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Review

Candida rugosa Lipases: Molecular Biology and Versatility in Biotechnology

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This review describes how the versatile *Candida rugosa* lipases (CRL) have extended the frontiers of biotechnology. As evidenced by the current literature, CRL claims more applications than any other biocatalyst. This review comprises a detailed discussion on the molecular biology of CRL, its versatile catalytic reactions, broad specificities and diverse immobilization strategies. It also discusses its role in the food and flavour industry, the production of ice cream and single cell protein, biocatalytic resolution of life-saving pharmaceuticals, carbohydrate esters and amino acid derivatives unobtainable by conventional chemical synthesis, potent biocide making, biosensor modulations, eco-friendly approach and bioremediation, biosurfactants in detergent making, and recently, cosmetics and perfumery. © 1998 John Wiley & Sons, Ltd.

KEY WORDS — amines and amides; biodetergents; biocides; bioremediation; biosensors; *Candida rugosa*; carbohydrate esters; cosmetics and perfumery; food and flavour; immobilisation; isoenzymes; lipases; molecular biology; pharmaceuticals; single cell protein; specificity; tanning; ultra-structure

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INTRODUCTION

Extracellular lipases (EC 3.1.1.3) produced by microorganisms are often investigated because of their many different uses in biotechnological processes.[14] A worldwide initiative has been taken up in the screening of lipase-producing organisms and their utilization in the swift-growing biotechnology for human welfare.[55,83] It is this enthusiasm which led the investigators to introduce *Candida rugosa* lipases (CRL) to the bioindustry.[10,15] Literature surveys reveal *C. rugosa* as the most extensively studied microorganism with respect to its lipase secretion.[12,18] Being a non-sporogenic, pseudo-filamentous, unicellular, and non-pathogenic

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yeast, *C. rugosa* (synonymous to *Candida cylindracea*) synthesizes and secretes a mixture of lipase isoenzymes that have been studied by several authors, commencing from the commercial preparation of the enzymes and aimed at their purification and characterization, in order to promote the growing 'Lip-tech' industry.[3,5,46,69,81]

C. rugosa secrets a mixture of enzymes, possessing at least five isoenzymes, encoded in a 'lipase minigene family' as testified by gene cloning. Each enzyme has a single polypeptide chain consisting of 543 amino acids and an apparent molecular weight of 60 kDa with a well-defined catalytic triad and an overarching flap at the active site.[81] CRL has been used in a wide range of catalytic reactions in both aqueous and water-restricted environments which include non-specific and stereo-specific hydrolysis, reversal of hydrolysis *via* esterification, trans-esterification and inter-esterification.[15,55,83] So far, no lipase is available with as broad a range of specificity (substrate, positional, fatty acid and stereopreference) as attributed to CRL. It has a general lack of discrimination towards ester bonds in the glycerides or primary and secondary esters, and accepts different saturated as well as unsaturated esters with varying chain-length of acid and alcohol moieties. To compensate for its increasing price, CRL is mostly employed in the immobilized form, being entrapped in durable and cheap supports with prolonged life.[19,85] The increasing current knowledge on the ultrastructure and kinetics of CRL has resulted in the improved efficiency of hydrolysis vs. synthesis, enhanced specificities, and novel entrapment techniques that have greatly revolutionized the CRL-based bio-industry.[133]

The positionally non-specific biocatalysis of fat- and lipid-containing substrates made CRL attractive for the core of dairy-based food industries, mainly for the synthesis of flavour and aroma as valuable food additives.[47,145] However, the scope of CRL broadened with its exploitation in detergent formulations as a bio-surfactant and also in the tanning industry (together with other major hydrolytic enzymes[91]). Modern eco-friendly policies, which have attained tremendous momentum in the last 25 years, have provided new opportunities for the exploitation of CRL, especially in the manufacture of fine chemicals, including biopharmaceuticals and biopesticides.[23,40] Optical resolution of fine chemicals is an important aspect, which could be extended to the synthesis of pure enantiomers with maximum optical purity and stereospecific hydrolysis and synthesis of valuable compounds.[22] Moreover, in recent years, the same principle has been employed for the production of efficient carbohydrate esters and amino acid derivatives.[71,108] Quantitative estimation of lipids in clinical samples and organic synthesis has led to the development of biosensors which contain a lipase, especially non-specific CRL.[48,125] As an alternative technology, lipases can be infused (infiltrated) into the intact organisms or cells to hydrolyse excess lipids (a challenging approach, yet to be tested in patients), and to extract cell-bounded lipids from industrial organisms and oil seeds.[86,132] In the bioremediation of lipid-tainted water, factory and restaurant effluents, lipases are used either in the *ex situ* or *in situ* form and useful products are obtained from these wastes—bypass technology.[15,17] The growth of cells (*in situ*) on effluent wastes, with the aim of producing single cell proteins (SCP) is a developing area which requires more attention.[13,76]

This review on *C. rugosa* lipases is prepared in the light of current literature which spans mainly the last 5 years. It deals with the molecular biology, catalytic activity, specificity and immobilization of CRL, as well as the challenging roles for CRL in the 'Lip-Tech' industry, both now and in the future.

MOLECULAR BIOLOGY OF CRL

Lipase iso-enzymes

Purification and characterization of multiple extracellular lipases of yeast (*C. rugosa*, *C. antarctica*), moulds (*Geotrichum candidum*, *Rhizopus miehei*, *Penicillim cyclopium*, *Aspergillus niger*, *A. oryzae*) and bacteria (*Chromobacterium viscosm*) have become rampant in microbial molecular biology.[1,81] The asporic yeast, *C. rugosa*, secretes several closely related exolipase isoforms. There have been contrasting reports on the number of lipase isoforms (two, three or five) and their molecular weight (MW).[46,78,119,128] The genes encoding five of them have been cloned and sequenced so far.[46] Each protein is encoded by a separate gene. All the genes encode 534 amino acids and their respective protein products have an apparent MW of 60 kDa.[21,46,128]

Lipases of *C. rugosa* (CRL) and *G. candidum* (GCL) have been studied together, since they show great similarities in many aspects.[81] They possess different lipase-encoding genes (lipase pseudogene

Table 1. Pairwise identity of LIP proteins.[81]

	LIP1	LIP2	LIP3	LIP4	LIP5
LIP1	100	—	—	—	—
LIP2	79	100	—	—	—
LIP3	88	82	100	—	—
LIP4	81	83	84	100	—
LIP5	81	77	86	78	100

Total amino acid identity = 66%; total amino acid similarity = 84%.

Table 2. Proteins predicted on the basis of nucleotide sequences as compared with proteins isolated from commercial preparations.

Gene	Amino acid mature protein	MW (kDa)	pI	N-glycosylation sites	
LIP1	534	57·223	4·5	3	291, 314, 351
LIP2	434	57·744	4·9	1	351
LIP3	534	57·291	5·1	3	291, 314, 351
LIP4	534	57·051	5·7	1	351
LIP5	534	56·957	5·5	3	314, 351

family), which might account for proteins differing in their amino-acid sequences and possibly in their biochemical and enzymatic properties.[46] As for the physiological relevance of lipase isoenzymes for the producing organisms, one could speculate that the availability of related but non-identical enzymes would improve the adaptation to different substrate sources. This view was supported by the recent characterization of GCL isoenzymes with different substrate specificities.[21]

Lotti *et al.*[81] isolated and elucidated five lipase sequences of *C. rugosa*, and termed *LIP1*, *LIP2*, *LIP3*, *LIP4* and *LIP5* (Table 1). The sequences of the 5′ upstream region of all these *LIP*s are preceded by canonical TATA and CAAT boxes, elements characteristic of eukaryotic promoters. The experimental data of Lotti *et al.*[81] (Table 2) show that *LIP* sequences constitute a family of functional genes (*LIP* mini-family) coding for isoenzymes closely related in their sequences. It is likely that such lipases might be produced in different amounts and/or according to the growth conditions. The product of the *LIP1* gene seems to be the major *C. rugosa* lipase, since it has been preferentially isolated by several laboratories working in the field.[3,27,128] Very recently, Fusetti *et al.*[43] succeeded in expressing the *LIP1* gene in *Saccharomyces cerevisiae* grown in fed-batch culture, using the plasmid pEMBLY ex-4. This recombinant lipase has 534 amino acids.[81]

In spite of the high sequence homology (84% similarity) in the whole penta protein lipase mini-family, the predicted lipase isoenzymes maintain distinctive biochemical features such as differences in sugar content, isoelectric points and local features of their hydrophobicity profiles[81,119] (Table 2). As depicted in Figure 1, each of the five lipases can be distinguished from the other enzymes of the family by the occurrence of unique amino acids along the peptide chains. The availability of a family of highly related but not

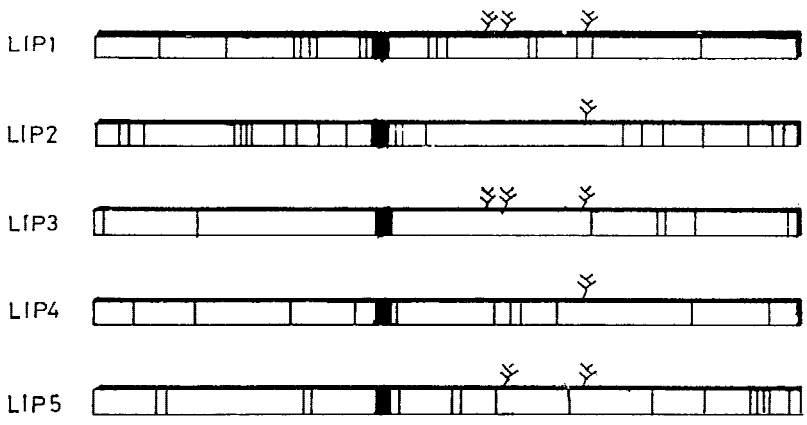

Figure 1. A comparative profile of the schematic representation of five lipase proteins. Bars mark the position of amino acids unique for each enzyme sequence. Glycosylation sites are shown as branched marking (Lotti *et al.*[81]).

identical lipase sequences provides an interesting tool for the study of the structural and functional significance of particular regions and positions within the protein.[81]

Ultra-ultrastructure

The tertiary structures of many microbial lipases have already been published. They include *Rhizomucor miehei*, *Rhizopus oryzae*, *Humicola lanuginosa*, *Penicillium camembertii*, *G. candidum*, *C. rugosa*, *C. viscosum*, *Pseudomonas aeruginosa*, *P. cepacia*, *P. glumae*, *Bacillus subtilis*, etc.[25,124] X-ray crystallography of *C. rugosa* lipase was published by Grochulski *et al.*[46] at high resolution.

Three-dimensional structure analysis

All lipases sequenced to date share sequence homologies, including a significant region **His**-X-Y-Gly-Z-**Ser**-W-Gly or Y-Gly-**His**-**Ser**-W-Gly (W, X, Y and Z denote unspecified amino acid sequence residues) that is conserved in all such enzymes.[85] With regard to their amino acid sequences, CRL appears to be significantly related to the lipases produced by the mould *G. candidum* (GLC) and to several esterases and acetylcholine esterases.[81,120] CRL contains a **Ser**-**His**-**Glu** catalytic triad and displays a high degree of sequence identity (>40%) that extends through the whole length of the polypeptide chain (Figure 2). This similarity does not apply to the loops building the flap, i.e. the surface structure covering the enzyme active site. Within the CRL family, the following are strictly conserved: (i) residues of the catalytic triad (S209, H449, E341); (ii) residues involved in the formation of disulphide bridges (C60–C97 and C268–C277); (iii) residues forming salt bridges (R37–E95 and E172–R279); (iv) residues classified as invariable positions within the lipases/esterases/acetylcholine esterases family; and (v) residues proposed to form the oxyanion hole upon interaction with the substrate (G124 and A210).[81,85]

The alignment between CRL and GCL shows that four regions of CRL could show differences when compared with the corresponding regions of the template structure of GCL (Figure 2). Two of them (62–91 and 294–300) are indeed found to be different. A comparative profile of the structures published by Grochulski *et al.*[46] indicates that these two lipases show major differences between amino acid residues 122–129 (Figure 2). Parts of the molecules identified by the X-ray analysis revealed that three loops encompassing amino

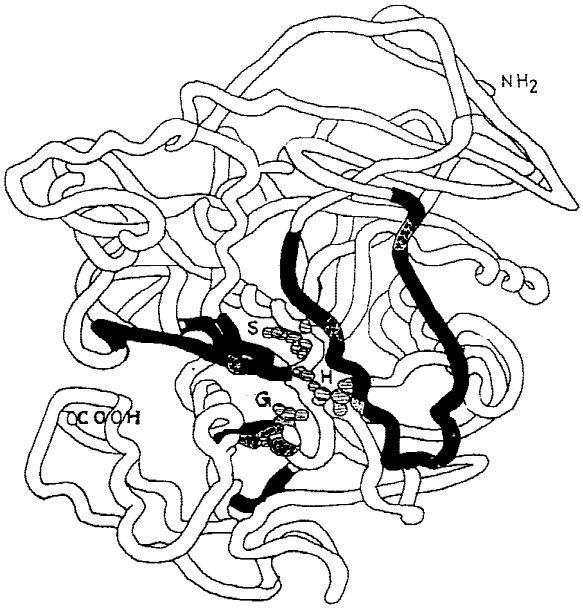

Figure 2. Ribbon representation of the Lip model for CRL and GCL, O regions of CRL homologous to GCL; Sequence and conformational differences between the two fungal lipases are shown in black; catalytic triad residues (S, H and G) are hatched and variable residues are stippled. N′ and C′ of the protein are also shown (Lotti *et al.*[81]).

acids 62–92, 121–129 and 296–299 assume significantly different conformations in CRL and GCL, which are otherwise very similar in structure.[46]

The flap/lid that canopies the active site has 31 amino acids, mainly hydrophobic on the side directed towards the active site, and hydrophilic on its external face, containing the solvent when the enzyme is in the closed inactive form and stabilized by interaction with the hydrophilic surface of the molecule in the open form.[81] As inferred from sequence alignments, the catalytic triad is formed by Ser-209, His-449 and Glu-341[46] (Figure 2). Nucleophilic serine of this triad is embedded in the characteristic super-secondary structural motif and is considered to be essential for the catalytic activity.[3,85] This putative hydrolytic site is buried under a lid composed of short helical fragments of a long surface loop stabilized by extensive hydrophobic and electrostatic interactions. The activation of this enzyme requires a strong conformational rearrangement of this flap. Otherwise, the catalytic triad is not accessible to the substrate done by the presence of water-lipid interface dramatically enhances the hydrolytic activity.[81]

The flap regions are quite variable in the five CRL: 13 positions are identical, eight carry conservative substitution, and four are non-conservative but maintain the hydrophobic or hydrophilic character.[81] The remaining positions differ considerably. In conclusion, all the five lipases reported so far have an individuality in terms of the active site and the flap—the central processing unit of this catalyst. Analysis of the substrate specificity of these lipases and further characterization, resolution of their three-dimensional structures and definition of the role of single amino acids, or groups of amino acids, needs further study to obtain a deeper insight into these industrial catalysts.[21,25,46,85]

As with many pseudogenes, deviations from the universal genetic code have also been reported for several micro-organisms, including *C. rugosa*. Kawaguchi *et al.*[81] reported a typical example of this divergence in the *C. rugosa* genome, in which the universal codon for leucine (CUG) is used to code for serine. This conclusion was based on the observation that: (1) the amino acid composition and the partial amino acid sequences of an extracellular lipase from this yeast agreed with those deduced from the complementary DNA (cDNA), if CUG was assumed to specify serine; and (2) serine, but not leucine was incorporated into a polypeptide in a cell-free translation system from *C. rugosa* in the presence of a synthetic oligomer.

LIPASE SPECIFICITY

Enzymes work to modify specific chemical bonds usually at specific sites on a molecule, in contrast to ordinary chemical reactions that occur at random in response to the laws of thermodynamics.[113] Enzymes permit control of products produced and also increase yield by reducing side-products; these advantages are coupled with mild reaction conditions and low waste treatment costs.[113]

CRL has a broad spectrum of specificities. The specificity of lipase is controlled by the molecular properties of the enzyme, structure of the substrate and factors affecting binding of the enzyme to the substrate.[56] Types of specificity are: (I) substrate: different ratio of lipolysis to TG, DG and MG by the same enzyme or separate enzymes from the same source; (II) positional: (a) primary esters, (b) secondary esters, (c) all three esters or non-specific hydrolysis; (III) fatty acid: preference for specific fatty acids; (IV) stereospecificity: faster hydrolysis of one primary Sn ester as compared to its counterpart and (V) combination of all the four specificities mentioned above. The industrial viability of CRL has been exploited in all these areas.

Substrate specificity of CRL is mainly due to the occurrence of multiforms of lipases, an indication of an evolutionary strategy of better adaptive value for the maximum utilization of the available substrate.[119,128] Rua *et al.*[119] studied the behaviour of two forms of lipases (A and B) on the rate of hydrolysis using *p*-nitrophenyl butyrate as substrate and observed that both the lipases showed striking variations in their K_m values. The behaviour of lipase-B could be consistent with an enzyme which still obeys Michaelis-Menten kinetics but with a K_m value for *p*-nitrophenyl butyrate much higher than the ester solubility under the experimental conditions. With triolein, both the forms showed relatively similar and slow activity up to 20 mM substrate, but higher substrate concentrations showed much enhancement of hydrolysis by Lip-B.[119] Studies of Shaw and Chang[128] on the effect of specificity on different acyl chain length with *p*-nitrophenol ester hydrolysis also confirm the findings of Rua *et al.*[119]

The non-specificity of CRL is well documented.[20,35,60,143] Benzonona and Esposito[20] found that CRL was very active towards long chain triglycerides at pH 8·0 and 30°C. Under these conditions, the three chains of the glycerol moiety were completely hydrolysed (Figure 3). A comparison between the proportions of the fatty acid chains in intact olive oil and cocoa butter and the proportions of the fatty acids liberated after a limited hydrolysis of these lipids shows that the CRL liberates all types of acyl chains, regardless of their position in the glycerol.[20] However, palmitic acid and oleic acid chains are liberated before stearic acid when they are present together in a given glyceride. Since 1,3-dihexadecyl-ether-2 oleoyl-glycerol is readily hydrolysed, it can be concluded that CRL is able to attack secondary ester groups of glycerol without the help of an isomerase. The positional specificity of a wide range of substrates like trioleoyl glycerol,[106] vegetable oil,[85] milk and creams[60] was also reported with CRL.

Fatty-acid preference of CRL is widely employed in lipase biotechnology. Many studies revealed that CRL has a preference for short-chain fatty acids.[57,58,73,104] Liu *et al.*[80] deduced the selectivity from trans-esterification of two or more different fatty acids, fatty alcohols and fatty acid

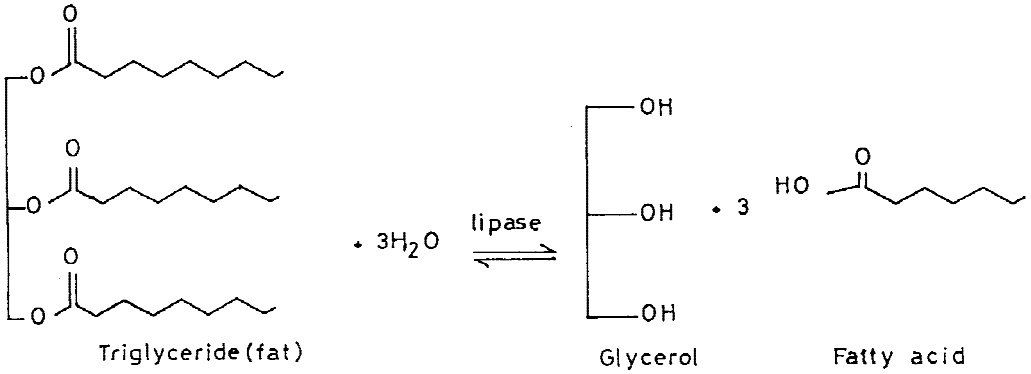

Figure 3. Enzymatic reaction of CRL catalysing hydrolysis or synthesis of a triglycerol substrate.

esters with another acyl ester or fatty alcohol. Butyl stearate and butyl palmitate were used as acyl donors trans-esterified by lauryl, and products were separated by HPLC. The lipase tended to select short-chain fatty acids. Osada *et al.*[104] synthesized triglycerides using fatty acids C18:1, C18:2, C18:3, C18:4, C18:5 and C18:6. Higher-chain-length fatty acids with more double bonds produced less triglycerides. In ester synthesis, CRL preferred short-chain fatty acids and alcohols *viz.* propionic acid, butyric acid, butanol, hexanol and pentanol, which impart quality flavours in food stuffs.[57] Jenssen and Halling[58] studied the esterification of sulcatol and several saturated fatty acids catalysed by CRL. Measurements made in toluene (based on V_m/K_m) showed a high preference for C4, C8, C10 and C12 fatty acids.

In the stereospecific esterification of racemic naproxen with trimethylsilyl methanol in iso-octane by CRL, improvements in (S) naproxen ester productivity and enzyme solubility were demonstrated by adding bis-(2-ethylhexyl)sodium sufosuccinate as the best surfactant.[137] In another study, stereospecific esterification of racemic methanol in a solvent system (hexane, iso-octane, *n*-heptane, cyclohexane, benzene, toluene, methylene chloride and chloroform) was investigated using acid anhydrides (acetic propionic or butyoic acid) as acylating agents. The (−) methanol was preferentially esterified with a yield as high as 64%.[142] S-stereopreference was also employed in the synthesis of a number of optically active non-steroidal anti-inflammatory drugs: naproxen, ibuprofen, suprofen.[128] Haalck *et al.*[49] noticed that trigycerols and 2-*o*-alkyl analogues were affected by the chain length of the *sn*-1 and *sn*-3 acyl groups. As a whole, multifacetedness of CRL in industries lies in its broad specificities.

HYDROLYSIS *VS.* SYNTHESIS

Potential industrial applications of CRL include production of fatty acids and glycerol *via* hydrolysis of oils and fats, modification of composition and physical properties of triglyceride mixtures by inter-esterification and trans-esterification (Figure 4), and synthesis of chemicals in organic solvents.[31,33,50] The non-hydrolytic (esterification) properties can occur mainly in a water-restricted environment. Lipases bind at the interface between aqueous and organic phases, and catalyse hydrolysis at this interface.[12,90] This binding not only places the lipase close to the substrate, but also increases the catalytic power of the lipase, a phenomenon called interfacial activation.[65,81] In the environment of organic solvents, CRL catalyse the synthesis of new esters and also modification of saccharides, peptides or the formation of optically active enantiomers for fine chemical production.[96,147]

The hydrolysis of fats and oils is an equilibrium reaction, and therefore it is necessary to change the direction of the reaction to ester synthesis by modifying the reaction conditions[55,75] (Figure 3). The equilibrium between to and fro reactions in this case is controlled by the water content of the reaction mixture so that, in a non-aqueous environment lipases catalyse ester synthesis. Another potent role is to deprotect (by hydrolysis) the ester-protecting groups in synthetic intermediates so as to exploit the ability of lipases to catalyse hydrolysis under mild conditions.[65] In

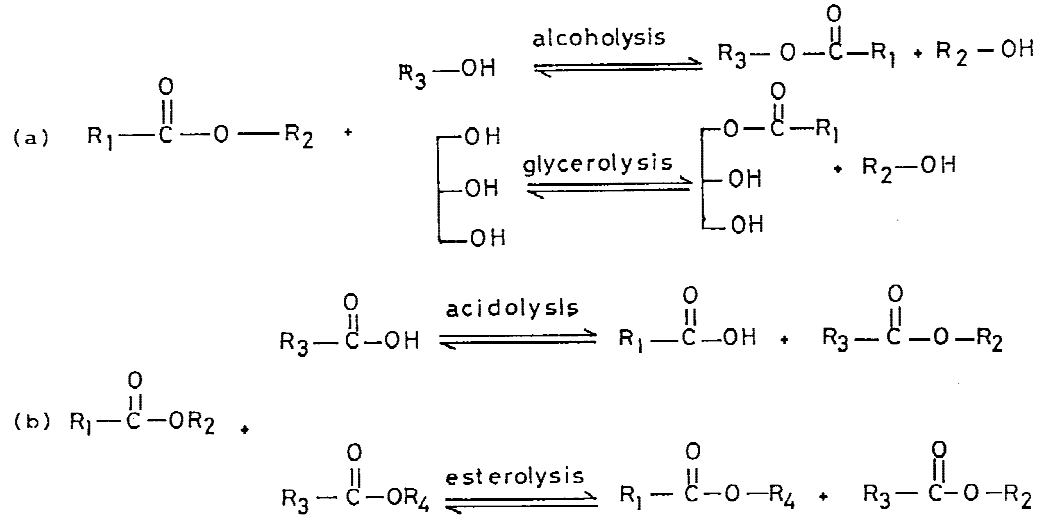

Figure 4. Industrially important reactions catalysed by CRL. (a) Trans-esterification involves the transfer of an acyl group to an alcohol (alcoholysis) or glycerol (glycerolysis). (b) Inter-esterification described the transfer of an acyl group to a fatty acid (acidolysis) or a fatty acid ester (esterolysis).

fact, CRL were found to catalyse the hydrolysis of a wide range of unnatural esters, and many of these hydrolyses are enantioselective, thus enabling chemists to generate pure enantiomers in organic synthesis (Figure 5a,b). These starting materials are required for the next generation of pharmaceuticals, which will be enantiomerically pure to maximize their potency and to minimize side products, for instance, production of optically pure ibuprofen.[66]

The advent of inter- and trans-esterification reactions has revolutionized lipase biotechnology.[53,107] In trans-esterification, the acyl doner is an ester (Figure 4a). Trans-esterification involving fats and oils can be specified further depending on the type of acyl acceptors. Alcoholysis and glycerolysis refer to the transfer of an acyl group from a triglyceride to either an alcohol or glycerol, respectively.[55] In inter-esterification, the acyl group is exchanged between a triglyceride or a fatty acid ester, the former being acidolysis and the latter being esterolysis (Figure 4b). Inter-esterification requires a controlled quantity of water, in addition to the amount needed for the enzyme to maintain the active hydrated state. As the presence of too much water will decrease the amount of ester synthesis products, the water content should be carefully adjusted (whereupon inter-esterification predominates over hydrolysis), to achieve the accumulation of the desired reaction products.[15,84]

IMMOBILIZED LIPASE

Soluble enzymes are preferentially used in entrapment technology because of their proven advantages, which include: (a) enzymes can be reused; (b) processes can be operated continuously and can be readily controlled; (c) products are easily separated; (d) effluent problems and materials handling are minimized; (e) enzyme properties (like activity and thermostability) can be altered favourably; and (f) the process is more cost effective.[14,85]

The costs of producing lipases necessary to catalyse versatile reactions which contribute to biotechnological processes are often prohibitive.[8,85] Hence processes that do not require the physical presence of lipase in the final product and that use feedstocks that are fluids (or that can be treated as such) are more economic if the lipase is employed in an immobilized form. A vivid illustration of the applications of immobilized enzymes was presented by Katchalski-Katzir,[62] which deals with immobilization techniques, immobilized enzyme reactors and immobilized enzymes in the food, pharmaceutical and chemical industries.[62]

Entrapment of enzymes is based on the coupling of enzymes to the lattices of a polymer matrix or enclosing them in semi-permeable membranes, tight enough to prevent the leakage of protein, while allowing the diffusion (mass transfer) of substrates and products to and fro.[34,37] Both natural

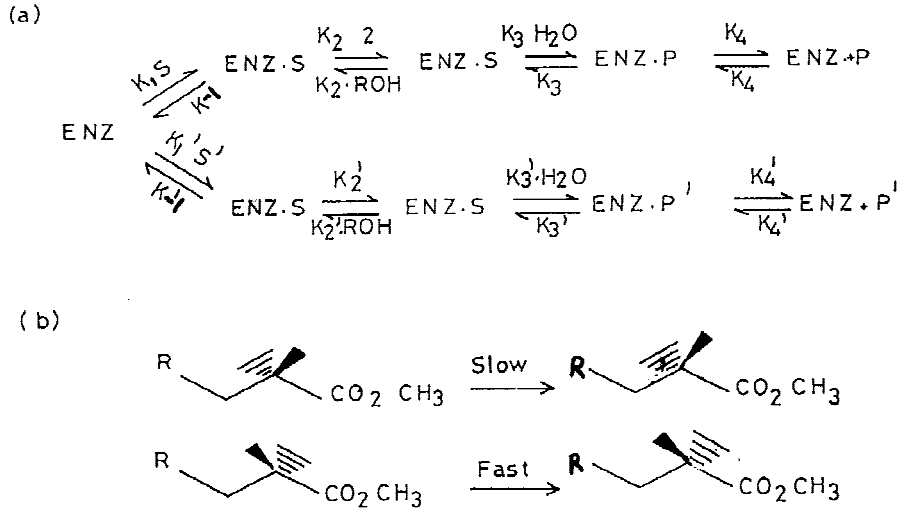

Figure 5. General mechanism in the CRL-mediated catalytic resolution of fine chemicals. (a) Asymmetric catalysis (ENZ, enzyme; S, substrate; P, product; K, reaction constants). (b) Kinetic resolution of enantiomer possessing a chiral centre.

Table 3. Commonly used agents for entrapping CRL and types of reactions with products.

Agent	Reaction	Product	Reference
Celite	Stereoselective esterification	2-(4-chlorophenoxy) propanoic acid	42
Nylon fiber	Stereospecific hydrolysis	Methyl methoxyphenyl glycidate	24
Co-polymer of methyl acrylate and methyl methacrylate	Hydrolysis	Free fatty acids	145
Polymethyl methacrylate	Esterification	Fatty ester	10
Lyotropic liquid crystal	Esterification	Butyl butyrate	41
Glass bead	Esterification	Citronellol ester with valeric acid	53
Kieselguhr	Esterification	Ester of isoamyl alcohol with butyric acid	64
Colloidal liquid aphrons	Hydrolysis	*p*-nitrophynil derivatives	82
Chitin	Hydrolysis	Free fatty acid	141
Silica gel	Hydrolysis	Glycerol	121
Cross-linked enzyme crystals	Hydrolysis/synthesis	Esters	110, 139

and synthetic polymers are used as matrices in many immobilization techniques. Commonly used modern immobilizing agents for entrapping CRL and the reactions involved are shown in Table 3.

Gel entrapment involves entrapping the enzyme within the meshes of a cross-linked polymeric network; such supports can be obtained from monomeric, oligomeric or polymeric precursors by changing the solubility variables, which include solvents, temperature, enzyme strength and pH.[24] Direct use of pure lipases which perform catalysis for a long time has been very limited due to their low molecular stability and high cost. Cross-linked enzyme crystals (CLEC) offer not only a pure but also very active and stable form of the enzyme.[110,139] Nucleophile (alcohol) selectivity studies showed that lipase immobilized on XAD_7 and celite were more accessible to C_{3-12} alcohols. Lipase immobilized on polymethylmethacrylate had a marked preference towards C_{3-10} alcohols.[10] Immobilization in lyotropic liquid crystals, consisting of polyoxyethylene-*n*-alkyl-ester-type non-ionic surfactants offers further potential for esterification reactions.[41] Lipase entrapped in methyl-substituted organic silicates formed on Kieselguhr such as Celite-545 and Hyflo Super-Cel, revealed the thermostability of lipase[64] up to 65°C. The findings of Kawakami[64] were very

significant for the detergent industry, which needs non-specific thermotolerant lipases.[64]

In view of the reusability of the enzyme, many different methods of immobilization have been applied. Covalent methods have been used to entrap lipase to polysaccharides, carboxymethyl cellulose and polyacrylamide gels.[114,126] Another method for the immobilization of lipases is the use of photo-cross-linkable resins.[110] Finally, adsorption has been used to immobilize lipases to amphiphilic gels of different types of membranes, as in cellulose membrane reactors.[114] More recently, attention has been concentrated on the surfactant-coated lipases for their superior performance and stability.

LIPASES IN FOOD AND FLAVOUR MAKING

Versatile CRL have an unchallenged role in modern food technology.[15] The use of enzymes to improve traditional chemical processes of food manufacture has been developed in the past few years, owing to the economic and technological advantages that it offers. Diverse products and processes (ranging from juices, baked food, fermented vegetables, dairy enrichment, desirable flavours in cheese and allied food stuffs, and the inter-esterification of fats and oils to produce modified glycerides unobtainable by chemical inter-esterification) have been manufactured by free or immobilized *C. rugosa* lipases.[18,130]

Fats and oils

The major part of the lipase-mediated food industry embodies biocatalysis of lipids, especially fats and oils.[84,88] During storage, one of the most important changes that occurs in the lipid fraction is the hydrolysis of triglycerides catalysed by lipases retaining free fatty acids, which are very important for the characteristic flavour of these products.[73] Likewise, the desired moiety of the triglyceride can be deleted or replaced under controlled esterification and ester interchange reactions.

Fragrance development in dairy products is dependent on the release of volatile fatty acids. The catalytic functions of CRL on cattle, sheep and goat milk fats reveal that CRL yielded a high amount of volatile branched-chain fatty acids, non-selectively, compared with only small quantities of volatile straight-chain fatty acids.[54,73] Considerable efforts have been made, notably in Japan and Europe, to commercialize lipase-catalysed trans-esterification and inter-esterification of relatively valuable food products.[83,84] Ester exchange for vegetable oil modification involving *C. rugosa* lipase suggests that it can effectively modify triolein in vegetable oils with high industrial qualities. The Unilever company has obtained a series of patents for the inter-esterification of fats and glycerides. These processes afford efficient means for the inter-esterification of fats suitable for use in emulsion and other fat-based food products such as margarine, artificial creams and ice creams.[15,55]

Fermented foods

Traditionally, fermented foods from fruits, vegetables, cereals, root crops, legumes and oil seeds have been used all over the world as baked or cooked stuffs.[72,146] An important result of the fermented fruits and vegetables is that the products so obtained can be stored and used as food supply during off-seasons. The bioconversion caused by fermentation contributes to the character and organoleptic properties of the fermented products.[38,146] In many cases, fermentation also contributes to the digestibility and nutritional value of the final products.[97] For instance, soyabeans can be stored relatively well in the dry state, but as such they are not readily consumable by humans, even when cooked. When converted to temp, they become a base material for several delicious, easily digestible and nutritional food items, which provide many millions of people with a vauable and affordable source of proteins.[118,146]

Ice cream

Flavour- and fragrance-rich ice creams have a large market in modern life. Production of flavour esters which contain short-chain or medium-chain fatty acids to be used in ice cream has been investigated by many authors.[73,134] However, the flavour compounds produced in solvent-free systems have the advantages (products will not contain solvents as impurities) of being safe for making delicious creams.[102] Ester-interchange reactions are now employed to obtain the desired flavour esters.[87]

SINGLE CELL PROTEIN

Exploitation of microbes for the production of valuable products has age-old history, but

Table 4. Important CRL-catalysed pharmaceuticals.

Target	Precursor	Reaction	Reference
Ibuprofen	Fatty alcohol	Esterification	44
Trifluorophenanthryl ethanol	Chloroacetate ester	Alcoholysis	63
Aspirin-like pro-drugs	Acetoxy and phenethylbenzoates	Hydrolysis	29
Acetic acid derivatives	α-Substituted acetic acid methyl esters	Hydrolysis	101
Androstane	Acetylated androstane	Hydrolysis	9
Prostaglandin	Polyunsaturated fatty acid containing oil	Hydrolysis	140
Naproxen	Trimethyl silyl methanol	Esterification	131, 137
Carbapenem antibiotics	Pyrolidine dione derivatives	Esterification	122
Ketoprofen	(R,S)chloroethyl ketoprofen	Hydrolysis	71
Inhibitor drugs	Tricarboxylic acid alkenyl ester	Esterification	127
Propanolamines	Epoxide and 2-propylamine	Esterification	59
Solketal	Trifluoroethyl butyrate	Esterification	138
Aconitium alkaloid	Vinyl acetate and mesodiols	Esterification	52
Anti-HIV carbovir	Azabicylo hept-en-ones	Esterification	99

production of SCP is an emerging technology by which microbes can be grown in valueless waste to produce value-added products.[16,17] Lee and Lee[76] of Hoseo University have very recently isolated a thermotolerant *C. rugosa* strain from Sudan which may be used for the continuous production of SCP from sugar beet stillages, a by-product of the ethanol production process.[76] The yeast contains 45·1% crude protein, 36·5% actual protein and 5·6% RNA. Such proteins, which are deficient in sulphur-containing amino acids (cysteine), can be suitably enriched and used as a staple food source. Recombinant-DNA technology is the best tool to achieve this objective. In many fermentation industries, especially the fat and oil industry, the treatment of waste is of crucial significance.[19,72] Much attention is needed in the treatment of high-strength molasses waste-water, molasses stillage, brewery waste and pharmaceutical wastes. However, *C. rugosa* was a failure in these treatments,[72] although the organism grew well in oil-rich wastes.[13,15] So far, the focus on *C. rugosa* has been mainly on its lipase-producing capacity. The potential of *C. rugosa* for the copious secretion of proteins, including lipases, would gradually provide an avenue for the large-scale production of SCP using this yeast.[16,18]

PHARMACEUTICALS

One of the challenging targets to a biopharmacist is to build lipase-catalysed bioactive components by hydrolysis vs. synthetic reactions (Figure 3). In addition to *in situ* racemization for optically pure enantiomers (Figure 5), CRL should also be capable of catalysing synthetic reactions which lead to the production of innumerable life-saving drugs (Table 4). Efficient kinetic resolution processes are in vogue for the preparation of optically active non-steroidal anti-inflammatory drugs (ibuprofen, naproxen, ketoprofen), the potential virucide carbovir (which can be applied against HIV) and for the enantiospecific synthesis of alkaloids, secondary alcohols, antibiotics, biochemical inhibitors and prodrugs (Table 4).

Conventional chemical synthesis of drugs containing a chiral centre generally yields equal mixtures of enantiomers. During the past decade, many studies have shown that racemic drugs usually have the desired therapeutic activity residing mainly in one of the enantiomers, and the other enantiomer may interact with different receptor sites, which will create untoward side effects.

CRL have made a significant contribution in pharmaceuticals as well as agrochemicals, mainly due to their (S)-stereospecificity.[135] Goto *et al.*[44] observed that S-(+)-ibuprofen was selectively transferred to the ester form by esterification reactions held in dry homogeneous organic media by CRL. Similar reports are available on a number of allied drugs.[15] Stereospecific esterification of racemic naproxen with trimethyl silyl methanol in iso-octane showed improvements in (S)-naproxen ester productivity by CRL.[137] Yasufuku and Ueji[143] observed that alteration of temperature during the esterification reaction drastically

Table 5. Important CRL-catalysed pesticides and their sources.

Product	Precursor	Catalysis	Reference
Nikkomycin-B	*p*-Siloxybenzaldehyde	Esterification	2
Perhydrofuro(2,3b)furan	2-Methoxy tetrahydrofuran-3-carboxylate	Alcoholysis	40
1,3,4-Oxadiazole-2(3H)-ones	2-(4-(Isopropoxy)phenyl) propionic acid methyl ester	Hydrolysis	23
Hormone analogs	2-(4-Methoxybenzyl)-1-cachohexyl acetate	Hydrolysis	147
Vinyl glycine	Racemic vinyl glycine	Hydrolysis	51
Morpholines and triazole	Chiral alcohol intermediate	Hydrolysis	22
Pyrethroids	Racemic ester	Hydrolysis	92
Fenpropimorph	4-Tert-butylbenzyl bromide and methyldiethyl malonate	Acylation	7
Venturicidin	Aglycone	Hydrolysis	97

changes the enantiomeric values and also the stereopreference. Ihara and co-workers,[52] while studying the products of chiral building blocks for the synthesis of aconitium alkaloids with vinyl acetate and meso-diols, found that CRL could yield the alkaloids in over 96% enantiomeric excess. Enantioselective inter-esterification and trans-esterification have great significance in pharmaceuticals for selective acylation and deacylation.[131]

CARBOHYDRATE ESTER SYNTHESIS

Lipase-mediated organic synthesis has found new avenues in the production of carbohydrate esters of fatty acids, glyceroglycolipids and glycosylated products like sphingomyelins, cerebrosides or gangliosides.[98,135] Utilization of carbohydrate esters has become significant in fine chemical manufacturing. Asahi Electrochem of Japan produced maltose- and lactose-like sugar fatty acid esters using CRL.[6] Production of these esters involves reacting the disaccharides or their analogues (with at least one primary alcohol group) and a $C_{8–22}$ fatty acid such as myristic, stearic, linolenic or hydroxystearic acid. The sugars include acetyl or amino or acetyl amino maltose/lactose. The Lion group obtained a patent on a new process for esterification/trans-esterification involving the synthesis of methylglyceride fatty acid monoester suppressing the formation of diesters and high esters.[79] Methyl glyceride is widely used as a surfactant in many biotechnological formulations. The stereospecificity of CRL is used for the stereospecific resolution of a mixture of carbohydrate monoesters, which attracted the attention of industry by dint of its involvement in the formation of pharmaceutical, food and cosmetic products.[4] Broad substrate specificity is shown towards various monosaccharides (glucose, galactose, fructose) and methyl alpha-glucoside, sorbitol, xylitol, N-acetyl, D-glucosamine, glucosamine, glucuronic and galacturonic acid, and ascorbic acid.[108]

AMINES AND AMIDES

A novel enzymatic method has been developed for protecting amines and carbamates as substrates for lipase.[103] The symmetrical structure of the homocarbamates gives a single carbamate product, making the process very simple. The carbamate is easily deprotected or converted to the *n*-methyl derivative using $LiAlH_4$, providing a new method for chemoenzymatic amine methylation. Rakels *et al.*[115] reported that lipase-catalysed simultaneous hydrolysis and amidation led to a significantly improved amide yield and enantiomeric excess in racemic methyl 2-chloropropionate hydrolysis and aminolysis. Lalonde[71] has made pure and well-defined CLEC for the production of enantiomerically pure amines, amino acids and peptides.

BIOCIDES

As shown in Table 5, CRL are very frequently used for racemic mixture resolution, *via* both hydrolysis and acylation reactions. The lipase-catalysed resolution of 2-(4-isopropyloxy)phenyl) propionic acid, an intermediate in the synthesis of a chiral acaricide, was described by Bosetti *et al.*[23] with hydrolytic reactions in a stirred emulsion of the oily substrate, over 95% enantiomeric excess stereospecificity with (S)-enantiodiscrimination.

Optically pure (S)-1,3,4-oxadiazole-2 (3H)-ones obtained showed high ovicidal activity against *Tetranychus urticae*, and insect juvenile hormone analogues useful as insecticides, were obtained in high purity by enzyme-mediated hydrolysis of the respective *cis*- and *trans*-2-(4-methoxybenzyl)-1-cyclohexylacetates.[147]

Enzymatic synthesis of chiral building blocks for the production of potent pesticides is one of the successful methods in the CRL-mediated biocide (pesticide) industry.[2,97] Franssen *et al.*[40] have succeeded in the resolution of 2-methoxy-3-carbomethoxy tetrahydrofuran, a chiral building block for perhydrofuro-furan insecticides. Optically pure enantiomers of triazole and morpholine fungicides could be produced by transesterification of enzymatically prepared chiral alcohol intermediates.[22] Both triazole and morphalines (R-isomer) were active against many agricultural fungal pathogens.

The main focus of fine and intermediate chemical manufacturers is in obtaining new products and processes with new possibilities to make valuable and effective biocides.[131] In the field of biotechnology, much attention has been focused on the use of lipases as enantioselective biocatalysts in organic media. However, the flexibility of the lipase molecule is restricted in organic solvents and therefore the resulting enzyme conformation probably recognizes one of the enantiomers in the mixture much more selectively. A new process for producing chiral compounds of high optical purity comprises: reacting chiral and non-chiral reactants in super-critical CO_2 in the presence of lipase so that principally only one of the optically active isomers of the chiral starting material reacts to produce a new critical compound with more optical purity.[89]

An efficient 'Kazlauskas active site' kinetic model was validated for CRL-catalysed alcoholysis, esterification and acidolysis reactions in organic medium by Franssen *et al.*[40] Crude CRL lipase was used to resolve C_3-stereoisomers of the furo-furan building block methyl-2-methoxy-tetrahydrofuran-3-carboxylate by alcoholysis using *n*-butanol in octane, and the substrate was converted into a mixture of *cis*- and *trans*-butyl esters and unconverted methyl esters without significant inactivation.[40] The enantiomeric ratio was well over 100, allowing isolation of product with over 98% enantiomeric excess at 45% conversion. The enantioselectivity of CRL depends considerably on the substrates used and the water content of the system, but it does not depend on the degree of purification of lipase.[40,106]

BIOSENSORS

Biological micro-devices or biosensors with enzymatic intensification are available for the quantitative determination of triglycerides in the fats and oil industry, in food and in chemical diagnosis.[48,116] A promising new method involves the use of lipase, where the glycerol liberated during lipid hydrolysis is oxidized by glycerol-dehydrogenase.[125] NADH formed during the reaction is measured by fluorescence spectroscopy. Non-specific lipase with high specific activity has been selected to allow rapid liberation of glycerol.[125]

An important analytical use of lipases in the determination of lipids is notably for clinical purposes. As far as the physicians are concerned, this principle enables them to diagnose patients with cardiovascular complaints very precisely.[93] Lipase biosensors are developed not only for the diagnosis of clinical samples but also for use in the food and drinks industry, pollutant analysis (contamination by pesticide), and pharmaceutical industry.[144] Very recently, a CRL biosensor has been developed by Pittner *et al.*[112] as an enzyme-labelled probe, which is useful as a bioassay reagent, as components of biosensors or DNA probes.

The major objective in constructing a lipase biosensor should be that the substrate has to be presented in a manner that meets the interfacial constraints of lipase activity and to facilitate the repeated reuse of the biocatalyst by immobilization. In fact, immobilization of lipase as CLEC was found to be very useful for detecting an analyte in a fluid.[111,136] Furthermore, the advent of modern computers has revolutionized the biosensor technology, from the oldest electrode, to modern miniaturized optically active models, through calorimetric determination.[68,74]

BIOREMEDIATION

Bioremediation for waste disposal is new in lipase biotechnology. Oil spills created during transportation and subsequent refining of crude, oil-wet soil collected from road-sides and shore sand, lipid-tinged wastes in lipid processing factories and restaurants and lipid-tainted clothes may all be

efficiently managed by lipases.[15] In fact, CRL are used as a mixture along with other hydrolytic enzymes like proteases, amylases and cellulases.[32] For waste management, CRL are used either in *ex situ* form (purified lipases) or *in situ* (growing whole cells in the target waste) state.[15]

Bioremediation aims not only at the eradication of wastes but also its useful management. Nisshin Oil Mills of Japan converted waste edible oil into valuable transparent liquid soap by hydrolysing it with CRL in the presence of an emulsifier.[100] *C. rugosa* cells can be mixed with lipid-containing waste water, containing animal or vegetable fat and oil, or higher fatty acids, and the lipid assimilated.[39] The yeast has high lipase activity, hydrolyses animal or vegetable lipid, and has superior oxidation capacity on higher fatty acids. The yeast may be used for direct treatment of lipid-containing waste water released from oil manufacturing, the food industry, or restaurants.[39] Another oil company of Japan, Kurita-Water[70] devised a simple process for treating waste-water-containing lipids and oils.[70] Biotechnological methods for improvement and modification of animal waste fats from fowl lipid or beet tallow can be achieved by *in situ* utilization of *C. rugosa* or by enzymatic glycerolysis.[11,12] Degradation of unsaturated polyester waste by CRL, after slicing it into small particles, is yet another area. The low molecular weight reusable products so obtained may be employed especially in moulding articles reinforced with glass fibres, e.g. vehicle parts, bathroom equipment, casings for electrical equipment, kitchen sinks, boat hulls, etc.[15]

BIODETERGENTS

Detergents comprise surfactants (i.e., surface-active agents) and builders which are inorganic compounds that create a favourable chemical background for the actions of surfactants.[28,91] The old organic surfactants are now replaced by much more active biosurfactants, which are proteinaceous in nature.[26] The marketing success of surfactants with trypsin led to the development of other types of surfactant proteases, and the introduction of amylases, cellulases, and lipases for various detergent applications.[28] However, the cost of enzymes remains a major obstacle to their substantial use in the surfactant industry.[123] Intensive screening programmes have resulted in the introduction of highly thermostable biocatalysts capable of performing at elevated temperature for hundreds of hours without substantial loss of activity, making enzymatic processing cost-efficient in the manufacturing of many speciality products.[123] To be a suitable additive in detergents, lipases should be both thermotolerant ($\sim$60°C), alkalophilic (pH 7–11), and capable of functioning in the presence of the varying components of washing formulations such as proteinases, which may cleave lipase proteins and surfactants (known lipase inhibitors) without losing activity or stability. Moreover, they should also be stable in the surfactants during storage and when active in the washing machine.[91]

The advent of recombinant-DNA technology and protein engineering greatly influenced the biosurfactant industry. Unilever, Cosmo Oil and Procter & Gamble are prominent in this field.[15,30] The activity, stability, priority and efficiency of enzymes can be improved *via* genetic engineering. The application of genetic engineering may lead to a significant ecological advantage, i.e. the energy demand for biotransformation by a genetically engineered enzyme could be reduced by over 60%, compared to the natural (unmodified) enzyme. Utilization of genetic engineering tools, such as site-specific mutagenesis and recombinant-DNA technology, for the production of functionally stabilized enzymes can further reduce environmental pollution.[67]

Use of additives such as specific ligands and chemical modifications (e.g. cross-linking by bifunctional reagents, strengthening of hydrophobic reactions by non-polar reagents, introduction of new polar or charged groups leading to additional ionic and hydrogen bonds and hydrophilization of the protein surface to reduce unfavourable surface hydrophobic contacts with water) are some of the means to modify the efficiency of the enzymes.[36]

TANNING

An enzyme preparation for leather processing is new in the tanning industry.[61] As in detergents, a mixture of hydrolytic enzymes which contains lipase, pepsin, chymotrypsin, elastase, papain, aminopeptidase, choline esterage, and amylase is also employed for the production of ready-to-tan hides.[117] The enzymatic process for the production of hides and skins ready for tanning involves the steps of soaking, washing, dehairing and bathing in an aqueous solution containing the enzyme

mixture. In this alternate technology, no surfactant is used in the soaking stage and in subsequent washing, dehairing and bathing. This method permits minimum use of detergents in the tanning industry.[117] A Russian patent[95] describes another technology which improves the quality of intermediate sheepskin products by increasing the strength and elasticity of the skin and reducing rigidity, with a reduced use of surfactants. One of the pre-requisites for biological detergents in tanning is that they should work under alkaline conditions.[45] Genetically engineered non-specific lipases which are functionally stabilized could offer much in this regard.[36]

COSMETICS AND PERFUMERY

The intense interest of technologists in the tailoring of lipases for use in the cosmetic industry is mainly due to their activity as softeners and in aroma production, which are major factors in cosmetics.[94,105] Glyceride esters, polyunsaturated fatty acids and mixed acid-type polyesters are the main components in CRL-mediated cosmetics.[140] McCrae *et al*.[87] formulated esterification reactions for the synthesis of costly cosmetic esters from fatty acids and miscible primary and secondary alcohols.

CONCLUSIONS

C. rugosa has GRAS (generally regarded as safe) status and no adverse effect on human or other forms of life has been reported as a result of traditional, or even open fermentation practices using both *in situ C. rugosa* or *ex situ* purified lipases, e.g. for the production of food and flavours, as well as SCP. A fascinating field, not yet exploited, is meat technology, especially for flavouring sausages. Moreover, the pulping, plastic and lubricant industries could be modernized by the introduction of lipases. Infusion of lipase into living cells would have radical effects, if it could be applied *in vivo* on patients suffering from chronic cardiovascular diseases.

The versatility of CRL has stimulated its unprecedented demand in the world market. It presents a challenge to scientists to improve both the quality and quantity of CRL as much as possible in order to bridge the gap between demand and production. Quantitative enhancement can be achieved by strain improvement and site-specific mutagenesis of the lipase genes of *C. rugosa* or by cloning and expression of those genes in a surrogate organism for overproduction of lipases. Optimization of reaction parameters by the molecular restructuring of the enzyme through genetic adjustments would yield functionally stabilized CRL with maximum quality. Finally, we hope that this review will stimulate further novel investigations into *C. rugosa* and its lipases.

REFERENCES

1. Aires-Barros, M. R. and Cabral, J. M. S. (1991). Selective separation and purification of two lipases from *Chromobacterium viscosum* using AOT reversed micelles. *Biotechnol. Bioeng.* **38,** 1302–1307.
2. Akita, H., Chen, C. Y. and Uchida, K. (1995). A formal synthesis of nikkomycin-B based on enzymatic resolution of a primary alcohol possessing two stereogenic centres. *Tetrahedron Asymmetry* **6,** 2131–2134.
3. Alberghina, L., Gnandori, R., Longhi, S., Lotti, M., Fusetti, F. and Vanoni, M. (1991). Molecular cloning of a lipase and of a lipase related gene from *Candida cylindricea*. In Alberghina, L., Schmid, R. D. and Verger, R. (Eds), *Lipases: Structure, Mechanism and Genetic Engineering. GBF Monographs* **16,** 231–235.
4. Andersen, O. (1993). Acyl monoesters of alkyl glucosides, a new class of mild and efficient surfactants. *J. Chem. Technol. Biotechnol.* **56,** 412–413.
5. Antonian, E. (1988). Recent advances in the purification, characterization and structure determination of lipases. *Lipids* **12,** 1101–1106.
6. Asahi Electrochem (1988). Production of maltose-like sugar fatty acid ester. PN: JP; J63222698.
7. Avdagic, A., Cotarca, L., Ruzic, K. S., Gelo, M. and Sunjic, V. (1994). An efficient chemoenzymatic synthesis of S-(−)-ferpropimorph. *Biocatalysis* **9,** 49–60.
8. Bailie, P., McNerlen, S., Robinson, E. and Murphy, W. (1994). An immobilized lipase reactor for the hydrolysis of fats and oils. *Appl. Biocatal.* **14,** 11–13.
9. Baldessari, A., Bruttomesso, A. C. and Gros, E. G. (1996). Lipase-catalyzed region selective deacylation of androstane derivatives. *Helv. Chim. Acta* **79,** 999–1004.
10. Basri, M., Yunus, W. M. Z. W., Yoong, W. S., Ampon, K., Razak, C. N. A. and Salleh, A. B. (1996). Immobilization of lipase from *Candida rugosa* on synthetic polymer beads for use in the synthesis of fatty esters. *J. Chem. Technol. Biotechnol.* **66,** 169–173.

11. Bednarski, W., Adamczak, M., Kowalewska-Piontas, J. and Zadernowski, R. (1994). Biotechnological methods for the upgradation and modification of animal waste fats. *Acta Biotechnol.* **14,** 387–393.
12. Benjamin, S. and Pandey, A. (1996). Optimization of liquid media for lipase production by *Candida rugosa*. *Bioresource Technol.* **55,** 167–170.
13. Benjamin, S. and Pandey, A. (1996). Lipase production by *Candida rugosa* on copra waste extract. *Indian J. Macrobiol.* **36,** 201–204.
14. Benjamin, S. and Pandey, A. (1997). Enhancement of lipase production during repeated batch culture using immobilized *Candida rugosa*. *Process Biochem*. **32,** 257–260.
15. Benjamin, S. and Pandey, A. (1998). *Candida rugosa* and its lipases—a retrospect. *J. Sci. Ind. Res.* **57,** 1–9.
16. Benjamin, S. and Pandey, A. (1997). Coconut cake—a potent substrate for the production of lipase by *Candida rugosa* in solid-state fermentation. *Acta Biotechnol.* **17,** 241–251.
17. Benjamin, S. and Pandey, A. (1998). Mixed solid substrate—a novel process for enhanced lipase production by *Candida rugosa* (submitted).
18. Benjamin, S. and Pandey, A. (1995). Lipase production by *Candida rugosa* in both solid and liquid fermentation—a comparative. *Proc. Louis Pasteur's Cent. Symp.* Trivandrum, India, p. 11.
19. Benjamin, S. and Pandey, A. (1996). Packed-bed bioreactor system for overproduction of lipase by immobilized *Candida rugosa*. *Proc. 37th Annual Conference*, AMI, Madras, India, p. 83.
20. Benzonana, G. and Esposito, S. (1971). On the positional and chain specificities of *Candida cylindracea* lipase. *Biochim. Biophys. Acta* **231,** 15–22.
21. Bertolini, M. C., Schrag, J. D., Cygler, M., Thomas, D. Y. and Vernet, T. (1995). Expression in yeast, characterization and comparison of lipases from *Geotrichum candidum*. *Yeast* **11,** Spec. Iss. S523.
22. Bianchi, D., Cesti, P., Golini, P., Spezia, S., Garavaglia, C. and Mirenna, L. (1992). Enzymatic preparation of optically active fungicide intermediates in aqueous and in organic media. *Pure Appl. Chem.* **64,** 1073–1078.
23. Bosetti, A., Bianchi, O., La-Porta, P., Bettarini, F. and Massimini, S. (1994). Chemo-enzymatic synthesis of optically pure 1,3,4-oxadiazol-2(3H)-ones with acaricidal activity. *J. Agric. Food. Chem.* **42,** 2596–2599.
24. Braun, B., Klein, E. and Lopez, J. L. (1996). Immobilization of *Candida rugosa* lipase to nylon fibres using its carbohydrate groups as the chemical link. *Biotechnol. Bioeng.* **51,** 327–341.
25. Cambillau, C. (1991). Crystallization and X-ray studies of lipases. In Alberghina, L., Schmid, R. D. and Verger, R. (Eds), *Lipases: Structure, Mechanism and Genetic Engineering. GBF Monographs* **16,** 17–26.
26. Carlsen, S. (1994). Lipolytic enzymes and their use in the oleochemical and detergent industry. *Fat Sci. Technol.* **96,** 408.
27. Chang, R. C., Chou, S. J. and Shaw, J. F. (1994). Multiple forms and functions of *Candida rugosa* lipase. *Biotechnol. Appl. Biochem*. **19,** 93–97.
28. Christensen, P. N. and Donelyan, V. (1990). Detergent enzymes. *Chim. Oggi* **8,** 41–43.
29. Cipiciani, A., Fringuelli, F. and Scappini, A. M. (1996). Enzymatic hydrolysis of acetoxy- and phenethyl benzoates by *Candida cylindracea* lipase. *Tetrahedron* **52,** 9869–9876.
30. Cosmo Oil (1995). Lipase useful in detergents, pulp production and synthetic reactions. PN: JP; 07227276.
31. Cudrey, C., Ferrato, F., Haalck, L., Paltauf, F., Rogalska, E. and Spener, F. (1994). Stereoselective hydrolysis of triglycerides and triglyceride analogs by animals and microbial lipases. *Prog. Biotechnol.* **9,** 137–140.
32. Dauber, S. R. and Boehnke, B. (1993). Wastewater process and apparatus treats mixture of activated and primary sludges. PN: DE; 4141832.
33. Davranov, K. (1994). Microbial lipases in biotechnology. *Prikl. Biokhim. Microbiol.* **30,** 527–534.
34. Eigtved, P. and Hansen, T. T. (1987). Ester synthesis with immobilized lipases. *Fat Sci. Technol.* **89,** 467–470.
35. Eyodorova, G. D., Arends, I. M., Dorokhov, V. V., Konchalovskaya, M. E., Sokolova, I. A. and Shelamova, S. A. (1986). On the positional specificity of some microbial lipases. *Parikl. Biokhim. Microbiol.* **22,** 630–634.
36. Fagain, C. D. and O'Kennedy, R. (1991). Functionally-stabilized proteins—a review. *Biotechnol. Adv.* **9,** 351–409.
37. Ferreiradias, S. and Dafonseca, M. M. R. (1995). Glycerolysis of olive oil: batch operational stability in hydrophilic polyurethane foams. *Bioprocess Eng.* **13,** 311–315.
38. Fleming, H. P. (1991). Mixed cultures in vegetable fermentation. In Zeikus, J. G. and Johnson, E. A. (Eds), *Mixed Cultures in Biotechnology*. McGraw-Hill, pp. 69–103.
39. Food Agr. Clean. Echo. Syst. Eng. Res. Ass. (1994). Food industry or restaurant lipid wastewater waste-disposal using lipophilic yeast. PN: JP; 06062837.
40. Franssen, M. C. R., Jongejan, H., Kooijmau, H., *et al*. (1996). Resolution of a tetrahydrofuran ester by *Candida rugosa* lipase (CRL) and an examination of CRL's stereochemical preference in organic media. *Tetrahedron Asymmetry* **7,** 497–510.

41. Frense, D., Lange, U. and Hartmeier, W. (1996). Immobilization of *Candida rugosa* lipase in lyotropic liquid crystals and some properties of the immobilized enzyme. *Biotechnol. Lett.* **18,** 293–298.
42. Fukui, T., Kawamoto, T., Sonomoto, K. and Tanaka, A. (1990). Long-term continuous production of optically active 2-(4-chlorophenoxy)-propanoic acid by yeast lipase in an organic solvent system. *Appl. Microbiol. Biotechnol.* **34,** 330–334.
43. Fusetti, F., Brocca, S., Porro, D. and Lotti, M. (1996). Effect of the leader sequence on the expression of recombinant *C. rugosa* lipase by *S. cerevisiae* cells. *Biotechnol. Lett.* **18,** 281–286.
44. Goto, M., Noda, S., Kamiya, N. and Nakashio, F. (1996). Enzymatic resolution of racemic ibuprofen by surfactant coated lipase in organic media. *Biotechnol. Lett.* **18,** 839–844.
45. Grant, W. D., Mwatha, W. E. and Jones, B. E. (1990). Alkaliphiles: ecology, diversity and applications. *FEMS Microbiol. Rev.* **72,** 255–269.
46. Grochulski, P., Li, Y., Schrag, J. D., *et al.* (1993). Insights into interfacial activation from an open structure of *Candida rugosa* lipase. *J. Biol. Chem.* **268,** 12,843–12,847.
47. Ha, K. J. and Lindsay, R. C. (1993). Release of volatile branched-chain and other fatty acid from ruminant milk fats by various lipases. *J. Dairy Sci.* **76,** 677–690.
48. Haalck, L., Kallabis, B., Schoemaker, M., Cammann, K. and Spener, F. (1991). Lipases for biosensors. *Fat Sci. Technol.* **93,** 415–416.
49. Haalck, L., Paltauf, F., Stadler, P., Steffens, U., Kovac, A. and Spener, F. (1994). Determination of the stereopreference of microbial lipases using triacyl glycerols and 2-o-alkyl analogs as substrates. *Fat Sci. Technol.* **96,** 429–430.
50. Handlesman, T. and Shoham, Y. (1994). Production and characterization of an extracellular thermostable lipase from thermophilic *Bacillus* species. *J. Gen. Appl. Microbiol.* **46,** 435–443.
51. Hoechst (1993). Production of optically active vinylglycine compounds. PN: EP; 529601.
52. Ihara, M., Suzuki, M., Hirahayashi, A., Tokunaga, Y. and Fukumoto, K. (1995). Preparation of chiral building blocks for synthesis of Aconitium alkalids. *Tetrahedron Asymmetry* **8,** 1829–1832.
53. Ikushima, Y., Satio, N. and Arai, M. (1996). Promotion of lipase-catalysed esterification on N-valeric acid and citronellol in supercritical carbon dioxide in the near-critical region. *J. Chem. Eng. Jpn.* **29,** 551–553.
54. Jachamanian, I., Schulte, E. and Mukherjee, K. D. (1996). Substrate selectivity in esterification of less common fatty acids catalysed by lipases from different sources. *Appl. Microbiol. Biotechnol.* **44,** 563–567.
55. Jaeger, K.-E., Ransac, S., Dijkstra, B. W., Colson, C., Van Heuvel, M. and Misset, O. (1994). Bacterial lipases. *FEMS Microbiol. Rev.* **5,** 29–63.
56. Jensen, R. G. (1983). Detection and determination of lipase (acylglycerol hydrolase) activity from various sources. *Lipids* **18,** 650–657.
57. Jenssen, A. E. M., Aaidya, A. M. and Halling, P. J. (1996). Substrate specificity and kinetics of *Candida rugosa* lipase in organic media. *Enz. Microb. Technol.* **18,** 340–346.
58. Jenssen, A. E. M. and Halling, P. J. (1994). Specificities of enzymes corrected for solvation depend on the choice of the standard state. *J. Am. Chem. Soc.* **16,** 9827–9830.
59. Kamal, A. and Rao, M. V. (1994). Enzyme catalyzed synthesis of (S)propanolamines. *Tetrahedron Asymmetry* **5,** 1881–1882.
60. Kanisawa, T., Yamaguchi, Y. and Hattori, S. (1982). Action of *Candida cylindracea* lipases on concentrated milk and cream. *J. Jpn. Soc. Food Sci. Technol.* **29,** 693–699.
61. Kao (1995). Preparation of enzyme composition for cleaners and leather processing. PN: JP; 07177884.
62. Katchalski-Katzir, E. (1993). Immobilized enzymes—learning from past successes and failures. *Trends Biotechnol.* **11,** 471–478.
63. Kato, K., Katayama, M., Fugii, S., Fukaya, H. and Kimoto, H. (1996). Optical resolution of 2,2,2-trifluoro-1-(9-phenanthryl) ethanol *via* enzymatic hydrolysis of its activated ester. *J. Ferment. Bioeng.* **81,** 206–211.
64. Kawakami, K. (1996). Enhancement of thermostability of lipase by the sol-gel entrapment into methyl substituted organic silicates formed on diatomaceous earth. *Biotechnol. Tech.* **10,** 491–494.
65. Kazlauskas, R. J. (1994). Elucidating structure–mechanism relationships in lipases. Prospects for predicting and engineering catalytic properties. *Trends Biotechnol.* **12,** 464–472.
66. Kim, M. G. and Lee, S. B. (1996). Enzymatic resolution of racemic ibuprofon by lipase catalyzed esterification reaction: effects of water contents and solid support. *J. Ferment. Bioeng.* **81,** 269–271.
67. Kottwitz, B., Upadek, H. and Carrer, G. (1994). Application and benefits of enzymes in detergents. *Chim. Oggi* **12,** 21–24.
68. Krause, J., Rohen, A. and Winter, B. (1990). Biosensors—hit or flop. *Chem. Ind.* (Ouesseldorf) **113,** 74–75.
69. Kreger-van Rig, N. J. M. (Ed.) (1984). *The Yeasts—A Taxonomic Study*. Elsevier, 785 pp.
70. Kurita-Water (1994). Lipid degradation and oil degeneration using lipase in a digeste. P: JP; 06246295.
71. Lalonde, J. (1995). The preparation of homochiral drugs and peptides using cross-linked enzyme crystals. *Chim. Oggi* **13,** 33–37.

72. Lange, C. R. (1993). Fermentation industry. *Water Environ. Res.* **65,** 400–402.
73. Langrand, G., Rondot, N., Triantaphilides, C. and Baratti, S. (1990). Short chain flavour esters synthesis by microbial lipases. *Biotechnol. Lett.* **12,** 581–586.
74. Laurinavicins, V., Kurtinaitiene, B., Gureviciene, V., Boguslavsky, L., Geng, L. and Skotheim, T. (1996). Amperometric glyceride biosenser. *Anal. Chim. Acta* **330,** 159–166.
75. Lazar, G. and Eirich, L. D. (1989). Hydrolysis of tallow, olive oil and coconut oil by extracellular, intracellular and membrane-bound microbial lipases. *Biol. Chem. Hoppe-Seyler* **370,** 985.
76. Lee, K. Y. and Lee, S. T. (1996). Continuous process for yeast biomass production from sugar beet a stillage by a novel strain of *Candida rugosa* and protein profile of the yeast. *J. Chem. Technol. Biotechnol.* **66,** 349–354.
77. Lelieveld, H. L. M., Bachmayer, H., Boon, B., *et al*. (1995). Safe biotechnology: Part 6, Safety assessment in respect of human health of micro-organisms used in biotechnology. *Appl. Microbiol. Biotechnol.* **43,** 389–393.
78. Linko, Y. Y. and Wu, X. Y. (1996). Biocatalytic production of useful esters by two forms of lipase from *Candida rugosa*. *J. Chem. Technol. Biotechnol.* **65,** 163–170.
79. Lion (1990). A method for preparing methylglycoside fatty acid monoester. PN: JP; J04016195.
80. Liu, S. D., Cao, S. G., Ding, Z. T., *et al*. (1995). A new method for estimating the selectivity for fatty acids and fatty alcohols in organic solvents. *Ann. N.Y. Acad. Sci.* **750,** 209–214.
81. Lotti, M., Tramontano, A., Longhi, S., *et al*. (1994). Variability within the *Candida rugosa* lipase family. *Protein Eng.* **7,** 531–535.
82. Lye, G. J., Pavlou, O. P., Rosjidi, M. and Stuckey, O. C. (1996). Immobilization of *Candida cylindracea* lipase on liquid aphrons (CLAs) and development of a continuous CLA—membrane reactor. *Biotechnol. Bioeng.* **51,** 69–78.
83. MacRae, A. R. and Hammond, R. C. (1985). Present and future application of lipases. *Biotechnol. Genetic Eng. Reviews* **3,** 193–217.
84. MacRae, A. R. and Staines, H. (1994). Evaluation of microbial lipases as fat splitting catalysts. *Fat Sci. Technol.* **96,** 430.
85. Malcata, F. X., Reyes, H. R., Garcia, H. S., Hill, C. G., Jr and Amundson, C. H. (1992). Kinetics and mechanisms of reactions catalysed by immobilized lipases. *Enzyme Microb. Technol.* **14,** 426–446.
86. McArdle, R. N. and Culver, C. A. (1994). Enzyme infusion—a developing technology. *Food Technol.* **48,** 85–86, 88–89.
87. McCrae, A., Roehl, E. L. and Brand, H. M. (1990). Bioesters. *Seifen. Oele. Fette. Wachse* **116,** 201–205.
88. McGee, H. (Ed.) (1986). *On Food and Cooking: the Science and the Lore of the Kitchen.* Unwin Hyman Ltd., 3 pp.
89. Michor, H., Marr, R., Gamse, T., Schilling, T., Klingsbichel, E. and Schwab, H. (1996). Enzymatic catalysis in supercritical carbon dioxide, comparison of different lipases and a novel esterase. *Biotechnol. Lett.* **18,** 79–84.
90. Mingarro, I., Gonzalez-Navarro, H. and Braco, L. (1996). Trapping of different lipase conformers in water-restricted environments. *Biochemistry* **35,** 9935–9944.
91. Misset, O., Lenting, H. B. M., Labout, J. J. M., Bolle, R., Oomen, A. J. A. M. and Mulleners, L. J. S. M. (1994). Applications of lipase in household detergents. *Fat Sci. Technol.* **96,** 421.
92. Mitsuda, S., Matsuo, N. and Nabeshima, S. (1992). Preparation of (−)-alpha-ethyl alcohol moieties of pyrethroid insecticides by lipase-catalyzed enantioselective hydrolysis. *Biosci. Biotechnol. Biochem.* **56,** 357–358.
93. Mitsubishi Elec. (1990). Blood analysis using enzyme electrode biosenser. PN: JP; J02145968.
94. Miyamoto, A., Shigeta, A., Tanaka, Y., *et al.* (1995). PN: EP; 65 8629.
95. Moscow Technol. Inst. Light. Ind. (1992). Treatment of sheepskin. PN: SU; 730163.
96. Mukherjee, K. D. and Kiewitt, I. (1996). Environment of very long chain mono-unsaturated fatty acids by lipase catalysed hydrolysis and transesterification. *Appl. Microbiol. Biotechnol.* **44,** 557–562.
97. Nagooi, T. K., Guo, Z. W. and Sih, C. J. (1990). Enantioselective biocatalysis—optically active intermediates for venturicidin synthesis. *Biocatalysis* **3,** 119–128.
98. Nakano, H., Kitahata, S., Shimada, Y., Nakamura, M. and Tominaga, Y. (1995). Esterification of glycosides by a mono- and diacylglycerol lipase from *Penicillium camembertii* and comparison of the products with *Candida cylindracea* lipase. *J. Ferment. Bioeng.* **80,** 24–29.
99. Nakano, H., Okuyama, Y., Iwasa, K. and Hongo, H. (1994). A facile lipase catalyzed resolution of 2-azabicyclo(2.2.1)hept-5-en-3-ones. *Tetrahedron Asymmetry* **5,** 1155–1156.
100. Nisshin Oil Mills (1993). Waste edible oil lipid hydrolysis using lipase. PN: JP; 05302100.
101. Noya, F. C., Ferorelli, S., Franchini, C., Scilimati, A., Sinicropi, M. S., Tortorella, P. (1996). *Candida cylindracea* lipase-mediated kinetic resolution of alpha-substituted alpha aryloxy acetic acid methyl esters. *Farmaco.* **51,** 293–296.
102. Oguntimein, G. B. (1994). Lipase-catalysed synthesis of flavour compounds in a solvent-free medium. *Meded. Fac. Landbouwwet, Rijksuniv. Gent*. **59,** 2377.

103. Orsat, B., Alper, P. B., Moree, W., Mak, C. P. and Wong, C. H. (1996). Homo carbonates as substrates for the enantioselective enzymatic protection of amines. *J. Am. Chem. Soc.* **118,** 312–313.
104. Osada, K., Takahashi, K. and Hatano, M. (1990). Polyunsaturated fatty acid glyceride preparation by microbial lipases. *J. Am. Oil Chem. Soc.* **67,** 921–922.
105. Osaka Fugi Jin (1995). Pack base agent utilizing extraction residue of plant ash and its preparation. PN: JP; 07082136.
106. Ota, Y., Itabashi, Y. and Hasuo, M. (1996). Measurement of positional specificity index of microbial lipases by chiral phase high-pressure liquid chromatography. *Biosci. Biotechnol. Biochem.* **60,** 145–146.
107. Pabai, F., Kermasha, S. and Morin, A. (1995). Lipase from *Pseudomonas fragi* CRDA 323: Partial apurification, characterization and interesterification of butter fat. *Enz. Microb. Technol.* **43,** 42–51.
108. Park, O. J., Park, H. G. and Yang, J. W. (1996). Enzymatic transesterification of monosaccharides and amino acid esters in organic solvents. *Biotechnol. Lett.* **18,** 473–478.
109. Patel, M. T., Nagarajan, R. and Kilara, A. (1996). Hydrolysis of milk fat by lipase in solvent-free phospholipid reverse micellar media. *J. Food Sci.* **61,** 33–38.
110. Persichetti, R. A., Lalonde, J. J. and Margolin, A. L. (1996). Enhancing the enantioselectivity: a look at *Candida rugosa* lipase and the importance of purity and reaction media. *Abstr. Pap. Am. Chem. Soc.* **211** meet Pt. 2, ORGN006.
111. Pittner, F. (1990). Immobilization of enzymes etc. PN: WO; 9012092.
112. Pittner, F., Kynclova, E., Schalkhammer, T., Ecker, B. and Wakolbinger, W. (1995). Enzyme-labelled probes and assay reagents. PN: AT; 9302071.
113. Posorske, L. H. (1984). Industrial scale application of enzymes to the fats and oil industry. *J. Am. Oil Chem. Soc.* **61,** 1758–1760.
114. Pronk, W., Boswinkel, G. and van't Riet, K. (1992). The influence of fatty acid and glycerol on the kinetics of fat hydrolysis of *Candida rugosa* lipase in a membrane reactor. *Biocatal.* **5,** 305–323.
115. Rakels, J. L. L., Schneiders, A. G. M., Straathof, A. J. J. and Heijnen, J. J. (1996). Improved kinetic resolution of enzyme-catalyzed parallel reaction. *Biocatal. Biotransform.* **13,** 179–188.
116. Rakhimov, M. M. (1986). Biological microdevices with enzymatic intensification. *Biofizika* **31,** 704–710.
117. Rohm (1992). Enzymatic treatment of hide and skin using lipase. PN: DE; 4119889.
118. Rombouts, F. M. and Nout, M. J. R. (1995). Microbial fermentation in the production of plant foods. *J. Appl. Bacteriol. Symp. Suppl.* **79,** 1085–1175.
119. Rua, M. L. and Ballesteros, A. (1994). Rapid purification of two lipase isoenzymes from *Candida rugosa*. *Biotechnol. Tech.* **8,** 21–26.
120. Rubin, B., Jamison, P. and Harrison, D. (1991). Crystallization and characterization of *C. rugosa* lipase. In Alberghina, L., Schmid, R. D. and Verger, R. (Eds), *Lipases: Structure, Mechanism and Genetic Engineering. GBF Monographs* **16,** 63–66.
121. Sanchez, E. M., Bello, J. F., Roig, M. G., Burguillo, F. J., Moreno, J. M. and Sinisterra, J. V. (1996). Kinetic and enantioselective behaviour of the lipase from *Candida cylindracea*: a comparative study between the soluble enzyme and the enzyme immobilized on agarose acid silica gels. *Enzyme Microb. Technol.* **18,** 468–476.
122. Sankyo, Amanopharm. (1995). Preparation of optically active 2,5-pyrolidine dione derivative with very high optical purity. PN: JP; 07322889.
123. Sarney, D. B. and Vulfson, E. N. (1994). Enzymatic synthesis of surfactants. *Med. Fac. Landbouwwet. Rijksuniv. Gent.* **59,** 2321–2330.
124. Schmid, R. D., Menge, U., Schomburg, D. and Spencer, F. (1995). Towards novel biocatalysts *via* protein design: the case of lipases. *FEMS Microbiol. Rev.* **16,** 253–257.
125. Schoemaker, M., Feldbruegge, R., Trau, D., Gruendig, B. and Spencer, F. (1994). Enzyme sensors for triglycerides and essential fatty acids. *Fat. Sci. Technol.* **96,** 420–421.
126. Seitai, Kino Riyo Kagakuchin (1987). Lipase immobilized membrane and its production. PN: JP; J62171685.
127. Seufer-Wasserthal, P., Mayrhofer, H., Wirth, I. and Poechlauer, P. (1995). Enzymatic resolution of asymmetric alcohol. PN: EP; 675205.
128. Shaw, J. F. and Chang, C. H. (1989). Characterization of three distinct forms of lipolytic enzyme in a commercial *Candida* lipase preparation. *Biotechnol. Lett.* **11,** 779–784.
129. Sih, C. J., Gu, Q. M., Fulling, G., Wu, S. H. and Reddy, D. R. (1988). The use of microbial enzymes for the synthesis of optically active pharmaceuticals. *Dev. Ind. Microbiol.* **29,** 221–229.
130. Stead, D. (1986). Microbial lipases: their characteristics, role in food spoilage and industrial uses. *J. Dairy Res.* **53,** 431–505.
131. Stinson, S. C. (1995). Fine and intermediate chemicals makers emphasize new products and process. *Chem. Eng. News* **73,** 10–26.
132. Sztajer, H. and Zboinska, E. (1988). Microbial lipases in biotechnology. *Acta Biotechnol.* **8,** 169–175.

133. Taipa, M. A., Aires-Barros, M. R. and Cabral, J. M. S. (1992). Purification of lipases. *J. Biotechnol.* **26,** 111–142.
134. Takahata, H., Uchida, Y., Ohkawa, Y. and Momose, T. (1993). Enzymatic resolution of N,N-dialkyl-3-hydroxy-4-pentanamides, unsuccessful in resolution by the Katsuki–Sharpless asymmetric epoxidation. *Tetrahedron Asymmetry* **4,** 1041–1042.
135. Takahashi, M., Koike, R. and Ogasawara, K. (1995). Lipase mediated resolution of inden-1-ol. *Chem. Pharm. Bull.* **43,** 1585–1587.
136. Toshiba (1989). New Biosensor Construction. PN: JP; J01102352.
137. Tsai, S. W., Lu, C. C. and Chang, C. S. (1996). Surfactant enhancement of (S)-naproxen ester productivity from racemic, naproxan by lipase in isooctane. *Biotechnol. Bioeng.* **51,** 148–156.
138. Vattiner, E. and Kanerva, L. T. (1994). Lipase-catalyzed transesterification in the preparation of optically active solketal. *J. Chem. Soc. Perkin Trans. 1* **23,** 3459–3463.
139. Vertex Pharm (1992). Use of cross-linked enzyme crystals as a novel form of enzyme immobilization. PN: WO; 9202617.
140. Wille, H. J. (1996). Hydrolysis of polyunsaturated acid triglycerides. EP: EP; 714983.
141. Williams, T. R. (1996). The immobilization of lipase on chitin. *Abstr. Pap. Am. Chem. Soc.* 211 meet, Pt. 1, CHED, 319.
142. Wu, W. H., Akoh, C. C. and Phillips, R. S. (1996). Lipase catalyzed stereospecific esterification of DL-menthol in organic solvents using acid anhydrides as acylating agents. *Enzyme Microb. Technol.* **18,** 536–539.
143. Yasufuku, Y. and Ueji, S. (1996). Improvement (5-fold) of enantioselectivity for lipase-catalyzed esterification of a bulky substrate at 57 deg in organic solvent. *Biotechnol. Tech.* **10,** 625–628.
144. Yissum Res. Dev. Hebrew Univ. (1991). Doped sol-gel glass. PN: EP; 439318.
145. Yunus, W. M. Z. W., Salleh, A. B., Basri, M., Ampon, K. and Razak, C. N. A. (1996). Preparation and immobilization of lipase on to poly(methyl acrylate methyl methaecrylate-divinyl benzene) beads for lipid hydrolysis. *Biotechnol. Appl. Biochem.* **24,** 19–23.
146. Zalacain, I., Zapelena, M. J., Astiasaran, I. and Bello, J. (1995). Dry fermented sausages elaborated with lipase from *Candida cylindracea*, comparison with traditional formulations. *Meat Sci.* **40,** 55–61.
147. Zarevuika, M., Rejzek, M., Wimmer, Z., Streinz, L. and Demnerova, K. (1993). Enzyme mediated hydrolysis of acetates. *Biotechnol. Lett.* **15,** 1139–1144.