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LIPASE CATALYZED ESTERS SYNTHESES IN ORGANIC MEDIA: A REVIEW

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ABSTRACT

Lipases (E.C. 3.1.1.3) from different sources have investigated for their hydrolytic as well as synthetic activity. The most desired characteristics of the lipase are its ability to utilize all mono-, di-, and tri-glycerides as well as the free fatty acids in transesterification, low product inhibition, high activity/ yield in non-aqueous media, low reaction time, resistance to altered temperature, pH, alcohol and reusability of immobilized enzyme. Clearly, lipases from different sources have different properties suitable for the process. Thus, there has been a search for an ideal enzyme which can work efficiently in organic media. Different strategies have been employed to improve the yield of the enzyme and to make the reaction time as short as possible. The optimum reaction conditions such as temperature, time duration and agitation speed are varied wholly and optimized with different combinations of the source of the lipase, the immobilization media, and alcohol used. Thermodynamic water activity (*aw*) determines the mass action effects of water on hydrolytic equilibrium and distribution of water between various phases that can compete in binding water. Thus water activity is a better parameter than water activity content to study the effect of water on enzymatic reactions in non-aqueous media as it eliminates the ambiguity due to the competition for binding water by immobilization support or other substances/impurities in the reaction mixture. During enzymatic reaction as the level of water increases, it increases the enzyme flexibility and the expressed activity. In case of the lipases, an increase in water content above an optimum level promotes the hydrolytic activity and the transesterification yield drops. Thus the present review enlightens various aspects of lipase catalysis in aqueous and non aqueous media for the synthesis of commercially important esters.

1. INTRODUCTION

The esterification and transesterification reactions by lipase catalysis in industrial biotechnology yield the products of commercial value. Till to date hundreds of different esters used in foods, flavors, additives and fragrances as well as in medicines have been synthesized by lipase-catalyzed reactions and their properties have been evaluated. However, as the real biocatalytic potential of microbial lipases in both aqueous and non aqueous media has been realized, the industrial fronts have shifted towards utilizing this enzyme for a variety of reactions of immense importance. Lipases possess the unique feature to act at an interface between aqueous and non-aqueous (i.e. organic) phase and this feature distinguishes them from esterases. In organic solvents lipase catalyzes the reverse reaction of synthesis or transesterification (Intra *et al.,* 2004) and shows high tolerance to different organic solvents and wide range of temperature and pH. Thermophilic lipases are of more interest in today's pharmaceuticals well as biotechnology industry as these are more resistant to thermal and chemical denaturation (Castro 2005). The high reaction temperature in the enzymatic process results in higher conversion rate, minimal risk of microbial contamination and higher solubility of the substrates. The major lipase producing organisms are *Bacillus prodigiosus*, *B. pyocyaneus* and *B. fluorescens* which represent today's best studied lipase producing bacteria now named *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively (Verma and Kanwar 2008; Guncheva and Zhiryakova 2011). Esterification, transesterification, alcohlysis and other hydrolytic reactions occur in mild conditions and are usually highly enantio- and regio-selective, and such properties are highly appreciated in fine chemical synthesis. Lipases from a large number of bacterial, fungal, plant and animal sources have been purified and generally used at commercial scale for lab as well as industrial use (Saxena *et al.,* 2003). Although the sources of lipase might be different but the natural function of all lipases is to catalyze the hydrolysis of ester linkage and to catalyze the esterification. Therefore, since they can catalyze hydrolysis, alcoholysis, esterification and transesterification they have a wide spectrum of biotechnological applications. They all show high specific activity towards glyceridic substrates. The increased availability and stability of commercially available lipases has resulted in an increased interest in the potential applications of lipase biocatalysis (Vulfson, 1994; Kumar and Kanwar 2011).

The catalytic properties of lipases have enabled their use in important applications in processes usually considered as pollution-prone. In addition, the move towards green

chemistry demands the development of biodegradable products, including polymers that can substitute for petroleum-derived products. These syntheses produce eco-friendly products that can, in turn, be degraded by lipases in aqueous media. Owing to their low cost and applicability to a broad range of substrates, lipases have become the most versatile class of biocatalysts in organic synthesis. They often operate at neutral, weakly acidic or weakly basic pH values and in many cases combine the high selectivity for the reactions they catalyze and the structures they recognize with a broad substrate tolerance. Lipases can accept a wide range of organic substrates and they work very well in organic solvents.

2. Bioeconomics of lipases

The production of such lipases at commercial level is usually achieved by employing the use of genetic engineering and protein engineering techniques. By gene expression in an appropriate microorganism such as a fungus, yeast, or bacterium (e.g., *Escherichia coli*), the maximum production of lipase can be achieved easily (Pamies and Backvall 2003). In the recent years, application of enzymes has emerged as an interesting alternative, since enzymatic synthesis has several advantages over chemical synthesis, such as high regio- and stereo-selectivity, mild reaction conditions, avoiding the use of toxic catalyst, and low energy consumption (Ganske and Bornscheuer, 2005). Reputed companies are involved in the production of lipase from bacterial as well as fungal sources at commercial scale by using techniques of recombinant DNA technology (Table 1).

Table 1. Commercially available lipases and the companies in the lipase production.

Source (Abbreviation)	Commercial lipase supplier	Application(s)	Reaction
Bacillus pumilus	Solvay, Belgium	Detergent	Hydrolysis
P. cepacia (PCL)	Amano P, P- 30, PS, LPL-80, LPL-200S/ Amano Pharmaceuticals, Japan	Organic synthesis	Synthesis
P. alcaligenes (PAL)	Lipomax/ Gist- Brocades, The Netherlands; Genencor International, USA	Detergent	Hydrolysis
tabP. glumae (PGL)	Unilever, The Netherlands	Detergent	Hydrolysis
Alcaligenes sp.	Lipoprotein lipase, Lipase PL, QL/ QLL, PLC/ PLG, QLC/ QLG/	Organic synthesis and	Synthesis

	Meito Sankyo Co. Japan	research	
C. viscosum (CVL)	*Chromobacterium viscosum* lipase/ Asahi Chemical Biocatalysts; Lipase 50P/ Biocatalysts, UK	Organic synthesis	Synthesis
Achromobacter sp.	Lipase AL, ALC/ ALG/ Meito Sankyo Co., Japan	Technical grade	Synthesis
Pseudomonas sp. (CVL)	Lipase K-10/ Amano Pharmaceuticals, Japan	Organic synthesis	Synthesis
Geotricumcandidum (GCL)	Chirazyme L-8 SP 524, Lipolase/ Boehringer Mannheim Novo-Nordisk		Synthesis
Candida antartica A (CAL –A)	Chirazyme L-5, SP526/ Boehringer Mannheim Novo-Nordisk		Synthesis
Candida rugosa(CRL)	Amano (lipase AY),Meito Sangyo, Japan		Synthesis
Yarrowia lipolytica (earlier *Candida lypolytica*, CLL)	Lipase L/ Amano		Synthesis
Cross-linked enzyme crystals (CLEC)	Altus	Ester	Synthesis
	Greasex (lipase)/ Novo Nordisk	Leather	
Thermomyces lanuginose	Lipolase/ Novozyme Nordisk	Detergent	Hydrolysis
Burkholderia cepacia (PCL, BCL)	Lipase PS/Amano		
P. stutzeri	Lipase TL/ Meito Sangyo (Japan)		

3. Advantages of lipase catalysis over chemical synthesis

In the times of industrial biotechnology, the leading representatives of the new environmentally friendly science promoted the idea of using biological systems to create more efficient and more selective processes for the conversion of raw materials into

industrial products, thereby substituting problematic chemical transformations. Therefore, the demand for industrial enzymes, particularly of microbial origin, is ever increasing owing to their applications in a wide variety of processes. Biocatalysis or biotransformation encompasses the use of biological systems to catalyze the conversion of one compound to another. The catalyst part of the biological system can thereby consists of whole cells, cellular extracts, or isolated enzyme(s). Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods. In the present scenario, enzymes such as proteases and amylases have dominated the world market owing to their hydrolytic reactions for proteins and carbohydrates. However, with the realization of the biocatalytic potential of microbial lipases in both aqueous and non-aqueous media in the last one and a half decades, industrial fronts have shifted towards utilizing this enzyme for a variety of reactions of immense importance. Major improvements have been achieved in the development and application of lipase as catalyst with the advancement in modern enzymology. Compared with conventional chemical synthesis from alcohols and carboxylic acids using mineral acids as a catalyst, the use of enzymes such as lipases to produce these high value-added fatty acid esters in solvent-free media may offer many significant advantages (Yadav and Lathi, 2003). Environmentally friendly and economic processes are the goals of every industry nowadays. The use of lipases for the synthesis of chiral drugs, cosmetic or nutritional compounds is well established since years ago. Lipid modification strategies for industry include processes such as fractionation, hydrogenation and interesterification. While each of the first two processes has specific uses and advantages, interesterification reaction offers the greatest potential application. It includes three approaches: acidolysis, alcoholysis and transesterification. Chemically, it can be induced by the use of alkali catalysts in a reaction which lacks specificity and offers little or no control over the positional distribution of fatty acids in the final product (Marangoni *et al.,* 1995). In the chemical synthesis, mineral acids are most commonly used to catalyze the esterification. Other agents such as tin salts, organo-titanates, silica gel, and cation-exchange resins are also employed. The classical acid catalysis may lead to unwanted side reactions. Although the metal salts minimize the side reactions, they require higher temperature (Kirk and Othmer, 1979). Normally, the fat and oil modifications carried out by the chemical inter-esterification are energy intensive and non-specific (Gupta *et al.,* 2003). The esterification by the lipases appears to be an attractive alternative to the bulk chemical routes. Lipases are being developed to carry out the transformations without the extreme temperature and pressure conditions which are essential for the traditional

industrial processes. Lipases display a high degree of specificity and enantio-selectivity for the esterification and transesterification reactions (Okahata *et al.,* 1995) which makes them a principle biocatalyst in the trans- and inter-esterification reactions for the synthesis of several useful acylglycerols. The use of lipase to carry out the esterification alleviates the need for a wide variety of the complex post-reaction separation processes, which may lower the overall operating costs.

4. Effect of organic solvents on lipase catalysis

In the modern enzymology the use of enzymes in organic media has been one of the most exhilarating topics among the researchers. Lipases are one of the versatile enzymes used in various fields such as food industry, nutritional and pharmaceutical sciences, chemical and detergent industries and clinical medicine because of their ability to catalyze various reactions involving a wide range of substrates. Despite such advantages the lipase technology has limited exploitation due to the facts such as high enzyme cost, requirement of high enzyme volume and microbial contamination. In nature, enzymes function in aqueous solutions. Therefore, it is not surprising that virtually all studies in enzymology thus far have used water as the reaction medium. Many of these limitations were due to the usage of lipase in water-rich media. To overcome these problems and to realize the full potential of lipases, lipase-catalyzed reactions in organic solvents have become recent subject of interest. Also, from the biotechnological standpoint there are numerous advantages of conducting enzymatic conversions in organic solvents (Figure 1) as opposed to water.

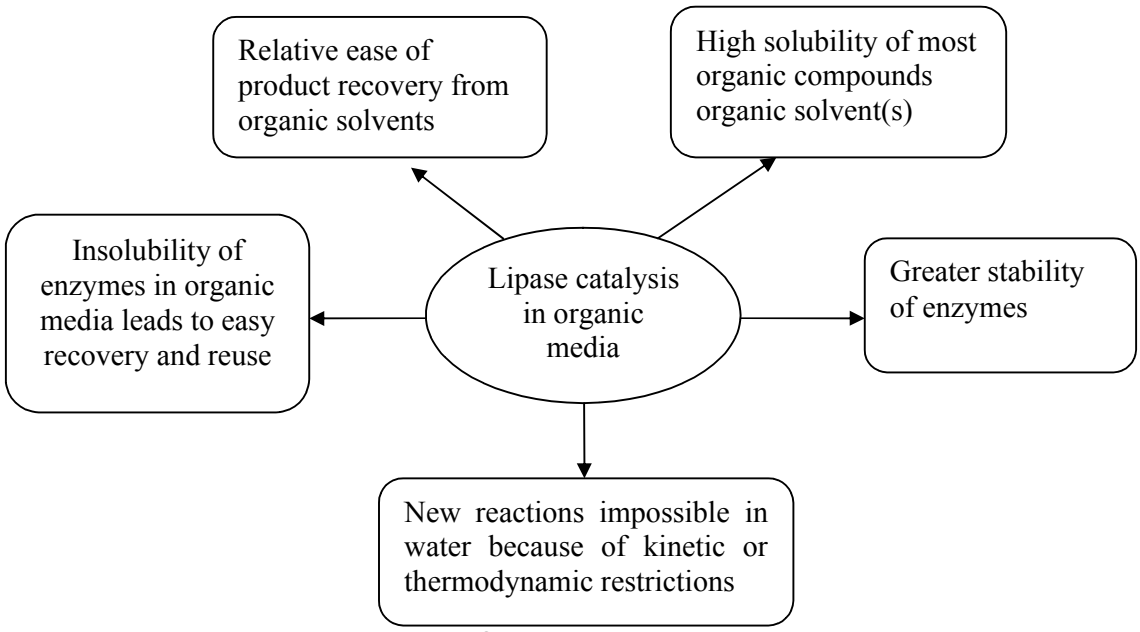

Figure 1. Advantages of lipase catalysis in organic solvent

Conventional wisdom dictates that water is required for enzyme action. This conclusion originates from the fact that water participates (directly or indirectly) in all non-covalent interactions maintaining the native, catalytically active enzyme conformation (Cantor, 1980; Creighton, 1983). Hence, the complete removal of water should drastically distort that conformation and inactivate the enzyme. Although this reasoning is undoubtedly correct, the real question is whether water is indeed required but how much water is crucial to retain the catalytic activity of lipase. It is hard to imagine that an enzyme molecule can "see" more than just a few monolayers of water around it. As long as this water is present around the enzyme molecules, the rest of water can probably be replaced with an organic solvent without adversely affecting the performance of the enzyme. Since the absolute amount of water contained in those few monolayers is very small, this situation is tantamount to an enzyme functioning in a nearly anhydrous organic medium. Stability of lipases in organic solvents makes their uses commercially feasible in the enzymatic esterification reactions. Lipase was found to work in the organic solvent because of its rigid conformation and interfacial activation properties. Many lipases are active in organic solvents where they catalyze a number of useful reactions including esterification (Chowdary *et al.,* 2001; Hamsaveni *et al.,* 2001; Kiran *et al.,* 2001a; Kiyota *et al.,* 2001; Krishna and Karanth, 2001; Krishna *et al.,* 2001; Rao and Divakar, 2001). Various factors for maintaining lipase activity in non-aqueous media have been considered such as tuning up of the lipases pH and it is accomplished while the enzyme is dissolved in the buffer prior to dehydration (lyophillization) before its suspension in organic solvent. Also the correct protonation state of the side chain of amino acids residues of lipase is important for retaining its catalytic activity (Saifuddin, 2008). A relevant practical example is the use of esterases and lipases to catalyze esterification in organic solvents (Chandel *et al.,* 2011; Kumar and Kanwar*,* 2011).

Enzymatic reactions in organic media are actually divided into two systems: reactions performed in organic solvent systems and in solvent-free systems. The solvent-free system, *i.e*. the reaction mixture comprising only liquid organic substrates (such as liquid oil) without any organic solvent, if it is possible, has high volumetric performance and economical advantages over the organic solvent system especially for large scale production. It is also desirable for the synthesis of food-grade products since very stringent safety regulations concerning organic solvent usage have to be observed in food industry. Non aqueous enzymology is concerned with the utilization and understanding of enzymes in essentially organic environments. Conventional biocatalysis is carried out in aqueous media, and it is not

surprising that most of the methods developed to study enzymes performance are water based. Recently, however the interest has been focused on using enzymes to catalyze reactions in organic media (Mander *et al.,* 2012). If an enzyme could function in an essentially organic environment, increased ease of product recovery, increased hydrophobic reactant solubility and reduced microbial contamination would be properties contributing to its wide applicability. Altering the solvent can have a dramatic effect on enzyme function, inability the production of catalysts by design via 'solvent engineering'. Also lyophilized enzyme powders suspended in organic solvents could catalyze a variety of novel catalytic functions *e.g*. esterification, transesterification, interesterification etc. The exclusion of water leads to an improved enzyme thermostability (as water is a reactant in many processes that irreversibly denature enzymes), and the reduction of undesirable side reactions that require water as a substrate (Castillo *et al.,* 2000). In organic solvent systems, however, lipases have also been shown to catalyze ester synthesis (Klibanov, 1989; Chand *et al.,* 1997). They have been employed in the modification of fats and oils both to provide novel materials of improved characteristics and to upgrade inexpensive raw-materials to valuable products by transesterification (West, 1988). Normally, the ester synthesis by the interesterification requires a suitable mixture of the non-polar solvents, but in the food application process, solvent-free systems are often preferred (Undurraga *et al.,* 2001). The enzymatic esterification of 2-(4-isobutyl phenyl) propionic acid (ibuprofen) was studied in different solvents at various water activities to measure the effects of these variables on the activity and selectivity of the enzyme. The hydrophobic solvents yielded higher reaction rates than the hydrophilic ones even at a constant water activity; however, better enantio- selectivity was observed (Dueret *et al.,* 1998).

5. Immobilization strategies of lipases

Immobilization of lipases onto various matrices and supports offers high chemical, mechanical and thermal stability as well as an improved enantioselectivity and stereo-selectivity. Many lipases except those obtained from thermophilic organisms are moderately stable at high temperature and pH. Ester exchange reactions take place at low water activity and make use of the law of mass action to drive the equilibrium in the direction of the synthesis by removing the water generated during the reaction (Macrae, 1983). Hence to exploit the potential of these enzymes to suit the industrial demand, it is essential to improve their features. Also the majority of current biocatalytic approaches rely on using free enzyme in reaction media or living cells, which set hurdles in the separation of product and enzyme.

The immobilization strategies may improve the stability and ease in recovery of product/ enzyme and enable the reuse of enzyme making the process more economical. Stability of lipase in various environments, like those of other enzymes, is too low to allow it to be used under the severe conditions often required for industrial applications. Many studies have examined the use of immobilization techniques to overcome these constraints (Balcao *et al.,* 1996). The immobilization of lipase renders lipase insoluble and, thus, it can then be easily separated from reaction mixtures (Table 3). In this manner, immobilized lipases can be reused after batch reactions (Kim *et al.,* 2000). Furthermore, immobilized lipases can be used as catalysts in continuous reaction systems, and the thermal and pH stabilities of lipase may also be increased by its immobilization on a carrier matrix (Park *et al.,* 2002). Enzyme immobilization gives an inherent advantage to isolate the biocatalyst from the reaction product and reuse it in order to increase the process productivity (Zou *et al.,* 2010; Gao *et al.,* 2010; Itoh *et al.,* 2010). The high cost of lipase however, makes enzymatically driven processes economically unattractive. The use of immobilized lipase is a possible solution to this problem because the enzyme can be recovered from the product and reused (Balcao *et al.,* 1996). The reuse of lipase provides cost advantages that are often an essential prerequisite for establishing a lipase-catalyzed process (Tischer and Wedekind, 1999; Kim *et al.,* 2000). Furthermore, easy separation of lipase from the product simplifies lipase applications and provides the basis for a reliable and efficient technology. Many methods have been used to immobilize lipases, including adsorption or precipitation onto hydrophobic materials (Wisdom *et al.,* 1984), covalent attachment to functional groups (Shaw *et al.,* 1990), polymer gels (Telefoncu *et al.,* 1990), adsorption in macroporous anion exchange resins (Rizzi *et al.,* 1992), microencapsulation in lipid vesicles (Balcao *et al.,* 1996) and sol-gel entrapment (Jaeger and Reetz, 1998; Krishnakant and Madamwar, 2001). *G. candidum* lipases A and B were immobilized on Accurel EP 100 porous polypropylene supports, precoated with ovalbumin to increase stability in organic solvents and at elevated temperatures (Charton and Macrae, 1992; Bosley and Clayton, 1994) used hydrophobic controlled pore glasses to immobilize *Rhizopus miehei* lipase (Reetz *et al.,* 1995). Although many techniques and carriers have been employed for immobilization of lipases, the activities and operational stabilities of the resultant catalysts, exhibited in ester synthesis, were rarely studied and compared in a systematic way. Due to economical considerations their application on an industrial scale requires their immobilization and thus re-usability.

Table 3. Immobilized lipase preparations used in ester synthesis

Immobilization strategies	Carrier/ support	Source of lipase	Ester(s)	Reference(s)
Adsorption	Celite, Silica, Amberlite, Nylon and Nitrocellulose membrane	Porcine pancreatic lipase (PPL), *Candida cylindracea* lipase (CCL). *Bacillus cereus, Candida rugosa, Candida cylindracea,* Commercial lipase (Steapsin)	Ethyl ferulate, isopropyl ferulate, pentyl isovalerates, Iso-propyl acetate, methyl adipate, ethyl propionate, iso-amylbutyrate butyl ferulate,	Kumar and Kanwar 2011; Sagiroglu and Telefoncu, 2004; Verma *et al.,* 2011; Rahman *et al.,* 2007; Carta *et al.,* 1992; Chandel *et al.,* 2011
Covalent bonding	Magnetic poly siloxane-polyvinyl Sepabeads	*Mucor meihei, Candida rugosa*	Butyl caprylate, pentyl octanoate	Bruno *et al.,* 2004 Zorica *et al.,* 2008
Entrapment	Gels formed from photo-cross linkable pre-polymers, poly-ethlene glycol, PVA-boric acid beads, cellulose acetate-TiO2 gel fiber	*Candida cylindracea lipase*, Novozyme 435 from *C. antarctica* and Lipozyme TL IM from *T. lanuginosus*, *Candida rugosa and Candida antartica*	Ethyl butyrate, aromatic esters of sugar alcohols, ethyl butyrate, geranyl acetate	Croitoru., 2011; Dave and Madamvar 2006; Jyh-Ping Chen, 1996
Cross-linked enzyme molecules	-	CLEC, porcine pancreatic lipase, *Burkholderia cepacia*	Lauryl laurate, ethyl butyrate	Gogoi *et al.,* 2006;

The kinetics of the immobilized lipase B from *Candidas antarctica* has been studied in organic solvents. This enzyme has been shown to be slightly affected by the water content of the organic media, and it does not seem to be subject to mass transfer limitations. On the other hand, some evidence indicates that the catalytic mechanism of reactions catalyzed by this lipase proceeds through the acyl-enzyme intermediate. Moreover, the fact that the immobilization support dramatically enhances the catalytic power of the enzyme, it does not interfere with the intrinsic solvent effect. Consequently, this enzyme preparation becomes optimum for studying the role played by the organic solvent in catalysis.

5.1 Adsorption

Adsorption is the easiest and also least expensive technique for immobilizing the biocatalyst (lipase). The non-covalent linkages mainly developed between the support/ carrier and lipase is mainly Vander wall forces, hydrogen bonds and hydrophobic interactions. Although the linkages have little effect on catalytic effect the enzyme may sometimes be leaked into the solution. Hence adsorption can be used in processes that are carried out in non-aqueous solvent, in which lipase is less soluble. A number of supports/ carriers have been used that included matrices such as alumina, silica, celite, ceramics, metal oxides, porous glass, Sepharose, Sephadex, cellulose, zeolites, polyethylene, polypropylene, polystyrene and nylons polyacrylates (Kumar and Kanwar 2011). The efficient adsorption depends on nature of carrier/ support such as pore size, hydrophobic/ hydrophilic balance, porous/ nonporous, surface chemistry and binding capacity. Porous particulate supports are superior to non-porous supports for immobilization of lipases due to their greater surface area. However, porous supports must have an internal morphology that allows not only the lipase binding but also an easy accessibility to substrate molecules in order to minimize diffusional limitation. It appears that pore sizes best suited for lipase adsorption are at least 100 nm in diameter. Lipase activity is also found to be greater with the hydrophobic support and also the conjugates so formed are more stable than those with hydrophilic supports. Simplicity of adsorption and use in immiscible solvents makes widespread use of adsorbed lipases on an industrial scale. Immobilization by adsorption has been most widely used for immobilization of various enzymes (Chaubey *et al.,* 2009; Yilmaz *et al.,* 2011).

5.2 Covalent binding

Covalent binding is another method of immobilization of enzymes on to different inorganic and organic carriers that appears to be more advantageous than other methods since diffusional restrictions to substrate or products are reduced considerably. In addition,

covalent immobilization offers the greatest advantage by increasing the stability of the enzyme and preventing it from leaking into solution (Girelli *et al.,* 2012). Among various immobilization methods available, covalent bonding has been widely studied (Byun *et al.,* 2004) Covalent bonding provides a powerful link between the lipase and its carrier matrix. Lipase immobilized through covalent bonding can be reused more often than other available immobilization methods, such as adsorption and entrapment. Inorganic materials have been successfully used for the immobilization of enzymes (Soares *et al.,* 2003). Silanization using appropriate organosilane agents to activate supports and subsequent covalent binding of an enzyme to the carrier by using a coupling reagent, *e.g*., glutaraldehyde, is a common method used in surface modification for immobilization (Cestari *et al.,* 2000). However, nowadays most of the catalysis is carried out in organic solvents; therefore the additional costs of activation of matrix or carrier can be overcome by using simple method of adsorption.

5.3 Entrapment

Lipase immobilization by entrapment is another strategy of trapping the enzyme, which however suffers from problems like diffusion and enzyme leakage, which may lead to decrease in activity of lipase. The above problem can be overcome by decreasing the size of immobilised particles and increasing the stirring rate. Alternatively lipase can be trapped within semi-permeable membranes.

5.4 Cross-linking of enzyme aggregates

Cross-linked enzyme aggregates are an interesting method to immobilize enzyme which uses no support. It involves the crystallization of pure enzymes followed by its chemical cross-linking. The biocatalyst preparation involves crystallization of the protein, and that is never a simple issue, although expert companies may produce them. Cross-linking of enzyme aggregates using a bi-functional reagent, to prepare carrierless macroparticles is an increasing interest in carrier-free immobilized enzymes (Margolin and Navia 2001). These are robust, highly active immobilized enzymes of controllable particle size, varying from 1 to 100 mm. Their operational stability and ease of recycling, coupled with their high catalyst and volumetric productivities, renders them ideally suited for industrial bio-transformations.

5.5 Crude extract of solid state fermentation

Solid-state fermentation is defined as the culture system involving solids in the absence or near absence of free water. With an increasing trend towards efficient utilization of agricultural and agro-industrial residue, and production of value-added agro-industrial products, SSF has attracted more attention in recent years (Pandey *et al.,* 2000). There has

been considerable interest to produce enzymes in SSF process (Mahanta *et al.,* 2008; Ruchi *et al.,* 2008). Ethyl esters synthesized from fatty acids are a large group of flavor and fragrance compounds widely used in food and beverage industries (Xu *et al.,* 2002) due to their special strong fruit flavor.

6. Lipases in organic synthesis

In fact the great potential of lipase has been realized in last two decades by pharmaceutical as well as food industry as lipases have occupied a prominent place among biocatalysts used in biotechnological applications. In a wide range of synthetic reactions lipases are employed for the production of organic compounds, which are difficult to prepare and to handle by conventional means. Lipases are serine hydrolases which do not require any cofactor. Due to this unique feature, they remain dissolved in oil-water interface and under the natural conditions they hydrolyse the triacyl glycerols which have low solubility in the water. In the presence of traces of water, they reverse the reaction leading to esterification and formation of glycerides from the fatty acids and glycerols (Ghosh *et al.,* 1996; Sharma *et al.,* 2001). The attention towards tremendous use of microbial lipase was exploited in the past decade leading to the easy hydrolysis/ synthesis of esters at ambient condition with an advantage of precise selectivity. Such reactions mediated by biocatalysts have advantages like mild reaction conditions, one step synthesis without protection and deprotection steps, and easy application to food processing (Arcos *et al.,* 1998; Gao *et al.,* 2000; Bornscheuer *et al.,* 2002). A lipase catalyzes a reversible reaction and the direction and equilibrium of the reaction is determined by the activities of the substrates, products, temperature and pressure (Kobayashi and Adachi, 2004). The stability of these enzymes in organic solvent has pushed them into frontier areas of organic synthesis leading to designing of novel drugs, surfactants, bioactive compounds and oleo-chemicals. The esters produced from short-chain fatty acids have applications as flavor constituents in food industry, while methyl or ethyl esters of long-chain acids are expected to be used to enrich diesel fuels. Lipase catalyzed ester synthesis requires the maintenance of a low concentration of water.

The available literature indicates variation in water concentration from 0.75 to 4% (w/v) in different type of esters synthesis. The esters produced from short-chain fatty acids have applications as flavoring agents in food industry (Vulfson, 1994). Esterification of lactic acid and alcohols using a lipase of *Candida antarctica* in hexane and esterification of five positional isomers of acetylenic fatty acids (different chain lengths) with *n*-butanol was studied by using eight different lipases (From *et al.,* 1997; Lie *et al.,* 1998). It has been noted

that an optimum pre-equilibrium water activity value was necessary for obtaining a high rate of esterification of (R, S)-ibuprofen by esterification of sulcatol and fatty acids in toluene was catalyzed by *C. rugosa* lipase (Arroyo *et al.,* 1999; Janssen *et al.,* 1999; Krishnakant and Madamwar, 2001). Biocompatibility, biodegradability, and environmental acceptability of bio-technically produced polyesters are desired properties in agricultural and medical applications (Evans and Sikdar, 1990). The use of lipase to carryout the esterification alleviates the need for a wide variety of the complex post-reaction separation processes, which may lead to lower the overall operating costs.

6.1 Esterification by lipases

Esters can be directly obtained from natural sources or may be synthesized by process of fermentation. However, these processes may not prove to be economical due to high cost processing and low yields. (Langrand, 1990). The other alternative may be chemical synthesis, in which unfortunately the product obtained so far may not be safe for human consumption. (Armstrong, 1989). Hence manufacture of esters, through lipase-catalyzed synthesis is thus gaining industrial importance (Table 2). Several high-purity esters have been manufactured for use in the cosmetic industry. Many of the esters, derived by using these reactions, resemble naturally occurring waxes of commercial importance.

Table 2. List of lipase catalyzed flavor and medically important esters

Name of ester	Application(s)	Lipase type	Reference(s)
Ethyl ferulate	Antioxidant ester	Steapsin *ex microorganism*	Kumar and Kanwar 2011
Isopropyl ferulate	Antioxidant ester	Steapsin *ex microorganism*	Kumar and Kanwar 2011
Butyl ferulate	Antioxidant ester	Lipolase100T, Novozyme 435	Chandel *et al.,* 2011
Ethyl acetate	Fruity odor	*Bacillus cereus*	Verma *et al.,* 2011
(Z)-Hexen-1-yl caproate	Green top-note	Rapeseed lipase	Liaquat 2011
Terpene esters	Fruity odour	*Candida antartica lipase B*	Patil *et al.,* 2011
Ethyl cinnamate	Antioxidant ester	*Bacillus coagulans*	Sharma *et al.,*2010
Ethyl valerate	Green apple ester	*Candid rugosa lipase*	Raghvendra *et al.,* 2010
Aromatic esters of arbutin	Flavor in food, pharmaceutical industries	*Penicillium expansum*	Yang *et al.,* 2010
2-Phenylethyl esters	Fruity floral esters	Lipase AYS “Amano”, Lipase A “Amano” 12,	Tan *et al.,* 2010

Aroma flavor esters (isoamyl acetate, ethyl valerate)	Banana flavor, green apple flavor and pineapple flavor	*Candida rugosa* lipase	Ozyilmaz and Gezer (2010)
Hyper-branched polyesters	Pharmaceutical industries,	CALB Novozym 435	Lopez-Lunaa *et al.,* 2005
Esters of ferulic acid (Vinyl ferulate)	Antioxidant esters	*Candida antarctica, Candida rugosa*	Chigorimbo-Murefua *et al.,* 2009
Geranyl acetate	Floral or fruity aroma	*Bacillus cereus*	Verma and Kanwar, 2008
Geranyl butyrate	Citrus notes	*Pseudomonas aeruginosa*	Verma *et al.,* 2008
Propyl oleate	Fruity flavor and	*M. circinelloides*	Szczesna-Antczak *et al.,* 2004
Ethyl laurate	Fruity floral flavor	*Bacillus coagulans*	Kanwar *et al.,* 2005
Isoamyl butyrate	Banana flavor	*Rhizomucor miehei*	Mestri and Pai, 1994
Isoamyl propionate	Banana flavor	*Rhizomucor miehei*	Chowdary *et al.,* 2002
Isoamyl isovalerate	Apple flavor	*Rhizomucor miehei*	Chowdary *et al.,* 2002
Isobutyl isobutyrate	Pineapple flavor	*Rhizomucor miehei*	Hamsaveni *et al.,* 2001
Methyl propionate	Fruity flavor	*Rhizomucor miehei*	Perraud and Laboret 1989
Glyceryl ferulate	Antioxidant, UV protecting ester	*Aspergilus niger*	Matsuo *et al.,* 2008
Ethyl butyrate	Pineapple flavor	*Candida cylindracea*	Yadav, Lathi 2003
Butyl isobutyrate	Sweet fruity odour	*Candida cylindracea*, *Aspergillus niger*	Welsh *et al.,* 1990

Due to commercial availability, low cost, high stereo selectivity and the possibility of use at large range of pH and temperature; lipases are among the most used biocatalysts in organic synthesis (Dalla-Vecchia *et al.,* 2005). They have been employed for direct esterification and transesterification reactions in organic media to produce esters having potential applications in fine chemicals, pharmaceuticals and agrochemicals industries (Kumar and Kanwar 2011).

6.2 Uses of lipases in food and pharmaceutical industry

The applications of enzymes in the food industry are many and diverse ranging from texturing to flavoring. Several traditional chemical markets are increasing with products

derived from bioprocesses or hybrid chemical/ bio-catalytic processes. Most of them are utilized for flavor development in dairy products and processing of other foods, such as meat, vegetables, fruit, baked foods, milk product and beer (Caugila *et al.,* 2008). The esterification by the lipases appears to be an attractive alternative to the bulk chemical routes. Lipases are being developed to carry out the transformations without the extreme temperature and pressure conditions which are essential for the traditional industrial processes (Sharma *et al.,* 2010; Kumar and Kanwar 2011). The chemical routes require more energy than the enzymatic route and have a low selectivity. Current applications of lipase biocatalysis include flavor enhancement of cheese, acceleration of cheese ripening, manufacture of cheese-like products, lipolysis of butter fat, and cream. While the addition of lipases primarily releases short-chain (C_4 and C_6) fatty acids that lead to the development of sharp, tangy flavor, the release of medium-chain (C_{12} and C_{14}) fatty acids tends to impart a soapy taste to the product. In addition, the free fatty acids take part in simple chemical reactions where they initiate the synthesis of other flavor ingredients such as aceto-acetate, *ß*-keto acids, methyl ketones, flavor esters and lactones (Falch, 1991; Vulfson, 1994). More recently, a whole range of microbial lipase preparations have been developed for the food, pharmaceutical as well as biodiesel production, such as those of *Mucor miehei*, *Aspergillus niger*, *A. oryzae and Candida antartica* origin (Yang *et al.,* 2009; Ji *et al.,* 2010; Yoo *et al.,* 2011) A range of cheese of good quality were produced by using individual microbial lipases or mixtures of several preparations. Enzyme-modified cheese is a cheese that has been incubated in the presence of enzymes at elevated temperatures in order to produce a concentrated flavor for use as an ingredient in other products such as dips, sauces, soups, and snacks. Lipases are used to break down milk fats and give characteristic flavors to cheeses (Verma and Kanwar 2010). Although microbial lipases are available for cheese-making, they are less specific in what fats they hydrolyze, while the animal enzymes are more partial to short and medium-length fats.

Hydrolysis of the shorter fats is preferred because it results in the desirable taste of many cheeses. Hydrolysis of the longer chain fatty acids can result in either soapiness or no flavor at all. The production of isopropyl myristate, isopropyl palmitate, and 2-ethylhexyl palmitate for use as emollient in personal care products like skin and sun-tan creams, and bath oils (Young and Bratzler, 1990). Wax esters have similar application in personal care products and are being manufactured enzymatically, using *C. cylindracea* lipase, in a batch bioreactor (Hoq *et al.,* 1985). Lipases have applications as industrial catalysts for the

resolution of racemic alcohols in the preparation of some prostaglandins, steroids, and carbocyclic nucleoside analogues. Lipases from *A. carneus* and *A. terreus* show chemo- and regio-specificity in the hydrolysis of peracetates of pharmaceutically important polyphenolic compounds (Parmar *et al.,* 1998). Lipases are also useful in the synthesis of the artificial sweetener sucralose by regio-selective hydrolysis of octa-acetylsucrose (Bornemann *et al.,* 1989; Sainz-Daiz *et al.,* 1997). The esters produced from short-chain fatty acids are used as flavoring agents in the food industry. Lipase immobilized on silica and micro-emulsion based organogels were widely applied for ester synthesis (Ghosh *et al.,* 1996; Sharma *et al.,* 2001). An accurate control of lipase concentration, pH, temperature and emulsion content are required to maximize the production of flavor and fragrance compounds.

6.3 Lipases in flavor ester synthesis

Some unique properties of lipase such as their specificity, temperature, pH dependency, activity in organic solvents and nontoxic nature leads to their major contribution in the food processing industries. Ethyl, isobutyl, amyl and isoamyl acetates are widely used flavor esters (Verma and Kanwar 2008). Hexyl acetate, a short chain ester with fruity odor is a significant green non-flavor compound widely used in food industry (Shieh and Chang, 2001). Hexyl butyrate synthesized by the immobilized lipase (Lipozyme IM-77) from *Rhizomucor miehei* was employed as flavor and fragrance in the food, beverage and pharmaceutical industry. It finds enormous interest as the natural flavoring compound rather artificial or synthetic (Chang *et al.,* 2003). Low molecular weight esters have a potential interest for the food industry as the flavor compounds. Direct esterification of citronellol and geraniol with short-chain fatty acids were catalyzed by the free lipase from *M. miehei* and high yields were obtained in n-hexane. (Laboret and Perraud 1999). High activity of lipase was recovered and the ester synthesis was maximized for the substrates containing excess acyl donor (Bruno *et al.,* 2004). The esters, which could be obtained from lipase mediated biotransformation processes, better serve the food industry's needs. Lipases have become an integral part of the modern food industry and are used in the preparation of a variety of products including fruit juices, baked food, vegetable fermentation and dairy enrichment.

The use of phytochemical-type molecules has emerged as a promising and pragmatic clinical approach: besides reducing cancer risk, these molecules constitute a wide family of natural compounds with a considerable range of important properties such as low toxicity, low cost, and effectiveness when orally administrated. Among phytochemicals, phenolic compounds such as phenolic acids, stilbenes, curcuminoids, vanilloids, chalcones, and flavonoids, are

one of the most numerous and ubiquitous groups of plant metabolites that are an integral part of the human diet (Haki and Rakshit, 2003). Phenolic acids and derivatives are a particular phenolic class known to display a wide variety of biological functions. Besides their high antioxidant capacity, phenolic acids have shown remarkable modulating properties in the carcinogenic process. Environment-friendly biocatalysts are now rapidly substituting the conventional harsh chemical methods for the synthesis of important fatty acid esters used in many chemicals, medicines, cosmetics and foods (Sharma *et al.,* 2001; Hasan *et al.,* 2006; Kanwar and Verma 2010). Esters are a major group of aroma compounds (Verma and Kanwar 2008; Liaw *et al.,* 2010). Various reaction conditions, such as biocatalyst concentration, substrates concentration, choices of solvents (*n*-alkanes), incubation time, temperature, molecular sieves (3Å × 1.5 mm), and water activity are need to be optimized for each of the lipase catalyzed organic synthesis.

6.4 Role of esters in biological systems

The property to synthesize the esters from the fatty acids and glycerol promotes its use in various ester syntheses. The esters synthesized by lipase finds applications in numerous other fields such as biodiesel production, resolution of the racemic drugs, fat and lipid modification. Lipase modifications such as the surfactant coating, molecular imprinting to suit for the non-aqueous ester synthesis have also been reported. Over the last decade, enzyme immobilization has become more important in industry, medicine, and biotechnology (Kumar and Kanwar 2011). Environment-friendly biocatalysts are now rapidly substituting the conventional harsh chemical methods for the synthesis of important fatty acid esters used in many chemicals, medicines, cosmetics, and foods.

7. Future perspectives of lipase in industry

Compared to enormous microbial lipases in general and yeast lipases in particular, their actual use in biotechnology industry at present is almost negligible. Hydrolysis of fats and oils is still being carried out by the conventional emulsion system and not by the use of lipases. The technology has not advanced to provide substitutes of the conventional chemical process. Introduction of new generation thermostable yeast lipases can tilt economic balance in favor of lipases. Novel lipases can also be used for the synthesis of whole range of amphoteric biodegradable surfactants namely amino acid based ester amides. The prospects of industrial application of lipases are bright. However it calls for discovering novel lipases by extensive screening and transformation of the known ones by genetic manipulations (Liu *et al.,* 2012). Factors such as immobilization and the nature of organic solvents used also

affect the catalytic efficiency of lipase. Thorough understanding of the factors can help in the development of tailor made lipases for specific application and in long run open up new vistas. Dozen of lipases are now commercially available nevertheless those employed in large scale industrial process and products are still limited to few cases (Sharma *et al.,* 2010; Patil *et al.,* 2011). This is mainly due to high price/ low availability or non-optimal operational features of naturally available enzymes. In future the use of lipases as industrial catalysts strongly rely on the production of recombinant enzymes with biochemical and catalytic features improved by protein engineering methods may be further explored and exploited. The expectation is that lipases will be as important industrially in the future as the proteases and carbohydrolases are currently.

8. CONCLUSION

Lipases being a versatile enzyme are exploited in various industries especially in the food processing industries. Lipase-mediated ester synthesis is an alternative technique to bulk chemical routes. Factors such as the nature of the solvent, *aw* and temperature greatly influence the enzyme catalyzed synthesis. The enzyme-mediated production of the esters was found to be more cost effective than the chemical synthesis. Although lipase catalyzed transesterification process has commercial significance, the utilization of the enzyme is not extensive because of its high cost. Increased understanding of the primary and 3D structure of lipase enzyme will pave the way for the variety of different applications. The future developments in low cost production and purification technologies would lower the cost of these enzymes for the increased commercial applications. The immobilized lipase offers high chemical, mechanical and thermal stability beside improved enantioselectivity and stereo-selectivity. Various novel reactor designs with this immobilized enzyme would offer effective and efficient bioconversion using lipase. The development of lipases with novel properties by the directed evolution and molecular technologies, strain improvement by the site directed mutagenesis and medium optimization for lipase overproduction holds a major area of research in the future. Enzymatic synthesis provides an attractive and environmentally more begin alternative to the conventional chemical approaches used for the production of high quality alkanolamides. Current commercial use of enzymes, together with new applications, will continue to play an important role in maintaining and enhancing the quality of life we enjoy today while protecting the environment for generations to come.

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