Research review paper

Production, purification, characterization, and applications of lipases

Rohit Sharma[a], Yusuf Chisti[b], Uttam Chand Banerjee[a],*

[a]*National Institute of Pharmaceutical Education and Research, Sector 67, SAS Nagar (Mohali), Punjab 160062, India*
[b]*Institute of Technology and Engineering, Massey University, Private Bag 11 222, Palmerston North, New Zealand*

Abstract

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. Lipases occur widely in nature, but only microbial lipases are commercially significant. The many applications of lipases include speciality organic syntheses, hydrolysis of fats and oils, modification of fats, flavor enhancement in food processing, resolution of racemic mixtures, and chemical analyses. This article discusses the production, recovery, and use of microbial lipases. Issues of enzyme kinetics, thermostability, and bioactivity are addressed. Production of recombinant lipases is detailed. Immobilized preparations of lipases are discussed. In view of the increasing understanding of lipases and their many applications in high-value syntheses and as bulk enzymes, these enzymes are having an increasing impact on bioprocessing. © 2001 Elsevier Science Inc. All rights reserved.

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1. Introduction

The use of enzyme-mediated processes can be traced to ancient civilizations. Today, nearly 4000 enzymes are known, and of these, about 200 are in commercial use. The majority of the industrial enzymes are of microbial origin. Until the 1960s, the total sales of enzymes were

* Corresponding author. Tel.: +91-172-214682; fax: +91-172-214692.
E-mail address: niper@chd.nic.in (U.C. Banerjee).

only a few million dollars annually, but the market has since grown spectacularly (Godfrey and West, 1996; Wilke, 1999). Because of improved understanding of production biochemistry, the fermentation processes, and recovery methods, an increasing number of enzymes can be produced affordably. Also, advances in methods of using enzymes have greatly expanded demand. Furthermore, because of the many different transformations that enzymes can catalyze, the number of enzymes used in commerce continues to multiply.

The world enzyme demand is satisfied by 12 major producers and 400 minor suppliers. Around 60% of the total world supply of industrial enzymes is produced in Europe. At least 75% of all industrial enzymes (including lipases) is hydrolytic in action. Proteases dominate the market, accounting for approximately 40% of all enzyme sales. Major fields of applications of enzymes are summarized in Table 1. Lipases are represented in most of these fields of applications.

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface (Martinelle et al., 1995) and do not hydrolyze dissolved substrates in the bulk fluid. A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. Lipases are serine hydrolases. Lipases display little activity in aqueous solutions containing soluble substrates. In contrast, esterases show normal Michaelis–Menten kinetics in solution. In eukaryotes, lipases are involved in various stages of lipid metabolism including fat digestion, absorption, reconstitution, and lipoprotein metabolism. In plants, lipases are found in energy reserve tissues. How lipases and lipids interact at the interface is still not entirely clear and is a subject of intense investigation (Balashev et al., 2001).

Because of their wide-ranging significance, lipases remain a subject of intensive study (Alberghina et al., 1991; Bornscheuer, 2000). Research on lipases is focussed particularly on structural characterization, elucidation of mechanism of action, kinetics, sequencing and cloning of lipase genes, and general characterization of performance (Alberghina et al., 1991; Bornscheuer, 2000). In comparison with this effort, relatively little work has been done on development of robust lipase bioreactor systems for commercial use.

Table 1
Fields of applications of enzymes

Scientific research: Enzymes are used as research tools for hydrolysis, synthesis, analysis, biotransformations, and affinity separations.

Cosmetic applications: Preparations for skin; denture cleansers.

Medical diagnostics and chemical analyses: Blood glucose, urea, cholesterol; ELISA systems; enzyme electrodes and assay kits.

Therapeutic applications: Antithrombosis agents, antitumor treatments, antiinflammatory agents, digestive aids, etc.

Industrial catalysis in speciality syntheses; brewing and wine making; dairy processing; fruit, meat, and vegetable processing; starch modifications; leather processing; pulp and paper manufacture; sugar and confectionery processing; production of fructose; detergents and cleaning agents; synthesis of amino acids and bulk chemicals; wastewater treatment; desizing of cotton.

Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Many lipases are active in organic solvents where they catalyze a number of useful reactions including esterification (Chowdary et al., 2001; Hamsaveni et al., 2001; Kiran et al., 2001a; Kiyota et al., 2001; Krishna and Karanth, 2001; Krishna et al., 2001; Rao and Divakar, 2001), transesterification, regioselective acylation of glycols and menthols, and synthesis of peptides (Ducret et al., 1998; Zhang et al., 2001) and other chemicals (Therisod and Klibanov, 1987; Weber et al., 1999; Bornscheuer, 2000; Berglund and Hutt, 2000; Liese et al., 2000; Azim et al., 2001). The expectation is that lipases will be as important industrially in the future as the proteases and carbohydrases are currently.

Lipases find promising applications in organic chemical processing, detergent formulations, synthesis of biosurfactants, the oleochemical industry, the dairy industry, the agrochemical industry, paper manufacture, nutrition, cosmetics, and pharmaceutical processing. Development of lipase-based technologies for the synthesis of novel compounds is rapidly expanding the uses of these enzymes (Liese et al., 2000). One limiting factor is a shortage of lipases having the specific required processing characteristics. An increasing number of lipases with suitable properties are becoming available and efforts are underway to commercialize biotransformation and syntheses based on lipases (Liese et al., 2000). The major commercial application for hydrolytic lipases is their use in laundry detergents. Detergent enzymes make up nearly 32% of the total lipase sales. Lipase for use in detergents needs to be thermostable and remain active in the alkaline environment of a typical machine wash. An estimated 1000 tons of lipases are added to approximately 13 billion tons of detergents produced each year (Jaeger and Reetz, 1998).

Lesser amounts of lipases are used in oleochemical transformations (Bornscheuer, 2000). Lipases can play an important role in the processing of γ-linolenic acid, a polyunsaturated fatty acid (PUFA); astaxanthine, a food colorant; methyl ketones, flavor molecules characteristic of blue cheese; 4-hydroxydecanoic acid used as a precursor of γ-decalactone, a fruit flavor; dicarboxylic acids for use as prepolymers; interesterification of cheaper glycerides to more valuable forms (e.g., cocoa butter replacements for use in chocolate manufacture) (Undurraga et al., 2001); modification of vegetable oils at position 2 of the triglyceride, to obtain fats similar to human milkfat for use in baby feeds; lipid esters including isopropyl myristate, for use in cosmetics; and monoglycerides for use as emulsifiers in food and pharmaceutical applications.

The increasing awareness of the importance of chirality in the context of biological activity has stimulated a growing demand for efficient methods for industrial synthesis of pure enantiomers including chiral antiinflammatory drugs such as naproxen (Xin et al., 2001) and ibuprofen (Lee et al., 1995; Ducret et al., 1998; Xie et al., 1998; Arroyo et al., 1999; Chen and Tsai, 2000); antihypertensive agents such as angiotensin-converting enzyme (ACE) inhibitors (e.g., captopril, enalapril, ceranopril, zofenapril, and lisinopril); and the calcium channel-blocking drugs such as diltiazem. Lipases are used in synthesis of these drugs (Berglund and Hutt, 2000).

This review reports on the production, purification, and characterization of lipases from different microbial sources. The various uses of lipases are discussed. Many

commercial lipases are used as immobilized enzymes and the methods of immobilization are discussed.

2. Applications of lipases

Lipases are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals (Rubin and Dennis, 1997a,b; Kazlauskas and Bornscheuer, 1998). Lipase can be used to accelerate the degradation of fatty waste (Masse et al., 2001) and polyurethane (Takamoto et al., 2001). Major applications of lipases are summarized in Table 2. Most of the industrial microbial lipases are derived from fungi and bacteria (Table 3).

2.1. Lipases in the detergent industry

Because of their ability to hydrolyzes fats, lipases find a major use as additives in industrial laundry and household detergents. Detergent lipases are especially selected to meet the following requirements: (1) a low substrate specificity, i.e., an ability to hydrolyze fats of various compositions; (2) ability to withstand relatively harsh washing conditions (pH 10–11, 30–60 °C); (3) ability to withstand damaging surfactants and enzymes [e.g., linear alkyl benzene sulfonates (LAS) and proteases], which are important ingredients of many detergent formulations. Lipases with the desired properties are obtained through a combination of continuous screening (Yeoh et al., 1986; Wang et al., 1995; Cardenas et al., 2001) and protein engineering (Kazlauskas and Bornscheuer, 1998).

Table 2
Industrial applications of microbial lipases (Vulfson, 1994)

Industry	Action	Product or application
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavoring agents in milk, cheese, and butter
Bakery foods	Flavor improvement	Shelf-life prolongation
Beverages	Improved aroma	Beverages
Food dressings	Quality improvement	Mayonnaise, dressings, and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish products; fat removal
Fats and oils	Transesterification; hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono-, and diglycerides
Chemicals	Enantioselectivity, synthesis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification, hydrolysis	Specialty lipids, digestive aids
Cosmetics	Synthesis	Emulsifiers, moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality
Cleaning	Hydrolysis	Removal of fats

Table 3
Some commercially available microbial lipases (Jaeger and Reetz, 1998)

Type	Source	Application	Producing company
Fungal	C. rugosa	Organic synthesis	Amano, Biocatalysts, Boehringer Mannheim, Fluka, Genzyme, Sigma
	C. antarctica	Organic synthesis	Boehringer Mannheim, Novo Nordisk
	T. lanuginosus	Detergent additive	Boehringer Mannheim, Novo Nordisk
	R. miehei	Food processing	Novo Nordisk, Biocatalysts, Amano
Bacterial	Burkholderia cepacia	Organic synthesis	Amano, Fluka, Boehringer Mannheim
	P. alcaligenes	Detergent additive	Genencor
	P. mendocina	Detergent additive	Genencor
	Ch. viscosum	Organic synthesis	Asahi, Biocatalysts

In 1994, Novo Nordisk introduced the first commercial recombinant lipase 'Lipolase,' which originated from the fungus *Thermomyces lanuginosus* and was expressed in *Aspergillus oryzae*. In 1995, two bacterial lipases were introduced — 'Lumafast' from *Pseudomonas mendocina* and 'Lipomax' from *P. alcaligenes* — by Genencor International (Jaeger and Reetz, 1998). Gerritse et al. (1998) reported an alkaline lipase, produced by *P. alcaligenes* M-1, which was well suited to removing fatty stains under conditions of a modern machine wash. The patent literature contains examples of many microbial lipases that are said to be suitable for use in detergents (Bycroft and Byng, 1992).

2.2. Lipases in food industry

Fats and oils are important constituents of foods. The nutritional and sensory value and the physical properties of a triglyceride are greatly influenced by 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. 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 the fatty acids with new ones. This way, a relatively inexpensive and less desirable lipid can be modified to a higher value fat (Colman and Macrae, 1980; Pabai et al., 1995a,b; Undurraga et al., 2001).

Cocoa butter, a high-value fat, contains palmitic and stearic acids and has a melting point of approximately 37 °C. Melting of cocoa butter in the mouth produces a desirable cooling sensation in products such as chocolate. Lipase-based technology involving mixed hydrolysis and synthesis reactions is used commercially to upgrade some of the less desirable fats to cocoa butter substitutes (Colman and Macrae, 1980; Undurraga et al., 2001). One version of this process uses immobilized *Rhizomucor miehei* lipase for the transesterification reaction that replaces the palmitic acid in palm oil with stearic acid. Similarly, Pabai et al. (1995a) described a lipase-catalyzed interesterification of butter fat that resulted in a considerable decrease in the long-chain saturated fatty acids and a corresponding increase in C18:0 and C18:1 acids at position 2 of the selected triacylglycerol.

Because of their metabolic effects, PUFAs are increasingly used as pharmaceuticals, neutraceuticals, and food additives (Gill and Valivety, 1997a; Belarbi et al., 2000). Many of

the 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 diglycerides are subsequently used to produce a variety of pharmaceuticals including anticholesterolemics, antiinflammatories, and thrombolytics (Gill and Valivety, 1997b; Belarbi et al., 2000). In addition, lipases have been used for development of flavors in cheese ripening, bakery products, and beverages (Kazlauskas and Bornscheuer, 1998). Also, lipases are used to aid removal of fat from meat and fish products (Kazlauskas and Bornscheuer, 1998).

2.3. Lipases in pulp and paper industry

'Pitch,' or the hydrophobic components of wood (mainly triglycerides and waxes), causes severe problems in pulp and paper manufacture (Jaeger and Reetz, 1998). Lipases are used to remove the pitch from the pulp produced for paper making. Nippon Paper Industries, Japan, have developed a pitch control method that uses the *Candida rugosa* fungal lipase to hydrolyze up to 90% of the wood triglycerides.

2.4. Lipases in organic synthesis

Use of lipases in organic chemical synthesis is becoming increasingly important. Lipases are used to catalyze a wide variety of chemo-, regio-, and stereoselective transformations (Rubin and Dennis, 1997b; Kazlauskas and Bornscheuer, 1998; Berglund and Hutt, 2000). Majority of lipases used as catalysts in organic chemistry are of microbial origin. These enzymes work at hydrophilic–lipophilic interface and tolerate organic solvents in the reaction mixtures. Use of lipases in the synthesis of enantiopure compounds has been discussed by Berglund and Hutt (2000).

The enzymes catalyze the hydrolysis of water-immiscible triglycerides at water–liquid interface. Under given conditions, the amount of water in the reaction mixture will determine the direction of lipase-catalyzed reaction. When there is little or no water, only esterification and transesterification are favored (Klibanov, 1997). Hydrolysis is the favored reaction when there is excess water (Klibanov, 1997). Lipase-catalyzed reactions in supercritical solvents have been described (Rantakyla et al., 1996; Turner et al., 2001; King et al., 2001).

2.5. Lipases in bioconversion in aqueous media

Hydrolysis of esters is commonly carried out using lipase in two-phase aqueous media (Vaysse et al., 1997; Chatterjee et al., 2001). Penreac'h and Baratti (1996) reported on the hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) in *n*-heptane by a lipase preparation of *P. cepacia*. Jaeger and Reetz (1998) used lipase entrapped in a hydrophobic sol–gel matrix for a variety of transformations.

Mutagenesis has been used to greatly enhance the enantioselectivity of lipases (Bornscheuer, 2000; Gaskin et al., 2001). For example, in one case, the enantioselectivity of lipase-catalyzed hydrolysis of a chiral ester (*P. aeruginosa* lipase) was increased from e.e. 2% to e.e.

81% in just four mutagenesis cycles. The lipase-acyl transferase from *C. parapsilosis* has been shown to catalyze fatty hydroxamic acid biosynthesis in a biphasic liquid/aqueous medium. The substrates of the reaction were acyl donors (fatty acid or fatty acid methyl ester) and a hydroxylamine. The transfer of acyl group from a donor ester to hydroxylamine (aminolysis) was catalyzed preferentially compared to the reaction of free fatty acids. This feature made the *C. parapsilosis* enzyme the catalyst of choice for the direct bioconversion of oils in aqueous medium (Vaysse et al., 1997). Yeo et al. (1998) reported a novel lipase produced by *Burkholderia* sp., which could preferentially hydrolyze a bulky ester, *t*-butyl octanoate (TBO). This lipase was confirmed to be 100-fold superior to commercial lipases in terms of its TBO-hydrolyzing activity.

2.6. Lipases in bioconversions in organic media

Enzymes in organic media without a free aqueous phase are known to display useful unusual properties, and this has firmly established nonaqueous enzyme systems for synthesis and biotransformations (Klibanov, 1997). Lipases have been widely investigated for various nonaqueous biotransformations (Therisod and Klibanov, 1987; Klibanov, 1990; Tsai and Dordick, 1996; Ducret et al., 1998; Dong et al., 1999; Kiran and Divakar, 2001).

2.7. Lipases in resolution of racemic acids and alcohols

Stereoselectivity of lipases has been used to resolve various racemic organic acid mixtures in immiscible biphasic systems (Klibanov, 1990). Racemic alcohols can also be resolved into enantiomerically pure forms by lipase-catalyzed transesterification. Arroyo and Sinisterra (1995) reported that esterification reaction in nonaqueous media using lipase-B from *C. antarctica* was stereoselective towards the *R*-isomer of ketoprofen in an achiral solvent such as isobutyl methyl ketone and (*S*+)-carvone.

In one study, a purified lipase preparation from *C. rugosa* was compared to its crude counterpart in anhydrous and slightly hydrated hydrophobic organic solvents. The purified lipase preparation was less active than the crude enzyme in dry *n*-heptane, whereas the presence of a small concentration of water dramatically activated the purified enzyme but not the crude enzyme in the esterification of racemic 2-(4-chlorophenoxy) propanoic acid with *n*-butanol (Tsai and Dordick, 1996).

Profens (2-aryl propinoic acids), an important group of nonsteroidal antiinflammatory drugs, are pharmacologically active mainly in the (*S*)-enantiomer form (Hutt and Caldwell, 1984). For instance, (*S*)-ibuprofen [(*S*)-2(4-isobutylphenyl) propionic acid] is 160 times more potent than its antipode in inhibiting prostaglandin synthesis. Consequently, considerable effort is being made to obtain optically pure profens through asymmetric chemical synthesis, catalytic kinetic resolution (Van Dyck et al., 2001; Xin et al., 2001), resolution of racemate via crystallization, and chiral chromatographic separations. Microorganisms and enzymes have proved particularly useful in resolving racemic mixtures. Thus, pure (*S*)-ibuprofen is obtained by using lipase-catalyzed kinetic resolution via hydrolysis (Lee et al., 1995) or esterification (Ducret et al., 1998; Xie et al., 1998). Similarly, 2-phenoxy-1-propanol was

resolved into its enantiomers using *Pseudomonas* sp. lipase by enantioselective transesterification (Miyazawa et al., 1998). Weber et al. (1999) reported solvent-free thioesterification of fatty acids with long-chain thiols catalyzed by lipases from *C. antarctica* and *R. miehei*. Also, solvent-free *trans*-thioesterification of fatty acid methyl esters with alkane thiols was reported (Weber et al., 1999).

2.8. Lipases in regioselective acylations

Lipases acylate certain steroids, sugars, and sugar derivatives with a high regioselectivity. Monoacylated sugars have been produced in anhydrous pyridine from triethyl carboxylates and various monosaccharides (Therisod and Klibanov, 1987). In contrast, Chen et al. (1995) used a lipase from *A. niger* to catalyze the regioselective deacylation of preacylated methyl β-ᴅ-glucopyranoside. Similarly, Kodera et al. (1998) reported regioselective deacetylation of preacetylated monosaccharide derivatives in 1,1,1-trichloroethane using a lipase modified with polyethylene glycol.

2.9. Lipases in ester synthesis

Lipases have been successfully used as catalyst for synthesis of esters. The esters produced from short-chain fatty acids have applications as flavoring agents in food industry (Vulfson, 1994). Methyl and ethyl esters of long-chain acids have been used to enrich diesel fuels (Vulfson, 1994). From et al. (1997) studied the esterification of lactic acid and alcohols using a lipase of *C. antarctica* in hexane. Esterification of five positional isomers of acetylenic fatty acids (different chain lengths) with *n*-butanol was studied by Lie et al. (1998), using eight different lipases. Arroyo et al. (1999) noted that an optimum preequilibrium water activity value was necessary for obtaining a high rate of esterification of (*R,S*)-ibuprofen. Janssen et al. (1999) reported on the esterification of sulcatol and fatty acids in toluene, catalyzed by *C. rugosa* lipase (CRL). Krishnakant and Madamwar (2001) reported using lipase immobilized on silica and microemulsion-based organogels, for ester synthesis.

2.10. Lipases in oleochemical industry

Use of lipases in oleochemical processing saves energy and minimizes thermal degradation during alcoholysis, acidolysis, hydrolysis, and glycerolysis (Vulfson, 1994; Bornscheuer, 2000). Although lipases are designed by nature for the hydrolytic cleavage of the ester bonds of triacylglycerol, lipases can catalyze the reverse reaction (ester synthesis) in a low-water environment. Hydrolysis and esterification can occur simultaneously in a process known as interesterification. Depending on the substrates, lipases can catalyze acidolysis (where an acyl moiety is displaced between an acyl glycerol and a carboxylic acid), alcoholysis (where an acyl moiety is displaced between an acyl glycerol and an alcohol), and transesterification (where two acyl moieties are exchanged between two acylglycerols) (Balcão et al., 1996).

3. Microorganisms producing lipases

Lipases are produced by many microorganisms and higher eukaryotes. Most commercially useful lipases are of microbial origin. Some of the lipase-producing microorganisms are listed in Table 4.

3.1. Isolation and screening of lipase-producing microorganisms

Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food (Sztajer et al., 1988), compost heaps, coal tips, and hot springs (Wang et al., 1995).

Lipase-producing microorganisms include bacteria, fungi, yeasts, and actinomyces. A simple and reliable method for detecting lipase activity in microorganisms has been described by Sierra (1957). This method uses the surfactant Tween 80 in a solid medium to identify a lipolytic activity. The formation of opaque zones around the colonies is an indication of lipase production by the organisms. Modifications of this assay use various Tween surfactants in combination with Nile blue or neet's foot oil and Cu^{2+} salts. Also, screening of lipase producers on agar plates is frequently done by using tributyrin as a substrate (Cardenas et al., 2001) and clear zones around the colonies indicate production of lipase. Screening systems making use of chromogenic substrates have also been described (Yeoh et al., 1986). Wang et al. (1995) used plates of a modified Rhodamine B agar to screen lipase activity in a large number of microorganisms. Other versions of this method have been reported (Kouker and Jaeger, 1987; Hou, 1994).

4. Production and media development for lipase

Microbial lipases are produced mostly by submerged culture (Ito et al., 2001), but solid-state fermentation methods (Chisti, 1999a) can be used also. Immobilized cell culture has been used in a few cases (Hemachander et al., 2001). Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production by submerged culture. Lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature, and the dissolved oxygen concentration (Elibol and Ozer, 2001). Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield; however, a few authors have produced good yields in the absence of fats and oils.

4.1. Effect of carbon sources

Sugihara et al. (1991) reported lipase production from *Bacillus* sp. in the presence of 1% olive oil in the culture medium. Little enzyme activity was observed in the absence of olive oil even after prolonged cultivation. Fructose and palm oil were reported to be the best

Table 4
Some lipase-producing microorganisms

Source	Genus	Species	Reference(s)
Bacteria (Gram-positive)	*Bacillus*	*B. megaterium*	Godtfredsen, 1990
		B. cereus	El-Shafei and Rezkallah, 1997
		B. stearothermophilus	Gowland et al., 1987; Kim et al., 1998
		B. subtilis	Kennedy and Rennarz, 1979
		Recombinant *B. subtilis* 168	Lesuisse et al., 1993
		B. brevis	Hou, 1994
		B. thermocatenulatus	Rua et al., 1998
		Bacillus sp. IHI-91	Becker et al., 1997
		Bacillus strain WAI 28A5	Janssen et al., 1994
		Bacillus sp.	Helisto and Korpela, 1998
		B. coagulans	El-Shafei and Rezkallah, 1997
		B. acidocaldarius	Manco et al., 1998
		Bacillus sp. RS-12	Sidhu et al., 1998a,b
		B. thermoleovorans ID-1	Lee et al., 1999
		Bacillus sp. J 33	Nawani and Kaur, 2000
	Staphylococcus	*S. canosus*	Tahoun et al., 1985
		S. aureus	Lee and Yandolo, 1986
		S. hyicus	Van Oort et al., 1989; Meens et al., 1997; van Kampen et al., 1998
		S. epidermidis	Farrell et al., 1993; Simons et al., 1998
		S. warneri	Talon et al., 1995
	Lactobacillus	*Lactobacillus delbruckii* sub sp. *bulgaricus*	El-Sawah et al., 1995
		Lactobacillus sp.	Meyers et al., 1996
	Streptococcus	*Streptococcus lactis*	Sztajer et al., 1988
	Micrococcus	*Micrococcus freudenreichii*	Hou, 1994
		M. luteus	Hou, 1994
	Propionibacterium	*Propionibacterium acne*	Sztajer et al., 1988
		Pr. granulosum	Sztajer et al., 1988
	Burkholderia	*Burkholderia* sp.	Yeo et al., 1998
		Bu. glumae	El Khattabi et al., 2000
Bacteria (Gram-negative)	*Pseudomonas*	*P. aeruginosa*	Aoyama et al., 1988; Hou, 1994; Ito et al., 2001
		P. fragi	Mencher and Alford,1967
		P. mendocina	Jaeger and Reetz, 1998
		P. putida 3SK	Lee and Rhee, 1993
		P. glumae	Frenken et al., 1993; Noble et al., 1994
		P. cepacia	Penereac'h and Baratti, 1996; Lang et al., 1998; Hsu et al., 2000

(continued on next page)

Table 4 (*continued*)

Source	Genus	Species	Reference(s)
		P. fluorescens	Maragoni, 1994; Lacointe et al., 1996
		P. aeruginosa KKA-5	Sharon et al., 1998
		P. pseudoalcaligenes F-111	Lin et al., 1995, 1996
		Pseudomonas sp.	Sin et al., 1998; Miyazawa et al., 1998; Reetz and Jaeger, 1998; Dong et al., 1999
		P. fluorescens MF0	Guillou et al., 1995
		Pseudomonas sp. KWI56	Yang et al., 2000
	Chromobacterium	*Ch. viscosum*	Rees and Robinson, 1995; Helisto and Korpela, 1998; Jaeger and Reetz, 1998; Diogo et al., 1999
	Acinetobacter	*Aci. pseudoalcaligenes*	Sztajer et al., 1988
		Aci. radioresistens	Chen et al., 1999
	Aeromonas	*Ae. hydrophila*	Anguita et al., 1993
		Ae. sorbia LP004	Lotrakul and Dharmsthiti, 1997
Fungi	*Rhizopus*	*Rhizop. delemar*	Klein et al., 1997; Espinosa et al., 1990; Haas et al., 1992; Lacointe, et al., 1996
		Rhizop. oryzae	Salleh et al., 1993; Coenen et al., 1997; Beer et al., 1998; Essamri et al., 1998; Takahashi et al., 1998; Hiol et al., 2000
		Rhizop. arrhizus	Sztajer and Maliszewska, 1989; Elibol and Ozer, 2001
		Rhizop. nigricans	Ghosh et al., 1996
		Rhizop. nodosus	Nakashima et al., 1988
		Rhizop. microsporous	Ghosh et al., 1996
		Rhizop. chinensis	Ghosh et al., 1996
		Rhizop. japonicus	Nakashima et al., 1988
		Rhizop. niveus	Kohno et al., 1994, 1999
	Aspergillus	*A. flavus*	Long et al., 1996, 1998
		A. niger	Chen et al., 1995
		A. japonicus	Satyanarayan and Johri, 1981
		A. awamori	Satyanarayan and Johri, 1981
		A. fumigatus	Satyanarayan and Johri, 1981
		A. oryzae	Ohnishi et al., 1994a,b
		A. carneus	Helisto and Korpela, 1998
		A. repens	Kaminishi et al., 1999
		A. nidulans	Mayordomo et al., 2000

Table 4 (*continued*)

Source	Genus	Species	Reference(s)
	Penicillium	*Pe. cyclopium*	Chahinian et al., 2000
		Pe. citrinum	Sztajer and Maliszewska, 1989
		Pe. roqueforti	Petrovic et al., 1990
		Pe. fumiculosum	Hou, 1994
		Penicillium sp.	Helisto and Korpela, 1998
		Pe. camambertii	Ghosh et al., 1996
		Pe. wortmanii	Costa and Peralta, 1999
	Mucor	*Mucor miehei*	Rantakyla et al., 1996; Lacointe et al., 1996; Plou et al., 1998
		Mu. javanicus	Ishihara et al., 1975
		Mu. circinelloides	Balcão et al., 1998
		Mu. hiemalis	Ghosh et al., 1996
		Mu. racemosus	Ghosh et al., 1996
	Ashbya	*Ashbya gossypii*	Stahmann et al., 1997
	Geotrichum	*G. candidum*	Sugihara et al., 1991; Ghosh et al., 1996
		Geotrichum sp.	Macedo et al., 1997
	Beauveria	*Beauveria bassiana*	Hegedus and Khachatourians, 1988
	Humicola	*H. lanuginosa*	Ghosh et al., 1996; Takahashi et al., 1998; Plou et al., 1998; Zhu et al., 2001
	Rhizomucor	*R. miehei*	Merek and Bednasski, 1996; Weber et al., 1999; Jaeger and Reetz, 1998; Dellamora-Ortiz et al., 1997
	Fusarium	*Fusarium oxysporum*	Rapp, 1995
		F. heterosporum	Takahashi et al., 1998
	Acremonium	*Ac. strictum*	Okeke and Okolo, 1990
	Alternaria	*Alternaria brassicicola*	Berto et al., 1997
	Eurotrium	*Eu. herbanorium*	Kaminishi et al., 1999
	Ophiostoma	*O. piliferum*	Brush et al., 1999
Yeasts	*Candida*	*C. rugosa*	Wang et al., 1995; Frense et al., 1996; Yee et al., 1995; Brocca et al., 1998; Xie et al., 1998
		C. tropicalis	Takahashi et al., 1998
		C. antarctica	Weber et al., 1999; Jaeger and Reetz, 1998; Arroyo et al., 1999
		C. cylindracea	Kamiya and Gotto, 1998; Helisto and Korpela, 1998
		C. parapsilosis	Lacointe et al., 1996
		C. deformans	Lacointe et al., 1996

(continued on next page)

Table 4 (*continued*)

Source	Genus	Species	Reference(s)
		C. curvata	Ghosh et al., 1996
		C. valida	Ghosh et al., 1996
	Yarrowia	*Y. lipolytica*	Merek and Bednasski, 1996; Pignede et al., 2000
	Rhodotorula	*Rho. glutinis*	Papaparaskevas et al., 1992
		Rho. pilimornae	Tahoun et al., 1985
	Pichia	*Pi. bispora*	Hou, 1994
		Pi. maxicana	Hou, 1994
		Pi. sivicola	Sugihara et al., 1995
		Pi. xylosa	Sugihara et al., 1995
		Pi. burtonii	Sugihara et al., 1995
	Saccharomyces	*Sa. lipolytica*	Tahoun et al., 1985
		Sa. crataegenesis	Hou, 1994
	Torulospora	*Torulospora globora*	Hou, 1994
	Trichosporon	*Trichosporon asteroides*	Dharmsthiti and Ammaranond, 1997
Actinomycetes	*Streptomyces*	*Streptomyces fradiae* NCIB 8233	Sztajer et al., 1988
		Streptomyces sp. PCB27	Sztajer et al., 1988
		Streptomyces sp. CCM 33	Sztajer et al., 1988
		Str. coelicolor	Hou, 1994
		Str. cinnamomeus	Sommer et al., 1997

carbohydrate and lipid sources, respectively, for the production of an extracellular lipase by *Rhodotorula glutinis*. When the two carbon sources were compared, palm oil at a concentration of 2% was found to yield 12-fold more lipase than the fructose medium (Papaparaskevas et al., 1992).

A specific activity of 7395 U/mg protein was observed for alkaline lipase (pH 8.5) produced by *P. fluorescens* S1K WI in a medium which contained emulsified olive oil as the carbon source (Lee et al., 1993). The enzyme showed a high lipolytic activity towards tricaproic (C6) and tricaprylin (C8) compared to the other triacylglycerols examined and preferentially hydrolyzed the ester bonds in positions 1 and 3 of triolein. Similarly, an alkaline lipase from *Penicillium expansum* yielded maximum activity when the biomass was grown in an oil-containing medium (0.1% olive oil) at pH 8.3 (Sztajer et al., 1993). Enzyme stability was enhanced by the addition of Tween 20 and lubrol PX (Sztajer et al., 1993). The enzyme had a preference for triacylglycerols but showed no positional specificity (Sztajer et al., 1993).

Production of a thermostable lipase from thermophilic *Bacillus* sp. strain Wai 28A 45, in the presence of tripalmitin at 70 °C, was described by Janssen et al. (1994). Media with tripalmitin, tristearin, and trimystin carbon sources were tested, and tripalmitin was found to be the best inducer of lipase activity. Gao and Breuil (1995) compared different plant oils for lipase production from the sapwood staining fungus *Ophiostoma piceae*. High levels of lipase activity were obtained when vegetable oils (olive, soybean, sunflower, sesame, cotton seed,

corn, and peanut oil) were used as the carbon source. Maximum lipase production occurred when olive oil was used. Similarly, a thermophilic *Bacillus* strain A30-1 (ATCC 53841) produced maximal levels of thermostable alkaline lipase when corn oil and olive oil (1%) were used as carbon sources (Wang et al., 1995). The lipase produced was active on triglycerides of C16:0 to C22:0 fatty acids and on natural fats and oils.

Gordillo et al. (1995) observed that lipase production from *C. rugosa* in batch culture was affected by the initial concentration of oleic acid — one of the major products of hydrolysis of the lipase inducers (oils, Tween 80, etc.) used. The maximum lipase/substrate yield was obtained at an initial oleic acid concentration of 2 g/L and the yield decreased at higher concentrations of oleic acid. Several other studies confirm enhanced lipase production when oils are used as enzyme inducers. Lin et al. (1996) produced an alkaline lipase from *P. pseudoalcaligenes* F-111 in a medium that contained both olive oil (0.4%) and Triton X-100 (0.2%). The addition of Triton X-100 enhanced the alkaline lipase production by 50-fold compared to using olive oil alone. The addition of various kinds of oils to the medium for *Rhizopus oryzae* increased both the lipase activity and cell growth up to three fold compared to results in a lipid-free medium (Essamri et al., 1998). Rapeseed and corn oil were the most suitable substrates for cell growth and lipase production (Essamri et al., 1998). The oil concentration for optimal biomass growth was 3%, but optimal production of lipase occurred at 2% oil concentration.

Because of their use in alkaline detergents, alkalostable lipases are especially sought after. An alkaline lipase was produced by *P. alcaligenes* M-1 in a medium with citric acid and soybean oil as substrates in the batch and fed-batch phases, respectively (Gerritse et al., 1998). This lipase had excellent capability for removing fatty stains in an alkaline environment. The gene encoding the alkaline lipase was isolated and characterized. Kim et al. (1998) reported production of a highly alkaline thermostable lipase by *Bacillus stearothermophilus* L1 in a medium that contained beef tallow and palm oil. This lipase was most active at 60–65 °C and pH 9–10. Activity assessments with synthetic substrates showed this enzyme to be especially active towards *p*-nitrophenyl caprylate (Kim et al., 1998).

The yeast *C. rugosa* has been shown to secrete an extracellular lipase (Lotti et al., 1998) whose production can be induced by adding fatty acids to the culture broth. This lipase is composed of several isoforms with slightly differing catalytic properties. Lipase production could be induced by adding oleic acid as the carbon source. In the same yeast, the production of a constitutive lipase was induced by using glucose as the carbon source (Lotti et al., 1998). *P. aeruginosa* KKA-5 produced the maximal lipase activity when castor oil (2%) was used as the carbon source at pH 6.9 (Sharon et al., 1998). This enzyme could cause up to 90% hydrolysis of castor oil and it was stable in alkaline conditions (pH 7–10). The maximum activity was obtained at pH 8.5 (Sharon et al., 1998).

One study explored 56 strains of molds for the ability to produce lipase (Costa and Peralta, 1999). A strain identified as *Pe. wortmanii* was determined to be the best lipase producer (Costa and Peralta, 1999). Maximum lipase production (12.5 U/mL) was obtained in a 7-day culture using olive oil (5% wt/vol) as the carbon source. The optimal pH and temperature for the crude lipase activity were 7.0 and 45 °C, respectively (Costa and Peralta, 1999).

A thermophilic bacterium, *B. thermoleovorans* ID-1, isolated from hot springs in Indonesia, showed extracellular lipase activity and high growth rates on lipid substrates at elevated temperatures (Lee et al., 1999). Using olive oil (1.5% vol/vol) as the sole carbon source, the isolate ID-1 grew rapidly at 65 °C (specific growth rate of 2.5 h^{-1}) and its lipase activity attained a maximum value of 520 U/L during the late exponential growth phase. The isolate ID-1 could grow on a variety of lipidic substrates such as oils (olive, soybean, and mineral oils), triglycerides (triolein, tributyrin), and synthetic surfactants (Tweens 20 and 40). In view of the reports reviewed, the production of lipase is mostly inducer-dependent, and in many cases, oils act as good inducers of the enzyme.

4.2. Effect of nitrogen sources

For an extracellular lipase of *Pe. citrinum*, Sztajer and Maliszewska (1989) obtained maximal production in a medium that contained 5% (wt/vol) peptone (pH 7.2). Nitrogen sources such as corn steep liquor and soybean meal stimulated lipase production but to a lesser extent than peptone. Urea and ammonium sulfate inhibited lipase synthesis (Sztajer and Maliszewska, 1989). Lipolytic activity (1120 U/L) was determined by titration of the free fatty acids released from olive oil incubated with the cell-free broth.

Thermostable lipase of *Pseudomonas* sp. KW1-56 was produced in a medium that contained peptone (2% wt/vol) and yeast extract (0.1% wt/vol) as nitrogen sources (Izumi et al., 1990). The lipase was purified by acetone precipitation and gel filtration. The purification factor was 13.9, but the overall recovery was only 2.9% (Izumi et al., 1990). The enzyme produced a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and its molecular mass was estimated at 33 kDa. The temperature optimum for the enzyme was 60 °C and more than 96% of the original activity remained after 24 h at 60 °C (Izumi et al., 1990).

Acremonium structum produced a large amount of lipase under stationary conditions in a medium containing 35% (wt/vol) soybean meal as the nitrogen source (Okeke and Okolo, 1990). Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used. One exception reported is *Rho. glutinis* (Papaparaskevas et al., 1992). Although good growth of *Rho. glutinis* seems to require organic nitrogen sources (e.g., yeast extract and tryptone), an inorganic nitrogen source such as ammonium phosphate appears to favor lipase production (Papaparaskevas et al., 1992). The enzyme produced had an optimal activity at pH 7.5. The half-life of the enzyme was 45 and 11.8 min at 45 and 55 °C, respectively (Papaparaskevas et al., 1992).

In agreement with other authors, Salleh et al. (1993) obtained maximal production of extracellular lipase by the thermophilic fungi, *Rhizop. oryzae*, when the medium contained peptone as the nitrogen source. Production of intracellular lipase by *Rhizop. oryzae* was not particularly sensitive to the organic nitrogen source used (tryptone, tryptic digest, corn steep liquor, polypeptone). In studies of thermostable lipase production from thermophilic fungi *Emericella rugulosa*, *Humicola* sp., *T. lanuginosus*, *Pe. purpurogenum*, and *Chrysosporium sulfureum*, use of yeast extract as the nitrogen source gave consistently high lipase production (Venkateshwarlu and Reddy, 1993).

A. oryzae produced maximal alkaline lipase in a medium that contained yeast extract (1%), polypeptone (2%), and soybean meal (3%) as nitrogen sources (Ohnishi et al., 1994a). The enzyme produced had an activity optimum at pH 7.5 and 10.0, respectively, with olive oil and tributyrin as substrates. A Brazilian strain of Pe. citrinum produced a maximal lipase activity of 409 IU/mL in a medium that contained yeast extract (0.5%) as the nitrogen source (Pimentel et al., 1994). A decrease in yeast extract concentration reduced the attainable lipase activity. Replacement of yeast extract with ammonium sulfate diminished lipase production (Pimentel et al., 1994). A. niger produced lipase in a lipid-free medium but required an inducer for improved production (Pokorny et al., 1994). Lipase production increased when the medium was supplemented with an inorganic nitrogen source (ammonium nitrate) (Pokorny et al., 1994). Similarly, the addition of ammonium sulfate and peptone to the medium enhanced lipase production by the fungus O. piceae (Gao and Breuil, 1995). The enzyme had optimal activity at 60 °C and pH 9.5 (Gao and Breuil, 1995).

Wang et al. (1995) reported production of a highly thermostable alkaline lipase by Bacillus strain A 30-1 (ATCC 53841) in a medium that contained yeast extract (0.1%) and ammonium chloride (1%) as nitrogen sources. The partially purified lipase preparation had an optimal activity temperature of 60 °C and the optimum pH was 9.5. This enzyme was stable to both hydrogen peroxide and alkaline protease (Wang et al., 1995). Cordenons et al. (1996) examined various nitrogen sources for producing extracellular lipase from Acinetobacter calcoaceticus. Use of amino acids and tryptone improved the lipase yield by a factor of 2 or 3 when compared to the use of ammonium, yeast extract, and protease peptone (Cordenons et al., 1996). However, lipase yield and stability could be improved by supplementing the preferred organic nitrogen source with ammonium (Cordenons et al., 1996). The extracellular lipase was measured using pNPP as the substrate (Vorderwiilbecke et al., 1992).

Lin et al. (1996) reported an extracellular alkaline lipase produced by P. alcaligenes F-111 in a medium that contained soybean meal (1%), peptone (1.5%), and yeast extract (0.5%). The lipase produced was unaffected by various detergents. The cationic surface active agents such as SDS, sodium tripolyphosphate, sodium dodecyl benzene sulfonate, and sodium alkyl benzene sulfonate did not affect the enzyme activity, suggesting that this enzyme is a good candidate for detergent applications.

For intracellular lipase production by the fungus Rhizop. oryzae, corn steep liquor (7%) was an optimal nitrogen source (Essamri et al., 1998). At concentrations greater than 7%, corn steep liquor caused a rapid decline in cell growth and lipase production. P. aeruginosa KKA-5 produced an extracellular lipase in a medium composed of polypeptone (4%) and yeast extract (0.05%) (Sharon et al., 1998). This enzyme was stable up to 45 °C. The lipase was highly stable in aqueous solutions of solvents such as methanol and ethanol, but was weakly inhibited in the presence of acetone (Sharon et al., 1998).

Hiol et al. (2000) isolated a lipolytic strain of Rhizop. oryzae that yielded a high extracellular lipase activity in a medium composed of corn steep liquor (4%) and peptone (1%) as nitrogen sources. The pH and temperature optima for the activity of this enzyme were pH 7.5 and 35 °C (Hiol et al., 2000). The enzyme was stable in a pH range of 4.5–7.5 and retained about 65% of its initial activity after 30-min incubation at 45 °C.

4.3. Effect of metal ions

Lipase production by a thermophilic *Bacillus* sp. was increased several fold when magnesium, iron, and calcium ions were added to the production medium (Janssen et al., 1994). Similarly, Pokorny et al. (1994) reported that lipase production by *A. niger* was enhanced in the presence of Mg^{2+}. Production of an extracellular lipase by *Aci. calcoaceticus* BD 413 was enhanced when the medium was supplemented with Mg^{2+}, Ca^{2+}, Cu^{2+}, and Co^{2+} (Kok et al., 1995). The enzyme hydrolyzed long acyl chain *p*-nitrophenol (*p*NP) esters, such as *p*NPP, and its optimal activity occurred between pH 7.8 and 8.8 (Kok et al., 1995). The *A. calcoaceticus* lipase was quite similar to *Pseudomonas* lipases.

Lipase production by *P. pseudoalcaligenes* F-111 was enhanced when a phosphate-containing medium was provided with Mg^{2+} (Lin et al., 1995). This alkaline lipase was most active and stable in the pH range 6–10 and its optimal reaction temperature was 40 °C. Lipase production by *Bacillus* sp. A 30-1 (ATCC 53841) required a complex medium that contained Ca^{2+}, Mg^{2+}, Na^+, Co^{2+}, Cu^{2+}, Fe^{2+}, K^+, Mn^{2+}, Mo^{2+}, and Zn^{2+} (Wang et al., 1995). The source bacterium, isolated from a mineral-rich hot spring (Yellowstone National Park), grew optimally at 60 °C (pH 9) (Wang et al., 1995).

Maximal lipase production by *P. pseudoalcaligenes* KKA-5 occurred at Mg^{2+} concentration of 0.8 M (Sharon et al., 1998). Exclusion of the magnesium ions from the medium caused approximately 50% reduction in lipase production (Sharon et al., 1998), but supplementing the medium with calcium ions did not affect lipase production. In one case, presence of Ca^{2+} was reported to enhance lipase production by the thermophilic *Bacillus* sp., RS-12 (Sidhu et al., 1998a,b). The bacterium grew optimally at 50 °C and did not grow below 40 °C. The enzyme production was growth-associated. Use of Tween 80 (0.5%) and yeast extract (0.5%) in the medium gave a maximal yield of the enzyme at 50 °C culture temperature.

5. Purification and kinetic characterization of lipases

Many lipases have been extensively purified and characterized in terms of their activity and stability profiles relative to pH, temperature, and effects of metal ions and chelating agents. In many cases, lipases have been purified to homogeneity and crystallized. Purification methods used have generally depended on nonspecific techniques such as precipitation, hydrophobic interaction chromatography, gel filtration, and ion exchange chromatography. Affinity chromatography has been used in some cases to reduce the number of individual purification steps needed (Woolley and Peterson, 1994).

Chartrain et al. (1993) purified a lipase from *P. aeruginosa* MB5001 using a three-step procedure. Concentration by ultrafiltration was followed by ion exchange chromatography and gel filtration. The purified lipase had a molecular mass of 29 kDa by SDS-PAGE. The enzyme exhibited maximum activity at 55 °C and had a pH optimum of 8.0. Lee and Rhee (1993) used ion exchange and gel filtration to purify a lipase from *P. putida* 3SK. The activity of the purified enzyme was inhibited by mercury ions and SDS (Lee and Rhee, 1993). Calcium ions and taurocholic acid stimulated the enzyme activity (Lee and Rhee, 1993).

Two types of lipases (Lipases I and II) were purified to homogeneity by Kohno et al. (1994), using column chromatography on DEAE-Toyopearl. Lipase I consisted of two polypeptide chains [a small peptide with sugar moiety (A-chain) and a large peptide of 34 kDa molecular weight (B-chain)]. Lipase II had a molecular mass of 30 kDa and a single polypeptide chain (Kohno et al., 1994). Ohnishi et al. (1994b) reported an *A. oryzae* strain that produced at least two kinds of extracellular lipolytic enzymes, L1 and L2. The enzyme L1 was purified to homogeneity by ammonium sulfate and acetone fractionation, ion exchange chromatography, and gel filtration. Lipase L1 was a monomeric protein (24 kDa molecular weight) and preferentially cleaved all the ester bonds of triolein.

An extracellular lipase from *Aci. calcoaceticus* BD 413 was purified to homogeneity using hydrophobic interaction fast performance liquid chromatography (FPLC) (Kok et al., 1995). The enzyme had an apparent molecular mass of 32 kDa on SDS-PAGE and an optimal activity pH of between 7.8 and 8.8 (Kok et al., 1995). Also, a lipase from *Pe. roqueforti* IAM 7268 was purified to homogeneity by a procedure involving ethanol precipitation, ammonium sulfate precipitation, and three chromatographic steps on different matrices (DEAE-Toyopearl 650 M, Phenyl Toyopearl 650 M, Toyopearl HW-60). The molecular mass of purified lipase was 25 kDa by electrophoresis (Mase et al., 1995). The enzyme had a high specificity towards short-chain fatty acid esters (Mase et al., 1995). A *Pichia burtonii* lipase was purified to homogeneity by a combination of DEAE-Sephadex A-50 ion exchange chromatography, Sephadex G-100 gel filtration, and isoelectric focusing (Sugihara et al., 1995). The purified enzyme was monomeric and had a molecular mass of 51 kDa by SDS-PAGE. The isoelectric pH of the enzyme was 5.8 (Sugihara et al., 1995). The enzyme had temperature and pH optima of 45 °C and pH 6.5, respectively (Sugihara et al., 1995).

Kim et al. (1996) purified a highly alkaline extracellular lipase of *Proteus vulgaris* by ion exchange chromatography. The purified lipase had a maximum hydrolytic activity at pH 10.0 and its molecular mass was 31 kDa by SDS-PAGE. Lin et al. (1996) purified an alkaline lipase from *P. pseudoalcaligenes* F-111 to homogeneity. The apparent molecular mass by SDS-PAGE was 32 kDa and the isoelectric pH was 7.3 (Lin et al., 1996). The enzyme showed a preference for C_{12} aryl and C_{14} acyl groups when using *p*-nitrophenyl esters as substrates. An extracellular lipase from *P. aeruginosa* KKA-5 was purified using ammonium sulfate precipitation and successive chromatographic separations on hydroxyl appetite (Sharon et al., 1998). After a 518-fold purification, the enzyme was homogenous electrophoretically and its molecular mass was estimated to be 30 kDa (Sharon et al., 1998). The enzyme was inhibited by SDS, an anionic surfactant; however, the cationic surfactants Triton X-100 and Tween 80 appreciably enhanced the enzyme activity (Sharon et al., 1998).

A lipase produced by *Staphylococcus epidermidis* RP 62A was purified to homogeneity by a combination of precipitation techniques, metal affinity chromatography, and gel filtration (Simons et al., 1998). The purified enzyme had a pH optimum of 6.0 and required calcium as a cofactor for catalytic activity (Simons et al., 1998). A recombinant lipase (rROL) produced by *S. cerevisiae* was purified by ethanol precipitation, butyl-Toyopearl 650 M chromatography, and Sephacryl S-100 HR gel filtration, to a single band by native PAGE (Takahashi et al., 1998). The band was found to consist of two proteins with molecular masses of 35 and 46 kDa, on SDS-PAGE.

A major and minor lipase from the fungus *O. piliferum* were copurified by hydrophobic interaction chromatography on octyl sepharose FF, followed by ion exchange chromatography on Q sepharose FF (Brush et al., 1999). This protocol resulted in a 1000-fold purification of the lipase. The major lipase had a molecular mass of approximately 60 kDa and a p*I* of 3.79. The minor lipase had a molecular mass of 5 kDa and a p*I* of 3.6. Diogo et al. (1999) reported the fractionation of *Chromobacterium viscosum* lipase using a polypropylene glycol Sepharose gel. Adsorption of the lipase on the gel depended on the salt concentration and the ionic strength of the mobile phase (Diogo et al., 1999). A mobile phase of 20% (wt/vol) ammonium sulfate in phosphate buffer produced total retention of lipase on the column. The lipase could be desorbed easily by decreasing the ionic strength of the buffer (Diogo et al., 1999).

An extracellular lipase from *Pseudomonas* sp. could be purified to homogeneity by extraction, Bio-gel P-10 chromatography, and Superose 12B chromatography (Dong et al., 1999). The overall purification factor was 37. SDS-PAGE indicated a molecular mass of 30 kDa for this lipase and its isoelectric point was pH 4.5. The pH and temperature optima for hydrolysis were pH 7.0–9.0 and 45–60 °C, respectively. The enzyme was stable between pH values of 6 and 12 and at less than 60 °C.

Two lipases were purified using a DEAE-Sephadex A-50 column and preparative electrophoresis (Kaminishi et al., 1999). The purified enzymes from *A. repens* and *Eurotrium hebariorum* NU-2 had molecular masses of 38 and 65 kDa, respectively, as determined by SDS-PAGE (Kaminishi et al., 1999). Lipase from *A. repens* had a pH optimum of 5.3 and temperature optimum of 27 °C. The NU-2 lipase had a pH optimum of 5.2 and a temperature optimum of 37 °C (Kaminishi et al., 1999). A three-step procedure involving ammonium sulfate precipitation, DEAE Sephacel ion exchange chromatography, and Sephacryl S-200 gel filtration chromatography was used to purify a lipase from a thermophilic *B. thermoleovorans* ID-1 to homogeneity (Lee et al., 1999). The protein was purified 223-fold. The molecular mass of the lipase was 34 kDa (SDS-PAGE). The enzyme showed optimal activity at 70–75 °C and pH 7.5. The enzyme retained 50% of its original activity after 1-h incubation at 60 °C and 30-min incubation at 70 °C (Lee et al., 1999).

Pe. cyclopium grown in stationary culture produced a Type I lipase specific for triacylglycerols (Chahinian et al., 2000). In agitated culture, the fungus produced a Type II lipase that was only active on partial acylglycerols (Chahinian et al., 2000). Lipase II was purified by ammonium sulfate precipitation and two chromatographic steps. The enzyme existed in several glycosylated forms (40–43 kDa molecular masses), which could be converted to a single protein of 37 kDa by enzymatic deglycosylation (Chahinian et al., 2000). Activity of Lipase II was maximal at pH 7.0 and 40 °C. The enzyme was stable between pH values of 4.5 and 7.0. Activity was rapidly lost at temperatures greater than 50 °C (Chahinian et al., 2000).

Hiol et al. (2000) purified an extracellular lipase produced by *Rhizop. oryzae* by ammonium sulfate precipitation, sulfopropyl Sepharose chromatography, Sephadex G-75 gel filtration, and a second sulfopropyl Sepharose chromatography step. The enzyme was purified 1200-fold and had a molecular mass of 32 kDa by SDS-PAGE and gel filtration (Hiol et al., 2000). The enzyme had an isoelectric point of pH 7.6. A thermostable lipase produced

by a thermophilic *Bacillus* sp. J 33 was purified to 175-fold by ammonium sulfate and phenyl Sepharose column chromatography (Nawani and Kaur, 2000). The overall recovery was 15.6%. The enzyme was shown to be a monomeric protein of 45 kDa molecular mass. The enzyme hydrolyzed triolein at all the positions.

Most of the lipase purification schemes described in the literature focused on purifying small amounts of the enzyme to homogeneity to characterize it. Little information has been published on large-scale processes for commercial purification of lipase. Most commercial applications of lipases do not require highly pure enzyme. Excessive purification is expensive and reduces overall recovery of the enzyme (Chisti, 1998).

In many cases, lipases appear to obey Michaelis–Menten kinetics (Guit et al., 1991; Malcata et al., 1992a). Michaelis–Menten kinetics are characterized by two parameters, K_m and v_{max}. The latter is the maximum rate of reaction and K_m is a measure of the affinity of an enzyme for a particular substrate. A low K_m value represents a high affinity. The K_m values of the enzyme range widely, but for most industrially relevant enzymes, K_m ranges between 10^{-1} and 10^{-5} M (Fullbrook, 1996). Pabai et al. (1995b) reported Michaelis–Menten parameters K_m and v_{max} of a purified lipase of *P. fragi* CRDA 323. The K_m and v_{max} values were 0.7 mg/mL and 0.97×10^{-3} U/min, respectively. For a *P. cepacia* lipase, Pencreac'h and Baratti (1996) reported K_m and v_{max} values of 12 mM and 30 μmol/min, respectively, when the substrate was *p*NPP. For a lipase of *Rho. glutinis*, the K_m values were 2.7 and 0.7 mM when the substrates were *p*-nitrophenyl butyrate and *p*-nitrophenyl laurate, respectively (Hatzinikolaou et al., 1999). Competitive inhibition of lipases by fatty acid substrates has been reported during esterification (Krishna and Karanth, 2001).

6. Thermostability of lipase

The rate of a reaction approximately doubles for each 10 °C increase in temperature. Assuming the enzyme is stable at elevated temperatures, the productivity of the reaction can be enhanced greatly by operating at a relatively high temperature. Consequently, thermal stability is a desirable characteristic of lipases (Janssen et al., 1994).

Thermostable lipases have been isolated from many sources, including *P. fluorescens* (Kojima et al., 1994); *Bacillus* sp. (Wang et al., 1995; Sidhu et al., 1998a,b); *B. coagulans* and *B. cereus* (El-Shafei and Rezkallah, 1997); *B. stearothermophilus* (Kim et al., 1998); *Geotrichum* sp. and *Aeromonas sobria* (Lotrakul and Dharmsthiti, 1997; Macedo et al., 1997); and *P. aeruginosa* (Sharon et al., 1998). The latter enzyme was significantly stabilized by Ca^{2+} and was inactivated by EDTA. This inactivation could be overcome by adding $CaCl_2$, suggesting the existence of a calcium-binding site in *P. aeruginosa* lipase.

One of the more notable thermostable enzyme was isolated by Wang et al. (1995) from a *Bacillus* strain. This enzyme had an activity maximum activity at 60 °C and retained 100% of the original activity after being held at 75 °C for 30 min. The half-life of the enzyme was 8 h at 75 °C (Wang et al., 1995). The enzyme retained at least 90% of the original activity after being incubated at 60 °C for 15 h (Wang et al., 1995). Other highly thermostable lipases have

been reported (Izumi et al., 1990; Janssen et al., 1994; Gao and Breuil, 1995; Kim et al., 1998; Lee et al., 1999).

An extracellular *Bacillus* lipase isolated by Sidhu et al. (1998a,b) had an activity optimum at 50 °C. The enzyme had a half-life of 15 min at 75 °C and it was stable to various oxidizing, reducing, and chelating agents. The enzyme was stable in the presence of surfactants and in organic solvents (Sidhu et al., 1998a,b). The crude lipase had an activity of 8.2 U/mL at 50 °C and pH 8.0. The activity was further enhanced by the presence of Ca^{2+}, Na^+, and Ba^{2+} (Sidhu et al., 1998a,b). Thermal stability of porcine pancreatic lipase has been discussed by Kiran et al. (2001b).

Thermal stability of a lipase is obviously related with its structure (Zhu et al., 2001). Thermostability is influenced by environmental factors such as pH and the presence of metal ions. At least in some cases, thermal denaturation appears to occur through intermediate states of unfolding of the polypeptide (Zhu et al., 2001). Mutations in the 'lid' region of the enzyme can significantly affect heat stability (Zhu et al., 2001). Attempts are being made to protein-engineer lipases for improved thermal stability.

Compared to the native enzyme, thermal and operational stability of many lipases can be significantly enhanced by immobilization (Xu et al., 1995; Reetz et al., 1996; Arroyo et al., 1999; Hiol et al., 2000). *C. antarctica* lipase B could be thermally stabilized by immobilization (Arroyo et al., 1999). The native enzyme and the covalently immobilized preparation appeared to follow different modes of thermal deactivation (Arroyo et al., 1999).

6.1. Effect of metal ions and chelating agents on lipase activity

Chartrain et al. (1993) observed that an extracellular lipase of *P. aeruginosa* MB5001 was strongly inhibited by 1 mM $ZnSO_4$ (94% inhibition) but was stimulated by adding 10 mM $CaCl_2$ (1.24-fold stimulation) and 200 mM taurocholic acid (1.6-fold stimulation). Mase et al. (1995) studied the effect of metal ions (1 mM concentration) on a purified lipase of *Pe. roqueforti* IAM7268. The lipase activity was not affected by Ca^{2+}, Mg^{2+}, Mn^{2+}, Na^+, K^+, Cu^{2+}, EDTA, *p*-chloro mercuribenzoic acid, and iodoacetate (Mase et al., 1995). In contrast, the enzyme was inhibited by Ag^+, Fe^{2+}, Hg^{2+}, and isopropyl fluorophosphate. In another similar study with metal ions (1 mM) and chelating agents, *P. pseudoalcaligenes* F-111 lipase activity was 60% inhibited by Fe^{3+} but not by Ca^{2+}, Hg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Co^{2+}, Cd^{2+}, and Pb^{2+} (Lin et al., 1996). Metal chelators (EDTA, *o*-phenanthrolin) did not significantly affect the alkaline lipase activity (Lin et al., 1996).

Sharon et al. (1998) reported a lipase of *P. aeruginosa* KKA-5 that retained its activity in presence of Ca^{2+} and Mg^{2+} but was slightly inhibited by Mn^{2+}, Cd^{2+}, and Cu^{2+}. Salts of heavy metals (Fe^{2+}, Zn^{2+}, Hg^{2+}, Fe^{3+}) strongly inhibited the lipase, suggesting that they were able to alter the enzyme conformation (Sharon et al., 1998). The effect of various metal ions on *S. epidermidis* lipase activity was reported by Simons et al. (1998). The enzyme needed calcium as a cofactor for catalytic activity (Simons et al., 1998). Biochemical characterization showed that this lipase was closely related to the lipase of *S. aurelis* NCTC 8530. Both the enzymes had a pH optimum of around 6.0 and were quite stable at low pH. Hiol et al. (2000) studied the effect of various compounds and enzyme

inhibitors on *Rhizop. oryzae* lipase. Among the metal ions, Fe^{2+}, Fe^{3+}, Hg^{2+}, and Cu^{2+} ions strongly inhibited the enzyme. Benzamidine and PMSF had no effect on the enzyme activity (Hiol et al., 2000).

7. Shear tolerance of lipases

Lipase-catalyzed reactions occur at interfaces and, therefore, the interfacial area and the quality of the interface are important influences on the observed reaction rate (Balashev et al., 2001). Use of intense mechanical agitation and emulsifiers in bioreactors helps in providing the requisite interfacial area. A combination of interface and agitation can be damaging to lipases. Intense agitation and liquid–liquid interfaces are especially frequent during lipase-catalyzed hydrolysis (Rooney and Weatherley, 2001). Gas–liquid interfaces occur in the production of lipases by aerobic submerged culture.

Shear-associated inactivation at gas–liquid and liquid–liquid interfaces has been reported for numerous enzymes (Chisti, 1999b) including lipases (Lee and Choo, 1989; Gordillo et al., 1995; Mohanty et al., 2001). The rate of interfacial denaturation increases with increasing temperature (Lee and Choo, 1989) and turbulence in the fluid (Chisti, 1999b). Denaturation generally obeys the first-order kinetics (Chisti, 1999b). The denaturation rate constant depends on the specific power input in the reactor and the amount of gas–liquid interface present (Mohanty et al., 2001). Additives such as polypropylene glycol can greatly reduce the rate of denaturation (Lee and Choo, 1989). Interfacial denaturation of lipases by unfolding apparently occurs without the molecule breaking into multiple peptides (Lee and Choo, 1989).

8. Multiple forms of lipases

Certain microbial and other lipases exist in multiple forms. Chang et al. (1994) identified multiple forms of CRL. The presence of Tween 80 and Tween 20 in the culture medium altered the relative abundance of the various forms of lipase in the medium, relative to when no additives were used (Chang et al., 1994). Two types of lipases, Lipases I and II, are known to be produced by *Rhizop. niveus* (Kohno et al., 1994). Lipases I and II differ in molecular weight and Lipase I appears to be converted to Lipase II by limited proteolysis (Kohno et al., 1994). *Geotrichum candidum* ATCC 34614 has been found to produce four different lipases (Sugihara et al., 1994). The main lipase (Lipase I) produced is nonspecific in positional specificity, whereas Lipase IV has unusual positional specificity (Sugihara et al., 1994).

Multiple forms of *C. antarctica* lipase have been reported (Arroyo and Sinisterra, 1995; Arroyo et al., 1999). Of these forms, lipase B is stereoselective towards the *R*-isomer of ketoprofen in an achiral solvent such as isopentyl methyl ketone and also in *S*(+)-carvone (Arroyo and Sinisterra, 1995). Martinelle et al. (1995) studied interfacial activation of *C. antarctica* lipases A and B (CALB) and compared them with the *Humicola lanuginosa* lipase. CALB displayed no interfacial activation, which indicated an absence of the lid

structure that regulates the access to the active site. The hydrolysis of the lipid *p*-nitrophenyl ester by lipases A and B of *C. rugosa* was characterized by Rodendo et al. (1995). Lipase A was maximally active on caprylate, whereas lipase B had maximal activity on laurate. The two enzymes were identical in other respects. Similarly, a commercial lipolytic preparation of *Ch. viscosum* was reported to contain two different lipases (Taipa et al., 1995).

9. Immobilization of lipases

Both native and immobilized lipases are available commercially. Lipases used in laundry detergents and many other applications are not immobilized; however, an increasing number of speciality applications of lipases in synthesis and biotransformation demand an immobilized biocatalyst for efficiency of use. Immobilization improves recyclability of expensive lipases. Also, immobilization can enhance enzyme stability and activity.

Many methods have been used to immobilize lipases, including adsorption or precipitation onto hydrophobic materials (Wisdom et al., 1984), covalent attachment to functional groups (Shaw et al., 1990), entrapment in polymer gels (Telefoncu et al., 1990), adsorption in macroporous anion exchange resins (Rizzi et al., 1992), microencapsulation in lipid vesicles (Balcão et al., 1996), and sol–gel entrapment (Jaeger and Reetz, 1998; Krishnakant and Madamwar, 2001). *G. candidum* lipases A and B were immobilized on Accurel EP 100 porous polypropylene supports, precoated with ovalbumin to increase stability in organic solvents and at elevated temperatures (Charton and Macrae, 1992). Bosley and Clayton (1994) used hydrophobic controlled pore glasses to immobilize *R. miehei* lipase. Reetz et al. (1995) employed sol–gel entrapment in silica gel to immobilize various lipases.

C. cylindracea lipase was immobilized on methyl acrylate divinyl benzene copolymer and its derivatives (Xu et al., 1995). The immobilized lipase had improved resistance to thermal denaturation than the native enzyme (Xu et al., 1995). Reetz et al. (1996) reported an immobilization procedure using alkyl silane precursors of the type R Si (OCH$_3$)$_3$ and mixtures of R Si (OCH$_3$)$_3$ and Si (OCH$_3$)$_4$ to immobilize *C. antarctica* lipase. This immobilization process provided highly active, chemically and thermally stable, heterogeneous biocatalysts (Reetz et al., 1996). Shin et al. (1997) prepared a celite-immobilized lipase of *A. oryzae* and used it for continuous esterification of *N*-protected amino acids with secondary alcohols in organic solvents (Shin et al., 1997). Jaeger and Reetz (1998) produced glutaraldehyde cross-linked microcrystals of CRL. These cross-linked crystals were used for the chiral resolution of commercially important compounds by ester hydrolysis.

Arroyo et al. (1999) covalently immobilized *C. antarctica* lipase B on Sepharose, alumina, and silica. This increased the thermal stability of the catalyst and modified its apparent mode of deactivation relative to the native enzyme. In one case, Amberlite IRC 50 was a suitable adsorbent for immobilizing the purified *Rhizop. oryzae* lipase (Hiol et al., 2000). Compared to other supports, Amberlite offered a high adsorption capacity and good long-term stability of the immobilized lipase. The stability of the immobilized enzyme was assessed by studying its capacity to esterify equimolar amounts of oleic acid and hexanol in cyclohexane at 30 °C (Hiol et al., 2000). The stability was further assessed by measuring the hydrolyzing activity of

the enzyme against trioctanoin. Repeated use of the immobilized lipase over a period of 3 weeks reduced its esterifying capacity by only 18% (Hiol et al., 2000). Over the same period, the hydrolyzing activity of the enzyme decreased by 80%.

For immobilization by adsorption on polymer membranes, hydrophobic membranes tend to load much more lipase than the hydrophilic membranes (Bouwer et al., 1997); however, at least for the hydrolytic reaction, the lipase immobilized on hydrophilic membranes generally appears to be much more active than the enzyme adsorbed on hydrophobic membranes (Bouwer et al., 1997). Use of hollow fiber and flat membrane reactors for biotransformations with immobilized lipases has been reported extensively (Balcão et al., 1996; Bouwer et al., 1997; Giorno et al., 1995, 1997; Guit et al., 1991; Malcata et al., 1991, 1992b; Xu et al., 2000; Xin et al., 2001). Also, packed bed lipase bioreactors have been used (Xu et al., 2001).

10. Sequencing and cloning of lipase gene

Early work on sequencing and cloning of lipase genes was discussed by Alberghina et al. (1991) and this subject continues to attract attention. Lipase genes from many micro-organisms and a higher animals have been cloned. The structural lipase gene from a gene library of *Aci. calcoaceticus* BD413 DNA was cloned in *Escherichia coli* phage M13 by Kok et al. (1995). The sequence analysis of 2.1-kb chromosomal DNA fragment revealed one complete open reading frame, lip A, encoding a mature protein with a predicted molecular mass of 32.1 kDa.

A recombinant plasmid expressing the alkaline lipase of *P. aeruginosa* IGB83 under the tac promoter was constructed by Leza et al. (1996). The plasmid was then cloned in *Xanthomonas campestris*, which efficiently produced and secreted the alkaline lipase. An optimization of culture conditions of recombinant *X. campestris* led to a 12-fold increase in lipase production relative to initial results in shake flasks (Leza et al., 1996).

Cloning of a lipase from *Rhizop. oryzae* DSM 853 has been reported (Beer et al., 1998). Apparently, the different lipase forms of *Rhizopus* sp. described in the literature result from different proteolytic processing and originate from the same gene. Epitope mapping studies using monoclonal antibodies directed against human pancreatic lipase (HPL) and various mutant lipases suggest that the beta 5′ loop from C-terminal domain may be involved in the interaction of HPL with a lipid/water interface (Bezzine et al., 1998).

The dimorphic yeast *C. rugosa* has an unusual codon usage that interferes with the functional expression of genes derived from this yeast in conventional heterologous hosts. CRL occurs in several different isoforms encoded by the *lip* gene family (Brocca et al., 1998). Of these lipases, the isoforms encoded by the gene *lip 1* is the most abundant (Brocca et al., 1998). The *lip 1* gene (1647 bp) was completely synthesized with an optimized nucleotide sequence to simplify genetic manipulation and allow heterologous expression in yeast (Brocca et al., 1998). The synthetic gene was functionally overexpressed in *Pi. pastoris*, allowing for the production of the specific isoform recombinant lipase at a level of 150 U/mL in the culture medium. The physiochemical and catalytic properties of the recombinant lipase

were compared with those of a commercial, nonrecombinant, CRL preparation containing the various isoforms.

According to Mileto et al. (1998), the lipase isoenzymes (CRLs) of the yeast *C. rugosa* share ca. 40% and 30% sequence homology with lipases of *G. candidum* and *Yarrowia lipolytica*, respectively. The domain of sequence conservation occurs in the N-terminal half of the protein. For the resolution of isoforms via heterologous expression, the *lip 1* gene, encoding the major CRL form, was expressed in *C. maltosa* — a related yeast with the same codon usage as *C. rugosa* (Mileto et al., 1998). A recombinant lipase was thus produced and secreted in an active form in the culture medium.

Production of *Pseudomonas* lipases requires correct folding and secretion through the membrane. A controllable expression of the gene *lip H*, encoding a lipase-specific foldase, is important for overexpression of lipase in the homologous host *E. coli* (Reetz and Jaeger, 1998). Construction of appropriate His-tagged fusion proteins permitted overexpression, secretion, and one-step purification of lipase from culture supernatants of the homologous host *P. aeruginosa*.

An efficient expression system for the previously only weakly expressed thermophilic lipase BTL-2 (*B. thermoatenulatus* Lipase II) has been developed for overexpression of the lipase in *E. coli* (Rua et al., 1998). The gene was subcloned in the pCVT-EXP1 (pT1) expression vector downstream of the temperature-inducible lambda promoter PL. Three different expression vectors were constructed. The expression vectors pT1-BTL2 and pT1-pre BTL 2 allowed comparable lipase expression levels of 7000–9000 U/g cells (Rua et al., 1998). Using the expression vector pT1-Omp ABTL2, the soluble lipase production levels were between 30,000 and 660,000 U/g cells, depending on the specific *E. coli* strain used to express the gene (Rua et al., 1998).

In *S. epidermidis* RP62A, the lipase gene (*geh SE1*) on the chromosome is immediately flanked by the ica AA′BC operon, which is involved in biofilm formation (Simons et al., 1998). This association has been claimed to suggest a possible role of lipase in staphylococcal colonization of the skin. The DNA sequence and the deduced lipase sequence revealed that *geh SE1* is very similar to the lipase sequence of *S. epidermidis* strain 9 and is organized as a preproenzyme. The part of *geh SE1* coding for the mature lipase was cloned and overexpressed as a fusion protein with an N-terminal histidine tag in *E. coli* (Simons et al., 1998). The lipase was purified and was shown to be biochemically closely related to the lipase of *S. aurelis* NCTC 8530 (Simons et al., 1998).

van Kampen et al. (1998) used site-directed mutagenesis and domain exchange to investigate the role of C-terminal domains of *S. hyicus* lipase (SHL) and *S. aureus* lipase (SAL) in substrate selectivity. A single point mutation coding for the substitution of Val for Ser 356 in SHL yielded an enzyme that retained full lipase activity, but with more than 12-fold lower phospholipase activity. Starting with this S356V variant of SHL, the C-terminal 40 amino acids were replaced by the corresponding SAL sequence. The resulting change in phospholipase/lipase activity ratio showed that in the C-terminal domain, Ser 356 mainly determines the phospholipase activity (van Kampen et al., 1998).

Rhizop. niveus lipase has a unique structure consisting of two noncovalently bound polypeptides (A-chain and B-chain). To improve this enzyme by protein engineering, Kohno

et al. (1999) developed a new expression system for producing the lipase in the yeast *Saccharomyces cerevisiae*. The efficient expression system used the strain ND-12 B and the multicopy plasmid pJDB 219.

A thermophilic lipase of *B. thermoleovorans* ID-1 was cloned and sequenced by Cho et al. (2000). The lipase gene coded 416 amino acid residues and contained the conserved pentapeptide Ala−X−Ser−X−Gly, as do other *Bacillus* lipases. For expression in *E. coli*, the lipase gene was cloned in pET-22b(+) vector with a strong T7 promoter (Cho et al., 2000). The lipase activity was approximately 1.4-fold greater than the activity with the native promoter. Pignede et al. (2000) isolated the *lip 2* gene from the lipolytic yeast *Y. lipolytica*. The gene encoded a 334-amino acid precursor protein. The secreted lipase was a 301-amino acid glycosylated polypeptide (Pignede et al., 2000).

The lip 2p protein is processed by the KEX 2-like endoprotease encoded by XPR6 (Pignede et al., 2000). Deletion of the *XPR6* gene resulted in the secretion of an active but less stable proenzyme. The proregion did not inhibit lipase secretion and activity and played an essential role in the production of a stable enzyme. The overexpressing strains correctly processed the gene, secreting 100-fold more activity than the wild type (Pignede et al., 2000).

A. oryzae produces at least three extracellular lipolytic enzymes, L1, L2, and L3. Of these, the L3 lipase (a triacylglycerol lipase) gene (provisionally designated *tglA*) was cloned (Toida et al., 2000). Nucleotide sequencing of the genomic DNA and cDNA revealed that the L3 gene (*tglA*) had an open reading frame of 954 nucleotides, including three introns of 47, 83, and 62 bp. The deduced amino acid sequences of the *tglA* gene implied a protein of 254 amino acid residues, including a single sequence of 30 amino acids that was homologous to a sequence of fungal cutinases. Three residues presumed to form the catalytic triad, Ser, Asp, and His, were conserved (Toida et al., 2000). The cloned cDNA of the *tglA* gene was expressed in *E. coli* to encode a functional triacylglycerol lipase (Toida et al., 2000).

11. Concluding remarks

As discussed here, lipases are versatile enzymes that are used widely. Lipases are becoming increasingly important in high-value applications in the oleochemical industry and the production of fine chemicals. Lipases are capable of regioselective and stereoselective biotransformations and allow resolution of racemic mixtures. Lipases with improved properties are being produced by natural selection and protein engineering to further enhance usefulness of these enzymes. Simultaneously, advances are being made in bioreactor and reaction technologies for effectively using the lipases. Various kinds of immobilized enzyme reactors and multiphase reaction systems have greatly influenced the processes that require catalysis by lipases (Balcão et al., 1996; Bouwer et al., 1997; Giorno et al., 1995, 1997; Malcata et al., 1991, 1992b; Xu et al., 2000, 2001; Xin et al., 2001). Lipase-based processing has a promising future; however, the rate of progress is slow. Factors posing limitations include a relatively high cost of lipases and a lack of enzymes with the optimal range of catalytic specificities and properties required in the various applications.

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