).

3.2.3. Disulfide bonds

This study's unique approach to irreversible enzyme immobilization is its capacity to create a stable covalent link between the enzyme and matrix that can be selectively broken down under mild conditions with the help of a suitable chemical such as dithiothreitol (DTT). Furthermore, thiol groups' reactivity can be managed by varying the pH, which produces high activity yields when disulfide bonds are created with the help of an appropriate thiol-reactive adsorbent (Batista-Viera et al., 2011). Under mild circumstances, enzymes with exposed nonessential thiol (SH) groups can be immobilized onto thiol-reactive substrates, producing reactive disulfides or disulfide oxides. One important benefit of this strategy is that the

bonds created between the thiol enzyme and the activated solid phases are reversible and can be removed with an excess of a low molecular weight thiol. This characteristic becomes essential when the enzyme breaks down more quickly than the absorbent, enabling subsequent reloading (Ovsejevi et al., 2013).

In conclusion, covalent interactions usually help maintain enzymes' structural stability. Some cost-related issues arise from the irreversible attachment of enzymes to the matrix, which necessitates discarding both the matrix and the enzyme once enzymatic activity falls. However, adopting reversible techniques is quite interesting, mainly due to financial considerations; the support can be renewed and reloaded with new enzymes when enzymatic activity declines. The price of support materials frequently significantly impacts the total cost of an immobilized catalyst. In bioanalytical systems, reversible enzyme immobilization is particularly useful for labile enzymes.

IV. MATERIALS USED FOR THE FABRICATION OF IMMOBILIZATION SUPPORT

Enhancing the immobilization of lipase involves the development of novel support materials characterized by optimal porosity, surface area, and a balanced hydrophobic/hydrophilic profile. These materials should possess specific attributes, as illustrated in **Fig.3**, such as thermal and chemical stability, inertness, renewability, heterogeneity, a strong affinity for lipase, interactive functional groups, physical robustness, availability, and cost-effectiveness (Rodríguez-Restrepo & Orrego, 2020; Zdarta et al., 2018). Moreover, an ideal support material would facilitate the binding of active lipase sites to substrate molecules and exhibit a distinct morphology to minimize diffusional limitations (Wong et al., 2009).

It is important to note that lipase, being an essential enzyme widely applied in biotechnology and various industrial processes, has garnered significant attention from researchers. Consequently, the development of new support materials for lipase can be categorized into three fundamental groups based on their properties.

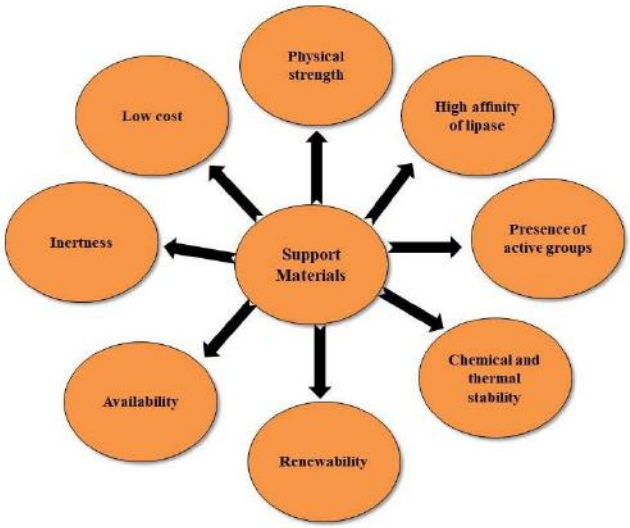

Fig.3: Properties of support materials used for lipase immobilization

4.1. Natural polymers as supports

Alginate: Alginate, extracted from the cell walls of brown algae, constitutes the calcium, magnesium, and sodium salts of alginic acid. Its utilization in immobilization is widespread, such as in the formation of xanthan–alginate beads, alginate–polyacrylamide gels, and calcium alginate beads. These configurations result in heightened enzyme activity and reusability. The stability of enzymes is further enhanced through cross-linking alginate with divalent ions, such as Ca^{2+} and glutaraldehyde. This cross-linking process, as documented by Flores-Maltos et al. (2011), contributes to the improved durability of the immobilized enzymes.

Chitosan and chitin: Natural polymers such as chitin and chitosan serve as effective supports for immobilization, as documented by Kapoor and Kuhad (2007). The binding of enzymes to chitosan involves the utilization of protein or carbohydrate moieties, as outlined by Hsieh et al. (2000). Notably, the combination of chitosan with alginate has demonstrated reduced leaching effects, attributed to physical and ionic interactions between the enzyme and the support, as observed by Betigeri and Neau (2002). Additionally, a wet composite comprising chitosan and clay has proven to be a reliable method for enzyme trapping due to its hydroxyl and amino groups, facilitating easy enzyme linkage, favorable hydrophilicity and high porosity. In bead form, chitosan exhibits superior enzyme entrapment, capturing twice the amount of enzymes, as highlighted by Chang and Juang (2007), emphasizing the high affinity of the chitin-binding domain of chitinase A1 from Bacillus circulans to chitin, which has been strategically employed for the retention of D-hydantoinase.

Collagen: Given its natural polymer composition, collagen has found application in immobilizing tannase, utilizing glutaraldehyde as a cross-linking agent, as detailed by Katwa et al. (1981). The utilization of Fe^{+3}-collagen fibers has demonstrated remarkable efficacy as a supporting matrix for catalase immobilization. Even after 26 consecutive reuses, significant catalase activity was retained, according to findings by Chen et al. (2011).

Carrageenan: Carrageenan, characterized as a linear sulfated polysaccharide, has consistently been employed in the immobilization of various enzymes, such as lipase, to enhance stability, as discussed by Tümtürk et al. (2007). Notably, this support exhibits pseudoplastic behavior, thinning under shear stress and promptly recovering viscosity once the stress is alleviated. (Jegannathan et al., 2010) achieved an encapsulation efficiency of 42.6% through the co-extrusion method, utilizing Carrageenan to support biodiesel production. Carrageenan has been recognized for its cost-effectiveness and durability as a support, demonstrating superior entrapment capabilities for lactic acid and agalactosidase enzymes, according to reports by Rao et al. (2008).

Gelatin: Gelatin, characterized as a hydrocolloid material abundant in amino acids, can adsorb up to ten times its weight in water, making it a noteworthy candidate for enzyme immobilization due to its extended shelf life. In a mixed carrier system with polyacrylamide, Emregul et al. (2006) found that cross-linking with chromium (III) acetate yielded superior results compared to chromium (III) sulfate and potassium chromium (III) sulfate. Additionally, combining calcium alginate with gelatin is an effective template for calcium phosphate deposition in enzyme immobilization. Moreover, when gelatin is paired with polyester films, it promotes a loading efficiency of 75%, a notable improvement compared to previous studies with 50% loading efficiency (Ates & Dogan, 2010; Shen et al., 2011).

Cellulose: Cellulose, the most abundant natural polymer, has found extensive applications in the immobilization of various enzymes, including fungi laccase, penicillin G acylase, glucoamylase, α-amylase, tyrosinase, lipase, and β-galactosidase (Huang et al., 2011; Klein et al., 2011; Namdeo & Bajpai, 2009). Notably, the storage capacity of Diethylaminoethyl (DEAE)-modified cellulosic supports has been demonstrated to be prolonged (Al-Adhami et al., 2002). Cellulose-coated magnetite nanoparticles, employed for starch degradation, showcased the development of a novel starch-degrading system when α-amylase was attached to cellulose dialdehyde-coated magnetite nanoparticles, as observed in the work by Namdeo and Bajpai (2009). Furthermore, the immobilization process using ionic liquid-cellulose film activated by glutaraldehyde exhibited enhanced formability and flexibility, as detailed by Klein et al. (2011).

Starch: Comprising linear amylase and branched amylopectin units, starch is an effective enzyme immobilizer. Hybrid supports, specifically those combining calcium alginate and starch, were utilized for the surface immobilization and entrapment of bitter gourd peroxidase in a study by Matto and Husain (2009). The entrapped enzyme exhibited enhanced stability in the presence of denaturants such as urea, attributed to internal carbohydrate moieties. Conversely, the surface-immobilized enzyme demonstrated superior activity. Industrial techniques widely adopted for a high product yield include radiation grafting of substances like acrylamide and dimethylaminoethyl methacrylate onto starch (anh Dung et al., 1995; Raafat et al., 2012).

Pectin: This heteropolysaccharide, combined with 0.2–0.7% glycerol acting as a plasticizer, mitigates the support's brittleness. It has been employed in immobilizing papain and developing novel materials for treating skin injuries, as discussed by Ceniceros et al. (2003). Pectin–chitin and pectin–calcium alginate supports have demonstrated improved thermal and denaturant resistance and enhanced entrapped enzyme catalytic properties. This enhancement is attributed to the formation of highly stable polyelectrolyte complexes between the enzyme and the pectin-coated support (Gomez et al., 2006; Satar et al., 2008).

Sepharose: The immobilization of amylase and glucoamylase has been achieved using CNBr-activated Sepharose-4B due to its porous nature and facile adsorption of macromolecules. Matrix modifications, such as the use of alkyl-substituted Sepharose with multipoint attachment between hydrophobic clusters of the enzyme and alkyl residues of the support, play a crucial role in preserving catalytic properties under extreme conditions such as pH extremes, high salt concentrations, and elevated temperatures (Hosseinkhani et al., 2003). An additional illustration of a modified Sepharose matrix involves Concanavalin A (Con A)–Sepharose 4B. In this case, the biospecific interaction between the glycosyl chains of the enzyme and Con A is pivotal in fabricating various biosensors (Mirouliaei et al., 2007).

4.2. Synthetic polymers as supports

Insoluble supports with porous surfaces, such as ion exchange resins/polymers, serve as effective platforms for enzyme entrapment. Renewable matrices like Amberlite and DEAE cellulose, known for their substantial surface areas, have been utilized for immobilizing α-amylase

(Kumari & Kayastha, 2011). In immobilizing white radish peroxidase, introducing glutaraldehyde and polyethylene glycol serves a dual purpose by acting as both an additive and a protective layer around the enzyme's active center. This protective layer prevents free radical attacks (Ashraf & Husain, 2010).

Various synthetic polymers have also found application as enzyme supports. Polyvinyl chloride, for instance, hinders thermal inactivation of cyclodextrin glucosyltransferase. Polyurethane microparticles, derived from polyvinyl alcohol and hexamethyl diisocyanate in a specific ratio, exhibit high enzyme loading and efficiency. UV-curable methacrylated/fumaric acid-modified epoxy is proposed for industrial applications, while polyaniline in emeraldine salt and emeraldine base powder forms facilitates the covalent binding of α-amylase. Other examples include glutaraldehyde-activated nylon for lipase immobilization and UV-activated polyethylene glycol with high porosity for wastewater treatment (Ashly et al., 2011; Pahujani et al., 2008; Romaskevic et al., 2010; Xiangli et al., 2010).

4.3. Inorganic materials as supports

Zeolites: Zeolites, also known as 'molecular sieves,' are crystalline solids with well-defined structures and shape-selective properties, extensively employed in molecular adsorption. Compared to microporous de-aluminized counterparts, microporous zeolites are more effective supports for α-chymotrypsin immobilization. This superiority stems from the increased presence of hydroxyl groups, fostering robust hydrogen bonds with the enzyme (Xing et al., 2000). Similarly, Na Y zeolite emerges as a preferred choice for lysozyme immobilization due to its heightened activity compared to alternative supports, as documented by Chang and Chu (2007). The heterogeneous surface of zeolites, characterized by multiple adsorption sites, is conducive to regulating interactions between enzymes and supports (Serralha et al., 1998).

Ceramics: The utilization of ceramic membrane for *Candida antarctica* lipase immobilization demonstrated the potential of this inert support for conducting hydrolytic and synthetic reactions while mitigating feedback inhibition, as elucidated by Magnan et al. (2004). Ceramic foams, incorporating both macro (77 nm) and micropores (45 µm), proved effective in reducing diffusion rates and enhancing specific surface area, as evidenced by the research of Huang and Cheng (2008). Another illustration involves toyonite ceramics, whose variable pore structure can be adjusted through diverse organic coatings, as explored by Kamori et al. (2000).

Celite: Celite, characterized as a highly porous diatomaceous material with bio-affinity properties, has found application in immobilizing enzymes such as lipase, polyphenol oxidases, and β-galactosidase. This preference arises from its cost-effectiveness, low polarity, and extensive adhesion area (Ansari & Husain, 2012; Khan et al., 2006; Liu et al., 2009). Notably, Celite exhibits resilience against elevated pH or temperature, urea, detergents, and organic solvents, as outlined by Khan et al. (2006). In the realm of x-transaminases immobilization within sol–gel matrices, Celite is an additive of choice due to its chemical inertness and interconnected pore structure, as highlighted by Koszelewski et al. (2010).

Silica: The effective removal of chlorolignins from eucalyptus kraft effluent has been achieved using enzymes such as lignin peroxidase and horseradish peroxidase (HRP) when immobilized on activated silica, as demonstrated by Dezott et al. (1995). Additionally, the enhancement of detergent cleaning performance is observed with α-amylase immobilized on silica nanoparticles. The utilization of these enzymes is attributed to their nano-sized structures, featuring a high surface area, ordered arrangement, and remarkable stability against chemical and mechanical forces, as explored by Soleimani et al. (2012). The reinforcement of enzyme and support bonds is achieved through surface modifications of silica, involving the amination of hydroxyl and reactive siloxane groups, as well as the addition of methyl or polyvinyl alcohol groups (Narsimha Rao et al., 2000; Pogorilyi et al., 2007; Shioji et al., 2003).

Glass: Glass, characterized as a highly viscous liquid, has been utilized to immobilize α-amylase. In this process, phthaloyl chloride-containing amino group functionalized glass beads demonstrated robustness and renewability, as evidenced by Kahraman et al. (2007). Another enzyme, nitrite reductase, found immobilization on controlled pore glass beads, which served as a biosensing device for continuous monitoring, as reported by Rosa et al. (2002). Furthermore, urease immobilized on glass pH-electrodes has proven to be an effective and stable biosensor, enabling the monitoring of urea levels as low as 52 µg/ml in blood samples, as highlighted by Sahney et al. (2005).

Activated carbon: Both natural and hydrochloric acid-modified activated carbon are valuable supports for enzyme adsorption, as discussed by Alkan et al. (2009). Recently, mesoporous-activated carbon particles have been employed in immobilizing acid protease and acidic lipases, featuring ample contact sites for enzyme immobilization. Notably, catalytic efficiency has been well-maintained even after 21 cycles of reuse, as indicated in the work of Ramani et al. (2012). Additionally, it has been observed that activated carbon with a high surface area (600-1,000 m^2 g^{-1}) and a significant fraction of its pore volume in the

300-1,000 Å range is well-suited for enzyme immobilization, as reported by Daoud et al. (2010).

Charcoal: The chemical modification of charcoal through the adsorption of papain with sulfhydryl groups has resulted in an increased number of active sites. This modified charcoal has been effectively applied to recover mercury from aqueous solutions and has proven efficient in industrial wastewater treatment, as documented by Dutta et al. (2009). Furthermore, charcoal supports have found application in the food industry, particularly for immobilizing amyloglucosidase in starch hydrolysis without the need for any crosslinking agent, achieving a remarkable 90% catalytic activity, as reported by Rani et al. (2000). The excellent adsorptive capacity of charcoal with minimal release of fine particulate matter has been underscored in earlier findings by Kibarer and Akovali (1996).

V. APPLICATIONS OF IMMOBILIZED LIPASES

Microbial lipases stand out as the primary choice for various biotechnological applications in today's industries, and it is anticipated that this market will experience further growth in the upcoming years. Substantial efforts are directed towards the advancement of new technologies to enhance the production and preservation (reuse) of microbial lipases. These enzymes find widespread use in diverse industrial sectors such as food, oil and fat, soap and detergent, paper and cellulose, leather, textiles, cosmetics, and biodiesel, showcasing their versatility. Immobilized lipases offer specific applications in industries. The various factors that impact enzyme immobilization and potential modifications to enhance their activity have been outlined in **Fig.4**.

As of 2016, most of the global market for technical enzymes is reported in Europe, the Middle East, and Africa (EMEA). Projections for 2021 indicate an expansion of this market into North America and the Asia-Pacific region, with annual growth rates of 6.8% and 7.9%, respectively. Furthermore, the technical enzyme market is anticipated to surpass the EMEA market to become the largest global market in the following years. This suggests that significant progress in process development within the enzyme industry will predominantly occur in the Asia-Pacific and North America regions by the specified year.

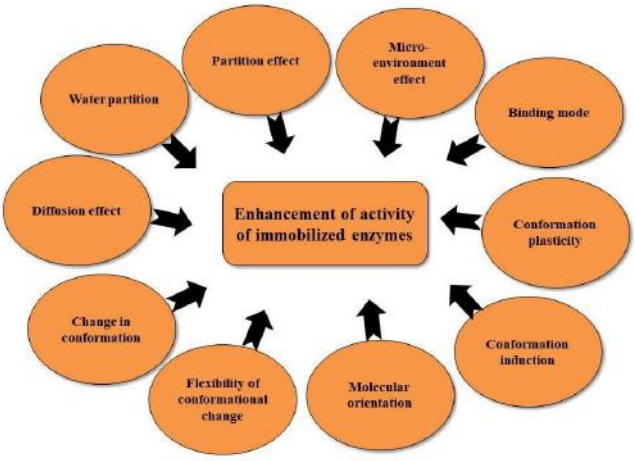

Fig.4: Determinants of enzyme immobilization and activity

5.1. Biosensors

An electrochemical biosensor is designed to offer analytical, quantitative, or semi-quantitative information by utilizing a biological recognition device in direct contact with an electrochemical signal transduction system. Today's prevalence of biosensors is attributed to their cost-effectiveness, rapid analysis, efficiency, sensitivity, and selectivity of enzymes for specific analytes, as highlighted by the International Union of Pure and Applied Chemistry in 2014.

Lipase-based biosensors have proven utility in detecting environmental pollutants, such as pesticides, as noted by Ma et al. (2018). In the food industry, biosensors play a crucial role in monitoring food quality, particularly the presence of triacylglycerol. Meanwhile, in the medical field, lipase and phospholipase sensors serve as diagnostic tools for detecting levels of triglycerides, cholesterol, and phospholipids in blood samples, according to Herrera-López (2012).

In a notable example by Zhang et al. (2014), an electrochemical biosensor was developed for monitoring tributyrin in human serum. Tributyrin, a triacylglycerol naturally present in butter, was detected using a biosensor constructed from polydopamine (PDA) and gold nanoparticles (GNPs), forming a hybrid material (GNPs@PDA) deposited on pretreated indium oxide and tin (ITO) electrodes. Lipase was then immobilized onto the GNPs@PDA. The cyclic voltammetry technique was employed for tributyrin determination in conjunction with the electrochemistry of lipase/GNPs/PDA/ITO electrodes. The optimized methodology utilized a scan rate of 50 mV s^{-1} in phosphate buffer (50 mM, pH 7.5, 0.9% NaCl), achieving a detection limit of 0.84 mg/dL. This biosensor exhibited excellent performance for determining tributyrin in human serum, showcasing potential applications in

clinical settings and related research for triglyceride determination in biological samples, offering fast, safe, and cost-effective results.

5.2. Structured lipids

Immobilized lipases serve as efficient and selective catalysts in synthesizing structured lipids. Their role in esterification and interesterification allows for the customization of lipid structures, leading to the production of fats and oil with improved nutritional, functional, and sensory properties. Structured lipids are effectively synthesized through lipase-mediated esterification and interesterification reactions (Kim & Akoh, 2015). Additionally, lipases play a vital role in enhancing the quality of food products within the food processing industry, primarily by modifying fats and oils. This enzymatic activity is applied to a diverse range of vegetable oils, including sunflower, coconut, olive, corn, and rice bran oil, all rich in omega-6 fatty acids. Similarly, lipases are utilized to modify oils from fish, linseed, walnuts, and milk, which are abundant in omega-3 fatty acids. Both omega-6 and omega-3 fatty acids are crucial for maintaining optimal health by regulating essential fatty acid levels within acceptable limits (Khan et al., 2023; Sangeetha et al., 2011). Additionally, phospholipases find industrial applications in processes such as egg yolk treatment for mayonnaise production, lecithin modification, and the oil-degumming step in refining vegetable oils. Novozymes 435 is an example of a lipase that demonstrates effectiveness in esterifying free fatty acids with octanol to produce octyl esters. Lipase-catalyzed transesterification, esterification, and epoxidation processes successfully modify vegetable oils, forming bio-lubricant components.

To enhance the industrial viability of lipases, immobilization techniques are employed, leveraging their inherent "interfacial" hydrophobicity. Various immobilization methods include adsorption on hydrophobic adsorbents like glass beads coated with hydrophobic materials, methylated silica, phenyl-Sepharose, poly-(ethylene glycol)-Sepharose, polypropylene particles, polypropylene hollow-fibers, nonwoven fabric, and nitrocellulose membranes (Sharma & Kanwar, 2014). Silica and solgels are also utilized, offering high compositional and morphological flexibility (Borza et al., 2015). For a more in-depth understanding of the diverse applications of lipases in oil and fat modifications, additional insights can be gained from the following literature (Ramani et al., 2010; Rodrigues & Fernandez-Lafuente, 2010).

5.3. Flavor production

The sensory experience of flavor and fragrance is pivotal in determining consumer preferences for various products. Consequently, synthesizing and utilizing these compounds are crucial for providing a distinct identity to a product. Flavor and fragrance are intentionally incorporated into formulations for food, beverages, pharmaceuticals, and personal care items. Ethers serve as primary contributors to flavor, but their high cost renders the production process impractical. Chemical methods, while an option, often result in the formation of undesirable compounds, limiting their application in the food and beverage industries (Aravindan et al., 2007; Dhake et al., 2013; Ray, 2012). An alternative approach involves enzymatic catalysis for flavor and fragrance synthesis, eliminating the need for toxic solvents and intricate product recovery procedures. Notably, lipase stands out among enzymes, capable of producing diverse flavors depending on the reaction medium. In the food industry, lipases find applications to enhance the organoleptic characteristics of products. They are employed in flavored dairy items to improve the taste of cheese, milk, and butter, contribute to aroma development in beverages, extend shelf life in baking, enhance quality in mayonnaise, facilitate cocoa butter processing, and serve various other purposes (Aravindan et al., 2007; Dhake et al., 2013; Ray, 2012).

To demonstrate the effectiveness of enzymatic synthesis in flavor production, (Sadighi et al., 2017; Silva et al., 2014) devised methods for immobilizing lipases from various sources, such as porcine pancreatic lipase (PPL) and *Thermomyces lanuginosa* lipase (TLL), respectively. Silva et al. (2014) employed polyhydroxybutyrate (PHB) particles to immobilize porcine pancreatic lipase (PPL) for synthesizing pineapple flavor through the esterification of butanol and butyric acid in a heptane medium. The optimized pH and temperature for hydrolysis reaction with immobilized PPL were at 8.5 and 50°C. In the esterification reaction, the optimum conversion reached approximately 93% after 2 hours. Operational stability tests suggested that this methodology could be a valuable tool for lipase immobilization for ester synthesis, with the biocatalyst retaining 63% of its initial activity after six cycles of esterification. In a separate study, Sadighi et al. (2017) utilized mesoporous silica nanoparticles (MCM-41) coated with polyethyleneimine (MCM-41 @ PEI) and modified with divalent metal ions (M = Co^{2+}, Cu^{2+}, or Pd^{2+}) to produce chelated silica nanoparticles (MCM 41 & M) for lipase immobilization. The MCM-41 @ PEI Co carrier demonstrated higher catalytic activity at 75°C, retaining 70% of its initial activity after 14 days of storage at room temperature. When applied to the synthesis of ethyl valerate (green

apple flavor) in the presence of valeric acid and ethanol, a reduction of 60% and 53%, respectively, was observed after 24 hours of incubation in n-hexane and dimethylsulfoxide medium.

These studies underscore the versatility of lipases in the food and pharmaceutical industries, showcasing their high yield in esterifying lipids for flavor production. Using non-polar substrates in organic chemical synthesis further highlights their broad applicability. Immobilization of enzymes proves crucial, enhancing storage stability and maintaining enzymatic activity across a wide range of temperatures and pH. Moreover, this approach reduces operational costs by facilitating the easy recovery of lipases for subsequent reuse, with minimal activity loss over multiple cycles.

5.4. Biodiesel production

Biofuels have emerged as a sustainable and renewable alternative to conventional fossil fuels, with biodiesel being a notable example. Biodiesel production involves the use of biomass, often comprising fats and vegetable oils, positioning it as a cleaner energy source compared to petroleum-based diesel fuels. The transformation of triacylglycerols found in oils and fats into esters through a process called transesterification is the key method for biodiesel synthesis. This reaction can be influenced by various factors, including the molar ratio of alcohol, catalyst type, presence of water and free fatty acid, temperature, time, and stirring speed (Vargas et al., 2018; Yücel et al., 2012). Enzymatic immobilization has proven to be a highly advantageous approach. Comparative studies reveal that the catalytic activities of the same lipase molecule can differ significantly based on the immobilization support used (Sankaran et al., 2016; Vargas et al., 2018; Yücel et al., 2012). Khosla et al. (2017) explored the potential of an extracellular lipase from Pseudomonas sp. ISTPL3 was isolated from Pangong Lake for the transesterification of lipids produced by the oleaginous chemolithotrophic bacterium Serratia sp. ISTD04, contributing to biodiesel production. Immobilizing the lipase on activated biochar resulted in a higher yield of fatty acid methyl esters (FAMEs) at 92.23%, compared to 87.81% for the non-immobilized lipase. The authors attribute this increase to the enhanced stability and catalytic activity achieved through lipase immobilization. Furthermore, the immobilized lipase retained 75.11% of its activity after three cycles of biodiesel production, highlighting the potential for cost reduction through lipase reuse and improved production efficiency.

Numerous other studies have also demonstrated favorable outcomes in immobilizing lipases from various microorganisms on different supports (Cruz-Izquierdo et al., 2014; Kalantari et al., 2013; Miao et al., 2018; Picó et al., 2018; Sankaran et al., 2016; Vargas et al., 2018; Xie & Huang, 2018; Yücel et al., 2012). These studies underscore the potential of immobilized enzymatic catalysts to enhance biodiesel production processes, offering advantages such as increased yield, improved lipase stability in reaction media, and cost reduction through the repeated use of lipases.

5.5. Treatment of wastewater

Industrial waste comprises organic and inorganic substances found in solvents or suspensions, varying in levels of harmfulness. Various effluent treatment processes aim to eliminate these characteristic residues from different industrial processes (Meng et al., 2015; Sarmah et al., 2018). Typically, these treatments involve physical, biological, and chemical stages, with the effectiveness directly impacted by the combination of treatments suitable for the specific waste type. Biological treatment, often following physical or chemical-physical treatment, employs microorganisms that produce enzymes capable of breaking down organic matter. In this process, microorganisms hydrolyze triacylglycerols in the extracellular medium through the action of lipases, resulting in the production of fatty acids and glycerol. Lipases can be utilized in various forms, such as in the crude form of a fermented broth or isolated, for pretreating effluents before anaerobic digestion. Alternatively, they can be used as an enzyme complex containing lipase and other enzymes to enhance treatment efficiency. These enzymes find extensive application in removing fats from treatment plant aerators utilizing activated sludge and are commonly employed in treating industrial effluents from sectors like food processing, textiles, paper and cellulose, tanneries, and automotive industries (Mendes et al., 2005; Meng et al., 2015; Ramani et al., 2013; Sarmah et al., 2018; Shen et al., 2013).

Recent trends indicate a growing interest in the study and application of marine lipases, driven by their ability to function in more extreme environments than other lipases. In this context, microorganisms are introduced into the reaction medium, and liquid fermentation is performed. The microorganisms utilize the lipids catalyzed by the lipases to obtain energy for maintenance and replication. Hassan et al. (2018) conducted studies involving isolating marine bacteria from the Mediterranean Sea for lipase production. They optimized cell production, immobilized the bacteria, and applied them in effluent treatment. Bacillus cereus HSS emerged as the most promising microorganism, exhibiting the highest lipolytic capacity. Immobilization through the adsorption of sponge cells and

recycling significantly increased lipase activity by 2.8-fold compared to free cells. The repeated reuse of immobilized *B. cereus* HSS maintained reasonable lipase activity. An economic study focusing on oily wastewater treatment demonstrated 87.63% efficiency in removing biological oxygen demand, a 90% removal of total suspended solids, and a 94.7% removal of oil and grease. This highlights the potential of immobilized microorganisms as a cost-effective method for wastewater treatment. The positive results suggest promising applications for the large-scale implementation of immobilized microorganisms, encouraging further research in this direction.

VI. CONCLUSION AND FUTURE PERSPECTIVES

Lipases are versatile tools and established biocatalysts in diverse industrial sectors due to their remarkable ability to catalyze reactions in aqueous and non-aqueous environments. The comprehensive overview presented in this review underscores the ongoing imperative for continuous development in the lipase immobilization process. Researchers are particularly concerned about its advancement, given its widespread application in numerous industrial-scale chemical reactions, spanning areas such as food, pharmaceuticals, cosmetics, and fuel production. The review further delves into lipase sources, categorization based on specificity, and various applications. Moreover, the lipase immobilization process brings significant advantages, including heightened chemical and thermal stability compared to the free form of lipase and ease of recovery and reuse. The diverse range of materials serving as supports for lipase immobilization, along with the methods employed in the immobilization process, not only confer crucial advantages but also provide ample scope for tailoring biocatalytic systems to specific chemical reactions and applications. The review discusses different support materials and immobilization techniques, highlighting the critical challenge of judiciously selecting the appropriate support material and immobilization method in this evolving field.

However, improving the lipase immobilization process comes with challenges, like the high cost of making lipases. So, future research should focus on using molecular biology and genetic engineering to make more microbial lipases and make the whole process cheaper. Also, from an economic standpoint, creating new supports from cheap waste biomass or natural materials that are easy to find can be a big advantage in using lipases in industries. Another way to cut costs is by using co-immobilization, where different enzymes, including lipase, are put together on the same material to do different jobs. In the future, we expect to see more reports about new materials with different properties and ways to use them in the lipase immobilization process.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31601433).

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