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IMMOBILIZATION OF LIPASE ON IRON OXIDE ORGANIC/INORGANIC HYBRID PARTICLES: A REVIEW ARTICLE

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Abstract. In last few decades the demand of lipase has been dramatically increase due to its useful use in numbers of biochemical industries. Varieties of natural and synthetic carriers and methodologies have been used to improve lipase activities by the process of immobilization in order to enhance its activities in term of its resistance to high temperature, pH and to increase its reusibity and storage capacity. Due to the expensive nature the recycling of the lipase has been the target of the researchers to decrease its cost in the industrial process. Magnetic iron oxide organic/inorganic hybrid nanoparticles as a support have been mostly used in the lipase immobilization due its large surface area, less toxicity, bio compatibility, easily functionalization, separation avoiding long time consuming process like filtration and centrifuging.

1. INTRODUCTION

Due to the hazardous effects of the chemical reagents recently used, the interest of the researcher in the green chemistry is being developed. In this scenario, the use of enzymatic processes has been highlighted due to their versatile and specific nature. These macromolecular biological catalysts have been used in the catalysis of different processes like organic synthesis, biomedical process, biosensor and in the green energy chemistry applications [1]. This emphasizes the need for better and improved enzymatic properties for the development of insoluble enzymes [2]. The process of Immobilization has enhanced stability of enzymes to thermal and pH changes and has also increased the reusibity of enzymes, ultimately leading to the lowering of the production cost in the industrial applications [3,4].

Lipases (triacylglycerol acyl hydrolase, EC 3.1.1.3) is a class of macromolecular enzymes that catalyze the hydrolysis of ester at an oil/water interface also catalyze the reverse reaction in organic media, esterifying fatty acids and glycerol into triglycerides, as well as catalyzing transesterification and alcoholysis reactions [5]. Different kinds of new research technologies are being utilized these days the immobilization of lipase. Immobilization is the physical confinement or localization of enzyme in a certain defined region of space with retention of their catalytic activities enhancing its storage and recycling capability [6].

Immobilization methods can be basically divided into chemical and physical methods. In the chemical method, formation of covalent bonds is achieved through ether, thio-ether, amide or carbamate bonds between the enzyme and support material are involved. Physical methods are interactions such as

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hydrogen bonds, hydrophobic interactions, van der Waals forces, affinity binding, ionic binding of the enzyme with the support material, or mechanical containment of enzyme within the support [7,8].

Superparamagnetic nanoparticles has been used in the valuable technologies[9] like, biosensing [10], drug delivery [11], magnetic resonance imaging (MRI) [12], magnetic inks [13] as these particles can attached to the drugs ,protein, enzymes antibodies, or nucleotides due to high magnetic properties [14].

The nanoparticles of the element with magnetic properties are iron nickel and cobalt [15]. During their applications the alarming concern is the toxic and expensive nature of the compounds of these magnetic elements [16]. Among these elements nanoparticles of the iron oxide have been mostly utilized in biochemical application like enzyme immobilization due to less toxic nature [17].

The technique of immobilizing lipase directly on ligand attached to the magnetic nanoparticles as a support has been widely used [18]. Magnetic particles of nano, meso, micro sizes have been modified with organic and inorganic materials to achieve functionalized particles as a matrix for lipase immobilization using coupling agents [19,20]. Hence magnetic particles of different size and morphologies have been the major interest of the researcher to be explored [21].

Enzymes may not always retain their activities after immobilization. Jordan et al (2011) found that after immobilization on the carrier enzymatic activity was lost. Several adsorbed protein were reported to show elution problems from ligand-immobilized magnetic nanoparticles [22]. The techniques like physical and chemical methods of immobilization and varieties of carriers are used in literature to enhance the immobilized lipase efficiency and to overcome the leakage and instability difficulties [23].

Of the novel matrixes, nanocrystals and oxides of metal are mostly used for the process of immobilization with good results [24]. Metallic nanoparticles have a good magnetization but, its toxicity and high reactivity limit its utilization in biomedicine and biotechnology fields. The problem can be solved by coating the particles with polymers or silica [25]. However; magnetic nanoparticles coated with polymers are unstable at high temperature. Like as the amylases, proteases and cellulases when immobilized on magnetic carriers, the effectiveness is less as compare to their soluble equivalents [26]. So selection of proper carrier for immobilization is needed to avoid all the defective suspects.

In the use of nanomaterials as carriers for enzyme immobilization some basic parameters like, immobilization yield, specific activity, recovered activity, less loss of activity and cost factor in operational processes must be considered [27]. Also in the process of immobilization in practice the particle size should be capable of separation by filtration or centrifugation, as well as may not be too large size so as to avoid diffusion limitations [28].

Thus, although there are hundreds of immobilization protocols but the design of new protocols that may permit to improve the enzyme properties during immobilization is still a challenging target. So for the deep analysis of immobilization procedure a comprehensive review pertaining to lipase immobilization and stabilization is needed.

The current study reviews the nature of magnetic particles capitalized in immobilization of lipase, the new developments in the immobilization, the different methodology and the magnetic carrier used to achieve stability of the lipase to reduce the cost factor by recycling which is the most challenging aspect of this research.

2. METHODS FOR THE SYNTHESIS OF SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLES AND ITS CHARACTERIZATION

The chemical pathways used in literature for the fabrications of iron oxide magnetic particles are classic synthesis using precipitation [29], sol-gel reaction [30] fluid stabilization, and surface modification for grafting biomolecules [31].One of the easy methods for the synthesis of particle is the following precipitation method [32]

$$Fe^{2+} + 2Fe^{3+} + 8OH^{-} \rightarrow Fe_3O_4 + 4H_2O, \quad (1)$$

$$Fe_3O_4 + 0.25O_2 + 4.5H_2O \rightarrow 3Fe(OH)^{3+}. \quad (2)$$

Due to the colloidal nature of magnetic iron oxide particles their synthesis is a complex process, so defining of experimental conditions to disperse it uniformly are needed. Similarly to controle suitable size and to reproduce it would be a successful procedure. So for the easy synthesis of the particles complicated steps like ultracentrifugation[33], size size-exclusion chromatography, [34] magnetic filtration or flow field gradient, should be not the part for the fabrication these super magnetic particles. Size controle of the particles is an important process [35]. The size may be affected by temperature, ionic strength, FeII/FeIII concentration ratio and na-

ture of the salts like perchlorates, chlorides, sulfates, and nitrates. Further to controle the size of the magnetic particles some chelating organic anions and polymer surface complexing agents have been proved to play effective roles [36].

The coordinating capacity of the iron oxide with water, leaving the hydroxyl easily available to be functionalized makes it amphoteric in nature with 6.8 as isoelectric point [37,38]. At zero charge point particles flocculate. The morphology of the particles can be controlled by temperature, time and concentration of the reactants [39]. Other important factors, affecting the morphology are addition of seeds and nature of the solvents [40].

Nude magnetic nanoparticles do not effectively interact with protein (enzyme), and a surface modification is required [41]. In the literature some modifications for the magnetic nanoparticles surface modification are reported as; by crosslinking with glutaraldehyde, coating with polymers, [42] coupled with compounds like as agarose[43]1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC)[44] and using chitosan with effective out comes in the process of immobilization [45].

3. BEHAVIOR OF LIPASE

Depending on the experimental condition the lipase exhibits two types of conformations which are in equilibrium [46]. In the lipase B from *Candida Antarctica* [47] the active center is not fully hidden to react but in most case it is not such, like lipases from *Thermomyces lanuginosus* [48] or *Rhizomur miehei* [49].The lipase is active in water oil interface ,the behavior is known as interfacial activation [50].As the process of immobilization may reduce the enzymatic activity, disturbing the confirmation that the active center may not be exposed to react freely, so optimum condition are to be searched out for the immobilization process [51]. The active center of the lipase is covered by polypeptide lid; the immobilization on the hydrophobic surface causes the opening of the lid, leading to the active behavior of the lipase [52]. The hydrophobic carriers which have been reported for the immobilization with enhanced activity are, such as octadecyl-sepabeads, octyl-agarose, oleic acid-coated magnetite, carboxylic surfactant grafted zirconia nanoparticles, SDS-bound nano-sized magnetite particles [53,54]. Similarly immobilization on hydrophobic surface with low ionic strength also favors the efficient process [55,56], the high ionic strength has a double negative effect, restricting lipase activity [57,58]. Immobilization leads to the dispersion of lipase also causing the rigidification of the stable confirmation of lipase on the support surface ultimately causing improved catalytic [59].

Another factor that affects the lipase activity is the inhibition. The immobilization causes the diminished enzyme inhibition [60,61], like Caldolysin from Thermus aquaticus strain T351 as reported for the first time [62].

Porosity of support also provides a protective layer to the immobilized lipase. So enzyme with in the porous body of the carrier will safe from the adverse effect produced by detergents or stirring process [63].

Mainly, three kinds of reactions catalyzed by lipase are: (1) Hydrolysis, (2) Esterification, and (3) Transesterification. The most commonly reported lipases in the literature and the abbreviations used are *Burkholderia cepacia* (CPL), *Candida antarctica* (CALB), *Candida rugosa* (CRL), *Rhizomucor miehei* (RML), *Rhizopus oryzea* (ROL), *Thermmmyces languginosus* (TLL) [64].

The supports used for the lipase immobilization may be inorganic carriers are silicas, titania, and hydroxyapatite or organic supports include compounds of natural origin, such as chitin, chitosan, cellulose and alginate, also the synthetic compounds, mainly polymers. Immobilization over comes the passive nature of free enzyme in organic solvents by enabling it to act in non-aqueous environments (organic solvents, surfactants, ionic liquids). Naturally free enzymes are catalytically efficient in the aqueous media and passive in organic medium [65]. The immobilization methods and the types of carriers used have been summarized in the graphical abstract in Fig. 1.

5. EFFECT OF IMMOBILIZATION CARRIERS

There are certain characteristic of the carrier which play key role in the performance of immobilized lipase. The most important one is the hydrophobicity, easy functionalization of the support, resistance to physical compression and microbial attacks, recylabliblity nontoxic nature and less expensiveness add to the valuability of the immobilization matrix [66].The reusability is achievable by immobilization on the magnetic carriers, which is less time consuming process as compare to the traditional centrifugal and chromatographic separation techniques [67]. Similarly the particle size and surface area, size to shape ratio are also the the important factors to be considered for the matrix used [68]. The macrobilogical molecules like lipase are

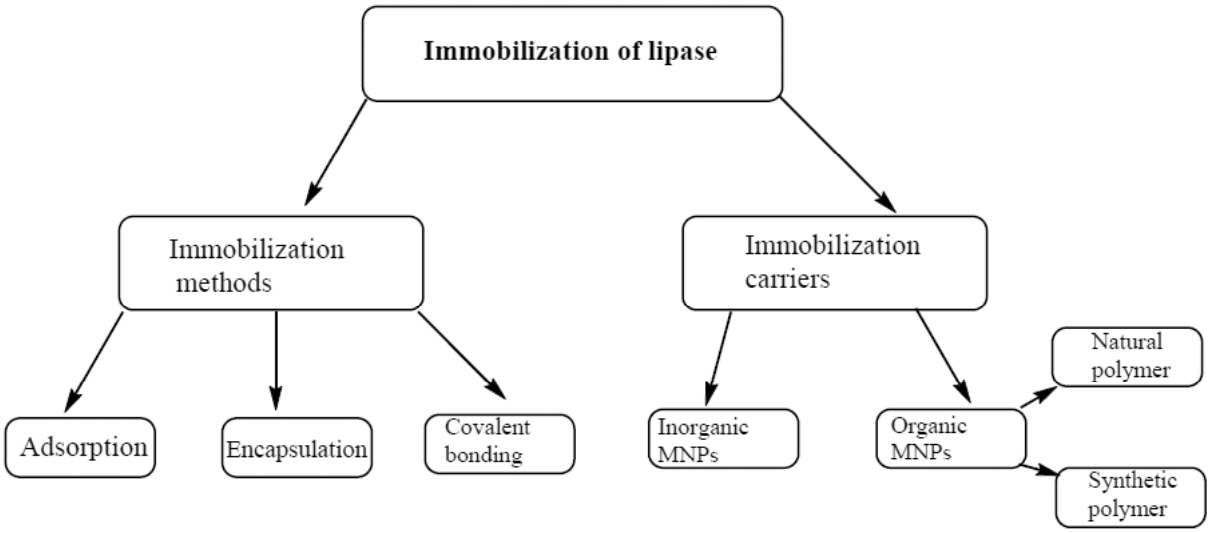

Fig. 1. Sketch showing methods and carriers used for immobilization of lipase.

used for long terms in the industries catalysis. So the mechanical stability of the carriers is needed [69]. For mechanical stability of the biocatalyst the particle size, pore distribution, and morphology of the carrier are also important [70]. Thus for the immobilization with high yield the selection of proper physical properties are the prerequisites [71].

5.1 Inorganic magnetic particles as carrier

Number of inorganic materials like silica of different morphologies MCM-41, and SBA-15 ([72-74]), zeolites are used for the immobilization in combination with magnetic particles[75]. In these iron oxides, magnetite (Fe_3O_4) and hematite (Fe_2O_3, weakly ferromagnetic or antiferromagnetic), are the best candidates, being commonly used for biolabelling and bio separation in the virtue of their biocompatibility [76].

Several approaches have been used to functionalize magnetic nanoparticles. Silane derivatives have mostly been used due its structural stability and due to easy functionlization, with -NH_2, -SH, - COOH and protection from oxidation [77]. All these properties recommend siloxane to be used in biomedical applications, including enzyme immobilization [78]. So the synthesis of magnetic core and silica shell has been widely reported due to its broad application [79].

T. Dang et al. (2012) synthesized core shell nanocomposites with magnetic core Fe_3O_4–SiO_2 which was modified with hydrophobic shell dimethyl octadecyl [3-(trimethoxysilyl) propyl] ammonium chloride. The efficiency of lipase with the hydrophobic layer was improved from 76% to 97% as compare to the Fe_3O_4–SiO_2, as the lipase performs well on the hydrophobic surface, by exposing its active site to interact [80].

Magnetic clay composites have also been found to be a good matrix for the lipase immobilization. Palygorskite (Pal), kaolinite (Kaol), and montmorillonite (Mt) are some the clay minerals which have been used with magnetic particles for immobilization of lipase. When lipase was immobilized on Fe_3O_4@Kaol & Fe_3O_4@ Pal the residual activity to hydrolyse the starch was 71.86% &71.86% respectively [81].

Naturally occurring clay material Halloysite [$Al_2Si_2O_5(OH)_4 \cdot 2H_2O$] (HNTs) due to its specific characteristics of high surface area, good biocompatibility and low cost has also been used for the immobilization of enzymes [82].

Resent researchers have discovered that porosity of the carrier is one of the many factors that affect the immobilization. If on one hand it increases the loading of lipase on the other hand it also safe guards the lipase from the negative effects like leakages during stirring, additionally protect it from the negative effects of detergents.

Due to the mass transfer capacity of the porous supports the enzymes immobilized generally have high catalytic activity [83]. Z. Ali et al. used the magnetic porous fibrous silica Fe_3O_4@ KCC-1, magnetic yolk shell fibrous porous silica with considerable increase in the catalytic activity of the immobilized lipase on these carriers with good recycling, thermal and storage ability [84,85].

Magnetic hollow porous silica was also used by means of multiple-mode adsorption based on both

hydrophobic and strong cation-exchange interactions, for the ultra-fast immobilization of lipase with a high loading of CRL (95.2 mg/g) [86]. Y. Dianyu et al. (2013) used the porous Phospholipase A1 (PLA1) onto magnetic Fe_3O_4/SiOx-g-P (GMA) nanoparticles for the process of immobilization [87].

5.2. Fe_3O_4@-Organic synthetic polymer materials

The natural and synthetic polymers which have good mechanical strength, large surface area, easy functionalization and resistant to environmental changes and good separation in their magnetic composites have been utilized in the process of lipase immobilization [88,89]. Synthetic polymers offering large areas for enzyme–support interactions are polystyrene, polyacrylate, polymethacrylates, polyacrylamide, polyamides, vinyl and allyl-polymers [90]. Other synthetic polymers reported in literature as enzyme supports are polyvinyl chloride cyclodextrin; polyurethane microparticles; UV-curable methacrylated/fumaric acid-modified epoxy; polyaniline and glutaraldehyde-activated nylon [91]. Porcine pancreas lipase (PPL) was immobilized covalently on Magnetic poly glycidylmethacrylate (GMA)–divinylbenzene (DVB)–methacrylicacid (MAA)) microspheres with active epoxy groups with activity yield and enzymatic activity [92]. Z.Ali et al. prepared Flower-like Fe_3O_4 microspheres by a fast solvothermal method micron-sized Fe_3O_4@polyglycidyl methacrylate (GMA) for the *candida rugosa* immobilization with enhanced catalytically activity [93].

Reports illustrate that lipase immobilized on microspheres of synthetic polymer prepared under magnetic field (MF) demonstrates increase activity and improved storage stability than those immobilized on microspheres prepared without MF. The study determined that microspheres synthesized with and without magnetic field (MF) had different morphology and surface area.Liu et al. used methacrylate (MA) and crosslinker divinylbenzene (DVB) to synthesize poly (methacrylate-divinylbenzene) magnetic microsphere in the presence of magnetic fluid via modified suspension polymerization. The results indicated that the immobilized *Candida cylindracea* lipase held high enzyme loading (34.0 mg g^{-1} carrier), high activity recovery (72.4%), and good stability in the recycling process [94,95].

Natural polymers like chitin, chitosan, alginate, collagen, carrageenan, gelatin, cellulose, starch, pectin have been used as supports for immobilization because these natural polymers are compatible with enzymes [96].

As the natural polymers are hydrophilic, with less interaction with enzymes, leading to weak bonding so they need proper functionalization, also weak mechanical stability may be overcome by cross linking. The derivatives of agarose, cellulose and cross-linked dextrans are the commonly used carriers for immobilization [97].

Chitosan is a nontoxic, biocompatible and gel-forming cationic compound which can easily be prepared in different geometrical configurations, such as membranes, beads, different size nanoparticles, fibers, hollow fibers or sponges. Another advantage of chitosan is that it can easily be chemically modified, which is possible due to the presence of modifiable functional groups ($–NH_2$ and –OH) on chitosan chains [198,99]. Metal-polyphenol film consolidated Fe_3O_4/chitosan hybrid microcapsules (MPP–Fe_3O_4/CS) were synthesized for the immobilization of lipase from *candida rugosa* with enhanced kinetic behaviors, stability and reusability [100].

Agarose as excellent natural support for enzyme immobilization has been used as in its magnetic composites due to its porous nature, hydrophilicity, easy functionalization, its good tendency for protein, no charged groups (which inhibits nonspecific adsorption of substrate and Products), and cheap availability [101,102]. Magnetic nanoparticles coated with polymers are most unstable at high temperature and the catalytic properties of the nanoparticles may further affect the polymer adversely [103].

It can be summarized that there are no universal carriers for all enzymes and their applications. Mostly, the choice of a carrier is determined by many factors such as the good surface area, high availability and low cost, high thermal stability and chemical resistance [104].

6. EFFECTS OF IMMOBILIZATION METHODS

For the efficient immobilization the selection of proper method is important**.** These methods may be lipase physical adsorption or the entrapment of lipase in the liposomal layer or in the porous body of the carriers. The other method may be the chemisorption by using covalent or condition bonding. The lipase may be cross linked using a bifunctional reagent. The matrix surface is functionalized in such a way to create electrophilic site on the carrier which can easily couple with the strong nucleophiles site on the proteins [105]. The

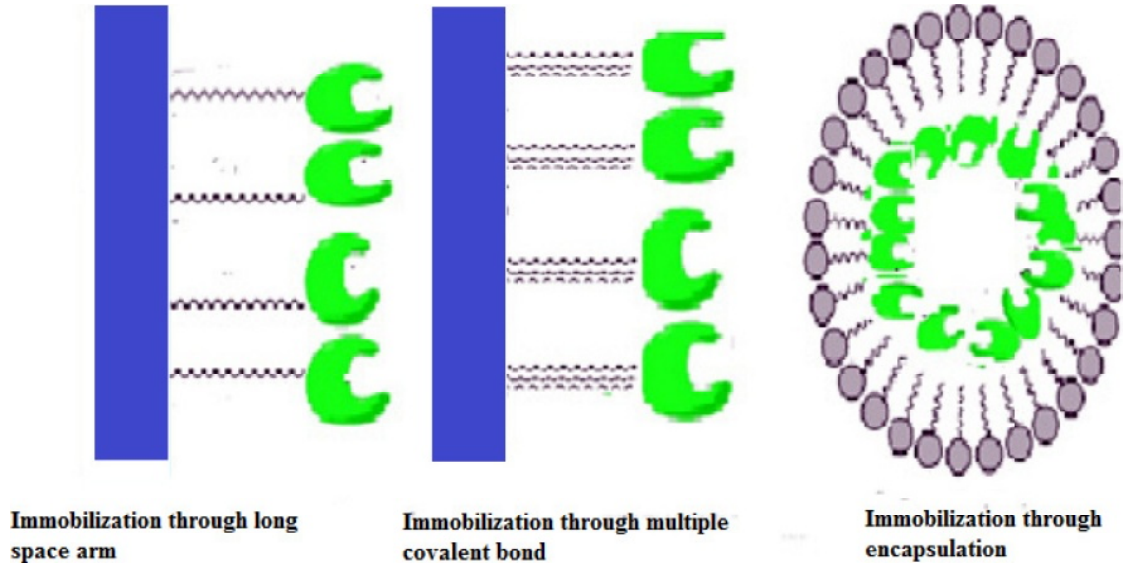

Fig. 2. Immobilization methods.

matrix may react directly or through an arm cross linker with amino group of lysine, thiol group of cysteine and carboxylic group of aspartic and glutamic acids which cause the rigidification of the stable configuration of enzyme [106].

The enzymes can be attached to the support through ether, thio-ether, amide or carbamate bonds [107] and the active tertiary structure of the lipase is retained [108]. A literature survey analyzed cross linker several enzymes including lactase, lipase, esterase, β-galactosidase, oxidase, dehydrogenase, a-chymotripsin, chloroperoxidase, penicillin G acylase, *L*-asparaginase, tyrosinase, horseradish peroxidase, chitosanase, papain, diastase levansucrase, amylase, streptokinase, dispase, dehalogenase, laccase and epoxide hydrolase have been immobilized onto magnetic particles for different purposes [109,110]. These methods of immobilization have been summarized graphically in Fig. 2.

6.1. Lipase immobilization via physical adsorption

Of the several methods used for preparing biocatalysts as lipase immobilization, adsorption is the cheapest [111]. The weak forces between enzyme and the support can only be of Van der Waals, hydrogen bonding and hydrophobic types, with minimum effect on catalytic activity [112]. Such weak interactions make easy recovery of immobilized enzymes by desorption from the support [113]. Wu et al. adsorbed lipase electrostatically on the magnetic chitosan nanoparticles with 129 mg/g loading and enzymatic activity was considerably increased [114].

The affinity of the lipase for the support is determined by the zeta potential which has been utilized in immobilization techniques) [115]. Hudson studied the zeta potential of the lipase and carrier and their compatibility with each other and found its significance role in immobilization [116].

As the interaction in the physical adsorption is weak which causes enzyme releasing, so strong adsorption is required which may be achieved by polymeric beds polyethylenimine (PEI), dextran sulfate (DS) which allow multipoint adsorption involving many areas of the protein surface without leading to the unwanted conformational distortion in the protein structure [117].

The physical adsorption may also be increased by increase in the hydrophilicity, correspondingly increase in the zeta potential. Wu et al. [118] coated magnetite nanoparticles with different lengths of alkyl chain designated as C3, C8, C18. The activity was found to increase for the immobilization from C8 to C18.The process of adsorption increased with the increase in hydrophobic character of carrier [119]. M. Kanagasabai et al. found increase in the activity of the immobilized lipase on NSM (ABS-NSM) as carrier, modified with more hydrophobic alkyl benzenesulfonate [120].

Physical interactions (adsorption) are weak and enzyme can easily washed out, which can be further enhanced under sever industrial conditions. This adsorption does not result in large loss of enzyme activity and can be carried out in mild conditions.

Despite many merits of technique, it also presents some other drawbacks. Like, the immobilized enzyme prepared by adsorption has poor operation stability; the amount of adsorbed enzyme is mostly affected by the other immobilization conditions as ionic strength, and pH; because of the weak forces between them [121].

6.2. Lipase Immobilization via encapsulation

The enzyme is called to be entrapped when it is enclosed within a polymeric network but the substrate and products can pass freely. The method has been utilized in the process of immobilization such as gel or fiber entrapping and micro-encapsulation. The limitation of this method is mass traversing through membrane or gel. The process of sol gel is a useful entrapment method as it encloses large amount the biological molecules, has thermal and chemical stability. The method is easy as it involve any covalent modification and the geometry can also be controlled [122,123].The disadvantage of the method is the transfer problems and leakage which need careful controle of the pore size [124].

P. Kristyna et al. reported immobilization lipase and β-galactosidase by the process of encapsulation using magnetic chitosan with enhanced enzymatic activity in the kinetic resolution of chiral alcohols and amines, recycling without any substantial loss in enantioselectivity [125,126]. Elif et al. immobilized *Candida rugosa* lipase by this encapsulation technique in the presence and absence of magnetic Fe_3O_4 nanoparticles [127].Magnetic carriers of celite, kaolin, chitosan, amberlite XAD 7, cyclodextrin, and calixarene have been used for the encapsulation of lipase [128,129].

Encapsulation method also suffers mass transfer limitations of substrate or analytes so restricting the use of this method. Other limitations are leakages if the pores size of the support is too large, low loading and abrasion of the material used for immobilization are also the demerits of the technique [130].

The process of entrapment must be differentiated from the support binding. Entrapment needs enzyme for the fabrication of the polymeric carrier. Enzyme is called to be entrapped when the enzyme is present during the synthesis of a silica sol–gel. The enzyme is not to be called entrapped when an enzyme is immobilized in prefabricated mesoporous silica [131].

6.3. Lipase immobilization via covalent bonding

Immobilization by covalent bonding is a method which is used to immobilize enzyme by binding the nonessential functionality of enzyme to the functional group of carrier via chemical bonds, under mild conditions, thus the conformation is maintained to be active for enzymatic reaction [132,133]. The commonly used coupling agents are glutaraldehyde [134], Epichlorohydrin [135],N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS). When *Candida rugosa* was immobilized on magnetic Fe_3O_4-chitosan nanoparticles using these linker, the highest activity obtained was 20 U/g,with good stability and reusability of the enzyme [136,137]. Due to the spacer-arm affect, and multi point attachment, these linkers cause increase in loading capacities and lipase activity as the arm spacer like Glu has also hydrophobic part which adds to the hydrophobic characteristics of the carrier, the unique characteristics of Glu [138].

Despite of the merits of the immobilization technique by covalent bonding, like making the lipase stable, recyclable and leak proof, the method has some drawbacks. It leads to some structural distortion of the lipase. As for the activation of carrier some harsh conditions are required which may adversely affect the structure of lipase [139]. Further after enzyme immobilization the carrier is to be discarded along with the expensive lipase. This method also leads to low yield along with the disadvantage of non-reversibility [140].

7. EFFECT OF SOLVENT ON IMMOBILIZATION

The activities of immobilizes lipase is also solvent dependent as reported in the literature. Adriano et al. (2011) worked on the different methods of immobilization lipase from *Penicillium Camembertii* (Lipase G) and also the effect of different solvents. Immobilization on the carrier MANAE-agarose-glutaraldehyde and glyoxyl-agarose in aqueous medium and organic solvents was studied also physical, covalent methods and ionic adsorption were compared.Immobilized derivative on epoxy-SiO_2-PVA composite using hexane was found to be with good results as compared to the ionic adsorption [141]. Similarly, G. Sabrieh et al. immobilized *Thermomyces lanuginosa* lipase (TLL) covalently on synthesised Fe_3O_4/ZnO core/shell magnetic nanoparticles, the effect of some organic solvents

was examined. These studies revealed that hydrophobic solvents such as isooctane or n-hexane were more suitable than others. In the presence of hydrophobic organic solvents, the hydrophobic surface of lipases increases upon lid opening causing availability of the active site resulting in high enzyme activity [142]. H. Bin et al. et al. immobilized lipase with amino fictionalized magnetic particles. Their study also analyzed the activity of lipase in the hydrophilic and hydrophobic solvents. The stability and high dispersity was found in the hydrophilic solvents like as water, ethanol, methanol and isopropanol which may be probably due to the high affinity between amino groups on magnetic nanoparticles and hydrophilic solvents [143].

8. EFFECTS OF DETERGENTS ON IMMOBILIZATION

It has been find out that surfactants add significantly to the increment in the activity of immobilized lipase. The surfactants have the capability to facilitate the open stable configuration of lipase, increasing the protein rigidity also making the active site of the protein to be easily accessible to the substrate in the presence of anhydrous media.

Using different concentrations of surfactants, anionic (AOT), cationic (CTAB) and non-ionic (Tween 80 and Triton X-100), Z. Wei et al. activated amino functionalized magnetic cross-linked enzyme aggregates to immobilize the lipase. Higher activity was shown by branched hydrophobic tails than those having straight chains [144].

A phenomenon called nonspecific binding is a random adsorption of bio components on noncomplementary materials. It has been shown to be successfully solved by application of specific detergents into reaction systems. It was found that aliphatic chain of tween molecule can prevent nonspecific binding of biomolecules. Effects of detergents on lipase conformational changes, to enhance their activity were studied as well. It was found that detergents when applied into enzymatic systems increase substrate concentration at the interface and reduce hydration shell around the ester bonds and thus increase catalytic activity of the protein. Simons et al. reported a conformational change occurring in *Staphylococcus aureus* lipase, which was induced by Triton X-100. Micelles of both non-ionic and ionic detergents induced the conversion of the pancreatic lipase from closed to open form in the study performed by Hermoso et al. [145]. Jutilla et al. examined conformational changes of *Humicola lanuginosa* lipase (HLL) induced by pentaoxyethylene octyl ether (C8E5), which resulted in enhanced lipolytic activity under defined conditions[146].

9. MAGNETIC CROSS-LINKED AGGREGATES (mCLEAs) AS CARRIER FREE IMMOBILIZATION SOURCE

To synthesize the carrier free macroparticles, enzyme aggregates have been utilized as a bifunctional reagent. When the carriers are used then the dilution of activity is an inevitable phenomenon leading to lower productivities and lower space-time yields. So the needed crosslinked enzyme crystals (CLECs), and cross-linked enzyme aggregates (CLEAs) can be capitalized as a carrier-free immobilized enzymes.So the problem of low concentration of enzyme activity and high cost factor can be avoided with the exclusion of carrier [147]. Numerous authors have suggested the combination of magnetic nanoparticles inside the CLEAS to enhance the enzyme properties. Mixing of magnetic nanoparticles and CLEAs allow easy isolation, reduced sizes [148], increases the enzyme loading, useful in biodiesel synthesis [149]. Cruz-Izquierdo et al. [150] fabricated magnetic cross-linked aggregates (mCLEAs) activated with glutaraldehyde.

10. CONCLUSION AND EXPECTATION

The magnetic iron oxide particles due to their small size and large surface area are versatile agents for manufacturing excellent matrices for immobilization of free lipase. Apart from these properties the magnetic particles also give mechanical strength and flexibility also their composites for lipase immobilization are environmentally compatible. The immobilization on the magnetic particles overcomes the challenges like high cost, escaping the long separation processes by centrifuging and chromatographic separation with better stability towards pH and heat as compare to free lipase. The size, shape and porosity of the magnetic particles have important role in determining the immobilization efficiency which need to be studied further to achieve small size with large surface area for the considerable loading of lipase. Different types of the inorganic and organic magnetic composites have been used which also to be searched out to resist the adverse environmental condition during the process of immobilization. The methods of immobilization like physical and chemical adsorption have been utilized

for the immobilization. New methods are to be discovered to overcome the leaking problem.

In all, many parameters will have influences on the properties of lipase during its immobilization. Particularly, immobilization methods, carrier materials, solvent and detergents used and enzyme loading amount have proven to be important for enzyme immobilization. Therefore, more research is required to select properly all these parameters for lipase immobilization for enhancing its activity. They are:
1) Increase the surface area of nanoparticles by using some novel methods with low coast.
2) Further research for increasing the activity and stability of immobilized lipase.
3) Design some novel structure carriers like Janus particles, smart polymers, etc.

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