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Recombinant Microbial Lipases for Biotechnological Applications

Claudia Schmidt-Dannert*

Institut für Technische Biochemie, Universität Stuttgart, D-70569 Stuttgart, Germany

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Abstract—Lipases, mainly of microbial origin, represent the most widely used class of enzymes in biotechnological applications and organic chemistry. Modern methods of genetic engineering combined with an increasing knowledge of structure and function will allow further adaptation to industrial needs and exploration of novel applications. Production of such tailored lipases requires their functional overexpression in a suitable host. Hence, this article describes the functional heterologous production of commercially important microbial lipases. Based on the knowledge of different lipases' substrate binding sites, the most suitable lipase for a particular application may be selected.

Introduction

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) catalyze the hydrolysis of water-insoluble esters and triglycerides at the interface between the insoluble substrate and water. Apart from their natural substrates, lipases catalyze the enantio- and regioselective hydrolysis and synthesis of a broad range of natural and non-natural esters.

Due to their extracellular nature, most microbial lipases can be produced in large quantities and are quite stable under non-natural conditions such as high temperatures and nonaqueous organic solvents employed in many applications. Their stability, inexpensive manufacturing, as well as their broad synthetic potential, make microbial lipases ideal biocatalysts for oleochemistry and organic synthesis. Applications of lipases in oleochemistry, in detergents, in the paper and food industry, and in organic synthesis, have recently been reviewed.[1–3] Molecular biology and biochemical properties of microbial lipases have also been summarized.[4,5]

Many microbial lipase genes have been cloned over the past few years, including important commercial lipases like *Candida rugosa* (formerly *C. cylindracea*), *Candida antarctica*, *Thermomyces lanuginosa* (formerly *Humicola*), *Rhizomucor miehei*, *Rhizopus delemar*, *Geotrichum candidum*, *Burkholderia cepacia* (formerly *Pseudomonas*), *Pseudomonas pseudoalcaligenes*, *Pseudomonas mendocina* (originally *P. putida*), and *Chromobacterium glumae* (identical to the lipase from *Burkholderia glumae*) (see Table 1 for references or sequence information). Recombinant production, however, has been limited until recently to only few microbial lipases, namely lipases from e.g. *C. antarctica* (lipase B),[6] *R. miehei*,[7] *T. lanuginosa*,[8] *P. pseudoalcaligenes*[9] and *P. mendocina*.[10]

Three-dimensional structures of more than ten microbial lipases have been solved since the elucidation of the first three-dimensional structure of the *R. miehei* lipase in 1990.[11] For various lipases distinct binding sites for the alcohol and acid moiety of esters were identified, and rational explanations for the stereoselectivity of these enzymes were proposed.[11]

Recombinant DNA technologies allow for the engineering of lipases for specific applications by altering its enantioselectivity, substrate specificity or general process performance. This can be done either by rational design or by a promising newer approach, directed evolution.[14] Rational design of lipases has been addressed mainly to the improvement of catalyst performance in laundry detergents.[1,12,13] Examples for rationally designed lipases regarding e.g. modified stereoselectivity, as it has been recently demonstrated for the lipase from *R. oryzae*,[15] are still limited. Directed evolution, however, proved to be a rapid and yet powerful method to alter enzyme properties, as recently reported for the lipases from *T. lanuginosa* and *P. aeruginosa,* in terms of wash performance in detergents[16] and enantioselectivity,[17] respectively.

* Present address: California Institute of Technology, Chemical Engineering 210-41, 120 E. California Blvd., Pasadena, CA 91125, USA. Tel.: 1-626-395-4151; fax: 1-626-568-8743; e-mail: csd@cheme.caltech.edu

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Table 1. Families of homologous microbial lipases, sequences and X-ray structures

Families	Sequence accession codes[a]	X-ray structure[b]
I. *Rhizomucor miehei* family		
Rhizomucor miehei	LIP_RHIMI [41726]	1TGL, 3TGL, 4TGL, 5TGL,
Penicillium camembertii	MDLA_PENCA [126918]	1TIA
Thermomyces lanuginosa	[299773]	1TIB
Rhizopus oryzae (R. delemar, R. niveus)	LIP_RHIDL [126331]	1TIC and homology model
II. *Candida rugosa* family		
Candida rugosa	LIP1_CANRU [417249]	1TRH, 1CRL, 1LPN, 1PLO, 1LPP, 1LPM,1LPS
Geotrichum candidum	LIP1_GEOCN [126299]	1THP
III. *Burkholderia cepacia* family		
Chromobacterium viscosum (Burkholderia glumae)	LIP_PSEGL [585408]	1TAH, 1CVL
Burkholderia cepacia	LIP_BURCE [1346459]	2LIP, 3LIP, 4LIP, 5LIP, 1OIL
Pseudomonas aeruginosa	LIP_PSEAE [266475]	Homology model
IV. Staphylococcus family		
Bacillus thermocatenulatus	[1321706]	No
Staphylococcus epidermidis	LIP_STAEP [547858]	No
Staphylococcus hyicus	LIP_STAHY [126334]	No
Staphylococcus aureus	LIP_STAAU [126333]	No
Others		
Aspergillus oryzae	1772353	No
Pseudomonas fluorescens	LIPA_PSEFL [126327], LIPB_PSEFL [1170792]	No
Pseudomonas fragi	LIP_PSEFR [126328]	No
Candida antarctica lipase B	LIPB_CANAR	1TCA, 1TCB, 1TCC, 1LBS, 1LBT
Candida antarctica lipase A[6]	No entry	No
Streptomyces exfoliatus	[3402115]	1JFR
Pseudomoas mendocina[10]	No entry	No
Pseudomonas pseudoalcaligenes[9]	No entry	No

[a] Sequence accession codes for the Swiss-Prot database at http://www.expasy.hcuge.ch/ are capitalized and for the NCBI gene bank at http://www.ncbi.nlm.nih.gov/ in brackets.
[b] Structure accession codes are given for the Protein Data Base at http://www.pdb.bnl.gov/

This article describes our efforts to create a set of recombinant genes for microbial lipases functionally overexpressed in suitable hosts, which allows us, in combination with our knowledge of the lipase binding site, to choose the most appropriate lipase for a specific application and to produce tailor-made enzymes via mutagenesis.

Structure of Microbial Lipases: Anatomy of Lipase Binding Sites

All lipases whose three-dimensional structures are known belong to the class of the α/β hydrolase fold family.[18] Together with other members of this family like serine esterases, thioesterases, dinelactone hydrolase and bromoperoxidase A2 they share a common fold composed of a central β-sheet of up to eight different β-strands connected by up to six α-helices and a common catalytic mechanism composed of five subsequent steps. A catalytic triad consisting of the residues serine, glutamate or aspartate, and histidine forms the active site of lipases.[19]

The structures of more than 25 serine esterases and lipases have been published in the Protein Data Bank. Table 1 summarizes the most important microbial lipases, classified in four main families according to their homology. With increasing knowledge of lipase structure and function, it turned out that lipases of different origin have considerably different substrate binding sites, which explains their varying substrate specificities. In order to understand the molecular basis of substrate specificity of lipases, lipases representing each of the four families have been systematically compared in terms of geometry and properties of the scissile fatty acid binding site.[20] Although these enzymes share similar architecture and catalytic mechanism as well as a large hydrophobic scissile fatty acid binding site when compared to esterases, all investigated lipases differ in their substrate specificities for the acyl moiety. According to the geometry of the binding site, three sub-groups can be defined as illustrated in Table 2. Based on this model, the most appropriate lipase for a particular reaction may be selected and, if necessary, tailored via mutagenesis. For example, a lipase with a tunnel-like

Table 2. Anatomy of microbial lipase binding sites: Subdivision of lipases according to the geometry of the scissile fatty acid binding site[20]

Anatomy of fatty acid binding site	Lipases
Group I Crevice-like binding site near the surface	*Rhizomucor meihei* family
Group II. Funnel-like binding site	*Burkholderia cepacia* family *Candida antarctica* lipase B
Group III. Tunnel-like binding site	*Candida rugosa* family

binding-site is more likely to accept substrates with long-chain fatty acids than bulky substrates. The opposite should apply to lipases with a crevice- or funnel-like binding site.

High-level Expression of Recombinant Microbial Lipases

Commercial lipases are usually manufactured from culture supernatants of lipase secreting yeast, fungi or bacteria, thus allowing for the inexpensive production of crude enzyme extracts. The quality of such crude preparations, however, can vary from lot to lot. It is well known that microorganisms, particularly yeasts and fungi, secrete lipases together with other hydrolytic enzymes as well as different lipase isoforms in varying amounts depending on the cultivation conditions.[21] Thus, any biocatalytic application using a crude lipase preparation bears the risk of non-reproducibility of results and unwanted side effects caused by unrelated enzymes. Purification of native enzymes from crude mixtures is often a cumbersome venture due to the hydrophobic nature of lipases and the presence of several similar isoforms. Use of recombinant DNA technology, however, facilitates the economic production of large quantities of pure lipases and the engineering of tailor-made enzymes for specific applications.

Hence, for functional production of recombinant lipases, enzymes representing different microbial lipase families with distinct lipase binding sites, as outlined in Table 2, were selected. Production of reasonable amounts of functional recombinant enzyme for application purposes had been achieved for none of the selected lipases at that time.

For the expression of lipases from either filamentous fungi or yeast we chose the methylotrophic yeast *Pichia pastoris*. *P. pastoris* has become increasingly attractive as a host for industrial-scale production of heterologous proteins.[22] As in *S. cerevisiae*, the secretion by Pichia cells is facilitated by a leader sequence fused to the expressed gene. Since *P. pastoris* does not secrete significant amounts of proteins into the medium naturally, the α-factor prepro-peptide from *S. cerevisiae* must be used for secretion. Genes of interest are placed under the control of the strong methanol-inducible AOX promoter on a shuttle-vector pPICZα (Invitrogen; pPICZαB and pPICZαC differ in their multiple-cloning sites), allowing for the cloning in *E. coli* and the chromosomal integration into *P. pastoris*. In contrast to *S. cerevisiae*, *P. pastoris* does not hyperglycosylate proteins and can provide up to 100-fold higher expression levels. However, for a directed evolution approach it would be necessary to rescue the gene from an isolated clone expressing the desired lipase variant. Therefore, all lipases expressed in *P. pastoris* were also expressed in *S. cerevisiae* under the control of the GAL10 promoter on the 2 μm plasmid-based vector pYES2 (Invitrogen) (Fig. 1).

For the expression of bacterial lipases, the genes were placed under the control of the strong temperature-inducible λ phage promoter P_L on the *E. coli* expression vector pCYTEXP1[23] (Fig. 1).

Rhizomucor family — lipase from *Rhizopus oryzae* (ROL)[24]

Over the last decade, more than 30 lipases were isolated from Rhizopus strains and many of them characterized.[25] Crude enzyme preparation of non-recombinant Rhizopus lipases are commercially provided by several suppliers, e.g. Amano N, D and L lipases derived from *R. niveus* (RNL), *R. delemar* (RDL), and *R. javanicus* (RJL). Rhizopus lipases, as well as the homologous lipase from *R. miehei* (>55% homology), have been used in a wide range of technical applications. Their high 1,3-regiospecificity make them particularly versatile enzymes in lipid modifications.

At present, the lipase genes from *R. oryzae* (ROL) DSM 853,[26] *R. niveus* IFO 9759[27] and *R. delemar*

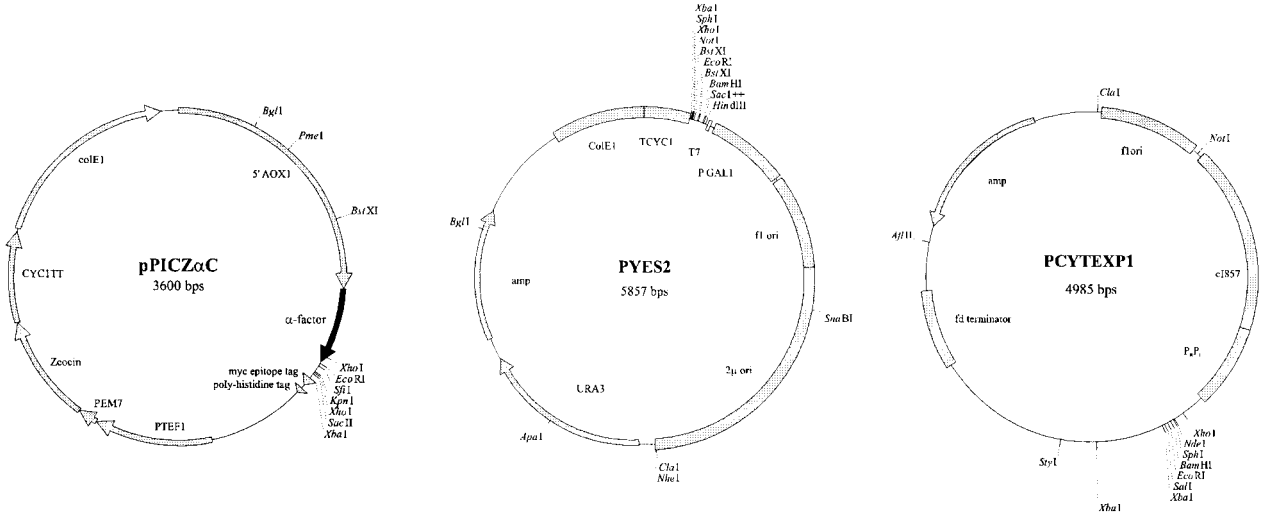

Figure 1. Expression vectors used for lipase overexpression in *P. pastoris* (pPICZαC), *S. cerevesiae* (pYES2) and *E. coli* (pCYTEXP1).

ATCC34612[28] have been cloned. All three lipases are organized as pre-pro-enzymes and have almost identical amino acid sequences; ROL differs only by two amino acid substitutions, His134Asn and Ile234Leu, supporting a recent taxonomic re-classification of all strains as *R. oryzae*.[29] Hence, different properties that have been observed in commercial Rhizopus lipase preparations are due to different production or formulation conditions affecting conformation and/or glycosylation or proteolytic cleavage rather than to actual sequence differences.

While *R. miehei* (RML) lipase has been manufactured in recombinant *Aspergillus oryzae* and is commercially available in immobilized form,[7] functional production of recombinant Rhizopus lipases has not been achieved yet. Although both ROL and RDL have been produced in good yields in *E. coli*,[26,30] formation of cytoplasmatic (RDL) or periplasmatic (ROL) inclusion bodies and hence, the necessity to refold the lipases in a tedious procedure, makes large-scale production in *E. coli* not economically feasible. Nevertheless, an effective small-scale refolding procedure was developed for the ROL, resulting in active lipase with good yields and a specific activity (10 000 U/mg) comparable to that of the native enzyme.[26,31]

To gain functional expression of ROL, we fused the gene encoding the mature lipase to the α-factor secretion signal in the Pichia expression vector pPICZαC. Following transformation into *P. pastoris*, we selected the best lipase producing clones under inducing conditions on minimal plates containing tributyrin and further cultivated them on a small-scale. After 48 h of induction with methanol, varying lipase activities of 30–110 U/mL were measured in the culture supernatants. The best clone was chosen for cultivation in a 500 mL scale, yielding 110 U/mL of secreted lipase with a specific activity of 14 U/mg after 4 days of induction. For comparison, expression of ROL under the control of the GAL1 promoter in *S. cerevisiae* resulted in only 1–5 U/mL lipase secreted in the culture supernatant. Separation of medium compounds was achieved by a two-step protocol consisting of an ultra-filtration through a 10 kDa membrane and a cation-exchange chromatography on SP-sepharose. The purified ROL had a specific activity of 8571 U/mg, comparable to 10 000 U/mg obtained for recombinant ROL expressed in *E. coli*[26] and native ROL.[31] The physicochemical properties of the purified lipase coincide with those of the recombinant ROL previously produced in *E. coli*, except of an increased thermostability (Table 3). SDS-PAGE analysis showed a single band of 30 kDa after silver staining, corresponding to the size reported for the native enzyme and also for the recombinant lipase refolded from inclusion bodies in *E. coli*. As expected, no glycosilation was seen after Endo-H treatment of recombinant ROL expressed in Pichia.

Production of larger amounts of ROL was obtained by fermentation in a simplified complex medium (1% (w/v) yeast extract, 2% (w/v) peptone, 4×10^{-5}% (w/v) biotin) at pH 5.5 and feeding of methanol in correlation to the carbon dioxide content in the off-gas. After 98 h of cultivation, 180 U/mL of ROL were obtained in the supernatant, corresponding to a productivity of 1837 U/l×h.

Pseudomonas family — lipase from *Burkholderia cepacia* (PCL)[32,33]

Lipases from Pseudomonas represent probably the largest group of microbial lipases at present, considering the quantity of publications on isolation, purification and cloning of various Pseudomonas lipases. A detailed review on molecular as well as biochemical properties is found in refs. 34–36. Besides their application in laundry detergents, e.g. lipase from *P. pseudoalcaligenes*,[1] lipases from Pseudomonas are widely used by organic chemists in enantioselective synthesis[1,2,37] which is reflected by the various Pseudomonas lipase preparations commercially available (e.g. Amano YS, P, AH; Fluka SAM-II).

Pseudomonas lipases can be divided into three homology groups (assigned as classes I–III), where class III is only distantly related to the other classes. Lipases of classes I + II, including lipases of *Burkholderia cepacia*, *Burkholderia glumae* (class II) and *P. aeruginosa* (class I), need a chaperone located downstream of the lipase gene for efficient secretion and folding of active lipase. Hence, manufacturing of these lipases is presently performed in recombinant Pseudomonas harboring a plasmid with the clustered lipase gene and its chaperone.

In view of simplified heterologous lipase production, both the lipase gene and chaperone of *B. cepacia* ATCC 21808 were subcloned in different *E. coli* expression vectors (pUC19, pET-20b(+), pCYTEXP1). High expression level of inactive PCL inclusion bodies (60%) in *E. coli* was only achieved with pCYTEXP1, though. Expression of chaperone, however, was not detected with any of the expression vectors, although the gene was placed in an optimal distance to the promoter and/or preceded by an ompA signal sequence, known to enhance protein expression. As expected, refolding of the lipase in the absence of its chaperone resulted in only 25 U/mg of active PCL under the best conditions.

Table 3. Physicochemical properties of overexpressed lipases

Lipase	MW [kDa]	Glykos.	pI	T_{opt} [°C]	T_{stab} [°C]	pH_{opt}	pH_{stab}
ROL	30	no	⩾9.3	30	45	8.0–8.5	6.0–7.5
GCL (lipase B)	66	3%	4.55	40–45	45	8.5–9.0	7.5–8.5
CRL	60	5%	3.9–4.0	30–40	50	6.5–7.5	8.0–8.5
PCL	33	no	n.d.	n.d.	n.d.	n.d.	n.d.
BTL-2	43	no	7.2	55–75	50	8.0–9.0	9.0–11.0

Sequence analysis of the chaperone revealed a high GC content (>90%) in the 5′ region of the gene and the presence of a putative membrane anchor at the N-terminus, which both may affect protein production in *E. coli*. Hence, the 5′ region of the gene was replaced by a synthetic fragment and the putative membrane anchor was removed by deleting the first 34 and 70 N-terminal amino acids, respectively. However, only the truncated genes resulted in an overexpression of the chaperone in *E. coli*. Fusion of the ompA signal sequence to the truncated genes in pCYTEXP1 led to a further increase of expression from 10 to 60%. Partial procession in *E. coli* was only seen for the 34 residues truncated ompA fused chaperone, though.

Employing these chaperones for refolding of denatured PCL recovered from inclusion bodies, it was possible to obtain for the first time highly active enzyme with a specific activity of up to 4 850 U/mg and a yield of 340,000 Units per gram of *E. coli* cells. Interestingly, while the ompA leader sequence had no influence on the foldase activity of the 70 residues truncated chaperone, it completely inhibited that of the 34 residues truncated chaperone. Our observation that the chaperones function also, when added in denatured form to the refolding mixture enabled us to develop a simple and fast, yet very efficient refolding procedure. Both, chaperone and lipase are denatured prior to refolding by lysing recombinant *E. coli* cells in 8 M urea followed by separation of cell debris. The thus obtained protein solution is directly used for refolding in water at 4°C for 12–24 h. Quantitative refolding is observed when both lipase and chaperone are present at equal concentrations of 10–15 μg/ml in the refolding mixture.

Candida rugosa family — lipases from *Geotrichum candidum* (GCL) and *Candida rugosa* (CRL)

***G. candidum* lipases A and B[38].** *G. candidum* CMICC 335426 secretes two homologous lipase isoforms (84% homology) named A and B with significant different substrate specificity.[39–42] While lipase B is highly specific for *cis*-Δ9 unsaturated fatty acids, lipase A is less specific, although both lipases prefer long-chain fatty acids as substrates. Its unique substrate specificity makes lipase B of particular interest for application in lipid modification.

Both lipases are found in the supernatant of *G. candidum* and their close physical and biochemical properties make purification difficult.[43,44] Cloning and separate expression of both isoforms is therefore the most reliable way to obtain pure isoforms. Functional production, albeit at very low level, in *S. cerevisiae* has been reported for lipases I (97% homology to lipase B) and II of *G. candidum* ATCC 34614.[45,46] The specificity for *cis*-Δ9 unsaturated fatty acids, however, seems to be more pronounced in the case of lipase B.[39,42,45]

As described for ROL, both mature lipase A and B genes were each fused to the prepro α-factor leader sequence and inserted into the vector pPICZαC for expression in *P. pastoris*. Ten positive clones for each expression vector were then selected and grown on a small scale under inducing conditions. After 48 h, lipase secretion of 1–23 U/mL and 1–50 U/mL was measured for different clones of lipase A and B, respectively. The best lipase-producing clones were chosen for further cultivation in a larger scale. Expression of lipase B in recombinant Pichia cells in a 400 ml scale resulted in 130 U/mL secreted lipase in the supernatant. SDS-PAGE analysis of the supernatant showed one single band of 66 kDa, corresponding to molecular sizes of 50–66 kDa reported for native lipase isoforms and the recombinant lipases I and II depending on the glycosilation.[39,43–46]

Separation of medium compounds was achieved by an ultra-filtration step through a 50 kDa membrane, thereby increasing the specific activity of the lipase solution 66-fold to 330 U/mg. In comparison, specific activities ranging from 270 U/mg to 2455 U/mg have been reported for the native lipase isoforms after multistep purification protocols.[39,43,47]

Deglycosylation of purified lipase B by Endo-H treatment resulted in a 2 kDa decrease in molecular weight, indicating a moderate carbohydrate content of 3% for the recombinant protein, similar to that determined for recombinant lipase I expressed in a hyperglycosylation-deficient mnn9 *S. cerevisiae* strain.[45] Up to 3-fold higher carbohydrate contents, however, have been reported for the native isoenzymes.[39,43,44] Substrate specificity and other physicochemical properties (Table 3) such as activity and stability of recombinant lipase B are comparable to those reported for the native enzyme.[39,43,44,48]

Optimization of the fermentation process allowed us to produce up to 200 000 U of recombinant lipase B per l of culture, which is comparable to the production level reported for the native lipase isoforms by *G. candidum*.[49] As in the case of ROL, introduction of lipase B fused to the α-factor signal sequence into pYES2 and transformation into *S. cerevisiae* yielded only 5–6 U/ml secreted lipase B.

Shortly after this work was published, the production of lipase I and II from *G. candidum* ATCC 34614 in *P. pastoris* was reported.[50] The obtained expression levels are comparable to those we obtained for lipase A and B.

***Candida rugosa* lipase[51].** Crude CRL preparations are among the commercial lipases most often employed in the hydrolysis and synthesis of a wide range of esters of commercial interest.[1,2] However, as in the case of the lipases from *G. candidum*, application of crude lipase preparation of *C. rugosa* suffers from the secretion of several isoforms by the yeast. Until now 7 different lipase genes with an average homology of 66% have been cloned.[52,53] Efforts to purify different lipase forms from commercial enzyme preparation identified the gene product of LIP1 as the major constituent and also demonstrated that the isoforms differ in their catalytic properties.[54] Variation in glycosylation, as well as cultivation-dependent expression, of these isoforms is thought to contribute to a further increase in heterogeneity of crude CRL preparations.

Cloning and expression of the LIP1 gene was hence considered to be the most suitable approach for the production of a homogeneous lipase preparation for biotechnological applications. However, several attempts to gain functional expression of LIP1 in *S. cerevisiae* failed.[55] The discovery that *C. rugosa* obeys a non-canonical codon-usage in which the triplet CUG, a universal codon for leucine, is read as serine, explained the observed difficulties in heterologous expression. In LIP1, 20 out of 47 serine residues, including that of the catalytic Ser209 are encoded by CTG.[52,53] A survey on *C. rugosa* lipases is found in a recent review.[56]

In a first attempt to overcome these problems in heterologous expression of LIP1, 8 CTG codons were selected for the replacement with universal codons. The selected codons were either highly conserved within the lipase/esterase family or considered of importance to maintain protein structure and function. To this end, 8 mutant genes containing an increasing number of restored Ser residues (2, 3, 5, 8) were created. However, recombinant *S. cerevisiae* cells still failed to secrete active LIP1, instead accumulating intracellular LIP1 independently of the number of Ser residues restored.

In a second approach for the functional expression of LIP1 in conventional yeast, we synthesized the complete gene of 1647 bps by the method of mutually priming long overlapping oligonucleotides. The nucleotide sequence was optimized in terms of heterologous expression in yeast and simplified genetic manipulations.

For secretion in either *P. pastoris* or *S. cerevisiae,* the synthetic codon-optimized gene encoding the mature lipase was fused to both the prepro and pre α-factor signal sequence and cloned into the expression vectors pPICZαB and pYES2, respectively (Fig. 1). In addition, the α-factor signal sequence was replaced by the natural leader sequence of LIP1 in both vectors.

All positive *P. pastoris* transformants as well as the *S. cerevisiae* transformants secreted lipase under inducing conditions as evidenced by halo-formation on tributyrin minimal plates. However, lipase secretion was much reduced in *P. pastoris* when the lipase gene was preceded by its natural leader sequence. A negative effect of the natural leader sequence on lipase secretion was not observed for *S. cerevisiae*.

Lipase secretion of recombinant *P. pastoris* and *S. cerevisiae* cells was further investigated in shaking-flask cultivation. After 5 days of induction, lipase secretion by recombinant *S. cerevisiae* cells was only 5–7 U/mL, compared to that by recombinant *P. pastoris* cells of 85 U/ml.

A single protein band of 60 kDa molecular weight, corresponding to that reported for the native lipase,[54] was seen after SDS-PAGE analysis of culture supernatant from lipase secreting *P. pastoris* cells. Deglycosylation by Endo-H treatment led to a 3 kDa decrease in molecular weight, suggesting a carbohydrate content of 5% for the recombinant protein, similar to 3.6–8% reported for the native isoforms.[54]

Fermentation of recombinant *P. pastoris* cells for 280 h in a one liter bioreactor yielded 150 U/mL lipase, corresponding to a productivity of 500–600 U/L×h. A two-fold lower productivity of 250 U/L×h has been reported for the secretion of native lipases by *C. rugosa*.[57] The physicochemical properties, as well as the substrate specificity, of the recombinant lipase were compared with those of a commercial lipase preparation and found to coincide (Table 3). Hence, LIP1 encodes in fact the major isoform present in commercial CRL preparations.

Staphylococcus family — lipase from *Bacillus thermocatenulatus* (BTL-2)[58–61]

The Staphylococcus lipase family, including lipase BTL-2 from *B. thermocatenulatus*, represents a group of large prokaryotic lipases with molecular weights of 40–45 kDa. Homologous lipase genes (70% homology) have been cloned and functionally expressed from different Staphylococcus strains.[62–64] Recent investigations of the physicochemical properties of Staphylococcus lipases revealed that lipases from *S. aureus* and *S. epidermidis* are closely related: both lipases prefer short-chain triglycerides. The lipase from *S. hyicus*, on the other hand, prefers phospholipids as substrate.[62] A three-dimensional structure, however, has not been solved yet.

Our attempts to clone a thermophilic lipase resulted in the isolation of a 43 kDa lipase named BTL-2 from an expression library generated from genomic DNA from the thermophile *B. thermocatenulatus*. Surprisingly, no sequence homology was found with other microbial lipases including those from other Bacillus strains, but instead BTL-2 shows a significant homology of 30–35% with the different Staphylococcus lipases. A first brief characterization of BTL-2, expressed at low level in *E. coli,* revealed its high activity at temperatures between 50 and 75°C and alkaline conditions. Lipase BTL-2 shares with lipases from *S. eperdimidis* and *S. aureus* the same preference for short chain triglycerides as substrate. However, activation by calcium as reported for the Staphylococcus lipases was not observed.

For the production of larger amounts of lipase needed for further characterization and any application, an efficient system for the overproduction of BTL-2 was developed. For this purpose, we placed the mature lipase gene either directly or N-terminal fused to the ompA signal sequence downstream of the strong temperature inducible λP_L promoter in pCYTEXP1 (Fig. 1). Expression levels of 54 000 U/g cells of soluble lipase were obtained for the mature lipase, while fusion to the ompA leader sequence resulted in a drastic increase of expression, yielding 660.000 U/g cells of soluble lipase. But due to the unprocessed ompA signal sequence more than 90% of the lipase remained insoluble, however active after cell breakage.

A two-step purification protocol consisting of a Butyl-Sepharose chromatography followed by a gel filtration

Table 4. Production of recombinant, microbial lipases in different expression systems

Lipase	Expression vector	Host	Expression level	Specific activity[a] [U/mg]
ROL	pPICZαC	*P. pastoris*	180 U/mL	8 571
	pYES2	*S. cerevisae*	< 5 U/mL	n.d.
GCL (lipase B)	pPICZαC	*P. pastoris*	200 U/mL	372
	pYES2	*S. cerevisae*	5–6 U/mL	n.d.
CRL	pPICZαB	*P. pastoris*	150 U/mL	n.d.
	pYES2	*S. cerevisae*	5–7 U/mL	n.d.
PCL	pCYTEXP1	*E. coli*	314 000 U/g cells	4 850
BTL-2	pCYTEXP1	*E. coli*		
	mature BTL2		54 000 U/g cells	55 000
	ompA-BTL2		$> 1.1 \times 10^6$ U/g cells	36 000

[a] Purified; after refolding in the case of *B. glumae* lipase.

resulted in pure highly active mature lipase with a specific activity of 55 000 U/mg. Cholate extraction of insoluble ompA-lipase followed by proteinase K cleavage of the ompA signal sequence allowed us to extract 1.1 million units of lipase per gram of cells with a purity of >95%. Physicochemical properties of recombinant BTL-2 are summarized in Table 3.

Summary

The choice of an appropriate expression system combined with genetic modifications to facilitate optimal expression as well as optimized fermentation conditions allowed us to produce large amounts of recombinant lipases previously not functionally produced. This is especially true for the lipase of *C. rugosa*, where only the synthesis of a codon-optimized lipase gene enabled the production of functional recombinant lipase. The methylotrophic yeast *P. pastoris* proved to be an excellent host for the overexpression of lipases from yeast, as well as from fungi resulting in the secretion of 100–200 U/mL lipase into the supernatant. Moreover, the absence of naturally secreted proteins by *P. pastoris* facilitated rapid purification of secreted recombinant lipases. In the case of recombinant lipase from *B. glumae* we demonstrated that inclusion body formation of a Pseudomonas lipases in *E. coli* must not necessarily render large scale production impossible, provided a simple chaperone mediated refolding process is available. Production of recombinant lipases in different expression systems is compared in Table 4 and their biochemical properties are summarized in Table 3. The diverse properties of these lipases, e.g. 1,3-regio-specificity of ROL, substrate specificity of GCL (lipase B) and the stability as well as the activity at elevated temperatures of BTL-2 should allow us to find the appropriate lipase for a specific application and to produce tailor-made lipases by either rational or random approaches, or both.

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