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Review: Immobilization and application of lipase

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Abstract: *The current demands of the world's biotechnological industries are enhancement in enzyme productivity and development of novel techniques for increasing their shelf life. Immobilized enzymes are more robust and more resistant to environmental changes than free enzymes in solution. Enzyme immobilization provides an excellent base for increasing availability of enzyme to the substrate with greater turnover over a considerable period of time. Several natural and synthetic supports have been assessed for their efficiency for enzyme immobilization. Future investigations should adopt logistic and appropriate entrapment techniques along with innovatively modified supports to improve the state of enzyme immobilization, providing new perspectives to the industrial sector. This paper reviews recent literature on enzyme immobilization by various techniques, as well as immobilization strategies and their application.*
Keywords*: Enzyme immobilization; Thermomyceslanuginosus lipase (TLL), carbon nanotube, magnetic nanoparticles, Applications.*

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I. Introduction

Lipases (triacylglycerol ester hydrolase, EC 3.1.1.3) are esterases belonging to sub class 1 of hydrolytic enzymes class 3 and have been assigned as sub-sub-class 3.1.1 due to their specificity for carboxylic acid ester bonds. They are known as carboxylic acid esterases and numbered as E.C.3.1.1 according to the classification recommended by enzyme commission of International Union of Biochemists[1]:(a) it should be activated by the presence of an interface, that is, its activity should sharply increase as soon as the triglyceride substrate forms an emulsion. This is commonly known as interfacial activation[2]. (b) It should contain a "lid" which is a surface loop of hydrophobic oligo-peptide covering the active site of the enzyme and moving away on contact with the interface and immediately the substrate enter the binding pocket[3]. However, these criteria are not the most appropriate for classification due to a number of exceptions. For instance, some lipases have a lid but do not exhibit interfacial activation[4]. In addition, there are microbial lipases lacking a lid that covers the active site in the absence of lipid-water interfaces. Consequently, lipases are simply defined as carboxyl-esterases catalysing the hydrolysis and synthesis of long-chain triglycerides[5].

However, there is no strict definition available for the term "long-chain," but glycerol esters with an acyl chain length greater than 10 carbon atoms can be regarded as a lipase substrate, with triglyceride being the standard substrate. Lipases differ from each other in length and architecture of the binding domain of the α/β-hydrolase fold proteins, conferring a wide range of substrate diversity. Typically, lipases are used to catalyse hydrolysis of triacylglycerol into glycerol and fatty acids, diacylglycerols, monoglycerols and glycerol[6, 7]. Lipase is widely present in animal, plant and microbial cells, In particular, microbial lipase is more easily prepared for current lipase applications[1]. In addition to hydrolysis, lipases can catalyse alcoholysis, acidolysis, esterification, transesterification, enantioseparation organic reactions and ammonia solution, as well as cooperation a ring-opening polymer, polymerization lactone and other complex organic reactions. (see Fig.1)[6, 7].

1.1 Properties and Sources of lipase

In plants, lipases are mostly present in food reserve tissues of growing seedlings especially in those which contain large amounts of triacylglycerols[8]. In animals, the lipases are found in pancreas, and on the surface of mucous cells,whereas in insects, these enzymes are found mostly in plasma, salivary glands, muscles and fat bodies[9]. The pH ranges of plant lipase is between 4.0 to 9.0, between 5.5 to 8.5 for animals and in insects, ranges somewhat higher than other two groups, from 6.0 to 10.0[10]. The temperature at which plant lipase is active is between 20 to 38°C, for animals it ranges between 37 to 60°C and microbial lipases are active between 37 to 55°C. Plant and animal lipases are activated in the presence of calcium and zinc, while they are inhibited in the presence of EDTA and Tween 80[11]. Microbial enzymes are inhibited in the presence of $FeCl_3$, $ZnCl_2$ and $HgCl_2$. It has been found that *P. pastoris* is the most successful expression system for lipase

production. These genetically modified organisms are capable of producing enzymes identical to those from original sources with higher efficiency, in industrial scale bioreactors and easily recovered compared to the original sources[6](as seen in Table 1).

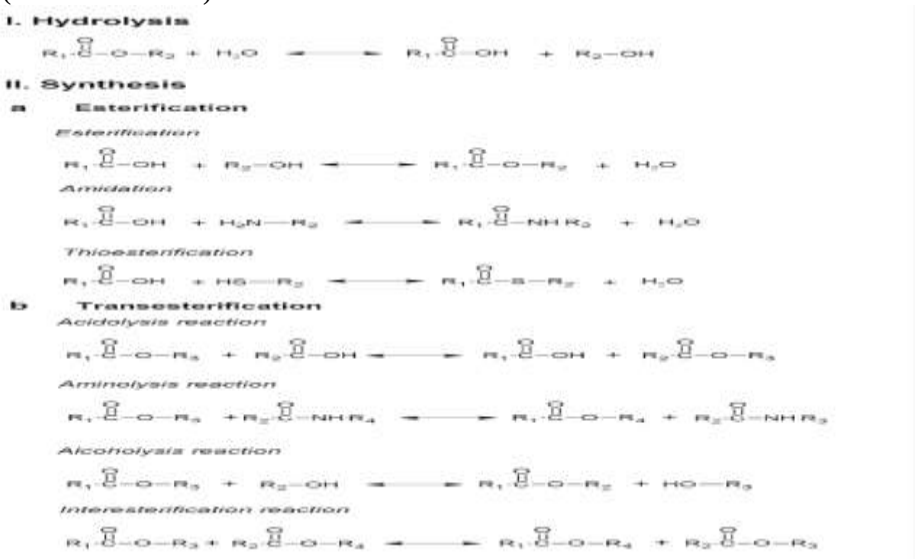

Fig. 1: Reaction catalyzed by lipase.[7]

Table1: Sources of industrially lipases and there successful expression systems

Original source	Expression system	Uses
Pseudomonas fluorescens ,	*P. pastoris*	Pharmaceutical industry
Thermomyceslanuginosus		Biodiesel processing
Bacillus cereus		Food processing
B. thermocatenulatus		Diary industry
Candida rugosa	S. *cerevisiae*	
C. antarctica		Butter and margarine production
C.thermophyla		
C. parapsilosis		
Rhizopusoryzae		
Yarrowialipolytica		
Pig pancreatic lipase		Pharmaceutical industry

1.2 Structure of lipase

The molecular weight of lipase is generally 19-60 kD[12]. Structurally, lipases are characterized by a common α/β hydrolase fold and a conserved catalytic triad. Although the amino acid composition of microbial lipase from different sources is different, its primary structure exhibits surprising diversity, but it has a similar catalytic activity centre and spatial structure. Most lipases possess the consensus motif G-X1-S- X 2-G and the structural framework of all lipases is represented by α/β hydrolase folds with the same catalytic mechanism[13, 14].Microbial lipases and esterases can be grouped into 15 superfamilies and 32 homologous families based on their structure, catalytic triad and residues in the oxyanion hole[15]. The α/β hydrolase fold is commonly composed of a central, parallel β-sheet of eight β-strands, except the second strand which is antiparallel (β2). Strands (β3– β8) are connected by helices arranged on the sides of the central β-sheet. There is a variation of the α/βfold in several lipases due to the number of α-helices and β-sheets, loop length and the composition of the substrate binding sites[12, 16].(see Fig. 2)

The core composition of the lipase is centred on up to eight β-folds (β1 – β8), connecting up to six α-helices (Aa – αF), where β2 is antiparallel,β3 – β8 is linked by α-helix (αA-αF), a specific structure that provides a stable stent for the active site of the enzyme-catalysed triad. The microbial lipase-catalysed triplets include nucleophilic, acidic and histidine residues. Most microbial lipase activity centres are Ser, Asp and His triplets. Typically, a Ser residue having a nucleophilic group is located after the β5 chain, the acidic amino acid residue (e.g., Glu or Asp) is located after β7 and the His residue is located afterβ8. The spatial structure of microbial lipases from different sources is mainly due to the number of β-bundle bundles and α-helices, as well as the β-fold angle and the spatial arrangement of α-helix[14, 17] .

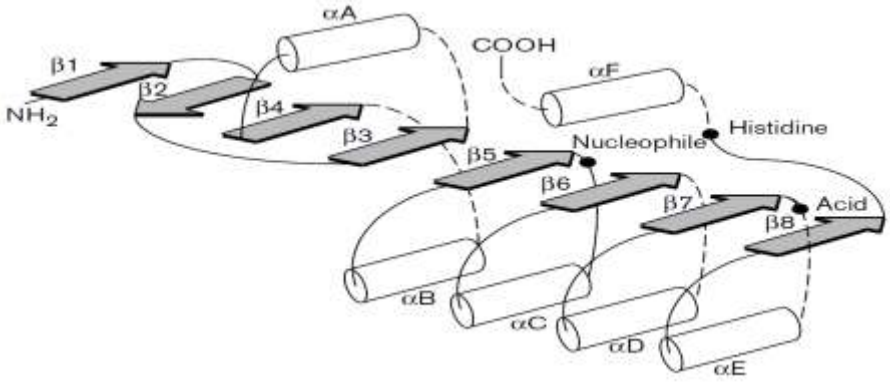

Fig. 2:Secondary structure topology diagram of the canonical α/β hydrolase fold.[8]

During the catalytic procedure of lipases, a tetrahedral intermediate is formed which is stabilized by a lipase oxyanion holeformed from hydrogen bonds with two amino acids residues. The first residue of the oxyanion hole is located in the loop between the β3-strand and the αA-helix in the N-terminal of the lipase. The second residue is the X2 residue of the consensus sequence G-X1-S- X2-G, which is located after the strand β5-sheet. Two oxyanion holes have been identified, G X and GG G X, based on the sequence surrounding the first residue[15]. They are responsible for the specificity of the lipases toward their substrates. The G X type normally hydrolyzes substrates with medium and long carbon chain lengths, while the GG G X type hydrolyzes short length lipases[18]. Most microbial lipase activity centres are covered by a structure called the "lid". The "lid" has an amphoteric molecular structure and the direction of the hydrophilic end and the hydrophobic end controls the closure of the "lid". The "lid" is composed of one or more α-helices joined to the main structure of the enzyme by a flexible structure. It is a mobile element, in the presence of the lipid/water interface, it uncovers the active site and thus, the substrate gains access to the active site[19].

When the lipase is in contact with the substrate, the hydrophobic end is exposed, the hydrophilic residue is hidden, the "lid" structure is opened and the enzyme catalytic activity centre is exposed. Also, in the catalytic active centre around the formation of electrophilic domains, there is increased enzyme affinity with the substrate and it maintains the stability of the transition state intermediates in the catalytic process, thus exhibiting catalytic activity.Most lipases occur in two main structural forms, the open form and the closed form(see Fig. 3)[8].

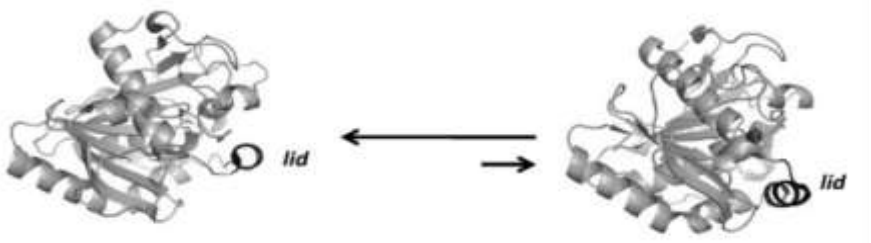

Fig. 3:3D Structure of the open and closed forms of lipase[8].

1.3*Thermomyceslanuginosus* lipase (TLL)

The lipase from *T. lanuginosus*(TLL) is a basophilicsingle chain protein consisting of 269 amino acids, of which, four are tryptophan residues (89, 117, 221 and 260). It has a molecular weight of 31,700 g/mol and isoelectric point of 4.4. The structure of TLL has been solved at 3.25Å and more recently, at 1.8Å[6]. The structure of TLL comprises an α/β hydrolase fold, where sevenstranded β-sheets are surrounded by a large N-terminal α-helix on the distal face. X-ray diffraction studies show that the active site of TLL includes the typical Ser146-Asp201-His258 catalytic triad. The α-helical surface loops constituting the lid cover the active site S146. This lid is also highly mobile in the crystal and the Trp at position 89 is reported to be important for the catalysis[20].Holmquist*et al*.[21] reported that site-directed mutagenesis at Trp89 decreases the activity of TLL,with the other Trp residues at positions 117, 221 and 260 providing structural stability.

The TLL is a thermostable enzyme widely used in industry, which can catalyse hydrolysis, esterification, alcoholysis and interesterification. In aqueous media, the enzyme is very stable, being active over the pH range of 7.0–11.0. It maintains activity reasonably well at 55–60°C, although the recommended temperature for applications is between 30°C and 40°C[22].

1.4 Application of lipase

Lipases are stable and extremely valuable catalysts, offering a promising alternative to many conventional, practical and industrial applications[23]. Lipases have wide specificity, recognizing various substrates, allowing their use as a catalyst for many different reactions pharmaceutical and drug production, in energy or food(Table 2)[24].

Table 2: Industrial applications of lipases and its sources

Industry		Function	Product or application
Cheese	*Aspergillusniger*	Cheese flavoring and ripening	Flavoring agents (acetoacetate,
	A. aoryzae		beta-keto acids, methyl ketones, flavor esters, and
	Candida rugosa,	Hydrolysis of milk fat Lipolysis and	lactones)
	C. antarctica	modification of butterfat and cream	
		Transesterification and Hydrolysis	Fragrance agents in
Fats and oils	*Porcine pancreas Pseudomonas* sp.		cheese, milk, and butter
	Rhizomucormiehei		Butter substitutes (cocoa butter) Glycerides for
	R. javanicus	Flavor improvement Control	butter and margarine
	Rhizopusoryzae	nonenzymatic browning	SL rich in PUFA
Baked products		Quality improvement	Low caloric triglycerols
confectionery	*Thermomyceslanuginosus*		Concentrate or purified FA
		Hydrolysis	Diglycerols for cooking oils
Detergents	*Acinetobactersp.*		Extend shelf-life
	Aspergillusoryzae	Synthesis Transesterification	Increase loaf volume
	Candida sp. Chromobacteriumsp.	Hydrolysis	Improve crumb structure
Pharmaceutis	*Pseudomonas mendocina*		Mayonnaise and dressings Emulsifiers
	T. lanuginosus		Fats removal (decomposition of lipids)
	Alcaligenessp.		Soap production
	Arthrobactersp.		Dish washing, dry cleaning
	Aspergillus sp.		solvents, liquid leather
	Burkholderiacepacia		cleaner, contact lens cleaning
	R. miehei		Building blocks for pharmaceuticals,
			agrochemicals, and pesticides Digestive aids

1.4.1 Application of lipase in the energy sector

Recently, owing to limited energy reserves and increasing environmental pressure due to green-house gases produced by fossil fuels and biodiesel as an alternative to fossil diesel[25]. lipases have attracted attention as a non-toxic, biodegradable and renewable alternative source of fuel and energy[26, 27].

Biodiesel productionis a technological field that challenges researchers to develop more efficient and environmental processes. Although traditionally homogenous alkali catalysis has been widely applied, this method requires complex estrus processes, including removal of inorganic salts from the product, recovery of glycerol containing saltand treatment of alkaline waste water[28].The process of producing biodiesel has been successfully intensified with newly developed processing techniques, including pyrolysis, dilution, partial emulsion, esterization and ester[29]. Indeed, TLL has been used to produce biodiesel from canola oil and methanol. TLL has been immobilized in hydrophilic polyurethane foams using polyglutaraldehyde, with a maximum methyl esters yield of 90% and the immobilized lipase lost little activity after repeated use[30].

1.4.2 Application of lipase in food industry

Lipase can catalyse the production of functional oils and fats. In triglycerides, unsaturated fatty acids are mostly in sn-2.The sn-1 3-position-specific lipase can be used to catalyse the transesterification reaction, while maintaining the sn-2 fatty acid. The sn-1,3 of the triglyceride is modified into short or medium carbon chain fatty acids and the resulting structural lipids (constitutional lipids)can provide essential fatty acids and be used as an energy source or to develop drugs.

Lipases have potential for use in health care as they can be used to obtain polyunsaturated fatty acids (PUFA) from oils and fats, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (see Fig. 4).With regard to health care, in Europe and the United states, DHA fish oil and products are currently used as food additives and supplements for the prevention of hypertension, heart disease, cancer and diabetes[31].The production of such PUFA involves mild reaction conditions, low energy consumption, less side effects and yields a highly pure product[32]. However, the packaging of the fatty acids, such as the 25 carbon EPA, DHA and γ-linolenic acid, as well as the physical and chemical methods of the preparation present several shortcomings[33] .

Fig. 4:EPA (Eicosapentaenoic Acid) and DHA (Docosahexaenoic Acid) are some of the fats that make up omega-3.

Furthermore, even though many studies have improved the catalytic performance of lipases, there remains certain restrictions in industrial applications, such as poor stability, narrow pH range, adaptability, sensitivity to the environmental conditions and the difficult separation of the free lipase from substrates and products[34]. In addition, most raw materials rely on the relatively costly deep-sea fish. In recent years, research has focussed on lipase from the raw material oil enrichment of functional oil[35]. Nonetheless, studies suggest that ω-3 fatty acids, such as DHA, are partially resistant to hydrolysis by lipases, making them more likelyto be partially detached from the monounsaturated and fatty acids present in fish oil[36]. Yan *et al*.,[27] used two different carriers and methods to immobilize the cellulite lipase to catalyze the hydrolysis of fish oil to prepare modified fats rich in DHA and EPA, substantially increasing the PUFA content. Kahveci*et al*.,[37] treated the lipase by molecular imprinting to improve its catalytic activity, selectivity and to enrich the PUFA. Wang *et al*.,[25] produced glycerol enriched with n-3 fatty acids using Novozym 435 as a catalyst for high crude acid oils. The reaction process included the synthesis of deacidification, polyunsaturated fatty acid ethyl ester catalysis, concentration and enzyme-catalyzed synthesis of polyunsaturated fatty acid glycerides. The results showed that the EPA and DHA content were 5.5% and 74.6% respectively, 1.21 and 2.71 times the content of the original fish oil. *T. lanuginosus* lipase, the subject of this report, has been used to catalyze the hydrolysis of emulsified tributyrin via two different steps[38].

1.4.3 Other use of lipase

Lipase catalysed synthesis of chiral drugs mainly includes pre-chiral substrate reactions and racemic compounds, such as the use of microbial lipase to produce ibuprofen, naproxen and other diaryl propionic acid drugs. Li *et al.*[9] immobilized with macroporous resin B. cepacia G63 catalyzed the synthesis of chitosan and different alcohols and synthesized chiral esters. The effects of different alcohols and solvents on their enantioselectivity were investigated. The results showed that Alcohol and heptane as acyl receptors and reaction medium, the enantioselectivity of the highest, and the addition of 18-crown-6 can significantly increase its E value.

Moreover, lipase can be used in the manufacture of various chemicals, including food, pesticides and medicines[6].TLL has been used in the solution of many rasidic mixtures of acids and alcohol. For example, higher motor resolutions were shown when the Z-4-trifenylmethoxy-2-3-epoxyptotane-1-first, and vinyl acetate was used as an asyl donor. It has also been reported that the TLL decision of nifedotoxyl nitrate activator dehydroperemidone, is a submitting of dehydroperemidone and an antihypertensive agent (R) -SQ 32926[39].The dyeing of wool has been enhanced with reactive dyes using TLL. The use of primary processing enzyme for dyeing wool can be conducted under mild temperature conditions, with increased dye consumption and a plus rate of dyeing rate[40].TLL can also be used in the treatment of sewage from the meat industry. Wastewater from the meat industry usually presents a high content of oils and fats, leading to reduced degradation. Enzymatic degradation by TLL may contribute to the enhanced hydrolysis of septic sewage, accelerate treatment and reduce oxygen demand from water[6].

1.5 Immobilization of lipase

Free lipase in catalytic reactionshas many disadvantages[41]as follows:

(1) Free enzyme is easily degraded, or agglomeration and inactivation.

(2) Free enzyme can't be separated from the reaction system, often leading to impure products, affecting product quality.

(3) Free enzyme is very sensitive to the environment, with poor tolerance to acid and alkali conditions and a narrow temperature tolerance, especially in non-aqueous catalytic systems, showing very low activity and operational stability.

(4) Free enzyme cannot be recovered and reused**.**

These drawbacks can generally be overcome by immobilization of the enzyme[23].According to PubMed database only in the first 6 months of 2010, According to PubMed database only in the first 6 months of 2010, many hundreds of papers on enzyme immobilization have been published.

Today, immobilized enzymes have been shown to be highly efficient for commercial use. They offer many advantages over enzymes in solution, including economic convenience, higher stability and the possibility to be easily removed from the reaction mixture leading to isolationof a pure product. An immobilized enzyme isattached to an inert, organic, or inorganic or insoluble material, such as calcium alginate or silica. Furthermore, the attachment of an enzyme to a solid support can increase its resistance to various environmental changes such as pH or temperature**.**

1.5.1 Commonly used immobilized carriers

The immobilization method determines the catalytic performance of the immobilized enzyme. Therefore, the selection and preparation of the carrier material with excellent comprehensive performance is a very important aspect of enzyme immobilization technology[42].here are various enzyme immobilization carrier materials, including glass beads[43], macroporous resin[10],mesoporous silica[44], sol-gel material[45], magnetic particles[42] carbon nanotube[46]. The support (carrier) can be a synthetic organic polymer, a biopolymer or an inorganic polymer (see Table).

Table 3: Classification of Supports

organic polymer	**Inorganic polymer**		
Natural polymers materials	**Synthetic polymers**	**Natural minerals**	**Processed**
Polysaccharides: cellulose, dextrans, agar, agarose, chitin, alginate	Polystyrene	Natural bentonite, silica	glass (nonporous and controlled pore), metals, controlled pore metal oxides
Proteins: collagen, albumin	Other polymers: polyacrylatepolymethacrylates, polyacrylamide, polyamides, vinyl, and allylpolymers		
Carbon			

1.5.1.1 organic polymers

Organic polymer materials play an important role in the study of enzyme immobilization, they can be used directly as enzyme immobilization carrier materials, but also modified to obtain a variety of different performances[47]. The specific activity of the immobilized enzyme is 21.7 times higher than that of the free enzyme, and the thermal stability of the immobilized enzyme is higher than that of the free enzyme by the method of molecular imprinting and interfacial activation. Organic solvent tolerance was also greatly improved. Biodiesel prepared by the alcoholysis of soybean oil in a solvent-free system with the immobilized enzyme as the catalyst, yielded 90% biodiesel after 8 h and maintained the initial vitality of 92% after 50 cycles[48]under the optimum reaction conditions.

An enzyme immobilized on a prefabricated supportby simple adsorptioncan be stabilized towards leaching and mechanical stress by deposition of a silicone coating formed from inexpensive readily available raw materials. For example, Novozyme 435 was coated with a silicone polymer obtained in a hydrosilylation reaction. The silicone was not only deposited as an external layer, but also permeated into the porous carrier. The resulting silicone coated Novozyme 435 exhibited high mechanical strength with excellent stability against leaching. Moreover, the high activity retention (92%) indicated that no significant diffusion limitations were caused by the silicone coating[49].

Ye *et al.*[50]anchored the natural macromolecular chitosan onto poly (acrylonitrile-maleic acid) (PANCMA) membranes to prepare bionic membranes with high biocompatibility. They reported a recovery rate of the enzyme of 54.1%, which was higher than that of the covalent binding immobilized enzyme (44.5%). Dizge*et al.*[51]treated TLL with covalent immobilized polystyrene-divinylbenzene (STY-DVB) as a hydrophobic porous material, and 85% of the enzyme was immobilized in the porous carrier on the surface.The immobilized enzyme was stored for 30 days and its initial catalytic activity was maintained after 15 cycles.

Yi *et al.*[52]studied the immobilization of CRL with chitosan particles of epoxidized scaffolds modified with amino acids and limited the amino acid content in the range of 15% to 60%. The immobilization rate of the

vector was 92.7%, which was much higher than that of the amino acid modified carrier. However, the recovery rate of the enzyme was only 10.4%, while that of the latter was 51%.

1.5.1.2 Inorganic polymers

A variety of inorganic supports have been used for the immobilization of enzymes, e.g., silica,[53] zeolites[54] and mesoporoussilicas[55] such as MCM-41, and SBA-15. One of the simplest and most inexpensive methods to immobilization an enzyme is by silica granulation. It is used in detergent formulations which release the enzyme into the washing liquid during washing. Granulation technology was used to immobilized CaL-B lipase on silica granules, by first adsorbing the lipase on silica powder followed by agglomeration[56].

The CaL-B silica granules can be used in a direct ester synthesis if the water is removed by e.g. evaporation under vacuum. Applying the granules in packed bed reactors also minimizes the contact time with high water concentrations. The CaL-B silica granules exhibited a similar activity to Novozyme 435 in the direct synthesis of the skin emollient, isopropyl myristate. In order to maintain stability in an aqueous environment the enzyme needs to be covalently attached to a functionalized silica support.

Nowadays,mesoporoussilicas, often referred to as nanosilicas, have several advantages as supports and are inert and stable at elevated temperatures. Moreover, the surface can be easily functionalized. The large pores and cages of these materials can accommodate relatively small enzymes.Covalent binding of a-chymotrypsin (EC 3.4.21.2) to a mesoporous sol-gel glass, which has been modified by reaction of surface hydroxyls with 3,3,3-trimethoxypropanal, afforded an immobilized catalyst with a half-life one thousand times that of the free enzyme[57]. Similarly, immobilization of *Mucorjavanicus* lipase on functionalized silica nanoparticles resulted in enhanced thermal stability and a high retention of activity over a wider pH range[47].

1.5.2 Commonly used immobilization method

Traditionally, four methods are used for enzyme immobilization, namely non-covalent adsorption and deposition, physical entrapment, covalent attachment, and bioconjugation. Several common fixation methods are shown in Fig. 5.

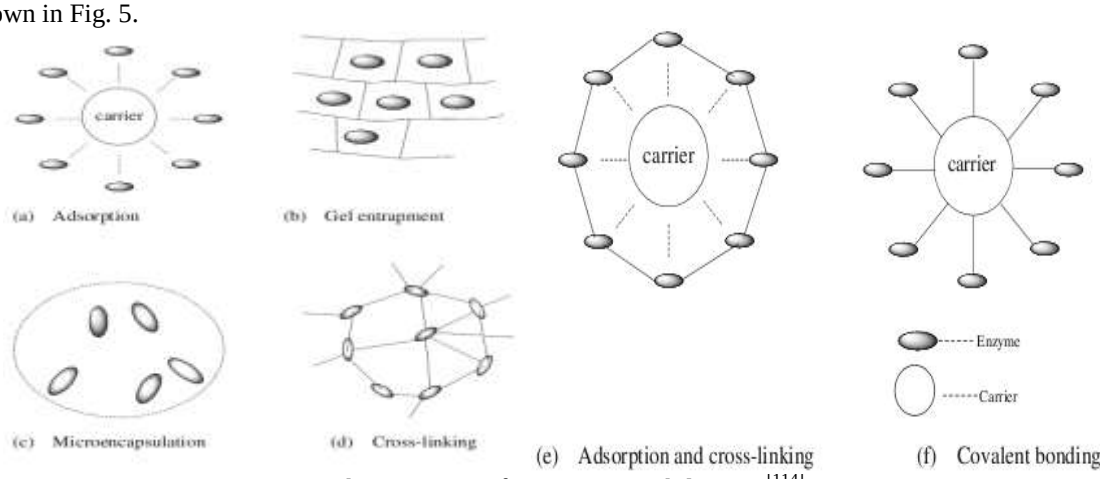

Fig. 5: The strategies of enzyme immobilization[114]

1.5.2.1 Physical adsorption method

Non-covalent methods of protein immobilization are widely employed and involve either passive adsorption onto hydrophobic surfaces or electrostatic interactions with charged surfaces[58]. The use of nitrocellulose membranes or polylysine-coated slides for electrostatic binding is perhaps the most common. Immobilization by adsorption is a mild, easy to perform process, usually preserving the catalytic activity of the enzyme[59].

Compared with other immobilization methods, physical adsorption has the advantages of a low cost and easy carrier, simple operation and mild preparation conditions, also the catalytic activity of the enzyme is less harmful to the enantioselectivity. Consequently, it is widely used in the field of immobilization of enzymes. Such methods are economically attractive, but may suffer from problems such as enzyme leakage from matrix when the interactions are relatively weak[60]. Silanized molecular sieves have also been successfully used as supports for enzyme adsorption owing to the presence of silanols on pore walls that facilitate enzyme immobilization by hydrogen bonding[61] .

Yu *et al*.[62]adsorbed and immobilized CRL on CRB02 macroporous resin and used it to catalyse the esterification of lauric acid with propanol in isooctane. It was found that the activity of the immobilized enzyme and the ratio of enantioselectivityof the free enzyme increased by 50% and 120%, respectively. Immobilized lipase with an alkylated modified polymethyl methacrylate macroporous resin as a carrier, in which the

octaalkyl-modified macroporous resin adsorbed and immobilized three different sources of lipase (*H. lanuginose* Lipase, *C. antarctica* lipase B, *R.mieheil*ipase), achieved high transesterase activity[63].

1.5.2.2 Covalent bonding

2.2 Covalent bonding

During covalent bonding, an active group on an enzyme molecule reacts with an active group on a carrier to form a covalent bond, thereby immobilizing the enzyme on the carrier. Covalent association of enzymes to supports occurs via their side chain amino acids like arginine, aspartic acid, and histidine, with the degree of reactivity based on different functional groups such as imidazole, indolyl and phenolic hydroxyl[64].

Bifunctional reagents are commonly used as a cross-linking agent in the preparation process, including glutaraldehyde, epichlorohydrin, hexamethylenediamine, carbonyl imidazole, maleic anhydride and so on[65]. The enzyme and carrier binding have high binding affinity, conferring good stability to the immobilized enzyme. Moreover, the use of peptide-modified surfaces results in higher specific activity and stability with controlled protein orientation[66]. Mendes *et al.*[67]activated glutaraldehyde, using epichlorohydrin or glycidylaldehyde as a crosslinking agent, and then, immobilized TLL and *P. fluorescens* lipase by multipoint covalent bonding (PFL) for use in a solvent-free system to catalyse the transesterification of palm oil with ethanol. They showed that the immobilized enzyme had high activity and thermal stability. In the course of experiment, the parameters were optimized by olive oil emulsification, achieving the highest activity of acetaldehyde-resin-immobilized TLLand improved thermal stability27-31 times higher than that of the free enzyme.

1.5.2.3 Entrapment

Entrapment is the caging of enzymes by covalent or non-covalent bonds within gels or fibres[23]. The best means of avoiding any negative influence on the structure of an enzyme is to encapsulate it. Proteins or enzymes can be extremely fragile and easily damaged by external agent such as proteases. Encapsulation of these fragile macromolecules is a possible strategy for preventing their aggression and denaturation[68].Sol-gel chemistry is a well-established technique to encapsulate biological species such as enzymes, antibodies, and other proteins in a functional state. Sol-gels have been used extensively in the immobilization of proteins and in particular, for the development of biosensors. Although sol-gels are porous, diffusion of substrate to the enzyme can be restricted and care has to be taken to minimize this effect[69].

In the same context, entrapment by mesoporous silica is attributed to its high surface area, uniform pore distribution, tunable pore size and high adsorption capacity[55]. Simultaneous entrapment of lipase and magnetite nanoparticles with biomimetic silica enhanced its activity in varying silane additives[70].

1.5.3 Magnetic nanoparticles

Recently, magnetic nanoparticles (MNPs) based on iron oxides, have attracted a lot of attention thanks to their multifunctional properties, such as biocompatibility, superparamagnetism, small size, low toxicity, simple preparation process and low cost[71]. Magnetic nanoparticles are a highly valuable substrate for the attachment of homogeneous inorganic and organic containing catalysts[72].

1.5.3.1 Magnetic nanoparticles fixed

Magnetic nanoparticles are a highly valuable substrate for the attachment of homogeneous inorganic and organic containing catalysts.In general, immobilized carriers and the immobilized enzyme are easily separated, but in comparison with the free enzyme, there were still resistance towards mass transitdue to the low catalytic efficiency[71]. If the nano-carrier achieves enzyme immobilization, this problem can be resolved[73].

1.5.3.2 Synthesis and application of magnetic nanoparticles

Magnetic nuclear magnetic nanoparticles are mainly iron, manganese, cobalt and chromium oxide. Because nanoparticles Small size, large specific surface area, high-purity metal magnetic nanoparticles in cases without special surface treatment, easy to oxidation or corrosion, so magnetic nanoparticles for oxide[74].

They have been applied in magnetic resonance imaging (MRI), biosensorsand as anti-cancer drugs carriers[75]. Due to their high specific surface area and easy separation from the reaction medium by the use of a magnet, they have been employed in enzyme-catalysed applications[76]. There are many methods for their preparation, which can be divided into co-precipitation method, precipitated silica, emulsion and a sol-gel method[77].

The most common method for the production of magnetite nanoparticles is the chemical co-precipitation of iron salts[74]. Specifically, magnetite (Fe_3O_4) is a common magnetic iron oxide having a cubic inverse spinel structure[72]. Co-precipitation is one of the most diffused and low-cost techniques due to its simplicity, hence it is an efficient chemical pathway for the preparation of various solid-state materials of interest[75]. The compound exhibits unique electric and magnetic properties based upon the transfer of electrons between Fe^{2+} and Fe^{3+} in octahedral sites[78]. Magnetic particles of less than 20nm aresuperparamagnetic,

instantly magnetized in the presence of an external magnetic field, there is no residual magnetismafter removal of the magnetic field, so they are easy to recycle and re-allocate the reaction system[79].

The advantages of magnetic nano-scale materials include a higher specific surface, lower mass transfer resistance, facility of reusability related to the selective separation under a magnetic field and modification on the surface with various activity groups, etc[73]. In general, their surface can be modified prior to use, increasing the biocompatibility and surface functionalized particle surface, it can also protect the magnetic nanoparticle structure from oxidation and reunion magnetic nanoparticles can solve each other[78]. Many modified materials including oleic acid, PEG, chitosan, polystyrene and methacrylicacidare used for functional surface modification of magnetic nanoparticles. In particular, thesilane coupling agent can make magnetic nanoparticles,bringing different active reactive groups and contribute to the nanoparticles in a non-aqueous solvent stable[80].

Surface modification of magnetic nanoparticles can effectively change the surface charge of magnetic nanoparticles to enhance dispersion and stability, but also selectively introducing many different activity groups; the resulting strong reactivity can increase the range of applications[81]. Typical strategies for immobilizing lipase onto MNPs rely on surface grafting via low molecular weight linkers or polymers containing amino or epoxy functional groups to which lipases are reacted via covalent conjugation methods[82]

Preparation of magnetic nanocomposites can be divided into two types, surface polymerization and surface adsorption[83]. The former utilizes the interaction between the magnetic nanoparticles and the coating material, adsorbs them to the surface, initiating the surface reaction, whereas the latter directly deposits the coating material on the surface of the magnetic nanoparticles. Surface polymerization yields more stable nanoparticles, which have more applications[78].In the same context, Sulek*et al.*[84]synthesized magnetic nanoparticles by hydrothermal coprecipitation to obtain γ-Fe_2O_3 magnetic particles with a diameter of 13 nm, coated them with aminosilane to bring the amino reaction group on the surface, and then activated them with glutaraldehyde to obtain a magnetic nanocomposite material for immobilizing cholesterol oxidase. In addition to inorganic materials such as silica, the polymer material, because its copolymerization or surface modification enables the surface of magnetic nanoparticles to possess a variety of active functional groups (e.g. -COOH, -NH_2, -OH, etc.), is relatively easy and widely used for the surface modification of magnetic nanoparticles.

Ren*et al.*[72]immobilized lipase with poly dopamine-coated magnetic nanoparticles as carriers. The enzyme loading reached 429 mg/g and the pH stability as well asthermostability of the immobilized enzyme were improved. In another study, Zhang *et al.*[85]prepared magnetic microspheres by copolymerization of vinyl acetate (VAC), acrylamide (AM) and acrylic acid (AA) in the presence of Fe_3O_4 nanoparticles, and adsorbed and covalently produced two types of immobilized lipase, with a maximal recovery rate of 87%. Tran *et al.*[86]synthesized core-shell type Fe_3O_4-SiO_2 nanoparticles and treated with dimethyloctadecyl [3-(trimethoxysilyl) propyl] chloride to obtain surface-hydrophobic magnetic nanoparticles, and as a carrier immobilized lipase; after treatment of nanoparticles.

Wang *et al.*[83]synthesized nanoparticles with a Fe_3O_4 core anda fluorescent SiO_2 shell and grafted with hyperbranchedpolyglycerol (HPG-grafted Fe_3O_4@SiO_2 nanoparticles) conjugated with folic acid. Significant preferential uptake of the folic acid-conjugated nanoparticles by human ovarian carcinoma cells (SKOV-3), as compared to macrophages and fibroblasts, were shown by *in vitro* studies.Lee *et al.*[87]studied the binding of the non-magnetic particles to the dodecyl groups via sulphate linkages, using sodium sulphonate to make the surface hydrophobic, and then, adsorbed the immobilized porcine pancreatic lipase, with the enzyme separated from the carrier by one dodecyl carbon chain.The results showed that immobilized enzyme activity was greatly improved in comparison to the free enzyme and could be separated by magnetic field and used repeatedly.

1.5.3.3 Immobilized lipase with magnetic nanoparticles as a carrier

In recent years, magnetic nanoparticles as carriers of immobilized enzyme have become more popular. Indeed, a variety of enzymes have been successfully fixed to the magnetic nanoparticles via immobilization methods including physical adsorption, ion complexation, covalent bonding and immobilization. The more commonly used physical adsorption method is simple and easy, with a relatively small loss in enzyme activity[88]. The obtained nanocomposites are widely used in the immobilization of biomolecules. Nevertheless, the nano-scale immobilization of enzyme is challenging as their small size makes them difficult to separate[70]. However, the use of an external magnetic field can easily separate them from the reaction system, so is widely used in homogeneous catalysis[89].

Zhang *et al.*[85]prepared several different hydrophilic/hydrophobic magnetic nanoparticles to adsorb immobilized lipase. The results showed that the hydrophobic surface of the magnetic microspheres was more efficiently immobilized. When the acrylamide monomer accounted for 14.3% of the total amount of polymerized monomers, the highest recovery rate of the immobilized enzyme was 87%. Wang *et al.*[90]immobilized lipase to synthetic surfaces via hydrophobic magnetic nanoparticles, greatly improving the immobilized enzyme activity, with easy separation and recovery for re-use, retaining 65% enzyme activity after seven cycles of repeated use.It is well known that the covalent bond binds with a stronger forcethus, the stability

of the obtained immobilized enzyme is better and therefore, more widely used. Covalent bond fixation usesbifunctional or multifunctional reagents, includingglutaraldehyde[91], epichlorohydrin[91], EDC and NHS[92], cyanuric chloride[93], 1-butanal[94]. as coupling agents to achieve covalent bonding(see Fig. 6).

Xie*et al.*[95] used magnetic chitosan microspheres as a carrier, glutaraldehyde as a cross-linking agent to covalently bond lipase, catalytic soybean oil and methanol transesterification reaction of biodiesel, achieving a rate of up to 87%, with no significant decline in enzyme activityafter four cycles. Sui *et al.*[92]used hydroxyl-functional magnetic nanoparticles as a carrier and carbodiimide as a coupling agent to covalently bond immobilized lipase, significantly improving thetemperature and pH stability.

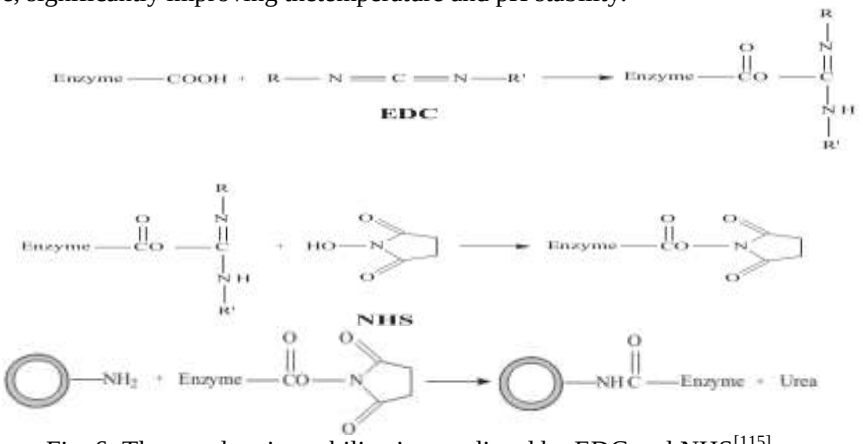

Fig. 6: The covalent immobilization mediated by EDC and NHS[115].

Lee *et al.*[87]surface hydrophobic magnetic nanoparticles adsorption immobilization of porcine pancreatic lipase, significantly increasing the enzyme activity in comparison to the free enzyme and it could be re-used by magnetic separation. Wu *et al.*,[82]adsorbed immobilized lipase with Fe_3O_4-chitosan magnetic nanoparticles, achieving a maximum adsorption rate of 129 mg/g and recovery of enzyme activity of 55.6%. Woo *et al.*[81]used four surface-complexes Cu^{2+} core-shell magnetic nanoparticles as the carrier, immobilizing the lipase by complexing with Cu^{2+}.The best immobilized enzyme could be reused three times. (see Fig. 7).

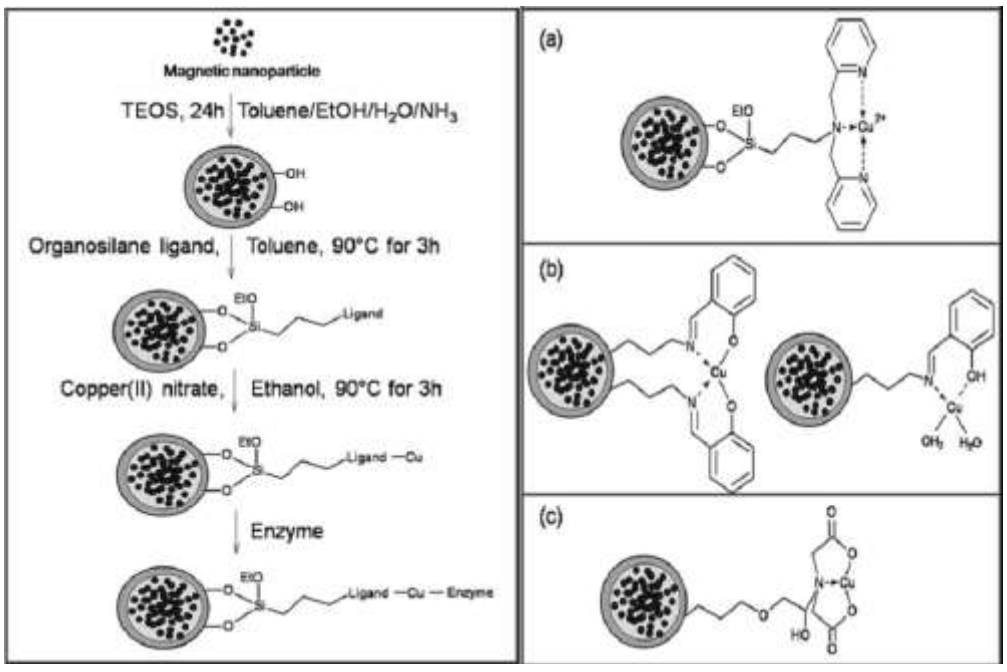

Fig. 7: Lipase immobilization by copper ion complexation[81].

1.5 Carbon nanotubes

A new form of carbon, Buckminster fullerene, was discovered in 1985 by a team headed by Korto[96]. Besides diamond, graphite, and fullerene, quasi-one-dimensional nanotubes are another form of carbon first

reported by Ijima in 1991, when he discovered multiwalled carbon nanotubes (MWCNTs) in carbon soot made by an arc-discharge method[97].

1.5.1 Classification of carbon nanotubes

Carbon nanotubes are classified into two types,single-walled carbon nanotubes(SWCNTs) and multiple-walled carbon nanotubes(MWCNTs) (see Table 4 and Fig.8)[98, 99]

Table 4: Comparison between SWCNT and MWCNT.

SWCNT	MWCNT
Single layer of graphene.	Multiple layer of grapheme
Catalyst is required for synthesis.	Can be produced without catalyst.
Bulk synthesis is difficult as it requires proper control over growth and atmospheric condition.	Bulk synthesis is easy
Not fully dispersed, and form bundled structures.	Homogeneously dispersed with no apparent bundled formation.
Resistivity usually in the range of 10^{-4}–10^{-3} Ω•m	Resistivity usually in the range of 1.8×10^{-5}–6.1×10^{-5} Ω•m
A chance of defect is more during functionalization.	A chance of defect is less especially when synthesized by arc-discharged method.
Characterization and evaluation is easy.	It has very complex structure.
It can be easily twisted and are more pliable.	It cannot be easily twisted.

Fig. 8 : : Molecular representations of (a) SWCNTs and (b) MWCNTs

1.5.2 Modification of Carbon Nanotubes

The chemical inertness of CNTs obstruct their ability to be processed, which has prevented the full realization of their potential. One disadvantage of the CNTs concerning their use in biochemistry and biomedical applications is that they are highly hydrophobic and generally form insoluble aggregates. Due to their low solubility in many solvents, it is also very difficult to isolate one carbon nanotube from another. Therefore, proper stabilization of CNTs mixtures is a prerequisite for technological applications[100, 101]

Gao*et al*,[102]reported that when CNTs were sonicated in organic solvents, they produce dangling bonds that will undergo further chemical reactions. Many efforts in recent years have led to the development of versatile chemical modification methodologies in order to solve the insolubility problem. The recent expansion in methods to chemically modify and functionalize carbon nanotubes has made it possible to solubilize and disperse carbon nanotubes in water, thus allowing for their facile manipulation and processing in physiological environments. The surface functionalization of CNTs by chemically attaching an organic functional groups will aid the carbon nanotube materials in becoming biocompatible, improving their solubility in physiological solutions and selective binding to biotargets[103]. It has been demonstrated that biological and bioactive species, such as proteins, carbohydrates, and nucleic acids, can be conjugated with carbon nanotubes[104, 105]. Both noncovalent and covalent strategies have been explored to engineer the interface between biological molecules and CNTs, with the goal of preserving the functional properties of the biomolecules. The biomolecule immobilization on the sidewall of the CNTs, and more interestingly, inside the CNTs, has been reported in both computational and experimental fields[106].

1.5.3 Immobilized lipase with CNTs as carrier

Nanomaterials can serve as excellent supporting materials for enzyme immobilization as they offer ideal characteristics for balancing the key factors that determine the efficiency of biocatalysts, including surface area, minimized mass transfer resistance and effective enzyme loading[107].

Carbon nanotubes are receiving a great deal of attention as alternative matrices for enzyme immobilization. CNTs are better support material for enzyme immobilization compared to common support like zirconia, silica, and epoxy. They are more stable under harsh conditions, providing higher loading of enzyme[108]. It was reported that small proteins such as metallothionein, cytochrome C, and -lactamase were able to adsorb on the internal, as well as outer walls, of CNTs after the ends were opened by treatment with concentrated nitric acid[109]. Non-covalent attachment preserves the unique properties of both enzymes and CNTs, but the immobilized protein can be gradually lost during the use of the CNT-enzyme complex[110]. Covalent conjugation provides durable attachment, but the enzyme structure may be more disrupted. Functionalization of CNTs with organic, polymeric and biological molecules can provide biocompatible nanotube composites with specific groups on their surface. No matter what method is used, the main challenge is promoting the stable attachment of enzymes, while maintaining their activity and function as closely as possible to their native state[111]. For the direct physical adsorption method, the interacting force between the protein or enzyme and CNTs is predominantly a hydrophobic interaction. The amount of enzymes adsorbed on CNTs are affected by various factors, such as the nature of the enzyme, the surface chemistry of the CNT and operation variables[100].

In a study byMohamad*et al*.[112]geranyl propionate was enzymatically synthesized from geraniol and propionic acid using *C. rugosa* lipase immobilized on acid functionalized multi-walled carbon nanotubes. The use of CRL-MWCNTs in n-heptane (log P 4.0) and alcohol/acid (molar ratio of 5:1) resulted in a 2-fold increased conversion frequency as compared to the free CRL for the production of geranylpropionatein addition to a noteworthy 2-fold enhanced thermal stability. Fan *et al*.[113]grafted a polyamidoamine (PAMAM) dendrimer onto magnetic multi-walled carbon nanotubes to combine magnetic properties with a large surface functionalized with amino groups, achieving an oriented-immobilization of RML on the obtained m-MWCNTs-PAMAM matrix, with esterification activity 27-fold higher than that of the free enzyme.Wang *et al*.[44]presented the first report of the use of lipase-adsorbed halloysitenanotubes as an efficient medium for the selective enrichment of lipase inhibitors from natural products. A simple and rapid approach was proposed to fabricate lipase-adsorbed nanotubes through electro-static interactions. The results showed that more than 85% lipase was adsorbed into nanotubes in 90 min.

II. Conclusion

In conclusion,enzymeimmobilization is one of the most promising techniques for highly efficient and economically competent biotechnological processes in the field of environmental monitoring, biotransformation and food production. The technology of immobilized enzymes is still going through a phase of evolution and maturation, mirrored in the development of the theory of how immobilized enzymes function and how the technique of immobilization is related to their primary structure through the formation and configuration of their three-dimensional structure with attributes like efficiency, quicker performance and multifarious use. Research should be focused on overcoming the current limitations related to immobilization technique to allow for more widespread application of such immobilized enzymes.

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