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Microbial lipases: production and applications

P.K.GHOSH, R.K. SAXENA*, RANI GUPTA, R.P. YADAV AND SHEBA DAVIDSON*

Lipases occupy a prominent place among biocatalysts and have a wide spectrum of biotechnological applications. Lipases are unique as they hydrolyse fats into fatty acids and glycerol at the water–lipid interface and can reverse the reaction in non-aqueous media. The stability of these enzymes in organic solvents have pushed them into the frontier areas of organic synthesis leading to the designing of novel drugs, surfactants, bioactive compounds and oleochemicals. In addition, lipase-catalysed trans-esterification and inter-esterification reactions have been exploited in the fat industry. Looking into the wide scenario of lipase applications, commercialization of lipase production is a prime area of interest for microbiologists, process engineers and biochemists. Research carried out in this field has revealed that microbes, especially fungi and bacteria, are the tools of choice for commercial production. Recently, the structure determination of a few microbial lipases has widened our knowledge about the unique mechanism of catalysis of this enzyme.

Introduction

General considerations

Lipases [triacyl glycerol acylhydrolases (EC 3.1.1.3)] have come into prominence because of new and novel applications in oleochemistry, detergent formulation, organic synthesis and nutrition. Consequently, the sourcing of the enzymes is also being expanded from the conventional animals and plants to microbial sources. The search for newer and rugged products is continuing to find materials

Dr R.K. Saxena, Dr Rani Gupta, R.P. Yadav and Sheba Davidson are in the Department of Microbiology, University of Delhi, South Campus, Benito Juarez Road, New Delhi-110021, India.

Dr P.K. Ghosh is an advisor in the Department of Biotechnology, CGO Complex, Lodhi Road, New Delhi-110003, India.

**To whom correspondence should be addressed.*

having specific application potential[1,2]. The development of technology for lipase production and synthesis of novel compounds using lipase-mediated reactions will result in their expansion into new areas and will have a major impact on a range of industries.

Lipases belong to the class of serine hydrolases and, therefore, do not require any cofactor. The natural substrates of lipases are triacylglycerols, which have very low solubility in water. Under natural conditions, lipases catalyse the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase where the enzyme remains dissolved (Figure 1). Under certain experimental conditions, such as in the presence of traces of water, they are capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol (Figure 2). The occurrence of lipase catalysed reactions at an interface between the substrate and the aqueous phase

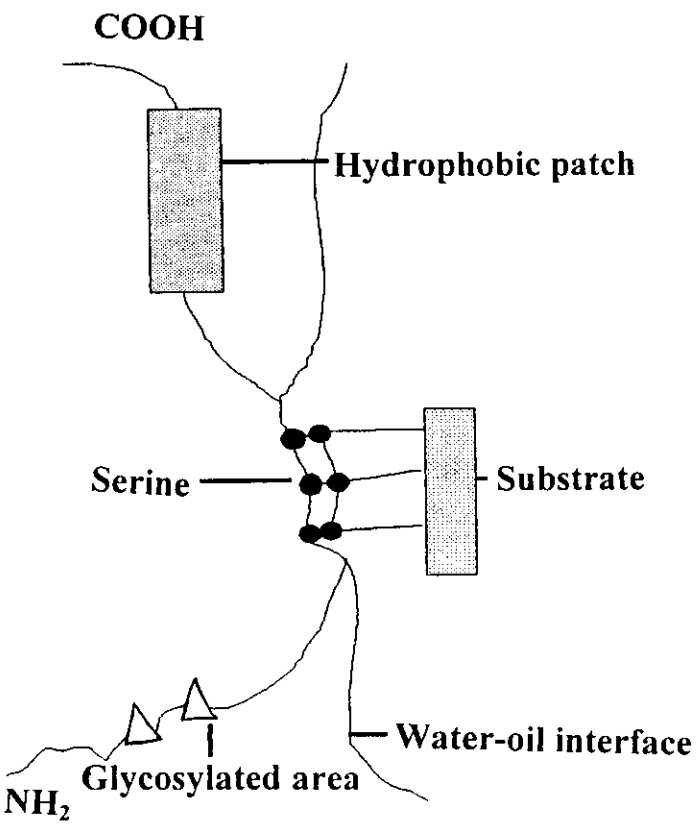

Fig. 1. *Diagrammatic representation of a lipase molecule showing its main features, substrate can be any triglyceride.*

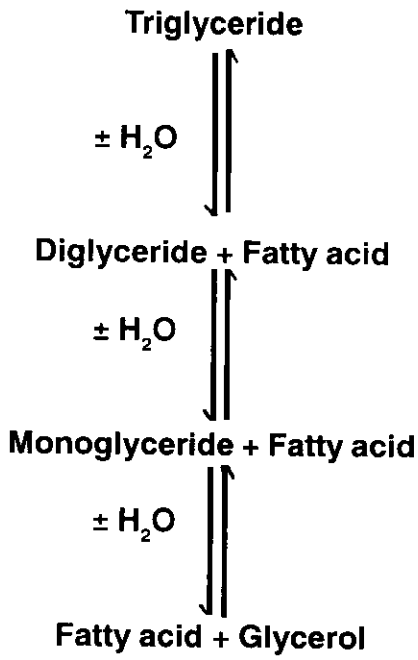

Fig. 2. The lipase reaction.

generates scientific challenges in the assay and kinetic analysis of the reaction[1].

Lipases act on ester bonds. The usual industrial lipases are special classes of esterase enzymes that act on fats and oils, and hydrolyse them in steps into the substituted glycerides and fatty acids, and finally on total hydrolysis into glycerol and fatty acids. In nature, the available lipases from various sources have considerable variations in their reaction specificities, this property being referred to as the enzyme specificity. Thus, from the fatty acid side, some lipases have an affinity for short-chain fatty acids (C_2, C_4, C_6, C_8 and C_{10}), some have a preference for unsaturated fatty acids (oleic, linoleic,linolenic *etc.*) while many others are non-specific and randomly split the fatty acids from the triglycerides. From the glycerol side of the triglycerides, the lipases often show positional specificity and attack the fatty acids at the 1 or 3 position of glycerol or at both these positions but not the fatty acid at the 2 position of the glycerol molecule except for the single report of Asahara *et al.* from *Geotrichum* spp.[3] (Figure 3). The 2-fatty acid monoglyceride, however, rearranges through acyl-migration randomly, pushing the fatty acid to the 1 or 3 position of the glycerol molecule; as acyl migration is a slow process and as

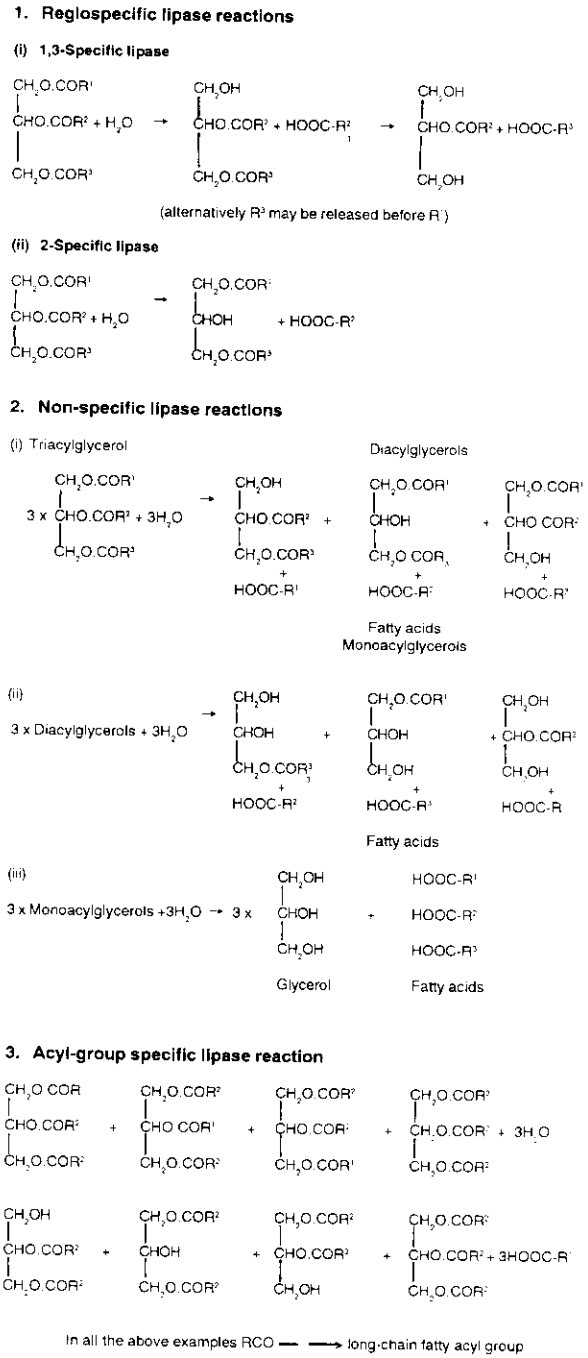

Fig. 3. *Lipase catalysed reactions.*

the available lipases do not act on glycerol 2-mono fatty acid esters, the rate of hydrolysis slows down and awaits the acyl migration to complete before enabling the lipase to attack the glyceride at the 1 and/or the 3 position. Interestingly, lipases function at the oil–water interface. The amount of oil available at the interface determines the activity of the enzyme. This interface area can be increased substantially up to a saturation limit by the use of emulsifiers as well as by agitation. The saturation limit depends upon the ingredients used and the physical conditions deployed. Thus, the activities of lipases can be pronouncedly increased by the use of agents and methods which increase the size of the emulsion micelles[4–6].

Lipases are not involved in any anabolic processes *in vivo*. Since these enzymes act at the oil–water interface, they can be used as catalysts for the preparation of industrially important compounds[1,7]. As lipases act on ester bonds, they have been used in fat splitting, interesterification (transesterification), development of different flavours in cheese, improvement of pallatability of beef fat for making dog food, *etc*. A current application involves the use of lipases in water deficient organic solvents for synthesising different value-added esters from organic acids and alcohols. Lipases which are stable and work at alkaline pH, say 8 to 11, which are usually the suitable wash conditions for enzyme-containing detergent powders and liquids, have also been found, and these hold potential for use in the detergent industry[1].

Extensive research work has been carried out on plant lipases[8], animal lipases[9,10] and microbial lipases, particularly bacterial and fungal lipases[4–7]. Although pancreatic lipases have been traditionally used for various purposes, it is well established that microbial lipases are preferred for commercial applications due to their multifold properties, easy extraction procedures and potential of unlimited supply[1]. In the present study, we shall briefly review the important areas of research in bacterial lipases and their properties, and will discuss at greater length, the fungal lipases describing their useful properties, applications and commercial usage.

Bacterial lipases

Compared to plant and fungal lipases, a relatively small number of bacterial lipases have been well studied and reviewed[11,12].

Generally, bacterial lipases are glycoproteins but some extracellular bacterial lipases are lipoproteins. The production of extracellular lipases from bacteria is often dependent on nitrogen and carbon sources, inorganic salts, presence of lipids, temperature and avail-

ability of oxygen. It was reported in 1979[13] that enzyme production in most bacteria is affected by certain polysaccharides. Most of the bacterial lipases reported are constitutive and inhibited by some serine hydrolase inhibitors. Most bacterial lipases are non-specific in substrate specificity and a few are thermostable[1].

Different genera of bacteria including *Streptomyces* spp. produce lipase but the following genera have been well exploited for lipase production: *Achromobacter* spp., *Alcaligenes* spp., *Arthrobacter* spp., *Pseudomonas* spp. and *Chromobacterium* spp.[7].

The bacterial genera *Staphylococcus* spp. and *Pseudomonas* spp. have been specifically investigated for commercial exploitation. Among the staphylococci, *S. aureus* and *S. hyicus* are two important species described for lipase production.

Lipases purified from *Staphylococcus aureus* and *S. hyicus* have molecular weights ranging between 34 and 46 kDa on SDS-PAGE. They are usually stimulated by divalent ions such as Ca^{2+} and the chelator EDTA acts as an inhibitor. The pH-optima of these enzymes varies between 7.5 and 9.0. Staphylococcal lipases are lipoprotein in nature[12].

Among pseudomonads, three important species of *Pseudomonas* i.e. *P. fragi, P. fluorescens* and *P. aeruginosa* have been exploited for production. The lipase from *P. fragi* was purified 68-fold by acidification of the culture supernatant, followed by ammonium sulphate precipitation and finally purified by sepharose CL-6B chromatography with 48% recovery. The purified lipase consisted of a single sub-unit with molecular weight 33 kDa as determined by SDS-PAGE. The lipase was inhibited by Zn^{2+}, Fe^{2+}, Al^{3+} whereas Ca^{2+} activated the hydrolysis of long-chain fatty acid esters[14]. The lipase gene of *P. fragi* has been cloned and sequenced.

The lipase from *P. fluorescens* has been purified by chromatography on DEAE cellulose and octyl-sepharose CL-4B. The purified lipase showed a molecular weight of 45 kDa. The lipase from *P. aeruginosa* excreted during the late logarithmic growth phase is associated with lipopolysaccharide[12]. The lipase was purified by isoelectric focussing in an agarose gel containing CHAPS (3-cholamidopropyl dimethyl ammonio-1-proponsulfonate). The enzyme exhibited a molecular weight of 29 kDa on SDS-PAGE.

Fungal lipases

Work on fungal lipases started as early as the 1950s and comprehensive reviews have been presented by Lawrence[15] and subsequently by Brockerhoff and Jensen[5] who discussed various aspects of these

enzymes. Since then, many workers have exploited fungi as valuable sources of lipase due to the following properties: thermal stability, pH stability, substrate specificity and activity in organic solvents. Fungal lipases have benefits over bacterial ones due to the fact that present day technology favours the use of batch fermentation and low cost extraction methods. In this regard, a good number of fungi have been screened for lipase production[16]. The chief producers of commercial lipase are *Aspergillus niger, A. terreus, A. carneus* (the latter two have been investigated in our laboratory), *Candida cylindracea, Humicola lanuginosa, Mucor miehei, Rhizopus arrhizus, R. delemar, R. japonicus, R. niveus* and *R. oryzae*[7].

Taxonomic distribution of fungal lipases

Fungal lipases which degrade lipids from palm oil were investigated[16]. Fungi with lipolytic enzymes important in biotechnology have been listed[17]. Some of the major fungal genera reported producing lipase include: *Mucor, Rhizopus, Candida, Geotrichum, Aspergillus, Penicillium* and *Humicola*.

Among the Mucorales, the lipolytic enzymes of the moulds *Mucor hiemalis, M. miehei, M. lipolyticus, M. pusillus, Rhizopus japonicus, R. arrhizus, R. delemar, R. nigricans, R. nodosus, R. microsporus* and *R. chinensis* have been studied in great detail. The thermophilic *Mucor pusillus* is well known as a producer of thermostable extracellular lipase. From a lipase producing strain of *M. miehei*, two isoenzymes with slightly different isoelectric points, but a high degree of antigenic identity, have been isolated.

Due to their 1,3-(regio)-specificity, lipases of the genus *Rhizopus* are especially suited for the conversion of triglycerides to the corresponding monoglycerides and interesterification reactions of fats and oils having food and pharmaceutical applications. *R. japonicus* lipase has been used to produce hard butter suitable for chocolate manufacture by 1,3-(regio)-specific interesterification of palm oil with methyl stearate. The lipases of the different *Rhizopus* spp. show maximum activity towards medium chain fatty acids (C_8–C_{10}) and their molecular weights range from 40 to 45 kDa. In the case of *R. delemar*, extracellular and intracellular lipase isoenzymes have been isolated.

Lipase producers within the order Entomophthorales include *Entomophthora apiculata, E. coronata, E. thaxteriana, E. virulenta, Basidiobolus* spp. and *Conidiobolus* spp.

The genera *Pichia, Hansenula* and *Saccharomyces* are also reported to produce lipase. Two kinds of cellbound lipases were purified from *Saccharomyces lipolytica*.

Some of the reported strains of *Candida* that produce lipase are *C. curvata, C. tropicalis, C. valida* and *C. pellioculosa. Candida* lipases known so far are non-specific towards the different ester bonds in triglycerides, with the exception of *C. deformans.*

The imperfect fungus *Geotrichum candidum* has been considered to be responsible for acid formation in dairy products by lipolysing fat. The *G. candidum* lipase features a clear cut specificity towards fatty acids with a *cis* double bond at C_9, therefore, this enzyme has been applied in the structural analysis of triglycerides.

Species of the mould *Aspergillus* are well known lipase producers. Most experiments reported deal with the lipase from *Aspergillus niger* whose intracellular and extracellular lipases are 1,3-regio-specific. *Aspergillus oryzae* was found to be an efficient host for the heterologous expression of the lipases from *Rhizomucor miehei* and *Humicola lanuginosa.* We have demonstrated that *Aspergillus terreus* produces a lipase that is thermostable (100% stable at 60°C and 70°C for 15 minutes), pH stable (5.5–10.5), 1,3-regio-specific and capable of trans-esterification and esterification in organic solvents. The enzyme catalyses deacetylation of peracetates of polyphenolic ketonic compounds at both the positions, *i.e. ortho* and *para* to the carbonyl function. The lipase also distinguishes between phenolic esters and the esters of aromatic acid and alcohol. Our strain of *A. carneus* produces a lipase that has an optimum pH value of 9.0 and is stable in various organic solvents and detergents and 1,3-regiospecific.

The lipase synthesised by *Penicillium roquefortii* is largely responsible for the development of the characteristic flavour of blue cheese. Lipolytic activity has also been detected in *P. camembertii*, the white surface mould of brie and camembert cheese. Lipases with a clear cut specificity for butyric acid have been isolated from a variety of strains of the genus *Penicillium* such as *P. cyclopium, P. verrucosum* var. *cyclopium* and *P. crustosum.* The *P. cyclopium* lipase has a much higher activity towards di- and mono-glycerides than triglycerides.

The lipase of *Humicola lanuginosa* DSM 3819 is suitable as a detergent additive because of its thermostability, high activity at alkaline pH and stability towards anionic surfactants. *H. lanuginosa* lipases show a high degree of hydrolytic activity with coconut oil and oils having a high content of lauric acid. The two lipases differ in their positional specificity[16].

Thus, it can be said that lipase producers are widespread in the fungal kingdom and are of much biotechnological interest in both research and applications.

Lipase production conditions

Optimisation of cultural parameters

Most of the microbial lipases are extracellular, being excreted through the cell membrane(s) into the culture medium. The amount of lipase produced is dependent upon several environmental factors, such as temperature, pH, nitrogen, carbon and lipid sources, agitation and dissolved oxygen concentration. A variety of media compositions have been worked out for different organisms, for example, *Pseudomonas fragi*, *Aspergillus wentii*, *Mucor hiemalis*, *Rhizopus nigricans* and *Mucor racemosus*[18,19].

Lipase production is generally stimulated by lipids and is usually coordinated with the availability of triglycerides. Certain inducers also have a profound effect on the stimulation of lipase production. The inducers include: triglycerides, free fatty acids, hydrolysable esters, bile salts and glycerol. The variable effects of addition of lipids on lipase production has been studied (Table 1). Evidence is, however, divided on lipids being considered as the true inducers[18].

Emulsification of culture media containing oil by gum acacia supported good growth and lipase production by *Rhizopus oligosporus*. Triolein, olive oil, tributyrin and oleic acid butylester were able to

Table 1. *The effect of fats and fatty acids on lipase production by various microbes.*

Lipid source	Organism	Effect on lipase production
Synthetic and natural lipids	*Aspergillus wentii*	Decrease
Olive oil	*Saccharomycopsis* sp. *Bacillus licheniformis*, *Staphylococcus* sp., *Mucor caseiolyticus*	Decrease
Olive oil, corn oil, butter oil	*Penicillium roqueforti*	Decrease
Olive oil, groundnut oil, cotton seed oil	*Pseudomonas mephitica*	Increase
Tributyrin and Trioctanoin	*P. fragi*, *M. freudenreichii*	No effect
Unsaturated fatty acids	*P. fragi*	Decrease
Oleic, linoleic and linolenic acids	*P. mephitica*	Increase

induce lipase in immobilised protoplasts whereas Tween 80 enhanced lipase activity in them. Lipase activity is strongly induced by a wide range of fatty acyl esters including triglycerides, spans, and Tweens and was repressed by long-chain fatty acids including oleic acid.

In general, it has been observed that the type of nitrogen source in the medium determines the production levels of lipases. High production of lipase in the case of *Pseudomonas* spp. generally occurs in a peptone supplemented medium although different peptones vary in their effectiveness[18,19]. However, growth and lipase production by a *Micrococcus* sp. were unaffected by a concentration of peptone between 0.5 and 2%. In *Aspergillus wentii, Mucor racemosus* and *Rhizopus nigricans*, lipase yield was maximum with peptone at 2% level.

Soybean meal extract in *Rhizopus oligosporus* culture medium supports good growth and lipase production. Physiological regulation of lipase activity by a thermotolerant strain of *Pseudomonas aeruginosa* (EF_2) under various conditions in batch, fed batch and continuous cultures supports the fact that nitrate generally stimulates production of lipase. In some cases, individual amino acids have been shown to play important roles. A strain of *Penicillium roqueforti* produces maximum amount of lipase when grown in 0.5% casitone–1% proflobroth. Lipase production was stimulated in *P. aeruginosa* by the addition of alanine to the medium. Milk is a good medium both for the growth of psychrotrophic bacteria and for lipase production. However, it is very susceptible to microbial spoilage and hence, not recommended for microbial cultivation.

The requirement for sugar as carbon source in addition to lipids varies with the organism. It has been shown that glucose is essential for production of lipase by *P. fragi*, whereas *P. aeruginosa* had no such requirement. The effect of sugars as the carbon source in fungi is presented in Table 2. It can be observed that in general, media supplemented with glucose stimulated maximum lipase production in case of all the fungi.

Polysaccharides such as glycogen, hyaluronate, laminarin, gum arabic and pectin stimulated production of lipase in *Serratia marcescens* and by *Saccharomycopsis lipolytica*. This might be due to the detachment of lipase from the oil surface. A similar mechanism may explain the stimulating effect of lecithin on lipase production as investigated in *Rhizopus japonicus*.

The initial pH of the growth medium is also important for lipase production. Maximum activity was observed at pH > 7.0 for *Pseudomonas fragi* and at pH 9.0 for *P. aeruginosa*. Development of

Table 2. *The effect of sugars as additives on lipase production in fungi.*

Organism	Sugar as additive
Mucor hiemalis	Glucose
M. racemosus	Glucose > mannitol, sucrose, raffinose, galactose, maltose, fructose, ribose, lactose
Rhizopus nigricans	Glucose > galactose, mannitol, fructose, sucrose, lactose, maltose
Aspergillus wentii	Glucose > mannitol, galactose, sucrose

acidity in media containing fermentable carbohydrates was accompanied by reduced lipase activity. It has been reported that at 20°C more lipase was produced at pH 7.0 than at pH 8.0 by nutrient broth cultures of *Saccharomycopsis lipolytica* and *M. caseiolyticus*. *B. licheniformis* and *A. wentii* showed maximum growth and lipase production at pH 6.0 when cultivated under shake conditions. It has been observed that *Mucor hiemalis* produced maximum lipase when the initial pH of the medium was kept at 4.0. *Rhizopus nigricans* showed maximum growth and lipase production at pH 6.0. *Mucor racemosus* isolated from butter exhibited maximum lipase production at 22°C and pH 5.0. The yield of lipase was maximum at pH 6.5 in the case of *Rhizopus oligosporus*[19]. Maximum lipase activity was reported at pH 6.5 in Tween 80-limited continuous cultures in the case of *Pseudomonas aeruginosa* EF_2.

In general, lipases are produced between 20 and 45°C. However, the optimum temperature for lipase production corresponds with the growth temperature of the respective microorganisms, for example, the best temperature for lipase production and growth in the case of *Rhizopus nigricans* was 30°C while for *Talaromyces emersonii*, it was around 45°C.

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)[18]. Vigorous aeration also greatly reduced lipase production by *Pseudomonas fragi*, *P. aeruginosa* and *Rhizopus oligosporus*. However, high aeration (shaking) was needed for high lipase activity by *P. mephitica* var. *lipolytica*. Changing the ratio of surface area to volume and hence, aeration of cultures of *P. fragi* had no effect on the quantity of lipase produced per cell, but increasing aeration by shaking resulted in both increased growth and lipase production, followed by a

rapid decrease of lipase activity as shaking continued. It has been reported that stationary conditions in *Talaromyces emersonii* and *Mucor racemosus* favoured maximum lipase production. However, *Aspergillus wentii* and *Mucor hiemalis* showed maximum lipase production when cultivated in shake cultures.

Lipase assay procedures

Lipases hydrolyse triglycerides and give rise to free fatty acids and glycerol. Therefore, the assay methods for these enzymes have generally been developed around the criteria of testing the free fatty acid formed, either spectrophotometrically or titrimetrically in broth cultures[20]. However, lipase activity can be detected as a clear zone on tributyrin agar plates where tributyrin is hydrolysed to form free fatty acids and glycerol[21].

Tributyrin plate assay and titrimetry are the most commonly used methods for screening of lipase producers and estimation of lipase activity. There are many other methods and procedures that have been developed using a variety of microorganisms. A brief survey of the literature on the different assay procedures for lipase activity has been summarised in the present text.

Spectrophotometric assay

A rapid colorimetric assay based on the colour change of the pH indicator, phenol red, as butyric acid is released from tributyrin has been developed for pancreatic lipase. A colorimetric assay for urinary lipase based on the hydrolysis of the colourless 8-carbon ester-β-napthyl caprylate to yield β-napthol was developed. A sensitive, rapid and reproducible copper soap colorimetric determination of fatty acid using the cupric acetate–pyridine method was simplified by eliminating the solvent evaporation and centrifugation steps by substituting isooctane for benzene. A colorimetric assay for *Rhizopus arrhizus* lipase using long-chain fatty acids and the ester of 1 mercapto-2,3-propandiol or 2-mercaptoethanol as substrates has been developed. *p*-Nitrophenyl palmitate is also used for lipase estimation in bacterial and eukaryotic systems[20].

A turbidimetric assay for *Lysobacter enzymogenes* measuring the increase in optical density at 500 nm caused by hydrolytic release of fatty acids from Tween-20 and their precipitation with $CaCl_2$ has been developed. A naturally occurring fluorescent 18-carbon fatty acid, *cis*-parinaric acid (9,11,13,15 – *cis*, *trans*, *trans*, *cis* octadecatetraenoic acid) has been used to develop another assay for *Candida cylindracea* and porcine pancreatic lipases.

Radiolabelling assay

The use of phorbol esters and diacylglycerol as substrates for assay of various mammalian lipases has been summarised in the literature. An improved, sensitive and reproducible lipase assay monitoring the binding of ^{63}Ni fatty acid using triolein as substrate has been developed for different lipases[22].

Fluorescence assay

A new sensitive fluorescent plate assay for bacterial lipase(s) using rhodamine B has been developed. The assay uses trioleoylglycerol as substrate and the assay can be used to identify lipase producing bacteria and other microorganisms. In the presence of rhodamine B and substrate, fluorescence by an unknown mechanism occurs when lipase producing colonies are exposed to UV light at 350 nm.

HPLC assay

A simple and sensitive method for measuring the released fatty acids from an emulsified triolein solution has been developed for lipase(s) from *Pseudomonas* sp., *Rhizopus arrhizus, Candida rugosa* and *Geotrichum candidum.*

Titrimetric assay

Titrimetric methods measure the rate of neutralisation of sodium or potassium hydroxide by released fatty acids as a function of time[23]. A newer method uses olive oil emulsified by periodic sonication without the addition of colloids or surfactants in order to obtain more natural conditions. The method works especially well in the case of lipases from *Chromobacterium viscosum, Candida rugosa, Aspergillus niger* and *Rhizopus arrhizus.* Although the assay is highly reproducible, it is time consuming.

Surface tension assay

The change in surface tension of the lipid monolayer caused by lipase mediated hydrolysis can also be used to determine lipase activity.

Monitoring lipase catalysed reactions

The catalysis is monitored by measuring the change in the following parameters: (a) free fatty acids (FFA) content; (b) monoglyceride, diglyceride and triglyceride contents and (c) full lipid profile.

The major problems in monitoring the above parameters are that

often the selected methods are time consuming; opportunities for error increase when there are many steps for handling the sample; and the presence of emulsifiers complicates extraction and isolation procedures. The variation in the pK_a values of fatty acids are from 4.5 for butyric acid to 8.5–9.0 for longer chain fatty acids like myristic or stearic acid, which factor may affect the full extraction of FFA depending upon the pH used in the assay; reaction mass is often not homogenous and, therefore, representative samples should be collected from well-stirred reaction vessels; and finally the presence of calcium and magnesium ions in the reaction mixture could affect the accuracy as they form insoluble salts with the FFA. It is often necessary to run blank samples to enable calculation of relative changes.

Monitoring total FFA

In the pH stat method, the reaction vessel is fitted with an agitator for good mixing, the probes of a pH meter are held inside the vessel and there is a way to measure the amount of alkali added or consumed in the system. The principle is that as lipolysis proceeds, the FFAs released tend to lower the pH of the reaction mixture which is restored by the addition of standard alkali solution. The amount of alkali consumed is proportional to the FFA released. The method is rapid and is used for screening enzymes and for determining dose requirements.

In the AOCS acid value method, a sample is drawn and mixed with isopropyl alcohol and toluene. The sample is then titrated with a standard solution of caustic potash to a phenolphthalein end point. This method is often used to measure FFAs in crude and refined fats and oils.

In the direct titration method[24], a sample collected is mixed with ethanol to emulsify the mixture. The sample is then titrated to thymol blue end point (pH 9.5). The method is rapid and is used to screen enzymes or estimate doses. However the data generated are only indicative, as many FFAs are not completely soluble in the solvent system.

The dole extraction and titration method is widely used to measure the full amount of FFAs in a wide spectra of fats and oils. In this method, the sample is mixed with heptane and isopropyl alcohol mixture at acidic pH to enable complete extraction of FFAs in the solvents. More heptane or water is added to break the emulsions. An aliquot of the extracted solvent layer is taken and mixed with alcohol and titrated with standard potassium hydroxide to thymol blue end point (pH 9.5). There are many steps involved in this method although it gives accurate results. The method is followed in de-

veloping various cheese flavours in dairy products, as such flavours are related with the nature and type of FFA in those products.

In the Folch method, a sample of the reaction mixture is acidified and repeatedly extracted with methylene chloride – methanol mixture. The organic phases are consolidated and the solvent evaporated. The residual fat/oil is then titrated with standard potassium hydroxide solution using alcohol as the solvent. This method is also quite tedious and time consuming although it is quite accurate.

The silicic acid titration and filtration method is followed for dairy products or other products containing large amounts of non-lipid materials like proteins, metal ions *etc*. In this method, the sample is acidified and mixed with silicic acid in powdered form using a mortar and pestle. The mixture is then extracted with methylene chloride – methanol and filtered. The filtrate is titrated with a standard solution of caustic potash using alcohol as the diluent. The silicic acid aids the binding of extraneous materials which may interfere with the titration method.

Monitoring of glycerides

During the production of monoglycerides (MG) through glycerolysis or the preparation of cocoa butter substitutes from palm oil by an interesterification reaction, the measurement of MG as well as triglycerides (TG) becomes very important. Several methods are available to monitor such reactions.

In the AOCS method for the measurement of alpha monoglycerides, the sample is extracted and oxidised with periodic acid; the excess periodic acid is back titrated. Periodic acid oxidises the 1, 2-adjacent alcohol of MG. The β-monoglycerides cannot be measured by this method; after measuring the MG value, an estimate of 5–8% of MG value is assigned to beta monoglyceride content.

In the NOVO industrial method, the total TG is measured by dissolving the sample in heptane and loading the extract onto activated alumina. The TG is then eluted using diethyl ether, solvent is evaporated and the residue measured by weighing to get the TG content. The TG can be further analysed by thin layer chromatography. This method gives insight into residual TG during and after the end of the lipolysis reaction.

Lipid profile determination

The methods narrated earlier do not give insight into the fatty acid composition or the triglyceride profile or the types of changes in the

lipid mixtures taking place during the course of lipase catalysed reactions. In many situations where the reactions are directed towards imparting specific characteristics to modified fats and oils, the quantitative information becomes essential. In such situations, the following analytical methods become relevant.

Fatty acid profiling

In the official AOCS method, the fatty acids are converted into their methyl esters and subjected to quantitation by gas chromatography.

In a separate procedure usually followed in the dairy industry, the free fatty acids are extracted from the sample, acidified with sulphuric acid mixed with silicic acid using petroleum ether containing 1% butanol. The eluate is directly analysed by gas chromatography.

Triglyceride profiling

The sample extracted using organic solvents is resolved by high-performance liquid chromatography (HPLC). In this method, the sample is extracted to isolate total lipids using organic solvents. Aliquots are spotted on TLC plates of activated silica gel using several solvent systems. After resolving the mono-, di and triglycerides on the plate one-dimensionally, the plate is dried and sprayed with cupric acetate or concentrated sulphuric acid, and charred to locate the lipid class spots. The spots could be quantitated by a densitometer.

In the HPLC method, the sample is treated to convert the free fatty acids to their respective methyl esters and the modified sample can be resolved over RPC–HPLC system.

As regards isolation of the lipases from the fermented broth, the usual process adopted is to filter the culture broth by centrifugation (at around 2,000*g*) when the broth handled is small,or by plate and frame filtration or rotary vacuum filtration as the case may be, when the broth volume is large (>10 kl). The clear filtrate can be directly concentrated by ultra-filtration in a hollow fibre Amicon model with say 30 kDa cutoffs, to 30–35 fold concentration; further concentration can be made by using membrane filters, say DDS-GRGIPP membrane from De Damske Sukkerfabrik. The concentrated mass may be dialysed in water to remove salts, and then freeze-dried. Alternatively, the clear filtrate can be saturated with ammonium sulphate to obtain a saturation of 50–90%, to obtain the proteins precipitated. The proteins can then be separated by high speed centrifugation (15,000–18,000*g*). The residues are then dissolved in small volumes of buffers to maintain the most stable pH and fractionated

over Sephadex Column (say G-200) to obtain the lipase fraction which can then be concentrated by ultrafiltration and freeze dried.

Analytical methods

Research has usually concentrated on the determination of extracellular lipase activity and protein concentration. Consequently, samples have been withdrawn from the growing cultures, filtered (through say glass wool) and frozen. Lipolytic activity has been determined with the continuous titrating pH stat method using standard sodium hydroxide (usually 0.1 N) as the titrant. Protein concentrations have been determined with say Bio-Rad protein assay method (Bio-Rad Lab., Richmond, C.A., USA). A unit lipase activity (U) relates to the release of 1 μmole of free fatty acid from emulsified olive oil or triolein or tributyrin per minute at specified temperature and pH values. Specific activity of lipases are expressed as units of lipolytic activity per microgram of extracellular protein.

Purification

Lipases have been purified from animal, plant, fungal and bacterial sources by different methods[25,26]. Most of the purification procedures reported involve a series of nonspecific techniques, such as ammonium sulphate precipitation, gel filtration and ion exchange chromatography. In recent years, affinity chromatographic techniques have come into use as this technique decreases the number of steps necessary for lipase purification. More recently, reversed-micellar and non-aqueous two phase systems, membrane ultrafiltration and immunopurification have also been used mainly for purification of lipases of microbial origin.

Purification of fungal lipases

Lipases secreted by various microbial sources have been purified from the culture filtrates by successive steps involving salt, organic solvent or polymer precipitation, gel filtration, ion exchange chromatography and hydrophobic interaction chromatography (*Mucor lipolyticus*, *M. javanicus*, *Penicillium cyclopium*, *P. camembertii*, *Neurospora crassa* and *Ustilago maydis*). 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.

A lipase produced by *Rhizopus delemar* was purified with a recovery of 3–4%. A lipase from *R. japonicus* NR 400 was purified to

homogeneity by chromatography on hydroxyapatite, octylsepharose and sephacryl S-200.

Novel purification technology

The usual procedures for lipase purification are sometimes troublesome and time consuming and usually result in a low final yield. Alternatively, novel purification techniques such as reversed micellar and the two phase system, membrane processes and immunopurification techniques have recently been applied to purification.

Properties of lipase

Substrate and reaction type

The natural substrates of lipase are triacylglycerols of long-chain fatty acids and the occurrence of the lipase reaction at an interface between the substrate and the aqueous phase is because of the reversible nature of the enzyme reaction. This is responsible for both hydrolysis and synthesis of reactants and products which is under the control of the water activity of the enzyme[1]. The enzyme acts on the substrate in a specific or non-specific manner resulting in either the complete hydrolysis of triglycerides into free fatty acids and glycerol or, along with triglycerides, monoglycerides, diglycerides, fatty acids and glycerol are also formed[1]. The kinetics of the lipolysis reactions have been discussed by Brockerhoff and Jensen[5]. 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 enzyme in aqueous solutions.

Under certain experimental conditions such as in the absence of water in a system, lipases are capable of reversing the reaction that leads to esterifiction and interesterification.

pH optima

The activity of lipase is pH dependent. Some lipases have shown considerable stabilty over a wide range of pH values. The ideal lipase would be the one which is active over the full pH range of 0–14; such a lipase may never be available in nature. Nevertheless, the lipases studied have usually shown profound stability at neutral pH or near the neutral pH range of 6.0 to 7.5, with considerable stability at acidic pH down to 4.0 to alkaline pH up to 8.0.

Extracellular lipases produced by *Aspergillus niger, Chromobacterium viscosum* and *Rhizopus* spp. are particularly active at

acidic pH. An alkaline lipase active at pH 11.0 has been isolated from *Pseudomonas nitroreducens*.

Temperature optima and thermal inactivation

The pancreatic lipases lose activity on storage at temperatures above 40°C but some microbial lipases are more resistant to heat inactivation. Thus, the enzymes produced by *Aspergillus niger*, *Rhizopus japonicus* and *Chromobacterium viscosum* are stable in solution at 50°C and the thermotolerant fungus *Humicola lanuginosa* excretes a lipase which is stable at 60°C. A strain of *Pseudomonas nitroreducens* produces a lipase which is stable at 70°C.

The temperature stability profiles determined by half-life values show maximum stability at lower temperatures. Thus, *Calvatia gigantea* lipase had values of half-life of 35.7, 46.4 and 22.9 at 45°C, 50°C and 55°C; results were similar for the lipases obtained from *R. japonicus*[27]. As regards the maximum lipase activities versus temperature, the highest activities for *C. gigantea* lipase and many others were at about 30–35°C. All these lipases were, however, derived from mesophilic sources. Thermophilic bacterial lipases obtained from organisms from Icelandic hot springs had higher lipase activity at 40–60°C. The characteristics of lipases obtained from different sources thus needs careful study at molecular level to enable insights into the protein structure and sequence for enabling the designing of thermostable lipases.

Activation and inactivation of the enzyme

Cofactors are not essential for the expression of lipase activity but divalent cations such as calcium stimulate the activity. It has been postulated to be based on the formation of calcium salts of long-chain fatty acids[1,7]. 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.

In *Humicola lanuginosa* S-38, sulphhydryl reducing agents like dithiothreitol did not alter enzyme activity, but rendered it more susceptible to heat inactivation. Inactivation is also accelerated by the addition of urea. Reducing compounds (cysteine, 2-mercapto-ethanol), chelating agents such as ethylene diamine tetraacetic acid (EDTA) and O-phenanthroline, and thiol group poisons (*p*-chloro mercuricbenzoate, monoiodoacetate) did not have a detectable effect on lipase in *Mucor pusillus*, suggesting that lipase is not a metallo-enzyme and does not require either free-SH group(s) or an intact S–S bridge for its activity. Spontaneous and cyclic AMP induced lipase formation is greatly enhanced in *Serratia marcescens*

SM-6 on exposure to glycogen, hyaluronate, pectin B and gum arabic.

Substrate specificity

Specificity of lipase is controlled by the molecular properties of the enzyme, structure of the substrate and factors affecting binding of the enzyme to the substrate[28].

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[28]. Simple alkyl esters of fatty acids are useful substrates for investigating fatty acid specificity, because they are liquids at normal assay temperatures and the alcohol group can be kept constant as the fatty acyl group is altered[1].

In general, lipases show little fatty acid specificity when incubated with most natural oils and fats. Exceptions occur when fish oils and milk fat are used as substrates[1]. The presence of double bonds close to the carboxyl groups in some fatty acids probably makes their esters resistant to attack by lipases. Tributyrin is hydrolysed slowly by some microbial lipases. In contrast, *Mucor miehei* lipase preferentially releases butyric acid from milk fat especially at low pH. 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. Substrate specificity of this lipase has been summarised. Lipases show both regio- and stereo-specificity with respect to the alcohol moiety of their substrates.

Lipases can be divided into two groups according to the regio – specificity exhibited with acylglycerol substrates[1] (Figure 3). Lipases in the first group show no regio-specificity and release fatty acids from all three positions of glycerol. These lipases catalyse the complete breakdown of triacylglyerol to glycerol and free fatty acid along with diacylglycerols and monoacyl glycerol as intermediates in the reaction. These intermediates do not accumulate because they are hydrolysed more rapidly than triacyl glycerol. Examples of the first group of lipases include lipase from *Candida cylindracea*.

The second group of lipases release fatty acids regiospecifically 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. The rate of hydrolysis of

triacylglycerol is normally faster than that of diacylglycerols and consequently substantial quantities of both diacylglycerols and monoacyl glycerol accumulate during the reaction. This is because 1,2-(2,3)-diacylglycerols and 2-monoacylglycerol are chemically unstable, and undergo acyl migration to give 1,3 diacylgycerol and 1-monoacylglycerol respectively[1]. Prolonged incubation of an oil with these enzymes will give complete breakdown of some of the acylglycerol with the formation of glycerol. Many extracellular microbial lipases such as those from *Aspergillus niger* and *Rhizopus arrhizus* show 1,3-regio-specificity. Lipases excreted by *Rhizopus japonicus, Mucor miehei, Humicola lanuginosa, Chromobacterium viscosum* and *Pseudomonas fluorescens* are also 1,3-regio-specific[1].

In general, the regio-specificity of the enzymes listed above is almost absolute and probably results from an inability of sterically hindered esters of secondary alcohols (*e.g.* those of the 2-position of glycerol) to enter the active sites of the enzymes. To date, there are no authentic reports of lipases which catalyse the release of fatty acids selectively from the central 2-position of acylglycerols, except for the report of Asahara *et al.*[3].

Partial stereo-specificity in the hydrolysis of triacyl glycerols has been observed in *Rhizopus arrhizus, R. delemar, Candida cylindracea* and *Pseudomonas aeruginosa*. Owing to this property, these enzymes can be used to isolate optically pure esters and alcohols.

Production of an extracellular microbial lipase possessing pronounced stereo-specificity in the hydrolysis of triacyl glycerols would be of considerable commercial interest. Most lipases attack triglycerides as readily as partially esterified glycerides, but an enzyme from a specific *Penicillium cyclopium* strain has been shown to attack monoglycerides most rapidly followed by di- and triglycerides respectively and it has been described as a partial glycerol ester hydrolase. Properties of some important commercially produced microbial lipases are illustrated in Table 3.

Applications of lipases

Several versions of microbial lipases have already been introduced commercially as indicated in Table 4.

Lipases in organic synthesis

The application of lipases as catalysts in organic synthesis has great advantage for synthetic chemists as they can act on a variety of

Table 3. *Properties of some microbial lipases.*

Organism	Specificity	Molecular weight (kDa)	Isoelectric point	pH optimum	Temp. optimum (°C)	Specific activity (U mg^{-1} lipase)
Chromobacterium viscosum	unspecific	30	7.3	6.5–7.0	70	22.75
Pseudomonas spp.	regio 1,3	32	4.5	7.8	47	7.80
Pseudomonas fluorescens	regio 1,3	32	4.5	7.0	50–55	3.05
Candida cylindracea	unspecific	120	4.2	7.2	45	53.22
Candida curvata	18:1 > 16.0 = 14:0	195	–	5.0-8.0	60	4
Candida deformans	regio 1,3	207	–	7.0	80	19
Aspergillus niger	regio 1,3	38	4.3	5.6	25	9.02
Geotrichum candidum	cis-Δ^9-unsaturated fatty acids	55	4.3	6.6	40	14.2
Humicola lanuginosa	unspecific	27.5	–	8.0	60	5.16
Mucor sp.	regio 1,3	–	–	7.0	35	0.39
Mucor miehei	regio 1,3	–	–	8.0	40	3.25
Penicillium cyclopium						
A lipase	unspecific	27	4.9	7.5	35	0.09(A+B)
B lipase	unspecific	36	4.1	5.8	40	–
Rhizopus arrhizus	regio 1,3	43	6.3	8		16.08
Rhizopus delemar	regio 1,3	41.3	4.2	5.6	35	2.20

substrates (Figure 4). These enzymes can show stereo- and regio-specificity and tolerate organic solvents in the incubation mixture. Both the activity of the enzyme and the identity of the product depend upon the solvents used, which may vary from aqueous buffer systems through biphasic emulsions and microemulsions, to organic solvents[27,28].

For synthetic purposes, crude enzyme preparations are often convenient. Because the catalyst is always an expensive factor in

Table 4. Some industrially available microbial lipases.

Strain	Manufacturer	Thermo-stability	Optimal pH	Positional specificity	Molecular weight (kDa)
Aspergillus niger	Amano Pharmaceuticals, Japan	50% at 60°C, 15 min	5.6	α, α′	38
Candida cylindracea (Candida rugosa)	Meito Sangyo, Japan	40% at 50°C, 10 min	7.0	α, β, α′	55
Pseudomonas fluorescens	Amano Pharmaceuticals, Japan	60% at 60°C 30 min	7.0	α, α′	31
Rhizopus japonicus	Osaka Saikin Kenkyusho, Japan	50% at 55°C, 30 min	5.0	α, α′	30
Rhizopus niveus	Amano Pharmaceuticals, Japan	60% at 50°C, 30 min	7.0	α, α′	N.A.
Rhizopus arrhizus	Sigma, USA	N.A.	7.0	α, α′	N.A.

a chemical reaction, strategies for enzyme recycling are being developed.

Synthetic strategies involving microbial lipases can be used to prepare molecules of high positional and configurational purity. Lipases can be used to create biologically active analogues of naturally occurring messenger molecules such as antagonists or inhibitors in biological systems.

Lipases catalyse the hydrolysis of water-immiscible triglycerides at the water–lipid interface. Under given experimental conditions, the amount of water in the reaction mixture will determine the direction of the lipase-catalysed reaction. With water absent, or present in trace quantities only, esterification and transesterification are favoured; with excess water, hydrolysis occurs.

(1) Bioconversions in aqueous media

A typical lipase catalysed reaction in aqueous media is ester hydrolysis. This enzymic conversion can be used for the synthesis of triglycerides as shown for the preparation of platelet-activating

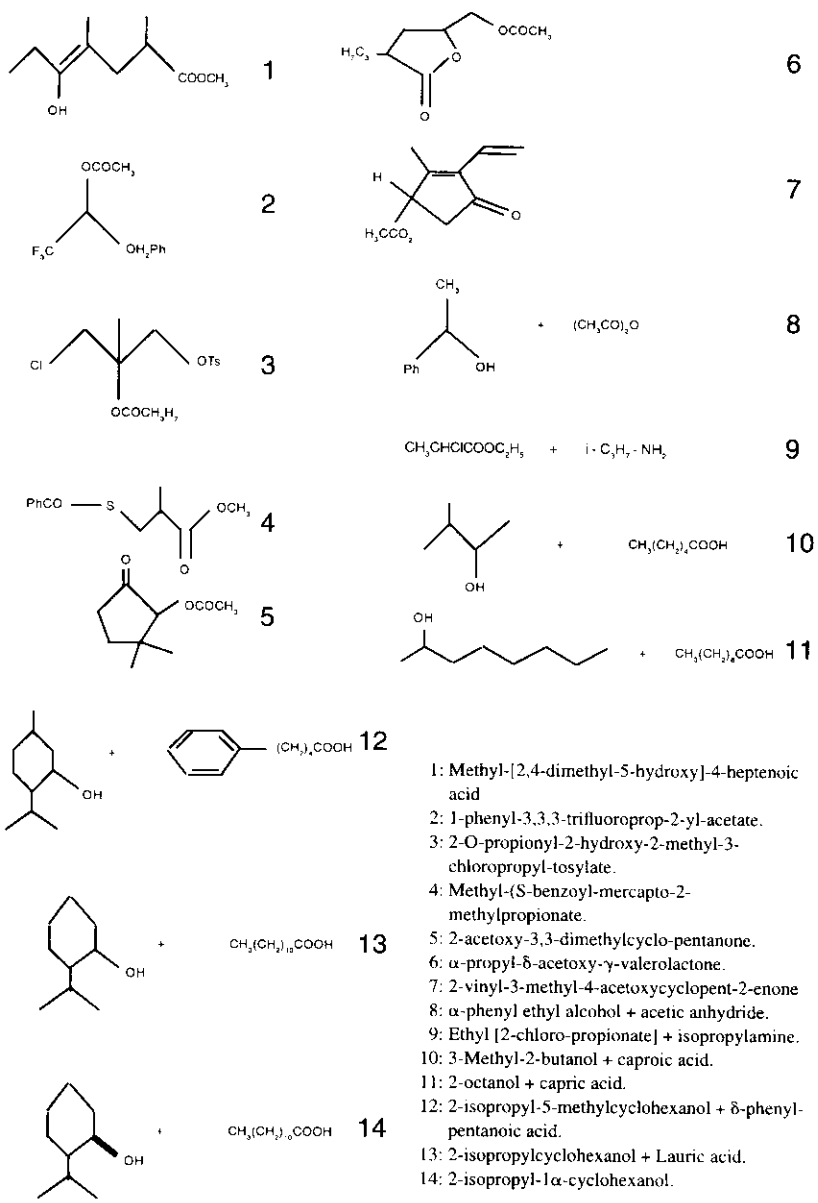

Fig. 4. *Types of substrates employed in lipase catalyzed synthesis.*

factor. Another application of the hydrolytic specificity of lipases is the partial hydrolysis of triglycerides to di- and mono-glycerides in the food industry where di and monoglycerides serve as biocompatible emulsifiers and food additives. These and other applications of lipases in industry and research have been discussed by many workers.

(2) Bioconversions in organic media

The synthetic potential of lipases in organic solvents has been widely recognised and is well documented in several publications. An important prerequisite for this development was the recognition that lipases work in organic solvents with low water content[29].

The main application of lipases in organic chemistry is the resolution of enantiomeric compounds, making use of the enantioselectivity of these enzymes (Figure 5). The use of organic solvents

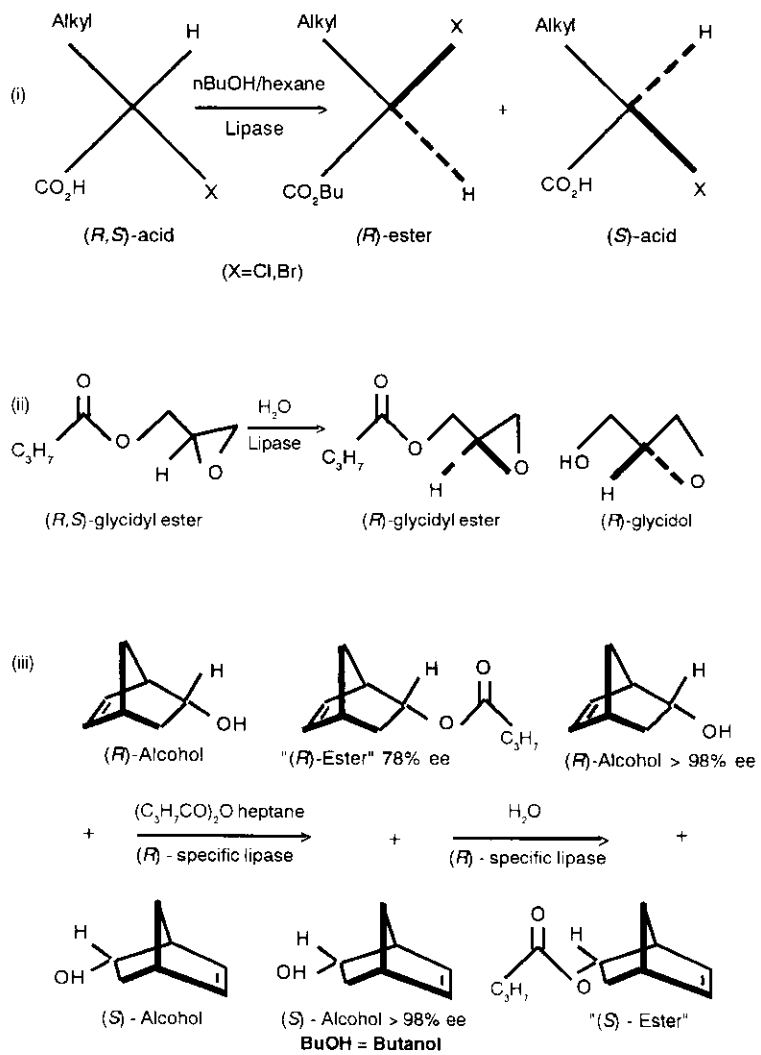

Fig. 5. *The resolution of racemic mixtures using microbial lipases.*

for lipase-catalysed resolutions has four main advantages in comparison with water as the solvent:

(a) Racemic mixtures of alcohols or acids need not be esterified before resolution into enantiomers.
(b) These enzymes are more stable in organic solvents than in water.
(c) Lipases used need not be immobilised for recovery, owing to their insolubility in organic solvents; they can be collected by filtration in their active state.
(d) Furthermore, substrates and products may be unstable in aqueous solution; in this case, reaction in organic solvents is essential for formation and isolation of the products.

The three main areas of lipase catalysed reactions in organic solvents include:

(i) Resolution of racemic alcohols

Racemic alcohols in non-aqueous media can be resolved in a biphasic system with lipase from *Candida cylindracea*. Trans-esterification reactions were performed in diethyl ether and heptane using porcine pancreatic lipase as the catalyst. Trans-esterification was also used to prepare useful synthons, such as optically active monoesters of 3-hydroxylglutarate and related compounds. Lipases have been used for the production of pure, biologically active S-enantiomer of sulcatol and for trans-esterification of cyanohydrin compounds, α-substituted cyclohexanol and epoxy esters.

(ii) Resolution of racemic acids by asymmetric esterification

The use of the stereo-selectivity of lipases for the resolution of racemic acid mixtures in immiscible biphasic systems has been demonstrated. Lipase from *C. cylindracea* has been applied to the resolution of 2-bromo and 2-chloropropionic acids, which are the starting materials for the synthesis of phenoxypropionic herbicides. *Pseudomonas fluorescens* lipase was used for asymmetric ring opening of substituted prochiral glutaric anhydrides of dicarboxylic acids. Porcine pancreatic lipase has been used for stereoselective lactonisations and polycondensations from prochiral hydroxy diesters. Macrocyclic lactones, such as (-) pyrenophorin can be synthesised stereoselectively by *Pseudomonas* spp. lipase from (w-1) – hydroxy alkanoic esters in anhydrous isooctane at 65°C.

(iii) Regioselective acylations

Lipase catalysed acylations are not limited to simple alcohols. Lipases also acylate with high regioselectivity certain steroids, sugars

and sugar derivatives such as monoacylated sugars. The regio-selective acylation of hydroxy groups in glycals, which are versatile chiral intermediates, is catalysed by lipases. Another example of the high regiospecificity of lipases is acylation reactions in anhydrous organic solvents for hydroxy steroids.

Lipases have been deployed for catalysing three major types of reactions namely trans-esterification (also called inter-esterification), hydrolysis and esterification (Figure 6).

As the net energy of a trans-esterification reaction is zero, these reactions are performed very easily. However, the other two types of reactions, especially esterification, are gaining significant industrial importance in the synthesis of value added esters used in the cosmetic

Transesterification Reactions:

(a) Acidolysis

$$R_1-\underset{\underset{O}{\|}}{C}-O\,R_2 + R_3-\underset{\underset{O}{\|}}{C}-OH \rightarrow R_3-\underset{\underset{O}{\|}}{C}-OR_2 + R_1-\underset{\underset{O}{\|}}{C}-OH$$

(b) Alcoholysis

$$R_1-\underset{\underset{O}{\|}}{C}-O\,R_2 + R_3-OH \rightarrow R_1-\underset{\underset{O}{\|}}{C}-OR_3 + R_2-OH$$

(c) Ester Exchange

$$R_1-\underset{\underset{O}{\|}}{C}-O\,R_2 + R_3-\underset{\underset{O}{\|}}{C}-OR_4 \rightarrow R_1-\underset{\underset{O}{\|}}{C}-OR_4 + R_3-\underset{\underset{O}{\|}}{C}-OR_2$$

(d) Aminolysis

$$R_1-\underset{\underset{O}{\|}}{C}-O\,R_2 + R_3-NH_2 \rightarrow R_1-\underset{\underset{O}{\|}}{C}-NH\,R_3 + R_2-OH$$

Hydrolysis

$$R_1-\underset{\underset{O}{\|}}{C}-O\,R_2 + H_2O \rightarrow R_1-\underset{\underset{O}{\|}}{C}-OH + R_2-OH$$

Ester Synthesis

$$R_1-\underset{\underset{O}{\|}}{C}-OH_2 + OH-R_2 \rightarrow R_1-\underset{\underset{O}{\|}}{C}-OR_2 + H_2O$$

Fig. 6. *Various lipase mediated reactions.*

industry, and the reaction is performed by controlling the water content in the reaction mixture.

(3) Trans-esterification reactions

When lipases are incubated with triglycerides, hydrolysis and resynthesis cause acyl migration between glyceride molecules. By controlling the quantity of water in the reaction system, it is possible to restrict the hydrolysis. The trans-esterification reactions can thus be made to dominate. If a nonspecific lipase is used, then the rearranged triglycerides become more or less the same as that obtained by chemical trans-esterification using sodium alkoxides as catalysts. However, by using 1,3-specific lipases, a mixture of triglycerides can be obtained which cannot be obtained by chemical methods as schematically illustrated in Figure 7.

Table 5 further demonstrates this selectivity through results obtained by trans-esterification of olive oil with stearic acid using *Rhizopus delemar* lipase as the catalyst. For this, a mixture of olive oil (2.5 g) and stearic acid (0.5 g) dissolved in 60–80°C petroleum ether (6 g) was stirred at 40°C for 24 hours with hydrated catalyst (250 mg) prepared from *R. delemar* lipase and kieselguhr.

Lipase catalysed trans-esterification reactions which are 1,3-specific are utilised for making cocoa butter substitutes using the cheaper midfraction of palm oil and stearic acid (Figure 8). The midfraction of palm oil contains 1, 3-dipalmitoyl-2-monoolein (POP) as

With chemical or nonspecific lipase catalysis:

$$\begin{bmatrix}A\\B\\A\end{bmatrix} + \begin{bmatrix}C\\B\\C\end{bmatrix} \rightarrow \begin{bmatrix}A\\A\\A\end{bmatrix} + \begin{bmatrix}A\\A\\B\end{bmatrix} + \begin{bmatrix}A\\B\\A\end{bmatrix} + \begin{bmatrix}A\\A\\C\end{bmatrix} + \begin{bmatrix}A\\C\\A\end{bmatrix} + \begin{bmatrix}A\\B\\C\end{bmatrix}$$

$$+ \begin{bmatrix}B\\B\\B\end{bmatrix} + \begin{bmatrix}B\\B\\A\end{bmatrix} + \begin{bmatrix}B\\A\\B\end{bmatrix} + \begin{bmatrix}B\\B\\C\end{bmatrix} + \begin{bmatrix}B\\C\\B\end{bmatrix} + \begin{bmatrix}B\\C\\A\end{bmatrix}$$

$$+ \begin{bmatrix}C\\C\\C\end{bmatrix} + \begin{bmatrix}C\\C\\A\end{bmatrix} + \begin{bmatrix}C\\A\\C\end{bmatrix} + \begin{bmatrix}C\\B\\C\end{bmatrix} + \begin{bmatrix}C\\C\\B\end{bmatrix} + \begin{bmatrix}C\\A\\B\end{bmatrix}$$

With 1,3-specific lipase catalysis:

$$\begin{bmatrix}A\\B\\A\end{bmatrix} + \begin{bmatrix}C\\B\\C\end{bmatrix} \rightarrow \begin{bmatrix}A\\B\\A\end{bmatrix} + \begin{bmatrix}A\\B\\C\end{bmatrix} + \begin{bmatrix}C\\B\\C\end{bmatrix}$$

Fig. 7. *Non-specific and 1,3-specific trans-esterification.*

Table 5. *Triglycerides formed by trans-esterification of a mixture of olive oil (5 parts) and stearic acid (1 part) using* R. delemar *lipase.*

Fatty acid	Amount in olive oil			Amount in trans-esterified oil		
	Total TG (%)	2-position (%)	1 & 3 position (%)	Total TG (%)	2-position (%)	1& 3 position (%)
16:0	16.6	3.5	23.2	13.7	3.2	18.9
16:1	1.8	1.3	2.0	1.6	1.6	1.6
18:0	2.0	1.0	2.5	15.6	0.7	23.0
18:1	66.8	72.0	64.2	56.6	72.2	48.0
18:2	12.8	22.2	8.1	12.6	22.3	7.7

Triglycerides formed by trans-esterification of a mixture of olive oil (5 parts) and stearic acid (1 part) using *R. delemar* lipase showing preferential substitution at 1 and 3 positions of glyceride 16:0 → Palmitic acid, 16:1 → Palmitoleic acid, 18:0 → Stearic acid, 18:1 → Oleic acid, 18:2 → Linoleic acid.

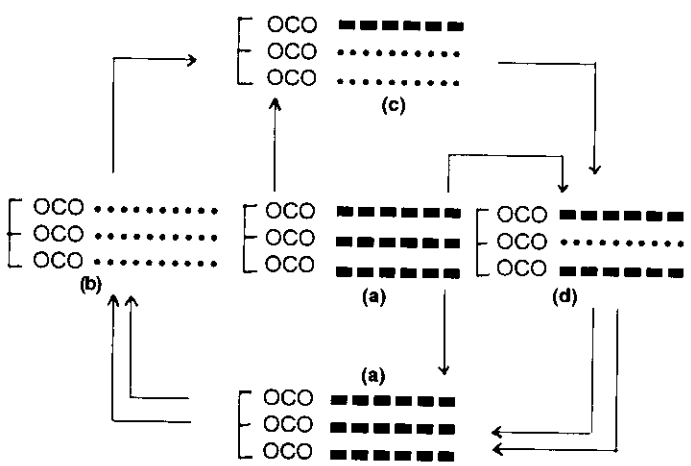

Fig. 8. *Application of lipases in trans-esterification.*

the major triglyceride, while 1-(3-) palmitoyl-3 (1-) stearoyl-2-monoolein (Post) and 1-(3-) distearyl-2-monoolein (Stost) are the main constituents of cocoa butter. Therefore, by trans-esterification between POP and stearic acid or POP and tristearin, valuable equivalents of cocoa butter have been made.

The midfraction of palm oil is obtained by double stage fractiona-

tion of the oils from n-hexane. The trans-esterification of the mid-fraction has been carried out at 37°C for 20 hours using one part of the midfraction, 0.7 parts of stearic acid dissolved in water saturated n-hexane (2.8 to 3 parts) and using 0.1 parts of celite-immobilized lipase (*Rhizopus arrhizus* lipase from Sigma of 400,000 units per mg activity adsorbed on celite by adding lipase solution in 0.2M phosphate buffer, drying it and hydrating it before use). The composition of the trans-esterified product approaches that of cocoa butter as is evident from Table 6. Sunflower and safflower seed oils have also been used for the production of cocoa butter substitutes in Japan.

(4) Hydrolysis

There is a large volume of literature on the hydrolysis of fats and oils by lipases used either in the pure form or in the immobilised form or in the cell bound form. The hydrolysis has usually been carried out in the conventional emulsion systems. The enzymatic hydrolysis has not however, replaced the conventional Colgate Emery Process. The saving in energy costs is not perhaps considered adequate to attract adoption of the lipase catalysed fat splitting process over the conventional chemical process. Some companies however, have been reported to have used lipases from say, *Candida cylindracea* for the splitting of oils, and the resulting fatty acids have been used for the production of soaps. It is claimed that the enzymatic method yielded soaps with better colour and odour and resulted in overall cost saving. Oils containing highly unsaturated or conjugated fatty acids are expected to be specially amenable to enzymatic hydrolysis processes. Currently, several investigations have been directed towards continuous hydrolysis of oils into fatty acids using membrane bioreactors or hollow fibre reactors.

The enzymatic method of fat splitting is expected to be industrially

Table 6. *Trans-esterified product from palm oil midfraction and different fatty acids.*

Fatty acid group	Palm oil midfraction (%)	Trans-esterified product (%)	Cocoa butter (%)
14:1	0.9	1.0	0.4
16:0	51.4	23.4	25.4
18:0	3.0	35.4	35.4
18:1	37.0	34.0	34.1
18:2	7.2	6.5	3.0
20:0	0.5	0.7	0.9

Trans-esterified product from palm oil midfraction and different fatty acids and its comparison with the composition of cocoa butter.

popular when the cost of energy rises or when special grades of susceptible fatty acid need to be produced in large quantities.

(5) Ester synthesis

Manufacture of esters, through lipase catalysed synthesis as well as alcoholysis, is gaining industrial importance. Several high-purity esters have been made for use in the cosmetic industry. The esters produced from short-chain fatty acids have applications as flavour constituents in the food industry, while methyl or ethyl esters of long-chain acids are expected to be used to enrich diesel fuels.

Lipase catalysed ester synthesis requires the maintenance of a low concentration of water. The available literature indicates a variation in water concentration from 0.75 to 4% (w/v) in the different types of ester synthesis. This condition is satisfied by using non-aqueous solvents like hexane.

Certain lipases also selectively esterify alcohols. Thus, *Rhizopus arrhizus* lipase of Novo converts selectively the geraniol into its acetate ester when a mixture of geraniol and nerol is subjected to esterification in hexane. Similarly a large number of lipases from various microbes are known to esterify a variety of alcohols with fatty acids (Table 7).

Table 7. *Lipase-catalysed synthesis of esters.*

Acid	Alcohol	Source of lipase	Conversion (%)
Erucic acid	methanol	*Pseudomonas* sp.	37
	octanol	*Rhizopus arrhizus*	74
	oleyl alcohol	*Humicola lanuginosa*	75
	oleyl alcohol	*Pseudomonas* sp.	79
	erucyl alcohol	*Humicola lanuginosa*	90
	erucyl alcohol	*Pseudomonas* sp.	84
	erucyl alcohol	*Mucor* sp.	41
2-Methyl -pentanoic acid	prenyl alcohol	*Candida cylindracea*	98
	2-methylbutan-1-ol	*Candida cylindracea*	92
Oleic acid	methanol	*Pseudomonas* sp.	37
	propan-2-ol	*Mucor* sp.	65
	2-ethylhexanol	*Candida cylindracea*	90
	octanol	*Rhizopus arrhizus*	90
	2-octyldodecanol	*Candida cylindracea*	73
	oleyl alcohol	*Candida cylindracea*	85
	erucyl alcohol	*Candida cylindracea*	86
	erucyl alcohol	*Mucor* sp.	81
	erucyl alcohol	*Pseudomonas* sp.	85

Applications of lipases in other industries

In modern industry, lipases have had their potential realised owing to their involvement in various industrial reactions either in aqueous or organic systems. It is realised that lipases are very important in the fields of detergents, pharmaceuticals, perfumes, flavour enhancers and texturising agents in cosmetic products as has been reviewed elsewhere[29,30] (Table 8).

Lipases in the dairy industry

Lipases are used extensively in the dairy industry for the hydrolysis of milk fat. Current applications include flavour enhancement of cheese, the acceleration of cheese ripening, the manufacture of cheese-like products and lipolysis of butter fat and cream. The addition of lipases that primarily release short chain (C_4 and C_6) fatty acids leads to the development of a sharp, tangy flavour, while 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

Table 8. *Important areas of industrial application of microbial lipases.*

Industry	Effect	Product
Bakery	Flavour improvement and shelf life prolongation	Bakery products
Beverages	Improved aroma	Beverages
Chemical	Enantioselectivity	Chirale building blocks and chemicals
	Synthesis	Chemicals
Cleaning	Hydrolysis	Removal of cleaning agents *e.g.* surfactants
Cosmetics	Synthesis	Emulsifiers, moisturising agents
Dairy	Hydrolysis of milk fat	Flavour agents
	Cheese ripening	Cheese
	Modification of butter fat	Butter
Fats and oils	Trans-esterification	Cocoa butter, margarine
	Hydrolysis	Fatty acids, glycerol, mono- and diglycerides
Food dressing	Quality improvement	Mayonnaise, dressings and whippings
Health food	Trans-esterification	Health food
Leather	Hydrolysis	Leather products
Meat and fish	Flavour development and fat removal	Meat and fish products
Paper	Hydrolysis	Paper products
Pharmaceuticals	Trans-esterification	Speciality lipids
	Hydrolysis	Digestive aids

simple chemical reactions where they initiate the synthesis of other flavour ingredients such as aceto-acetate, β-keto acids, methyl ketones, flavour esters and lactones.

More recently, a whole range of microbial lipase preparations has been developed for the cheese manufacturing industry such as those of *Mucor miehei*, *Aspergillus niger* and *A. oryzae*. A range of cheeses of good quality were produced by using individual microbial lipases or mixtures of several preparations (Table 9). Lipases are widely used for imitation of cheese made from ewe's or goat's milk. Addition of lipases to cow's milk generates a flavour rather similar to that of ewe or goat milk. This is used for producing cheese or the so-called enzyme modified cheese (EMC). EMC is a cheese that has been incubated in the presence of enzymes at elevated temperature in order to produce a concentrated flavour for use as an ingredient in other products such as dips, sauces, soups and snacks.

Lipases in household detergents

The usage of enzymes in washing powders remains the single biggest market for industrial enzymes. The world-wide trend towards lower laundering temperatures has led to much higher demand for household detergent formulations. Recent intensive screening programmes, followed by genetic manipulation have resulted in the introduction of several suitable preparations, for instance Novo

Table 9. *Some examples of the use of lipase in cheese making and accelerated cheese-ripening.*

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

Nordisk's Lipolase (*Humicola* lipase expressed in *Aspergillus oryzae*.

Lipase in the oleochemical industry

The scope for the application of lipases in the oleochemical industry is enormous as it saves energy and minimises thermal degradation during hydrolysis, glycerolysis and alcoholysis. Miyoshi Oil and Fat Co., Japan, reported the commercial use of *Candida cylindracea* lipase in the production of soap. The introduction of the new generation of cheap and very thermostable enzymes can change the economic balance in favour of lipase use.

The current trend in the oleochemical industry is a movement away from using organic solvents and emulsifiers. The major reactions of hydrolysis, alcoholysis and glycerolysis have been performed directly on mixed substrates, thus providing very high productivity. Hence, these processes have been run continuously, with a range of immobilised lipases. Enzymatic hydrolysis probably offers the greatest hope for successful fat splitting without substantial investment in expensive equipment and the expenditure of large amounts of thermal energy.

Lipases in the synthesis of structured triglycerides

The commercial value of fats depends on the fatty acid composition within their structure. A typical example of a high value asymmetric triglyceride mixture is cocoa butter. The potential of 1,3-specific lipases for the manufacture of cocoa-butter substitutes was clearly recognised by Unilever, and Fuji Oil, Comprehensive reviews on this technology, including the analysis of the product composition are available. In principle, the same approach is applicable to the synthesis of many other structured triglycerides possessing valuable dietetic or nutritional properties, for example, human milk fat. This triglyceride and functionally similar fats are readily obtained by acidolysis of palm oil fractions, rich in 2-palmitoyl glyceride with unsaturated fatty acid(s). Acidolysis, catalysed by 1,3-specific lipases, is used in the preparation of nutritionally important products which generally contain medium chain fatty acids. Lipases are being investigated extensively with regard to the modification of oils rich in high value polyunsaturated fatty acids such as arachidonic acid, eicosapentaenoic and docosahexaenoic acids. Substantial enrichment in the poly-unsaturated fatty acid content of the mono-glyceride fraction has been achieved by lipase catalysed alcoholysis or hydrolysis.

Lipases in the synthesis of surfactants

Polyglycerol and carbohydrate fatty-acid esters are widely used as industrial detergents and as emulsifiers in a great variety of food formulations (low fat spreads, sauces, ice-creams, mayonnaises). Enzymic synthesis of functionally similar surfactants has been carried out at moderate temperature (60–80°C) and with excellent regioselectivity. Adelhorst *et al.*[31] have performed solvent free esterification of simple alkyl-glycosides using molten fatty acids and immobilised *Candida antarctica* lipase. Fregapane *et. al.*[32] prepared mono- and di-esters of monosaccharides in high yields using sugar acetals as starting materials. (Poly)glycerol-based surfactants can also be produced enzymically at ambient temperatures and ultimately, in solvent-free processes. Mono- and di-glycerides can be prepared in batch mode and in membrane bioreactors. Lipases may also replace phospholipases in the production of lysophospholipids. *Mucor miehei* lipase has been used for the trans-esterification of phospholipid in a range of primary and secondary alcohols. Lipases may also be useful in the synthesis of a whole range of amphoteric biodegradable surfactants, for instance, amino-acid based esters and amides.

Lipases in the synthesis of ingredients for personal care products

Unichem International has recently launched the production of isopropyl myristate, isopropyl palmitate and 2-ethylhexyl palmitate for use as an emollient in personal care products like skin and sun-tan creams and bath oils. Wax esters have similar application in personal care products and are also being manufactured enzymically using *Candida cylindracea* lipase in a batch bioreactor.

Lipases in the synthesis of pharmaceuticals and agrochemicals

The utility of lipases in the preparation of chiral synthons is well recognised and documented. Several processes have recently been commercialised.

The resolution of 2-halopropionic acids, the starting materials for the synthesis of phenoxypropionate herbicides, is a process based on the selective esterification of (S)-isomers with butanol catalysed by porcine pancreatic lipase in anhydrous hexane. Another impressive instance of the commercial application of lipases in the resolution of racemic mixtures is the hydrolysis of epoxy ester alcohols. The reaction products, (R)-glycidyl esters and (R)-glycidol are readily converted to (R)- and (S)- glycidyltosylates which are very attractive

intermediates for the preparation of optically active ß-blockers and a wide range of other products. A similar technology has been commercialised to produce 2(R),3(S)-methylmethoxyphenyl glycidate, the key intermediate in the manufacture of the optically pure cardiovascular drug diltiazem[33].

Lipases have been found to be useful as industrial catalysts for the resolution of racemic alcohol in the preparation of some prostaglandins, steroids and carbocyclic nucleoside analogues. Regioselective modification of polyfunctional organic compounds is yet another rapidly expanding area of lipase application, particularly in the field of AIDS treatment. It is also useful in the synthesis of the artificial sweetener sucralose by regioselective hydrolysis of octaacetylsucrose[34].

Lipase in polymer synthesis

Recently, it has been found that the stereo-selectivity of lipase is useful for the synthesis of optically active polymers. These polymers are asymmetric reagents and are used as absorbents and in the area of liquid crystals. The use of chiral glycidyl tosylates for the preparation of ferro-electric liquid crystals has also been reported[35].

From the above, it is clear that lipases have great prospects in modern industry because the enzyme has a diversified commercial use both in terms of scale and processes. Lipases have been employed successfully in the food industry as well as in high-tech production of fine chemicals and pharmaceuticals.

Besides this, the enzyme has great potential in new fields. It has been reported that lipases have been used successfully in paper manufacturing; apparently, the treatment of pulp with lipase leads to a higher quality product and reduced cleaning requirement. Similarly, the enzyme has also been used in conjunction with a microbial cocktail for the treatment of fat rich effluents from an ice-cream plant. This could also be utilised in waste processing of many food industries.

Conclusions

Microorganisms, especially fungal species, are considered to be more promising sources of lipases due to their higher growth rates in simple media and simpler manipulation procedures. As the volume and application spectra of lipases become enhanced in the industry, the methods for maximising the production of lipases by microbes through the selection of proper species and through the standardisation of bioreactor conditions should be vigorously pursued.

The use of lipases in the edible oil industry has not yet been extensive as the unit costs of lipases are very high, and the comparatively high value-added products from this industry are limited. Moreover, the exact nature of oleochemical biotransformations in the heterogenous microaqueous environment, the operational data on the stability of enzymes as well as the engineering aspects of large-scale manipulation of production parameters are not fully resolved or optimised as a basic understanding in certain spheres is incomplete. Future research would elicit increased understanding and would enable a greater use of lipases in the oleochemical industry. The main advantage from lipase-catalysed reactions are their substrate selectivity, high reaction efficiency and mild reaction conditions. Among the types of reactions catalysed by lipases, trans-esterification technology has already been commercially applied, though on a limited scale, to produce products with comparatively higher added-value. Future developments are anticipated to lower the costs of these enzymes enabling the use of this technology for producing fatty acids of better colour and improved flavour characteristics or for the manufacture of natural fatty acids or alcohols or esters thereof of higher economic value.

The use of microbial lipases for improving the flavour in milk-based processed food and cheese would be intensified specially in the developing countries where future production of milk is expected to increase substantially during the coming decades.

There should be an intensive search for bacterial as well as fungal lipases for use in the detergent industry. Lipases which are substantially stable at pH of 8–11 would have considerable value for this purpose. Commercial success would, however, depend upon locating a cheaper product, economically affordable by the detergent manufacturers. Genetically mutated organisms with high copy number of target genes or recombinant organisms, with enhanced capacity to produce the enzyme, when developed would reduce the costs enormously.

Ester synthesis, using both long- and short-chain fatty acids and/or alcohols for use as flavours in the processed food industry should become an important industrial activity during the next decade. Very pure esters of terpene alcohols and other natural alcohols would be commercially made by using a lipase-catalysed esterification process for use in the perfume industry. Lipases would also be used for manufacturing waxes for diverse uses, utilising long-chain fatty acids and fatty alcohols. In the end, it is foreseen that the use of lipases, especially microbial ones, will increase significantly in diverse fields of application in the coming years.

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