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MINI-REVIEW

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Bacterial lipases: an overview of production, purification and biochemical properties

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Abstract Lipases, triacylglycerol hydrolases, are an important group of biotechnologically relevant enzymes and they find immense applications in food, dairy, detergent and pharmaceutical industries. Lipases are by and large produced from microbes and specifically bacterial lipases play a vital role in commercial ventures. Some important lipase-producing bacterial genera include *Bacillus*, *Pseudomonas* and *Burkholderia*. Lipases are generally produced on lipidic carbon, such as oils, fatty acids, glycerol or tweens in the presence of an organic nitrogen source. Bacterial lipases are mostly extracellular and are produced by submerged fermentation. The enzyme is most commonly purified by hydrophobic interaction chromatography, in addition to some modern approaches such as reverse micellar and aqueous two-phase systems. Most lipases can act in a wide range of pH and temperature, though alkaline bacterial lipases are more common. Lipases are serine hydrolases and have high stability in organic solvents. Besides these, some lipases exhibit chemo-, regio- and enantioselectivity. The latest trend in lipase research is the development of novel and improved lipases through molecular approaches such as directed evolution and exploring natural communities by the metagenomic approach.

Introduction

The advent of enzymology represents an important breakthrough in the biotechnology industry, with the worldwide usage of enzymes being nearly U.S. $ 1.5 billion in 2000 (Kirk et al. 2002). The major share of the industrial enzyme market is occupied by hydrolytic enzymes, such as proteases, amylases, amidases, esterases and lipases. In recent times, lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) have emerged as key enzymes in swiftly growing biotechnology, owing to their multi-faceted properties, which find usage in a wide array of industrial applications, such as food technology, detergent, chemical industry and biomedical sciences (Jaeger et al. 1994, 1999; Pandey et al. 1999). Lipases are hydrolases, which act under aqueous conditions on the carboxyl ester bonds present in triacylglycerols to liberate fatty acids and glycerol. The natural substrates of lipases are long-chain triacylglycerols, which have very low solubility in water; and the reaction is catalyzed at the lipid–water interface. Under micro-aqueous conditions, lipases possess the unique ability to carry out the reverse reaction, leading to esterification, alcoholysis and acidolysis. Besides being lipolytic, lipases also possess esterolytic activity and thus have a very diverse substrate range, although they are highly specific as chemo-, regio- and enantioselective catalysts (Jaeger et al. 1994, 1999; Jaeger and Reetz 1998; Kazlauskas and Bornscheur 1998; Pandey et al. 1999; Beisson et al. 2000; Gupta and Soni 2000; Jaeger and Eggert 2002). The catalytic potential of lipases can be further enhanced and made selective by the novel phenomena of molecular imprinting and solvent engineering and by molecular approaches like protein engineering and directed evolution (Reetz and Jaeger 1999; Jaeger et al. 2001). The properties of lipases that need to be improved are stability and turnover under application conditions. They need to be robust and versatile with respect to the range of substrates they can act on, but at the same time they should have a high specificity for the reactions they catalyze.

Lipases are serine hydrolases which act at the lipid–water interface. The catalytic triad is composed of Ser-Asp/Glu-His and usually also a consensus sequence (Gly-x-Ser-x-Gly) is found around the active site serine. The three-dimensional (3-D) structures of lipases reveal the characteristic α/β-hydrolase fold (Nardini and Dijkstra 1999).

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The growing importance of lipases within biotechnological perspectives can be easily envisaged by the number of recent review articles covering various aspects of this extremely versatile biocatalyst, such as biochemistry, assay protocols, molecular biology, purification approaches and biotechnological applications (Jaeger and Reetz 1998; Beisson et al. 2000; Gupta et al. 2003; Saxena et al. 2003). In this review, we present an overview on the fermentation, downstream processes and properties of bacterial lipases.

Sources of lipases

Lipases are ubiquitous in nature and are produced by various plants, animals and microorganisms. Lipases of microbial origin, mainly bacterial and fungal, represent the most widely used class of enzymes in biotechnological applications and organic chemistry. A list of the common bacterial lipase producers is presented in Table 1. The extracellular bacterial lipases are of considerable commercial importance, as their bulk production is much easier. Although a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains (Jaeger et al. 1994; Palekar et al. 2000). Of these, the important ones are: *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium* and *Pseudomonas*. Of these, the lipases from *Pseudomonas* bacteria are widely used for a variety of biotechnological applications (Jaeger et al. 1994; Pandey et al. 1999; Beisson et al. 2000).

Several products based on bacterial lipases have been launched successfully in the market in the past few years (Table 2). A number of such products are from *Pseudomonas* spp, such as Lumafast and Lipomax with their major application as detergent enzymes, while Chiro CLEC-PC, Chirazyme L-1 and Amano P, P-30 and PS have tremendous potential in organic synthesis.

Fermentation conditions

Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors, such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, agitation and dissolved oxygen concentration (Brune and Gotz 1992; Aires-Barros et al. 1994; Jaeger et al. 1994; Kim et al. 1996). A list of various fermentation conditions used with different bacteria is presented in Table 3.

The major factor for the expression of lipase activity has always been carbon, since lipases are by and large inducible enzymes (Lotti et al. 1998) and are thus generally produced in the presence of a lipid source such as an oil or any other inducer, such as triacylglycerols, fatty acids, hydrolyzable esters, tweens, bile salts and glycerol (Ghosh et al. 1996; Dharmsthiti et al. 1998; Shirazi et al. 1998; Bradoo et al. 1999; Rathi et al. 2001). However, their production is significantly influenced by

Table 1 Sources of bacterial lipases

Bacterium	References
Achromobacter sp.	Mitsuda et al. 1988
A. lipolyticum	Brune and Gotz 1992; Davranov 1994
Acinetobacter sp.	Wakelin and Forster 1997; Barbaro et al. 2001
A. calcoaceticus	Dharmsthiti et al. 1998; Jaeger et al. 1999; Pandey et al. 1999; Pratuangdejkul and Dharmsthiti 2000
A. radioresistens	Liu and Tsai 2003
Alcaligenes sp.	Mitsuda et al. 1988
A.denitrificans	Odera et al. 1986
Arthrobacter sp.	Pandey et al. 1999
Archaeglobus fulgidus	Jaeger et al. 1999
*Bacillus*sp.	Sidhu et al. 1998a, 1998b; Pandey et al. 1999; Sharma et al. 2002a; Nawani and Kaur 2000
B. alcalophilus	Ghanem et al. 2000
B. atrophaeus	Bradoo et al. 1999
B.megaterium	Hirohara et al. 1985
B.laterosporus	Toyo-Jozo 1988
B. pumilus	Jaeger et al. 1999
B.sphaericus	Toyo-Jozo 1988
B. stearothermophilus	Bradoo et al. 1999; Jaeger et al. 1999
B. subtilis	Jaeger et al. 1999
B. thaiminolyticus	Toyo-Jozo 1988
B. thermocatenulatus	Jaeger et al. 1999; Pandey et al. 1999
Brochothrix thermosphacta	Brune and Gotz 1992
Burkholderia glumae	Jaeger and Reetz 1998; Reetz and Jaeger 1998
Chromobacterium violaceum	Koritala et al. 1987
C. viscosum	Jaeger and Reetz 1998; Jaeger et al. 1999
Corynebacterium acnes	Brune and Gotz 1992
Cryptocoocus laurentii	Toyo-Jozo 1988
Enterococcus faecalis	Kar et al. 1996
Lactobacillus curvatus	Brune and Gotz 1992
L. plantarum	Lopes Mde et al. 2002
Microthrix parvicella	Wakelin and Forster 1997
Moraxella sp.	Jaeger et al. 1999
Mycobacterium chelonae	Pandey et al. 1999
Pasteurella multocida	Pratt et al. 2000
Propionibacterium acnes	Jaeger et al. 1999
P. avidium	Brune and Gotz 1992
P. granulosum	Brune and Gotz 1992
Proteus vulgaris	Jaeger et al. 1999
Pseudomonas aureofaciens	Koritala et al. 1987

Table 1 (continued)

Bacterium	References
P. fluorescens	Arpigny and Jaeger 1999; Pandey et al. 1999
P. fragi	Jaeger et al. 1994; Schuepp et al. 1997; Ghanem et al. 2000
P. luteola	Arpigny and Jaeger 1999; Litthauer et al. 2002
P. mendocina	Jaeger et al. 1999; Surinenaite et al. 2002
P. nitroreducens var. *thermotolerans*	Ghanem et al. 2000
P. pseudomallei	Kanwar and Goswami 2002
P. wisconsinensis	Arpigny and Jaeger 1999
Psychrobacter immobilis	Jaeger et al. 1999
Staphylococcus aureus	Simons et al. 1996; Jaeger et al. 1999
S. epidermidis	Simons et al. 1996; Jaeger et al. 1999
S. haemolyticus	Oh et al. 1999
S. hyicus	Jaeger et al.1999; Van Kampen et al.2001
S. warneri	Pandey et al.1999; Van Kampen et al.2001
S. xylosus	Pandey et al.1999; Van Kampen et al.2001
Serratia marcescens	Matsumae et al. 1993,1994; Pandey et al. 1999; Abdou 2003
Streptomyces exfoliatus	Arpigny and Jaeger 1999
Sulfolobus acidocaldarius	Jaeger et al. 1999
Vibrio chloreae	Jaeger et al. 1999

other carbon sources, such as sugars, sugar alcohol, polysaccharides, whey, casamino acids and other complex sources (Gilbert et al. 1991a; Lotrakul and Dharmsthiti 1997; Dharmsthiti and Kuhasuntisuk 1998; Ghanem et al. 2000; Rashid et al. 2001). Certain long-chain fatty acids, such as oleic, linoleic and linolenic acids, are known to support lipase production from various bacteria, such as *P. mephitica* (Ghosh et al. 1996). However, lipases from *P. aeruginosa* EF2 (Gilbert et al. 1991a) and *Acinetobacter calcoaceticus* (Mahler et al. 2000) are reported to be repressed in the presence of long-chain fatty acids, such as oleic acid. Yeo et al. (1998) used the fatty acid ester *t*-butyl octanoate (TBO) for the screening of lipase-producing bacteria from different soil samples. Of 279 strains isolated, *Burkholderia* YY62 was selected for its strong TBO-hydrolyzing activity. Kanwar et al. (2002) reported the production of a *Pseudomonas* sp. G6 lipase in the presence of *n*-alkane substrates, with a maximum production of about 25 units/ml when *n*-hexadecane was the sole carbon source. Production was enhanced to nearly 2.4-fold using tributyrin at a concentration of 0.05% in the production medium.*n*-Hexadecane and olive oil were employed as the carbon source for producing an alkaline lipase from *A. radioresistens* (Liu and Tsai 2003).

Besides carbon source, the type of nitrogen source in the medium also influences the lipase titers in production broth (Ghosh et al. 1996). Generally, organic nitrogen is preferred, such as peptone and yeast extract, which have been used as nitrogen source for lipase production by various *Bacillus* spp (viz. *Bacillus* strain A30-1, *B. alcalophilus*, *B. licheniformis* strain H1) and various pseudomonads (viz. *Pseudomonas* sp., *P. fragi*, *P. fluorescens* BW 96CC), *Staphylococcus haemolyticus*; (Wang et al. 1995; Khyami-Horani 1996; Pabai et al. 1996; Oh et al. 1999; Ghanem et al. 2000; Lanser et al. 2002; Sharma et al. 2002b), while tryptone and yeast extract have been used in the case of *S. haemolyticus* L62 (Oh et al. 1999). Inorganic nitrogen sources such as ammonium chloride and diammonium hydrogen phosphate have also been reported to be effective in some microbes (Gilbert et al. 1991a, 1991b; Bradoo et al. 1999; Dong et al. 1999; Rathi et al. 2001).

Divalent cations stimulate or inhibit enzyme production in microorganisms. Rathi et al. (2001) observed stimulation in lipase production from *Burkholderia* sp. in the presence of Ca^{2+} and Mg^{2+}. Sharma et al. (2002b) also reported stimulation in lipase production from *Bacillus* sp. RSJ1 in the presence of calcium chloride. However, most other metal ion salts were inhibitory to lipase production. Iron was found to play a critical role in the production of lipase by *Pseudomonas* sp. G6 (Kanwar et al. 2002).

In addition to the various chemical constituents of a production medium, physiological parameters such as pH, temperature, agitation, aeration and incubation period also play an important role in influencing production by different microorganisms. The initial pH of the growth medium is important for lipase production. Largely, bacteria prefer pH around 7.0 for best growth and lipase production, such as in the case of *Bacillus* sp. (Sugihara et al. 1991), *Acinetobacter* sp. (Barbaro et al. 2001) and *Burkholderia* sp. (Rathi et al. 2001). However, maximum activity at higher pH (>7.0) has been observed in many cases (Nashif and Nelson 1953; Gilbert et al. 1991a; Wang et al. 1995; Khyami-Horani 1996; Dong et al. 1999; Sharma et al. 2002b). The optimum temperature for lipase production corresponds with the growth temperature of the respective microorganism. For example, the best temperature for growth and lipase production in the case of *Bacillus* sp. RSJ1 was 50°C (Sharma et al. 2002b). It has been observed that, in general, lipases are produced in the temperature range 20–45°C. Incubation periods ranging from few hours to several days have been found to be best suited for maximum lipase production by bacteria. An incubation period of 12 h was optimum for lipase production by *A. calcoaceticus* and *Bacillus* sp. RSJ1 (Mahler et al. 2000; Sharma et al. 2002b) and 16 h for *B. thermocatenulatus* (Schmidt-Dannert et al. 1997). While maximum lipase was produced after 72 h and 96 h of incubation, respectively, in the case of the *Pseudomonas* spp*P. fragi* and *P. fluorescens* BW 96CC (Pabai et al. 1996; Dong et al. 1999).

Thus, bacterial lipases are generally produced in the presence of oil or any other lipidic substrate (viz. fatty acid esters, fatty acids, glycerol) as carbon in the presence of any complex nitrogen source. The requirement for metal ions varies with the organism. However, physical para-

Table 2 Commercial bacterial lipases, sources, applications and their industrial suppliers. *n.s.* Not specified

Commercial lipase	Source	Supplier	Application	References
Lumafast	*Pseudomonas menodocina*	Genencor International, USA	Detergent	Jaeger et al. 1994; Jaeger and Reetz 1998
Lipomax	*P. alcaligenes*	Gist-Brocades, The Netherlands; Genencor International, USA	Detergent	Jaeger et al. 1994; Jaeger and Reetz 1998
n.s.	*P. glumae*	Unilever, The Netherlands	Detergent	Jaeger et al. 1994
n.s.	*Bacillus pumilus*	Solvay, Belgium	Detergent	Jaeger et al. 1994
Chiro CLEC-PC, Chirazyme L-1	*P. cepacia*	Altus Biologics, Manheim	Organic synthesis	Jaeger and Reetz 1998
Amano P, P-30, PS, LPL-80, LPL-200S	*P. cepacia*	Amano Pharmaceuticals, Japan	Organic synthesis	Jaeger and Reetz 1998
Lipase AH	*P. cepacia*	Amano Pharmaceuticals, Japan	Organic synthesis	Jaeger and Reetz 1998
Lipase AK, YS	*P. fluorescens*	Amano Pharmaceuticals, Japan	Organic synthesis	Jaeger and Reetz 1998
Lipase 56P	*P. fluorescens*	Biocatalysts, UK	Biotransformations, chemicals	Godfrey and West 1996
Lipase K-10	*Pseudomonas* sp.	Amano Pharmaceuticals, Japan	Organic synthesis	Jaeger and Reetz 1998
Chromobacterium viscosum lipase	*C. viscosum*	Asahi Chemical Biocatalysts	Organic synthesis	Godfrey and West 1996
Lipase 50P	*C. viscosum*	Biocatalysts, UK	Biotransformations, chemicals	Godfrey and West 1996
Lipase QL	*Alcaligenes* sp.	Meito Sankyo Co., Japan	Organic synthesis	Jaeger and Reetz 1998
Lipoprotein lipase	*Alcaligenes* sp.	Meito Sankyo Co., Japan	Research	Godfrey and West 1996
Lipase PL, QL/QLL, PLC/PLG, QLC/QLG	*Alcaligenes* sp.	Meito Sankyo Co., Japan	Technical grade	Godfrey and West 1996
Alkaline lipase	*Achromobacter* sp.	Meito Sankyo Co., Japan	Research	Godfrey and West 1996
Lipase AL, ALC/ALG	*Achromobacter* sp.	Meito Sankyo Co., Japan	Technical grade	Godfrey and West 1996
Combizyme 23P (proteinase/lipase mix)	n.s.	Biocatalysts, UK	Waste treatment	Godfrey and West 1996
Combizyme 61P (proteinase/lipase mix)	n.s.	Biocatalysts, UK	Waste treatment	Godfrey and West 1996
Combizyme 209P (amylase/lipase/proteinase mix)	n.s.	Biocatalysts, UK	Waste treatment, grease disposal	Godfrey and West 1996
Greasex (lipase)	n.s.	Novo Nordisk	Leather	Godfrey and West 1996

Table 3 Fermentation conditions

Bacterium/mixture	pH	Temperature (°C)	Agitation (rpm)	Incubation period (h)	Carbon source	Nitrogen source	Reference
Acinetobacter sp.	7.0	25	n.s.	9	Tween-80/ olive oil	n.s.	Barbaro et al. 2001
A. calcoaceticus	6.8	30	250	12	Lactic acid, oleic acid	n.s.	Mahler et al. 2000
A. calcoaceticus LP009	7.0	15	200	n.s.	Tween-80	Tryptone, yeast extract	Pratuangdejkul and Dharmsthiti 2000
*Bacillus*sp.	7.0	28	Reciprocal shaking	80	Olive oil	Peptone, yeast extract	Sugihara et al. 1991
*Bacillus*sp. RSJ1	9.0	50	200	12	Tween-80/ olive oil	Peptone, yeast extract	Sharma et al. 2002b
*Bacillus*sp. strain 398	7.2	55	Reciprocal shaking	12	Glycerol	Polypeptone, yeast extract, beef extract	Kim et al. 1994
*Bacillus*strain A30-1 (ATCC 53841)	9.0	60	200	15–24	Corn oil	Ammonium chloride, yeast extract	Wang et al. 1995
B. alcalophilus	10.6	60	100	20	Maltose, soybean meal	Peptone, yeast extract	Ghanem et al. 2000
B. licheniformis strain H1	9.0	50	200	10	Glucose	Peptone, yeast extract, lab. beef extract	Khyami-Horani 1996
Burkholderia sp.	7.0	45	250	24	Glucose, mustard oil	NH_4Cl, $(NH_4)_2HPO_4$	Rathi et al. 2001
Geobacillus sp.	9.0	70	n.s.	n.s.	Tween-80/ olive oil	n.s.	Abdel-Fattah 2002
Pseudomonas sp.	9.0	30	150	72	Ground soybean, soluble starch	Corn steep liquor, $NaNO_3$	Dong et al. 1999
Pseudomonas sp.	n.s.	n.s.	n.s.	60	Soya peptone, cottonseed meal, groundnut oil	Soya peptone	Kulkarni and Gadre 1999
Pseudomonas sp. G6	8.0	34	n.s.	n.s.	*n*-hexadecane, tributyrin	n.s.	Kanwar et al. 2002
Pseudomonas sp. strain KB 700A (recombinant lipase)	7.0	37	n.s.	16	Casamino acids	Yeast extract	Rashid et al. 2001
P. aeruginosa	8.5	37	200	6	Tween-80	KNO_3	Gilbert et al. 1991a
P. aeruginosa LP602	7.2	30	200	48	Whey, soybean oil, glucose	Ammonium sulfate, yeast extract	Dharmsthiti and Kuhasuntisuk 1998
P. fragi,*P. fluorescens* BW 96CCI, *P. putida*	7.5	30	150	96	Dextrose, butter	Tryptone, yeast extract	Pabai et al. 1996
P. putida ATCC 795	7.5	27	150	72	Soybean flour, soluble starch, unsalted butter	Bacto-peptone	Pabai et al. 1995
P. putida 3SK	n.s.	30	500	24	Olive oil	n.s.	Lee and Rhee 1994
S. haemolyticus L62	7.0	37	n.s.	20	n.s.	Tryptone, yeast extract	Oh et al. 1999
*Bacillus*sp., *Pseudomonas* sp.	n.s.	30	150	24	Dextrose, triolein	Tryptone, yeast extract	Lanser et al. 2002
*Bacillus*sp., *Pseudomonas* sp., *Arthrobacter* sp., *Chromobacterium*sp., *Staphylococcus* sp., *Streptococcus* sp.	n.s.	28	200	5 days	Glucose, soybean oil	Asparagine	Koritala et al. 1987

meters such as pH, temperature, agitation and aeration influence lipase production via modulating the growth of the bacterium. Lipases are produced throughout bacterial growth, with peak production being obtained by the late log phase. The production period for lipases varies from a few hours to a few days.

Strategies for improving fermentation conditions: statistical design approach

When developing an industrial fermentation, designing a fermentation medium is of critical importance, because medium composition significantly affects product concentration, yield and productivity. For commodity products, medium cost can substantially affect the overall process economics. Designing the medium is a laborious, expensive and often time-consuming process involving many experiments (Kennedy and Krouse 1999). There is a general practice of determining optimal concentration of media components by varying one factor at a time. However, this method does not depict the net effect of total interactions among the various media components (Rathi et al. 2001). Thus, the emphasis has shifted towards medium optimization using response surface methodology (RSM). The factorial design of a limited set of variables is advantageous in relation to the conventional method of manipulation of a single parameter per trial, as the latter approach frequently fails to locate the optimal conditions for the process, due to its failure to consider the effect of possible interactions between factors. Moreover, the factorial design makes it possible to take advantage of practical knowledge about the process during the final RSM analysis (Kalil et al. 2000).

Optimization through factorial design and RSM analysis is a common practice in biotechnology. Various research workers have applied this approach, especially for the optimization of process parameters such as pH, temperature, aeration and others. Using the RSM approach, Mahler et al. (2000) reported that lactic acid used as carbon source does not have any significant effect on lipase production, while gum arabic increases the yield of extracellular lipase by 2- to 5-fold and oleic acid has a negative effect on lipase production from *Acinetobacter calcoaceticus*. An overall 2.4-fold increase in lipase production and a 1.8-fold increase in specific activity was obtained from *Burkholderia cepacia* after validation of RSM in shake-flasks (Rathi et al. 2002). Abdel-Fattah (2002) reported a 4-fold increase in lipase production in shake-flask cultures from a thermophilic *Geobacillus* sp., using a Box–Behnken experimental design. An empirical model was developed through RSM to describe the relationship between the tested variables, viz. Tween-80, olive oil, temperature, pH and enzyme activity. Lipase production from *P. fluorescens* NS2W was optimized in shake-flasks using a statistical experimental design (Kulkarni and Gadre 2002). Cell growth and lipase production were studied in shake-flasks and a 1-l fermentor, using the optimized medium. The optimized medium resulted in about a 5-fold increase in enzyme production, compared with that obtained in the basal medium. However, not many reports of the applicability of the RSM approach to the optimization of lipase production exist in the literature.

Purification strategies for bacterial lipases

Most of the commercial applications of enzymes do not always need homogeneous preparation of the enzyme. However, a certain degree of purity is required, depending upon the final application, in industries such as fine chemicals, pharmaceuticals and cosmetics. Besides, purification of the enzyme is a must for understanding the 3-D structure and the structure–function relationships of proteins (Taipa et al. 1992; Aires-Barros et al. 1994; Saxena et al. 2003).

For industrial purposes, the purification strategies employed should be inexpensive, rapid, high-yielding and amenable to large-scale operations. They should have the potential for continuous product recovery, with a relatively high capacity and selectivity for the desired product. Various purification strategies used for lipases have been reviewed several times (Antonian 1988; Taipa et al. 1992; Aires-Barros et al. 1994; Palekar et al. 2000; Saxena et al. 2003), highlighting clearly the importance of designing optimal purification schemes for various microbial lipases. The extent of purification varies with the order of the purification steps; and this aspect has been evaluated through different purification protocols pursued by various investigators.

Prepurification steps involve concentration of the culture supernatant containing the enzyme by ultrafiltration, ammonium sulfate precipitation or extraction with organic solvents. Precipitation often gives a high average yield (Aires-Barros et al. 1994) although with limited purification; and such enzyme preparations are apt for use in detergent formulations. However, for certain applications, such as synthetic reactions in pharmaceutical industry, further purification is needed. Since lipases are known to be hydrophobic in nature, having large hydrophobic surfaces around the active site, the purification of lipases may best be achieved by opting for affinity chromatography, such as hydrophobic interaction chromatography. The use of hydrophobic interaction chromatography has increased tremendously in the past few years (Kordel et al. 1991; Hong and Chang 1998; Imamura and Kitaura 2000; Queiroz et al. 2001). Affinity methods can be applied at an early stage, but as the hydrophobic matrices are expensive, alternatively ion exchange and gel filtration are usually preferred after the precipitation step (Schmidt-Dannert et al. 1994, 1996; Jose and Kurup 1999; Ghanem et al. 2000; Imamura and Kitaura 2000; Litthauer et al. 2002; Snellman et al. 2002; Abdou 2003).

The usual procedures for lipase purification are sometimes troublesome, time-consuming and result in low final yields. Novel purification steps are therefore needed to increase the overall enzyme yields and to reduce the

number of steps in the downstream processing. Since lipases are different from other enzymes in terms of their hydrophobic nature, interfacial activation phenomenon and activity in non-aqueous systems, some novel purification technologies have recently been applied for the purification of lipases. These include a reversed micellar system, membrane processes, immunopurification, hydrophobic interaction chromatography employing an epoxy-activated spacer arm as a ligand, column chromatography using polyethylene glycol (PEG)/Sepharose gel or poly (vinyl alcohol) polymers as stationary phases and aqueous two-phase systems (Saxena et al. 2003). Here, a brief description of some of these novel methods is provided.

Aqueous two-phase systems

The aqueous two-phase systems used in bioseparation are composed of two incompatible polymers (e.g. dextran vs PEG) in water solution or in a high salt concentration (e.g. phosphate). The partitioning of proteins in aqueous two-phase systems depends on the physico-chemical properties, e.g. protein hydrophobicity, charge and size. The partitioning is influenced by changing polymers, polymer molecular mass, or pH, or by the addition of salts or detergent to the system. The advantages of aqueous two-phase extraction lie in volume reduction, high capacity, rapid separations and mildness. The technique can be used early in the purification on process streams containing whole cells or cell debris. Compared with other separation techniques, two-phase extraction is relatively straightforward to scale-up. The aqueous two-phase system is an interesting technique with properties suitable for the separation and purification of macromolecules and particles that are difficult to purify with other existing techniques (Albertsson et al. 1990; Gupta et al. 1999). A number of examples of lipase purification using aqueous two-phase systems are available in the literature. For lipases, the hydrophobic nature of the enzyme is exploited in aqueous two-phase systems by employing detergents or surfactants during the purification. Terstappen et al. (1992) studied detergent-based aqueous two-phase systems for the purification of lipase from *P. cepacia* and found that all prokaryotic lipases showed a preference for a detergent-based coacervate phase. Queiroz et al. (1995) employed PEG/potassium phosphate aqueous two-phase systems for the extraction of *C. viscosum* lipase and concluded that lipase partitioning could be easily manipulated by modifying the separation conditions. Bompensieri et al. (1996) studied lipase purification from *Acinetobacter calcoaceticus* by aqueous two-phase systems using PEG, dextran, salt or a surfactant. Two lipases, one acidic and one neutral from *Bacillus stearothermophilus* SB1 were purified using PEG and salt, with the lipases preferentially partitioning to the PEG phase, due to hydrophobic interactions with ethylene groups of the polymer (Bradoo et al. 1999).

Reversed micellar systems

Liquid/liquid extraction of biomolecules using a reversed micelle is a promising method when traditional techniques with organic solvents are limited by protein denaturation and solubilization (Castro and Cabral 1988). Reversed micelles are water droplets within an organic solvent which are stabilized by a monolayer of surfactant molecules and can be formed by contacting an aqueous phase with an immiscible organic phase containing these surfactants. The inner cores contain an aqueous microphase which is able to solubilize bioproducts such as proteins. The selective separation and purification of a lipolytic preparation from *C. viscosum* (Vicente et al. 1990) was achieved in AOT-based reverse micelles with benzene as the organic solvent. The method involves a very simple procedure and requires two steps. The first step is based on the ability of reversed micelles to solubilize proteins from an aqueous phase into the water pool of the surfactant aggregates. In the second step, the solubilized proteins are back-extracted into a new aqueous phase by changing the interactions between the protein and the reversed micellar system. Selective solubilization of a mixture of proteins can be achieved by manipulating the parameters of the systems, both in the micellar and aqueous phases, the most important parameters being the pH and ionic strength of the aqueous phase. The pH value influences electrostatic interactions between the polar head groups of the surfactant and the charged protein. Hydrophobic interactions may also act on the transfer of proteins, especially the proteins, such as lipases, that bear a hydrophobic region on their surface. Although the reversed micelle seems to be a very promising technique for lipase purification, it is not much exploited by researchers, due to inefficient back-extraction protocols. However, its high efficiency during the biocatalytic reactions of lipases is very well documented (Skagerlind et al. 1992; Yamada et al. 1993).

Immunopurification

Immunopurification is one of the most efficient and selective protein-purification techniques, because of the high specificity of the antibody–antigen reactions. Highly specific antibodies can distinguish between very similar antigens, which are otherwise difficult to separate by conventional methods (Harlow and Lane 1988). Most immunopurifications are carried out with monoclonal antibodies or affinity-purified polyclonal antibodies, depending on the availability of the monoclonal antibody against the target protein and the type of contaminants present in the crude protein preparation. Bandmann et al. (2000) used IgG-affinity chromatography for the purification of the modified cutinase lipase variants produced in *Escherichia coli*. However, in spite of being an extremely selective and efficient purification technique, the high costs involved (particularly for the production of mono-

clonal antibodies) remain the major bottleneck in the extensive usage of this method.

Table 4 provides a comprehensive account of the purification strategies adopted for various bacterial lipases.

Properties of bacterial lipases

Lipases from several microorganisms have been studied extensively and, based on their properties, used in various industries. Various properties of bacterial lipases (viz. molecular weight, pH and temperature optima, stability, substrate specificity) are summarized in Table 5. However, a brief account of individual properties is presented in the following sections.

pH and temperature kinetics

Generally, bacterial lipases have neutral (Dharmsthiti et al. 1998; Dharmsthiti and Luchai 1999; Lee et al. 1999) or alkaline pH optima (Schmidt-Dannert et al. 1994; Sidhu et al. 1998a, 1998b; Kanwar and Goswami 2002; Sunna et al. 2002), with the exception of *P. fluorescens* SIK W1 lipase, which has an acidic optimum at pH 4.8 (Andersson et al. 1979). Lipases from *Bacillus stearothermophilus* SB-1, *B. atrophaeus* SB-2 and *B. licheniformis* SB-3 are active over a broad pH range (pH 3–12; Bradoo et al. 1999). Bacterial lipases possess stability over a wide range, from pH 4 to pH 11 (Kojima et al. 1994; Wang et al. 1995; Khyami-Horani, 1996; Dong et al. 1999).

Bacterial lipases generally have temperature optima in the range 30–60°C (Lesuisse et al. 1993; Wang et al. 1995; Dharmsthiti et al. 1998; Litthauer et al. 2002). However, reports exist on bacterial lipases with optima in both lower and higher ranges (Dharmsthiti and Luchai 1999; Lee et al. 1999; Oh et al. 1999; Sunna et al. 2002). Thermal stability data are available only for species of *Bacillus*, *Chromobacterium*, *Pseudomonas*and *Staphylococcus*. The thermostability of the enzyme from *Bacillus* sp. was enhanced by the addition of stabilizers such as ethylene glycol, sorbitol, glycerol, with the enzyme retaining activity at 70°C even after 150 min (Nawani and Kaur 2000). A few*Pseudomonas* lipases have been reported which are stable at 100°C or even beyond to 150°C with a half-life of a few seconds; (Andersson et al. 1979; Swaisgood and Bozoglu 1984; Rathi et al. 2001). A highly thermotolerant lipase has been reported from *B. stearothermophilus*, with a half-life of 15–25 min at 100°C (Bradoo et al. 1999).

Stability in organic solvents

Stability in organic solvents is desirable in synthesis reactions. From the available literature, it can be inferred that lipases are generally stable in organic solvents, with few exceptions of stimulation or inhibition. Acetone, ethanol and methanol enhanced the lipase activity of *B. thermocatenulatus* (Schmidt-Dannert et al. 1994), whereas acetone was inhibitory for *P. aeruginosa* YS-7 lipase and hexane for *Bacillus* sp. lipase (Sugihara et al. 1991). Lipase from *A. calcoaceticus* LP009 was highly unstable with various organic solvents (Dharmsthiti et al. 1998).

Effect of metal ions

Cofactors are generally not required for lipase activity, but divalent cations such as calcium often stimulate enzyme activity. This has been suggested to be due to the formation of the calcium salts of long-chain fatty acids (Macrae and Hammond 1985; Godtfredsen 1990). Calcium-stimulated lipases have been reported in the case of *B. subtilis* 168 (Lesuisse et al. 1993), *B. thermoleovorans* ID-1 (Lee et al. 1999), *P. aeruginosa* EF2 (Gilbert et al. 1991b), *S. aureus* 226 (Muraoka et al. 1982), *S. hyicus* (Van Oort et al. 1989), *C. viscosum* (Sugiura et al. 1974) and *Acinetobacter* sp. RAG-1 (Snellman et al. 2002). In contrast, the lipase from *P. aeruginosa* 10145 (Finkelstein et al. 1970) is inhibited by the presence of calcium ions. Further, lipase activity is in general inhibited drastically by heavy metals like Co^{2+}, Ni^{2+}, $Hg^{2+}$and $Sn^{2+}$and slightly inhibited by Zn^{2+} and Mg^{2+} (Patkar and Bjorkling 1994). However, the lipase from *A. calcoaceticus* LP009 was stimulated by the presence of Fe^{3+} and its activity was reduced by less than 20% on addition of various other ions (Dharmsthiti et al. 1998).

Lipase inhibitors

Lipase inhibitors have been used in the study of structural and mechanistic properties of lipases. Further, the search for lipase inhibitors is also of pharmacological interest. Lipase inhibitors are used for designing drugs for the treatment of obesity and the problem of acne. Following is an account of general inhibitors. Broadly, inhibitors of enzymes are classified as reversible or irreversible. The reversible inhibitors can be further classified as non-specific and specific reversible inhibitors.

Non-specific reversible inhibitors

Compounds that do not act directly at the active site, but inhibit lipase activity by changing the conformation of lipase or interfacial properties are defined as non-specific inhibitors. Surfactants (Iizumi et al. 1990; Patkar and Bjorkling 1994), bile salts (Borgstrom and Donner 1976; Wang et al. 1999) and proteins (Gargouri et al. 1984; Bezborodov et al. 1985) belong to this group of inhibitors. However, surfactants and bile salts activate the enzyme in some cases.

Table 4 Purification strategies for bacterial lipases. *Fold increase* is the ratio of specific activity of the final purified product to the initial specific activity; and *yield* is the ratio of initial enzyme titer to the final titer obtained after the purification process

Bacterium	Purification technique	Fold increase/yield	Reference
Acinetobacter spp			
A. calcoaceticus AAC323-1	Triton X-114-based aqueous two-phase partition	68-fold/81%	Bompensieri et al. 1996
A. calcoaceticus LP009	Ultrafiltration, gel filtration on Sephadex G-100	n.s.	Pratuangdejkul and Dharmsthiti 2000
A. radioresistens CMC-1	Ammonium sulfate, PD-10 column, Mono Q, phenyl-Sepharose CL-4B column chromatography	64-fold/13%	Hong and Chang 1998
Acinetobacter sp. RAG-1	Mono Q, butyl Sepharose column, elution with Triton-X 100	10-fold/22%	Snellman et al. 2002
Bacillus spp			
Bacillus sp.	Ammonium sulfate, acrinol treatment, DEAE-Sephadex A-50, Toyopearl HW-55F, butyl Toyopearl 650 M	7,760-fold/10%	Sugihara et al. 1991; Palekar et al. 2000
Bacillus sp.	Ammonium sulfate, phenyl Sepharose column	175-fold/15.6%	Nawani and Kaur 2000
Bacillus sp.	Acetone fractionation, two acetone precipitations, octyl-Sepharose CL-4B, Q-Sepharose, Sepharose-12	3,028-fold/20%	Imamura and Kitaura 2000
Bacillus sp. strain 398	Ammonium sulfate, DEAE-Sepharose, butyl Toyopearl, DEAE-Sepharose	10,300-fold/30%	Kim et al. 1994
Bacillus sp. THL027	Ultrafiltration, Sephadex G-100	2.6-fold/n.s.	Dharmsthiti and Luchai 1999
B. alcalophilus	50% ammonium sulfate, Sephadex G-100	111-fold/5%	Ghanem et al. 2000
B. pumilus	Ammonium sulfate fractionation, gel filtration on Sephadex G-100	75-fold/n.s.	Jose and Kurup 1999
B. stearothermophilus (recombinant lipase)	CM-Sepharose, DEAE Sepharose	11.6-fold/62.2%	Kim et al. 2000
B. thermocatenulatus	Calcium soap, hexane extraction, methanol precipitation, Q-Sepharose (ion exchange)	67-fold/11%	Schmidt-Dannert et al. 1994
B. thermocatenulatus (recombinant lipase)	Cell breakage with heat precipitation, S-Sepharose, Q-Sepharose, phenyl-Sepharose	329-fold/49%	Schmidt-Dannert et al. 1996
Chromobacterium spp			
C. viscosum	Alginate (macroaffinity ligand), elution by NaCl, 0.5 K	1.76-fold/ 87%	Sharma and Gupta 2001
C. viscosum Lipase A	AOT-isooctane reverse micelle system	4.3-fold/91%	Vicente et al. 1990
C. viscosum Lipase B	AOT-isooctane reverse micelle system, back-extraction from micellar phase by 2.5% ethanol at pH 9.0	3.7-fold/75%	Vicente et al. 1990
Pseudomonas spp			
Pseudomonas sp. G6	Silicone 21 defoamer, ammonium sulfate (60% saturation) fractionation	n.s./83%	Kanwar et al. 2002
Pseudomonas sp.	Extraction, Bio-gel P-10 chromatography, Superose 12B chromatography	37-fold/64.3%	Dong et al. 1999
Pseudomonas sp. KWI-56	Acetone precipitation, gel filtration by HPLC	14-fold/4%	Iizumi et al. 1990
Pseudomonas sp. ATCC 21808	Q-Sepharose, octyl-Sepharose, elution with isopropanol	159-fold/56%	Kordel et al. 1991
Pseudomonas sp. Yo103	Ammonium sulfate precipitation, DEAE- cellulose, Sephadex G-200	62-fold/3.7%	Kim et al. 1997
P. aeruginosa	Ammonium sulfate precipitation, hydroxyapatite column chromatography	518-fold/n.s.	Sharon et al. 1998
P. aeruginosa EF2	Ultrafiltration, anion-exchange chromatography (Mono-Q), gel filtration (Superose) FPLC	31-fold/18%	Palekar et al. 2000
P. cepacia	Polyoxyethylene detergent C14EO6-based aqueous two-phase partitioning	24-fold/76%	Terstappen et al. 1992
P. fluorescens	Ultrafiltration, ammonium sulfate precipitation, DEAE-Toyopearl 650 M, phenyl Toyopearl 650 M	6.1-fold/42%	Kojima et al. 1994

Table 4 (continued)

Bacterium	Purification technique	Fold increase/yield	Reference
P. luteola	Two-phase partitioning, anion exchange, exclusion chromatography	17-fold/16%	Litthauer et al. 2002
P. pseudo-alcaligenes F-111	Acetone precipitation, Sephadex G-100 chromatography, fractogel phenyl 650 M chromatography, Sephadex G-100 chromatography	144-fold/15%	Lin et al. 1996
P. pseudomallei	Ammonium sulfate, Sephadex G-150	n.s.	Kanwar and Goswami 2002
P. putida 3SK	DEAE-Sephadex A-50, Sephadex G-100	5.3-fold/21%	Lee and Rhee 1993
Serratia marcescens	Ion-exchange chromatography, gel filtration	n.s./45.4%	Abdou 2003.
Staphylococcus spp			
S. haemolyticus	80% ammonium sulfate, DEAE-Sepharose CL-6B column, CM-Sepharose CL-6B, resource S column (ion-exchange chromatography)	n.s./42%	Oh et al. 1999
S. warneri 863	Nickel–NTA affinity chromatography, hydroxyapatite column (HIC)	n.s./40%	Van Kampen et al. 2001
His_6-*S. aureus* (recombinant lipase)	Protamine sulfate, ammonium sulfate, nickel nitrilotriacetate, hydroxyapatite	42-fold/41%	Simons et al. 1996

Specific inhibitors

Specific inhibitors are those compounds, which directly interact with the active site of the enzyme. Such inhibitors can be either reversible or irreversible. Specific reversible inhibitors include: (1) boronic acid derivatives, which form reversible but long-lived complexes with the active-site serine of lipases (Lolis and Petsko 1990) and (2) substrate analogues including triacylglyceride analogue glycerol triether, which is also a competitive inhibitor of pancreatic lipase (Lengsfeld and Wolfer 1988). However, the affinity of this compound for the enzyme is not high enough, compared with the substrate, and hence it is difficult to obtain useful information from these analogues. Specific irreversible inhibitors generally react with the amino acids at or near the active site and thus inhibit the catalytic activity. Further, such inhibitors may also disturb sulphydryl bonds and thus modify the protein conformation.

Lipases belong to the class of serine hydrolases with the catalytic triad as Ser-His-Asp/Glu. Therefore, serine inhibitors are potential irreversible active-site lipase inhibitors, e.g. phenylmethylsulfonyl fluoride (PMSF), phenylboronic acid, diethyl*p*-nitrophenyl phosphate. In contrast, the lipase from *A. calcoaceticus* LP009 was not inhibited by PMSF (Dharmsthiti et al. 1998). Generally, lipases are not sulphydryl proteins; and thus in most lipases neither free –SH nor S–S bridges are important for their catalytic activity. This is substantiated by the use of 2-mercaptoethanol,*p*-chloromercuric benzoate and iodoacetate, which have no detectable effect on lipase from *C. viscosum* (Sugiura et al. 1974), *S. aureus* 226 (Muraoka et al. 1982) and *A. calcoaceticus* LP009 (Dharmsthiti et al. 1998). Further, EDTA does not affect the activity of most lipases (Gilbert et al. 1991b; Sugihara et al. 1991; Kojima et al. 1994). However, it is inhibitory to lipases from *P. aeruginosa* 10145 (Finkelstein et al. 1970), *Pseudomonas* sp. nov. 109 (Ihara et al. 1991), *Bacillus* sp. THL027 (Dharmsthiti and Luchai 1999) and *A. calcoaceticus* LP009 (Dharmsthiti et al. 1998). Tryptophan residues play an important role in maintaining the conformation of lipases (Patkar and Bjorkling 1994). Modification of tryptophan residues in lipases from *P. fragi* CRDA 037 (Schuepp et al. 1997) and *P. fluorescens* (Sugiura et al. 1977) by *N*-bromosuccinimide leads to decreased lipase activity.

Substrate specificity

Microbial lipases may be divided into three categories: namely nonspecific, regiospecific and fatty acid-specific, based on the substrate specificity. Nonspecific lipases act at random on the triacylglyceride molecule and result in the complete breakdown of triacylglyceride to fatty acid and glycerol. Examples of this group of lipases include those from *S. aureus*, *S. hyicus* (Davranov 1994; Jaeger et al. 1994),*Corynebacterium acnes* (Hassing 1971) and *Chromobacterium viscosum* (Jaeger et al. 1994).

In contrast, regiospecific lipases are 1,3-specific lipases which hydrolyze only primary ester bonds (i.e. ester bonds at atoms C1 and C3 of glycerol) and thus hydrolyze triacylglyceride to give free fatty acids, 1,2(2,3)-diacylglyceride and 2-monoacylglyceride. Extracellular bacterial lipases are regiospecific, e.g. those from *Bacillus* sp. (Sugihara et al. 1991; Lanser et al. 2002), *B. subtilis* 168 (Lesuisse et al. 1993), *Bacillus* sp. THL027 (Dharmsthiti and Luchai 1999), *Pseudomonas* sp. f-B-24 (Yamamoto

Table 5 Properties of bacterial lipases

Source	Molecular weight, pH, temperature optima	pH, temperature stability	Substrate specificity	Comments	Reference
Acinetobacter calcoaceticus	30.5 kDa, pH 8.0, 30–40°C, pI 5.5	n.s.	Enzyme hydrolyzes tri-, di-, monoacylglycerols	Enzyme is stimulated by deoxycholate, while inhibited by Hg^{2+} and *p*-hydroxymercuribenzoate	Brune and Gotz 1992
Acinetobacter calcoaceticus LP009	23 kDa, pH 7.0, 50°C	Stable at pH 4–8, temperatures lower than 45°C	n.s.	Enzyme inactivated with EDTA, enzyme stability enhanced with Triton X-100, Tween-80 or Tween-20	Dharmsthiti et al. 1998; Pratuangdejkul and Dharmsthiti 2000
Acinetobacter sp. RAG-1	33 kDa, pH 9.0, 55°C	Active at temperatures up to 70°C	Hydrolyzes wide range of *pnp* esters, but preference for medium-length acyl chains (C6, C8)	Lipase stabilized by Ca^{2+}, strongly inhibited by EDTA, Hg^{2+} and Cu^{2+}, retains 75% activity after exposure to organic solvents	Snellman et al. 2002.
Alcaligenes sp.	n.s., pH 9.0, 50°C	65% residual activity at 60°C after 10 min	Enzyme hydrolyzes natural fats and oils	n.s.	Brune and Gotz 1992
*Bacillus*sp.	22 kDa, pH 5.6–6.2, n. s., pI 5.1	Stable over pH 5.0–11.5, stable at 65°C for 30 min at pH 5.6	Tricaprylin, tricaprin, 1,3-regiospecific lipase	70% inhibition by Cu^{2+}, Hg^{2+}, Zn^{2+}	Sugihara et al. 1991
*Bacillus*sp.	45 kDa, n.s., n.s.	Stable for 12 h at 60°C	Triolein hydrolyzed at all positions; broad fatty acid specificity	Ethylene glycol, sorbitol, glycerol act as thermostabilizers	Nawani and Kaur 2000
*Bacillus*sp. strain 398	50 kDa, pH 8.2, 65°C	Stable over pH 4–11, stable up to 60°C, 50% residual activity at 65°C after 30 min	Tricaprylin among triacylglycerides; *pnp* caproate among *pnp* esters	n.s.	Kim et al. 1994
*Bacillus*strain A30-1 (ATC-C 53841)	65 kDa, pH 5.0–9.5, 60°C, pI 5.1	90–95% residual activity after 15 h at pH 5.0–10.5, half-life of 8 h at 75°C	High activity on tricaprin and trilaurin among various triacylglycerides; corn, olive, cottonseed, coconut, soyabean, wheatgerm oil among other oils	Stable to hydrogen peroxide and an alkaline protease which are detergent ingredients	Wang et al. 1995
*Bacillus*sp. THLO27	69 kDa, pH 7.0, 70°C	Stable over pH 6.0−8.0, 80% residual activity after 1 h at 75°C	Preference for C4–C12 fatty acid; 1,3-regiospecific	Enzyme sensitive to EDTA; it is a metalloenzyme	Dharmsthiti and Luchai 1999
B. alcalophilus	n.s., pH 10.6, 60°C	Stable at pH 10.0–10.5, 80% activity at pH 11.0 after 1 h; stable at 60°C for 1 h, 70% residual activity at 75°C	n.s.	150% activation in presence of 50 mM Ca^{2+}	Ghanem et al. 2000
B. licheniformis strain H1	n.s., pH 10.0, 55°C	Stable at alkaline pH 9–11, 65% residual activity at pH 12 after 30 min at 4°C, retained 100% activity after 15 min at 70°C	n.s.	Activity enhanced (120%) in presence of 10 mM Ca^{2+}, 55% residual activity in presence of Cu^{2+} or Fe^{3+}	Khyami-Horani 1996
B. pumilus B26 (recombinant lipase)	n.s., pH 8.5, 35°C	n.s.	Hydrolyzes various long triacylglycerols (C14–C18) and triolein (C18:1)	Exhibits Ca^{2+}independent thermostability and catalytic activity	Kim et al. 2002
B. subtilis 168	19 kDa, pH 9.9–10.0, 35°C	Stable at pH 12; 100% activity after 30 min. at 40°C	Preference for C8 fatty acid; 1,3-regiospecific	Ca^{2+}stimulated activity; lipase shows a tendency to aggregate	Lesuisse et al. 1993

Table 5 (continued)

Source	Molecular weight, pH, temperature optima	pH, temperature stability	Substrate specificity	Comments	Reference
B. thermo-catenulatus	n.s., pH 8.0–9.0, 60–80°C	Stable at pH 9–11 for 12 h at 30°C, 48.5% residual activity at 60°C for 30 min	Tributyrin,*pnp* caprate	n.s.	Schmidt-Dannert et al. 1996
B. thermo-oleovorans ID-1	34 kDa, pH 7.5, 75°C	n.s., half-life at 70°C 30 min	Broad	$Ca^{2+}$and Zn^{2+}enhanced activity	Lee et al. 1999
Burkholderia sp. lipase	30 kDa, pH 11.0, 90–100°C	Stable at pH 6.0–12.0, half-life of more than 12 h at 90–100°C	High rate of hydrolysis towards mustard oil, linseed oil, neem oil, and almond oil, preference for long chain (>C12) triacylglycerides)	Stable in organic solvents, activated in presence of $CaCl_2$, $MgCl_2$, $BaCl_2$, stable to bleaches and proteases which are detergent ingredients	Rathi et al. 2000, 2001; Bradoo et al. 2002
Pseudomonas sp. KWI-56	33 kDa, pH 5.5–7.0, 60°C, pI 5.0	Stable at pH 4–10; stable up to 60°C at pH 7.0 for 24 h	Triacylglycerides (C10–C14), whale wax	n.s.	Brune and Gotz 1992
Pseudomonas sp. (PSL)	30 kDa, pH 7.0–9.0, 45–60°C, pI 4.5	Stable at pH 6–12 after 4 h at 40°C; stable at 25–50°C for 30 min	n.s.	Activity enhanced in presence of Ca^{2+} (250%) and Bi^{3+}(154%), inhibition by Fe^{2+}, Fe^{3+}, Al^{3+}, Zn^{2+}, Mn^{2+}	Dong et al. 1999
Pseudomonas sp. strain KB 700A (recombinant lipase)	n.s., pH 8.0–8.5, 35°C	70% decrease in activity after 5 min at 60°C	Highest activity for *pnp* caprate, 20-fold higher activity towards 1 (3) position than 2 position	Activation by Ca^{2+},Mn^{2+}, Sr^{2+}, detergents while inhibited in presence of EDTA	Rashid et al. 2001
P. aeruginosa EF2	29 kDa, pH 9.0, 50°C, pI 4.9	n.s., half-life at 45°C 360 min, at 70°C 2.1 min	Preference for C18 fatty acid; 1,3-regiospecific	Forms aggregates; $Ca^{2+}$and Na^{+}increased the activity	Gilbert et al. 1991b
P. aeruginosa LP 602	n.s., pH 8.0, 55°C	90% residual activity at pH 8 after 5 h; 50% residual activity at 55°C after 2 h	High activity towards melted butter, castor, coconut oil	Insensitive towards EDTA	Dharmsthiti and Kuhasuntisuk 1998
P. cepacia DSM 50181	n.s., pH 5.0, 60°C, pI 7.1	Stable over pH 2.0−12.0, n.s.	n.s.	n.s.	Dunhaupt et al. 1991
P. fluorescens AK 102	33 kDa, pH 8.0–10.0, 55°C, pI 4.0	pH 4.0−10.0, stable below 50 ºC for 1 h; 100%	Broad	Enzyme stable in anionic surfactants	Kojima et al. 1994
P. fluorescens MC50	55 kDa, pH 8.0–9.0, 30–40°C	Stable over pH 6.0–9.0	Triacylglycerols	Inhibited by EDTA, Ca^{2+} stabilized enzyme at 60°C	Brune and Gotz 1992
P. fluorescens NS2W	n.s., pH 9.0, 55°C	Stable over pH 3–11 with more than 70% residual activity; stable up to 60°C with more than 70% residual activity for at least 2 h	n.s.	n.s.	Kulkarni and Gadre 2002
*P. fragi*22.39B	33 kDa, pH 9.0, 65°C, pI 6.9	Stable up to 51°C at pH 9.0 for 24 h; stable over pH 6.5–10.5 at 30°C for 24 h	Triacylglycerols, methyl oleate, Tween, Span, 1,3-regiospecific	Inhibited by Zn^{2+}, Fe^{2+}, Fe^{3+}, cationic surfactants Ca^{2+} enhances hydrolysis of C14-C18	Brune and Gotz 1992
P. luteola	n.s., n.s., 55°C	Half-life of 84 min. at pH 12.25; half-life of 116 min at 65°C	Preference for medium-chain saturated and unsaturated fatty acids	Inhibited by Sn and Zn	Litthauer et al. 2002

Table 5 (continued)

Source	Molecular weight, pH, temperature optima	pH, temperature stability	Substrate specificity	Comments	Reference
P. mendocina 3121-1	62 kDa, pH 7.2–9.5, 50–65°C	Different for different substrates	Hydrolyzes*pnp* butyrate, Tween-80, olive oil	pH and temperature kinetics, effect of various metal ions and EDTA depended on the nature of the substrate.	Surinenaite et al. 2002
P. multocida	n.s., pH 8.0, n.s.	n.s.	Tweens specific for Tween-40	n.s.	Pratt et al. 2000
P. pseudoalcaligenes F-111	32 kDa, pH 6.0–10.0, 40°C, pI 7.3	Stable over pH 6.0–10.0, stable up to 70°C	High activity towards linseed, soybean oil, preference for C12, C14 *pnp*esters	Lipolysis greatly inhibited by diisopropyl fluorophosphate	Lin et al. 1996
Serratia marcescens	52 kDa, pH 8.0–9.0, 37°C	70% activity after 24 h at pH 8, high activity at 5°C, 15% activity at 80°C	Michelis-Menten constant 1.35 mM on tributyrin	n.s.	Abdou 2003
Staphylococcus aureus	46 kDa, pH 6.5, n.s.	n.s.	Preference for short chain triacylglycerides and pnp esters (caprate)	n.s.	Paiva et al. 2000
S. hyicus	46 kDa, pH 8.5, n.s.	n.s.	Preference for phospholipids, neutral lipids, *pnp* esters irrespective of chain length	n.s.	Simons et al. 1996
S. haemolyticus	45 kDa, pH 8.5–9.5, 28°C, pI 9.7	Stable at pH 5–11 for 24 h; stable at 50°C in presence of Ca^{2+}	High activity on tributyrin, tripropionin, trimyristin, *pnp* caprylate	n.s.	Oh et al. 1999
S. warneri lipase 2	45 kDa, pH 7.0, n.s.	Stable at pH 6–8 for 24 h	High activity for*pnp* butyrate	Ca^{2+}-dependent	Van Kampen et al. 2001

and Fujiwara 1988, 1995), *P. aeruginosa* EF2 (Gilbert et al. 1991b) and *P. alcaligenes* 24 (Misset et al. 1994).

The third group comprises fatty acid-specific lipases, which exhibit a pronounced fatty acid preference.*Achromobacterium lipolyticum* is the only known bacterial source of a lipase showing fatty acid specificity (Davranov 1994). However, lipases from *Bacillus* sp. (Wang et al. 1995), *P. alcaligenes* EF2 (Gilbert et al. 1991a, 1991b) and *P. alcaligenes* 24 (Misset et al. 1994) show specificity for triacylglycerides with long-chain fatty acids, while lipases from *B. subtilis* 168 (Lesuisse et al. 1993), *Bacillus* sp. THL027 (Dharmsthiti and Luchai 1999), *P. aeruginosa* 10145 (Finkelstein et al. 1970), *P. fluorescens* (Sugiura et al. 1977), *Pseudomonas* sp. ATCC 21808 (Kordel et al. 1991), *C. viscosum* (Horiuti and Imamura 1977) and *Aeromonas hydrophila* (Angultra et al. 1993) prefer small- or medium-chain fatty acids. Lipase from *S. aureus* 226 shows a preference for unsaturated fatty acids (Muraoka et al. 1982).

Another important property of lipases is their enantio-/stereoselective nature, wherein they possess the ability to discriminate between the enantiomers of a racemic pair. Such enantiomerically pure or enriched organic compounds are steadily gaining importance in the chemistry of pharmaceutical, agricultural, synthetic organic and natural products (Reetz 2001). Mostly lipases from *Pseudomonas* family fall in this category (Reetz and Jaeger 1998). The stereospecificity of a lipase depends largely on the structure of the substrate, interactions at the active site and the reaction conditions (Lavayre et al. 1982; Cambou and Klibanov 1984; Muralidhar et al. 2002). A number of examples of biocatalysis by lipases leading to the synthesis of important enantiomers are available in the literature. The lipase from *P. cepacia* is a popular catalyst in organic synthesis (Kazlauskas and Bornscheuer 1998) for the kinetic resolution of racemic mixtures of secondary alcohols in hydrolysis, esterification and transesterification (Petschen et al. 1996; Takagi et al. 1996; Schulz et al. 2000). Lipases from*Pseudomonas* spp are used for the synthesis of chiral intermediates in the total synthesis of the antimicrobial compound chaungxixmyxin and the potent antitumor agent epothilone. Lipases are also used in the efficient production of enantiopure (*S*)-indanofan, a novel herbicide used against grass weeds in paddy fields. The synthesis of flavor and fragrance compounds such as menthol has been reported, using lipase from *B. cepacia* (Jaeger and Eggert 2002).

Thus, bacterial lipases are highly robust enzymes, since they are active over a wide range of pH and temperature. They belong to the group of serine hydrolases and are not sulfahydryl proteins. They may be regiospecific or non-specific towards triacylglycerols. Some lipases also possess fatty acid-specificity with reference to the carbon-chain length. Besides these features, the enantioselective nature of lipases provides them with an edge over other hydrolases, particularly in the field of organic chemistry and pharmaceuticals.

Novel developments in the field of lipases

Directed evolution of enzymes

In the past few decades, biocatalysts have been successfully exploited for the synthesis of complex drug intermediates, specialty chemicals and even commodity chemicals in the pharmaceutical, chemical and food industries. Recent advances in recombinant DNA technologies, high-throughput technologies, genomics and proteomics have fuelled the development of new catalysts and biocatalytic processes. In particular, directed evolution has emerged as a powerful tool for biocatalyst engineering (Zhao et al. 2002), in order to develop enzymes with novel properties, even without requiring knowledge of the enzyme structure and catalytic mechanisms. The approach of directed evolution has been reviewed several times by a number of researchers (Arnold 1996; Reetz and Jaeger 1999; Petrounia and Arnold 2000; Tobin et al. 2000; Jaeger et al. 2001).

Table 6 Directed evolution of lipases. *ee* Enantiomeric excess

Microbial source	Type of lipase	Strategies employed	Change in property	Reference
B. cepacia	Lipase (intermediate for synthesis of Paclitaxel used for cancer treatment)	–	Increase in*ee* value >99.5%; Bristol-Myers Squibb, USA	Liese et al. 2001
B. plantarii	Lipase (intermediate for pharmaceuticals and insecticides)	–	Increase in*ee* value >99%; BASF, Germany	Liese et al. 2001
P. aeruginosa	Lipase	Random mutagenesis (substitution of Ser for Asn-163, Pro for Leu-264)	Increase in thermal stability of the enzyme	Shinkai et al. 1996
P. aeruginosa	Lipase	Error-prone PCR for random mutagenesis	Increase in*ee* from 2% to >90% for*p*-nitrophenyl, 2-methyldecanoate	Jaeger and Reetz 2000
Serratia marcescens	Lipase (intermediate in the synthesis of dilitazem)	–	Increase in*ee* value >99.9%; Tanabe Seiyaku Co., Japan; DSM, The Netherlands	Liesse et al. 2001

In the field of lipase research, directed evolution has been employed for the creation of enantioselective catalysts for organic synthesis (Table 6). The first and most comprehensive study with respect to directed evolution of an enantioselective enzyme was performed with a lipase from *P. aeruginosa* (Jaeger et al. 2001). They applied this approach of directed evolution in combination with a newly developed screening method to generate lipases with improved enantioselectivity. A bacterial lipase from *P. aeruginosa* was evolved towards a model substrate, 2-methyldecanoic acid *p*-np ester, to yield in a lipase mutant showing >90% enantiomeric excess, as compared with 2% for the wild-type lipase (Jaeger and Reetz 2000). Recently, this group has also used a *B. subtilis* lipase as the catalyst in the asymmetric hydrolysis of meso-1,4-diacetoxy-2-cyclopentene, with the formation of chiral alcohols (Jaeger et al. 2001).

Metagenome approach

Microbial diversity is a major resource for biotechnological products and processes. The biosphere is dominated by microorganisms, yet most microbes in nature have not been studied. This is mainly due to the fact that, historically, the only way to reliably characterize a microorganism was by isolation of a pure culture. However, the vast majority of microbes present in a single environmental niche are not culturable in the laboratory and it is estimated that, on average, less than 1% have ever been identified (Lorenz et al. 2002). An alternative approach is to use the genetic diversity of the microorganisms in a certain environment as a whole (the so-called "metagenome") to encounter new or improved genes and gene products for biotechnological purposes (Henne et al. 2000). The sequencing of large metagenomic DNA fragments has fortuitously revealed numerous open reading frames, many of them encoding enzymes such as chitinase, lipase, esterase, protease, amylase, Dnase, xylanase, etc. (Lorenz et al. 2002). Henne et al. (2000) screened environmental DNA libraries prepared from three different soil samples for genes conferring lipolytic activity on *E. coli* clones and identified four clones harboring lipase and esterase activities. Bell et al. (2002) described a PCR method suitable for the isolation of lipase genes directly from environmental DNA, using primers designed on the basis of lipase consensus sequences.

Conclusions

Lipases are the biocatalysts of choice for the present and future, owing to their properties such as activity over a wide temperature and pH range, substrate specificity, diverse substrate range and enantioselectivity. Their importance is increasing by the day in several industries, such as food, detergents, chemicals, pharmaceuticals, etc. However, the commercial exploitation of lipases is still in its infancy, due to the economics of the lipase industry. Thus, there is a need today to develop production and downstream-processing systems which are cost-effective, simple and not time-consuming. The growing demand for lipases has shifted the trend towards prospecting for novel lipases, improving the properties of existing lipases for established technical applications and producing new enzymes tailor-made for entirely new areas of application. This has largely been possible due to outstanding events in the field of molecular enzymology. The number of novel microbial lipases being cloned and biochemically characterized is on the rise. Rational protein engineering, by way of mutagenesis and directed evolution, has provided a new and valuable tool for improving or adapting enzyme properties to the desired requirements. The upcoming trend to access novel natural sequenced space, via the direct cloning of metagenomic DNA, is significantly contributing to the screening and identification of hitherto unexplored microbial consortia for valuable biocatalysts. However, the success of these techniques demands the development of faster high-throughput screening systems. Thus, the modern methods of genetic engineering combined with an increasing knowledge of structure and function are allowing further adaptation to industrial needs and the exploration of novel applications.

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