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Production of Native and Recombinant Lipases by *Candida rugosa*

A Review

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Abstract

The yeast *Candida rugosa* produces multiple lipase isoenzymes sharing high sequence homology but with some differences in their catalytic properties. The regulation of *C. rugosa* lipase (CRL) synthesis and secretion in *C. rugosa* obeys a complex pattern. Fermentation processes for both wild-type and mutant *C. rugosa* strains are available for lipase production. Native CRL preparations have been extensively used for biotransformations. However, their inherent mixture of isoforms with variable profiles complicates interpretation and brings into question the reproducibility achieved between preparations. Although heterologous *CRLs* gene expression had been hampered owing to a nonuniversal codon usage, recent advances have made heterologous CRLs available. This will expand and improve the industrial utility of CRLs even further. The purpose of this review is to provide a summary of the recent advances on the production of native and recombinant lipases by *C. rugosa*.

Index Entries: *Candida rugosa*; lipase; isoenzymes; fermentation; heterologous expression.

Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) make up a wide family of enzymes whose natural catalytic function is to hydrolyze ester bonds in triglycerides to produce diglycerides, monoglycerides, glycerol, and fatty acids. The hydrolysis is an equilibrium reaction controlled by

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water content in the reaction medium. Thus, by modifying the reaction conditions, ester synthesis is also possible *(1)*. These reactions proceed with high regio- and/or enantioselectivity, which confers these enzymes their enormous biotechnologic potential. Their advantages also include stability in organic solvents, no requirement for cofactors, broad substrate specificity, and a high enantioselectivity *(2)*.

The most commercially important fields of application of lipases in the last three decades have been in the oleochemical industry, detergent manufacturing, and food industry. Furthermore, the enantioselectivity of lipase catalysis has promoted a spectacular increase in its potential use on an industrial scale, particularly in pharmaceutical applications. However, other biotechnologic industrial applications are emerging in fields such as flavor development in food, biomedical, biocides, biosensors, bioremediation, tanning industry, wastewater treatment, cosmetic, and perfumery. One cannot forget the possibility of modernizing classical industries such as pulping and paper, plastic, and lubricants by the introduction of lipases. Other potential applications are not in the market because the cost of producing lipases necessary to catalyze these versatile reactions is often prohibitive *(3)*. Many excellent reviews on industrial applications of lipases show the interest in these aspects *(1,2,4–10)*.

Mechanism of Lipolysis and Lipase Assay

Lipases are characterized by a drastically increased activity when acting at the lipid-water interface or emulsified substrates *(11)*. The mechanism of lipase adsorption to an aqueous-organic interface has been studied using various techniques *(12–14)*. The results have demonstrated that lipolytic activity depends on both the physical-chemical properties of the interface and the available interfacial area *(15,16)*. This relationship is a complex one because adsorption of lipase at the interface is a dynamic process and its physical-chemical properties change continuously with time as a function of environmental factors including the appearance of reaction products. The presence of different additives in lipase preparations (carbohydrates, detergents, other proteins, and so on) can have an important impact on the lipase activity assay *(17)*. When analyzing the lipolytic activity of partially purified native lipase preparations from a microorganism secreting different isoenzymes with different substrate specificities, significant differences in measured activities may be observed *(18)*.

Generally, lipase activity is tested on triacylglycerols composed of long-chain fatty acids, with the most common substrate being emulsions of triolein or purified olive oil *(19)*. Lipolytic activity is correlated to the generated fatty acids in the hydrolysis of the triacylglycerol detected by titration with an absolute control of pH. However, titrimetric methods tend to be highly time-consuming. In addition, lipase reaction rate varies directly with the surface area of the emulsified substrate used.

Chromogenic substrates such as *p*-nitrophenol derivatives (generally lauric or palmitic) can also be used. However, their application is under

criticism because these compounds are not considered specific for lipase since they can also be hydrolyzed by esterases *(20)*. The use of triacylglycerols or *p*-nitrophenol derivatives composed of short-chain fatty acids (butyric, propionic, acetic) presents the same problem.

A recent alternative is the colorimetric substrate 1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-resorufin ester, which is marketed by Roche as a specific lipase chromogenic substrate *(21)*. Other alternatives have been described in the literature *(1,21)*.

Overall, comparison of lipolytic activities is very difficult because the use of different emulsified substrates, assay conditions, and techniques results in different interfacial area, which greatly affects the enzymatic activity.

Source of Lipases

Pancreatic lipases obtained from pig and human pancreas are the best known and most often investigated of all lipolytic enzymes. Lipases can also be obtained from higher plants such as castor bean and rapeseed. Nevertheless, they are found more abundantly in microbial flora comprising bacteria, filamentous fungi, and yeast *(1,5,22–24)*.

Although practically all microorganisms are potential lipase producers, White and White *(25)* reported 34 commercially available triacylglycerol lipases from 34 different sources, including 18 from fungi and 7 from bacteria. Among them, *Candida antarctica*, *Thermomyces lanuginosus* (formerly named *Humicula lanuginosa*), *Rhizomucor miehei*, *Rhizopus arrhizus*, *Pseudomonas alcaligenes*, *Pseudomonas mendocina*, *Burkholderia cepacia* (formerly known as *Pseudomonas cepacia*), *Chromobacterium viscosum*, *Geotrichum candidum*, and *Candida rugosa* are the most widely used in biotechnology *(2)*.

C. rugosa has been called the most frequently used organism for lipase synthesis *(6)*. Currently, *C. rugosa* lipase is commercialized by a number of companies (e.g., Amano, BDH, Biocatalysts, Roche [formerly Boehringer Mannheim], Fluka, Genzyme, Meyto-Sangyo, Sigma).

Candida rugosa

C. rugosa (formerly *Candida cylindracea*) is an imperfect hemiascomycetous nonsporogenic, pseudofilamentous, unicellular, and nonpathogenic yeast. *C. rugosa* has generally regarded as safe status, and no adverse effect on human or other forms of life as a result of traditional or even open fermentation practices has been reported. The most widely used wild-type strain for both genetic and production studies is ATCC 14830, which is also a type strain for this organism.

C. rugosa lipase Gene Family

C. rugosa ATCC 14830 produces several closely related lipases. In 1990, Kawaguchi and Honda *(26)* were the first to report the nucleotide sequence

Table 1
Lipases Predicted on Basis of Nucleotide as Compared with Native and Recombinant Lipases Isolated from Commercial and Noncommercial Source Sequences[a]

Gene	Mol wt (kDa)	p*I*	N-glycosylation sites: No.	N-glycosylation sites: Residues	N-terminal sequence (mature protein)
lip1	57.223	4.50	3	291, 314, 351	APTATLANGDTITGLNAIINEA
lip2	57.744	4.90	1	351	APTATLANGDTITGLNAI**V**NE**K**
lip3	57.291	5.10	3	291, 314, 351	APTA**K**LANGDTITGLNAIINEA
lip4	57.051	5.70	1	351	APTATLANGDTITGLNAIINEA
lip5	56.957	5.50	3	291, 314, 351	APTATLANGDTITGLNAIINEA

Protein	Mol wt (kDa)	p*I*	Degree of glycosylation (%)	N-terminal sequence	Source	Ref.
Lip3 (LipA)	60.0	5.50	8 ± 3	APTA**K**LANGD	Sigma	*18*
Lip1, -4, or -5 (LipB)	60.0		3.6 ± 0.8	APTATLANGD		
4 isoforms		4.80 4.84 4.95 5.04				
2 isoforms		4.70 4.80	2.5	APTATLANGDTITGLNAI**I** (identical peptide maps)	Sigma	*35*
Lip1 (major isoform)	ND	4.40	ND	(crystal structure determination)	Sigma	*41*
Lip3 (separation of mono- and dimeric forms)	ND	ND	ND	(crystal structure determination)	Roche (formerly Boehringer Mannheim); commercialized as cholesterol esterase from *C. cylindracea*	*42*

Lip1 (2 isoforms: CEL1 and CEL2)	60.0	ND	5.0	APTATLANGDTITGLNAI**I**N (identical peptide maps, N-termini of peptides sequenced)	Sigma	*30*
Lip2 (CEL2)	58.0	ND	1.7	APTATLANGDTITGLNAIVN		
Lip2	57.7	ND	3.5	APTATLANGDTITGLNAI**V**NE**K** APTA**K**L	*C. rugosa* ATCC14830 grown in oleic acid	*31*
Lip3	62.0		5.3			
rLip1	60.0	3.90 4.00	5.0		*P. pastoris*	*150*
rLip1	70.0	ND	18.5		*C. maltosa*	*34*
rLip4	57.0	ND	0		*E. coli*	*46*

[a]Adapted from ref. *28*. ND, not determined.

of a lipase gene of a mutant derived from this strain. In the following years, the genes for five of such lipases (*lip1* to *lip5*) were identified in strain ATCC 14830 and sequenced *(27)*. All of these genes code for 534 amino acid–long proteins with mol wts of approx 60 kDa. Their amino acid sequences show 66% amino acid identity and 84% similarity among themselves; the lipase isoforms differ in terms of predicted p*I* and degree of glycosylation *(28)* (Table 1). Two additional sequences (*lip6* and *lip7*) with unique restriction patterns were amplified by polymerase chain reaction (PCR) using consensus oligonucleotide primers designed based on the DNA sequences of *lip1* to *lip5* genes *(29)*. However, the putative *lip6* and *lip7* genes have not been fully isolated and characterized.

C. rugosa lipases (CRLs) share weak sequence similarities to other lipases, with the exception of those from *G. candidum*, with which they share 40–44% identity over 544 amino acids aligned. In addition, they have significant similarity to acetylcholinesterases from *Torpedo californica* and *Torpedo marmorata* (32% identity over a 475–amino acid overlap).

Multiplicity of extracellular lipases in *C. rugosa* has been attributed to a change in gene expression, heteroglycosylation, or other posttranslational modifications. The first evidence of the expression of multiple *lip* genes was inferred from the comparison of restriction maps from CRL cDNAs *(26)*. In addition, three different Lip isoenzymes have been identified in different native CRL preparations: Lip1, Lip2, and Lip3 (Table 1) *(18,30,31,32)*. More recently, studies using the reverse transcriptase (RT)-PCR technique have proved the functional expression of the five *lip* genes *(33)*, as discussed later. The regulatory sequences found upstream of *lip* genes have not been extensively characterized. Analysis of 5' regions of *lip1* to *lip5* genes showed the presence of TATAA and CAAT boxes enclosed in conserved sequence regions *(27)*. CAAT and TATAA boxes are implicated in the initiation of transcription in eukaryotes. Upstream sequences have also been analyzed for the presence of upstream activation sequences such as the oleate response element (ORE). This element also exists in the upstream regions of genes encoding enzymes relating to the β-oxidation pathway and fatty acid metabolism, and proteins relating to peroxisomal biogenesis. However, ORE sequences are quite degenerate and their identification upstream of the *lip* genes is not completely unambiguous. Nevertheless, it can be concluded than some *lip* genes are preceded by sequences that match the ORE consensus motif better than others do *(34)*.

Besides the fact that multiple *lip* genes are expressed in *C. rugosa* depending on the physiologic conditions *(33)*, there is some evidence suggesting that the heterogeneity of Lip forms secreted by this organism may also be partially owing to heteroglycosylation of a given isoenzyme *(31,35)*.

Similarly, the multiplicity of extracellular lipases in other fungi has been attributed to multiple factors, including a change in gene expression, variable glycosylation, partial proteolysis, or other posttranslational modifications *(36–40)*.

Three-Dimensional Structure of CRLs: Studies on Role of Glycosylation on CRL Catalysis

The crystal structures of two CRL isoforms, Lip1 *(41)* and Lip3 *(42)*, have been determined (see ref. *43* for a recent review). These two lipases share 88% sequence identity, yet they show some differences in their substrate specificity *(18)*.

CRLs belong to a large protein family that contains mostly lipases/esterases. This family has a characteristic fold called the α/β-hydrolase fold that comprises a central mostly parallel β-sheet with several helices on both sides of the sheet. The structures of the two lipases Lip1 and Lip3 are very similar. The active site of CRLs is composed of residues Ser209, His449, and Glu341, which form a catalytic triad similar in arrangement to those of serine proteases. CRL has been crystallized in the presence and absence of inhibitors under several conditions and its structure determined in all these states. These structures revealed that the enzyme undergoes a conformational rearrangement that involves a single, 26-residue-long surface loop (lid). This loop extends over the active site and sequesters it from the solution environment ("closed state" conformation). On a change in external conditions (substrate, inhibitors, or a less polar solvent), the loop swings to the side leading to the "open state" conformation. This rearrangement is accompanied by profound changes in the surface characteristics in the vicinity of the active site. Several hydrophobic side chains, which in the closed state reside under the loop, and those on the loop's internal side, now become largely exposed to the solvent, and to the lipase natural substrate (water/lipid interface) *(43)*. The carbohydrate attached at Asn351, whose position is conserved in all five lipase isoenzymes, provides stabilization for the open conformation of the flap *(44)*. Recent site-directed mutagenesis studies have provided experimental evidence of the importance of glycosidic chains in the activation of Lip1; that is, an Asn351Gln Lip1 mutant has a severe negative effect on enzyme activity. Introduction of a Gln to replace Asn351 is likely to disrupt a stabilizing interaction between the sugar chain and the residues of the inner side of the lid in the enzyme-active conformation *(45)*.

Tang et al. *(46)* have reported the expression in *Escherichia coli* (i.e., deglycosylated) of a functional Lip4. The specific activity of the purified recombinant Lip4 was not compared with that of a purified native Lip4. Nevertheless, recombinant Lip4 had specific activities (using triolein or olive oil), 15–30% of those of a commercial crude CRL preparation (Sigma), whereas for tributynin, the activity of Lip4 was only one-seventh that of the commercial preparation. This would be in agreement with the studies on deglycosylated Lip1 mutants *(45)*. Both Lip1 and Lip3 have three putative glycosylation sites at Asn291, -314, and -351, whereas Lip 4 has only one (Asn351). In Lip 1, only Asn314 and -351 seem to be glycosylated *(45)*.

Given the relevance of glycosylation in CRL function, further characterization of the role of glycosylation in lipase catalysis, and a glycosylation

pattern characterization of the native lipase isoforms and the corresponding recombinant isoforms in relation to their performance as catalysts, will be crucial in the application of these enzymes in biotransformations. In addition, studies on how culture conditions may affect protein glycosylation in *C. rugosa* and other heterologous hosts will have to be taken into account. Thus, simple and reliable methods to assess and monitor the degree and heterogeneity of glycosylation of CRLs produced under different expression systems and fermentation conditions are particularly relevant in subsequent biotechnologic applications of these enzymes (e.g., production of fine chemicals).

The three-dimensional structure of Lip1 and other lipases has helped researchers to understand its kinetic properties (e.g., the two lipase conformations probably correspond to states at opposite ends of the pathway of interfacial activation), including the properties shared by many lipases and esterases, namely common enantiopreference in many chiral reactions catalyzed by these enzymes.

Physiology of Lipase Production

Culture Growth Conditions

C. rugosa fermentations have been performed mainly in submerged cultures. The fermentation temperature is up to 30°C and the pH is maintained between 5.5 and 7.0 *(47,48)* or even under noncontrolled pH *(49)*. Tsujisaka et al. *(50)* have shown that a shortage of oxygen in the fermentation broth restricts lipase production by *G. candidum*. This was also observed in *C. rugosa* lipase production *(51,52)* where a set point of 20% saturation of dissolved oxygen concentration was enough to ensure lipase production and to avoid formation of byproducts such as ethanol. The use of 100% dissolved oxygen saturation resulted in an extremely long lag phase *(53)*, probably because an excess of dissolved oxygen limited cell growth *(54)*.

Carbon Sources

The carbon sources initially tested to obtain lipolytic enzymes from microorganisms were a mix of an easily assimilable substrate to support growth (e.g., glucose, sucrose), and a lipidic or related substance as inducer of the lipase production. Natural oils, fatty acids, fatty esters, sterols, bile salts, Tween, and Span were some of the most common inducers used early in the *C. rugosa* system *(55)*. However, many of the screening experiments were made in shake flasks, where culture conditions are poorly controlled. Furthermore, some of these inducer substrates may interfere with the lipolytic activity assay *(17)*, making the interpretation of shake-flask screening experiments difficult. Hence, these experiments must be considered as preliminary.

A range of different carbon sources, mainly carbohydrates, alcohols, acids, and lipids, have been screened for their capacity to support growth

and lipase production by *C. rugosa*. Some substrates, typically carbohydrates and acids nonrelated to fats, support high growth, but very low lipase production is usually obtained *(55)*.

It has been shown that among lipidic substances, fatty acids are the most effective inducers. Studies using different fatty acids showed that fatty acids with an odd number of carbons did not induce enzyme production *(56)*. Although caproic (C_6) and caprylic (C_8) acids were better inducers than oleic acid ($C_{18:1}$), they had a toxic effect on microbial growth at concentrations above 0.5–1 g/L. Fatty acids with a number of carbons lower than six did not have any effect on lipase production *(56)*. Palmitic acid (C_{16}) increased the level of lipolytic activity detected and promoted high growth; however, its use had operational problems because of its insolubility *(57)*. The solubility of fatty acids in water must be taken into account in addition to their concentration in the culture medium. In general, fatty acids with higher solubility support higher cell growth but lower or no lipase production. Overall, among fatty acids, oleic acid has been reported as one of the best inducers of lipase production *(56)*.

The effect of different vegetable oils on secretion of lipase from *C. rugosa* has also been studied. Production increased when the relative percentage of C18:n fatty acid esters in the respective vegetable oils was increased, showing the importance of these substances in the synthesis and secretion of the enzyme *(47)*. Among vegetable oils, olive oil has also been referred as one of the best inducers of lipase production *(53,58)*.

To elucidate some aspects of the induction effect of lipid-related substrates on lipase production, mixed carbon sources consisting of a soluble compound selected for its growth-promoting capacity and a fatty acid selected as inducer of the enzyme production were used *(57)*. This strategy should have made it possible for the microorganism to use both substrates in a sequential or simultaneous way, depending on its metabolism. However, no significant improvement was obtained when compared with previous results in which a sole lipid-related substrate was used. Although biomass production was higher in most cases, no increase in lipolytic activity was observed. This suggested a possible competing effect of some soluble carbon sources or a close relation between extracellular lipase activity production and consumption of fatty acids. Increased activity relative to biomass was observed when sodium lactate, oleic, palmitic, or stearic acids were used. This could indicate a possible no-repressor effect of these carbon sources *(57)*.

When glucose and oleic acid were used as a mixed carbon source, they were consumed in a sequential pattern, and it was also shown that lipase production is repressed by the glucose present in the defined medium *(57)*. These results agree with those of Nahas *(58)* for *Rhizopus oligosporus*, Baillargeon et al. *(59)* for *G. candidum*, Rapp *(60)* for *Fusarium*, and Lotti et al. *(61)* for *C. rugosa*, but differ from those obtained for *C. rugosa* by Chang et al. *(62)*, who did not report a repressive effect of glucose.

Nitrogen Sources

Different forms of nitrogen from inorganic and organic sources have been used for the production of lipases, especially ammonium sulfate and urea. In batch cultures, no differences were observed in terms of biomass yield and lipase production when these nitrogen sources were used in an excess of three- to fourfold of the minimum stoichiometrically necessary, as calculated from the microorganism elemental composition *(51)*. However, in continuous culture, urea and oleic acid at a concentration of 8 g/L and an agitation rate of 500 rpm produced a very stable emulsion after 20 h. The formation of this emulsion drastically reduced the accessibility of urea to the microorganism, and, hence, lipase production was suppressed. Changes in cell morphology and elemental composition of the microorganism were detected. The formation of emulsion was not observed when ammonium sulfate was used as nitrogen source *(63)*.

Wang et al. *(52)* worked in batch mode testing different strategies such as the utilization of different carbon sources, nitrogen limitation, or pure oxygen supply. When olive oil was used together with glucose and complex nitrogen sources (peptone, yeast extract, and malt extract), the lipolytic activity was higher than when using olive oil and urea as the sole carbon and nitrogen sources, respectively.

The metabolism of nitrogen in *Candida* sp. has been little studied. Gradova et al. *(64)* investigated the kinetics of ammonium transport and specific features of regulation as an aspect of catabolite nitrogen repression.

Other common components of defined media are phosphate and sulfate salts, as well as micronutrients *(57,65)*. In addition, a nondefined medium using yeast and malt extract has been used *(52,66)*.

Regulation of Lipase Synthesis

The mechanisms regulating lipase biosynthesis vary widely in different microorganisms. Results obtained with *Calvatia (67)*, *Rhizopus (68)*, *Aspergillus (69)*, and *Rhodotorula (70)* showed that lipase production seems to be constitutive and independent of the addition of lipidic substrates, although their presence enhanced the level of lipase activity produced. By contrast, it has been suggested that long-chain fatty acids such as oleic acid participate in the expression of lipase genes in *G. candidum*, by controlling induction at the level of transcription *(71)*. Carbohydrates have been described to act as repressors of lipase biosynthesis in *Fusarium* sp. *(60)*.

The synthesis and secretion of lipases in *C. rugosa* have been studied with carbon sources that are known to affect the production of lipase in two opposite ways: glucose (repressor) and oleic acid (inducer). In these studies, lipase production was monitored both by enzyme activity and by immunodetection with specific antibodies. However, the use of polyclonal antibodies did not allow distinguishing among isoenzymes since all CRL proteins display similar surface epitopes. These studies showed that, according to their regulation, lipase-encoding genes might be grouped in

two classes, one of which is constitutively expressed and the other is induced by fatty acids. The synthesis of inducible enzymes is inhibited at the level of transcription by the addition of glucose, and, conversely, oleic acid appears to hinder the synthesis of the constitutive lipase *(61)*. These results are strongly suggestive of the existence of a complex pattern of regulation controlling gene expression in which different genes may be switched on and off according to the composition of the medium *(61)*.

Growth conditions on oleic acid supporting high-level expression in both batch and continuous culture give rise to the intracellular accumulation of enzyme, possibly owing to the existence of a rate-limiting step in the transport of the newly synthesized protein related to the crossing of the cell wall *(61)*. This intracellular accumulation was related to microbial growth. High specific growth rates led to high intracellular accumulation of lipase. The addition of glucose after oleic acid consumption stopped lipase synthesis but allowed for the secretion of the intracellular lipase previously secreted to the extracellular medium *(57)*.

Recently, the use of the competitive RT-PCR technique has been demonstrated as a feasible alternative to obtain quantitative information on the expression levels of *lip* genes at the transcriptional level owing to its high sensitivity and specificity *(33)*. In that study, a differential transcriptional control of *lip* genes was shown to result in multiple profiles of lipase proteins in strain ATCC 14380. In particular, *lip1* and *lip3* were shown to be highly and constitutively expressed, whereas the other inducible genes showed significant changes in mRNA expression under different culture conditions (i.e., different carbon sources). The abundance of lip mRNAs was found to be (in decreasing order) *lip1*, *lip3*, *lip2*, *lip5*, and *lip4*. This is in agreement with the fact that only Lip1, Lip2, and Lip3 have been identified and purified from native CRL preparations. However, Lip2 and Lip3 were detected in commercial (Sigma) preparations only in small amounts *(18,30)*, and Sánchez et al. *(31)* reported Lip2 and Lip3, but not Lip1, as the major isoforms secreted by *C. rugosa* in pilot plant fed-batch fermentation growing on oleic acid. These results seem to indicate that the expression profile of *C. rugosa* ATCC 14380 can also be altered by different culture conditions besides the inducer carbon source. Moreover, mutant *C. rugosa* strains used for industrial production of native CRLs may secrete altered isoenzyme profiles, as discussed later *(26)*.

These studies clearly show that different inducers may change the expression profile of individual genes. A differential transcriptional control of *lip* genes had been previously suggested from several studies on the relationship between culture conditions of *C. rugosa* and the lipase/esterase profiles secreted by this organism *(48,61,62,72)*.

Since Lip isoenzymes seem to have differences in their catalytic properties *(18,30,46)*, the production of different lipase isoforms by *C. rugosa* growing under different growth conditions may be physiologically significant for this organism, enabling it to grow under different environments.

Such multicomponent gene families have been observed in other yeasts. For instance, in *G. candidum*, two lipase genes have been reported to be expressed under different growth conditions and to produce isoenzymes differing in their substrate specificities *(73,74)*. Furthermore, there is some evidence suggesting that *Yarrowia lipolytica* may have a multigene lipase family *(75)*.

Cell Morphology

It has been described that oleaginous yeasts accumulate lipids when there is a deficiency of a particular nutrient, normally nitrogen, and an excess of carbon sources. Thus, when the limiting nutrient is exhausted cell proliferation ceases, and the excess of carbon source continues to be assimilated by the cell and channeled into lipids *(76)*. This could explain the lipidic accumulations observed when a reduced N:C source ratio is provided to the microorganism, and, as a consequence, the nitrogen percentage in the chemical formulae of the microorganism decreases *(53,63)*.

On the other hand, a typical feature of the yeast *C. rugosa* is the formation of pseudohyphae. Both shape and length of the cells, organized in chains, change according to culture conditions. For example, when some oxygen shortage was detected, the pseudohyphaes were very long *(53)*. This fact can be very useful as a control factor indicating the limiting conditions of system aeration. Cells grown on glucose were more rounded and they formed short chains of four to five cells. Additionally, large vacuoles were observed in the interior of the cells. In the earlier phase of cultivations, cells were arranged in longer chains forming aggregates with attached droplets of olive oil. When droplets of olive oil were consumed, e.g., in the stationary phase, the cells were predominantly not aggregated, and intracellular lipidic accumulations were observed. A similar behavior was observed when oleic acid was used as the sole carbon source; that is, long cell chains corresponding to the formation of pseudohyphae were observed (Fig. 1A). When saccharides, oxidized acids, or alcohols were used as a carbon source, or there were nitrogen-limiting conditions or operational problems (formation of emulsion), shape and length of the cells changed and they became predominantly rounded or ellipsoidal *(63)* (Fig. 1B). When this morphology was present, lipase production ceased. Changes in conditions of bioreactor operation involving different rheologic properties (e.g., conditions of aeration and agitation, baffle configuration) may lead to unfavorable formation of emulsion or changes in the drop size of the organic substrate. This provokes an evolution in the morphology of the cells from pseudophypae (lipase producers) to individual ellipsoidal cells *(77)*.

Improved *C. rugosa* Strains for Industrial Production of Native CRLs

Several patents describe the production of lipases from wild-type *C. rugosa* cultured on different carbon sources (e.g., kerosene, polyoxyethylene sorbitan monoesterate, *n*-paraffin, or starch) *(78–80)* in batch

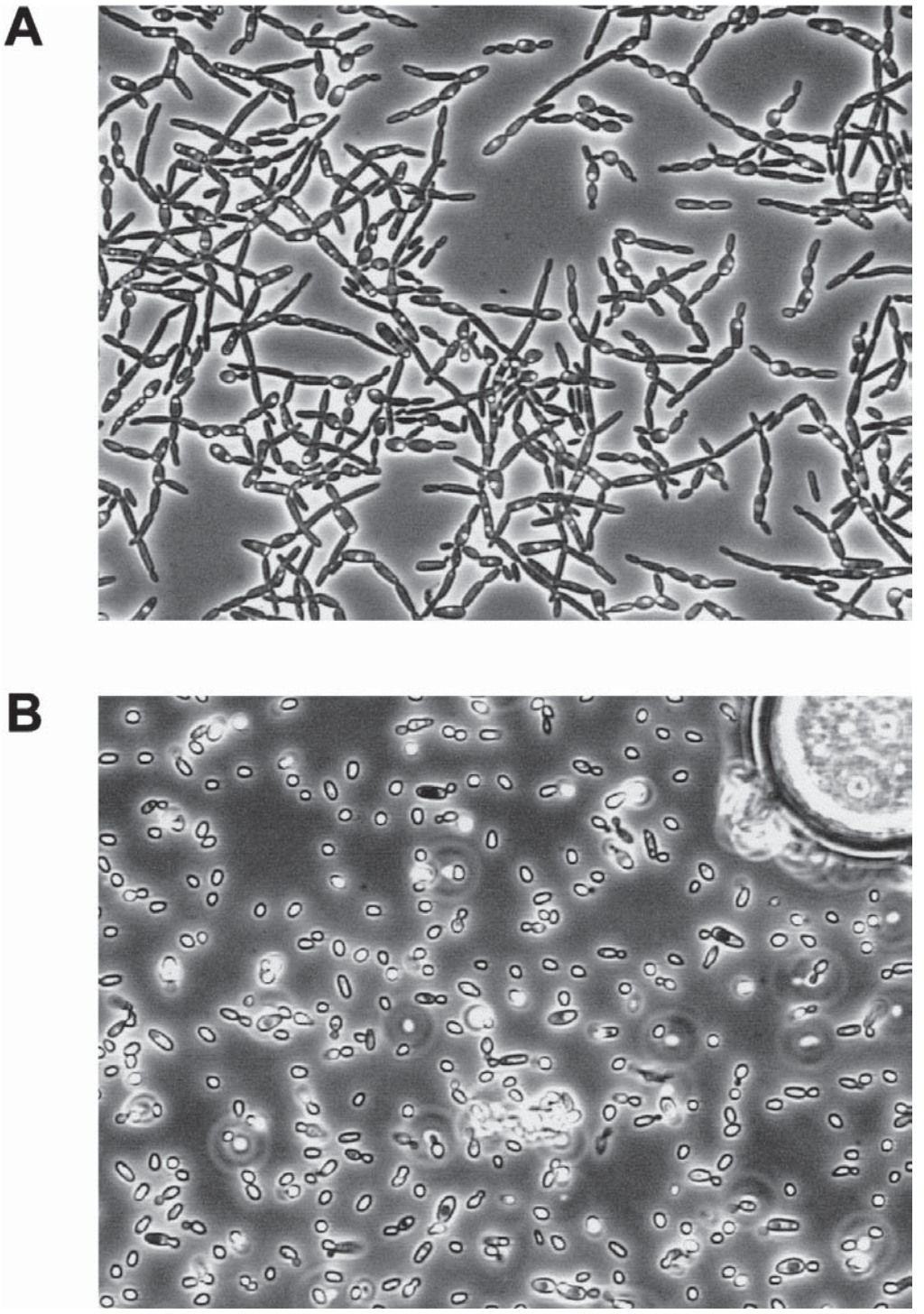

Fig. 1. **(A)** *C. rugosa* cells growing on oleic acid as sole carbon source under optimal operation condition. **(B)** *C. rugosa* cells growing on oleic acid as sole carbon source under operational problems.

or fed-batch cultures. In addition, deregulated mutants derived from the same strain may be used for CRL production *(26,81–84)*. Since regulatory elements among *C. rugosa lip* genes have been found to be very complex, such mutations could also have resulted in altered lipase isoenzyme profiles with modified substrate specificities. For instance, Kawaguchi and Honda *(26)* from Meito-Sangyo reported a *C. rugosa* MS-5 mutant strain (derived from ATCC 14830 strain) that produced a large amount of Lip I and a negligible amount of Lip II isoenzyme compared with the wild-type strain, when growing in soybean oil. cDNAs with different restriction maps were isolated. The most abundant cDNA specie (22 of 24 clones isolated from a cDNA library) corresponded to Lip1 (i.e., Lip I could be assigned to Lip1).

Native CRL Production: Fermentation Technology

The difficulty of monitoring and controlling key variables and process parameters (e.g., immiscible substrate concentration), together with the complexity of the induction mechanisms of lipase production, implies that industrial processes for lipases production by *C. rugosa* are often based on empirical procedures. Additionally, culture media used in industrial processes are often undefined; that is, they come from renewable sources with variable composition. Therefore, to guarantee the quality and quantity of the enzyme produced, empirical procedures currently used must be substituted for a stricter monitoring and control of the main parameters and variables involved in microbial lipase production. This is particularly relevant for native CRL production because of the complexity of the system.

Monitoring and Control

Monitoring and control of the CRL production process is not an easy task to be performed. Production of the enzyme is carried out under aerobic conditions. Therefore, a highly dynamic system of four phases—biomass, gas phase, aqueous phase, and organic phase (insoluble carbon source inducer)—is required. On-line analysis of some of the main variables of the system such as biomass, substrate, and product is not easily available.

There are three different ways to enhance the operative procedures in biotechnologic processes, which have been developed simultaneously. The first one is to increase the number of sensing devices available *(85,86)*. Accordingly, an on-line lipolytic activity analyzer, which used triolein as substrate, was developed and applied to lipase production *(87)*. This analyzer developed for the determination of the lipolytic activity present in the aqueous phase has been improved by changing the substrate of the catalytic reaction, reducing the volume of both reagents and sample, and using a sequential injection analyzer *(88)*. An important interfacial lipase adsorption-desorption phenomenon was observed between the aqueous and organic phases in the culture broth. Therefore, the lipolytic activity levels detected in the presence of organic interfaces were lower than the actual

levels excreted by the microorganism. Nevertheless, the determination of the adsorption law of lipase to organic phase allowed for the determination of the total extracellular lipolytic activity *(89)*.

On-line measurements from off-gas analysis, such as carbon dioxide evolution rate (CER), oxygen uptake rate (OUR) and respiratory quotient (RQ), can be made with a high degree of accuracy *(90–92)*. These measurements may give indirect valuable information about the physiologic state of the cells. Mass spectrometry to obtain CER, OUR, and RQ data have been applied in *C. rugosa* cultures *(93,94)*.

However, there is no standard method for direct on-line measurement of biomass and substrate, because of the tetraphasic nature of the culture broth. Secondly, indirect measurement or estimation of variables and parameters, which constitutes the so-called software sensors, is an attractive alternative *(95)*. This approach uses several mathematical techniques, such as the extended Kalman filter *(96,97)*, observer-based estimators *(98–100)*, recursive prediction error *(101,102)*, and neural networks *(103–105)*.

Biomass, substrate, and specific growth rate have been estimated from on-line measurable variables such as glucose by using an extended Kalman filter *(106)*. Biomass and specific growth rate have also been estimated from measurement of CER by mass spectrometry *(94)*. These estimated variables allow the estimation of oleic acid concentration in the culture broth when considering a constant yield of biomass/substrate during the fermentation. Alternatively, substrate concentration can be estimated with a previously developed structured model of lipase production using a recursive prediction error algorithm for parameter estimation, and an adaptive observer for state estimation *(107)*. However, this methodology needs a better understanding of the biologic system and a more complex mathematical support.

Dissolved oxygen and cumulative carbon dioxide produced have been applied to estimate biomass and lipase activity by using neural networks *(52)*.

Thirdly, the use of more powerful control techniques is being applied to cope with the specific characteristics of biologic processes, such as high nonlinearity and time-dependent varying parameters *(108–114)*.

Gordillo et al. *(65)* developed and implemented a feedback control strategy for CRL production. It was based on the estimation of biomass concentration and specific growth rate from the measurement of indirect variables such as CER by means of mass spectrometry techniques. A substrate addition controller was used to maintain the specific growth rate at the desired value by adjusting the substrate feeding rate.

Reaction Schemes and Mathematical Modeling

Del Río et al. *(115)* demonstrated the diauxic growth of *C. rugosa* on olive oil that proposes a reaction scheme for lipase production. Two stages could be observed in the consumption of the olive oil: the first one was related to the glycerol depletion without lipase production, and the second one was associated with the fatty acids consumption when the enzyme

appeared in the medium. According to this observation, the initial presence of a small quantity of lipase would be sufficient to hydrolyze the triglyceride to glycerol and fatty acids. The yeast would preferably be using glycerol, which would repress the assimilation of fatty acids. Therefore, production of high levels of lipase would be associated with the consumption of fatty acids. Similar results have been obtained by Sokolovska et al. *(53)*, who used olive oil and oleic acid for lipase production. It has been observed that the uptake of oleic acid by *C. rugosa* is favored by the presence of extracellular lipases *(63)*.

Based on the observations and hypothesis just described, Serra et al. *(116)* calibrated and validated a model for lipase production on olive oil and free fatty acids in batch fermentation. The model development process helped to obtain a threefold increase in lipase production when oleic acid was used as substrate instead of the olive oil initially used. However, Sokolovska et al. *(53)* did not observe significant differences in lipase production using these substrates.

Montesinos et al. *(107)* developed a simple structured mathematical model for lipase production by *C. rugosa* in batch fermentation. The model describes the system according to the following qualitative observations and hypothesis (Fig. 2): Lipase production is induced by extracellular oleic acid present in the medium. The acid is transported into the cell, where it is consumed, transformed, and stored. Lipase is then excreted to the medium, where it is distributed between the available oil-water interface and the aqueous phase. Cell growth is modulated by the intracellular substrate concentration. Model parameters were determined in a calibration step, and then the whole model was experimentally validated with good results. This model was later modified to be applied from batch to fed-batch and continuous lipase production *(117)*. Finally, it was exploited in simulations and for the design of new operational conditions as discussed next.

Operational Strategies

Reactor technology used for the production of *C. rugosa* lipase includes various types and sizes of reactors. Operation mode ranges from standard batch cultures to continuous process, including different fed-batch strategies with different levels of complexity.

Batch fermentations have been mainly used in the CRL production processes. Gordillo et al. *(48)* studied the effect of oleic acid concentration in batch mode and obtained the maximum lipase/substrate yield and specific productivity for an initial oleic acid concentration of 2 g/L. At higher concentrations, up to 8 g/L of oleic acid, specific productivity decreased. It seems that lipase production was under substrate inhibition. This effect was also detected when olive oil was used as the carbon source *(53)*. Lipase production was not observed below 1 g/L of oleic acid.

The behavior of typical batch fermentation is shown in Fig. 3 *(118)*. The pattern of extracellular lipase production seems to be growth associated, as observed in other microorganisms *(119)*. The maximal specific

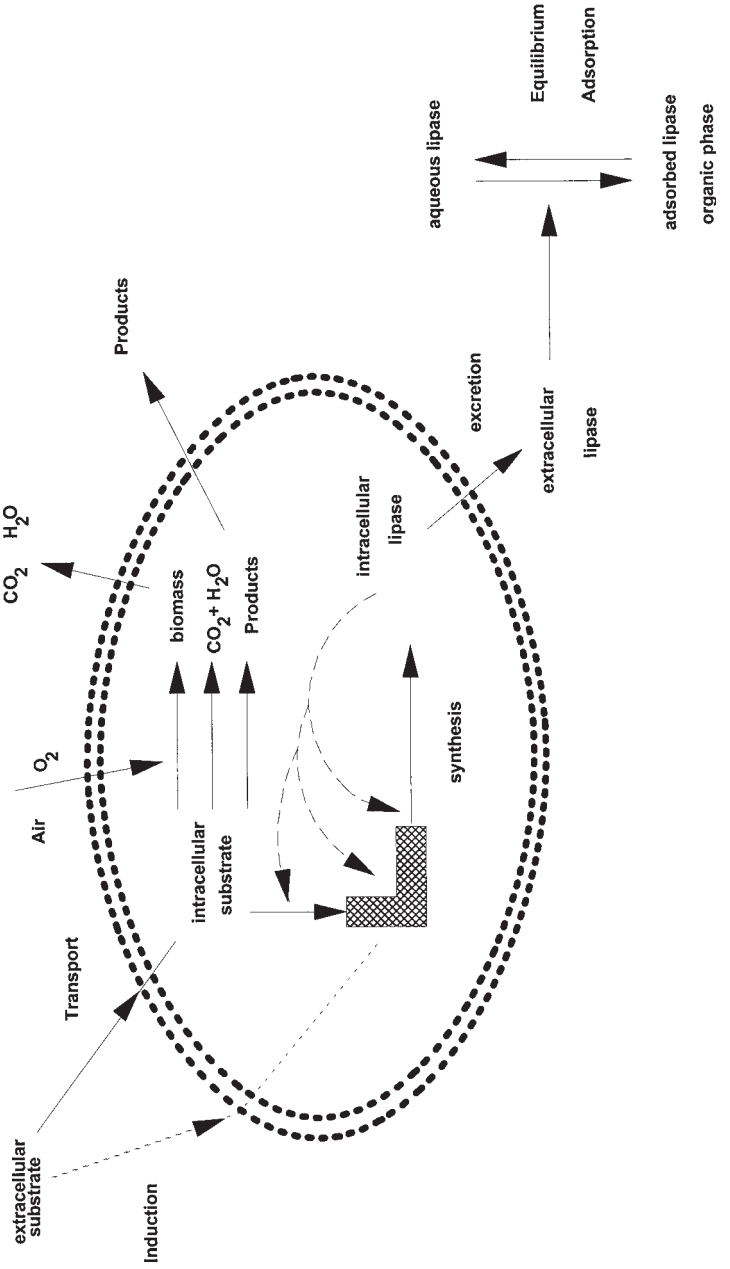

Fig. 2. Qualitative description of lipase production by *C. rugosa*.

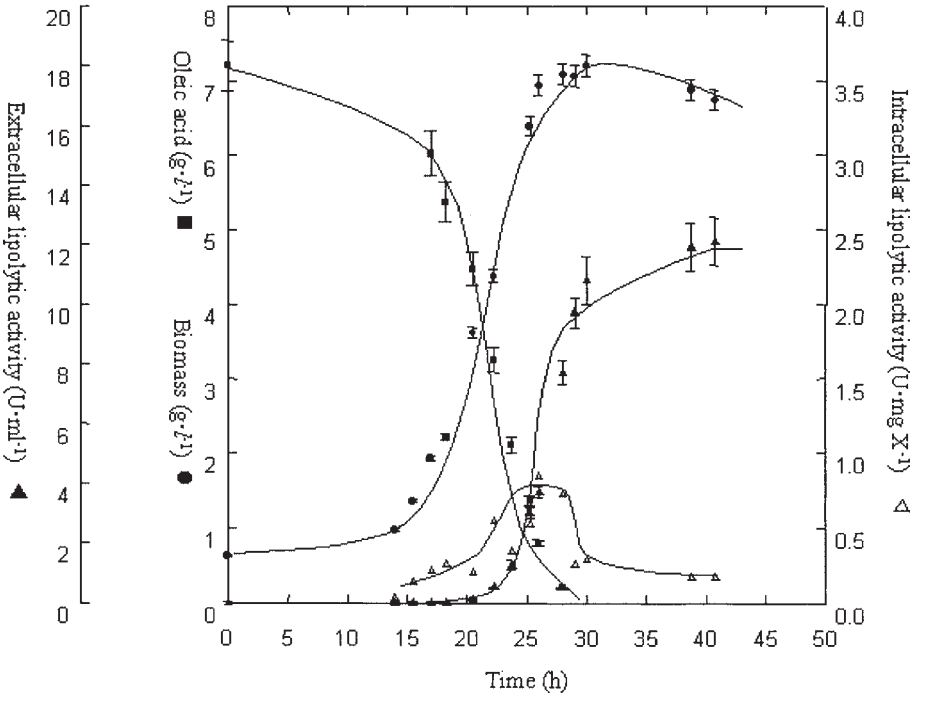

Fig. 3. Evolution of key fermentation parameters in an 8 g/L oleic acid batch fermentation by *C. rugosa*.

growth rate was 0.15 h^{-1}, similar to that reported by Sokolovska et al. *(53)* (0.20 h^{-1}). The slight delay observed between growth and extracellular enzyme detection has been attributed to a limitation in product excretion through the cell wall and to the lipase adsorption to the organic-aqueous interphase. Intracellular lipase activity is growth associated. A maximum of intracellular lipase activity is observed in the mid-exponential phase. At the end of the batch, when carbon source is depleted, intracellular lipase activity levels remain constant. Lipase synthesis stops at the beginning of the stationary phase, and the enzyme that still remains inside the cell is not longer excreted, probably owing to energy-limiting conditions *(48)*. Lipidic accumulations in the microorganisms were detected by microscopic observation during fermentation, and they gradually decreased until the stationary phase was reached.

Sokolovska et al. *(53)* investigated the influence of aeration and substrate (olive oil and oleic acid) in batch fermentation. They found that air enriched with pure oxygen appeared to be most suitable for lipase production. Additionally, they found that the presence of fats in the culture broth did not affect the volumetric mass transfer coefficient of oxygen in the system.

Continuous production of lipase is a complex process because of the tetraphasic nature of the culture broth *(63)*. Continuous experiments with oleic acid as substrate were conducted at dilution rates from 0.03 to 0.15 h^{-1},

and the effective maximal specific growth rate was about this latter value *(117)*. It was found that high lipase concentrations were obtained at low dilution rates, since intracellular accumulation of lipase takes place at higher dilution rates. Maximum productivity was obtained at intermediate dilution rates, owing to the limiting factor mentioned before.

Batch bioreactors account for the bulk of all industrial production. However, fed-batch cultures are increasingly implemented in industrial processes since they have several advantages over batch and continuous operations. However, control schemes of fed-batch processes are faced with several difficulties in maintaining favorable conditions in a continuously changing environment within the reactor. Montesinos et al. *(117)* simulated the best conditions (both mode and strategy of operation) to produce lipase. They found that best lipase productivity was obtained in continuous culture, whereas the highest predicted lipase activity was obtained in fed-batch cultures with a prefixed substrate-feeding rate, either in terms of substrate/biomass ratio or of specific growth rate.

Thereafter Gordillo et al. *(65)* worked on two different fed-batch operational strategies. A constant substrate feeding strategy showed that maximum aqueous lipolytic activity was reached at low substrate feeding rates, whereas lipases tended to accumulate inside the cell at higher rates of substrate addition. In the constant specific growth rate strategy, a feedback control strategy was developed based on the parameter and state estimation from the measurement of CER by means of mass spectrometry techniques *(94)*. Higher levels of aqueous lipolytic activity at low specific growth rates were observed. By using these fed-batch strategies, lipase production was enhanced 10-fold compared to a batch operation.

The results of a comparative study on both operational mode and strategy based on experimental data of *C. rugosa* fermentation growing on oleic acid are given in Table 2 *(65)*. Productivity and specific productivity were calculated in batch cultures. These calculations were based only on the exponential growth phase, to avoid the influence of different inocula between batches. In fed-batch cultures, productivity was calculated using the time of oleic acid addition. Best results in terms of specific productivity were obtained in continuous operation, whereas maximum productivity and maximum lipase production were obtained in controlled fed-batch operation at constant specific growth rate. Mass balances performed throughout the process indicated that lipase represents about 20% of the total protein excreted at maximum fed-batch production conditions, which corresponds to 30 mg/L in the culture broth.

Stability of Lipase in Submerged Cultures

Deactivation owing to the surface's forces on the gas/liquid interface, or owing to shear stress at typical fermentation conditions, was not observed *(48)*. Moreover, lipases secreted by *C. rugosa* seem to be significantly stable in terms of proteolytic degradation. No extracellular protease activity has been detected or described in *C. rugosa* cultures under lipase-

Table 2
Comparison of Main Results Obtained for Both Different Operational Mode and Strategies of *C. rugosa* Fermentation Growing on Oleic Acid[a]

	Extracellular lipolytic activity (U/mL)	$Y_{P/S}$ (U/[mL·mg])	$Y_{P/X}$ (U/[mL·mg])	Productivity (U/[mL·h])	Specific productivity (U/[mgX·h])
Batch (8 g/L)	12.1	1.5	1.8	0.80	0.09
Batch (4 g/L)	10.5	2.6	3.1	0.80	0.20
Batch (2 g/L)	7.2	3.6	4.2	0.72	0.35
Batch (1 g/L)	1.8	1.8	1.8	0.25	0.22
Batch (0.5 g/L)	0.21	0.4	0.3	0.04	0.03
Continuous ($0.025\ h^{-1}$)	27.0	13.5	13.5	0.68	1.68
Continuous ($0.08\ h^{-1}$)	10.0	5.0	5.0	0.88	2.00
Constant feeding ($0.2\ g/[h^{-1}\cdot L]$)	55.0	6.4	12.0	1.60	0.35
Constant feeding ($0.4\ g/[h^{-1}\cdot L]$)	58.0	6.9	9.8	3.25	0.55
Constant feeding ($0.68\ g/[h^{-1}\cdot L]$)	33.2	2.5	3.4	2.21	0.23
Constant μ ($0.015\ h^{-1}$)	117.0	12.0	22.2	1.70	0.32
Constant μ ($0.04\ h^{-1}$)	94.1	11.6	21.3	5.10	1.13
Constant μ ($0.08\ h^{-1}$)	52.5	7.0	11.5	4.03	0.86

[a]$Y_{P/S}$, lipase to substrate yield; $Y_{P/X}$, lipase to biomass yield.

producing conditions. This also facilitates the subsequent downstream processing of native CRLs.

Solid-State Fermentation

Solid-state fermentation offers several advantages over liquid fermentation, including higher product titers, simpler media, concentrated product, easier isolation techniques, and minimal generation of liquid waste *(5)*. Although solid-state fermentation has been used in the production of several industrial enzymes such α-amylase, cellulase, and β-glucosidase, very few reports are available on the production of *C. rugosa* lipase by solid-state fermentation. Rao et al. *(120,121)* studied the most important variables affecting lipase production using rice bran as a solid substrate *(120,121)*. Although they reported that the maximum activity obtained was higher than in submerged cultures, the different lipase analysis and units used in both cultures greatly invalidate the comparison. Coconut oil cake has also been used as a solid substrate with good performance *(122)*. Nevertheless, the use of solid-state fermentation for the production of lipases is still far from being applied on an industrial scale.

Immobilized Cell Cultures

The immobilization of *C. rugosa* cells on a solid support for industrial production of lipase could offer several advantages, such as improvement of microbial cell stability, higher dilution rates without culture washout, and facilitation of continuous operation in a bioreactor. Both the process control and downstream operations are significantly simplified when compared with conventional batch processing.

Ca-alginate gels and a range of matrices with different Ca-alginate/polyurethane foam ratios have been tested as solid supports in a fluidized-bed bioreactor in batch and continuous strategies. Mixed supports with increased polyurethane ratios did not show significant differences in terms of affinity for substrate droplets. Furthermore, cell growth and, hence, lipase production on beads appeared to be limited to their surface *(123)*. Thus, diffusion limitations of both organic substrates and products seem to be the great problem in these systems. The use of gum arabic as emulsifier agent greatly enhanced lipase production, proving that the system was strongly limited by substrate availability *(123)*. The use of other supports such as polyurethane and polyacrylamide did not improve the performance of the system, confirming the importance of support-substrate contact. Lipase production was higher in free-cell systems than in equivalent immobilized-cell systems, probably owing to strong mass transfer limitations in the latter case *(124)*. Other fermentation strategies using immobilized-cell systems did not improve the performance compared to free-cell cultures *(125)*. Overall, *C. rugosa*–immobilized cell cultures do not seem to be a promising alternative from an industrial point of view.

Scale-Up

It has been shown that dissolved oxygen concentration greatly influences lipase production in submerged cultures *(51–53)*. Therefore, oxygen transfer efficiency (in terms of k_La) is one of the main parameters to consider when scaling up the process.

As described earlier, culture media used for the production of lipases have a complex nature, frequently involving oil in water emulsions. The organic phase in the growth medium disperses, forming microscopic drops. Thus, the drop size of the disperse phase is a measure of the extent of emulsification of the system concerned, suggesting that it could be used as a key scale-up parameter. In particular, Dalmau et al. *(126)* found that by providing the same initial oleic acid drop size, referred as specific interfacial area, similar enzyme activities were obtained. This allowed the establishment of a specific criterion for adaptation to a different scale of bioreactor. Constant feeding rate fed-batch fermentations were carried out in a pilot plant bioreactor (70 L) using this criterion, and high levels of lipase activity and productivity were obtained *(127)*.

Downstream Processing of Native CRLs

Recovery of CRLs

Lipases are extracellular enzymes. Moreover, *C. rugosa* secretes a relatively small number of endogenous proteins (Fig. 4). Hence, relatively simple and efficient recovery processes of the fermentation product may be used. Sánchez et al. *(128)* reported a pilot-plant scale recovery process involving a centrifugation step followed by a microfiltration step (0.45 μm) to remove most of the biomass. The proteins from the cleared supernatant were then concentrated by ultrafiltration (10-kDa cutoff) and subsequently dialyzed against phosphate buffer (pH 7.4). Finally, the concentrate thus obtained was lyophilized without further addition of any stabilizing/preservative agent. The overall recovery yield of this process was >90% *(127)*. Many commercially available crude enzyme preparations are obtained by trichloroacetic acid precipitation of the culture supernatant *(129)*.

Batch-to-Batch Reproducibility in Native Crude CRL Preparations

Traditionally, culture conditions in fermentation are optimized for the maximal production of enzyme activity units. Catalytic applications indicate that quality is as important as quantity in enzyme preparations, since different culture conditions might result in the production of heterogeneous compositions of isoenzymes, which display different catalytic properties.

Crude enzyme preparations from various suppliers or different batches have been reported to show variations in their catalytic action and stereospecificity in several applications *(130,131)*. Chang et al. *(62)* reported that three commercial CRLs differed in protein composition, which accounted for the difference in their catalytic efficiency and specificity.

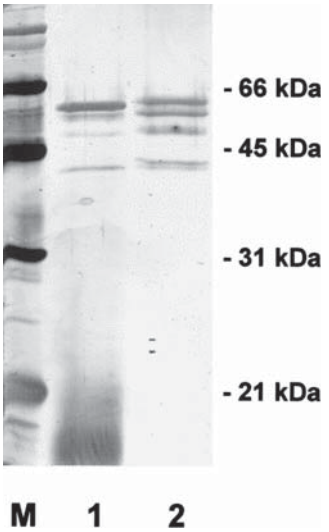

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of a commercial crude CRL preparation (Sigma), lane 1, and of CRL-UAB crude preparation (see text), lane 2. Lane M, molecular weight standards, indicated in kilodaltons; 12% acrylamide gel, silver stained.

Differences in catalytic performances of partially purified lipase forms in preparations containing different combinations/ratios of lipase isoforms have also been reported *(18,72,132)*.

Researchers working with commercial CRLs from Sigma type VII *(18)* and Biocatalyst *(72)* have shown that the specific lipolytic activity ratios of partially purified lipase fractions were similar. However, these ratios were about three times lower than those reported by Veereregavan et al. *(133)* and Brahimi-Horn et al. *(134)* working with CRL (Sigma Type VII). These differences could result from variations from batch to batch from the same supplier, but they could also be owing to the different protocols of purification used (e.g., presence of different amounts of impurities). Gordillo et al. *(65)*, producing lipases under different fed-batch strategies, obtained higher specific activities when working at a constant specific growth rate. A possible explanation for this observation may be related to differences in post-translational modifications that the protein may suffer as a function of operational strategy *(135,136)*.

Characterization of Components of Crude CRL Preparations: Lipase Isoenzyme Profiles and Coproduction of Esterases and High Molecular Weight Polysaccharides

Most of the characterization studies of CRLs have been performed using commercially available crude CRL preparations, mainly those from Sigma. In these preparations, Lip1 has been identified as the major isoenzyme (about 70% of the total lipase) *(18,30,41)*. Lip2 *(30)* and Lip3 *(18)*

have also been identified as minor isoforms in this preparation. However, commercial CRLs may have been obtained from industrial strains, which can produce altered isoenzyme profiles as a result of mutations *(26)*. In addition, in crude CRL preparations (CRL-UAB) obtained from fed-batch cultures of *C. rugosa* wild-type strain ATCC 14830 grown in synthetic medium and oleic acid as the sole carbon source *(127)*, two major lipase components of 62 and 57.7 kDa were detected *(31)*. These were identified as Lip3 and Lip2, respectively. Interestingly, Lip1, the major isoform present in Sigma crude lipase preparation, could not be identified. Lip2 and Lip3 were present in similar amounts. Furthermore, there is now clear evidence that growth conditions (e.g., carbon source) affect the lipase isoenzyme profile secreted by *C. rugosa (33)*.

Two 43-kDa esterases have also been identified in commercial (Sigma) crude CRL preparations *(137)*. This component has been identified in Sigma and CRL-UAB preparations *(31)*. The esterase components may have a significant effect in some reactions using crude CRL preparations and therefore explain some of the differences in their catalytic properties observed among different batches and partially purified lipase preparations. In addition, the presence of an esterase component in crude CRL preparations may lead to misinterpretations of the results obtained when using such preparations in biocatalysis. Interestingly, CRL-UAB and Sigma preparations had similar behavior in terms of esterase activity but were dramatically different in the case of lipolytic activity *(127)*.

A 50.4-kDa protein has also been identified in CRL-UAB preparations, and it seems to be present in Sigma CRLs as well, as observed in Fig. 4. However, its N-terminal did not show any apparent similarities to other sequences in the Swiss-Port and translated GenBank and EMBL databases, and its function/activity remains unknown *(31)*.

High molecular weight polysaccharides with emulsifying properties, which are capable of stabilizing oil-in-water emulsions, are commonly cosecreted with lipases by many lipase-producing microorganisms (e.g., the yeast *Yarrowia lipolytica*) when these are grown in the presence of water-immiscible carbon sources *(138,139)*. Similarly, a high molecular weight polysaccharide has also been detected in CRL production, which appears to be noncovalently—but tightly—bound to the lipase component *(127)*. The secreted polysaccharide/protein ratio seems to vary strongly, depending on the culture conditions (e.g., carbon source, cultivation time) and genetic background of the strain. For instance, the carbohydrate content of a crude lipase preparation from an oleic acid–grown *C. rugosa* ATCC 14830 strain was about 4 mg/mg of protein, a value clearly higher than that determined for commercial CRL preparations (e.g., Sigma; 0.63 mg/mg of protein) *(31)*.

These macromolecules may have a significant influence on the functional properties of CRLs, as described for other lipases (e.g., alteration of the substrate specificity *[140,141]*, stability *[142]*, or adsorption to solid support *[143,144]*). Additionally, the presence of carbohydrates modifies

the microenvironment of the enzyme and maintains the hydration of the biocatalyst *(128,145)*, which may affect the catalytic performance of CRLs in organic media.

Purification of CRLs

Hydrophobic interaction chromatography (HIC) has been shown to be useful as a first chromatographic step of native lipase and esterase purification from crude CRL preparations *(18,31,146)*. Recent observations have proved that CRL and other lipases strongly and selectively adsorb to hydrophobic matrixes such as octyl-agarose, a phenomenon that is simultaneous to a superactivation of these enzymes *(144)*. In addition, HIC allowed exploitation of the potentially different properties in terms of hydrophobicity between proteins and high molecular weight polysaccharides *(31)* and between lipase isoenzymes *(18,30)*. However, separation of CRL isoforms to homogeneity requires the exploitation of rather small differences in their physicochemical properties (hydrophobicity, molecular weight, and p*I*), which results in time-consuming purification protocols with very low yields not appropriated for industrial applications. For instance, purified Lip3 is commercialized by Roche (sold as cholesterol esterase from *C. cylindracea*, standardized with bovine serum albumin) for analytical purposes. Recombinant production of the CRL isoenzymes is the most promising strategy to allow for industrial production of pure CRL isoenzymes, as discussed next.

Heterologous Expression of CRLs

Commercial preparations of native CRLs are widely used in industrial applications. However, they contain several different Lip isoenzymes, as well as other components such as esterases *(18,30,31,137)*, as already discussed. Pure isoenzymes can be obtained from purification of these preparations, but yields are very low. Thus, the recombinant production of CRL isoenzymes is the most promising strategy for future industrial applications of pure/defined CRL isoenzyme preparations.

The heterologous production of different CRLs in suitable hosts allows the production of highly pure Lip isoenzymes in high yields, thus providing enzyme preparations with reproducible and defined reactivity profiles for biotransformations applications. In addition, the availability of heterologous expression systems for CRLs makes it easier to characterize different isoenzymes, which are otherwise difficult to obtain by chromatographic resolution of the various forms obtained from the natural sources. Furthermore, it allows the generation of new CRL derivatives by mutagenesis for further industrial applications as well as for studies on structure-function relationships.

C. rugosa and some other *Candida* species have an unusual codon usage in which the triplet CUG, a universal codon for leucine, is read as serine *(147)*. In this organism, the CUG triplet accounts for about 40% of the total

serine codons *(27)*. This hampers the functional expression of genes derived from this yeast in a conventional heterologous host. For instance, in the *lip1* gene, 20 of its 47 serine residues, including the catalytic Ser209, are encoded by CUG triplets. As a consequence, the heterologous expression of *lip1* in *Saccharomyces cerevisiae* resulted in an inactive lipase *(148)*. Furthermore, the exchange of most of the CUG codons by universal serine triplets (UCN, AGY) is required for the expression of a functional Lip1 protein in heterologous hosts *(149,150)*. As an alternative approach, the *lip1* gene has been completely synthesized with an optimized nucleotide sequence and subsequently functionally expressed in *S. cerevisiae* and *Pichia pastoris (150–152)*. The recombinant Lip1 expressed in *P. pastoris* had a similar degree of glycosylation as native Lip1 (Table 1). Additionally, Lip1 expression levels were 12- to 17-fold higher in *P. pastoris* than in *S. cerevisiae* shake-flask cultures *(150)*. The substrate specificity of the *P. pastoris*–produced Lip1 was compared with that of a commercial crude CRL (Sigma) using various triacylglycerides differing in acyl group chain length (C2–C18) and various methyl esters differing in acyl group chain length (C6–C18). Both recombinant and native lipase preparations showed higher lipolytic activity toward middle-chain (C8 and C10) triacylglycerides and methyl esters *(150)*.

Furthermore, this system for functional expression of Lip1 has been proven to be suitable for the expression of Lip1 mutants (e.g., deglycosylated mutants) for studies on structure-function relationships *(152)*, as well as future directed evolution/gene shuffling of *lip1*. An alternative approach to circumvent the nonuniversal use of a serine codon in heterologous expression of *lip* genes is a host with similar codon usage—*Candida maltosa (34)*. The recombinant Lip1 produced in this host was heavily hyperglycosylated (Table 1). However, no systematic studies on its catalytic properties have been reported so far. A preliminary characterization of the activity of the recombinant enzyme shows that the enzyme has a preference for substrates with short (C8–C10) chain length *(34)*, in good agreement with the results obtained with the Lip1 expressed in *P. pastoris* and the commercial (Sigma) crude preparation *(150)*.

More recently, Tang et al. *(46)* have reported the functional expression in *E. coli* of a *lip4* gene in which all 19 CUG codons had been converted to a universal serine codon by site-directed mutagenesis. Interestingly, they were able to produce active Lip4, in spite of the enzyme being deglycosylated (Lip4 has one potential site for N-glycosylation, Asn351, which is conserved within the Lip family) (Table 1). In addition, recombinant Lip4 had higher lipase activities toward long-chain esters (C12–C18) and lower activities toward tributyrin, triolein, and olive oil when compared to a commercial CRL crude preparation (Sigma), in which Lip1 is the major isoform (Lip2 and Lip3 have also been identified) *(18,30)*. The catalytic properties of recombinant Lip1 have been reported to coincide with those of the commercial preparation *(150)*, strongly supporting that Lip1 and Lip4 have different substrate specificities. Thus, it appears that local sequence divergences among Lip enzymes indeed provide the structural

bases for a tuning in CRL catalysis. This is also in agreement with previous studies reporting differences in catalytic performances of partially purified native CRL forms, i.e., preparations containing different combinations/ratios of lipase isoforms *(18,72,132)*.

Among the expression systems tested so far, *P. pastoris* has proved to be the most promising in terms of yields/costs and product quality. *P. pastoris* allowed for higher expression levels of Lip1 than in *S. cerevisiae*, easier downstream processing than in *E. coli*, and better product quality (degree of glycosylation, functionality) when compared to *Candida maltosa*– or *E. coli*–expressed Lips.

Native CRL preparations have been extensively used in biotransformations. Heterologous expression of functional CRLs will expand and improve the industrial utility of these enzymes even further by making available cost-effective pure CRL isoenzymes, as well as allowing the generation of new enzyme derivatives by directed evolution.

Patents on CRL Production

Haruo, M., Sumitaka, K., Susumu, A., Mineo, N., Norio, S., and Teru, T.
Lipase overproduction by a mutant of *C. cylindracea*.
JP patent 58051889, issued March 26, 1983.
Assignees: Meito-Sangyo Co., Ltd., Japan.

A mutant of *C. cylindracea* (*rugosa*) that does not assimilate xylose is used to produce lipase with an activity higher than 800 U/mL in the culture medium. A method for the production of lipase comprises the incubation of the mutant strain in a culture medium followed by its recovery from the medium and purification. The culture medium contains a carbon source (glucose, galactose, fructose, sorbitol, and so on), a nitrogen source (soybean powder, yeast extract, and so on), and minerals. The fermentation is carried out for 1–4 d at 20–30°C under aerobic conditions. The mutant strain (FERM 6137, 6134, 6135, or 6136) is derived from *C. cylindracea* (*rugosa*) ATCC 14830 by treating with *N*-methyl-*N*'-nitrosoguanidine, ultraviolet rays or X-rays, ethyl methane sulfonic acid, methyl methane sulfonic acid, and so on. The mutant strain does not assimilate xylose and overproduces lipase.

Osamu, Y., Takuo, S., and Koji, H.
Preparation of protoplast of lipase-producing microorganism.
JP patent 61104781, issued May 23, 1986.
Assignees: Nisshin Oil Mills Ltd.

A method is described for the preparation of protoplasts and/or spheroplasts of lipase-producing microorganisms by using one or more of the following enzymes: cellulase, polygalacturonase, zymolyase, chitinase, and lysozyme. Lipase can be easily extracted from the microorganism by removing its cell wall using these enzymes. The lipase-producing organism belonging to the *Candida* genus, such as *C. cylindracea* (*rugosa*) or *C. para-*

lypolytica, is cultured in a potato sucrose liquid medium at room temperature with shaking. Cells in the logarithmic growth phase are treated at 20–30°C for 1–24 h with the enzymes in weak acid or neutral buffer containing an osmotic pressure regulator to produce protoplast. The enzymatic removal of the cell wall facilitates the extraction and separation of the lipase in the cell by simply dispersing the cells in a low-tonic liquid such as water.

Yoshio, K. and Tamio, M.
Hydrolysis of fat and oil.
JP patent 63304992, issued December 13, 1988.
Assignees: Amano Pharmaceuticals Co. Ltd., Japan.

A process to efficiently hydrolyze fats and oils by using lipase produced by a *C. cylindracea* (*rugosa*) mutant is disclosed. The process comprises the cultivation of *C. cylindracea* (*rugosa*) strain U-3 (FERM P-9365), a mutant of *C. cylindracea* (*rugosa*) ATCC 14830, at 20–30°C for 2–4 d under aerobic conditions. Lipase recovery from the culture broth filtrate includes extraction, salting out, and dialysis steps. The enzyme has the following physical and chemical properties: The optimum pH is about 7.0 and is stable over a pH range between 4.0 and 7.0. The optimum temperature is at least 40°C and is stable up to 50°C at pH 7.0. The enzyme shows high hydrolyzing activity on short-chain fatty acid esters, and it can be used to hydrolyze fats and oils with high melting points (e.g., beef tallow).

Susumi, K., Toshimitsu, N., and Hideki, F.
Production of triglyceride using lipase.
JP patent 01257485, issued October 13, 1989.
Assignees: Kanegafuchi Chem. Ind. Co. Ltd.

Production of a triglyceride involves reacting a fatty acid or fatty acid ester and a glycerol or a partial glyceride in the presence of a mixture of a 1,3-specific lipase and a nonspecific lipase. The lipase is selected from solid lipase preparations, lipase-containing microorganisms, dried extracts of lipase-containing microorganisms, immobilized lipase-containing microorganisms, and dried and immobilized extracts of lipase-containing microorganisms. The 1,3-specific lipase may be derived from *C. rugosa* ATCC 10571 or *Corynebacterium acnes*, among others. The nonspecific lipase may be derived from *Aspergillus niger* IFO 6341 or *Rhizopus delemar* IFO 4697, among others.

Masahide, N. and Tadashi, F.
Method for purifying enzyme.
JP patent 01080286, issued March 27, 1989.
Assignees: Nippon Oils & Fats Co. Ltd., Japan.

A new purification procedure involves subjecting a commercial lipase preparation obtained from a *C. cylindracea* (*rugosa*) culture to column chromatography for fractionation into stereospecific 1,3-diglyceride lipase and

2-monoglyceride lipase components. Lipase purification comprises an ion exchange (e.g., using DEAE-Sephadex® or SP-Sephadex®) and/or gel filtration (e.g., using Sephadex G-150 or G-100) chromatographies. The chromatographic resins are treated with MacIlvaine buffer solution (0.01 *M* citric acid; 0.02 *M* disodium phosphate; pH 3.8), filled in a column having a diameter of 1–10 cm and length of 50–200 cm, and used for column chromatography. The temperature is preferably below 10°C to prevent lipase inactivation.

Toshimitsu, N. and Hideki, F.
Culture process to activate lipase activity in microbial cell.
JP patent 02049582, issued February 19, 1990.
Assignees: Kanegafuchi Chem. Ind. Co. Ltd., Japan.

A method for producing lipase comprises culturing an immobilized lipase-producing microorganism in a support while controlling the substrate feeding rate at a specific constant level. The support is preferably a lipophilic high molecular weight porous material. The organism is preferably *C. cylindracea* (*rugosa*) ATCC 10571, ATCC 14830, ATCC 20116, ATCC 20263, ATCC 20306; *R. delemar* IFO 4697; or *A. niger* IFO 4343. A medium is charged with 5–40% vol of a microorganism-support material and inoculated with a lipase-producing microorganism. A substrate (e.g., polypeptone) is supplied to the medium at a rate of 0.03–1 g of nutrient/(g of cell·h). The substrate concentration is preferably maintained at 0.2–8.0%, especially 0.2–2.0%. By regulating the substrate supply rate, the nutrients for lipase production can be effectively utilized and lipase production can be optimized. Immobilized microorganisms may be dried. The process is effective in reducing the production cost of an immobilized lipase catalyst to be used in lipase reactions such as ester interchange reaction, ester-synthesis reaction, and hydrolysis reaction.

Tamio, M.
Decomposition of fats and oils by enzyme.
JP patent 02086787, issued March 27, 1990.
Assignees: Amano Pharmaceuticals Co. Ltd., Japan.

A method to efficiently hydrolyze fats and oils (e.g., coconut oil) uses a lipase produced by a new mutant of *C. cylindracea* (*rugosa*) with a higher substrate specificity for short-chain fatty acid esters (less than 18C) than for long-chain fatty acid esters (greater than 18C). Previously isolated lipases from *C. cylindracea* (*rugosa*) have shown higher substrate specificity for long-chain fatty acid esters and have been used for hydrolysis of oil and fat containing long-chain fatty acids (e.g., oleic acid, stearic acid, linoleic acid, olive oil, tallow). A new mutant strain with lipase of higher substrate activity for short- and intermediate-chain fatty acids is preferably used. This strain, *C. cylindracea* (*rugosa*) E-118 (FERM P-10282), is generated by mutation of *C. cylindracea* (*rugosa*) ATCC 14830. The enzyme is suitable for

hydrolysis of oil and fat rich in short- and intermediate-chain fatty acids (e.g., coconut oil, palm kernel oil).

Cobbs, C. S., Barton, M. J., Peg, L., Goswami, A., Malick, A. P., Hamman, J. P., and Calton, G. J.
C. rugosa lipase and isoenzyme.
WO patent WO9015146, issued December 13, 1990.
Assignees: Rhone-Poulenc, Bridgewater, NJ.

A lipase or lipase isoenzyme from *C. rugosa* is used to stereoselectively hydrolyze esters, esterify acids, or transesterify esters in aqueous/organic systems. The enzyme may be purified by immobilization of an enzyme mixture. The procedure comprises the purification of two lipase isoenzymes from *C. rugosa* by isoelectric focusing or ion-exchange chromatography on sulfopropyl-derivatized polymers of *N*-acryloyl-2-hydroxy-1,3-propanediol, crosslinked dextran with *N*,*N*'-methylenebisacrylamide, or agarose.

Rúa Rodríguez, M. L. and Ballesteros Olmo, A.
Method for purifying two isoenzyme lipases from *C. rugosa*.
WO patent WO9401542, issued January 20, 1994.
Assignees: Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain.

A method for the purification of two extracellular lipases present in a commercial crude lipase from *C. rugosa* is disclosed. The method comprises a single hydrophobic interaction chromatography step on an agar matrix to yield lipase A and lipase B fractions. The crude lipase preparation is dissolved in buffer with a pH of 6.0–8.0 (preferably 0.25 *M* phosphate buffer) and passed through a hydrophobic interaction chromatography column (2 cm in diameter) containing a phenyl agarose gel (4% agarose content). The column is washed with the same buffer to remove impurities, washed with 50-fold diluted buffer to elute lipase B, and then with di- or polyalcohol (polyethylene glycol) mixed with buffer (1:1) to elute lipase A, which is further concentrated by ultrafiltration and molecular exclusion chromatography with Sephadex G25 to remove the alcohol.

Brocca, S., Schmidt-Dannert, C., Lotti, M., Alberghina, L., and Schmid, R. D.
Synthesis and functional expression of a *C. rugosa* Lip1 gene coding for a major industrial lipase.
WO patent WO9914338, issued March 25, 1999.
Assignees: Unilever N. V., Rottherdam, The Netherlands.

C. rugosa has an unusual codon usage that hampers the functional expression of genes derived from this yeast in a conventional heterologous host. Two procedures for functional overexpression of the *C. rugosa lip1* gene in a heterologous host are disclosed. In a first example, the *lip1* gene was systematically modified by site-directed mutagenesis to gain func-

tional expression in *S. cerevisiae*. As an alternative approach, the gene (1688 bp) was completely synthesized with an optimized nucleotide sequence in terms of heterologous expression in yeast and simplified genetic manipulation. The synthetic gene was functionally overexpressed in *P. pastoris*. The recombinant lipase was produced at a high level and purity, accounting for 90–95% of the secreted proteins.

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