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Review

Tune to immobilize lipases on polymer membranes: Techniques, factors and prospects

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ABSTRACT

The review is looking forward to explain different strategies, several prime controlling factors of enzyme immobilization on polymeric membranes. Lipases acts in the presence of interfaces, has attracted membrane researchers and biotechnologists to synthesize variety of polymeric membranes as efficient carriers. The immobilization is proved by different analytical tools. The differences in stability and activity of bound lipases with respect to free lipase are compared. In terms of practicability set up details of membrane bioreactors are discussed. It has also shed light on different applications (viz. oil, food, medical and pharmaceutical, and waste treatment) of lipase immobilized membranes.

Contents

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1. Introduction

Enzymes are biological catalysts and also termed as “catalytic machinery” of the living systems as most of the reactions catalysed by these protein molecules. They accelerate or catalyze various reactions by reducing the activation energy needed to these reactions without consumed up in living as well as in non-living systems (Hartmeier, 1986). Most such reactions would not take place at a useful rate without them and in general 10^6 times faster than un-catalyzed reactions. Their use as biocatalysts for industrial applications is on the verge of significant growth (Schmid et al., 2001). In 1878, Wilhelm Kuhne, first time used the term “enzyme”. It is derived from a Greek word, which means “in yeast” for close relation with yeast activity (Lengler et al., 1999).

The active center of enzyme is a small part of the surface area and is composed of a binding site and a catalytic site. Binding of substrate occurs at binding site and at catalytic site the catalysis takes place. It folds to bind substrate by weak electrostatic interactions and makes easy the bond reactivity (Bailey and Ollis, 1994).

The conformation of an enzyme depends upon the enzyme environment as it is made by interactions between the constituent amino acid residues. Enzymes have three-dimensional structure of folded protein. This structure is responsible for the nature and specificity of their catalytic behaviour, which is analyzed by the sequence of its amino acids.

Enzymes (biological catalyst) have some advantages over chemical catalysts as (i) substrate specificity, they exhibit remarkable substrate specificity and unique regiospecific and stereospecific selectivity, (ii) high efficiency, (iii) mild processing conditions, (iv) avoids disposable problems, (v) low capital cost (Bailey and Ollis, 1994; Malcata and Hill, 1991).

The enzymes are divided into six major categories considering their activities to catalyze chemical reactions. It is as follows:

1. Oxido-reductases: catalyses redox reactions. It is of dehydrogenase, oxidase, peroxidase, oxygenases viz. lactate dehydrogenase, glucose oxidase, horsh radish peroxidase, lactate oxygenases.
2. Transferases: catalyses the transfer of functional group/entity viz. transaminases, trans aldo/ketolases.
3. Hydrolyases: catalyses hydrolytic reaction viz. lipase, alkaline phosphatase, cholesterol esterase.
4. Lyases: cleavage of C–C, C–O, C–N and other bonds and formation of C=C bond or ring, viz. pyruvate decarboxylase.
5. Isomerases: catalyses isomerization. It can be of racemases, epimerases, mutases, *cis*-trans isomerases viz. maleate isomerase, glucose isomerase.
6. Ligases: condensation of two substrates with splitting of ATP, pyruvate carboxylase.

2. Immobilization of enzymes

Use of enzymes is still limited due to high cost of enzyme isolation and purification for their single use. The enzymes are labile in nature so their isolation from natural environment can cause denaturation and diminished activity. The low pH, temperature and chemical stability in organic solvents also restricts the use of free enzymes. Moreover the separation of products in presence of free enzymes is tedious. These drawbacks of the free enzymes are overcome through immobilizing them. Therefore, immobilization of enzymes is one of the useful techniques to improve the application of enzymes at the industrial level (Saleem et al., 2003; Yucel, 2012).

2.1. Definition and advantages

The immobilized enzyme is a system/preparation in which an enzyme is confined in relatively defined region of space with the retention of their catalytic activities for repetitive uses (Katchalski-Katzir et al., 2000). The immobilization is favored over the free enzymes considering the following points. [a] Reusability, permits reuse of the single batch of immobilized enzymes, [b] Facilitates easy separation and purification of products [c] Enhances thermal and chemical stabilities of enzymes [d] Economical, [e] Enhances control of reaction and the product quality by choosing proper technique of immobilization, conversion yields can be improved [f]

Reactor development, possibilities to develop continuous, large-scale commercial processes having high efficiency in reactor, as opposed to free enzymes where most small-scale, batch scale is the usual technique.

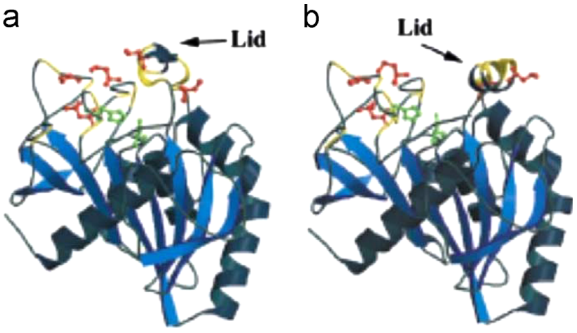


Fig. 1. Three-dimensional structures of lipase: (A) closed form, (B) open form. Reproduced from Noinville et al. (2002).

2.2. Choosing lipase as enzyme and polymeric membranes for support matrix

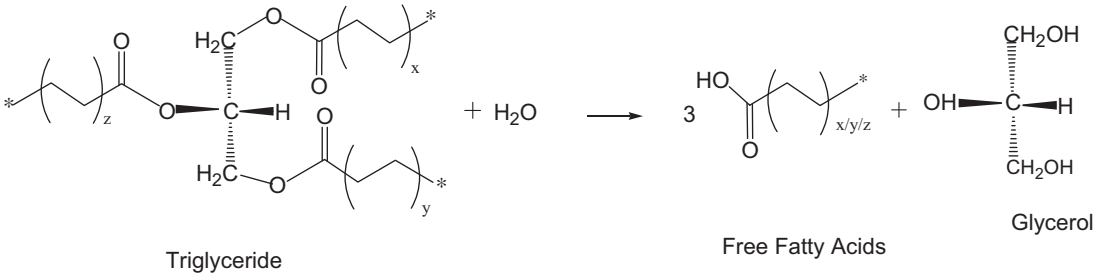
In this present review lipases are focus of attention as they are widely applicable in terms of various industrial applications.

2.3. Lipases: The special attraction

- Abundance in nature- they are produced in high yields from plants, animals and micro-organisms (fungi and bacteria). In particular, microbial lipases find immense applications because they can be extracted easily and in high yields.
- Ability to function in non-aqueous, aqueous systems and near interfaces. They do not have the requirement of cofactors or coenzymes in most cases.
- Needs interfacial activation for full catalytic activity (Sarda and Desnuelle, 1958).
- Show superb chemoselectivity, regioselectivity and stereo-selectivity.

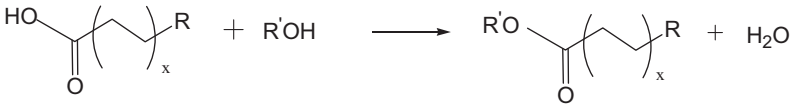
Lipase catalyzed reactions:

(i) Hydrolysis



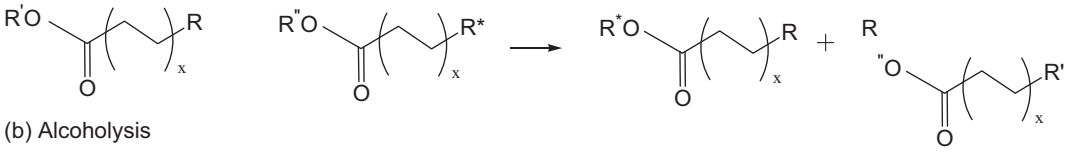
(ii) Synthesis

(A) Esterification

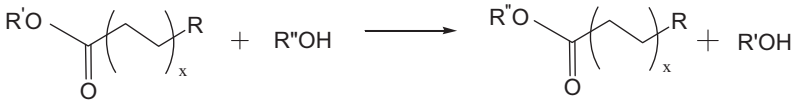


(B) Transesterification

(a) Interesterification



(b) Alcoholysis



(c) Acidolysis

Scheme 1. General reaction scheme of hydrolysis, esterification, transesterification, interesterification, alcoholysis and acidolysis.

- Produce a wide range of products with high potential and purity and they do not catalyze side reactions.

Lipases or triacylglycerol hydrolases (EC 3.1.1.3) are enzymes having an inherent capability to catalyze carboxy ester bonds cleavage and produce tri, di, monoacylglycerols and fatty acids (Bayramoglu et al., 2002; Hayes, 2004). Lipases found in higher plants, animals and microorganisms play a significant part in the lipid metabolism (Balcao et al., 1996a).

Same primary sequence homologies are found in all lipases. It includes significant regions His-X-Y-Gly-Z-Ser-W-Gly or Y-Gly-His- Ser-W-Gly (where X, Y, Z and W indicate generic amino acid residues). They have α–β hydrolase fold and a catalytic triad.

They contain an oxyanion hole and three pockets. These three pockets are used in fatty acids holding of the substrate at positions Sn-1, Sn-2 and Sn-3 (Jaeger et al., 1999). The serine residue is protected by a flap or α-helical lid structure i.e. called closed conformation of lipase molecule, but after restructuring, an electrophilic region (the oxyanion hole) around the abovementioned serine residue forms. This reconfiguration occurs by the contact with interface so that hydrophobic residues expose outside and hydrophilic ones inside hide (open form). It is seen in Fig. 1, carboxylic side-chains Asp-96, Asp-201, Asp-254, Glu-87, Glu-210 (in red) lie at the periphery of the external hydrophobic region (in yellow). The catalytic triad (in green) becomes accessible after opening the lid. The rotation of the lid exposed hydrophobic residues of the amphiphilic helix at the external surface of the Lipases (Noinville et al., 2002).

Lipases acquire many potential applications as they can catalyze numerous reactions such as hydrolysis, esterification, interesterification, transesterification, alcoholysis, acidolysis. The general reaction scheme is as follows (Scheme 1).

Lipases are well known hydrolase type of enzymes, having the catalytic ability to hydrolyze the ester bond between the fatty-acyl side chains and the lipid backbone of fat/oil into glycerol and fatty acids products. While in esterification reaction water and ester synthesis occurs. So, esterification mixtures include substrates and lipases and water is the only by-product of the reaction (Schmid et al., 2001). Transesterification processes viz. alcoholysis, acidolysis and interesterification give rise to alcohol, acid or ester, respectively. Lipases are active in organic solvents and can catalyze synthesis (esterifications) as well as the reverse reaction of synthesis (hydrolysis). So their ability is used in the synthesis of many desired products.

2.4. Lipase reactions over chemical method

Lipase catalyzed reactions resemble closely the pathways designed by the nature for the metabolism of living beings, and so the reaction mechanisms and processes associated therewith may be viewed as more environment friendly than bulk synthesis (Balcao et al., 1996b). Even though lipase catalyzed reactions can also be carried out using inorganic, metal derived catalysts, but attraction is generated for these enzymes due to many useful points.

The physiochemical conventional Colgate-Emery process requires high pressure and temperature for fat splitting (Brady et al., 1988; Shinota et al., 1967). In this process, special splitter column, which can tolerate high temperature, pressure and corrosive acids, is needed so the capital investment is high. On the other hand, for lipase catalyzed reactions as low activation energies concerned, mild reaction conditions (viz. temperature and pH) are required. So, enzymatic process requires low energy and thermal changes to reactants and products are less. Highly corrosion-resistant vessels are not desired as there are not harsh environmental conditions.

Fatty acids produced in conventional procedure cannot be used as they are acquired. They have to be redistilled to remove color and by-products. So, the process becomes energy consuming and gives rise to a variety of undesirable side reactions, such as polymerization of highly unsaturated fatty acids and production of ketones and hydrocarbons. In one another technique i.e. alkaline hydrolysis, acidification of soaps are done to produce the fatty acid products (Murty and Bhat, 2002). The products of bioprocesses in which lipases are used as catalyst have better odor and color, and usually pure so downstream processing is not required to purify them (Gandhi, 1997).

2.5. Speciality of polymeric membranes

There are different supports for lipase immobilization. They are generally divided into two main categories viz. inorganic and organic supports. Inorganic supports provide higher chemical resistances, but have some limitations in terms of flexibility and mass transfer. On the other hand though organic supports are less resistant to the medium but they are cheaper and provide wider variety of functionalities. In other words polymer materials have the wider applications as they are having different chemistry and low costs. Moreover, their properties can be tuned easily. Amongst polymer beads, powders, foams and membranes, the later is attractive to researchers (Gupta et al., 2012; Handayani et al., 2012; Kuo et al., 2012; Orregoa et al., 2010).

Large surface area: Membranes have large surface area in terms of surface as well as pores for efficient lipase immobilization.

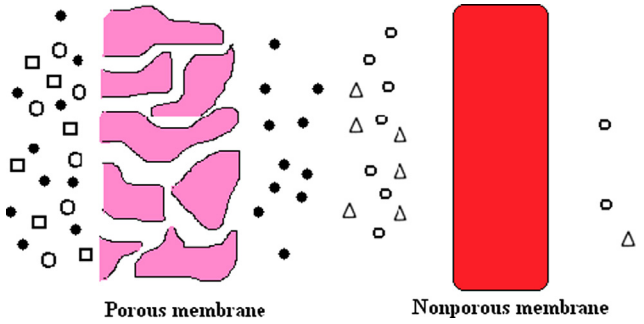


Fig. 2. Types of membranes.

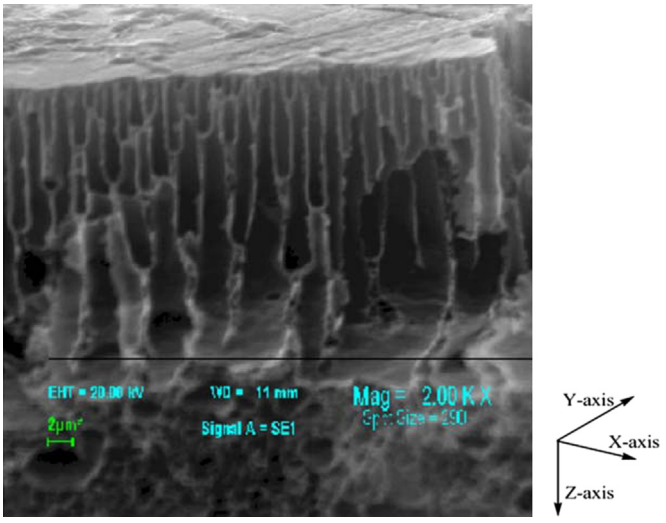


Fig. 3. Cross section morphology of asymmetric membrane.

Porosity: They have good porosity and allow lipase binding. Substrates are easily accessible to lipases through pores so minimize diffusional limitations.

Good mechanical strength: They can be easily prepared in different geometrical configurations if they exhibit good mechanical strength and rigidity.

Tailor made: They can be easily modified by functional groups through surface modification technologies for covalent coupling with lipases.

Enzyme-immobilized membrane reactors: In these reactors membranes have two roles; they are used as a catalytic support as well as a selective barrier for selective mass transfer through the membrane (Giorno et al., 1995; Molinari et al., 1994; Sirkar et al., 1999).

2.5.1. *Types of membranes*

To make the best use of the membranes for the particular applications it is necessary to have an idea about the "built-in" configuration of the membranes. These membranes may have either "non-porous" or "porous".

i. Non-porous membranes
ii. Porous membranes

The non-porous membranes are low flux membranes if they are not prepared very thin. The dense membrane properties are incorporated into the top "skin" layers of asymmetric membranes. This type of membranes consists of a dense film through which permeates are transported by diffusion under the driving force (Fig. 2).

The simplest form of porous membrane is a polymer film with cylindrical pores or capillaries. However, more commonly microporous membranes have a more open and random structure, with interconnected pores (Fig. 2). The separation of particles is mainly depends on molecular size and membrane pore size distribution. Their structure may be symmetric, i.e., the pore diameters do not vary over the membrane cross section, or they can be asymmetrically structured.

The asymmetric membranes have different layers with varying structures and permeabilities. A typical asymmetric membrane structure contains an open, much thicker porous substructure having a relatively dense, extremely thin skin layer on top as shown in Fig. 3. The polymer dense phase atop, channels and macro- voids are beneath it. The dense skin layer (also called the permselective layer), determines the fluxes and selectivities of these membranes. On the other hand, the more porous sublayer serves only as a mechanical support and has little effect on the separation property of membrane. The asymmetric nature along the axis perpendicular to the plane of the film, the *Z*-axis is depicted from the cross-section morphology (Fig. 3). These membranes have the advantages of higher fluxes, and almost all commercial processes use such membranes. We had also immobilized lipase on polysulfone polymer globules having the same asymmetric nature and applied them for oil hydrolysis (Gupta et al., 2010).

3. Different techniques of immobilization

The design of an efficient lipase immobilized system is an art. Different methods to immobilize lipases (Bhushan et al., 2008; Chiou et al., 2007; Gao et al., 2009; Ghiaci et al., 2009; Lei et al., 2009; Serra et al., 2008; Tischer and Wedekind, 1999; Wang et al., 2007) are divided into three main categories (Fig. 4): (i) physical methods, where weaker interactions are employed, (ii) chemical methods, where covalent bonds are formed with lipases and support matrix and (iii) entrapment, where lipase molecules are entrapped in the pores of the support. Table 1 shows the different techniques, support membranes and sources of lipases used for immobilization.

The efficient method should be based on mainly mild chemical conditions (pH, temperature, pressure) during immobilization. The large surface area of matrix also influences immobilization during the contact with enzyme. The chemical stability in the reaction medium (aqua, organic solvent or two phase system) as well as high lipase loading also demands the better technique for immobilization. The suitable method has the ability to minimize barriers

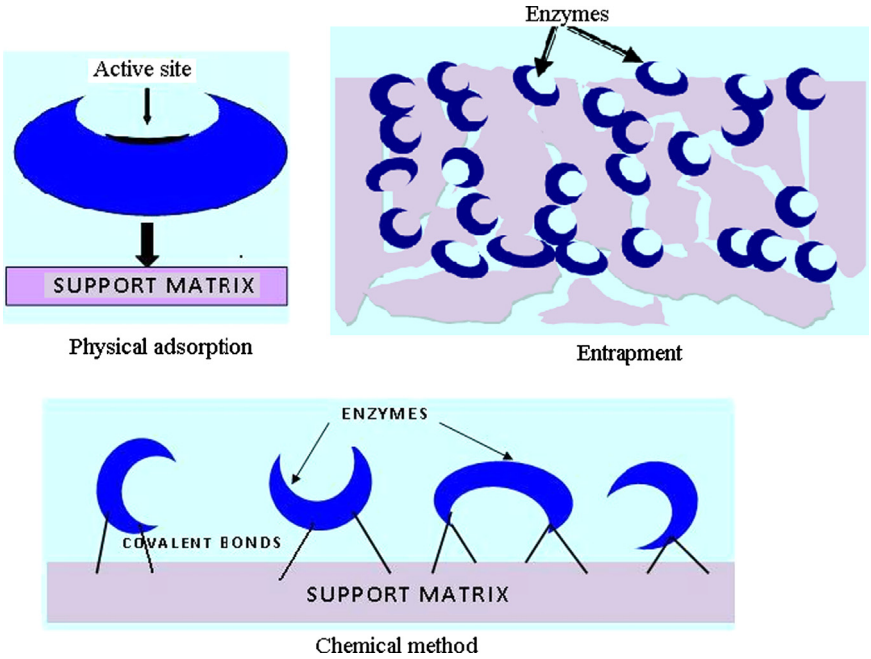


Fig. 4. Schematic presentation of enzyme through different routes of immobilization.

Table 1
Different techniques, membranes, sources of lipases for immobilization and their applications.

S. N.	Lipase source	Support membrane	Technique used for immobilization	Application	Refs.
1.	*Thermomyces lanuginose* *Pseudomonas fluoroscens* *Rhizomucor miehie*	Polypropylene	Adsorption	Synthesis of methyl and isopropyl fatty acid esters by alcoholysis	Soumanou and Bornscheuer (2003)
2.	*Rhizomucor miehie*	Polysulfone Polypropylene	Adsorption	Production of structured lipids	Xu et al. (2000)
3.	*Candida rugosa*	Cuprophane	Adsorption	Palm oil hydrolysis	Knezevic and Obradovic (2004b)
4.	*Candida rugosa*	Electrospun polysulfone	Adsorption	Olive oil hydrolysis	Wang et al. (2006)
5.	*Candida rugosa*	Polypropylene membrane modified with hydrophobic polypeptides	Adsorption	Olive oil hydrolysis	Deng et al. (2004a)
6.	*Candida rugosa*	Polyvinylidine difluoride Polysulfone	Adsorption	Olive oil hydrolysis	Tsai and Shaw (1998)
7.	*Candida rugosa*	Polypropylene	Adsorption	Enrichment of γ-linolenic acid in borage oil	Huanf et al. (1997)
8.	*Candida sps.*	Polypropylene	Adsorption	Production of structured triacylglycerols	Estrella et al. (2007)
9.	-	PVC Collagen, CA, PTFE membrane	Adsorption	Sunflower oil hydrolysis	Rucka et al. (1990), Rucka and Turkiewicz (1990a)
10.	*Candida sp.*	Surfactant modified cotton membrane	Adsorption	Synthesis of 2-ethylhexyl palmitate	Tan et al. (2006b)
11.	*Candida Antarctica, Rhizopus oryzae, Rhizopus delemar*	Polypropylene	Adsorption	Synthesis of 2-monoacylglycerols rich in polyunsaturated fatty acids by ethanolysis of fish oil	Muñío et al. (2008)
12.	*Rhizopus delmar*	Polypropylene	Adsorption	Structured triacylglycerols rich in DHA by acidolysis of tuna oil	Ko et al. (2006)
13.	*Rhizopus oryzae*	PVA/Chitosan And composite membrane	Adsorption with glutaraldehyde or epichlorohydrin	Membrane reactor for the synthesis of monoglyceride (MG) by hydrolysis of palm oil	Tan et al. (2002)
14.	*Candida rugosa*	Regenerated cellulose Polyethersulfone	Adsorption, filtration, periodate oxidation Adsorption, filtration	Synthesis of butyl oleate	Hilal et al. (2006)
15.	*Candida antarctica* *Porcine pancreas*	Polypropylene, polyamide, cellulose, nitrocellulose	Adsorption Filtration + Crosslinking with glutaraldehyde	Biphasic organic solvent-water system	Holownia and Noworyta (2002)
16.	*Candida rugosa*	CA/PTFE composite membrane	Filtration	Chiral separation of racemic ibuprofen	Wang et al. (2007), Wang et al. (2008)
17.	*Candida cylindracea*	Polyetherimide Nylon	Filtration	Membrane reactor for babassu oil hydrolysis	Mercon et al. (1997)
18.	*Candida rugosa*	Polyamide Polysulfone	Cross flow filtration	Optical resolution of racemic naproxen methyl ester	Giorno et al. (2003)
19.	*Candida rugosa*	Cuprophane	Filtration	biphasic oil/aqueous hollow-fibre bioreactor for palm oil hydrolysis	Knezevic et al. (2004a)
20.	*Candida rugosa*	Cuprophane	Ultrafiltration	Palm oil hydrolysis	Knezevic and Obradovic (2004b)
21.	*Candida cylindracea*	PAN containing 7% methylacrylate	Filtration	Membrane reactor for the production of diltiazen chiral intermediate for a drug diltiazen	Lopez and Matson (1997)
22.	*Candida rugosa*	PVA/PTFE composite membrane	Filtration	Biphasic membrane reactor for olive oil hydrolysis	Xu et al. (2006a)
23.	*Candida rugosa*	PS membrane	Adsorption	Hollow fiber reactor for palm and olive oil hydrolysis	Shamel et al. (2007)
24.	*Candida rugosa*	PS membrane	Ultrafiltration and crosslinking	Olive oil hydrolysis	Wang et al. (2008)
25.	*Candida rugosa*	CA/PTFE composite membrane	Filtration and Precipitation of crosslinked lipase aggregates	Esterification of oleic acid with n- butanol	Hilal et al. (2004)
26.	*Rhizopus oryzae*	Polyethylene (radiation+grafting GMA with diethylamine)	Covalent attachment with glutaraldehyde	Membrane reactor for esterification of lauric acid	Goto et al. (2006)
27.	*Candida rugosa*	Electrospun Polyacrlylonitrile	Covalent attachment by amidination reaction (C2H5OH/Dry HCl)	p-nitrophenol palmitate hydrolysis	Li et al. (2007)
28.	*Candida rugosa*	Polypropylene with UV curing of a blend of HEMA terminated polyurethane prepolymer and GMA	Covalent attachment with glutaraldehyde	Membrane reactor for esterification of oleic acid with octanol	Pujari et al. (2006)
29.	*Candida antarctica*	Alpha alumina ceramic membrane +gelatin/polyethyleneimine	Covalent attachment with glutaraldehyde	Butyl butyrate synthesis	Lozano et al. (2002)
30.	*Candida rugosa*	PAN membrane grafted with maleic acid	Covalent coupling with EDC/NHS	Comparison of hydrolytic activity in aqueous and organic media	Ye et al. (2005)

for mass transport of substrate and product. In terms of industrial applications the chemically and mechanically robust immobilized matrix is preferable. Of course, the efficiency of the immobilized matrix depends on the target applications.

3.1. Physical method

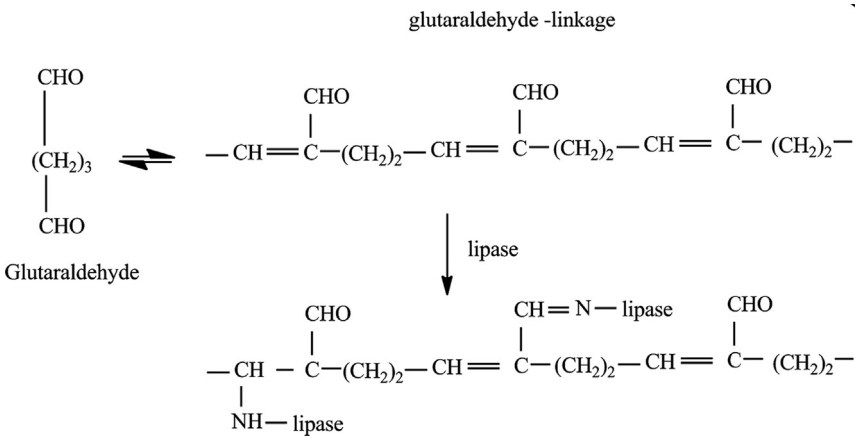
Immobilization of lipases by adsorption occurs through weak forces viz. Vander Waals, H-bonds, and hydrophobic–hydrophilic or ionic interactions. It is a simple, economical and little time consuming technique to prepare biocatalytic systems. The method is directly based on the inherent properties of the membrane material, so the support as well as lipase does not require any specific modification, so enzyme activity is not damaged (Bickerstaff, 1997). It also provides a large surface area to the substrate (compared to other technique), during the reaction and large lipase loading capacity (Gao et al., 2009; Alloue et al., 2008; Brigida et al., 2008; Cunha et al., 2008; Holownia and Noworyta, 2002; Palocci et al., 2007; Secundo et al., 2008; Wang et al., 2006; Ye et al., 2007).

The affinity of lipase adsorption for a membrane surface generally increases with their hydrophobicity. One more advantage is their improved activity after adsorption on hydrophobic support as the surface resembles the interface their natural substrates and creates the conformational changes in lipases (open form) (Noinville et al., 2002) (Fig. 1). This method is very useful to achieve interfacial activation of lipase to make them in open form, which is accessible to the substrate molecules (Sarda and Desnuelle, 1958).

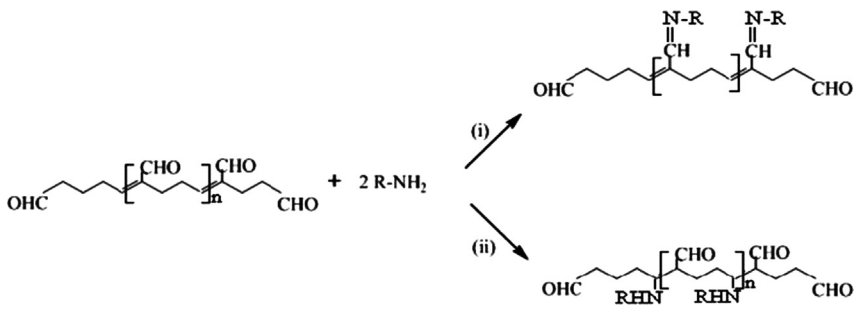
Lipase adsorption to the solid polymer matrix is mostly described by the Langmuir isotherm (Malcata et al., 1990). The lipase distribution on the polymeric surface could be best described by Freundlich (Mojović et al., 1998) or even the Redlich–Peterson models (Duri and Yong, 2000). Fig. 4 shows the sketch of enzyme immobilized on support by physical adsorption method. To stable adsorbed lipases on membranes some crosslinking agents are also used. It generally involves the residues (i.e. ε-amino group of lysine residues) which are not involved in catalysis (Weetall, 1974). Examples are there for crosslinking agents glutaraldehyde, epichlorohydrin, adipoyldichloride (Kilinc et al., 2002; Oliveira et al., 2012; Pahujani et al., 2008; Tan et al., 2002; Wu et al., 2006). But, glutraldehyde is a versatile agent to react with several functional groups of proteins, such as amine, thiol, phenol, and imidazole (Habeeb and Hiramoto, 1968). The glutaraldehyde shows many structures in aqueous solution from its simplest form monomeric dialdehyde form to dimer, trimer, and polymer (Migneault et al., 2004). Scheme 2 shows the mechanism of lipase and glutaraldehyde crosslinking by oligomer formation.

The reaction between enzyme and glutaraldehyde involves the conjugate addition of protein amino groups to ethylenic double bonds (Michael-type addition) of the α,β-unsaturated oligomers found in the commercial aqueous solutions of glutaraldehyde that are usually used (Scheme 3, reaction ii) (Richards and Knowles, 1968). However, Monsan et al. (1975) proposed a slightly different mechanism in which an addition reaction occurred on the aldehydic part of the α, β-unsaturated polymers (and poly-glutaraldehyde) to give a Schiff base (imine) stabilized by conjugation (Scheme 3, reaction i).

Besides many advantages, this method has some drawbacks. The risk of lipase desorption from the membrane support is high due to relatively weaker enzyme-support interactions. Activity of lipases after adsorption varies due to their orientation during the immobilization which is undecided. If the active sites of the immobilized lipases are exposed to the substrate, the activity can be highly enhanced. On the other hand, when the enzyme adsorbs using the active sites, the activity can be partial or totally lost.



Scheme 2. The mechanism of lipase and glutaraldehyde crosslinking by oligomer formation.



Scheme 3. Schiff base (i) and Michael type (ii) reactions of glutaraldehyde with. Reproduced from Migneault et al. (2004).

3.2. Chemical/covalent coupling

To avoid the limitations of adsorption method the "chemical/covalent coupling" method is exploited by many researchers. In this case, the attachment of lipases takes place using a chemical reaction or linkage to the membrane surface if it originally has the functional groups or to activated or monomer grafted membrane surfaces. The covalently bonded membranes usually have the better resistance to changes in pH, ionic strength, temperature, good stability to reuse, minimize leaching problem and prevents reversible unfolding. Like physical adsorption the binding of lipases to membrane surface is not site-specific, so orientation of the lipases is not influenced during immobilization (Mozhaev, 1993). Thus, it has the advantages of better retention activity, better hydraulic properties, and rendering better accessibility.

The amine of lysine or arginine, carboxyl from aspartic or glutamic acid, hydroxyl of serine or threonine and sulfydryl of cysteine (Bickerstaff, 1997). and terminal amino and carboxyl groups of the polypeptide chains are used in immobilization. Fig. 4 depicts the bonding occurs between the support surface and the lipases.

Recent studies have shown that numerous functional groups, including amines, chlorides, thiols, carboxylic acids, vinyl alcohols and phenolic are used for the modification of support surfaces by tailor made grafting for immobilization. The surface modification can be done by several techniques viz. chemical treatment, photo-irradiation, high energy radiation techniques (Bequet et al., 2002; Bhattacharya and Misra, 2004; Bhattacharya and Ray, 2009; Chennamesetty et al., 2006; Gupta et al., 2009; Liu and Martin, 1991; Morao et al., 2005; Ulbricht and Schwarz, 1997; Yogesh et al., 2007).

Modified surface of having –COOH, –CHO, –OH, –CN and $–NH_2$ groups can be covalently attached with lipases. Different techniques using carbodimide, periodate, cyanogen bromide, amidation, azo and Schiff's base formation reaction are there in the literature for the modification of membrane surface (Handayani et al., 2012; Bachmann et al., 2007; Bolivar et al., 2007; Foresti and Ferreira, 2007; Gallego et al., 2007; Geng et al., 2003; Hilal et al., 2006; Li et al., 2007; Palomo et al., 2002; Yucel et al., 2007).

The reaction schemes related to periodate & amidination are sketched in (Fig. 5).

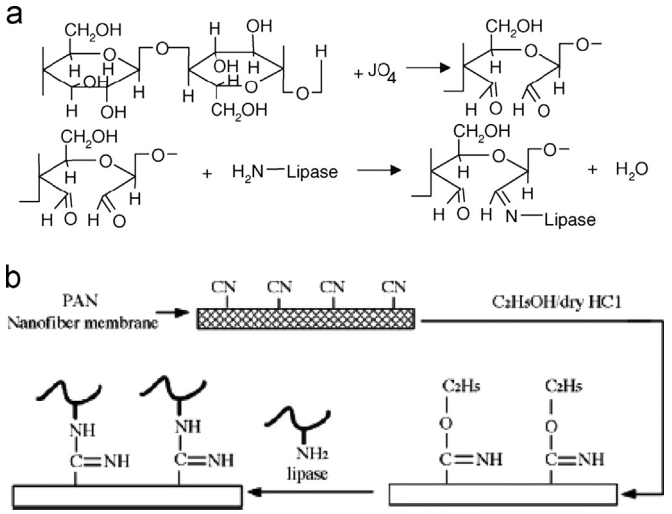


Fig. 5. Schematic illustration of covalent lipase immobilization on (a) cellulose C030F membrane by periodate (reproduced from Li et al. (2007)) and (b) PAN nanofibrous membrane by amidination reaction. Reproduced from Hilal et al. (2006).

Feasibility of chemical covalent coupling can be also done by modifying the lipase through chemical reaction. But, the enzyme modification is accomplished by highly reactive non-group specific chemicals which can easily alter the structure and the catalytic activity of the enzyme.

Though it is better method than adsorption in many aspects, it does not skip the limitations. It include possible losses in lipase activity since, the methods must be carried out at different pH ranges and with chemicals, which may not be good for lipase activity.

3.3. Entrapment

It is signified as "physical trapping" of the enzymes into membrane pores. It is especially applicable to very labile biomolecule like enzymes, which may degrade or lose activity at extreme conditions (viz. temperature, pH, harsh reagents). Lipase immobilization by entrapment is based on porosity of the membrane which retains lipases within the pores and provides substrate/product diffusion. The entrapped/encapsulated enzymes are free in solution but, their movement is restricted by the structure of the support (Bhushan et al., 2008; Alloue et al., 2008; Wang et al., 2008).

The entrapment of lipases depends upon the nature of the membrane matrix. If the pore is open to the surface then feasibility of lipase entrapment is there without adding external force, but, leaching possibility is there. For asymmetric membrane where pores/channels are covered by thin dense layer the entrapment of lipases into the membrane matrix can be done by pressurized conditions (Fig. 4). As lipase molecules are retained within a finely controlled porous structure tight enough the enzyme leaching into the surrounding medium is minimized. Moreover, the accessibility of active sites and penetration of the substrate in and product out are also there (Wang et al., 2007; Long et al., 2005a, 2005b). Encapsulation of lipases is another mode of entrapping technique. In this mode, lipases are immobilized within microcapsules prepared from polymers.

As the entrapment method is chemical independent one thus lipase activity is not affected. It is the simple one and high protein loading is possible. Furthermore, packing of lipase in the pores enhances enzyme stability, as the constraining structures help in protecting the enzymes from denaturing factors, (like pH, temperature, and organic solvents).

The method could not escape its limitations. The weak bonding between lipases and membrane surfaces can be easily disrupted and results the leakage of enzyme. It restricts the large molecular weight substrates to diffuse in the vicinity of enzymes. The support acts as an additional resistance to mass transfer so restricts the access to the immobilized enzymes (Holownia and Noworyta, 2002).

4. Factors influencing immobilization

The lipase immobilization is controlled by different factors. They are as follows:

4.1. Nature of membrane (support matrix)

The support provides a physical barrier to the free mobility of the enzyme molecules. In order to maximize lipase immobilization, proper choice of support matrix is necessary.

It needs the following properties for efficient immobilization.

- Proper functionality—Presence of the proper functional groups which are ready to attach lipase molecules and also promote their activity and stability.

- High surface area and porosity—It should have high surface area as possible, with better accessibility to the functional sites and enough large pores to allow lipase diffusion into the membrane pores.
- Hydrophobicity and hydrophilicity—The immobilization depends upon the nature of membrane matrix. The hydrophobic nature leads to better immobilization. It also can cause an enhanced stability or super activation of the immobilized lipases. Various membrane materials from hydrophobic to hydrophilic have been reported. In hydrophobic membranes made of polypropylene, (Malcata and Hill, 1991; Hoq et al., 1985a, 1985b, 1985c; Malcata et al., 1992), polytetrafluoroethylene, (Goto et al., 1992) polyvinylchloride (Rucka et al., 1990), polyvinylidene difluoride (Tsai and Shaw, 1998) or polyetherimide (Mercon et al., 1997). Hydrophilic membranes are made of cellulose (Guit et al., 1991; Pronk et al., 1988; Van Der Padt et al., 1990; Van Der Padt, 1993), acryl (Taylor et al., 1986), polyamide (Giorno et al., 1995; Molinari et al., 1994; Giorno et al., 1997),] or poly-acrylonitrile (Guit et al., 1991).
- Insolubility—The membrane should be insoluble to avoid the loss of lipases and to protect the enzyme molecules from contact with undesirable contaminants.
- Mechanical stability—The membrane should be stable enough to withstand shear forces that may be used in the chemical processes.

4.2. Source of lipases

Lipase is produced from different sources viz.: plants, animals and microbes. Plant lipases are not used commercially due to their low activity but animal and microbial lipases are used extensively. Some disadvantages are also associated with animal lipases. They cannot be used in the processing of vegetarian food and have undesirable effect. They are also likely to contain residual animal viruses, hormones, etc. So, microbes are the major source of lipases. The microbial lipases have attracted great attention due to their potentials, such as high production, good stability and many stereo specific properties (Tan et al., 2002).

The lipases are subdivided into three sub-groups according to their coordination–substrate site. One type of lipases with a hydrophobic, crevice-like binding site and a lid located on the surface of the enzyme corresponds to *Rhizomucor* family. Another type of lipases corresponds to *Pseudomonas*, *Candida antarctica* and mammalian pancreas and cutinase family, which has an active site and a funnel like lid. *C. antarctica* lipase B exhibits a very small lid and a funnel like binding site. The last type corresponds to *Candida rugosa* family and it has an active site at the end of a tunnel containing the lid in its external part. These peculiar structures affect the coordination of the substrate. Lipases from *R. miehei* catalyze the reaction in position either 1 or 3—rather than in position 2 of the triglyceride while lipases from *C. rugosa* have serine in the catalytic triad and attack all positions of the triglycerides (1, 3 and 2). The lipases having active site on the surface shows the highest potential of substrate coordination due to decreased steric energy. On the hand lipases with active sites at the end of a tunnel are comparatively less active (Ayestaa et al., 2007).

Table 2
Equilibrium values for the amount of protein adsorbed from preparation of lipase. Reproduced from Balcao et al. (1996a).

Source of lipase	Adsorbed amount (mg/cm^2)	Carrier	pH	T(^{0}C)
A. niger	65.5	Poly(propylene)	7.0	35
A. niger	41.4	Poly(propylene)	7.0	27
A. niger	39.7–109.2	Poly(propylene)	7.0	40
C. cylindracea	36.3–102.1	Poly(propylene)	7.0	40
C. rugosa	38.8	Cellulose	NA	30
C. rugosa	0.2	Cellulose	3.6–5.6	40
C. lipolytica	43.2–137.3	Poly(propylene)	7.0	40
G. candidum	34.8–97.2	Poly(propylene)	7.0	40
H. lanuginose	27–98.8	Poly(propylene)	7.0	40
M. javanicus	39.7–111	Poly(propylene)	7.0	40
P. camembertii	28.5–66.9	Poly(propylene)	7.0	40
P. roquefortii	41.7–76.6	Poly(propylene)	7.0	40
R. oryzae	28.4–66.9	Poly(propylene)	7.0	40
R. delmar	27.1–91.4	Poly(propylene)	7.0	40
Rhizopus sps.	9.7–12.2	Poly(tetrafluoroethylene)	8.0	37
Rhizopus sps.	2.8–3.4	Poly(vinyl chloride)	8.0	37

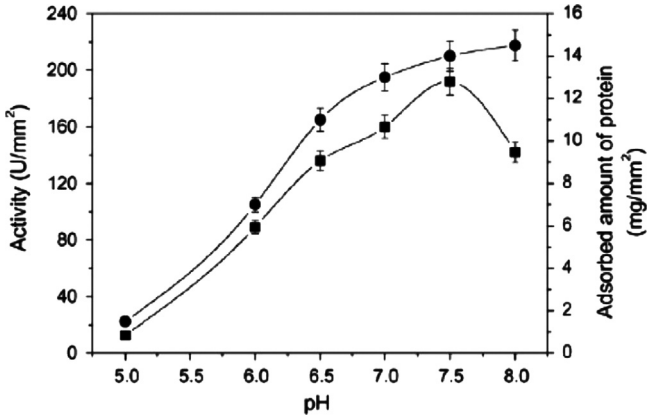


Fig. 6. Effect of immobilization pH on the activity of the immobilized lipase and the adsorbed amount of protein on the chitosan-tethered membrane: (■) activity of the immobilized lipase; (●) adsorbed amount of protein. Reproduced from Ye et al. (2007).

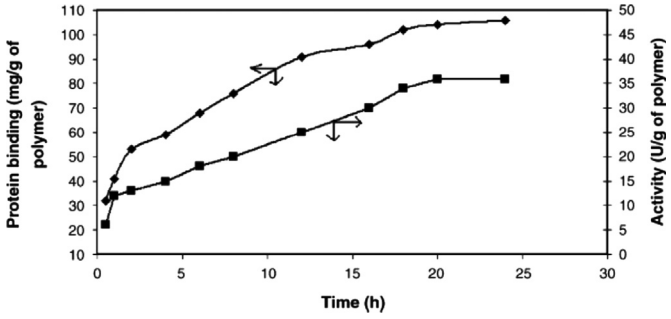


Fig. 7. Protein binding and activity as a function of coupling time for lipase immobilized on DEA (72/63) interface. Reproduced from Abrol et al. (2007).

Table 3
Amount of immobilized protein and immobilization yield at various initial concentration of protein. Reproduced from Deng et al. (2005).

Initial concentration of protein (μg/ml)	Amount of immobilized protein (mg/cm^2)	Immobilization yield (%)
22.80	68.68	27.42
57	114.29	18.26
114	210.99	16.85
171	251.65	13.41
228	260.32	10.40

Adsorption behavior of lipases from different sources is dissimilar in terms of the method of immobilization and the material of support membrane. In Table 2 the adsorbed amount of lipase from different sources are presented (Balcao et al., 1996a).

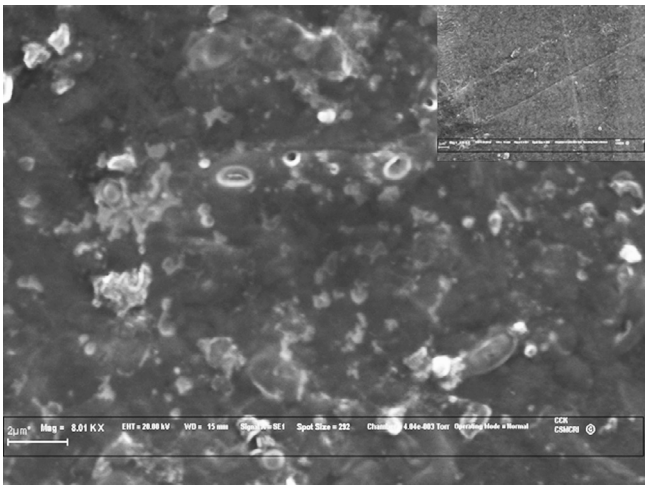


Fig. 8. Scanning Electron Micrograph of membranes PS–PVA after lipase immobilization, and virgin PS in inset. Reproduced from Gupta et al. (2010).

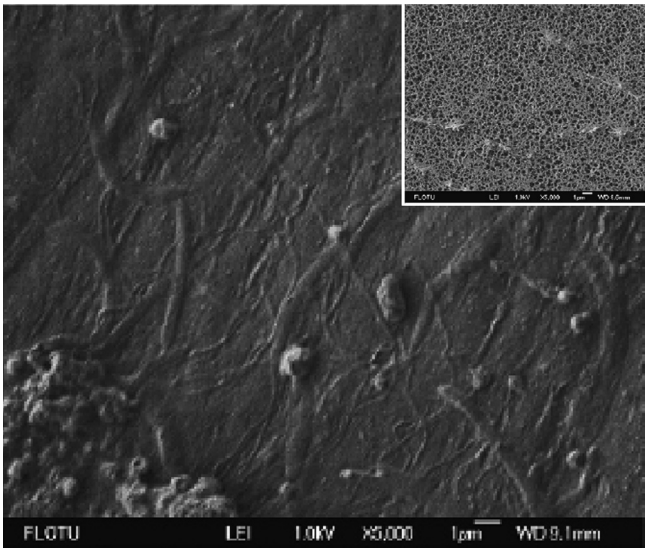


Fig. 9. Membrane surface before and after lipase immobilization, CA layer (facing the PTFE layer) before lipase immobilization in inset and CA layer (facing the PTFE layer) after lipase immobilization. Reproduced from Wang et al. (2007).

4.3. Immobilization conditions (pH, temperature, lipase concentration)

Immobilization conditions also affect amount of lipase immobilization viz. pH and temperature of immobilization medium, time of immobilization and lipase concentration.

The immobilized amount of lipase increases with the increment of pH value. But the maximum activity is attained with lipases immobilized at pH 7.5 or 7.0 near neutral range. pH of immobilization medium also depends upon the functional groups of membrane surface. For the polymeric material in which, the conformational stability is independent upon pH, the immobilization depends on the conformity of lipase molecules only. But for the polymer material i.e. chitosan whose behavior is altered in different pH medium, for them polymer materials as well as lipase conformation is important. Chitosan is a polycationic natural macromolecule and it can electrostatically couple with the anionic materials. Lipase whose isoelectric point is 4.6, should exhibit net negative charge during the pH value range of 5–8. Thus, with the increment of the pH value the adsorbed amount of lipase increases (Fig. 6) (Ye et al., 2007).

The enzymatic activity varies with different pH values and the denatured trend increases under high pH value. Therefore, there is an optimum pH value for the enzyme activity during the enzyme immobilization process.

The variation of the immobilization temperatures has minimal effect on immobilization amount. It may be due to independent behavior of hydrophobic interactions (in physical adsorption) and chemical bonds (in covalent coupling) to temperature (Kaewthonga et al., 2005).

Fig. 7 shows the variation of lipase loading and the corresponding activity with time. An increase in the time period leads to an increase in the lipase binding and activity. It is leveled off after a certain time. This is due to overloading of the immobilized lipase on the pore space of the membrane, which results as low diffusion of substrates (Wang et al., 2006; Pahujani et al., 2008).

The amount of immobilized lipase depends directly on lipase concentration However, the specific activity of immobilized lipase is found to decrease beyond a particular limit.

The amount of immobilized lipase with the increased lipase concentration is shown in Table 3 (Deng et al., 2005). It is seen that the increment of lipase concentration enhanced the driving force for the immobilization so; adsorbed amount of protein increased, but an opposite trend for immobilization yield is observed. Activity is also decreased with high lipase concentration, may be due to embedding of active sites of lipase molecules during the immobilization process by increased stacking or by dymers formation which involves the enzyme active site and diffusion limitations is also high.

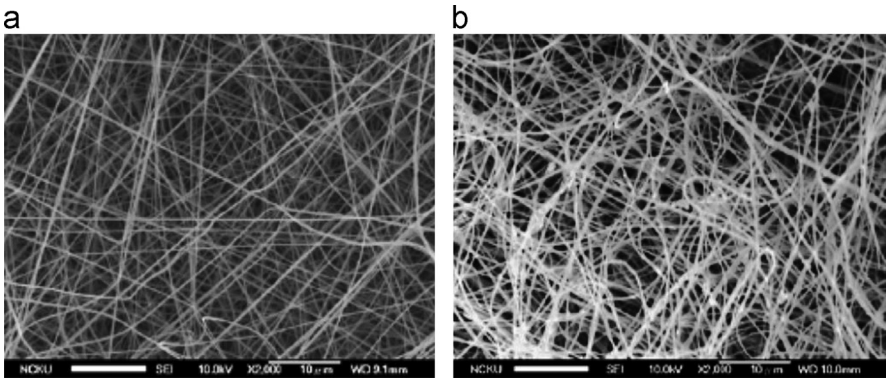


Fig. 10. Scanning electron micrographs of PAN nanofibrous membrane, (a) original nanofiber; (b) lipase-immobilized nanofiber, Magnification: 2000 ×. Reproduced from Hilal et al. (2006).

5. Evidences of immobilization

Lipase immobilization on membranes is evidenced by different some analytical tools. It may be classified as

(1) Direct
(2) Indirect methods

Surface immobilization can be proved by different analytical tools. SEM, AFM (topographic features), XRD (nature of surface through diffraction), Porometry (change in pore conformation), contact angle (nature of surface), water permeability and theses are termed as direct tools whereas protein test (such as Lowry, Bradford, etc.) in solution and mass balance, activity studies are indirect tools.

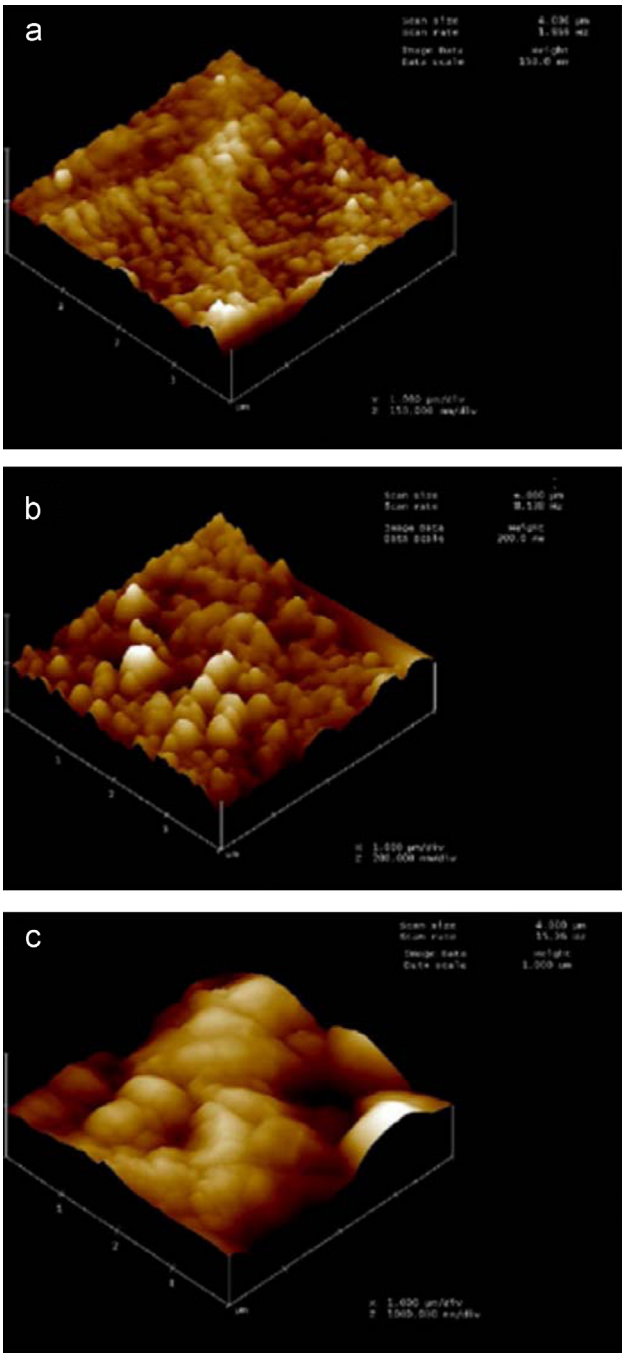


Fig. 11. 3D AFM images of (a) original PP, (b) DEA (72/63)-EA, (c) DEA (72/63)-EA with ABL lipase, isolated from Arthobacter sp. immobilized. Reproduced from Abrol et al. (2007).

5.1. Direct method

5.1.1. Micrographs

Scanning electron micrographs are the visual evidences of immobilization (Fig. 8). The evidences of distinct spots related to aggregates of protein are on PS-PVA membrane (Gupta et al., 2010). Wang et al. (2007) showed the network pore structure for composite hydrophilic cellulose acetate (CA)/hydrophobic polytetrafluoroethylene (PTFE) membrane which is packed after lipase immobilization (Fig. 9).

The change in the morphology of the poly-acrylo nitrile (PAN) nanofiber is reflected from Fig. 10 though the diameter. The morphologies of the nanofibers are not changed substantially. The lipase aggregates are shown on the surface of the nanofibers in spotted form (Hilal et al., 2006).

Fig. 11 shows the AFM images of virgin, amine treated PP fiber membrane. It is shown (Table 4) that there is a big difference in surface roughness parameters due to lipase immobilization (Abrol et al., 2007).

Table 4
Roughness parameter values (RMS) from AFM data of original, modified PP and lipase immobilized DEA-EA hollow fiber membranes. Reproduced from Abrol et al. (2007).

Membrane type	Surface roughness, RMS (nm)
PP	10
DEA (72/63)-EA	24
DEA (72/63)-EA with immobilized lipase	126

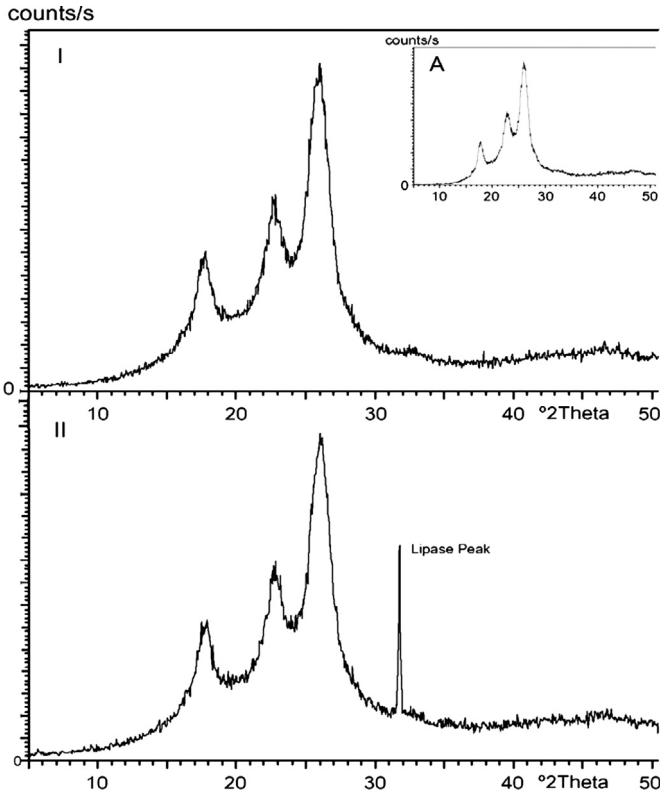


Fig. 12. XRD pattern of unmodified PS (I) and lipase immobilized on PS membranes (II). Embedded one (A) is for polyester non-woven fabric. Reproduced from Gupta et al. (2008).

5.1.2. X-ray diffraction pattern

As lipase is known for its crystallanity, the immobilization can be proved by X-ray diffraction studies. It is a rapid analytical technique primarily used for phase identification of a crystalline material.

Fig. 12 shows the X-ray diffraction pattern of the lipase immobilized as well as virgin PS membrane reinforced in non-woven polyester fabric. The peaks of virgin membranes are due to the polyester fabric embedded as A in the figures (Gupta et al., 2008). The immobilization has been proved by the additional peak in the figure. Free lipase XRD is also shown in Fig. 13.

Similar studies are also reported in the modified membrane systems viz. PS-PVA-lipase and PS-Hz-lipase (Gupta et al., 2010).

5.1.3. Capillary flow porometry

The asymmetric membranes are favored for lipase immobilization because of their wide applicability in terms of membrane reactors. The porosity of the asymmetric membranes is developed because of the thin polymer dense phase and polymer lean phase (Fig. 3). Considering it as cylindrical pores, the porosity is determined from capillary flow porometer.

Actually due to lipase immobilization of the membranes the pore conformations are altered from the virgin ones. Thus by comparing the porometric data before and after immobilization (Table 5), it is understood that lipase immobilization occurs on the membranes. Gupta et al. (2008) reported the evidences in terms of asymmetric PS and PES. It is observed that pore diameter (at maximum pore size distribution) reduces and the corresponding bubble point pressure (the pressure needed to empty the largest pore) increases. Bubble point pressure is defined as the least pressure required emptying the largest pore of a membrane and the diameter of the largest pore is termed as bubble point diameter.

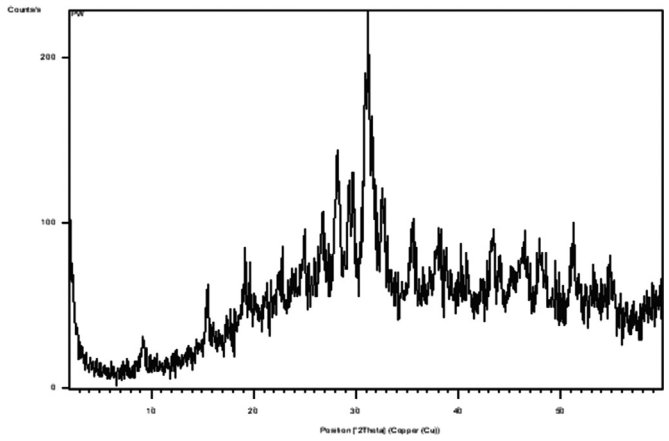


Fig. 13. XRD pattern of powder lipase.

Table 5
Porometric result before and after lipase immobilization. Reproduced from Gupta et al. (2008).

Membrane	Before immobilization		After immobilization	
	Bubble pt. press (MPa)	Diameter at maximum size distribution (μm)	Bubble pt. press (MPa)	Diameter at maximum size distribution (μm)
PS	0.41	0.1033	0.804	0.0562
PES	0.54	0.0793	0.566	0.075

Table 6
Contact angle data before and after lipase immobilization. Reproduced from Gupta et al. (2008).

Membrane	After immobilization contact angle(°)		
	Advancing	Receeding	Mean
PS	84.2	69.3	76.7
PES	82.1	72.1	77.1

Table 7
Water permabilities before and after lipase immobilization.

Membrane	Polymer concentration (% w/w in DMF)	Lipase immobilized amount (mg/cm^2)	Pure water permeability (l/m^2/h)	
			Before immobilization	After immobilization
PS-I	15	2.05	42.9	7.63
PS-II	18	1.35	17.16	5.72

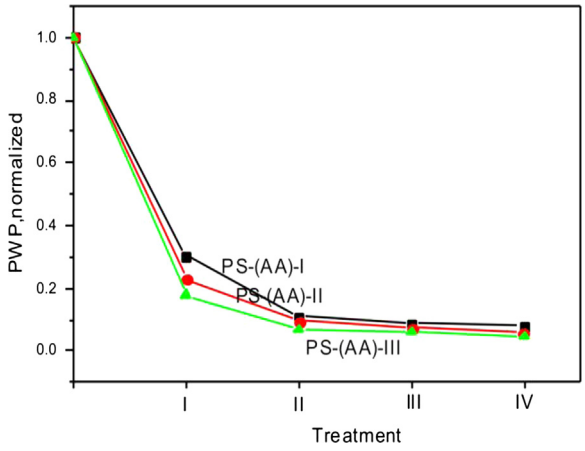


Fig. 14. Normalized pure water permeability (PWP) for the modified membranes with different modification stage. (I. after acrylic acid grafting, II. after methanol treatment, III. after hydrazine treatment and IV. after lipase treatment) PS-(AA)-I and PS-(AA)-II and PS-(AA)-III meant for PS membranes modified with 1, 5 and 10% Acrylic acid.

5.1.4. Contact angle

Contact angle measurements are used to demonstrate the relationship between the physical properties and chemistry of surfaces. The higher contact angle of surface signifies the hydrophobicity. The immobilization on the polymer membrane surface changes the properties of the polymer surface. After immobilization the decrease in contact angle suggests that the surface is relatively hydrophilic compared to unmodified one (Table 6). The decrease in contact angle is relatively more for Polysulfone suggests that more hydrophobic surface prefers immobilization compared to Polyether sulfone membrane (Gupta et al., 2008).

5.1.5. Water permeability

The immobilization is surface or entrapment phenomenon of the membranes. The immobilization changes the pores conformation. Thus the basic function viz. permeability of the membranes is altered. Table 7 features the change in water permeability after lipase immobilization. It is observed that 82.2 and 66.6 % decrease for PS-I and PS-II, respectively, and it correlates with the lipase amount. The higher lipase content blocks the pore conformation better.

In Fig. 14 the water permeability changes is reflected from the modified PS membranes. The different steps of modification including lipase immobilization show the reflection of water permeability change. Though the grafting step alters the permeability in greater extent, the immobilization step also shows its capability. PS-(AA)-II shows higher pore blocking and decrease 22.8% water permeability due to 1.53 mg/cm^2 lipase where as PS-(AA)-I shows decrease 10.9% in water permeability for 1.4 mg/cm^2 lipase (step III to IV).

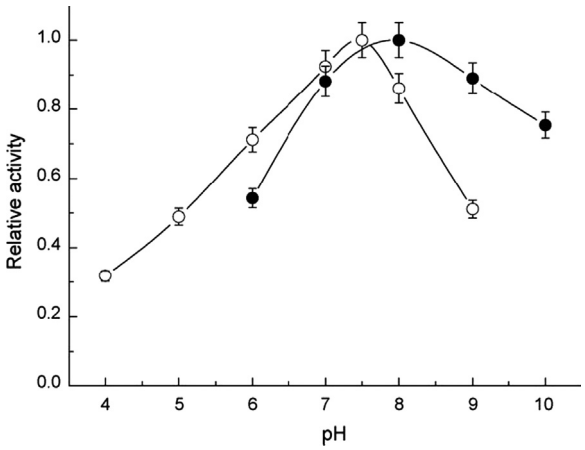


Fig. 15. Effect of pH on the activity of free and immobilized lipases: (O) free lipase, (●) immobilized lipases. Reproduced from Xu et al. (2006b).

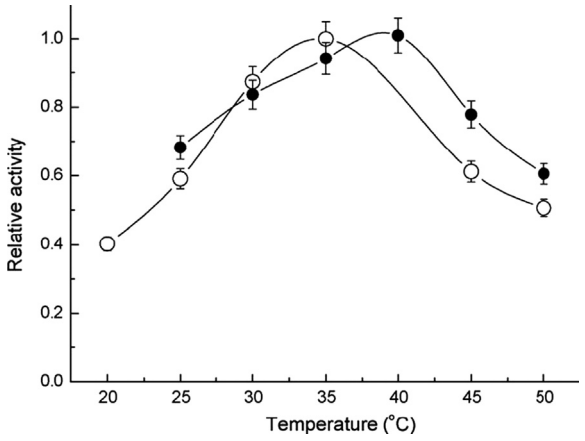


Fig. 16. Effect of temperature on the activity of free and immobilized lipases: (O) free lipase, (●) immobilized lipases. Reproduced from Ye et al. (2007).

5.2. Indirect method

The amount of lipase immobilized on polymeric matrix is estimated from the indirect way using Lowry or Bradford method.

5.2.1. Lowry method

The immobilized lipase amount on polymeric membrane matrix is estimated by using Lowry analytical method (Wang et al., 2008; Lowry et al., 1951; Bryjak and Trochimczuk, 2006). Two steps are involved in the particular method: (1) Biuret reaction which reduces Cu^{+2} to Cu^{+}, (2) Cu^{+} reduces the Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate). The coloured solution is detected at 750 nm and standard curve is plotted using Bovine serum albumin protein.

The amount of lipase immobilization is calculated from the difference of lipase concentration in the solution before and after immobilization. It is mathematically presented as follows for membranes.

$$w = (C_1 - C_2) \times \frac{V}{A} \quad (1)$$

where w is the total immobilized amount (mg/cm^2), C_1 and C_2 are the initial concentration of free lipase and decant after immobilization, respectively, (mg/ml). V is the volume of enzyme solution in buffer, which is taken for immobilization (ml), A the area of the membranes (cm^2).

5.2.2. Bradford method

It is another analytical method for quantifying proteins (Deng et al., 2005; Bradford, 1976; Rodrigo et al., 2006). The absorbance maximum of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The immobilization amount on membranes is determined by Eq. (1).

5.2.3. Activities for model compounds

As lipase is treated as hydrolase type of enzyme, the hydrolytic activities of immobilized lipase can also be treated as the evidence of immobilization. The quantification of free fatty acid content in the medium is the measurement of hydrolytic activity. The hydrolytic activity of lipase is assayed with olive oil emulsion method and it is treated as standard method (Soares et al., 1999).

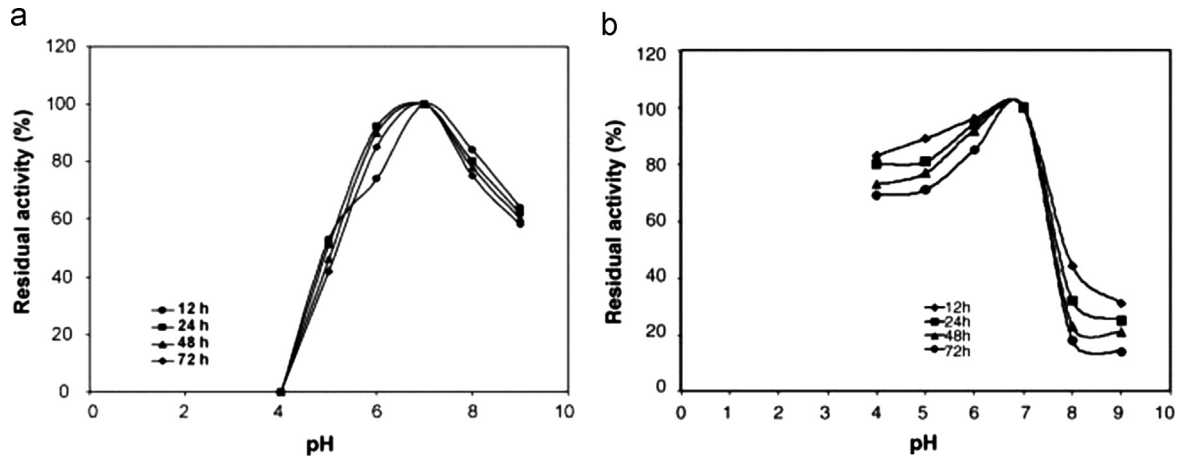


Fig. 17. (a) pH stability of free lipase (b) pH stabilities of lipase immobilized on DEA (72/63) interface. Reproduced from Abrol et al. (2007).

Several methods are employed in the literature viz. by titration, calorimetry, spectroscopy and GC-mass analysis from esters of released fatty acid.

6. Post-immobilization effects on lipase properties on

6.1. Reaction parameters (pH and temperature)

The activity range in terms of pH and temperature is shifted for the immobilized matrix. It is found that the optimum pH for the free lipase is about at neutral range, whereas those for the immobilized lipases shifted to the alkaline region at about 8.0 or 9.0 (Fig. 15) (Xu et al., 2006a, 2006b). It may be reason that the active sites of immobilized lipases become more exposed to solvent than in free lipases so, as a result proton transfer to the amino acid residues at the active site become less hindered (Ye et al., 2007; Duinhoven et al., 1995; Ting et al., 2006). The observed pH shift is also an effect of the fact that after immobilization only bulk pH can be measured, not the one at microscopic level.

In temperature variation the activity range is shifted towards higher range for immobilized matrix. The increase in optimum temperature may be caused by the changes in physical and chemical properties of the enzyme by immobilization. After immobilization the conformational flexibility of the immobilized enzymes reduces, for this reason they need higher temperature to form the proper conformation to recognize and bind the substrate molecules (Fig. 16). In other words there is the restriction in the diffusion of the substrate and products at low reaction temperature for the immobilized one (Ye et al., 2007).

6.2. Stabilities (thermal and pH)

It is already marked that immobilization is advantageous in terms of stability (i.e. thermal and chemical). It is seen (Fig. 17) that the pH profiles of the immobilized lipases are broader than that of the free enzyme which loses activity at pH 4.0. It means that the immobilization preserved the enzyme activity over a wider pH range (Abrol et al., 2007). The reason behind this fact is that immobilization physically protects lipase molecules so their protein conformation stabilizes and their thermal and pH stability slightly improves (Deng et al., 2004a, 2004b). However the stability depends upon the methods of immobilization.

In (Fig. 18) thermal stabilities of free and immobilized lipases are shown. It is observed that the free lipase loses its initial activity in 100 min at 50 °C heat treatment, whereas the immobilized ones retain their activities after 120 min at 50 °C. Of course the mode of attachment shows the variation of activity. The chemical bonding shows better activity in comparison to adsorption (Ye et al., 2007). The conformational integrity of the immobilized enzyme structure is better after immobilization so this may be the reason for increased thermal stability (Abrol et al., 2007; Liu and Chang, 2007).

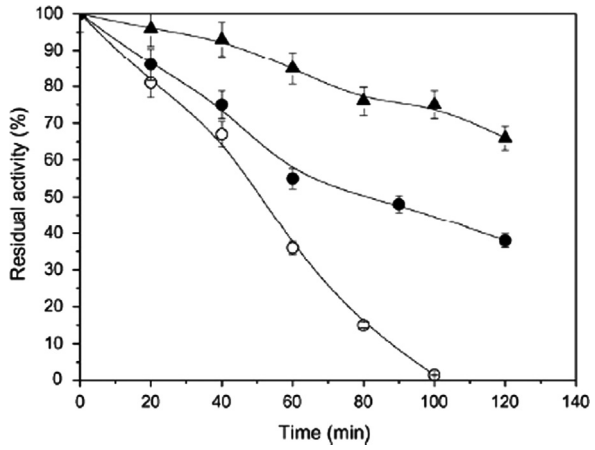


Fig. 18. Thermal stability of the free and immobilized lipases: (O) free lipase; (●) immobilized lipase by adsorption; (♠) immobilized lipase by chemical bonding. Reproduced from Ye et al. (2007).

6.3. Kinetic parameters (V_{max} and K_m)

The kinetic parameters (K_m and V_{max}) based on Michaelis–Menten Equation (Eq. (2)) signifies the physical interpretation of the affinity and maximum reaction velocity towards the substrate.

$$v = \frac{V_{max}S}{K_M + S} \quad K_m = \frac{k_2 + k_{-1}}{k_1} \tag{2}$$

v is the reaction rate, V_{max} is the maximum reaction rate, S is the substrate concentration, K_m is the Michaelis–Menten constant.

Three linear transformations (Lineweaver–Burk, Eadie–Hofstee, Hanse–Woolf) (Schultz, 1994) are introduced (Table 8) to get the above kinetic parameters. Researchers are mainly preferred Lineweaver–Burk plot as it is simple and accurate as well.

Kinetic parameters, the Michaelis–Menten constant K_m and V_{max}, for the free, the chemically bounded, and the adsorbed lipases are shown in Table 9 (Ye et al., 2007). After immobilization K_m of enzymes increases on the other hand V_{max} decreases. Immobilized lipases by chemical bonding method have higher K_m value than by adsorption. It means that it is easy to form substrate–enzyme complex for the adsorbed lipase compared to chemically bonded lipases. The increase in K_m after immobilization means that the affinity of enzyme towards its substrate decreases whereas the decrease in V_{max} indicates activity loss. The decrease in affinity may be attributed to the structural change of the enzyme introduced by immobilization so that accessibility of the substrate to the active site is low (Ayhan et al., 2002). Interaction of enzyme and the functional groups on the membrane surface and use of harsh reagents that can cause huge deformations in enzyme conformation causes loss of activity after immobilization. V_{max} is only affected by diffusional constrains if the enzyme has a K_m value that is higher than the diffusion under these conditions.

Table 8
Three linear transformations of Michaelis–Menten equation.

Tranformations	Equation	Plot
Lineweaver–Burk	$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$	$\frac{1}{v} \approx \frac{1}{S}$ V_{max} from intercept, K_m from slope
Hanse–Woolf	$\frac{S}{v} = \frac{S}{V_{max}} + \frac{K_m}{V_{max}}$	$\frac{S}{v} \approx S$ K_m from intercept, V_{max} from slope
Eadie–Hofstee	$v = \frac{K_m v}{S} + V_{max}$	$v \approx \frac{v}{S}$ V_{max} from intercept, K_m from slope

Table 9
Activity and kinetic parameters of the free and immobilized lipases. Reproduced from Ye et al. (2007).

Sample	V_{max} (U/mg)	K_m (mM)	Specific activity (U/mg)	Activity retention (%)
Free lipase	46.4	0.45	42.1	100
Lipase immobilized by chemical bonding	21.2	1.43	18.7	44.5 ± 1.8
Lipase immobilized by adsorption	26.2	1.08	22.8	54.1 ± 2.0

7. Towards practicability (membrane bioreactor)

The immobilized lipases are useful in different applications. In terms of continuous, large scale commercial processes different bioreactors (viz. membrane, hollow fiber) can be configured (Xu et al., 2006a; Shamel et al., 2007). The membrane reactor with lipase immobilized on the membranes is a promising approach to many lipase catalyzed processes. The different bioreactors set up are shown in Figs. 19 and 20.

In flat sheet membrane reactors, the immobilized membranes are used as catalyst as well as means of separation in a single step (Tan et al., 2002; Xu et al., 2006b; Lu et al., 2007). These can be monophasic or biphasic (Pujari et al., 2006; Prazer and Garcia, 1993). In monophasic reactor only one phase is there and bi phasic reactor has two phases viz. organic and aqueous. Two identical cylindrical channels have the two phases and they are separated by a biocatalytic membrane having lipase immobilized on it (Fig. 19). These channels are connected to feed and receive reservoirs. Lipase immobilized side of membrane is in contact with feed (substrate) solution. Recirculation of both feed and receiver phases are done by two peristaltic pumps. Substrate molecules diffuse through the lipase immobilized membrane where the esterification/hydrolysis reaction takes place.

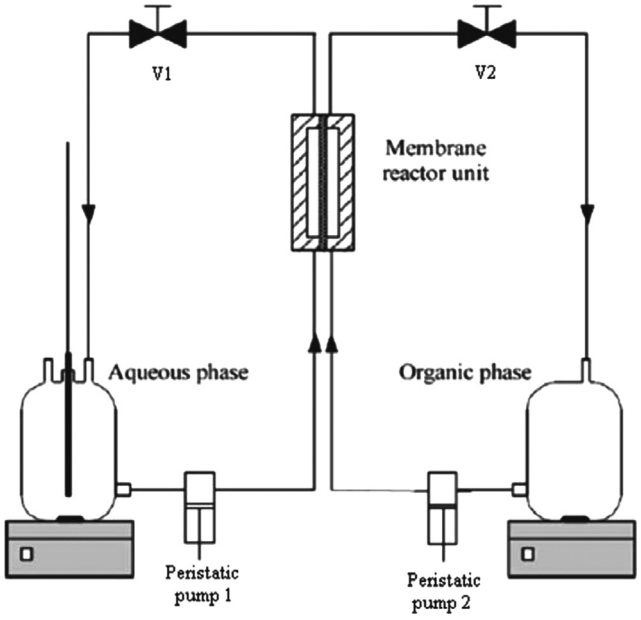


Fig. 19. Scheme of the experimental set-up of the biphasic membrane reactor. Reproduced from Xu et al. (2006b).

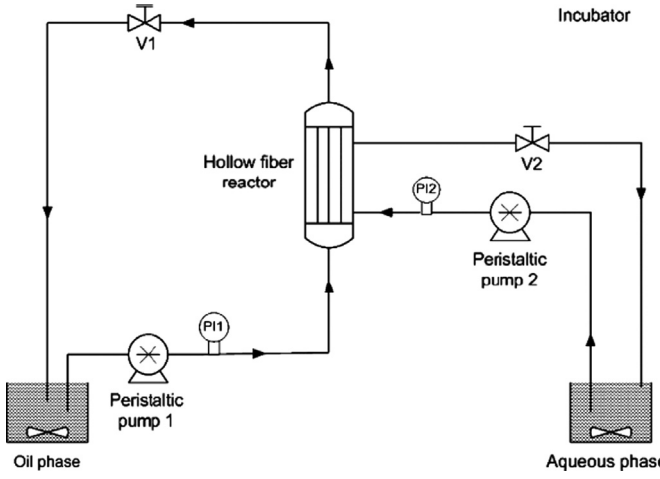


Fig. 20. Process flow diagram for the hollow fibre reactor system. Reproduced from Shamel et al. (2007).

In hollow fiber reactor, hollow fiber membranes are used as a carrier to immobilize lipase. Hollow fibers are cylindrical in shape and hollow in nature. Compared to flat sheet it has high specific surface area. The high specific area is of advantageous in terms of simultaneous reaction and continuous operation. As there is high feasibility of lipase immobilization because of its high surface area the extent of hydrolysis/esterification is more. A schematic diagram of the experimental set-up is shown in Fig. 20. In oil hydrolysis the oil phase is circulated in the lumen side of the reactor while buffer solution is circulated, co-currently, in the shell side. Analysis of the changes in free fatty acids concentration is used to determine the activity and production rate.

In the reactors the productivity and quality can be increased with decreased product recovery cost. They are used to enhance the productivity of enzymatic processes by improving substrate/enzyme contact. These reactors have easy control, straightforward scaling up, high lipase loading, prolonged lipase activity, high flow rates, low pressure drop, short residence time and high operational stability with low internal and external diffusional resistances and reduced costs (Lopez and Matson, 1997).

8. Applications in

Immobilized lipases show their potential in different type of industries. Some of the applications are ensemble in the following.

8.1. Oil industry

The oil industry is the source of various industries viz. soap production, detergents, cosmetics, pharmaceuticals and food. Hydrolysis of oils produces fatty acids and glycerol as shown in Scheme 1. In this regard hydrolysis of different oils like palm oil (Shamel et al., 2007; Knezevic et al., 2004a; Knezevic and Obradovic, 2004b), olive oil (Wang et al., 2007; Xu et al., 2006a, 2006b; Deng et al., 2004a; Shamel et al., 2007; Pugazhenthi and Kumar, 2004), sunflower oil (Rucka and Turkiewicz, 1990a), babassu oil (Mercon et al., 1997) is exploited. Hoq et al. (1985a, 1985b, 1985c) demonstrated a hydrophobic hollow fiber membrane system for fat hydrolysis whereas, Pronk and Van't Riet (1991) presented a hydrophilic hollow fiber membrane system for the hydrolysis of soybean oil. Shamel et al. (2007) used a lipase immobilized hollow fiber membrane for the hydrolysis of the palm and olive oils.

8.1.1. Enrichment of triglycerides and fatty acids

Lipases are used for enriching the triglycerides with poly unsaturated fatty acids (PUFA) in oils. Borage oil is enriched with γ-linolenic acid by selective hydrolysis of oil (Huanf et al., 1997). Lipases can also be used for selective enrichment of acid, (i.e. selective esterification of fatty acids) from oils. Huanf et al. (1997) used this technique for borage oil with *n*-butanol and acidolysis of γ-linolinic acid. Synthesis of 2-monoacylglycerol rich in polyunsaturated fatty acids is done by ethanolysis of fish oil (Muñío et al., 2008) and synthesis of structured triacylglycerols rich in docasahexanoic acid (DHA) at position 2 by acidolysis of tuna oil (Estrella et al., 2007; Ko et al., 2006).

8.1.2. Biodiesel production

Immobilized lipases also have good potential in terms of biodiesel production. Biodiesel is produced by transesterification of oils or fats by using chemical catalysts or biocatalysts i.e. lipase.

Process using immobilized lipase as the biocatalyst is preferred because it is "greener". Methyl and isopropyl fatty acid esters, used as biodiesel fuels are synthesized by alcoholysis of natural vegetable oils using lipase (Tan et al., 2006a). To achieve better production of biodiesel the other bye product (glycerol) is removed in continuous process (Shamel et al., 2007). Lipase catalyzed the reaction for soybean oil, canola oil, hydrogenated palm oil/palm oil blend, yellow grease and brown grease, salad oil, waste oil from Beijing with methanol as substrate (Cao et al., 2008; Nie et al., 2006). A lipase from *Candida* sp., suitable for transesterification of fats and oils to produce fatty acid methyl ester (FAME), immobilized on a cheap cotton membrane (Tan et al., 2006a; Nie et al., 2006; Ganesan et al., 2009).

8.2. Food industry

Apart from the oil industry, the immobilized lipases can also be useful in other food applications.

8.2.1. Synthesis of structured lipids

The immobilized lipases with their abilities in hydrolytic and esterification reaction are capable of preparing different structured lipids. The structured lipids have long-chain fatty acids at the sn-2 position and short- or medium-chain fatty acids at the sn-1, 3 positions of glycerol backbone. They have drawn attention for nutritional development and have beneficial effects on immune function, nitrogen balance, and improved lipid clearance from the bloodstream (Christophe, 1998; Xu et al., 1997).

In other sense regiospecificity of lipases is a key for the modification of oils and fats to produce high-value added products, viz. cocoa butter equivalents, human milk fat substitutes, and other specific-structured lipids (Xu, 2000; Xu et al., 2000).

Cocoa butter is an important major constituent of the chocolate formulations and confectionaries. Cocoa butter is composed of predominantly ($>70\%$) symmetrical triacylglycerols with oleic acid in the sn-2 position, mainly POP, POSt, and StOSt. Here, P, O and St stand for palmitic acid, oleic acid, and stearic acid, respectively, Jensen and Jensen (1992), Winter et al. (1993).

8.2.2. Flavor development

Immobilized lipases also have the potential to enrich flavour to food items. Actually different free fatty acids and esters act as flavors as well as flavor precursors. Thus it results more acceptability. For example butyl laurate is recognized for apricot and peach flavors, ethyl butyrate for pineapple flavor, terpenyl esters for fragrance preparations, butyl oleate for different flavor (Hilal et al., 2006, 2004). In chocolate industry, the free fatty acid contribution to flavor milk chocolate, caramels, toffees and butter creams is awesome.

8.3. Medical and pharmaceuticals

Immobilized lipases are very commonly used in pharmaceutical industry because of their capability in drug synthesis and regioselective property. Esterification of octanoic and decanoic acids forms respective triglycerides and have the potential in dissolving gallstones in humans. Mono, di and tri glycerides of octanoic and decanoic acids, 2-ethylhexyl palmitate, 2-ethylhexyl palmitate and isooctyl palmitate are having the uses in pharmaceuticals (Tan et al., 2006b).

8.3.1. Enantiomeric separation

In the pharmaceutical industry, stereoselective interactions are extremely important as one isomer has the desired properties of the compound, whilst the other isomer may be inactive or sometimes harmful. Lipases can also catalyzed resolution of racemic mixtures. Synthetic compounds that exhibit chirality are usually found as a mixture having equal amounts of both the forms i.e. isomers. The kinetic resolution of chiral molecules is catalyzed by lipases. In the process, the active site of lipase is found fit for only one enantiomer to bind properly for the reaction while the second enantiomer is left unreacted.

(*S*)-(+)-2-(6-methoxy-2-naphthyl) propionic acid (naproxen) belongs to the 2-aryl propionic acid derivatives family and widely used as non-steroidal anti-inflammatory drug in the treatment of headaches and minor pains (Wyss-Corary and Mucke, 2000). It is well known that lipases preferentially hydrolyze the (*S*)-naproxen methyl ester into (*S*)-(+)-2-(6-methoxy-2-naphthyl)-propionic acid (naproxen) from the (*R*,*S*)-naproxen methyl ester mixture. (*S*)-naproxen shows 28 times higher activity with respect to the (*R*)-isomer. (*S*)-naproxen is prepared by the enantioselective hydrolysis of racemic naproxen ethoxyethyl ester (Battistel et al., 1991), and racemic naproxen methyl ester by using immobilized lipase (Giorno et al., 2003; Li and Sakaki, 2008), Multiphasic membrane reactor having lipase immobilized is presented in Fig. 21 which is applied for the resolution of (*R*, *S*)-naproxen methyl ester mixture by hydrolysis and separation Giorno et al., 2003.

The other route to prepare (*S*)-naproxen ester is from racemic naproxen using enantioselective esterification (Chang and Tsai, 1997, 1999; Giorno et al., 2007; Sakakia et al., 2001; Tsai and Wei, 1994a, 1994b).

Ibuprofen, 2-(4-isobutylphenyl) propyl ionic acid, is also widely used as a non-steroid anti-inflammatory drug to treat headaches and minor pains. Chiral separation of ibuprofen carried out by the asymmetric hydrolyzation of racemic ibuprofen ester by lipase is one of the most important methods. A special microstructure in the composite hydrophilic cellucose acetate (CA)/hydrophobic polytetrafluoroethylene (PTFE) membrane (Wang et al., 2007), hydrophilic polyacrylonitrile (PAN) (Long et al., 2005a) are prepared for lipase immobilization and applied chiral separation of ibuprofen. Hydrolysis of 1-heptyl-ibuprofen ester and 2-ethoxyethyl-ibuprofen ester is done for the chiral separation using lipase immobilized polymeric membrane (Long et al., 2005b).

A diltiazen chiral intermediate is formed by immobilized lipase and it is used in the production of diltiazem, a drug used in the treatment of hypertension and angina (Lopez and Matson, 1997; Zhao et al., 2010).

The immobilized lipase is also used for the enantiomeric separation of pure (*S*)-3-hydroxy-γ-butyrolactone (Lee et al., 2008).

8.4. Biosensors

Lipases are also used in biosensors and find application in the fat and oil industry, food technology and beverage industry for the determination of fatty acids. They are also used in environmental control and pollution analysis especially pesticide contamination and in clinical diagnosis for the quantitative determination of triacylglycerols for the control of lipid level in the blood of patients with cardiovascular complaints (Starodub, 2006). Biosensors can be chemical, biological or electronical in nature but usage of biological one is cheaper and less time-consuming. *C. rugosa* lipase is used in potentiometric biosensors and applied for the detection of organophosphorus pesticide; methyl-parathion and tributyrin (Kartal et al., 2007). Lipases may also be immobilized onto pH/oxygen electrodes in combination with other enzymes like glucose oxidase. These can function as lipid biosensors and may be used in triglycerides as well as in blood cholesterol determination.

8.5. Production of high value products

As it is seen immobilized lipases have the potential to produce different free fatty acids and glycerol. The free fatty acids have the

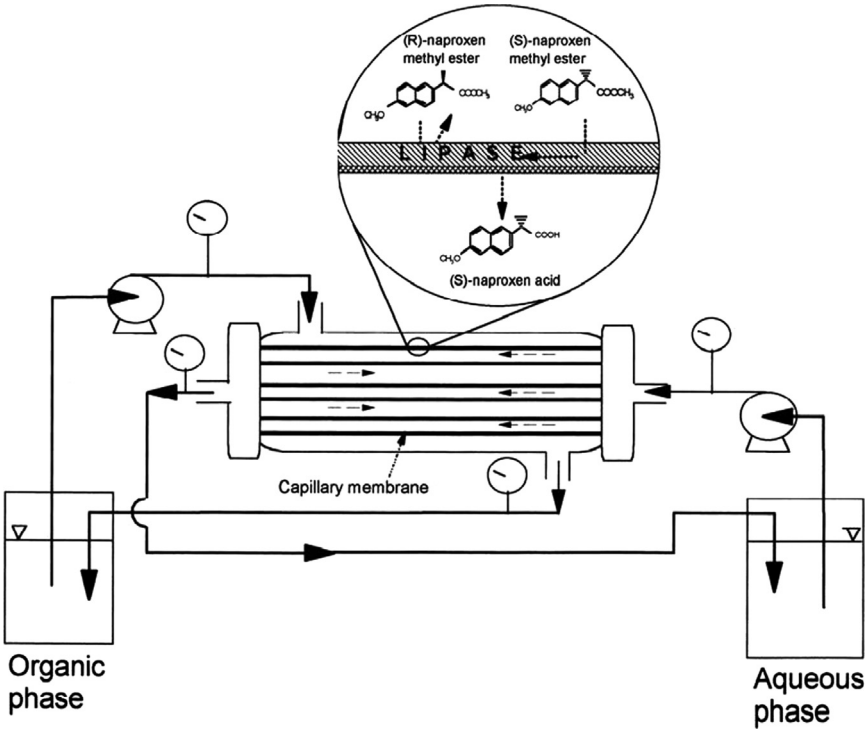


Fig. 21. Schematic diagram of reaction and separation process in multiphase enzyme membrane reactor with sponge layer of membrane as immobilization site. Reproduced from Giorno et al. (2003).

scope in different high value products viz. fatty alcohols, dicarboxylic acids, emulsifying agents for polymers, coatings, adhesives, specially lubricating oils, paints, varnish, soaps, shampoos, cosmetics and other personal care products. Glycerol is used as plasticizer in the manufacture of varnish, in cosmetic industries, preparation of explosives and propellants (Hoq et al., 1985b, 1985c). Butyl laurate and oleate are also used as plasticizers, lubricants etc. Isooctyl palmitate is recognizes as low temperature plasticizer for poly-vinyl chloride, vinyl chloride copolymer and synthetic rubber. Wax esters are widely used in cosmetics (Decagny et al., 1998; Willing, 1996). The special features of the wax esters are non-toxic, better fat soluble property, excellent wetting behavior. These make them suitable for cosmetic formulations (cleansers, conditioners and moisturizers) (Peter and Robert, 2001). Methyl and isopropyl fatty acid esters can also find application in cosmetic industry (Tan et al., 2006b).

8.6. Waste treatment

Bioremediation for waste disposal is a new avenue in lipase biotechnology. As an example, contaminants such as olive oil from oil mills are degraded by lipases for wastewater treatment. Lipases are also exploited for the treatment of wastes from fast-food restaurants for the removal of fats, oils and greases (Jeganathan et al., 2007). Immobilized lipases are used in activated sludge and other aerobic waste processes. In this case thin layers of fats are continuously removed from the surface of aerated tanks to permit oxygen transport. This skimmed fat rich liquid is digested with lipases. The enzymatic treatment plants where triglycerides are hydrolyzed by immobilized bacteria generating lipase with reasonable results up to 90% removal Gandhi, 1997; Tschocke, 1990.

8.7. Structural analysis of triglycerides

Lipases are very specific for hydrolysis towards Sn-1, 2 and 3 positions of fatty acids attached to glycerol backbone. So their regiospecificity and substrate selectivity is very advantageous in the structural determination of triglycerides, and specific and defined set of mono and/or di triglycerides can also be synthesized.

9. Conclusions

Immobilization of lipases is surely breakthrough that involves the synthesis of many products. Immobilization has many potential advantages over their free counterpart in terms of stability towards harsh environmental conditions (pH, temperature and organic solvents). The selection of proper immobilization method and support is very crucial to prepare good immobilization system with enhanced enzyme loading, good activity and stability to decrease the enzyme biocatalyst cost in industrial biotechnology. Several attractive concepts for lipase immobilization and bioreactor designs for lipase catalyzed process have been developed over the last decade for specific applications in the pharmaceutical, oleochemical and food industry.

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