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Lipase applications in food industry

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Lipases are the most pliable biocatalyst and bring about a wide range of bioconversion reactions, such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis. Lipases can act on a variety of substrates including natural oils, synthetic triglycerides and esters of fatty acids. They are resistant to solvents and are exploited in a broad spectrum of biotechnological applications. Lipase catalyzed transesterification, hydrolysis and esterification are the important class of reactions for food technology applications in fats and oil industry, dairy industry, pharmaceuticals and bakery industry. Lipases are very peculiar as they hydrolyse fats into fatty acids and glycerol at the water-lipid interface and can reverse the reaction in non-aqueous media. Novel biotechnological applications, like biopolymer synthesis, biodiesel production, treatment of fat-containing waste effluents, enantiopure synthesis of pharmaceuticals and nutraceutical agents, have been established successfully. The present article extends the frontier of lipase technology towards food processing applications and discusses the important characteristics of lipases and its sources. Various methods of lipase immobilization for food technology applications, various assay methods for lipase, production of lipase by submerged and solid state fermentation strategies, and various purification methods available have been discussed in detail.

Keywords: ester, fatty acids, food applications, lipases, lipase sources, triglycerides

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Introduction

Much of the early interest in enzymology was developed by scientists like Pasteur, Payen and Persoz, who were associated with food, wine and beer industries[1]. The presence of lipase in bacteria had been observed as early as 1901 AD for *Bacillus prodigiosus* (now *Serratia marcescens*), *B. pyocyaneus* (now *Pseudomonas aeruginosa*), *B. fluorescens* (now *P. fluorescens*) and *Staphylococcus pyogenesaucreus* (now *S. aureus*) by the microbiologist Eijkmann[2]. Lipases (triacylglycerol acylhydrolases EC: 3.1.1.3) are ubiquitous enzymes, which are found in animals, plants, fungi and bacteria, and are of considerable physiological significance and industrial potential. Lipases are serine hydrolases[3] and contain the consensus sequence G – X_1 – S – X_2 – G as the catalytic moiety, where G – glycine, S – serine, X_1 – histidine and X_2 – glutamic or aspartic acid[4]. The biological function of lipase is to catalyze the hydrolysis of triacylglycerols to give free fatty acids, diacylglycerols, monoacylglycerols and glycerol. Lipases posses the unique feature of acting at the interface between an aqueous and a non-aqueous phase. They synthesize esters from glycerol and long chain fatty acids when the water activity is low. A "true lipase" will split emulsified esters of glycerine and long chain fatty acids, such as triolein and tripalmitin. The growth of application of commercial enzymes is very significant and promising, particularly in food industry during the past 35 years[5].

The panorama of lipase utilization encompasses many other industries. It has wide range of applications in oleochemical, detergent, organic industries, leather industry, environmental management, cosmetics and perfume industry, biomedical applications and biosensors[3,6]. At present, lipases are used for the generation of enantiomerically enriched primary and secondary alcohols and to a lesser extent for chiral carboxylic acids and secondary amines[7].

Esters of short and medium chain carboxylic acids and alcohol moieties synthesized by lipase play a relevant role in the food industry as flavour and aroma constituents. Esterification by lipases appears to be an attractive alternative to bulk chemical routes. In fact, ester synthesis using lipase can be performed at room

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temperature, pressure and at neutral *p*H in reaction vessels operated either batch wise or continuously. Products obtained by these methods are more pure compared to the products obtained by alternative chemical means, because chemical catalysis may sometimes leads to non-specific and unwanted by-products and also requires high temperature, pressure and frequent regeneration of catalyst. Use of lipases alleviates complex downstream processes and thus, leads to reduction in overall operation costs. However, the major drawback with this system is the low conversion when compared to complex chemical processes.

The industrial enzyme market for non-therapeutic use, such as food, detergents, textiles, leather, pulp and paper industry, reached US $ 1 billion in 2000[8]. Lipases are produced by several microorganisms, namely bacteria, fungi, archea, eucarya as well as by animals and plants. Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases[3]. Microbial lipases are of special interest because of their stability in organic solvents and their lack of requirement for cofactors, their broad substrate specification and their high enantioselectivity[8]. In 1994, Nova Nordisk introduced the first recombinant commercial lipase, Lipolase®, which originated from the fungus *Thermomyces lanuginosus* (formerly *Humicola lanuginosa*) and was expressed in *Aspergillus oryzae*[9]. In 1995, Genencor International produced two bacterial lipases, Lumafast® from *P. mendocina* and Lipomax® from *P. alcaligenes*. Nova Nordisk markets a range of enzymes for various industrial purposes among which most of the enzymes are utilized mainly in food processing industries. Table 1 comprises some of the commercial lipases produced by Nova Nordisk and their applications in food processing industries[10].

Lipases are indispensable for the bioconversion of lipids (triacylglycerols) from one organism to another and within the organisms, and they possess the unique feature of acting at an interface between the aqueous and non-aqueous (*i.e.* organic) phase; this feature distinguishes them from esterases[6]. This property of lipases implies that the kinetics cannot be described by the Michaelis-Menten equation, which is valid only if the catalytic reactions take place in a homogenous phase. Some important reactions catalyzed by lipases are listed below[11].

The applications of enzymes in the food industry are many and diverse, ranging from texturing to flavouring. Several traditional chemical markets are increasing with products derived from bioprocesses or hybrid chemical/biocatalytic processes. In this review the various microbial sources, substrates and properties of lipase are discussed, with emphasis on recent developments of lipases in food processing industries.

Table 1—Commercial lipases produced by Nova Nordisk[10]

Brand name	Mechanism	Application
Lipopan ®	Hydrolysis and oxygen uptake	Baking industry
Lipozyme ®	Interesterification	Oils and fats industry
Novazym ® 27007	Hydrolysis	Pasta/noodles
Palatase	Hydrolysis	Dairy industry
Novozyme ® 871	Emulsification	Pet food industry

Microbial Sources for Lipase

Lipases occur widely in nature, but microbial lipases are commercially significant because of low production cost, greater stability and wider availability than plant and animal lipases. They may originate from fungi, molds or bacteria and most of them are formed extracellularly. This ready availability has created an enormous spin-off with respect to the enantioselective hydrolysis and formation of carboxyl esters[7].

The enormous biotechnological potential of microbial lipases are related to their exquisite chemoselectivity, regioselectivity and stereoselectivity[12,13]. They are readily available in large quantities because many of them can be produced in high yields from microorganisms[6]. The crystal structures of many lipases have been solved, considerably facilitating the design of rational engineering strategies. Finally, lipases do not usually require cofactors nor do they catalyze side reactions.

Most of the industrial microbial lipase is derived from fungi and bacteria[14]. Fungi are preferable because fungal enzymes are usually extracellular, facilitating extraction from fermentation media[15]. In modern processes fungal genes are expressed in bacterial systems. Some lipase producing microorganisms and their applications in various food processes were listed in Table 2[16-35]. Though several microbial lipases are produced commercially, the high cost of lipase seems to be a major factor in its successful application. This may be overcome by immobilization for reuse of the enzyme[13].

Table 2—Microbial sources and their applications in food processing

Microorganisms	Food applications	References
Aspergillus oryzae	Synthesis of saturated fatty acids	16
Aspergillus sp.	Flavour synthesis	17
Bacillus subtilis	Bread making	18
Candida rugosa	Flavour synthesis	19
C. antarctica, C. cylindracea AY30, Humicola lanuginosa, Pseudomonas sp. *& Geotrichum candidum*	Synthesis of lipophilic antioxidants	20
C. parapsilosis	Hydroxamic acids (food additive)	22
C. rugosa	Cheese production	23,24
Chromobacterium viscosum	Synthesis of aroma and flavour compounds	25
Geotrichum sp.	Bread making	26
Mucor miehei	Geraniol ester synthesis	27
M. miehei & C. antarctica	Synthesis of short chain flavour thio ester in solvent free medium	28
M. miehei & Rhizopus arrhizus	Production of flavour esters	29
Penicillium camembertii	Production of glycerol-glycolipids	30
P. chrysogenum	Food industry waste treatment	31
P. roquefortii, A. niger & R. arrhizus	Fragrance development in dairy production	32
Pseudomonas sp.	Production of PUFA	33
Pseudomonas sp.	Food processing and oil manufacture	34
Rhizomucor meihei	Surfactants for baking industry, Dairy products, Noodles	35
Staphylococcus warneri & S. xylosus	Production of flavour esters	33

Production of Lipases

Microbial lipases are produced mostly by submerged culture but solid state fermentation can also be used. Generally the lipase production is organism specific and it is released during the late logarithmic or stationary phase[14,37]. The cultivation period also varies with the microorganism and fast growing bacteria were found to secrete lipase within 24 h. The production of lipase is mostly inducer dependent, and in many cases oils act as good inducers of the enzyme[14]. Certain other inducers also have a profound effect on the stimulation of lipase production. They include triglycerides, free fatty acids, hydrolysable esters, bile salts and glycerol[37]. The organisms are normally grown in a complex nutrient medium containing carbon (oil, sugars, mixed carbon sources), nitrogen and phosphorous sources and mineral salts. Compounds such as olive oil, oleic acid and span 80 seem to play an essential role in lipase synthesis. Lipases production by *B. sphaericus* has been investigated and maximum lipase activity, *i.e*. about 5.2 μmoles/mL.min, was obtained by using an optimized medium with sesame oil as an inducer[38]. The yeast *Candida rugosa,* which is an industrial producer of lipase, has been shown to secrete extracellular lipase upon induction by fatty acids. Lipase production may also be induced by adding oleic acid as the carbon source. This lipase is composed of several isoforms with slightly differing catalytic properties. In the same yeast, the production of a constitutive lipase was induced by using glucose as carbon source[14]. Reychang *et al*[36] revealed that the presence of tween 80 and tween 20 in the culture medium not only promoted lipase production but also changed the production of multiple forms in cultured *C. rugosa*. Various non-conventional carbon sources, like beef tallow, wool scour effluent, whey and *n*-hexadecane, are also used to produce lipase. Other significant factors influencing lipase production include nitrogen sources, initial broth *p*H, growth temperature and dissolved oxygen concentration[14].

Lipase production by a thermophilic *Bacillus* species was increased several fold when magnesium, iron, and calcium ions were added to the production medium. Lipase production by *Bacillus* species A 30-1 (ATCC 1841) required a complex medium[39] that contained Ca^{2+}, Mg^{2+}, Na^{+}, Co^{2+}, Fe^{2+}, K^{+}, Mn^{2+}, Mo^{2+} and Zn^{2+}. Tween 80 and tween 20 increased lipase productivity by 2.9 and 5.7 fold, respectively with *p*-nitrophenyl butyrate as substrate. Lipase produced in the presence of tween 20 had much greater thermal stability than that of lipase produced either in the absence or in the presence of tween 80[36]. Generally bacterial lipases are glycoproteins but some extracellular bacterial lipases are lipoproteins. Lipases from *S. aureus* and *S. hyicus* are stimulated by divalent ions, such as Ca^{2+}, and the chelator EDTA acts as an inhibitor. The *p*H optima of these enzymes vary between 7.5 and 9.0.

Fungal lipases have benefits over bacterial lipases for the fact that present day technology favours the use of batch fermentation and low cost extractive methods. Lipase producers are widespread in the fungal kingdom and are of much biotechnological interest in both research and applications. The main fungal producers of commercial lipases are *A. niger,*

A. terreus, A. carneus, C. cylindracea, H. lanuginosa (*T. lanuginosus*)*, Mucor miehei, Rhizophus arrhizus, R. delemar, R. japonicus, R. niveus* and *R. oryzae.* Media supplemented with glucose stimulated maximum lipase production in case of all these fungi. Polysaccharides, such as glycogen, hyaluronate, laminarin, gum arabic and pectin, stimulated the production of lipase in *Serratia marcescens* and *Saccharomycopsis lipolytica*. This might be due to the detachment of lipase from the oil surface. A similar effect was found with lecithin on *R. japonicus*[37].

Aeration has a variable effect on lipase production by different organisms. The degree of aeration appears to be critical in some cases since shallow layer cultures (moderate aeration) produced much more lipase than shake cultures (high aeration). Lipase production using *P. fragi, P. aeruginosa* and *R. oligosporus* was reduced by vigorous aeration. Increased growth and increased lipase production, followed by a rapid decrease of lipase activity was observed in *P. fragi* with continuous shaking[37].

Bacterial lipases from various *Bacillus* species were over expressed in *E. coli* using conventional overexpression systems; however, many enzymes (e.g. from *Pseudomonas* sp. and *Burkholderia* sp.) were not amenable to these systems[2,40]. A study by Tang *et al*[41] revealed that the fusion with *Trx*, transformation into AD A9A (DE3) and recombinant expression at low temperature are reliable and simple techniques for the production of recombinant lipase. Moreover, this strategy avoids contamination of the active enzyme with inactivated protein from the inclusion bodies.

Rivera *et al*[42] studied the lipase production by several filamentous fungi in solid state fermentation (SSF). *Penicillium candidum, M. miehei* and *P. camembertii* were selected to be the high yielding lipase producers with low protease levels and high dairy flavour generation. The kinetics of lipase production by *P. candidium* by SSF was compared with submerged fermentation. Lipase production started earlier in SSF, generated a two fold higher maximum concentration and was quite stable on the 5^{th} d of incubation. On the other hand, lipase production by submerged fermentation reached its maximum after 2 d of fermentation and the maximum activity decreased rapidly after the 5^{th} d of incubation. These results make SSF an interesting alternative for microbial production of lipase. The solid state fermentation offers lower production cost of lipases when compared to the submerged fermentation as the medium employed can be obtained at low cost and the down stream processing is also easier.

Purification of Lipases

Novel purification technologies are available to obtain homogeneity of lipase from a large number of bacteria and fungi, and from a few plant and animal sources. The purification of lipases normally involves several steps depending upon the purity desired for food application.

Since most of the microbial lipases are extracellular, the fermentation process is usually followed by the removal of cells from the culture broth, either by centrifugation or by filtration. The cell-free culture broth is then concentrated by ultrafiltration, ammonium sulphate precipitation or extraction with organic solvents. About 80% of the purification schemes attempted have used a precipitation step, 60% of which use ammonimum sulphate and 35% use ethanol, acetone or an acid, followed by a combination of several chromatographic methods, such as gel filtration and affinity chromatography. The final step of gel filtration normally yields a homogenous product. Purification methods for lipases from various sources are listed in Table 3[17,31,43-52].

After these pre-purification steps, novel purification technologies like (i) membrane separation processes, (ii) immunopurification, (iii) hydrophobic interaction chromatography using epoxy-activated spacer arm as a ligand and polyethylene glycol immobilized on sepharose, (iv) polyvinyl alcohol polymers as column chromatography stationary phases, and (v) aqueous two phase systems are commonly employed[53]. The use of hydrophobic interaction chromatography was generally found to result in satisfactory enzyme recovery and fold purification.

An acid resistant lipase from *A. niger* has been purified from crude commercial preparations by size exclusion on Bio-gel-p-100 and ion exchange on Mono-Q. The lipase produced by *R. delemar* was purified with a recovery of 3-4%. The lipase from *R. japonicus* NR400 was purified to homogeneity by chromatography on hydroxyapatite, octylspharose and sephacryl S-200[37].

Methods for Lipase Assay

A number of assay protocols are employed for lipase assay due to the wide substrate specificity of

Table 3—Purification methods for lipase

Microorganism	Purification method	Reference
***Pseudomonas* spp.**		
P. fluorescens HU380	Phenyl-toyopearl fractionation (batch wise), DEAE – sepharose column chromatography, Superdex – 200 HR FPLC	43
Pseudomonas sp.	Biogel P-10 chromatography, Superose 12B chromatography	44
P. fluorescens strain 2D	Ammonium sulphate precipitation, Hydrophobic interaction chromatography	45
P. aeruginosa	Ammonium sulfate precipitation, Hydroxyapatite column chromatography	46
P. aeruginosa EF2	Ultrafiltration, Anion exchange chromatography (MonoQ), Gel filtration (superose) FPLC	47
***Bacillus* spp.**		
Bacillus sp.	Ammonium sulfate, Acrinol treatment, DEAE-sephadex A-50, Toyopearl HW-55F, Butyl toyoperal 650M	47
Bacillus sp.	Acetone fractionation, Two acetone precipitations, Octyl-sepharose CL-4B, Q-Sepharose, Sepharose-12	48
B. thermoatenulatus	Calcium soap, Hexane extraction, Methanol precipitation, Q-sepharose (ion exchange)	17
***Rhizopus* spp.**		
R. japonicus NR400	Hydroxy apatite, Octyl-sepharose and Sephacryl S-200	49
R. arrhizus	Ammonium sulfate fractionation and Sephadex G-100 gel filtration	50
R. oryzae	Acetone precipitation (80%), Sephadex G-100	51
***Penicillium* spp.**		
P. chrysogenum	Ultrafiltration, Phenyl-sepharose, Mono Q HR5/5 and PD-10 column on sephadex G-25	31
P. citrinum	Extraction and back-extraction using AOT reversed micelles in isooctane and phenyl – superose column	52

lipases. Determination of lipase activity at the lipid-water interface is also indicative of free lipase. As with all reactions catalyzed by enzymes, activity measurements can be carried out using various physico-chemical methods by monitoring the disappearance of the substrate or by the release of the product. Numerous methods are available for measuring the hydrolytic activity as well as the detection of lipase. The methods may be classified as[9]: (i) Titrimetry, (ii) Spectroscopy (Photometry, Fluorimetry and Infrared), (iii) Chromatography, (iv) Radio activity, (v) Interfacial tensiometry, (vi) Turbidimetry, (vii) Conductimetry, (viii) Immunochemistry, and (ix) Microscopy. The general triacylglycerol hydrolysis reaction catalyzed by lipases can be written as:

Triacylglycerols	→ Diacylglycerols	→ Monoacylglycerols	→ Glycerol
	+ Free fatty acids	+ Free fatty acids	+ Free fatty acids

This reaction shows that the activity of lipases can thus be assayed by monitoring the release of either free fatty acids or glycerol from triacylglycerols or fatty acid ester. The extensive reviews by Beisson *et al*[12] and Gupta *et al*[9] describe in detail the various methods available for lipase assay. Today the most widely used lipase assay protocol is the titrimetery assay using olive oil as a substrate because of its simplicity, accuracy and reproducibility. A few spectrophotometric assays are based on methods which render colour to fatty acids released after hydrolysis of triacylglycerols. A unit lipase activity[13] relates to the release of 1 μmole of free fatty acid from emulsified olive oil or triolein or tributyrin per min at specified temperature and *p*H values. Specific activity of lipases is expressed as units of lipolytic activity per microgram of extra cellular protein.

Properties and Characteristics of Lipases

Lipases are reported to be monomeric proteins, having molecular weight in the range of 19-60 kDa. The physical properties of the lipases mainly depend on factors such as the position of the fatty acid in the glycerol backbone, the chain length of the fatty acid, and its degree of unsaturation. These features also affect the nutritional and sensory value of a given triglyceride[40]. Many lipases are active in organic solvents where they catalyze a number of useful reactions including esterification[14].

Lipase activity generally depends on the availability of large surface area and requires extreme mild conditions. Several structural studies on lipases have provided clues to the understanding of hydrolytic activity, interfacial activation and stereoselectivity[6]. A survey by Linko and Wu[19] on

major commercial lipases revealed that *Aspergillus* lipases were highly selective for short-chain acids and alcohols; *C. rugosa* lipases for propionic acid, butyric acid, butanol, pentanol and hexanol; and *M. miehei* and *R. arrhizus* lipases for long-chain acids and acetates[21]. This information on enzyme catalyses has been valuable for the production of flavour esters[54].

Increase in lipase activity depends on the concentration of ammonium sulphate solution used during the purification process[53]. The kinetics of the lipolysis reactions have been discussed by Brokerhoff and Jensen[55].

The activity of lipase is *p*H dependent. Some lipases are stable over a wide range of *p*H values. Lipases are generally stable at or near a neutral *p*H; some are stable up to *p*H 4.0 and 8.0[37]. Extracellular lipases produced by *A. niger, Chromobacterium viscosum* and *Rhizophus* species are particularly active at acidic *p*H. An alkaline lipase active at *p*H 11.0 has been isolated from *P. nitroaeducens*[37].

Lipases are capable of reversing the reaction that leads to esterification and interesterification under certain experimental conditions, such as in the absence of water[37]. Cofactors are not essential for the expression of lipase activity but divalent cations, such as calcium, stimulate the activity. The lipase activity is inhibited drastically by Co^{2+}, Ni^{2+}, Hg^{2+} and Sn^{2+} and is slightly inhibited by Zn^{2+}, Mg^{2+}, EDTA and SDS[37, 56]. Spontaneous and cyclic AMP induced lipase formation is greatly enhanced in *S. marcescens* SM-6 exposure to glycogen, hyaluronate, pectin B and gum arabic[37].

The temperature stability profiles of lipases, determined by half-life values, show maximum stability at lower temperatures. The maximum lipase activity of *Epipactis gigantea* and many other lipases were found in the range of 30-35°C. Thermophilic bacterial lipases obtained from organisms from Icelandic hot springs had higher lipase activity at 40-60°C[37,57].

Lipases may be divided into two groups according to the region-specificity exhibited with acyl glycerol substrate. Lipases from the first group show no regiospecificity and release fatty acids from all three positions of glycerols. The second group lipases release fatty acids regio-specifically from the outer 1 and 3 positions of acylglycerols. These lipases hydrolyse triacylglycerol to give free fatty acids 1,2-(2,3)-diacylglycerols and 2-monoacylglycerol. Partial stereo-specificity in the hydrolysis of triacylglycerols has been observed in *A. arrhizus, R. delemar, C. cylindracea* and *P. aeruginosa*. Owing to this property these enzymes may be used to isolate optically pure esters and alcohols[37,58].

The use of organic media at low water activity offers a unique possibility to tune stereoselectivity through variation of the solvent. Since enzymes possess delicate and soft structures, any solvent may exert a significant influence on the catalytic properties of an enzyme. Thus, an enzyme's specificity may be altered by varying the properties of the solvents. The stereoselectivity of an enzyme catalyzed reaction is also true for lipases. It can be controlled by choosing the appropriate organic solvent[7]. Rooney and Weatherely[39] studied the lipase catalyzed hydrolysis of high oleate sunflower oil in a stirred liquid-liquid reactor and observed maximum enzyme activity at the oil-water interphase, in turn maximum overall rate of reaction.

Substrates for Lipase

Glycerides are the natural substrate for lipases; they possess a chiral alcohol moiety. It was understood that lipases were particularly useful for the resolution or asymmetrization of esters bearing a chiral alcohol moiety.

General guidelines for the design of substrates for lipase were formulated by the IUPAC[7]:

i. The center of chirality should be located as close as possible to the site of the reaction (i.e. the ester carboxyl group) to ensure optimal chiral recognition. Thus, esters of secondary alcohols are usually more selectively transformed than those of primary alcohols.
ii. There is wide tolerance for the nature of both substituants R_1 and R_2 but they should differ in size and/or polarity to aid the chiral recognition process. They may also be linked together to form cyclic structures. Polar groups, such as carboxylate, amide or amine—which would be heavily hydrated in an aqueous environment—are not tolerated and, if they are required, they should be protected with a lipophilic unit.
iii. The alkyl chain of the acid moiety (R_3) should be preferably of straight chain nature, possessing at least three to four carbon atoms. Reaction rates may be improved by using 'activated' esters bearing haloalkyl groups, e.g. $Cl – CH_2 –$ and $Cl – (CH_2)_2 –$ for Type I and II, respectively (Fig. 1).

iv. The remaining hydrogen atom in both substrate types must not be replaced by a substituent, since esters of tertiary alcohols and tri substituted carboxylates are usually not accepted by lipases.
v. The stereo-chemical preference of the most commonly used lipases (e.g. from *Pseudomonas* sp. and *Candida* sp.) for esters of secondary alcohols follows an empirical model generally referred to as "Kazlauskas rule". These guidelines may be followed to get fair accuracy to obtain the desired product.

The concept of lipase interfacial activation arises from the fact that their catalytic activity generally depends on the aggregation state of the substrates[6]. The occurrence of lipase reaction at an interface between the substrate and the aqueous phase is because of the reversible nature of the enzyme reaction. The enzymes acts on the substrate in a specific or nonspecific manner, resulting in either the complete hydrolysis of triacylglycerides into free fatty acids and glycerol or, along with triglycerides, monoacylglycerides, diacylglycerides, fatty acids and glycerol are also formed. Strong interactions with hydrophobic substrates at an interface are probably caused by hydrophobic patches on the other lipase surface. Such patches may also be responsible for self association behaviour shown by the enzymes in aqueous solutions[37]. Substrate specificity of lipases is often crucial to their application for analytical and industrial purposes. Specificity is shown both with respect to either fatty acyl or alcohol parts of their substrates[58]. Fig. 2 shows the diagrammatic representation of a lipase molecule with its substrate (triglyceride).

In general, lipases show little fatty acid specificity when incubated with most natural oils and fats, and high specificity when fish oil and milk fats are used as substrates. The presence of double bonds close to the carboxyl groups in some fatty acids probably makes their esters resistant to attack by lipases. In contrast, *M. miehei* lipase preferentially releases butyric acid from milk fat, especially at low *p*H. Many microbes produce two or more extracellular lipases with differing fatty acid specificities, especially with respect to short-chain fatty acids. *Geotrichum candidum* produces a lipase, which shows pronounced specificity for the hydrolysis of esters of a particular type of long-chain fatty acid. Lipases show both regiospecificity and stereospecificity with respect to alcohol moiety of their substrates[37,58]. The selective

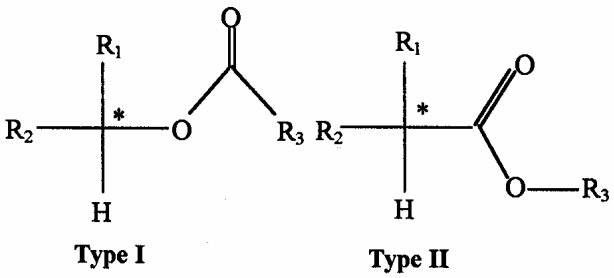

Fig. 1—Types of substrate for lipase

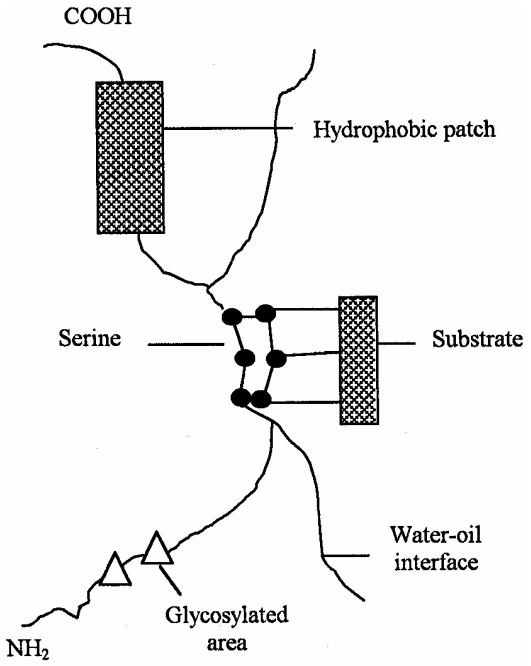

Fig. 2—Diagrammatic representation of a lipase molecule showing its main features; the substrate can be any triglyceride[37]

enhancement of lipase catalyzed reactions by designing a suitable medium is still a largely empirical, despite the tremendous amount of data published to date[7].

Molecular Modification

Modern genetic engineering approaches with protein engineering may help in maximizing the enzyme production for specific industrial need. The most suitable lipase for specific applications can be obtained based on their substrate binding sites. Prior knowledge on structure function relationship is required. The review of Schmidt-Dannert[59] on recombinant microbes for lipase overexpression provides a set of information to produce lipase for special applications. The most appropriate enzyme for particular reaction can be tailored via mutagenesis.

Protein engineering of lipases was studied based on the sequence information since the mid of 1980s. *P. mendocina* lipase was the first enzyme to be engineered[60]. Thermostability of lipases can be engineered by altering the amino acid sequence when designing for food application. A patent was registered on *H. lanuginosa* lipase with increased temperature stability. Many lipases have been engineered for thermostability, protease stability and for oxidative stability[60]. *A. niger* was developed as an important transformation host to overexpress food enzymes since it has been considered GRAS (Generally Recognized As Safe) by the US Food and Drug Administration[59]. Among the isoforms lip 1 of *C. rugosa* was found to be most prominent. The lip 1 gene was systematically modified by site-directed mutagenesis to gain functional expression in *Saccharomyces cerevisiae*[61]. Downstream from lip A gene of *P. cepacia*, an open reading frame lim A was identified. Only in the presence of this lim A, lip A gene gets expressed in *E. coli, B. subtilis* and *Streptomyces lividans*[62]. Baker's yeast strain transformed with plasmids containing LIP 1 and LIP 2 genes from *Geotrichum* sp. secreted high levels of lipase 1, but secretion of lipase 2 was comparatively higher. Addition of this lipase 2 enriched the bread dough formulations[26].

For the first time Sanchez *et al*[18] expressed a bacterial lipase gene (from *B. subtilis*) in baker's yeast, *S. cerevisiae*. Lipase B that exhibited unique substrate specificity for long-chain *cis*-9 unsaturated triacylglycerols were overexpressed in *Pichia pastoris*[63]. Fickers *et al*[64] developed a non-genetically modified mutant strain *Yarrowia lipolytica* CBS6303 with high productivity by chemical mutagenesis. Utility of *A. oryzae* as a host for the production of recombinant lipases was explained by Huge-Jensen *et al*[65]. Schmidt-Dannert[59] described the approaches for the production of recombinant lipase in different expression systems. Accumulation of active recombinant *G. candidum* lipase in *P. pastoris* without any contaminating protein was reported by Holmguist *et al*[66].

The non universal codon of *C. rugosa* was concerted to express universal serine triplets by site directed mutagenesis to gain expression of functional lipase in heterologous hosts[67]. Protein engineering of purified *C. rugosa* lipase (CRL) isoforms allows the tailoring of enzyme function. This involves computer modeling based on available 3-D structures of lipase isoforms. Lid swapping and DNA shuffling techniques could be used to improve the enantioselectivity, thermostability and substrate specificity of CRL isoform and increase their application in food processes[67]. A completely synthesized *C. rugosa* lipase gene over expression in *P. pastoris* with high purity was reported by Brocca *et al*[61].

Food Technology Applications

The majority of enzymes used in industry are for food processing, mainly for the modification and breakdown of biomaterials[68]. A large number of fat-clearing enzymatic lipases are produced on an industrial scale. Most of the commercial lipases produced are utilized for flavour development in dairy products and processing of other foods, such as meat, vegetables, fruit, baked foods, milk product and beer[69].

Phospholipases have found industrial applications in egg yolk treatment for the production of mayonnaise and other emulsifiers, in lecithin modification, and for the oil-degumming step in the refining of vegetable oils. Introduction of a microbial phospholipase (Lecitase Nova) has significantly improved the economy of enzymatic degumming of vegetable oils. In this process, the phospholipids are hydrolyzed and rendered more water soluble, hence facilitating their washout[70]. The function of phospholipase in egg yolk treatment is to hydrolyze egg lecithin, iso-lecithin, which improves the emulsifying capacity and heat stability. The egg yolk thus produced can be useful in the processing of custard, mayonnaise, baby foods, dressings and in dough preparation. It can also be applied in the processing of sauces, like hallandise, bernaise and cafe de paris[71].

Lipases have been successfully used as a catalyst for the synthesis of esters. The esters produced from short-chain fatty acids are used as flavouring agents in the food industry. Lipase immobilized on silica and microemulsion based organels were widely applied for ester synthesis[14,37]. Accurate control of lipase concentration, *p*H, temperature and emulsion content are required to maximize the production of flavour and fragrance[6]. Table 4 describes some food processing applications of microbial lipases[14]. Uhling[72] has explained the preparation of lipase modified butter fat which found wide application in various food processes. Chocolates with coco butter substitutes, bread, structured lipids like human milk fat replacers, low calorie health oils, nutraceuticals,

Table 4—Lipase applications in the food industry[14]

Food industry	Action	Product of application
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat.	Development of flavouring agents in milk, cheese and butter
Bakery foods	Flavour improvement	Shelf-life propagation.
Beverages	Improved aroma	Alcoholic beverages, e.g. sake, wine
Food dressings	Quality improvement	Mayonnaise, dressings and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavour development	Meat and fish product, fat removal
Fats and oils	Transesterification, hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono and diglycerides

EMC, etc. are few examples for lipase mediated food products. Since it is a new technology, more research on usage of lipase to develop new commercial food products must be promoted. Oil from soybean is hydrolyzed by lipase in making Koji, a traditional Asian food[73]. Another soybean fermented food Tempeh utilized lipase from *R. oligosporous*[74]. This Tempeh forms a base material for many delicious, easily digestible and nutritious food preparations, providing a good number of human populations with a valuable and affordable source of protein. In addition to Tempeh, Kenkey and Mave (African food) coupled with fermented vegetables and *salads* should be noted in this context[6].

Dairy Industry

Lipases are extensively used in the dairy industry for the hydrolysis of milk fat. The dairy industry uses lipases to modify the fatty acid chain lengths, to enhance the flavours of various cheeses. Current applications also include the acceleration of cheese ripening and the lipolysis of butter, fat and cream[14, 37].

The free fatty acids generated by the action of lipases on milk fat endow many dairy products, particularly soft cheeses with their specific flavour characteristics. The traditional sources of lipases for cheese flavour enhancement are animal tissues, especially pancreatic glands (bovine and porcine) and pre-gastric tissues of young ruminants (kid, lamb and calf). A whole range of microbial lipase preparations have been developed for the cheese manufacturing industry from *M. miehei*, *A. niger, A. oryzae* and several others. In some cases microbial lipases have successfully replaced pre-gastric lipases. A range of

Table 5—Examples of lipase in cheese production[37]

Cheese type	Lipase source
Romano	Kid/lamb pre-gastric
Domiati	*Mucor miehei*
Feta	
Camembert	*Penicillium camemberti*
Mazarella	Calf/kid pre-gastric
Parmesan	
Provolone	
Fontina	*Mucor miehei*
Ras	
Romi	
Roque fort	*Penicillium roqueforti*
Cheddar	*Aspergillus oryzae/A. niger*
Manchego	
Blue	

cheeses of good quality was produced by using individual microbial lipases or mixtures of several preparations. Enzyme modified cheese (EMC) is produced when cheese is incubated in the presence of enzymes at elevated temperature in order to produce a concentrated flavour by lipase catalysis for use as an ingredient in other products, such as dips, sauces, soups and snacks. The concentration of fat is 10 times higher in EMC to that of normal cheese[5,14,37,45,69]. Table 5 describes the use of lipase in cheese making and accelerated cheese ripening[37]. The free fatty acids take part in simple chemical reactions that initiate the synthesis of other flavour ingredients, such as aceto-acetate, β-keto acids, methyl ketones, flavour esters and lactones[37].

Cocoa butter (CB) contains palmitic and stearic acids and has a melting point of approximately 37°C, leading to its melting in mouth which results in a cooling sensation. In 1976, Unilever filed a patent describing a mixed hydrolysis and synthesis reaction to produce a cocoa butter substitute using an immobilized lipase. This technology was commercialized by Quest-Lodrs Croklaan, based on immobilized *R. miehei* lipase, which carries out a transesterification reaction replacing plamitic acid with stearic acid to give the desired stearic–oleic–stearic triglyceride[14,40].

Unduranga *et al*[75] analyzed the production of a cocoa butter equivalent (CBE) through enzymic interesterification of palm oil midfraction (POMF) with stearic acid in a solvent-free system using Nova lipase Lipozyme as a catalyst. Studies were carried out both in batch and continuous packed bed reactors. POMF was found to be a suitable raw material for the production of CBE, whose composition is close to

that of CB. The overall process is, however, uneconomical due to the cost of the purification steps.

By using lipase catalyzed hydrolysis and alcoholysis of ester bonds in vitamin A and E esters, their level in different food formulae can be determined. The supercritical fluid extraction (SFE) method using immobilized *C. antarctica* preparation is more beneficial to the oxidation prone vitamins A and E. This extraction methodology should be applicable for the determination of vitamins D_2/D_3, K_{53} and β-carotene in milk powder and infant formulae[13,69].

Gastric lipases have been used to accelerate ripening and flavour development of many cheese types, including cheddar, provolone and ras cheeses. Lipase addition enhances the rate of fatty acid liberation, which accelerates flavour development relative to control. These studies indicated that liberated fatty acid profiles of the accelerated process were identical to the control and the total quantities of short-chain liberated fatty acids (C_4 to C_6) were important for the development of typical cheddar cheese flavour during ripening. The addition of calf lipase and increasing the ripening temperature (from 7° to 53°C) result in a significant increase in the liberation of fatty acids. The disadvantage with this is that the lipase continues to be active after ripening and can cause the development of strong rancid flavour. When a cock-tail of fungal protease and lipase were used, cheddar cheese developed a highly soluble proteins and free fatty acids and displayed better flavour within three months of ripening. The level of enzyme added to accelerate cheese ripening is also very important. High levels of enzyme during ripening may result in excessive enzymatic reactions that impart undesired characteristics and reduce the yield. Adaptation of liposome technology for accelerated cheese ripening reduces bitterness and losses in yield[76].

Bacterial intracellular enzymes are released by cell lysis and contribute to flavour through lipolysis and other enzymatic actions. Microcapsules of cell free extracts encapsulated in milk fat can be added to carryout milk clotting. Cheeses made with intact capsules contain substantially more enzymatic end products than the one obtained by direct enzyme addition. The capsule stability can be improved by encapsulating in a high melting fraction of fat[76].

Inherent milk lipase in cheese, made from unpasturized milk, affects considerable lipolytic action. The cultures and secondary flora, such as the *P. roqueforti* and *P. camembertii* in Blue-vein and Camembert cheeses respectively, are lipolytic and produce lipases, which are responsible for lipolysis. In addition, lipases are usually added to Italian cheese, viz. paramesan, provolone, and romano, to intensify their flavour[76]. During ripening, there is a steady increase in the concentration of liberated fatty acids and total soluble nitrogen. Lipases release the fatty acids from triglycerides, thereby triggering the development of cheese flavour[77].

The introduction of conjugated linoleic acid (CLA) in dairy foods has been made possible through the immobilization of lipases[78]. Lipases and proteases have been used to accelerate ripening both individually and as a "cocktail". The enzymes may be added as such or they may be encapsulated. During cheese ripening, a series of enzymatic reactions proceed very gradually, modifying the fresh, mechanically worked curd to the desired final ripe cheese texture and flavour. The enzymes, lipases, proteases and lactase hydrolyze lipids, proteins and lactose, respectively in order to raise the level of flavour moieties and/or flavour processors[76].

Fats and Oil Industry

Fats and oil modification is one of the prime areas in food processing industry that demand novel economic and green technologies[79]. Fats and oils are important constituents of foods. Lipases allow us to modify the properties of lipids by altering the location of fatty acid chains in the glyceride and replacing one or more of these with new ones. In this way, a relatively inexpensive and less desirable lipid can be modified to a higher value fat[14]. Lipases catalyze the hydrolysis, esterification and interesterification of oils and fats. Among the lipolytic conversion of oils and fats, esterification and interesterification are used to obtain value added products, such as specialty fats and partial glycerides by using positional and fatty acid specific lipases, and have greater industrial potential than fatty acid production in bulk through hydrolysis. Venkata Rao and Laxmanan[13] constructed an immobilized lipase membrane reactor for fat and oil hydrolysis, which yielded products that require less downstream processing, thus reducing the overall processing cost. The removal of phospholipids in vegetable oils (de-gumming) using highly selective microbial phospholipases is also a recently developed environmental friendly process[80].

Using granulation to immobilize lipases, it is possible to produce a food grade, cost effective, immobilized 1,3-regioselective (lipozyme TL 1M) lipase targeted for the interesterification of commodity oils and fats for the production of frying fats, shortenings and margarine components[70,81]. Lipase catalyzed interesterificaiton of fats and oils to produce modified acylglycerols cannot be obtained by conventional chemical interesterification[6].

Buisman *et al*[20] used immobilized lipases from *C. antarctica* (CAL-B), *C. cylindracea* Ay30, *H. lanuginosa*, *Pseudomonas* sp. and *G. candidum* for the esterification of functionalized phenols for synthesis of lipophilic antioxidants to be used in sunflower oil. There are many studies on the hydrolysis of fats and oil by lipases used either in the pure form, in the immobilized form or in the cell bound form[37].

The use of triacylglycerol lipase obtained from genetically modified *A. oryzae* as a processing aid in the oils and fats industry for oil de-gumming, and in the food industry to improve emulsifying properties was technologically approved by Australia New Zealand Food Authority (ANZFA) in 2002[68]. A new process for immobilizing lipases based on the granulation of silica has dramatically simplified the process and lowered the process cost. Such innovative methods are now widely implemented for the production of commodity fats and oils with no content of trans-fatty acids[84].

A mathematical basis for the design and operation of a continuous, packed bed rector was developed for the interesterification of soybean oil, which contains 22.7% oleoyl and 54.3% linoleoyl moieties as molar acyl moieties in hexane, with oleic acid using an immobilized *Sn*-1,3-specific lipase (Lipozyme IM) from *M. miehei*. The rate of change in oleoyl and linoleoyl moiety compositions in soybean oil decreased due to the loss of catalytic activity of Lipozyme IM. In an organic solvent, Cossignani *et al*[82] studied the lipase catalyzed acidolysis of soybean oil with oleic acid to increase oleic acid content. Lipase from the same source (*R. miehei*) was used to carryout acidolysis and a product having minimal modification of *Sn*-2 position fatty acid composition was obtained[85]. In the physical refining of vegetable oils, the degumming step can be carried out with a phospholipase. The economy of enzymatic degumming has significantly been improved by introduction of a microbial phospholipase (Lecitase Novo)[70].

Novozym 435 (*C. antarctica* lipase) catalyzed glycerolysis of commercial oils and fats to produce monoacylglycerides (MGs) was investigated using a tetra ammonium based ionic liquid as the reaction medium. A 90% yield of monoglycerides and nearly 100% conversion of triglycerides in this ionic liquid were achieved, which were markedly higher as compared to yields in normal solvents. Excellent operational stability of the lipase and the reversibility of ionic liquid were also observed in consecutive reactions. This provides a new environmentally benign 'solution' to the enzymatic modification of fats and oils with industrial potentials[86].

Tailored vegetable oils with nutritionally important structured triacylglycerols and altered physico-chemical properties have a big potential in future. Microbial lipases may be exploited for retailoring of vegetable oils. Cheap oils may be upgraded to nutritionally important structured triacylglycerols, like coco butter substitutes, low caloric triacylglycerols, and PUFA and oleic oil- enriched oils. It is possible to change the physical properties of natural oils to convert them into margarines and hard butter with higher melting points, or into special low caloric spreads with short or medium chain fatty acids. Normally fat and oil modifications are carried out chemically by using directed interesterification. This process is energy intensive and non-specific. Lipase mediated modifications are likely to occupy a prominent place in oil industry for tailoring structured-lipids since enzymatic modifications are specific and can be carried out at moderate reaction conditions[79].

Kajilal *et al*[81] synthesized a structured lipid (SL) from natural vegetable oils so that it would contain EFAs and natural antioxidants. They incorporated behenic acid into the Sn-1 and Sn-3 positions of sunflower oil through a transesterification reaction catalyzed by lipase. The synthesized product delivered 5.36 kCal/g and had an improved plastic nature, which increased their potential food applications. Such foods are a special type of trans-free solid fat. The SL was fed to rats and the results compared to the control group, which was fed with sunflower oil. No differences were observed in the amount of food consumed, which indicates the palatability and taste of the SL was quite similar to the native sunflower oil. Enzymatic interesterification

can also be used to produce oils and fats containing nutritionally important polyunsaturated fatty acids (PUFAs), such as eicospentaenoic and docosa-hexaenoic acids[87]. Even though use of immobilized lipase in the interesterification of triglycerides was first described in 1980s, the process was not commercially viable then because of the high cost[84].

Lipases as Biosensors for Food Industry

Immobilized lipases are fast, efficient, accurate and cost effective as sensors for the quantitative determination of triacylglycerol. This application is important in the food industry, especially in fats and oils, beverages, soft drinks, pharmaceutical industries and also in clinical diagnosis[88]. The basic concept of using lipase as biosensors is to generate glycerol from the triacylglycerol in the analytical sample and to quantify the released glycerol by a chemical or enzymatic method[6].

Wei *et al*[89] developed a method for the determination of organophosphorous pesticides with a surface acoustic wave impedance sensor by lipase hydrolysis. This method is also used to determine the dichlorvous residues in the root, stem and blade of Chinese cabbage. Lipases may be immobilized onto *p*H/oxygen electrodes in combination with glucose oxidase, and these function as lipid biosensors and may be used in triglycerides and blood cholesterol determinations[90].

Flavour Enhancement by Lipases

The production of low molecular weight esters as flavour compounds by biotechnological processes has potential interest for the food industry. The use of naturally available substrates and enzymes is an essential part of the process design. Laboret and Perrand[91] performed direct esterification of citronellol and geraniol with short-chain fatty acids, catalyzed by free lipase from *M. miehei*, which gave high yields in *n*-hexane. The initiative to scale-up applications was also attempted, using a reactor that allowed the production of ester in quantities up to 100 cm^3.

M. miehei lipase was immobilized on magnetic polysiloxane-polyvinyl alcohol particles by covalent binding with extended high activity. The performance of the resulting immobilized biocatalyst was evaluated in the synthesis of flavour esters using heptanes as solvent, in which ester synthesis was maximized for substrates containing excess acyl donor. The biocatalyst selectivity for the carbon chain length was found to be different between organic acids and alcohols. High reaction rates were achieved for organic acids with 8 or 10 carbon chain length, whereas increase in carbon chain length from 4 to 8 for alcohol lowered the esterification yields. Esterification performance was also dependent on the alcohol structure, with maximum activity occurring for primary alcohol, whereas 40% reduced rate was observed for secondary and tertiary alcohols[92].

Hexyl esters, green note flavour compounds, are widely used for flavour and fragrance in food, beverage and in pharmaceutical industries[93]. There is a growing demand for natural flavours containing "green note" represented by hexanol (C_6 alcohols) derivates. Hexyl butyrate is of special interest as it represents a model of flavour ester. Owing to its 'natural' character, the biosynthesis of such esters by lipase catalyzed chemical reactions under mild conditions has much current commercial interest. The ability of immobilized lipase (Lipozyme IM-77) from *R. miehei* to catalyze the transesterification of hexanol and tributyrin was investigated by Chang *et al*[94]. They concluded that reducing the amounts of co-substrate (tributyrin) would reduce the production cost of synthesize hexyl butyrate. While, the transes-terification of hexanol with triacetin, catalyzed by immobilized lipase from *M. miehei* (Lipozyme IM-77), was studied by Shieh and Chang[91]. The results showed that reaction temperature and substrate molar ratio were the most important parameters and the content of added water had less effect on conversion. This optimization study has commercial potential. Methyl ketones are major group of compounds which contribute to cheese flavour and are derived from the fatty acids liberated by lipolysis. These fatty acids have been shown to be enzymatically oxidized by secondary flora into methylketones through the β-oxidation pathway. In addition, the oxy fatty acids found in milk fat may also be the source of methylketones[76].

Bakery Industry

In baking industry, there is an increasing focus on lipolytic enzymes. Recent findings suggest that (phospho) lipases can be used to substitute or supplement traditional emulsifiers since the enzymes degrade polar wheat lipids to produce emulsifying lipids *in situ*[10,83,95]. Lipase was primarily used to enhance the flavour content of bakery products by liberating short-chain fatty acids through esterifi-cation. Along with flavour enhancement, it also prolonged the shelf-life of most of the bakery

products. Texture and softness could be improved by lipase catalyzation[92].

An artificially expressed lipase in *A. oryzae* was used as processing aid in the baking industry[96]. All hydrolytic enzymes, including lipase, were found to be effective in reducing the initial firmness and increasing the specific volume of breads[97]. Recombinant yeast with *Geotricum* LIP 2 gene expressed a protein, which exhibited similar biochemical properties. Fermented dough prepared with this recombinant yeast rendered the bread with higher loaf volume and more uniform crumb structure[26]. Yeast with bacterial lipase gene LIP A resulted in higher productivity of enzyme and found use in bread making as a technological additive[18]. Increased butter falvour for baked goods was generated by hydrolysis of butterfat with suitable lipase[72].

Dietetics

With increasing consumer awareness of the risks associated with high fat intake, there is growing demand for low caloric fats and fat replacers, but these cannot be exposed to high temperatures. The majority of reduced caloric fats and fat substitutes available today contain fatty acids that are not naturally present in edible oils and fats but may match the chemistry and function of natural fats. The drawback is that such products lack nutritionally important essential fatty acids (EFA).

A positional analysis of the structured triglycerols formed showed an increase in preference of the lipase action for the primary positions compared to the secondary positions. The targeted structured triglycerols with palmitoyl moieties in the Sn-2 position and medium chain acyl moieties in the Sn-1,3 positions should be useful in food formulation for infant nutrient and clinical as well as parental nutrition applications. The use of a good grade papain containing lipase ensures ready acceptance of such products[54].

Osborn and Akoh[98] has discussed the modification of vegetable oil for the production of infant formula with highly absorbing TAGs. It is composed of MCFA (Medium Chain Fatty Acids) and PUFAs in the same positions and amounts as those found in human milk. Structured lipids (SLs) containing palmitic, oleic, stearic and linoleic acids, resembling human milk fat (HMF), were synthesized by enzymatic acidolysis between tripalmitin, hazelnut oil fatty acids and stearic acid. Commercially immobilized Sn-1, 3-specific lipase, lipozyme RM IM, obtained from *R. miehei* was used as the biocatalyst for the acidolysis. For both oleic and stearic acids, the incorporation level increased with reaction time. The SLs produced in this study by Sahin *et al*[99] have potential use in infant formulae. They also state that greater collaboration between industry and academia will hasten and increase successful commercialization of enzymatic processes[101].

Other Food Processing Industries

In recent times, lipases have been commonly used in the production of a variety of products, ranging from fruit juices to vegetable fermentation[6]. Lipases facilitate the removal of fat from meat and fish products[14]. Cao *et al* reported a lipase-catalyzed solid phase synthesis of sugar fatty acid esters[102]. Pandey *et al*[6] reviewed the direct conversion of alkane diols into their monoesters and diesters, which has strong implications for the food and pharmaceutical industries, because the products have non-ionic surfactant activity and can be used as monomeric units in cross-linking in polymerization. The processing of sausages with microbial lipases is an emerging are of meet technology[14].

An interesting finding is the addition of lipase to noodles, resulting in significantly softer textural characteristics in noodles despite having the relatively low levels of the substrate acylglycerols present in the formulations[101].

In confectionary, 1,3-regioselectivity of lipases was exploited in the process development of a fat production containing high concentration of 1,3-disteraroyl-2-monoloein[87]. This fat could be used as a substitute for shea stearine in the formulation of cocoa butter equivalents. Fats designed to inhibit bloom formation in chocolate products have also been produced by these types of enzyme esterification reactions[87].

C. rugosa lipases have many applications in the food and flavour industry, in the production of ice cream and single cell protein, biocatalytic resolution of life saving pharmaceuticals, carbohydrate esters and amino acid derivatives not obtainable by conventional chemical synthesis[103].

Immobilized lipase from *C. antarctica* (Novozym 435) has been applied to perform the enzymatic esterification of bioactive compounds with fatty acids. Various bioactive compounds, like vitamins,

secondary metabolites such as kojic acid from plants and microorganisms, can be acylated to generate products useful in the cosmetic, pharmaceutical, fine chemical, food and feed industries. A convenient scaleable procedure for the downstream processing of the ester product comprises hexane-solid extraction of the unreacted lauric acid and water ethyl acetate extraction of the unreacted pyridoxine, yielding lauric acid-pyridoxine monoester as a white powder with more than 90% purity, which is soluble in vegetable oil[100]. Regioselective modification of polyfunctional organic compounds is yet another rapidly expanding area of lipase application. The enzyme has also been used in conjugation with a microbial cocktail for the treatment of fat rich effluents from ice cream plants. This could also be utilized in waste processing of many food industries[37].

Lipases for Pharmaceutical Application

Microbial lipases are used to enrich PUFAs from animal and plant lipids, and their mono and diacylglycerides are used to produce a variety of pharmaceuticals[44]. PUFAs are increasingly used as food additives, pharmaceuticals and nutraceuticals because of their metabolic benefits. Many PUFAs are essential for normal synthesis of lipid membranes and prostaglandins. Microbial lipases are used to obtain PUFAs from animal and plant lipids, such as menhaden oil, tuna oil and borage oil. Free PUFAs and their mono and diacylglycerides are subsequently used to produce a variety of pharmaceuticals[14]. Liposomes are used in the medical field to optimize the action of drugs by transporting them to target areas, thus circumventing drug waste inactivation and anatomical barriers[19].

Considerable effort is being made to obtain optically, pure compounds, which are pharmacologically more active than its antipode. Profens, a class of nonsteroidal anti-inflammatory drugs, are active in the (s)-enantiomer form. Lee *et al*[104] and Xie *et al*[105] synthesized pure (s)-ibuprofen using lipase-catalyzed kinetic resolution via hydrolysis and esterification, respectively.

In addition to racemization *in situ*, lipases are also capable of catalyzing synthetic reactions, which has led to the production of life saving drugs. Efficient kinetic resolution processes are available for the preparation of optically active homochiral intermediates for the synthesis of nikkomycin-B, non steroid anti-inflammatory drugs (naproxen, ibuprofen, suprofen and ketoprox), the potential anti viral agent lamivudine, and for the enantiospecific synthesis of alkaloids, antibiotics, vitamins, and anti-arteriosclerotic, anti tumour and antiallergic compounds[6].

Nutraceuticals are food components that have health benefits beyond traditional nutritional value. Novel biotechnology tools, like immobilization, have also been applied for the isolation and incorporation of such food components in ordinary foods. Successful synthesis of nutraceuticals has been reported by employing immobilized lipases, such as those from *C. antarctica* and *Lactobacillus ruteri*[19]. Lipases are also used in the synthesis of the artificial sweetener sucralose by regioselective hydrolysis of octaacetylsucrose[2].

Some limiting problems for such processes are: (1) insufficient enantioselectivity, (2) limited enzyme activity, (3) difficulties in recycling the lipase, and (4) inherent practical limitations of the kinetic resolution arising from the fact that 50% conversion is the maximum possible[19].

Safety Evaluation

It is important that the lipases produced by microbial sources do not exhibit any toxicity when used in food applications. The evaluation of safety involves mainly testing for acute, subacute and subchronic oral toxicity and mutagenic potential[6]. Lipase G from *P. camembertii*, which is used as a processing aid in the food industry, was subjected to various safety evaluations. As a result, it was classified as a nonpathogenic and as safe for the enzyme production worker and the consumer[106]. No safety concern was identified with lipase derived from *R. oryzae* under controlled fermentation conditions and the toxicological test results concluded that it could be used as a food additive[107]. *A. niger* enzymes are considered GRAS with a restriction that the new and unknown isolates should be checked for ochratoxin A production before they are developed as production organisms[108]. At high levels *R. miehei* lipase expressed in *A. oryzae* showed consequential effects upon body weight and energy metabolism[109]. Lipase D from *R. oryzae*, which is used for selective hydrolysis of triglycerides and for interesterification of edible fats and oils, exhibits no adverse effect when used as described in the processing of dietary fatty acids and glycerides of fatty acid[110]. A lipase artificially expressed in *A. oryzae*, which is used as a

processing aid in the baking industry, appears safe for consumers and no occupational health precaution in manufacture was recommended due to its low environmental impact[96]. Enzyme preparation from *C. rugosa* is considered safe to workers and consumers when employed in the production of flavours[111].

Conclusion

The tremendous potential of lipases in food and allied technology applications shows the need to develop novel cost-effective technologies for increased production, scaling up and purification of this versatile enzyme. The large number of hydrolytic applications, like flavour development in dairy products (cheese, butter and margarine), alcoholic beverages, milk chocolate, etc., is a promising field of lipase enzyme. Production of diet control foodstuff, meat technology and the processing of sausages are some areas in food industry with commercial potential. The applications of lipases are broadening rapidly and new applications are still to be explored in food industries. The properties of lipases are being improved by protein engineering and genetic engineering to widen their applications in extreme conditions. Various innovations in the immobilization of enzymes play a vital role in using this enzyme as an effective and efficient biocatalyst in food processing technology. Other than the food industry, lipases have been applied in the synthesis of fine chemicals, biodiesel production, the production of biopolymeric materials, the detergent industry, organic synthesis, the paper and pulp industry, the synthesis of ingredients for personal care products, the synthesis of surfactants and of structural triglycerides, the oleochemical industry, agrochemicals production, the pesticide industry, and in environmental management. The characterization and application of lipases to catalyze reactions with commercial potential will significantly broaden the spectrum of industrial biotechnology.

To cater to the needs of these enzymes in industries, novel lipase genes have to be isolated and the existing lipases are to be engineered for desired properties. The engineered lipases can be evolved by directed evolution, ultra high-throughput screening system based on electrospray ionization mass spectrometry (ESI-MS) and by phase display techniques. The rapid boom in the future prospects of lipase technology is evident from the large number of patents, publications and research reports in the recent years and indications are that this growth will be sustained for many years.

References

1 Jain J L, *Biochemistry*, 1st edn (S Chand & Company Ltd., New Delhi) 1979, 197- 224.

2 Jaeger K E & Eggert T, Lipases for biotechnology, *Curr Opin Biotechnol*, 13 (2003) 390-397.

3 Akimoto M, Nagashima Y & Sato D, A kinetic study on lipase-catalyzed interesterificaiton of soybean oil with oleic acid in a continuous packed bed reactor, *Appl Biochem Biotechnol*, 81 (1999) 131-142.

4 Svendsen A, Sequence comparisons within the lipase family, in *Lipases: Their structure, biochemistry and applications*, edited by P Y Woolley & S B Petersen (Cambridge University Press, Cambridge) 1994, 1-21.

5 Anishetty S & Gowtham P, *Lipases*, AU-KBC Research Centre, (www.au-kbc.org).

6 Pandey A, Benjamin S, Soccol C R, Nigam P, Krieger N *et al*, The realm of microbial lipases in biotechnology, *Biotechnol Appl Biochem,* 29 (1999) 119-131.

7 IUPAC, *Pure Appl Chem,* 69 (1997) 1613-1632.

8 Godfrey T & West S, Introduction to industrial enzymology, in *Industrial enzymology*, 2nd edn, edited by T Godfrey & S West (Stockton Press, New York) 1996, 1-8.

9 Gupta R, Rathi P, Gupta N & Bradow S, Lipase assay for conventional and molecular screening: An overview, *Biotehnol Appl Biochem*, 37 (2003) 63-71.

10 Nova Nordisk, *www.novanordisk.com*

11 Alberghina L, Schmid R D & Verger R, *Lipases: Structure, mechanism and genetic engineering* (John Wiley & Sons, New York) 1991, 25-45.

12 Beisson F, Tiss A, Riviere C & Verger R, Methods for lipase detection and assay: A critical review, *Eur J Lipid Sci Technol*, 102 (2000) 133-153.

13 Venkata Rao P & Laxmanan C M, Lipase enzyme technology and its potential applications in the oils and fats industry, *Indian Chem Eng*, 33 (1991) 7-29.

14 Sharma R, Chisti Y & Banerjee U C, Production, purification, characterization and applications of lipases, *Biotechnol Adv*, 19 (2001) 627-662.

15 Jaeger K E, Dijkstra B W & Reetz M T, Bacterial biocatalysts: Molecular biology, 3-dimensional structures and biotechnological applications of lipases, *Annu Rev Microbial,* 53 (1999) 315-351.

16 Toida J, Arikawa Y, Kondou K, Fukuzawa M & Sekiguchi J, Purification and characterization of triacylglycerol lipase from *Aspergillus oryzae*, *Biosci Biotechnol Biochem*, 62 (1998) 759-763.

17 Schimidt-Dannert C, Sztajer H, Stocklein W, Menge W & Schimid R D, Screening purification and properties of thermophilic lipase from *Bacillus thermocatenulatus, Biochem Biophys Acta,* 1214 (1994) 43-53.

18 Sanchez M, Prim N, Rendez-Gil F, Pastor F I J & Diaz P, Engineering of baker's yeasts, *Escherichia coli* and *Bacillus* hosts for the production of *B. subtilis* lipase A, *Biotechnol Bioeng*, 78 (2002) 339-345.

19 Linko Y Y & Wu X Y, Biocatalytic production of useful esters by two forms of lipase from *Candida rugosa*, *J Chem Technol Biotechnol,* 65 (1996) 163-170.

20 Buisman G J H, Van-Heltersen C T W, Kramer G F H, Veldsnik J W, Derksen J T P *et al*, Enzymatic esterification of functionalized phenols for the synthesis of lipophilic antioxidants, *Biotechnol Lett*, 20 (1998) 131-136.

21 Kovac A, Stadler P, Haalck L, Spener F & Paltauf F, Hydrolysis and esterification of acylglycerols and analogs in aqueous medium catalyzed by microbial lipases, *Biochim Biophys Acta*, 1301 (1996) 57-66.

22 Vaysse L, Dubreucq E, Pirat J L & Galzy P, Fatty hydroxamic acid biosynthesis in aqueous medium in the presence of the lipase-acyltransferase from *Candida parapsilosis*, *J Biotechnol,* 53 (1997) 41-46.

23 Kanisawa T, Yamaguchi Y & Hattori S, Action of *Candida cylindracea* lipases on concentrated milk and cream, *J JPN Soc Food Sci Technol*, 29 (1982) 693-699.

24 Dorfman P, Greene J & Anne McGee, Milk and diary products, in *On food and cooking-The science and lore of the kitchen*, 1st edn, edited by Harold J McGee (Unwin Hyman Ltd., London) 1986, 56-85.

25 Carlile K, Rees G D, Robinson B H, Steer T D & Svenson M, Lipase-catalyzed interfacial reactions in reverse micellar systems, *J Chem Soc Faraday Trans,* 92 (1996) 4701-4708.

26 Monfort A, Blasco A, Sanz P & Prieto J A, Expression of LIP1 and LIP2 genes from *Geotrichum* species in baker's yeast strains and their application to the bread making process, *J Agric Food Chem*, 47 (1999) 803-808.

27 Karra C M, Pulvin S, Touraud D & Thomas D, Enzymatic synthesis of geraniol esters in a solvent-free system by lipases, *Biotechnol Lett*, 18 (1996) 1083-1087.

28 Cavaille L D & Combes D, Lipase synthesis of short-chain flavour thioesters in solvent-free medium, *Biocatal Biotransform,* 15 (1997) 265-279.

29 Rathi R, Goswami V K, Sahai V & Gupta R, Statistical medium optimization and production of a hyperthermostable lipase from *Burkholderia cepacia* in a bioreactor, *J Appl Microbiol*, 93 (2002) 930-936.

30 Nakano H, Kitahata S, Shimada Y, Nakamura M & Tominaga Y, Esterification of glycosides by a mono- and di-acylglycerol lipase from *Penicillium camembertii* and comparison of the products with *Candida cylindracea* lipase, *J Ferment Bioeng*, 80 (1995) 24-29.

31 Ferrer M, Plon F J, Nuero O M, Reyes F & Balleserol A, Purification and properties of a lipase from *Penicillium chrysogenum* isolated from industrial wastes, *J Chem Technol Biotechnol*, 75 (2000) 569-576.

32 Ha J K & Lindsay R C, Release of volatile branched-chain and other fatty acids from ruminant milk fats by various lipases, *J Dairy Sci,* 76 (1993) 677-690.

33 Talon R, Montel M C & Berdague J L, Production of flavour esters by lipases of *Staphylococcus warneri* and *S. xylosus*, *Enzyme Microb Technol,* 19 (1996) 620-622.

34 Jane Gilbert E, *Pseudomonas* lipases: Biochemical properties and molecular cloning, *Enzyme Microb Technol*, 15 (1993) 634-645.

35 Herrgard S, Gibas C J & Subramaniam S, Role of electrostatic network of residues in the enzymatic action of *Rhizomucor miehei* lipase family, *Biochemistry*, 39 (2000) 2921-2930.

36 Chang R C, Chow S J & Shaw J W, Multiple forms and function of *Candida rugosa* lipase, *Biotechnol Appl Biochem*, 19 (1994) 93-97.

37 Ghosh P K, Saxena R K, Gupta R, Yadav R P & Davidson S, Microbial lipases: Production and applications, *Sci Prog*, 79 (1996) 119-157.

38 Manikandan K, *Optimization of media for lipase production by Bacillus sphaericus.* M Tech Thesis, Annamalai University, Annamalai Nagar, 2004.

39 Rooney D & Weatherley L R, The effect of reaction conditions upon lipase catalyzed hydrolysis of high oleate sunflower oil in a stirred liquid-liquid reactor, *Process Biochem*, 36 (2001) 947-953.

40 Jaeger K E & Reetz M T, Microbial lipases form versatile tools for biotechnology, *Trends Biotechnol*, 16 (1998) 396-403.

41 Tang S J, Sun K H, Huan G, Chang T Y & Lee G C, Recombinant expression of the *Candida rugosa* lip 4 lipase in *Escherichia coli, Protein Expr Purif*, 20 (2000) 308-313.

42 Rivera-Munoz G, Tinoco-Valencia J R, Sanchez S & Farrer A, Production of microbial lipases in solid state fermentation system, *Biotechnol Lett*, 13 (1991) 277-280.

43 Kojima Y & Shimizu S, Purification and characterization of the lipase from *Pseudomonas fluorescens* HV380, *J Biosci Bioeng,* 3 (2003) 219-226.

44 Dong H, Gao S, Han S P & Cao S G, Purification and characterization of a *Pseudomonas* species lipase and its properties in non-aqueous media, *Biotechol Appl Biochem,* 30 (1999) 251-256.

45 Makhzoum A, Owusu-Apenten R K & Knapp J S, Purification and properties of lipase from *Pseudomonas fluorescens* strain 2D, *Int Dairy J*, 6 (1996) 459-472.

46 Sharon C, Furugoh S, Yamakido T, Ogawa H I & Kato Y, Purification and characterization of a lipase from *Pseudomonas aeruginosa* KKA-5 and its role in castor oil hydrolysis, *J Ind Microbiol Biotechnol,* 20 (1998) 304-307.

47 Palekar A A, Vasudevan P T & Yan S, Purification of lipase: A review, *Biocatal Biotransform*, 18 (2000) 177-200.

48 Mamura S & Kitaura S, Purification and characterization of monoacylglycerol lipase from the moderately thermophilic *Bacillus* species H-2575, *J Biochem,* 127 (2000) 419-425.

49 Suzuki M, Yamamoto H & Mizugaki M, Purification and general properties of a metal-insensitive lipase from *Rhizopus japonicus* NR 400, *J Biochem,* 100 (1986) 1207-1213.

50 Chattopadhyay M, Banik A K & Raychaudhuri S, Production and purification of lipase by a mutant strain of *Rhizopus arrhizus, Folia Microbiol*, 44 (1999) 37-40.

51 Razak C N A, Salleh A B, Musani R, Samad M Y & Basri M, Some characteristics of lipases from thermophilic fungi isolated from palm oil mill effluent, *J Molecular Catalysis B: Enzymatic*, 3 (1997) 153-159.

52 Krieger N, Taipa M A, Aires-Barros M R, Melo E H M, Lima-Filho J L *et al*, Purification of the *Penicillium citrinum* lipase using AOT reversed micelles, *J Chem Technol Biotechnol,* 67 (1997) 77-85.

53 Saxena R K, Sheron A, Giri B & Davidson S W, Purification strategies for microbial lipases, *J Microbial Methods*, 52 (2003) 1-18.

54 Langrand G, Rondot N, Triantaphylides C & Barthi J C, Short chain flavour esters synthesis by microbial lipase, *Biotechnol Lett*, 12 (1990) 581-586.

55 Brockerhoff H & Jensen R, *Lipolytic enzymes*, (Academic Press, New York) 1974, 29-85.

56 Godtfredsen S E, Microbial Lipases, in *Microbial enzymes and biotechnology*, edited by W M Fogarty & E T Kelly (Elsevier Applied Sciences, Amsterdam) 1990, 255-273.

57 Zaks A & Klibanov A M, Enzyme-catalyzed processes in organic solvents, *Proc Natl Acad Sci USA*, 82 (1985) 3192-3196.

58 Macrae A R & Hammond R C, Present and future application of lipases, *Biotechnol Genet Eng Rev*, 3 (1985) 193-219.

59 Schmidt-Dannert C, Recombinant microbial lipase for biotechnological applications, *Bioorg Med Chem*, 7 (1999) 2123-2130.

60 Svendsen A, Lipase protein engineering, *Biochem Biophys Acta*, 1543 (2000) 223-238.

61 Brocca S, Schmidt-Dannert C, Lotti M, Alberghina L & Schmid R D, Design total synthesis and functional over expression of the *Candida rugosa* lip 1 gene coding for a major industrial lipase, *Protein Sci*, 7 (1998) 1415-1422.

62 Jorgensen S, Skov K W & Diderichsen B, Cloning, sequence and expression of a lipase gene from *Pseudomonas cepacia*: Lipase production in heterologous hosts requires two *Pseudomonas* genes, *J Bacteriol,* 173 (1991) 559-567.

63 Catoni E, Schmidt-Dannert C, Brocca S & Schmid R D, Over expression of lipase A and B of *Geotrichum candidum* in *Pichia pastoris*: High level production and some properties of functional express lipase B, *Biotechnol Tech,* 11 (1997) 689-695.

64 Fickers P, Nicaud J M, Destain J & Thonar P, Overproduction of lipase by *Yarowia lipolytica* mutants, *Appl Microbiol Biotechnol,* 63 (2003) 136-142.

65 Huge-Jensen B, Anderson F, Christensen T, Christensen M, Thim L *et al*, *Rhizomucor miehei* triglyceride lipase is processed and secreted from transformed *Aspergillus oryzae*, *Biotechnol Bioeng*, 59 (1998) 122-127.

66 Holmquist M, Tesseier D C & Cygler M, High level production of recombinant *Geotrichum candidum* lipases in yeast *Pichia pastoris, Protein Expr Purif,* 11 (1997) 35-40.

67 Akoh C C, Lee G C & Shaw J F, Protein engineering and applications of *Candida rugosa* lipase isoforms, *Lipids,* 39 (2004) 513-526.

68 Australia Newzealand Food Authority, Lipase from genetically modified *Aspergillus oryzae*, Final Assessment Report, Application A41, May 2002.

69 Nagodawithana T & Reed G, *Enzymes in food processing*, 3rd edn (Academic Press, San Diego) 1993, 56-68.

70 Pearce S W, Nielsen P, Christensen M W, Holm H C & Clausen K, 2002. *Lipases for food ingredients*, paper presented in *Enzyme uses in the food industry*, Annual Meeting and Food Expo (Session 94), Anaheim, California, 17-20 November 2002.

71 Reimerdes E H, Franke K & Sell M, *Influencing functional properties of egg yolk by using phospholipases*, paper presented at Conf on Food Structure & Food Quality, held on 3-7 October, 2004 (German Institute of Food Technology, Cork) 2004.

72 Uhling H, *Industrial enzymes and their applications*, 2nd edn, (John Wiley & Sons, New York) 1998, 332-334.

73 Ang Y-K W C, Liu Ke S, Huang Y-W, *Asian foods-science and technology*, (Technomic Publication Co., Lancaster, Pa) 1999, 181-183.

74 Shurtleff W & Aoyagi A, *The book of Tempeh—A cultured soy food* (Ten Speed Press, Berkeley) 2001, 169-170.

75 Undurranga D, Markovits A & Erazo S, Coco butter equivalent through enzymic interesterification of palm oil, *Process Biochem*, 36 (2001) 933-939.

76 Custry F, Fernander N & Shahani K M, *Role of lipases and other enzymes in flavour development,* paper presented at 24th Annu Miles-Marschall Italian Cheese Seminar, held on September 16-17, 1987 (Madison, WI) 1987, 536-537.

77 Maia M M D, de Morais M M C, de Morais Jr M A, MeloE H M & Filho J L L, Production of extracellular lipase by the phytopathogenic fungus *Fusarium solani* FS1, *Rev Microbiol*, 30 (1999) 304-309.

78 Baianu I C, Lozano R, Prisecaru & Lin H C, *Novel techniques and their applications to agricultural biotechnology, health foods and medical biotechnology* (University of Illinois at Urbana, IL, USA) 2003.

79 Gupta R, Rathi P & Bradoo S, Lipase mediated upgradation of dietary fats and oils, *Crit Rev Food Sci Nutr*, 43 (2003) 635-644.

80 Clausen K, Enzymatic oil-degumming by a novel microbial phospholipase, *Eur J Lipid Sci Technol*, 103 (2001) 333-340.

81 Kajilal S, Prasad R B N, Kaimal T N B & Raosh G, Synthesis and estimation of caloric value of structured lipid-potential reduced calorie fat, *Lipids*, 34 (1999) 1045-1055.

82 Cossignani L, Damiani P, Simonetti M S & Manesh J, Biocatalyzed acidolysis of soybean oil triacylglycerols to increase oleic acid content, *J Chromatogr A,* 1052 (2004) 167-170.

83 Kirk O, Borchert T V & Furlerng C C, Industrial enzyme applications, *Curr Opin Biotechnol*, 13 (2002) 345-351.

84 Christensen M W, Andersen L, Kirk O & Holm H C: Enzymatic interesterification of commodity oils and fats: Approaching the tones scale, *Lipid Technol News*, 7 (2001) 33 -37.

85 Tan T & Yin C, The mechanism and kinetic model for glycerolysis by 1,3 position specific lipase from *Rhizopus arrhizus*, *Biochem Eng J*, 25 (2005) 39-45.

86 Guo Z & Xu X, New opportunity for enzymatic modification of fats and oils with industrial potentials, *Org Biomol Chem,* 3 (2005) 2615-2619.

87 Macrae A, *Enzymatic interesterification*, Science Lecture Paper Series (Society of Chemical Industry, Befford, UK) 2000.

88 Kynclova E, Hartig A & Schalkhammer T, Oligonucleotide labeled lipase as a new sensitive hybridization probe and its use in bio-assays and biosensors, *J Mol Recognit*, 8 (1995) 139-145.

89 Wei W, Wang R H, Wie L H & Yao S Z, Rapid determination of dimethoate with surface acoustic wave impedance sensor system, *Anal Lett*, 30 (1997) 2641-2661.

90 Hasan F, Shah A A & Hameed A, Industrial applications of microbial lipases, *Enzyme Microb Technol*, 39 (2006) 235-251.

91 Shieh C J & Chang S W, Optimized synthesis of lipase-catalyzed hexyl acetate in *n*-hexane by response surface methodology, *J Agric Food Chem*, 49 (2001) 1203-1207.

92 Loboret F & Perraud R, Lipase-catalyzed production of short-chain acids terpenyl esters of interest to the food industry, *Appl Biochem Biotechnol*, 82 (1999) 185-198.

93 Kazlauskas R J & Bornscheuer U T, Biotransformations with lipases, in *Biotechnology*, 1st edn, vol VIII, edited by H J Rehm, G Pihler, A Stadler & P J W Kelly (VCH, New York) 1998, 37-192.

94 Chang S W, Shaw J F & Shieh N, Optimization of enzymatically prepared hexyl butyrate by lipozyme 53M-77, *Food Technol Biotechnol,* 41 (2003) 237-242.

95 Collar C, Martilnoz J C, Andrew P & Armero E, Effect of enzyme association on bread dough performance: A response surface study, *Food Sci Technol Int*, 6 (2000) 217-226.

96 Greenough R J, Perry C J & Stavnsbjerg M, Safety evaluation of a lipase expressed in *Aspergillus oryzae*, *Food Chem Toxicol*, 34 (1996) 161-166.

97 Keskin S D, Sumnu G & Sahin S, Usage of enzymes in a novel baking process, *Mol Nutr & Food Res*, 48 (2004) 156-160.

98 Osborn H T & Akoh C C, Structured lipids—Novel fats with medical, nutraceutical, and food applications, *Comp Rev Food Sci Food Safety*, 1 (2002) 93-103.

99 Sahin N, Akoh C C & Karaali A, *Lipase-catalyzed acidolysis of tripalmitin with hazelnut oil fatty acids and stearic acid to produce human milk fat substitutes*, paper presented to 96th AOCS Annual Meeting of EXPO, held on May 1-4, 2005 at Salt Palace Connection Centre, Salt Lake City, Utah (AOCS, USA) 2005.

100 Zarevucka M, Zalska Z, Rejzek M, Streinz L, Wimmer Z *et al*, Lipase-mediated hydrolysis and esterification, *Enzyme Microb Technol*, 17 (1995) 866-869.

101 Undurraga D, Markovits A & Erazo S, Cocoa butter equivalent through enzymic interesterification of palm oil midfraction, *Process Biochem*, 36 (2001) 933-939.

102 Coa L, Fischer A, Bornschever V T & Schmid R D, Lipase catalyzed solid phase preparation of sugar fatty acid esters, *Biocatal Biotransform,* 534 (1997) 269-283.

103 Benjamin S & Pandey A, *Candida rugosa* lipases: Molecular biology and versatility in biotechnology, *Yeast*, 14 (1998) 1069-1087.

104 Lee W M, Kim K J, Kim M G & Lee S B, Enzymatic resolution of racemic ibuprofen esters: Effect of organic cosolvents and temperature, *J Ferment Bioeng*, 6 (1995) 613-615.

105 Xie Y C, Liu H Z & Chen J Y, *Candida rugosa* lipase catalyzed esterification of racemic ibuprofen and chemical hydrolysis of S-ester formed, *Biotechnol Lett*, 20 (1988) 455-458.

106 Kondo M, Ogawa T, Matsubara Y, Mizutani A, Murata S *et al*, Safety evaluation of lipase G from *Penicillium camembertii*, *Food Chem Toxicol*, 32 (1994) 685-696.

107 Coenen T M, Aughton P & Verhagen H, Safety evaluation of lipase derived from *R. oryzae*: Summary of toxicological data, *Food Chem Toxicol,* 35 (1997) 315-322.

108 Schuster E, Dunn-Coleman N, Frisvad J & Dijik P V, On the safety of *Aspergillus niger*—A review, *Appl Microbial Biotechnol,* 59 (2002) 426-435.

109 Broadmeadow A, Clare C & de Boer A S, An overview of the safety evaluation of the *Rhizomucor miehei* lipase enzyme, *Food Addit Contam*, 11 (1994) 105-119.

110 Flood M T & Kondo M, Safety evaluation of lipase produced from *Rhizopus oryzae*: Summary of toxicology data, *Regul Toxicol Pharmacol,* 37 (2003), 293-304.

111 Flood M T & Kondo M, Safety evaluation of lipase produced from *Candida rugosa*: Summary of toxicology data, *Regul Toxicol Pharmacol*, 33 (2001) 157-164.