# OVEREXPRESSION AND PROTEIN ENGINEERING OF LIPASE A AND B FROM *GEOTRICHUM CANDIDUM* CMICC335426

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# Zusammenfassung

## EINLEITUNG

Lipasen sind Triacylglycerinester-Hydrolasen, die die Triglyceride an der Grenzfläche zwischen hydrophiler wäßriger und hydrophober Fettphase hydrolysieren.

Lipasen aus den verschiedensten Organismen wurden im Laufe der letzten Jahre isoliert und die entsprechenden Gene identifiziert (Antonian 1988; Khachatourians and Hui 1995).

Anhand der dreidimensionalen Strukturen einer Reihe von Lipasen konnte geklärt werden, daß Lipasen zur Familie der Serinhydrolasen gehören, die sich durch ein aktives Zentrum mit einer katalytischen Triade aus Serin, Histidin und Asparaginsäure bzw. Glutaminsäure auszeichnen (Cygler, Schrag *et al.* 1993).

Weitere strukturelle Merkmale dieser Enzymklasse sind der sogenannte „α/β-hydrolase-fold“ und die Anwesenheit eines „Deckels“ über der Substratbindungstasche, der sich bei Kontakt mit einer Öl-Wasser-Grenzfläche öffnet und so den Zugang zum aktiven Zentrum ermöglicht („interfacial activation“).

Bei Lipasen handelt es sich je nach Abstammung um eine Enzymklasse mit einem weiten Anwendungsspektrum abhängig von der unterschiedlichen Substratspezifität.

Für Lipasen gibt es verschiedene Anwendungsmöglichkeiten, beispielsweise als Waschmittelbestandteile sowie in der Lebensmittelindustrie, wo die Umsetzung von Fetten und Ölen im Mittelpunkt steht. Weiterhin gewinnen Lipasen für die Darstellung von enantiomerenreinen Verbindungen in der pharmazeutischen Industrie und Synthesechemie an Bedeutung.

Der Schimmelpilz *Geotrichum candidum* produziert zwei unterschiedliche extrazelluläre Lipasen, die für den Stamm CMICC 335426 Lipase A und B, bzw. für ATCC 34614 Lipase I und II genannt werden (Sidebottom, Charton *et al.* 1991; Bertolini, Laramee 1994).

Obwohl die Homologie der verschiedenen Lipasen sehr hoch ist (84% für A und B bzw. 97% für B und I) zeichnen sie sich jedoch durch ein unterschiedliches Substratspektrum aus.

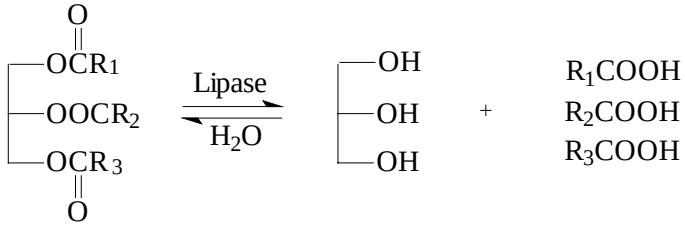



Abb. 1. Reaktionsschema für eine lipasekatalysierte Hydrolyse von einem Triacylglycerol. Im Falle der Lipase B aus *Geotrichum candidum* mit R= $-(CH_2)_8CH=CH(CH_2)_nCH_3$ folgt eine spezifische Reaktion.

Lipase B und I zeigen eine hohe Spezifität für Fettsäureester von *cis*-Δ9 ungesättigten Verbindungen. Im Falle der Lipase B scheint diese Spezifität jedoch ausgeprägter zu sein (Charton and Macrae 1992; Charton and Macrae 1993) (Abb.1).

Im wachsenden Interesse an Produkten mit reduziertem Fettgehalt und insbesondere mit geringerem Gehalt an gesättigten Fettsäuren liegt die Bedeutung der Lipase B von *Geotrichum candidum*. Somit stellt der Einsatz der Lipase B wegen ihrer hohen Spezifität eine gute Möglichkeit dar, das Fettsäure-Profil des Öls zu modifizieren.

Darüber hinaus haben beide Lipasen, die im Kulturüberstand von *Geotrichum candidum* gefunden werden, ähnliche physikalische und biochemische Eigenschaften, die eine Aufreinigung erschweren (Hedrich, Spener *et al.* 1991; Jacobsen and Poulsen 1992).

Um die reinen Isoformen zu erhalten, wurden die einzelnen Lipase-Gene kloniert und exprimiert. Auf dieser Weise können die beiden Isoformen getrennt eingesetzt sowie auf ihre Substratspezifität untersucht werden.

## ERGEBNISSE

### Klonierung und Fermentation

Die Lipasegene wurden zuerst hinter die α–Faktor Signalsequenz aus *Saccharomyces cerevisiae* fusioniert, anschließend in einen für die Transformation der Hefe S*accharomyces cerevisiae* geeigneten Vektor (pYES2) kloniert und dann in diese Hefe transformiert. Die α–Faktor Signalsequenz führt zur Sekretion des Proteins ins Medium. Nach Induktion mit Galaktose wurde die Lipaseaktivität im Kulturmedium der rekombinanten Klone bestimmt. Allerdings lag die Ausbeute von 6-8 U/(ml Medium) unter den Erwartungen.

Deshalb wurden im nächsten Schritt die Lipasegene in zwei Vektoren zur Expression in der methylotrophen Hefe *Pichia pastoris* kloniert: pPICZαC mit einem methanolinduzierbaren Alkoholoxidase-Promotor und pGAPZαC mit dem konstitutiven Promotor der Glyceraldehyd-3-Phosphat-Dehydrogenase. Die Lipasen wurden erfolgreich in *Pichia pastoris* exprimiert und in hoher Ausbeute ins Kulturmedium sekretiert. Da ausschließlich die rekombinanten Proteine von dieser Hefe sekretiert werden, liegen die Proteine bereits in nahezu reiner Form im Medium vor.

Die Fermentationen der rekombinanten Lipase B unter Kontrolle des AOX-Promotors wurden unter verschiedenen Kultivierungsbedingungen durchgeführt. In einem komplexen Medium aus Hefeextrakt, Pepton und Methanol wurden 300.000 U Lipase/(l Kultur) erreicht. Das ist vergleichbar mit der Ausbeute der homologen Produktion in *Geotrichum candidum*.

Das beste Ergebnis (600.000 U/l Kultur der reinen Lipase B, Abb. 2) wurde in einer Hochzelldichtefermentation in billigerem synthetischem Medium erzielt, in dem Glycerol als C-Quelle und Methanol für die Induktion der Lipaseproduktion eingesetzt wurden. Da die Zellen in der Lage sind, mit Glycerol höhere Zelldichten zu erreichen (Abb. 3), nimmt die Lipaseexpression auch zu.

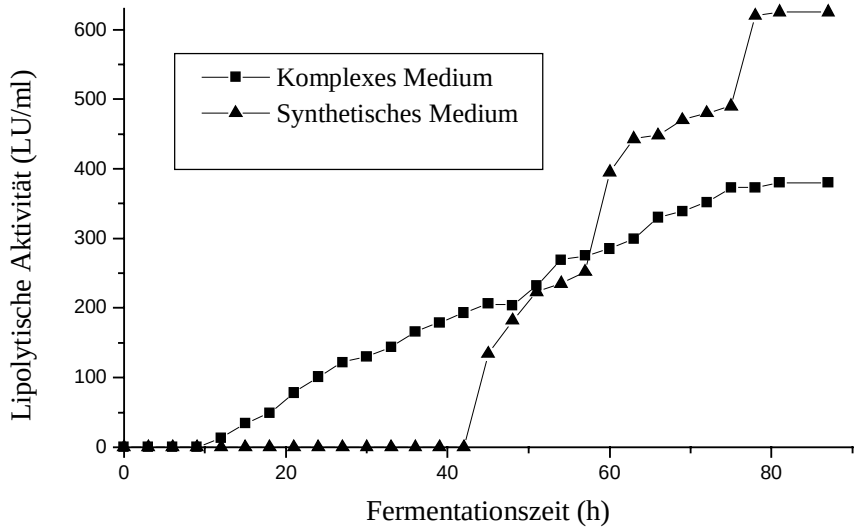



Abb. 2. Lipolytische Aktivität der Lipase B aus *Geotrichum candidum* im Kulturüberstand von *Pichia pastoris*, fermentiert in komplexem und synthetischem Medium.

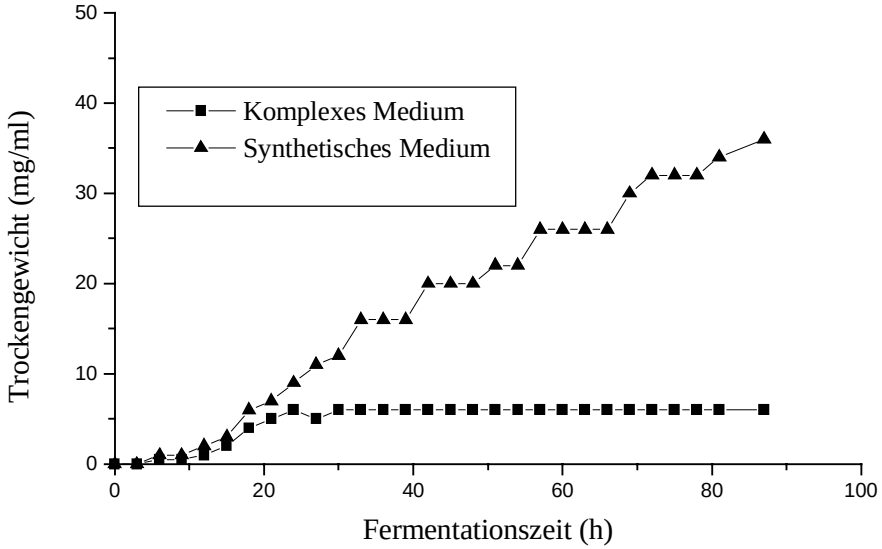



Abb. 3. Trockengewicht der mit dem Gen der Lipase B transformiertem *Pichia pastoris*, fermentiert in komplexem und synthetischem Medium.

Die rekombinanten Lipasen A und B wurden hinsichtlich Temperatur- und pH-Einfluß charakterisiert. Die Ergebnisse sind in Tab. 1 dargestellt.

Tabelle 1. Eigenschaften der rekombinanten Lipasen A und B von *G. candidum*, exprimiert in *Pichia pastoris*.

| **Parameter** | **Lipase A** | **Lipase B** |
|---|---|---|
| Temperatur-Optimum | 40 °C | 40 °C |
| Bereich der vollständigen Temperatur-Stabilität nach 24 Stunden Inkubation bei pH 8.0 | bis 30 °C | bis 30 °C |
| pH-Optimum | 8.0 | 8.0 |
| Bereich der vollständigen pH-Stabilität nach 24 Stunden Inkubation bei 30 °C | 6.0-9.0 | 8.0 |
| Gehalt an umgesetzten gesättigten Fettsäuren in einer Fettsäuremischung (%) | 49% | 5% |

Diese Eigenschaften sind ähnlich wie die der nativen Lipasen A und B von *Geotrichum candidum*. Die besondere Substratspezifität der Lipase B für *cis*-Δ9 ungesättigte Substrate konnte insofern bestätigt werden, da sie nur 5% der gesättigten Fettsäuren hydrolysiert, im Gegensatz zu 49% im Fall der Lipase A.

### Erhöhung der Thermostabilität

Ein weiteres Ziel dieser Arbeit war die Erhöhung der Thermostabilität der Lipase B, was von besonderem Wert in einer industriellen Anwendung ist.

Dazu wurde zuerst eine Zufallsmutagenese mittels Error-prone PCR durchgeführt, um Zufallsmutationen im Lipase-Gen zu erzeugen. Anschließend wurden die mutierten Lipase-Gene in einen pYES2 Vektor ligiert und in *Saccharomyces cerevisiae* transformiert. Auf diese Art erhielt man eine Mutantenbibliothek bestehend aus etwa $10^5$ mutierten Klonen. Trotz des niedrigeren Expressionsniveaus wurde *S. cerevisiae* gegenüber *Pichia pastoris* als Wirt für die Herstellung der Klonbank bevorzugt, da das transformierte Plasmid ins Genom von *P. pastoris* integriert wird, und so die Isolierung des Plasmids und die Identifizierung der eingeführten Mutationen erschwert.

Zur Bestimmung der Lipase-Thermostabilität der verschiedenen Mutanten wurde eine Hochdurchsatz-Screening-Methode auf der Grundlage eines Fluoreszenz-Tests entwickelt,

basierend auf der hydrolytischen Spaltung von 4-Methylumbelliferyl Oleate (4-MUO) und anschließender Freisetzung einer fluoreszierenden Verbindung. Die mit den Lipase-Mutanten transformierten Hefeklone wurden in Mikrotiterplatten mit selektivem Medium vermehrt. Die Zellen wurden durch Zentrifugation abgetrennt und der Kulturüberstand mit der sekretierten rekombinanten Lipase B aus *G. candidum* für die Thermostabilitätsbestimmung verwendet. Die lipolytische Aktivität des Überstandes wurde vor und nach 30 Minuten Inkubationszeit bei 50 °C mit 4-MUO bestimmt. Das Verhältnis der beiden Messungen ergibt eine Größe für die Thermostabilität der verschiedenen Lipasemutanten, die mit dem entsprechenden Wert für den Wildtyp verglichen werden kann, um so eine verbesserte Thermostabilität nachweisen zu können.

Die Parameter, die eine Rolle in der Reproduzierbarkeit des Screening-Assays spielen, wie die Substrat- und Lipasemenge, Zeit, Temperatur und pH Wert der Inkubation wurden untersucht und optimiert. Demnoch war die Fehlerrate des Tests im Vergleich zu den erwarteten Unterschieden in der Thermostabilität der Mutanten zu groß. Aus 1000 untersuchten Mutanten identifizierte Proteinvarianten mit scheinbar höherer Thermostabilität erwiesen sich nach Isolierung der Plasmide und Sequenzieren als Wildtyp.

Eine andere Technik, die zur Erhöhung der Thermostabilität eines Proteins angewendet werden kann, ist die punktgerichtete Mutagenese.

Es gibt noch keine generelle Regel oder Strategie, die in jedem Fall angewendet werden kann, denn die Faktoren, die die Stabilität beeinflussen können, müssen für die verschiedenen Proteine jeweils neu ermittelt werden.

Erfolgreich angewendete Ansätze berücksichtigen unter anderem die Einführung weiterer Disulfid-Brücken, H-Bindungen oder gezielte Mutationen, die die Stabilität der Tertiärstruktur erhöhen oder eine kompaktere Struktur des Proteins ermöglichen.

Die zu mutierenden Reste können entweder durch Vergleich der Aminosäuresequenzen, beispielweise aus mesophilen und thermophilen Organismen identifiziert werden, oder durch Analyse von Röntgenstrukturen.

Im Fall der Lipase B aus *Geotrichum candidum* wurden zwei Ansätze angewendet: Die Einführung einer Disulfid-Brücke und die Erhöhung der Anzahl der Salz-Brücken an der Proteinoberfläche.

Um die geeignetsten Positionen für die Mutationen herauszufinden, wurde die Röntgenstruktur der Lipase B mit denen der homologen *Candida rugosa* Lipase (CRL) und *Torpedo californica* Acetylcholinesterase (AChE) verglichen. Dabei fiel sofort auf, daß in

allen drei Proteinen zwei Disulfid-Brücken konserviert sind, während eine dritte nur in der AChE anwesend ist (Abb. 4) (Shimada, Sugihara *et al.* 1990).

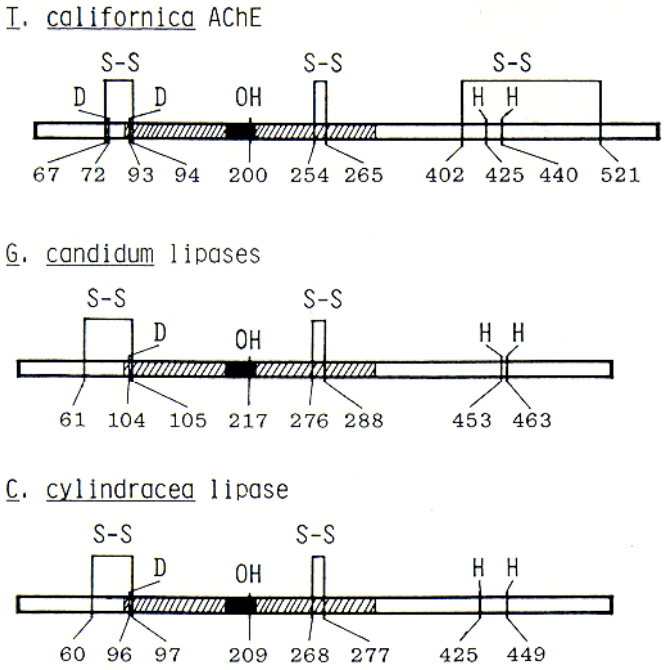



Abb. 4. Schematische Darstellung der *T. californica* AChE, *G. candidum* Lipase B, *C. cylindracea* (jetzt umbenannt in *C. rugosa*) lipase. Die Positionen der Disulfid-Brücken sind als S-S dargestellt. Gefüllte und schraffierte Regionen zeigen Bereiche mit 70% bzw. 30% Sequenz-Homologie (Shimada, Sugihara *et al.* 1990). Ser: OH; Asp: D und His: H.

Trotz des Sequenz-Unterschiedes wurde durch strukturelle Analyse gefunden, daß die Struktur-Homologie zwischen AChE und GCL B in der Region der Disulfid-Brücke sehr hoch ist, und die Aminosäuren der GCL B, die den zwei Cysteinen der AChE entsprechen, im richtigen Abstand zueinander für eine mögliche Disulfid-Brücke vorliegen.

Eine Disulfid-Brücke in dieser Position kann die Thermostabilität der Lipase durch eine Abnahme der Konformationsentropie des “Backbone” im denaturierten Zustand und durch die Fixierung der beweglichen Helix am C-Terminus erhöhen.

Zwei weitere Cysteine wurden dann in die Lipase durch die Mutationen S430C und I533C eingeführt. Die Messungen der Thermostabilität der Doppelmutante zeigte eine deutliche Destabilisierung, da sie nach 30 Minuten Inkubation bei 50 °C nur 11% Restaktivität gegenüber 26% des Wildtyps zeigte.

Verantwortlich für die Destabilisierung der Mutante könnte die Wechselwirkung der Helices E384-L393 und I530-T538 sein, da sich aufgrund der eingeführten Disulfid-Brücke die

beiden Helices zueinander verschieben und diese sich so nicht mehr im idealen Abstand zueinander befinden.

Die entsprechenden Reste, die in GCL B interagieren könnten, sind in der AChE weiter voneinander entfernt. Deswegen wurden die Aminosäuren L393 und N535 durch kleinere wie Alanin oder Valin ersetzt.

Außerdem wurde das hydrophobe L393, das sich auf der Proteinoberfläche befindet, in das hydrophile Glutamin mutiert.

Die Thermostabilität der verschiedenen Mutanten wurde untersucht. Die Restaktivität von drei der sechs Mutanten nach 30 Minuten Inkubation bei 50 °C betrug nur 42% der des Wildtyps nach Inkubation, gegenüber 35% des Werts, der bei der S430/I533 Mutante erhalten wurde (Abb. 5).

Es handelte sich also nur um eine geringfügige Steigerung der Thermostabilität gegenüber der Mutante mit eingeführter Disulfid-Brücke.

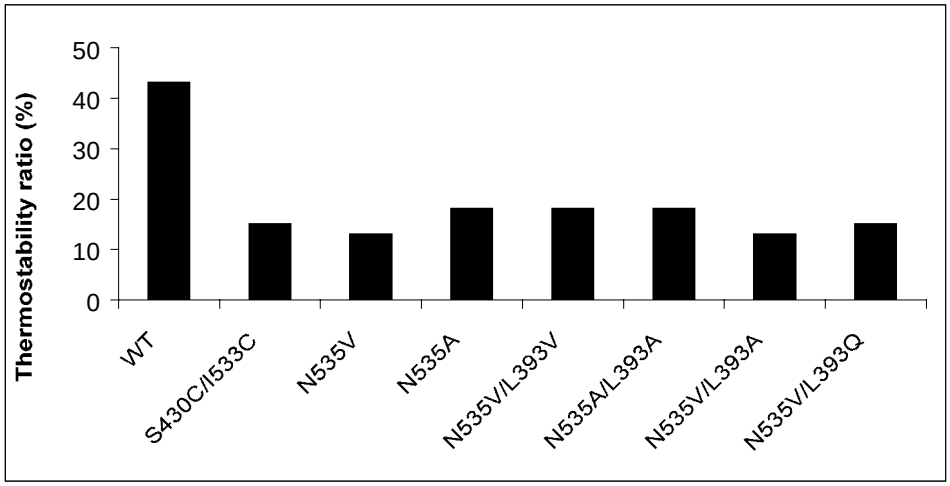



Abb. 5. Thermostabilität der verschiedenen Mutanten mit der eingeführten Disulfid-Brücke im Vergleich zur Wildtyp-Lipase.

Die Destabilisierung muß daher von einem Einfluß herrühren, der nicht durch Computer Modeling vorhersagbar ist.

Literatur-Recherchen ergaben, daß bestimmte Aminosäuren bzw. die Substitution mit bestimmten Aminosäuren vorwiegend in thermophilen Proteinen vorgefunden werden. Beispielsweise fanden Menendez-Arias und Dekker (Menendez-Arias and Argos 1989; Dekker, Yamagata *et al.* 1991), daß das Arginin/Lysin Verhältnis in thermophilen Proteinen größer als in mesophilen ist, was als Hinweis für die stabilisierende Rolle des Arginins

gewertet wurde.

Im Vergleich zu Lysin besitzt Arginin ausgeprägtere Möglichkeiten zur Bildung von Salz-Brücken. Diese Ursache kann in der längeren Seitenkette des Arginins gefunden werden, die weiter entfernte Aminosäuren erreichen kann, sowie in der Möglichkeit zur Delokalisation der positiven Ladung aufgrund der Guanidinium Gruppe.

In dieser Arbeit wurde eine Suche nach der Sequenzhomologie zwischen der AChE aus *Torpedo californica*, der *Candida rugosa* Lipase und der *G. candidum* Lipase B durchgeführt, wodurch die übereinstimmenden Positionen von Arginin in der AChE und CRL bezüglich GCL B bestimmt wurden. Durch Struktur-Analyse sollte herausgefunden werden, in welchen Positionen der Lysin/Arginin Austausch möglicherweise zur Bildung von stabileren Salzbrücken führt, ohne gleichzeitig ein sterisches Hindernis zu erzeugen.

Drei Lysine wurden bestimmt, bei denen die längere Seitenkette des Arginins von Nutzen sein könnte, um Salzbrücken zu anderen Aminosäuren (in diesem Fall Glu, Asp) ausbilden zu können.

Die Mutationen K40R, K239R und K372R wurden in das Lipasegen von GCL B eingeführt und anschließend die mutierten Proteine in *Pichia pastoris* exprimiert. Die anschließenden Meßergebnisse zeigten jedoch, daß die Mutationen keinen Einfluß auf die Thermostabilität hatten. Tatsächlich bleiben die Thermostabilitätsänderungen innerhalb der Fehlerrate der Messungen und es wurden keine Unterschiede in der Inaktivierung der drei Mutanten und des Wildtyps festgestellt.

So führten die Arginine nicht zur gewünschten Stabilisierung, woraus geschlossen werden muß, daß die Ursache für das Entfalten des Proteins in anderen Bereichen der Lipase zu finden ist.

Als Alternative zur Erhöhung der Thermostabilität wurde GCL B auf verschiedenen Matrixen, mittels Absorption, ionischer und kovalenter Bindung, sowie durch Quervernetzung immobilisiert.

Mit jeder der verwendeten Matrixen wurde eine Erhöhung der Thermostabilität der Lipase B erzielt. Die Halbwertszeit des Enzyms bei einer Temperatur von 50 °C konnte von 30 Minuten für die gelöste Lipase auf bis zu mehreren Stunden für die immobilisierte gesteigert werden (Abb. 6).

Eine Steigerung der Ausbeute an immobilisierter Lipase konnte durch den Einsatz von weniger Enzym pro Matrixmenge und durch Zugabe des Lipase Substrates Triolein zum Immobilisierungsansatz erreicht werden.

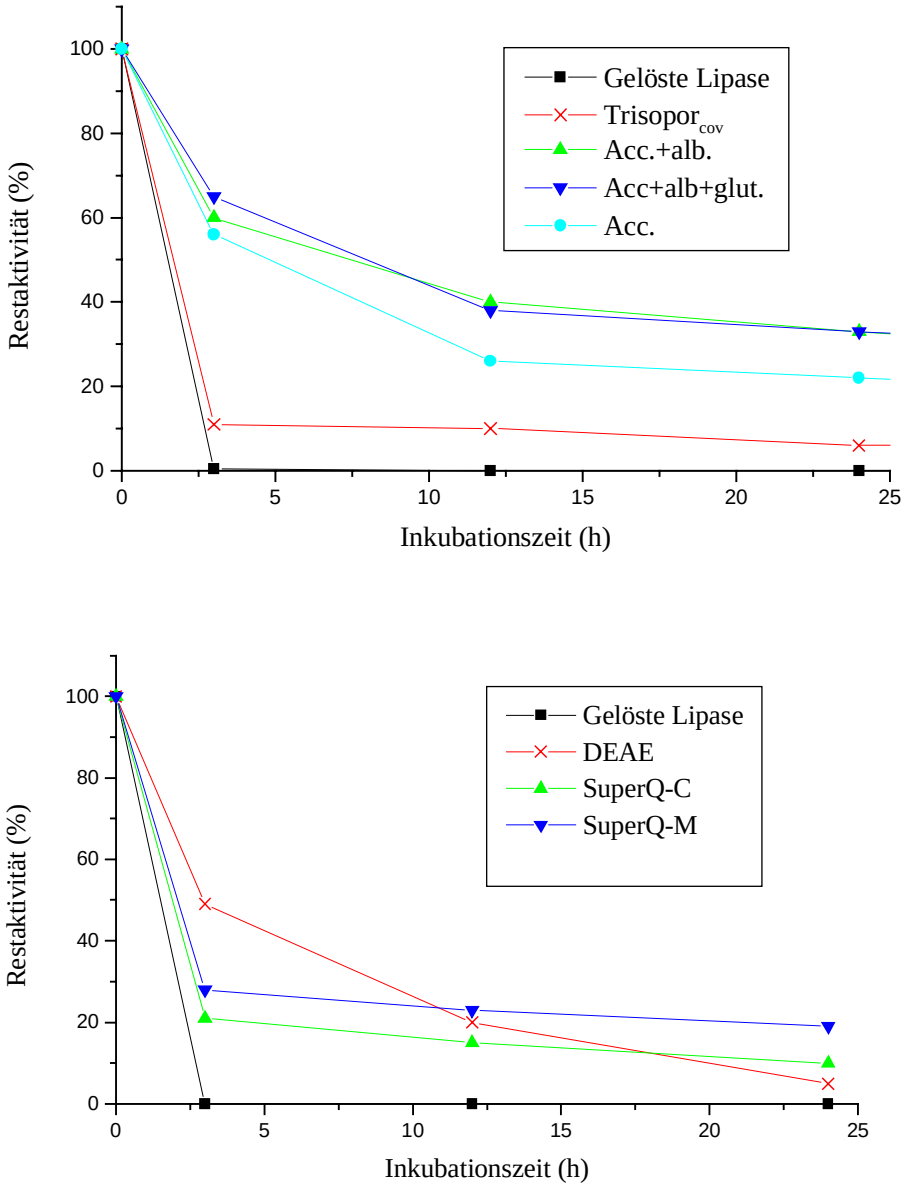



Abb. 6. Inaktivierung der auf verschiedenen Matrixen immobilisierten Lipase bei 50 °C im Vergleich zur Wildtyp-Lipase.

Die besten Ergebnisse wurden durch Immobilisierung auf einer mit Albumin vorbehandelten Polypropylenmatrix, Accurel, erhalten. In diesem Fall konnte die Halbwertszeit auf 5 Stunden gesteigert werden, ein um den Faktor 10 besserer Wert als bei der löslichen Lipase B.

Die Charakterisierung der auf Accurel + Albumin immobilisierten Lipase ergab ein unverändertes Temperaturoptimum bzgl. der nicht immobilisierten Lipase, wohingegen das pH-Optimum zu höheren Werten verschoben wurde. Die gelöste Lipase zeigt 100% relative

Aktivität im Bereich von pH 8.0-9.0, die immobilisierte nur bei pH 9.0.

Die Überexpression der Lipase B in *Pichia pastoris* ermöglicht zusammen mit der entwickelten Immobilisierungsmethode eine billige Nutzung des Enzyms in industriellem Maßstab. Beispielsweise kann die immobilisierte Lipase in einem kontinuierlich betriebenen Reaktor eingesetzt werden, so daß der Gehalt an gesättigten/ungesättigten Fettsäuren zur Modifikation der Zusammensetzung von Ölen in einem industriell interessanten Maßstab variiert werden kann.

## Abbreviations

| | |
|---|---|
| Å | Angström (= 0.1 nm) |
| AChE | Acetylcholinesterase |
| Ads | Adsorption |
| AOX | Alcohol oxidase |
| APS | Ammonium persulfate |
| bp | Base pairs |
| BPB | Bromophenol blue |
| BCA | Bicinchoninic acid |
| BSA | Bovine serum albumin |
| cov | Covalent immobilization |
| CRL | *Candida rugosa* lipase |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleoside triphosphate |
| DTT | Dithiotreitol |
| EDTA | Ethylendiamine tetraacetic acid |
| endo H | Endo-β-*N*-acetylglycosaminidase H |
| FA | Fatty acid |
| FFA | Free fatty acid |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GCL | *Geotrichum candidum* lipase |
| h | Hours |
| HEPES | 4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid |
| IEF | Isoelectric focusing |
| kb | Kilobases |
| kDa | Kilodalton |
| LU | Lipolytic unit |
| min | Minute(s) |
| 4-MUO | 4-Methylumbelliferyl oleate |
| OD | Optical density |
| ON | Overnight |

| | |
|---|---|
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| PEG | Polyethylenglycol |
| pI | Isoelectric point |
| PUFA | Polyunsaturated fatty acid |
| PVDF | Poly-vinylidenfluoride |
| rpm | Rotations per minute |
| RT | Room temperature |
| SAFA | Saturated fatty acid |
| SDS | Sodium dodecyl sulfate |
| TEMED | N,N,N',N'-Tetramethyl-ethylendiamine |
| TCA | Trichloroacetic acid |
| $T_m$ | Melting temperature |
| TE | Tris-EDTA |
| TR | Thermostability ratio |
| Tris | Tris-(hydroxymethyl)-aminomethane |
| U | Unit |
| Vol. | Volume |
| WT | Wild type |

# 1 INTRODUCTION

## 1.1 Lipases

Lipases (EC 3.1.1.3) are triacylglycerolester hydrolases, that catalyze the hydrolysis of triglycerides at the interface between the insoluble substrate and water (Fig.1). Apart from their natural substrates, such as water-insoluble esters and triglycerides, lipases catalyze the enantio- and regioselective hydrolysis and synthesis of a broad range of natural and synthetic esters (Bornscheuer 1995) (Santaniello, Ferraboschi *et al.* 1993).

In nature the lipases gain a very important role catalyzing the first step in the metabolism of fats and oils, which could not be burned off without being first hydrolyzed.

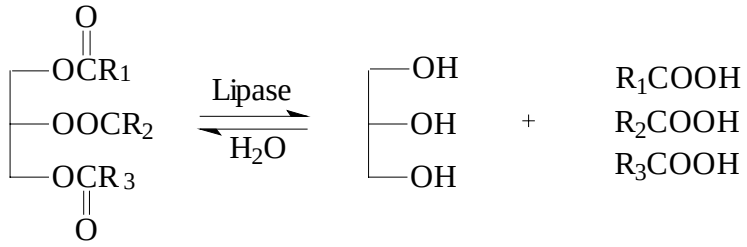



Fig. 1. Hydrolysis of a triglyceride catalyzed by a lipase.

Lipases from many different sources have been isolated, in the last few years the corresponding genes were also identified, and the amino acid sequences of more than 50 lipases from bacteria, fungi, mammalians, etc. were determined (Antonian 1988), (Khachatourians and Hui 1995).

The origins influence the structural characteristics of the lipases, giving rise to a catalyst class with a broad range of possible applications depending on the different substrate specificity. There are two kinds of substrate specificity: one regarding the hydrolyzed position in a triglyceride (regio-specificity) and one relative to the characteristics of the fatty acid released (substrate-specificity).

In the first case, there are lipases such as the one from *Rhizopus delemar* (Joerger and Haas 1993) which are 1,3-regio specific, that means they hydrolyze only the external fatty acids of a triglyceride; in contrast the lipase from *Penicillium expansum* is not specific and hydrolyzes the three positions of a triglyceride with the same velocity (Stöcklein, Sztajer *et al.* 1993). The lipase from *Penicillium camembertii* shows a different selectivity as it reacts only on mono- and diacylglycerols, whereas the one from *Bacillus stearothermophilus* reacts only on the

monoacylglycerols.

In the second kind of specificity the length of the available fatty acids determines whether a substrate will be hydrolyzed or not. For instance, the lipase from *Bacillus sp*. (Sugihara, Tani *et al.* 1991) prefers unsatured fatty acids with a middle chain length, and the lipases A and B from *Candida rugosa* prefer the short chain and the long chain fatty acids respectively (Rua, Diaz-Maurino *et al.* 1992). An unusual specificity is known for the lipase B from *Geotrichum candidum*, which reacts only on substrates with a *cis*- double bond in position 9, such as triolein.

### 1.1.1 Structure

Lipases with known three-dimensional structure span a wide range of molecular weight, from ~19 kDa (cutinase) to ~60 kDa (*Candida rugosa* lipase). All of them, with the exception of pancreatic lipases, consist only of one domain. The active site for lipases and for esterases is a catalytic triad formed by Ser-His-Asp/Glu (Cygler, Schrag *et al.* 1993) as in the serine proteases. The Ser is contained in a well conserved motif Gly-X-Ser-X-Gly, where X can be His or Tyr, located in a turn between a β-strand and an α-helix (Woolley and Petersen 1994); an exception was found for the lipases from *Bacillus subtilis* and *Bacillus pumilis* (Dartois, Baulard *et al.* 1992), where the first Gly is substituted by an Ala. The other two residues of the triad are also well conserved, although at least the acidic residue can move to a different sequence position without loss of activity (maintaining a similar relative orientation with respect to the His) (Rubin and Dennis 1997).

The three-dimensional structures of the first three lipases (*Rhizomucor miehei* lipase (Brady, Brzozowski *et al.* 1990), human pancreatic lipase (Winkler, D'Arcy *et al.* 1990), *Geotrichum candidum* lipase (Schrag, Li *et al.* 1991a, b) revealed that the catalytic triad is not exposed on the protein surface, as in the serine proteases, but is covered by surface loops and is not accessible to the substrate. The crystallization of the *Rhizomucor miehei* lipase with and without inhibitors (Brzozowski, Derewenda *et al.* 1991), and later of the *Candida rugosa* lipase with phosphonyl and sulfonyl inhibitors (Grochulski, Li *et al.* 1993; Grochulski, Bouthillier *et al.* 1994; Grochulski, Li *et al.* 1994), showed a large rearrangement of a single loop (lid or flap), relative to the uncomplexed structure. In aqueous media the predominant conformation for lipases is the closed form, with an unoccupied active site, which is shielded from the solvent by one or more loops forming the lid. The external part of the lid is hydrophilic, so this conformation is stabilized by electrostatic interactions. In a water-lipid

emulsion the movement of the lid allows the access to the active site for the lipolytic substrate (open conformation), exposes the nucleophilic serine and at the same time a large hydrophobic surface. This leads to the activation of the lipase.

Another common feature for lipases and many esterases is the α/β-hydrolase fold, consisting of a parallel β sheet and a number of helices that flank the sheet on both sides (Fig. 2).

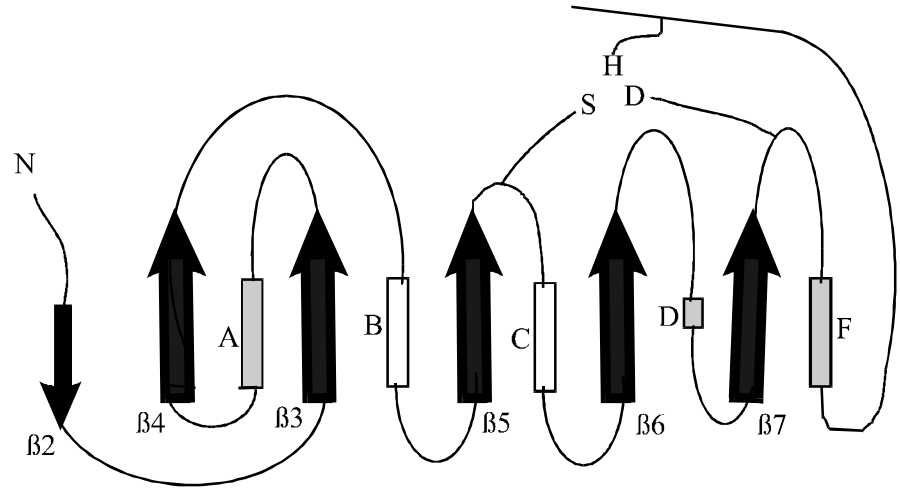



Fig. 2. The common lipase fold. Arrows indicate ß-strands and rectangles α-helices. Strands are numbered according to the nomenclature of the α/ß-hydrolase fold (Ollis, Cheah *et al.* 1992). The strands ß3-ß7 and helices B and C occur in all lipases, ß2 and helices A, D and F occur in most of them.

The minimal fragment of this fold common to all lipases (Ollis, Cheah *et al.* 1992) consists of 5 stranded β sheets and two α-helices. Helix A is present in all lipases except for those of the *R. miehei* family, and helix D is usually in the form of a distorted turn. The residues of the active site are positioned in the following way: Ser is located after strand β5, His after strand β8 and the acid amino acid after strand β7. The location of the lid is not conserved in this structure.

### 1.1.2 Mechanism of action

What distinguishes lipases from esterases is the so-called “interfacial activation”. The lipolytic activity is almost zero for substrate concentrations below the critical micellar concentration and increases rapidly for substrate concentrations above this value. This was explained by the movement of the lid and the following activation of the lipase, after contact with the hydrophobic substrate at the interface between water and lipid. In lipases not showing interfacial activation, such as the one from *Pseudomonas aeruginosa* (Misset,

Gerritse *et al.* 1994) or the pancreatic lipase from guinea pigs, the lid was found to be absent or a deletion in the lid-domain was present.

The hydrolysis of the fats starts with the formation of a tetraedrical serine-acyl intermediate, in which the carbonyl carbon of the substrate binds to the oxygen of the Ser of the active site. The nucleophilicity of the –OH group of the Ser is increased from the His, which accepts its proton when the Ser attacks the carbonyl carbon of the substrate (Fig. 3).

The resulting positive charge of the His is stabilized by the electrostatic interaction with the negative charge of the Asp (Stryer 1989). Properly located hydrogen bond donors, forming the so-called oxyanion hole, another important component for the lipolytic enzymes, help to stabilize this state in which the carbonyl oxygen bears a partial negative charge. In some lipases, such as the one from *Candida rugosa*, the oxyanion hole is already formed in the closed form, whereas in others, as in the one from *Geotrichum candidum*, the hole is present only in the open form, resulting from the movement of the external loops. The second phase, the deacylation, is the reverse of the first phase, with water substituting the alcohol (Fig. 3).

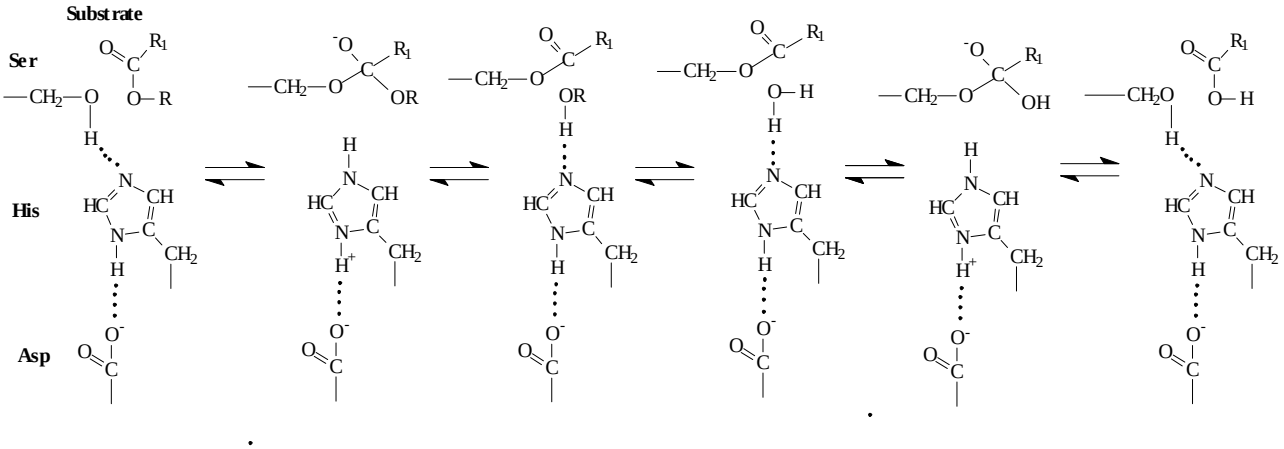



Fig. 3. Mechanism of action of a lipase, analogous to that of a serine protease.

### 1.1.3 Industrial applications of lipases

Lipases find a great application in the alimentary, cosmetic and pharmaceutical industry as well as in the detergents industry. The advantages of these biocatalyzed reactions with respect to the chemical processes are the substrate regio- and stereo specificity and the mild reaction conditions, which lead to a remarkable decrease in the down-stream processing and in the whole production costs. Besides, the wide spectrum of available lipases with variable

specificity allows the choice of a determined catalyst as a function of the desired reaction product (Ruttloff 1994).

For this reason the lipases gain more and more importance also in the organic synthesis (Björkling, Godfredsed *et al.* 1991; Harwood 1989).

Terpen alcohols, diols, polyalcohols and also acylamine can be esterified through lipase with different fatty acids giving rise to wax esters, emulgators, detergents or odorous and strongly flavored compounds (flavors esters) (Gillies, Yamazaki *et al.* 1987; Klibanov 1989; Langrand, Triantaphylides *et al.* 1988; Montet, Pina *et al.* 1989).

In the cheese production the hydrolysis of milk fat is very important for developing of the aroma; the fat is hydrolyzed by the natural lipases present in the milk or by addition of lipase preparations; the latter is especially used in the production of specially flavored cheeses, like Provolone, Gorgonzola, Roquefort and Cheddar (Arbige, Freund *et al.* 1986; Neshawy, Abdel-Baky *et al.* 1983; Luck and Haag 1991; Rothe, Ruttloff *et al.* 1986). The use of lipases is also very common for the improvement of the aromas of butter fat or cream and to produce concentrated aromas (Boudreaux 1987; Klibanov 1989).

Very important economically is the use of lipases in the production of cocoa-butter or cocoa-butter surrogates, which, starting from cheap substrates like palm oil, leads to an expensive product, that for its low melting point, is in high demand in the confectionery and pharmaceutical industry.

In the pharmaceutical industry, lipases are used for the asymmetric synthesis of chiral drugs; the enantiomers of a chiral drug may exhibit marked differences in biological activity, toxicity, drug elimination and metabolism. Two examples are the production of β-blockers, containing an aryl-oxi-propanolaminic group, from which only the S-enantiomer is active and the enantiomerically pure lipolytic hydrolysis of cyclopentendiol derivatives in the synthesis of prostaglandines.

### 1.1.4 Lipase from *Geotrichum candidum*

The fungus *Geotrichum candidum* produces two different extracellular lipases. According to the strain these lipases are called A and B from *Geotrichum candidum* CMICC 335426 and I (homologous to B) and II from *Geotrichum candidum* ATCC 34614 (Sidebottom, Charton *et al.* 1991).

Although the sequence homology between the different lipases is very high (84% between lipase A and B, and 97 % between lipase B and I), they exhibit a different substrate specificity

(Charton and Macrae 1992). Particularly, lipase B and I from strains CMICC 335426 and ATCC 34614, respectively, show a remarkably high specificity for *cis*-Δ9 unsaturated substrates having long fatty acyl chains. This specificity seems to be even more pronounced in the case of lipase B (Sidebottom, Charton *et al.* 1991; Charton and Macrae 1992; Bertolini, Schrag *et al.* 1995).

The structural basis of the different substrate preferences of *G. candidum* lipase B is still obscure, although, while this work was carried out, Holmquist *et al.* (Holmquist, Tessier *et al.* 1997) identified some residues essential for differential fatty acyl specificity of *Geotrichum candidum* lipases I and II at the active site entrance (Ile357/Leu358) and at the bottom (Cys379/Ser380) in the active site cavity of GCL I. The replacement in GCL I of these residues by their equivalent amino acids from GCL II (Ala357/Phe358/Phe379/Phe380) led to a specificity profile very similar to that of GCL II. The reverse mutations in GCL II recovered only a fraction of the specificity observed in GCL I, showing that there are other residues, not yet identified, which contribute to the peculiar substrate specificity of lipase I and B.

Concerning the regio-specificity all the lipases from *Geotrichum candidum* cleave both the inside and outside ester bonds of triglycerides indiscriminately.

In 1989, the lipase genes from *Geotrichum candidum* ATCC34614 were identified, cloned and the amino acid sequence was deduced (Shimada, Sugihara *et al.* 1989; Shimada, Sugihara *et al.* 1990). In 1994, the sequence of the lipases from other *Geotrichum candidum* strains was also determined (Bertolini, Laramée *et al.* 1994). The proteins consist of 544 amino acids, with a total relative molecular mass of about 60 kDa, preceded in the unprocessed form by a signal sequence of 13 amino acids.

A sequence alignment of *G. candidum* lipases and other lipases revealed no overall sequence similarity, except for the one from *Candida rugosa* (45% overall homology). However, the amino acid sequence showed remarkable sequence similarity to two esterases, the *Torpedo californica* acetylcholinesterase, and the human cholinesterase (Slabas, Windust *et al.* 1990): 30% homology was found in the central area and about 70% homology in a 28 amino acids region around the active site Ser. Besides, the catalytic triad Ser-His-Glu, the G-X-S-X-G consensus sequence, the acidic residues adjacent to the catalytic site and the salt bridges that stabilize the surface loops are conserved in these proteins. As for the disulfide bridges, two are conserved in the same position for these homologous proteins, while a third at the C-terminus of the *Torpedo californica* AChE was not present in the lipases from *Geotrichum candidum* and *Candida rugosa*.

The three-dimensional structure of a lipase from *Geotrichum candidum* was first reported with a low resolution in 1979 (Hata, Matsuura *et al.* 1979) and later with higher resolutions (1.8 Å) (Schrag, Li *et al.* 1991a; Schrag, Li *et al.* 1991b; Schrag and Cygler 1993).

The lipase consists of a single domain molecule, with $\alpha/\beta$ structure with eight stranded $\beta$-sheets in which six consecutive strands are parallel and six helices pack against the sheet.

A feature of the lipases from *C. rugosa* and *G. candidum* that differs from the other lipases is the shape of their binding sites. Whereas in other lipases the scissile acyl chain lies on the surface of the protein, the polypeptide chain folds over this site in the case of *C. rugosa*, forming a deep tunnel, lined with hydrophobic residues and penetrating toward the center of the molecule (Grochulski, Bouthillier *et al.* 1994; Ghosh, Wawrzak *et al.* 1995; Rubin and Dennis 1997). The mouth of this tunnel is located near the catalytic Ser. In the closed conformation the end of the tunnel is covered by the lid.

The production in high yields of the specific lipase B is very important from an industrial point of view. In fact, there is a growing interest in low fat products, and preferably products and liquid oils low in saturated fatty acid content (Napier 1997). As shown in a recent study, the replacement of satured fatty acids by mono- and polyunsatured ones is more effective in preventing coronary diseases in women than is the reduction of the overall fat intake (Hu, Stampfer *et al.* 1997). Commercially available oils such as sunflower oil and soy bean oil generally contain 11-15% saturated fatty acids; the use of the selective lipase B from *Geotrichum candidum* offers one way of modifying the oil fatty acid profile: the cheap sunflower oil can be selectively hydrolyzed to produce a very low saturated fatty acid concentrate. After separation from the partial glyceride fraction, these unsaturated free fatty acids can be converted into triglycerides by a non specific lipase-catalyzed esterification.

## *1.2 Random and site-directed mutagenesis*

In the last few years, random mutagenesis (directed evolution) has emerged as an alternative to the rational approach for engineering an enzyme for a wide range of applications, especially if the protein structure is not known or if the mutations, causing the improvement of the desired protein, cannot be predicted from the protein structure.

Like the natural evolution of proteins, directed evolution alternates between creation of diversity and selection. The selection should be simple enough, that it can be carried out over several generations, represented in this case by cycles of mutagenesis and selection. Multiple cycles are necessary since it is statistically unlikely to find a collection of favorable mutations

in a single round. Thus, a stepwise approach should be used, just like in nature allowing evolution over many generations. This technique was applied successfully in many cases to enhance the thermostability, the stability or activity in artificial environments (Moore and Arnold 1996; Moore, Jin *et al.* 1997; Kano, Taguchi *et al.* 1997), to change the substrate specificity or the enantioselectivity of a catalyst (Zhang, Dawes *et al.* 1997).

Error-prone PCR has been extensively used to introduce point mutations randomly in the DNA, according to the protocol of Leung *et al.* (Leung, Chen *et al.* 1989); it provides control of mutagenic rates by varying the concentration of $Mn^{2+}$ and dNTPs. The DNA-shuffling, developed by Stemmer (Stemmer 1994a; Stemmer 1994b), consists of creating new libraries by recombining genes with different combinations of mutations derived from a set of homologous natural sequences; for this method, the availability of two or more enzymes with an overall homology of more than 80% is required. Other methods to introduce random mutations are: chemical mutagenesis, UV irradiation, mutator strains or poisoned nucleotide analogues, but these methods cannot be limited to a gene or to a region of it. As the mutations will be introduced in the whole plasmid, it is necessary to reclone the gene in a new plasmid.

The mutagenic rate is, of course, very important for the success of the directed evolution and can be determined by parameters such as the proportion of clones that are inactive.

The other mutagenesis technique, the site-directed, has provided in the past enormous insight into the architecture and design of proteins by allowing the change of specific amino acids and testing their influence on the parameter of interest, which may include stability, folding kinetics or functional properties of the protein.

### *1.3 Immobilization*

The first attempt to immobilize a biocatalyst dates back to 1953 (Grubhofer and Schleith 1953), while in 1969 an immobilized enzyme was used for the first time in an industrial process; since then this technique has gained more and more importance, and now a wide variety of immobilized enzymes are employed in the food, pharmaceutical and chemical industries (Katchalski-Katzir 1993). To immobilize means to greatly restrict the freedom of movement of an enzyme with respect to the soluble enzymes; this causes a series of advantages (Benjamin and Pandey 1998):

- Possibility of separation of the catalyst from the reaction mixture and its reuse
- Possibility of continuous processes in reactors also with different enzymes in series
- Products can be easily separated

- Higher stability of the catalyst
- Positive changes in the enzyme features (pH and T optimum)
- Process is more cost effective.

In fact the incremental costs of using an immobilized biocatalyst in a continuous process are more than 20 times lower than those with a traditional one, arising primarily from the cost of the relatively large amount of non-reusable enzyme required by the latter process.

The main disadvantages can be:

- Loss of activity due to immobilization
- Limitation in substrate's diffusion
- Possible leakage of the biocatalyst from the support.

### 1.3.1 Immobilization methods

**Adsorption**

It is the simplest method and involves reversible surface interactions between enzyme and support material (Messing 1976; Woodward 1985). The forces involved are mostly hydrophobic. Among the advantages are: it is a cheap, fast and simple process; no chemical changes to support or enzymes are necessary; it is a reversible immobilization. Disadvantages are the leakage of the enzyme from the support, the possible steric hindrance by the support and the nonspecific binding. Inorganic (active carbon, silica, etc.) or organic (natural or synthetic polymers) compounds can be used as supports.

**Ionic binding**

It is based on electrostatic interactions (ionic and hydrogen bonding) between differently charged ionic groups of the matrix and of the enzymes (Ruttloff 1994). The advantages and disadvantages are the same of the adsorption process, but through ionic binding the enzyme conformation is influenced more than through adsorption and less than through covalent binding. Different anion exchange materials, like DEAE-Cellulose or DEAE-Sephadex are used as matrices.

**Covalent binding**

This method is based on the formation of covalent bonds between a support material and some functional groups of the amino acid residues on the surface of the enzyme (usually -$NH_2$ of lysine or arginine, -COOH of aspartic or glutamic acid, -OH of serine or threonine and -SH of cysteine) (Messing 1976; Cabral and Kennedy 1991; Srere and Uyeda 1976). Usually, the

support has to be first activated by a specific reagent, to make its functional groups strongly electrophilic; these groups are then allowed to react with strong nucleophilic groups of the enzyme. The reaction involved is usually the formation of isourea or diazo linkage, a peptide bond or an alkylation reaction, and must be carried out under mild reaction conditions to avoid deactivation of the enzyme. The advantage of this method is the strength of the bond and the consequent stability of the immobilization; the disadvantages are the higher costs and the lower yields, as the enzyme conformation and of course activity will be strongly influenced by the covalent binding.

**Cross-linking**

This type of immobilization is support-free and involves joining the enzymes to each other to form a three-dimensional structure (Broun 1976). The bond is formed by means of a bi- or multifunctional reagent, such as glutardialdehyde or toluene diisocyanate. The disadvantages are the very low immobilization yields, the absence of mechanical properties and the poor stability.

**Entrapment**

Using this method, the enzyme is free in solution, but restricted in movement by the lattice structure of a gel (Bickerstaff 1995; O'Driscoll 1976). There are different methods for the entrapment such as temperature induced gelation, polymerization by chemical/photochemical reaction, ionotropic gelation of macromolecules with multivalent cations. Entrapment is mainly used for immobilization of cells, but has the inevitable disadvantage that the support will act as a barrier to mass transfer.

**Encapsulation**

Encapsulation of enzymes (or cells) can be achieved by enveloping the biological components within various forms of semipermeable membranes, usually microcapsules varying from 10-100 µm in diameter (Groboillot, Boadi *et al.* 1994; Broun 1976). Large proteins cannot pass out of or into the capsule, but small substrates and products can pass freely across the semipermeable membrane. A disadvantage is the acute diffusion problem, an advantage can be the coimmobilization of different enzymes/cells to suit particular applications.

Bioreactors with lipases immobilized on different matrices have been used for hydrolysis, transesterifications, ester synthesis, alcoholysis reaction (for a review see Balcao *et al.* (Balcao, Paiva *et al.* 1996)) for the production of different products of industrial interest. The lipases were immobilized by various methods, such as adsorption, entrapment and covalent

binding using different supports. The yields of active immobilized lipases so far reported are considerably lower than those reported for many other enzymes, being often only a few percent (Mustranta, Forssell *et al.* 1993). Immobilized lipases were often used for the hydrolysis of triacylglycerides (Charton and Macrae 1993; Kimura, Tanaka *et al.* 1983; Brady, Metcalfe *et al.* 1988; Pronk, Boswinkel *et al.* 1992; Pronk, Kerkhof *et al.* 1988; Kang and Rhee 1989; Kang and Rhee 1989).

### *1.4 Aim of the work*

The focus of this work was on the high yield expression of active lipases A and B from *Geotrichum candidum* as single isoenzymes.

In the past few years there have been many attempts at separating lipase A from B and the I from II (Sugihara, Shimada *et al.* 1990; Veeraragavan, Colpitts *et al.* 1990; Phillips and Pretorius 1991; Hedrich, Spener *et al.* 1991; Baillargeon and McCarthy 1991; Jacobsen, Olsen *et al.* 1990; Jacobsen and Poulsen 1992; Sugihara, Shimada *et al.* 1994), but having these close physical and biochemical properties, the purification was complicated and time consuming. It was understood that the cloning and expression of the single lipase genes would be the easiest and most reliable way of obtaining the pure isoforms which also allows their substrate specificities to be studied.

In this work, *Saccharomyces cerevisiae* and *Pichia pastoris* were chosen as expression systems for the lipases A and B from *Geotrichum candidum*. Both being organisms generally recognized as safe (GRAS), they allow the utilization of the expressed proteins also in the alimentary field.

For industrial processes it would be of advantage to have a more stable catalyst, because this would allow the use of higher temperatures which impart such benefits as increased substrate solubility, decreased viscosity of the medium, lower risk of microbial contaminations or higher rates of concurrent non-enzymatic reactions.

The second part of this work was then aimed at enhancing the thermostability of lipase B, which is, from an industrial point of view, more interesting than lipase A, due to its peculiar specificity. An improvement of the thermostability of an enzyme can be obtained through different techniques such as protein immobilization or mutagenesis of the appropriate gene. Three different approaches were used in this work to get a more stable enzyme: random mutagenesis, site-directed mutagenesis and protein immobilization.

# 2 MATERIALS AND METHODS

## *2.1 Instruments*

| **Used Instrument** | **Characteristics** | **Company** |
|---|---|---|
| Agarose gel electrophoresis | DNA Sub Cell™, Mini Sub™ DNA Cell, Mini Sub™ Cell GT | BioRad |
| | Video Copy Processor P66E | Mitsubishi |
| | BWM 9X Monitor | Javelin Electronics |
| | UV-lamp table | MWG-Biotech |
| Balances | Basic, MC1 Research RC 210 D | Sartorius |
| | Precision Advanced | OHAUS® |
| Centrifuges | Eppendorf Centrifuge 5417 C | Eppendorf |
| | Eppendorf Centrifuge 5415 R | Eppendorf |
| | Eppendorf 5810 R | Eppendorf |
| | Universal 30 F | Hettich |
| | KR 22 i (Rotor: AK 500-11, 155 mm) | Jouan |
| | G412 | Jouan |
| | Sorvall RC - 5B (Rotor: SA 600) | Du Pont Instruments |
| DNA-Sequencer | 373A DNA Sequencer | Applied Biosystems |
| Elektroporation | Gene Pulser®, Pulse Controller | BioRad |
| Fermentor | Labfors 5l und 2 l | Infors HT |
| Fluorimeter | Fluostar | BMG |
| FPLC | LKB PumpP-500 | Pharmacia |
| Homogenisator | Ultra-Turrax T25 | Janke & Kunkel |
| Incubators | HT - Incubator (30°C, 37°C) | Infors AG |
| | Certomat R Incubator (42°C) | Braun |
| | WTE | Binder |
| | UM 500 | Memmert |
| Freeze dryer | Lyovac GT 2 | Finn-Aqua |
| Microscope | Axiolab E | Zeiss |
| Mixer-Mill | MM 2000 | Retsch , Haan |

| | | |
|---|---|---|
| PAGE | Minigel-Twin G42<br>Model 583 Gel Dryer<br>Phast System | Biometra®<br>BioRad<br>Pharmacia |
| PCR | Mastercycler Gradient<br>DNA Thermocycler<br>Hybaid Omnigene<br>Robocycle® Gradient 40 | Eppendorf<br>Perkin Elmer<br>MWG-Biotech<br>Stratagene® |
| pH-Meter | Digital pH Meter pH525 | WTW |
| pH-Stat | DL21<br>DG-111SC<br>LX-400 | Mettler-Toledo,<br>Urdorf, Switzerland<br>Epson |
| pH-Stat | Impulsomat 614, pH-Meter 620,<br>Dosimat 665<br>Water bath B3<br>X/Y-Printer L200 | Metrohm<br>Metrohm<br>Haake-Fisons<br>Linseis |
| Pipetting Robot | Biomek 2000 | Beckman |
| Power Supplier | Power Pac 3000, Power Pac 300,<br>Model 200/2.0 Power Supply | BioRad |
| Protein Blot | Trans Blot SD | BioRad |
| Protein Sequencer | 491 Protein Sequencer<br>140 C Microgradient System<br>Power Macintosh 7200/90 | Applied Biosystems<br>Applied Biosystems<br>Apple Macintosh |
| Sonifier | Sonifier 250<br>Sonorex Super RK 514 H | Branson<br>Bandelin |
| Spectrophotometer UV/VIS | Ultrospec 3000 | Pharmacia Biotech |
| Thermomixer | Thermomixer 5436 | Eppendorf |

The structures comparison was carried out on a Silicon Graphics Workstation, Indigo 2, using the program Insight II (MSI, San Diego, USA). The structures were obtained from the Protein Data Bank (PDB) (Bernstein, Koetzle *et al.* 1977), using the entries: 1THG (Schrag and Cygler 1993) for GCL B, 1LPN (Grochulski, Bouthillier *et al.* 1994) for CRL and 1ACK (Harel, Schalk *et al.* 1993) for AChE.

## *2.2 Plasmids and Microorganisms*

| **Organisms** | **Relevant Genotype / Phenotype** | | **Company** |
|---|---|---|---|
| *Escherichia coli* DH5α | $F^-$ *end*A1 *hsd*R17($rk^-$, $mk^+$) *sup*E44 *thi*-$\lambda^-$ *gyr*A96 *rel*A1 Δ(*arg*F- *laczya*)U169 | | BRK |
| *Epicurian Coli XL1-Blue* supercompetent cells | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI$^q$ZΔM15 Tn10(Tet$^r$)]* | | Stratagene |
| *Saccharomyces cerevisiae* INVSC2 | *his3, ura3* | | Invitrogen |
| *Saccharomyces cerevisiae* WCG4a | *Matα, his3-11, 15leu2-3, 112 ura3* | | (Heinemeyer, Gruhler *et al.* 1993) |
| *Pichia pastoris* GS115 | *his 4* $Mut^+$ | | Invitrogen |
| **Plasmid** | **Relevant Marker** | **Induction** | **Company** |
| pYES2 | Ampicillin resistance Ura3 | Galactose | Invitrogen |
| pPICZαC | Zeocin resistance | Methanol | Invitrogen |
| pGAPZαC | Zeocin resistance | Constitutive expression | Invitrogen |

### *2.3 Enzymes, Antibiotics, Antibodies*

| | |
|---|---|
| Ampicillin (sodium salt) | Fluka Chemie, Buchs, Schweiz |
| Polyclonal antibodies against the lipase from *Geotrichum candidum* | Kind gift of Unilever, Colworth House, England |
| Anti-rabbit IgG-POD | Boehringer Mannheim, Mannheim, Germany |
| Klenow fragment polymerase | MBI Fermentas, St. Leon-Rot, Germany |
| Calf intestine alkaline phosphatase | MBI Fermentas, St. Leon-Rot, Germany |
| T4-DNA-ligase | BioLabs New England, Beverly, MA USA<br>Boehringer Mannheim, Mannheim, Germany |
| Vent/Taq/Pfu-DNA-polymerases | BioLabs New England, Beverly, MA USA<br>Stratagene, Heidelberg, Germany |
| Restriction enzymes | BioLabs New England, Beverly, MA USA<br>Boehringer Mannheim, Mannheim, Germany |
| Zeocin | Invitrogen, Leek, The Netherlands |

### *2.4 Chemicals, Biochemicals, etc.*

| | |
|---|---|
| Accurel EP100 | Enka AG, Obernburg, Germany |
| 30% (m/v) acrylamide in water with 0.8% bisacrylamide | Roth, Karlsruhe, Germany |
| Agarose | Serva Feinbiochemika GmbH, Heidelberg, Germany |
| Aluminium-oxide-60 | Merck, Darmstadt, Germany |
| Asahi Pan hemofilter | Asahi Medical, Tokyo, Japan |
| BCA protein assay reagent | Pierce, BA oud-Beijerland, NL |
| Black microtiter plates (FluoroNunc Module, MaxiSorp Surface) | Nunc, Wiesbaden, Germany |
| Cellulose acetate and nitrocellulose filters | Sartorius AG, Göttingen, Germany |
| Chemicals for DNA-sequencing | Roth GmbH, Karlsruhe, Germany |
| Chemicals for protein sequencing | Applied Biosystems, Weiterstadt, Germany |
| Chemicals for preparation of media | Difco, Augsburg, Germany |
| Gel extraction kit | Qiagen, Hilden, Germany |

| | |
|---|---|
| Filter for microdialyses (0.025 µm, 13 mm) | Millipore, Bedford, MA, USA |
| Matrices and columns for protein purification | Pharmacia BioProcess Technology AB, Uppsala, Sweden |
| Matrices for anion exchange chromatography used for protein immobilization | Toso Haas, Stuttgart, Germany |
| Membranes (PVDF, nitrocellulose) and filter paper for blotting | BioRad, Hercules, CA, USA |
| 4-Methylumbelliferyl oleate | Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany |
| Microtiterplates (96 wells) | Greiner GmbH, Frickenhausen, Germany |
| Mini and Midi plasmid kit | Qiagen, Hilden, Germany |
| Organic solvents | Merck, Darmstadt, Germany |
| Phast-Gels: gel buffers, standard | Pharmacia LKB, Freiburg, Germany |
| Primers | MWG, Ebersberg, Germany<br>ARK, Darmstadt, Germany |
| QuikChange Site-Directed Mutagenesis Kit | Stratagene, Heidelberg, Germany |
| Robot tips | Beckman, CA, USA |
| Rotiphorese NF-10XTBE | Roth, Karlsruhe, Germany |
| Sigma lipase substrate | Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany |
| Standard for agarose gels | Gibco BRL GmbH, Eggenstein, Germany |
| Standard for SDS-PAGE | Bio-Rad, München, Germany |

All other chemicals were obtained from Fluka, Neu-Ulm.

### *2.5 Oligos used for the PCR reactions*

| Oligo name | Use | Sequence |
|---|---|---|
| GCLA1 | Sequencing lipase A | 5'-CACTTTGCTTGACAAAGC-3' |
| GCLA2 | Sequencing lipase A | 5'-TATCATAAAGGGGACCTC-3' |
| GCLA3 | Sequencing lipase A | 5'-GATTCCTGGGTGGTGATG-3' |
| GCLA4 | Sequencing lipase A | 5'-TTGCGCTGGTCGTGCAGA-3' |
| GCLA5 | Sequencing lipase A | 5'-ATTTGCTCAGTATGCCGG-3' |

| | | |
|---|---|---|
| GCLA6 | Sequencing lipase A | 5’-ACTTGCTGCGGAGACACTC-3’ |
| GCLA7 | Sequencing lipase A | 5’-GTTAAGAAGTGGTTGCAG-3’ |
| GCLA8 | Sequencing lipase A | 5’-TACAGCGACAAAACACG-3’ |
| GCLA9 | Sequencing lipase A | 5’-CAATGTGAACATTGGCCC-3’ |
| GCLA10 | Sequencing lipase A | 5’-TCATGGTGGTTGGCAAAG-3’ |
| GCLB1 | Sequencing lipase B | 5’-GCCTTTTCTTTGCTTGAC-3’ |
| GCLB2 | Sequencing lipase B | 5’-ATAAAGAGGGCCTCTAAG-3’ |
| GCLB3 | Sequencing lipase B | 5’-ATTCTTGGGTGGTGATGC-3’ |
| GCLB4 | Sequencing lipase B | 5’-TCGTGCAGACCAGCGTTG-3’ |
| GCLB5 | Sequencing lipase B | 5’-GATTTGCTCAGTATGCCG-3’ |
| GCLB6 | Sequencing lipase B | 5’-ACTTGCTGCGGAGACAAG-3’ |
| GCLB7 | Sequencing lipase B | 5’-CCCCATGTTAAGAAGTGG-3’ |
| GCLB8 | Sequencing lipase B | 5’-ACAAAACACGATCAAGCG-3’ |
| GCLB9 | Sequencing lipase B | 5’-ATACTACGTGGACCTTGG-3’ |
| GCLB10 | Sequencing lipase B | 5’-TGGTGGTTGGCAAACGAG-3’ |
| PYES1 | Sequencing | 5’-CGTGAATGTAAGCGTGAC-3’ |
| PYES2 | Sequencing | 5’-GCAGCTGTAATACGACTC-3’ |
| AOX1 | Sequencing | 5’-GACTGGTTCCAATTGACAAGC-3’ |
| Pme | PCR for cloning | 5'-CTG ACA GTT TAA ACG CTG TCT TGG-3' |
| Prepro | PCR for cloning | 5'-RAGAACGGCCGTGGGGGCCGCTTCAGCCTC TCTYTTCTCGAG-3' |
| Pre | PCR for cloning | 5'-RAGAACGGCCGTGGGGGCCGCTAATGCGGA GGATGCTGC-3' |
| Beginning | Random mutagenesis | 5’-AGCGGCCCCCACGGCCGTTCTTAATGGCAAC GAGGTC-3’ |
| End | Random mutagenesis | 5’-ACCGAAGAGAGTAACGTCAG-3’ |
| S430C | Point mutations | *Bse*RI site destroyed<br>5’-CACTGATTTGCTGTTCCAG(TGTCCTCGTC)GTGTTATGC-3’<br>cys |
| S430Crev | Point mutations | 5’-GCATAACACGACGAGG**ACA**CTGGAACAGCA AATCAGTG-3’ |

| | | |
|---|---|---|
| I533C | Point mutations | *Bst*BI site introduced<br>5’-CGACTTTAGAATCGAGGGA**TGT**TCGAACTTT<br>GAGTCTGACG<br>cys |
| I533Crev | Point mutations | 5’-CGTCAGACTCAAAGTTCGA**ACA**TCCCTCGAT<br>TCTAAAGTCG-3’ |
| L393A/V | Point mutations | 5’-GATCGTGTTTTGTCG**G(C/T)T**TACCCCGGCT<br>CTTGGTCG-3’<br>ala/val |
| L393A/Vrev | Point mutations | 5’-CGACCAAGAGCCGGGGTA**A(A/G)C**CGACAAA<br>ACACGATC-3’ |
| L393Q | Point mutations | 5’-GATCGTGTTTTGTCG**CAA**TACCCCGGCTCTT<br>GGTCG-3’.<br>gln |
| L393Qrev | Point mutations | 5’-CGACCAAGAGCCGGGGTA**TTG**CGACAAAAC<br>ACGATC-3’ |
| N535A/V | Point mutations | 5’-GAATCGAGGGATGTTCG**G(C/T)T**TTTGAGTCT<br>GACGTTA-3’<br>ala/val |
| N535A/Vrev | Point mutations | 5’-GTAACGTCAGACTCAAA**A(A/G)C**CGAACATC<br>CCTCGATTC-3’ |
| K40R | Point mutations | 5’-GGTGACTTGCGGTTC**AGA**CACCCCCAGCCT<br>TTCACTG-3’<br>arg |
| K40Rrev | Point mutations | 5’-CAGTGAAAGGCTGGGGGTG**TCT**GAACCGCA<br>AGTCACC-3’ |
| K239R | Point mutations | 5’-GACAACACCTACAACGGA**AGA**CAGCTTTTC<br>CACTCTGCC-3’<br>arg |
| K239Rrev | Point mutations | 5’-GGCAGAGTGGAAAAGCTG**TCT**TCCGTTGTAG<br>GTGTTGTC-3’ |
| K372R | Point mutations | 5’-CCACTACTCCCCATGTT**AGA**AAGTGGTTGA<br>AGTACATTTG-3’<br>arg |
| K372Rrev | Point mutations | 5’-CAAATGTACTTCAACCACTT**TCT**AACATGGG<br>GAGTAGTGG-3’ |

## *2.6 Molecular-biological methods*

### 2.6.1 Isolation of plasmid-DNA from *Escherichia coli* with Mini/Midi-Prep kit (Qiagen)

For preparation of extremely pure plasmid DNA to be used for PCR, sequencing, restriction analysis, etc., were utilized these kits, based on the lysis of cells in a basic environment (Birnboim and Doly 1979), followed by specific binding of the DNA to ion-exchange columns.

After separation of the cells from an overnight culture by centrifugation, the plasmid isolation was done according to the instructions of the manufacturer.

### 2.6.2 Mini-plasmid extraction for fast tests

For applications which do not need pure nor much plasmid DNA (analytical digestions of plasmids, etc.) the FlexiPrep method was used (Birnboim and Doly 1979).

The cells were collected from 1.5 ml of ON-culture by centrifugation for 30 sec at 14000 rpm. The cell pellet was resuspended in 200 µl of solution 1 (Tris-HCl, pH 7.5, 100 mM; EDTA 10 mM; RNase I 400 µg/ml); the cells were then lysed by addition of 200 µl solution 2 (NaOH 1 M; SDS 5.3% w/v) and mild mixing. After adding 200 µl of solution 3 ($K^+$ 3 M, $Ac^-$ 5 M) and inverting the tube more times, the proteins and the genomic DNA were precipitated and collected by centrifugation (5 min, 14000 rpm, RT). The plasmid DNA, remaining in the supernatant, was precipitated with isopropanol, and then resuspended in 20 µl TE-buffer (10 mM Tris-HCl buffer pH 7.5, 0.1 mM EDTA).

### 2.6.3 Precipitation of plasmid DNA with ethanol

1 vol. of DNA solution was mixed with 1/10 vol. 3 M NaAc, pH 4.8 and 2.5 vol. ethanol (100%, -20 °C); the solution was vortexed, kept at –20 °C for 20 min (alternative: -70 °C) to allow the precipitation. After centrifugation (14000 rpm, 30 min, 4 °C) and removal of the supernatant, the pellet was washed with ethanol (70%, -20 °C), then dried and resuspended in TE-buffer.

### *2.6.4* Precipitation of plasmid DNA with isopropanol

0.7 vol. isopropanol was added to 1 vol. of the DNA solution. Centrifugation (14000 rpm, 15 min, RT) followed 15 minutes precipitation at RT. The supernatant was removed and the

pellet washed with ethanol (70%, -20°C), dried and resuspended in TE-buffer.

### 2.6.5 Electrophoresis to separate DNA fragments

The agarose-gel-electrophoresis was carried out according to the protocol reported in Maniatis *et al.* (Sambrook, Fritsch *et al.* 1989). Depending on the molecular weight of the DNA fragments to separate, 0.8-2% gels were used. The buffer TAE (40 mM Tris/HCl, 20 mM acetic acid, 2 mM EDTA, pH 8.3) was used as electrophoresis buffer, and a mixture of glycerine 30% (m/v), bromophenolblue (BPB) 0,2% (m/v), EDTA 25 mM, pH 7.5 was used as 6x-DNA-loading buffer. The voltage applied was 120 V for 1% gels and 100 V for 2% to avoid generation of high temperatures. The DNA was made visible by ethidium bromide staining, and the bands on the gels were photographed under an UV-lamp or cut from the gel.

### 2.6.6 DNA-isolation from an agarose gel with QIAquick gel extraction kit

The extraction of the DNA from the agarose gel was carried out according to the instructions of the manufacturer. The method is based on the specific binding of the DNA on a silica gel matrix.

### 2.6.7 DNA digestions and ligations

The DNA digestions with restriction enzymes were performed under the reaction conditions specific for every enzyme, as suggested by the manufacturers.
To transform sticky in blunt ends, the Klenow enzyme was used, following the instructions of the manufacturers.
To avoid self-ligation of vectors cut only with an enzyme, the 5‘-phosphate groups were eliminated by treatment with alkaline phosphatase, according to the manufacturer’s instructions.

### 2.6.8 Automatic DNA sequencing

For the DNA sequencing, the 373A DNA Sequencer and the Dye Terminator Cycle Sequencing Kits with AmpliTaq-DNA-Polymerase (Applied Biosystems) were used, based on the enzymatic chain terminator technique, developed by Sanger (Sanger, Nicklen *et al.* 1977). The four ddNTPs are incorporated during the PCR in the amplified DNA fragments. They were not radioactive- but dye-labeled, and could be detected from specific emission after excitation through 2 argon lasers with emission bands of 488 and 514 nm (Prober, Trainor *et*

*al.* 1987; Freeman and Baehler 1990).

The PCR mixture was composed of the Dye Terminator Cycle Sequencing Kit and the AmpliTaq®DNA Polymerase, FS as follows:

| | |
|---|---|
| Terminator Ready Reaction Mix | 8 µl |
| Sequencing primer | 3,2 pmol |
| DNA | 300 -500 ng |
| $ddH_2O$ | ad 20 µl |

The PCR consisted of the following steps:

| **Steps** | **Denaturation** | **Annealing** | **Extension** | **Cycle number** |
|---|---|---|---|---|
| 1 | 96 °C, 1 min | - | - | 1 |
| 2 | 95 °C, 40 sec | 55 °C, 40 sec | 72 °C, 4 min | 25 |
| 3 | - | - | 72 °C, 4 min | 1 |

The PCR products after precipitation with ethanol were resuspended in 4 µl formamid: EDTA, 25 mM, pH 8.0 = 4 : 1, and before loading them onto the gel, were denatured for 5 minutes at 95 °C.

The gel was prepared by mixing 30 g urea with 6 ml Rotiphorese® NF-10x-TBE-buffer, 9 ml acrylamide/bis 40% (29:1) and 23,5 ml $ddH_2O$. To allow the polymerisation 24 µl TEMED and 180 µl 10% (m/v) APS were added, after degassing and filtration of the mixture.

### 2.6.9 Polymerase chain reaction (PCR)

The PCRs were carried out in order to amplify a specific DNA fragment (Mullis, Faloona *et al.* 1986). For the standard PCR the components listed below were pipetted in a PCR test tube:

| **Component** | **Final concentration** |
|---|---|
| 10x polymerase buffer | 1x |
| dNTP-Mix (each 2,5 mM) | 0,2 mM |
| Primer a | 100 pmol |
| Primer b | 100 pmol |
| Template DNA | ≅ 0.1 µg |
| DNA-polymerase (Vent, Taq or Pfu) | 1 or 2 U |
| $MgCl_2$ 25 mM (for the Taq polymerase) | 2 mM |
| $ddH_2O$ | to 50µl |

The cyclic reaction, composed of three steps (denaturation at 95 °C, annealing, extension at 72 °C), was repeated up to 25 times in a Thermocycler.

The optimal parameters, the annealing temperature in particular, had to be determined empirically depending on the fragment to be amplified. For standard PCR the annealing temperature was set at 55 °C.

### 2.6.10 Error prone PCR

Error prone PCR was carried out to generate random mutations throughout the length of the amplified fragment.

Low fidelity conditions, as described by Leung *et al.* (Leung, Chen *et al.* 1989) were used:

3 µl template (5-15 ng)
1 µl primer 1 (50 pmol/µl)
1 µl primer 2 (50 pmol/µl)
4 µl Taq polymerase (1 U/µl)
8 µl $dNTP_{mix}$ (0.25 mM for one of the four NTPs and 2.5 mM for the other three)
10 µl Taq buffer (10X)
8 µl $MgCl_2$ 25 mM
65 µl $ddH_2O$

The following PCR cycle was repeated 25 times, after an initial denaturation step at 95 °C for 4 minutes:

| | |
|---|---|
| 1.5 min | 95 °C |
| 1.5 min | 50 °C |
| X min | 72 °C |

The extension temperature depends on the length of the fragment to be amplified, and is usually 1 minute per kb. 4 PCR reactions were carried out, using a different nucleotide in defect each time. The DNA from the reaction mixture was precipitated with sodium acetate (1/10 vol.) and ethanol 100% (2.5 vol.) and then resuspended in water. The desired product was extracted from a gel, in order to remove the template. The fragment obtained in this way was used for ligations or for direct transformation in yeast.

### 2.6.11 Quik-change kit for site-directed mutagenesis

The point mutations were introduced in the genes using the QuikChange Site-Directed Mutagenesis Kit, Stratagene, according to the manufacturer's instructions.

## *2.7 Microbiological methods*

### 2.7.1 Media

For *Escherichia coli*:

**LB**

| | |
|---|---|
| Trypton | 10 g/l |
| Yeast extract | 5 g/l |
| NaCl | 5 g/l |
| Agar (for plates) | 14 g/l |

When required 100 mg/l ampicillin (filter sterilized) or 25 mg/l Zeocin was added for selection.

For *Saccharomyces cerevisiae*:

**YEPD Medium**

| | |
|---|---|
| Glucose (separately autoclaved) | 20 g/l |
| Peptone | 20 g/l |
| Yeast extract | 10 g/l |
| Agar (for plates) | 14 g/l |

**Synthetic Medium**

| | |
|---|---|
| Glucose | 20 g/l |
| Yeast nitrogen base (filter sterilized) | 6.7 g/l |
| Amino acids | |
| (depending on the used vector and strain) | 50 mg/l |
| Phosphate buffer | 0.1 M, pH 6.0 |
| (Agar for plates) | 14 g/l |

Galactose (20 g/l) was added instead of glucose to induce the production of the recombinant protein when the pYES2 vector was used.

For *Pichia pastoris*:

**YEPG and YEPM**

| | |
|---|---|
| Glycerol (separately autoclaved) | 10 g/l |
| Peptone | 20 g/l |

| | |
|---|---|
| Yeast extract | 10 g/l |
| Potassium phosphate buffer | 0.1 M, pH 6.0 |
| Agar (for plates) | 14 g/l |

For YEPM 0.5 % methanol was added instead of glycerol.

When required 100 mg/l Zeocin was added for selection.

**YEPDS**

| | |
|---|---|
| Glucose (separately autoclaved) | 20 g/l |
| Peptone | 20 g/l |
| Yeast extract | 10 g/l |
| Sorbitol | 182 g/l |
| Agar | 14 g/l |
| Zeocin | 100 mg/l |

**BMGY and BMMY**

| | |
|---|---|
| Glycerol (separately autoclaved) | 10 g/l |
| Peptone | 20 g/l |
| Yeast extract | 10 g/l |
| Biotin (filter sterilized) | 0.4 mg/l |
| Potassium phosphate buffer | 0.1 M, pH 6.0 |
| Yeast nitrogen base (filter sterilized) | 13.4 g/l |
| Agar (for plates) | 14 g/l |

For BMMY 0.5 % methanol was added instead of glycerol.

**Synthetic Medium for *P. pastoris'* fermentation**

Batch:

| | |
|---|---|
| Glycerol | 31 g/l |
| Methanol | 1.0 g/l |
| $NH_4Cl$ | 7.63 g/l |
| $K_2HPO_4$ | 0.59 g/l |
| $KH_2PO_4$ | 2.81 g/l |
| Egli vit | 2.00 g/l |
| Egli met | 10.00 g/l |
| $MgSO_4*7H_2O$ | 0.59 g/l |
| Histidine | 0.04 g/l |
| Structol (antifoam) | 0.40 g/l |

Feed:

| | |
|---|---|
| Glycerol | 341.0 g/l |
| Methanol | 85.0 g/l |
| $CaCl_2$ | 1.0 g/l |

| | |
|---|---|
| $(NH_4)_2SO_4$ | 7.63 g/l |
| $K_2HPO_4$ | 4.0 g/l |
| $KH_2PO_4$ | 4.0 g/l |
| Egli Vit. | 4.0 g/l |
| Egli Met. | 16.0 g/l |
| $MgSO_4$*7 $H_2O$ | 6.0 g/l. |

**Egli vit**: 0.05 g/l biotin, 5.0 g/l thiamine, 47.0 g/l myo-inositol, 1.2 g/l pyridoxine, 23 g/l D-pantothenic acid. The pH was adjusted to 4.0 with NaOH 6.0 N.
**Egli met**: 60 g/l Titriplex EDTA*2$H_2O$, 11.0 g/l $CaCl_2$*2$H_2O$, 7.5 g/l $FeSO_4$, 2.8 g/l $MnSO_4$, 2.7 g/l $ZnSO_4$, 90 g/l $CuSO_4$, 0.9 g/l $CoCl_2$, 0.5 g/l $NaMoO_4$, 0.5 g/l KI, 6.8 g/l $H_3BO_3$. The pH was adjusted to 4.0 with NaOH 6.0 N.

### 2.7.2 Plates with tributyrin

To identify the production of lipase from the different yeast clones directly from the plates, 10 ml tributyrin were emulsified with an Ultraturrax in 1 l medium before autoclaving.
The plates were poured, and the yeast was plated on them. The presence of secreted lipase is shown through the appearance of a transparent halo around the positive clones.

### 2.7.3 Transformation of *Escherichia coli*

#### *2.7.3.1 Transformation by heat shock*

To produce competent cells, 50 ml of LB medium were inoculated 1:100 with an ON culture and shaken at 37 °C until they reached an $OD_{578}$ =0.5-0.7. The cells were collected by centrifugation (10 minutes, 3600 rpm, 4 °C), resuspended in 2 ml TSS solution (10% PEG 6000, 5% DMSO, 50 mM $MgSO_4$, in LB-Medium) and incubated on ice for 5 minutes. 200 µl of this suspension were aliquoted in test tubes, to which the DNA to be transformed was added. After 20 minutes on ice, the cells were subjected to heat shock at 42 °C for 45 seconds, before addition of 1 ml LB and 36 µl of 20% glucose and incubation for 1 hour at 37 °C. The cells were then spread on selective plates.

#### *2.7.3.2 Transformation by electroporation*

1 l of cell culture was grown until it reached an $OD_{578}$=0.5-1.0. The flask was kept on ice for 15-30 minutes and the cells were harvested by centrifugation in a cold rotor at 4000 xg for 15 minutes. The pellet was resuspended in 1 l of a cold, low ionic strength wash medium such as HEPES 1 mM, pH 7 or water, and then harvested again by centrifugation. The pellet was

resuspended in 0.5 l of cold wash medium and centrifuged again. It was then resuspended in 20 ml of 10% glycerol, centrifuged again and resuspended to a final volume of 2 to 3 ml in 10% glycerol. The cell concentration should be about $3x10^{10}$ cells/ml. The cells were placed on ice. 40 µl of the cells suspension were mixed with the DNA and kept on ice for 1 minute. The DNA was in precedence dialysed for 1 hour on small filters (Millipore) against water in order to reduce the ionic strength of the mixture to be electroporated. The transformation of the cells was carried out, employing the Gene Pulser Electroporator (BioRad) with the following parameters: 25 µF of capacity, 1.5 kV and a resistance of 200 Ω. The mixture of cells and DNA was transferred to a cold 0.2 cm electroporation cuvette and an electric pulse was given. After the pulse the cells were grown in LB medium for 1 h and then spread on selective plates.

### 2.7.4 *Saccharomyces cerevisiae* transformation

Different transformation protocols were applied, using two *S. cerevisiae* strains: INVSC2 ($ura^-$, $his^-$) and WCG4a ($ura^-$, $leu^-$, $his^-$). Different media were also used to plate the cells after transformation: complex medium (without uracil) with glucose or galactose (2 g/l dropout powder, 1.7 g/l yeast nitrogen base without aminoacids, 5 g/l ammonium sulphate, 20 g/l sugar), minimal medium with glucose or galactose (yeast nitrogen base 7 g/l, sugar 20 g/l) and minimal medium with galactose and tributyrin (yeast nitrogen base 7 g/l, sugar 20 g/l, 10 ml/l tributyrin).

The dropout powder was composed of: 2.0 g L-alanine, 2.0 g L-arginine, 2.0 g L-asparagine, 2.0 g L-aspartic acid, 2.0 g L-cysteine, 2.0 g L-glutamine, 2.0 g L-glutamic acid, 2.0 g L-glycine, 2.0 g myo-inositol, 2.0 g L-isoleucine, 2.0 g L-methionine, 2.0 g L-phenilalanine, 2.0 g L-proline, 2.0 g L-serine, 2.0 g L-threonine, 2.0 g L-valine, 0.2 g PABA.

Uracil, adenine, tryptophan, histidine, lysine, leucine were missing and were added depending on the strain used.

#### *2.7.4.1 Transformation of yeast with lithium acetate and dimethyl sulfoxide (Hill, Donald et al. 1991)*

The yeast cells were grown in 100 ml YEPD until they reached an $OD_{600}$=1-2, and then were harvested by 2 minutes centrifugation at 3600 rpm. The pellet was washed with 10 ml lithium acetate and centrifuged again. The cells were resuspended in 1 ml lithium acetate, and 100 µl

of this suspension were mixed to circa 5 µg of DNA. After 5 minutes at room temperature, 280 µl of a PEG 4000 (50%) solution were added to the cells and the suspension was incubated for 45 minutes at 30 °C. 43 µl of DMSO were added and a heat shock for 5 minutes at 42 °C was performed. The cells were then harvested by centrifugation and spread on plates of selective medium.

#### *2.7.4.2 Transformation of yeast with lithium acetate (Gietz and Schiestly 1995)*

The yeast cells were grown in 50 ml YEPD until they reached an $OD_{600}$=1-2, and then harvested by 5 minutes centrifugation at 3600 rpm. The pellet was washed twice with water and once with 0.1 M lithium acetate pH 7.5 in a TE solution (10 mM Tris, 1 mM EDTA pH 7.5). The pellet was then resuspended in 250 µl 0.1 M lithium acetate pH 7.5 in TE, and 50 µl of this suspension were mixed to circa 1 µg of DNA, 20-40 µg salmon sperm and 300 µl 50% PEG 3350 in a LiAc/TE solution (0.1 M LiAc, 10 mM Tris, 1 mM EDTA, pH 7.5). The suspension was incubated for 30 minutes at 30 °C and then for 20 minutes at 42 °C. The cells were then harvested by centrifugation and spread on plates of selective medium.

#### *2.7.4.3 Saccharomyces cerevisiae Easy-Comp transformation kit (Invitrogen)*

The kit was used according to the manufacturer's instructions.

#### *2.7.4.4 Electro-transformation of Saccharomyces cerevisiae and Pichia pastoris*

For the preparation of competent cells, 200 ml YEPD were inoculated with approximately 200 µl of an ON culture and were grown for about 24 hours at 30 °C till $OD_{600}$ = 1.3 – 1.5. The cells were then collected by centrifugation at 4000 rpm for 5 minutes, the pellet was washed twice with 200 ml cold, sterile water and once with 40 ml cold, sterile sorbitol 1 M, and finally resuspended in 400 µl of cold sorbitol. The cells were divided in aliquots of 80 µl each, mixed with 5-10 µg plasmid DNA, transferred to electroporation cuvettes (2 mm) and kept for 5 minutes on ice.

The electroporation was then carried out with a resistance of 200 Ω, a capacity of 25 µF and a potential difference of 1500 V. Immediately after electroporation 1 ml of cold sorbitol was added to the cells, and they were incubated for 2 hours at 30 °C before plating on selective medium.

Before transforming the plasmid DNA in *Pichia pastoris*, it was linearized, using *Pme*I when the vector pPICZαC and *Avr*II for pGAPZαC, in order to facilitate the integration in the genomic DNA.

### 2.7.5 Expression of recombinant proteins in *Pichia pastoris* GS115

*Pichia pastoris* is a methylotrophic yeast, which means it is able to grow on methanol as a sole carbon source, producing an alcohol oxidase (AOX) able to oxidise the methanol to formaldehyde as the first step of the alcohol metabolism. The production of AOX is regulated by a methanol inducible promoter, and corresponds to 30% of the total cellular proteins produced by the cells. This same promoter is present in the vector pPICZαC to regulate the expression of the heterologous proteins. When this vector was used, the cells were grown first in BMGY until they reached $OD_{600}$=2–6, and then transferred in BMMY to induce the production of the recombinant protein. Each day 0.5% of methanol was added to the culture to replace what had been consumed.
In the case of the vector pGAPZαC, containing the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene, the expression is constitutive, so that the recombinant protein is continuously produced when the cells are grown in YEPD, without need of induction.

### 2.7.6 Yeast cell extracts

The following method was used both for *Saccharomyces cerevisiae* or for *Pichia pastoris*. The breakage of the cells wall occurs mechanically, due to violent shaking of the cells in the presence of glass beads.
10 ml of an overnight culture were harvested by centrifugation, transferred to an Eppendorf tube, and resuspended in 500 µl PBS buffer (8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$ were dissolved in 1 l water and the pH was adjusted to 7.4). About 500 mg of glass beads (diameter: 1.0-1.5 mm) and 0.01 mg/ml pepstatin to inhibit the protease action were also added. The cell breakage was carried out with a „mixer mill“ (Retsch), for 30 minutes, frequence 1800 $min^{-1}$, amplitude 100 (Schütte and Kula 1988), through vibrational movements. The soluble cellular components were in this way released. The samples were then centrifuged for 5 minutes and the supernatants used for further analysis.

### 2.7.7 Yeast DNA isolation

1 ml of cell extract was treated with 0.15 ml SDS-TE buffer (2% SDS, 100 mM Tris-HCl pH 9, 10 mM EDTA) for 5 minutes at 60-65 °C, 0.15 ml 5 M KAc were added, the samples were incubated on ice for 30 minutes and then centrifuged for 5 minutes at full speed. The supernatant was transferred to a new tube and the DNA precipitated with NaAc and

isopropanol. The pellet, collected by centrifugation, was then washed with 80% ethanol, dried and redissolved in 50 µl TE-RNase (100 µg/ml) pH 8.

The extracts obtained from *Saccharomyces cerevisiae*, containing the genomic and the plasmid DNA could be directly used for transformation of *E. coli*, for the reisolation of the plasmid.

Reisolation of the plasmid of transformed *Pichia pastoris* is not possible as it integrates in the genome.

### 2.7.8 PCR on DNA extracted from yeast

To test if the yeast clones, inoculated from the transformation plates, contain the gene coding for the recombinant protein a PCR was carried out, using two primers at the extremities of the gene to be amplified and as template the DNA isolated from yeast.

The following cycle was repeated 25 times:

1.5 min 95 °C
1.5 min 55°C
1.5 min 72 °C.

Loading the PCR mixture on a gel, a band corresponding to the length of the gene will be seen if the transformants inoculated contain the right gene.

### 2.7.9 Glycerin cultures in microtiter plates

The yeast cells were grown in microtiter plates in 100 µl of medium in each well for two days at 30 °C. 100 µl of glycerol were then added to each well, and the plates were immediately frozen at –80°C until use.

### 2.7.10 Fermentations

#### *2.7.10.1 Cultivations in 1 l scale*

The cultivations of *Pichia pastoris* producing lipase B from *Geotrichum candidum* were carried out in a 1 l bioreactor at 30 °C. The cultivation was maintained at constant pH by adding 2 M HCl or 2 M NaOH. Samples were taken at constant intervals and were assayed for lipolytic activity, $OD_{600}$ and wet-weight.

- Activity assay

The lipolytic activity of the supernatant was determined at the pH-stat using triolein as

substrate, at 37 °C, pH 7.5.

- Wet-weight

The wet weight was measured after centrifuging 2 ml culture, discarding the supernatant, washing the pellet with an isotonic solution (0.7% NaCl), weighing the vessel with the pellet and subtracting from the value obtained the weight of the empty vessel.

#### *2.7.10.2 Cultivations in 6 l scale*

The cultivations were carried out in a 15 l bioreactor filled with a maximum of 6 l medium. The cultivation was maintained at constant pH by adding 2 M HCl or 2 M NaOH. Samples were taken automatically every three hours and immediately cooled and kept at 4 °C before being assayed for lipolytic activity, $OD_{600}$ and dry-weight.

- Activity assay

The activity assay was based on the hydrolysis of triglycerides by the enzyme. 1 ml of the Sigma lipase substrate (olive oil triglycerides) was mixed with 29 ml buffer pH 9.0 (0.005 M Tris, 0.04 M NaCl, 0.02 M $CaCl_2 \cdot 2H_2O$). A suitable volume of sample solution (min 1 µl, max 1 ml) was added, and the amount of fatty acids released at 30 °C, was titrated with 0.05 M NaOH.

In this way, one unit of lipase activity is defined as the amount of enzyme liberating 1 µmol of fatty acid per minute from a triglyceride substrate at pH 9.0, 30 °C.

- Dry-weight

The dry-weight was measured by centrifuging the samples, washing the pellet with an isotonic solution (0.7% NaCl), drying it at 110 °C for 4 hours and then weighing it.

## *2.8 Protein methods*

### 2.8.1 Polyacrylamide gel electrophoresis (PAGE)

For polyacrylamide gel electrophoresis under denaturing conditions, according to Laemmli (Laemmli 1970), 12.5% gels were usually employed. The stacking gel (4%) was prepared with:

| | |
|---|---|
| 30% (m/v) acrylamide in water with 0.8% bisacrylamide | 0.52 ml |
| 4x stacking gel buffer (Tris 12.11 g, SDS 0,8 g, $ddH_2O$ ad 200 ml, pH 6.8 with HCl) | 1.00 ml |
| $ddH_2O$ | 2.47 ml |

4 µl TEMED and 40 µl APS 10 % (m/v) were added to start the polymerization.

The resolving gel (12,5 %) was prepared in the same way:

| | |
|---|---|
| 30% (m/v) Acrylamide in water with 0.8% Bisacrylamide | 3.33 ml |
| 4x resolving gel buffer (Tris 36.46 g, SDS 0.8 g, $ddH_2O$ ad 200 ml, pH 8.8 with HCl) | 2.00 ml |
| $ddH_2O$ | 2.67 ml |
| TEMED | 4 µl |
| APS 10 % (m/v) | 40 µl |

The electrode buffer was composed of:

| | |
|---|---|
| Tris | 6.0 g |
| Glycin | 28.8 g |
| SDS | 2.0 g |
| $ddH_2O$ | ad 2 l |
| pH 8,3. | |

The protein samples (around 10-20 µg of protein) were mixed 1:1 with the SDS-loading buffer (Tris 100 mM, DTT 200 mM, SDS 4%, BPB 0.2%, Glycerin 20%), denatured for 5 minutes at 95 °C, cooled on ice and then loaded on the gel.

The standard was a mixture of different proteins of well known molecular weight, as reported in the table below:

| **Protein** | **Molecular weight** |
|---|---|
| Phosphorylase B | 97.4 kDa |
| Serum albumin | 66.2 kDa |
| Ovalbumin | 45.0 kDa |
| Carbonic anhydrase | 31.0 kDa |
| Trypsin inhibitor | 21.5 kDa |
| Lysozyme | 14.0 kDa |

The proteins were separated at 24 mA of current for each gel for 1 hour. The gel was stained for 1 hour with a Coomassie solution (0.1% Coomassie brilliant blue R250, 30% methanol, 10% acetic acid), and then treated for 1-2 hours with the destaining solution (30% methanol, 10% acetic acid). To conserve the gel, it was dried at 80 °C under vacuum.

### 2.8.2 PAGE with the Phast-System

The Phast-System from Pharmacia was used to separate smaller amounts of protein (10 - 50 ng) under denaturing and native conditions, and to determine the isoelectric point. It was

carried out following the manufacturer's instructions. The gels were automatically silver stained (Butcher and Tomkins 1986) with the Phast-gel staining unit.
The Coomassie staining solution for Phast gels was composed of a 0.1% PhastGel Blue R solution in 30% methanol and 10% acetic acid in distilled water; the destaining solution consisted of 30% methanol and 10% acetic acid.

### 2.8.3 Western blotting

For the protein transfer on nitrocellulose membranes a semidry blot apparatus (Bio-Rad) was used. After SDS-PAGE, the gel and a nitrocellulose membrane of the same size of the gel were equilibrated for 30 minutes in a transfer buffer (20% methanol, 192 mM glycine, 25 mM Tris-HCl pH 8.3). Four pieces of filter paper were also soaked in the same buffer, and two of them were put on the bottom of the instrument, corresponding to the anode. The membrane was then laid on these two first layers, then the gel and at the end the other two filter papers, always without air bubbles. The apparatus was closed with the cover corresponding to the cathode. 15 V for 30 minutes were then applied, in order to let the proteins, bound to the SDS, move to the anode, that means to the membrane. At the end of the blotting, the membrane was saturated for 30 minutes in 1% (m/v) BSA in TS buffer (0.85% NaCl, 10 mM Tris-HCl pH 7.5). The reaction of the specific proteins with the antibody against the lipase from *Geotrichum candidum* occurred ON at 4 °C, followed by a washing of the membrane two times with 0.3% Tween 20 in TS for 20 minutes and three times with TS for 10 minutes. The membrane was then incubated with 2 U of the secondary antibody (anti-rabbit IgG-POD) conjugated to a horse-radish peroxidase in 1% albumin/TS for 1 hour at RT, and then washed three times with TS buffer. The proteins which bound specifically to the antibody were put in evidence as blue spots on the membrane, after reaction with 5.6 mM 4-chloro-1-naphtol (dissolved in methanol), 0.6 M citrate phosphate buffer pH 6.0 and 3.4 mM $H_2O_2$.

### 2.8.4 Automatic N-terminal sequencing of proteins

The determination of the N-terminal protein sequence was done according to the method of Edman (Edman 1950). The amino-terminal amino acids were chemically released from the rest of the protein, and as phenylthiodantoin derivative identified through their retention time in a connected HPLC. The use of an automatic sequencer (Protein Sequencers 491, Applied Biosystem) allows the repetition of this cycle up to 50 times.

The protein to be sequenced was first subjected to SDS-PAGE in order to separate it from other proteins and blotted on a PVDF membrane (Matsudaira, 1987).

Three filter papers of the same size as the gel were incubated for 5-10 minutes in three different buffers (anode buffer 1: Tris 0.3 M, methanol 10% (v/v), pH 10.4; anode buffer 2: Tris 25 mM, methanol 10% (v/v), pH 10.4; cathode buffer: Tris 25 mM, 6-aminocapronic acid 40 mM, methanol 10% (v/v), pH 9.4). The PVDF membrane was incubated for a few seconds in methanol 100%, and then for 2-3 minutes in anode buffer 2. All the material was arranged in the blotting device in the following way:

**Lid = Cathode**

5. Filter in cathode buffer
4. SDS-Gel
3. PVDF-Membrane
2. Filter in anode buffer 2
1. Filter in anode buffer 1

**Bottom = Anode**

15 V were applied for 20-30 minutes between the two poles, and, after the blotting, the membrane was incubated for a few seconds in the staining solution (Coomassie Blue R-250 0.025% (m/v), methanol 40% (v/v), and then in the destaining solution (methanol 50%).

The blotted membrane was washed with water, dried, and the desired protein band was cut and kept at –20 °C until sequenced.

### 2.8.5 Determination of protein concentration

The determination of protein concentration was carried out using the "BCA Protein Assay Reagent Kit" (Pierce). The method involves the reaction of proteins with $Cu^{2+}$ in alkaline medium giving $Cu^{+}$ ions which form a complex with bicinchoninic acid (BCA) that absorbs at 562 nm. The assay was carried out following the manufacturer's instructions allowing the detection of protein concentrations between 5 and 250 mg/ml.

### 2.8.6 Protein precipitation

To concentrate the proteins before loading them on the gels, 1 volume of the protein solution was precipitated with 1 volume of 48% TCA. After 5 minutes of centrifugation, the pellet was washed three times with acetone, air-dried and then resuspended in the desired buffer.

### 2.8.7 Endo-β-N-acetylglycosaminidase H digestion

Protein samples were incubated for 12 h with endo-β-N-acetylglycosaminidase H (25 mU/mg protein) at 30 °C in a 50 mM potassium acetate buffer, pH 5.5, containing 0.5 mM phenylmethylsulphonyl fluoride to prevent proteolysis. For the deglycosylation of denatured samples, the protein was first incubated with 0.01% (w/v) SDS at 95 °C for 3 min.

### 2.8.8 Determination of the lipase activity at the pH-Stat

Lipase activity was routinely measured using triolein as substrate. 20 mM triolein were emulsified in distilled water, containing gum arabic (20 mg/ml) as stabilizer, using a homogenizer (Ultraturrax T25) for 7 min at maximum speed. 20 ml of the substrate solution were heated to 37 °C and adjusted to pH 7.5. After the addition of 10-200 µl of the enzyme solution the pH, which tends to drop for the production of oleic acid due to the enzymatic hydrolysis of triolein, was kept constant by addition of NaOH 0.01 M with a pH-Stat. As every mole of freed fatty acid will be titrated with 1 mole of NaOH, connecting the system to a printer which reports the NaOH consumed with the time, the lipase activity can be calculated. One unit was defined as the amount of enzyme which released 1 µmol fatty acid per minute.

The activity is calculated with the following formula:

$$A = C_{NaOH} \cdot f_x \cdot f_y \cdot \frac{1}{V_E} \cdot \frac{\Delta x}{\Delta y}$$

A [$U \cdot ml^{-1}$] = Activity

$C_{NaOH}$ [$mol \cdot l^{-1}$] = NaOH concentration

$f_x$ [$\mu l_{NaOH} \cdot mm^{-1}$] = Parameter correlated to the consume of NaOH

$f_y$ [$mm \cdot min^{-1}$] = Parameter correlated to the reaction time

$V_E$ [ml] = Volume of NaOH consumed

$\Delta x/\Delta y$ [$mm \cdot mm^{-1}$] = Slope of the curve NaOH consumed as function of the time

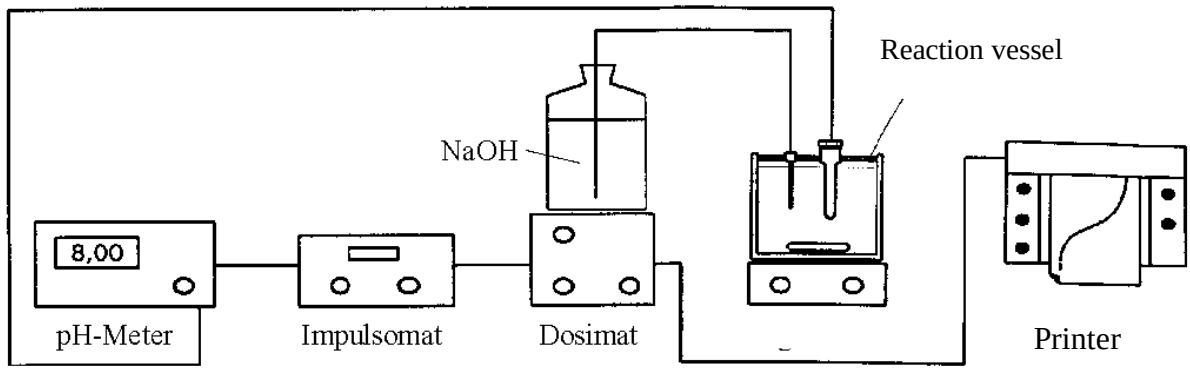



Fig. 4. Schematic representation of the pH-Stat.

For each measurement, the autohydrolysis of the triolein in the measuring conditions was tested and subtracted from the obtained value. The autohydrolysis plays a role especially at higher temperature and pH.

### 2.8.9 Determination of the substrate specificity

The method was based on the hydrolysis of an oil by the lipase at pH 9.0 at 30 °C in a pH-Stat. The oil contained triglycerides with a roughly even distribution of saturated fatty acids with chain length from C8 to C20 plus C18 unsaturated. Randomization of the acids over all the triglycerides and all positions was achieved by chemical interesterification with sodium methoxide as catalyst. The fatty acids liberated by the lipase during the assay reaction were titrated with NaOH 0.1 M. The enzyme reaction was stopped at calculated low conversions (<10%) by adding concentrated phosphoric acid. The products were separated by extraction with diethylether/heptane (1:1). The organic layer was collected and evaporated under nitrogen. An aminopropyl column was used to separate the liberated fatty acids from the substrate oil. The composition of the liberated fatty acids was determined by GC analysis, as reported below.

#### *2.8.9.1 GC analysis*

GC set-up

| | Prepressure | FID Pressure |
|---|---|---|
| $H_2$ | 3.5 bar | 1.3 bar |
| Air | 4.0 bar | 1.9 bar |

$N_2$ 3.5 bar 1.9 bar

He (carrier) 4.0 bar

Capillary column (CP-FFAP-CB): length = 25 m; internal diameter = 0.53 mm; FD = 1.00 µm

Column flow: 11.8 ml/min

Sample volume: 1 µl

Detector temperature: 270 °C

Injector temperature: 250 °C

Oven program

Temperature 1: 110 °C

Time 1: 0 min

Rate 1: 20 °C/min

Temperature 2: 250 °C

Time 2: 18 min

The free fatty acids were identified by using the retention times of the free fatty acids from a reference standard. The area data were provided from the integrated chromatogram. The response was corrected according to the response factor of each fatty acid (Table 1). The response factors were analyzed with the reference standard for every batch.

Table 1. Response factor of FID detector for the different fatty acids.

| Fatty acid chain length | Common name | Response factor |
|---|---|---|
| C8:0 | Caprylic acid | 0.9412 |
| C10:0 | Capric acid | 1.0177 |
| C12:0 | Lauric acid | 0.9248 |
| C16:0 | Palmitic acid | 0.9457 |
| C18:0 | Stearic acid | 0.9496 |
| C18:1 | Oleic acid | 0.9203 |
| C18:2 | Linoleic acid | 0.9261 |
| C18:3 | Linolenic acid | 0.8714 |
| C20:0 | Phytanic acid | 0.8714 |

The amount of the single released fatty acid was calculated using the formula:

$$\textbf{FFA (mg)} = \frac{\text{area of component X mass internal standard}}{\text{area of internal standard X response factor}}$$

The total of all corrected area values were renormalized to 100% to give the % weight of each

FFA in the reaction mixture.

### 2.8.10 Effect of temperature and pH on lipase stability and activity

The effect of pH on the lipase activity was determined by pH-stat assay at 37 °C using triolein as substrate, as reported above. The effect of pH on lipase stability was determined by incubating aliquots of lipase solution at 30 °C in Britton Robinson buffers at different pHs. Residual activity was measured by pH-Stat assay at different incubation times.
The optimum temperature for enzyme activity was determined using triolein as substrate, at pH 7.5 and various temperatures. The effect of temperature on lipase stability was determined by incubating aliquots of lipase solution in Britton Robinson buffer pH 8.0 at various temperatures. Residual activity was measured by pH-Stat assay at different incubation times.

### 2.8.11 Immobilization techniques

#### *2.8.11.1 Adsorption*

The supernatant of a lipase expressing culture of *Pichia pastoris* was used for all the immobilization procedures. The lipase activity of the solution, measured at pH 8.0, 37 °C on triolein, was 3600 U/ml. For the immobilization, the matrix was incubated overnight with mild stirring at room temperature or at 4 °C with the lipase solution. The pH was buffered with phosphate buffer 10 mM, pH 7.0, and in some cases 1% triolein was added to the immobilization mixtures. If not otherwise specified, 1 g of support was treated with 1800 U of lipase.
The matrices used were pre-treated in the following way:

- **Celite**: was washed with water and ethanol 95% in order to remove fines, and dried at 80 °C. It was then mixed with 5% $HNO_3$ and stirred at 80 °C for 4 hours. The acid-washed support was washed with distilled water until the pH of the filtrate was neutral. The celite was then dried ON at 80 °C, and kept at RT until use.
- **Accurel** (polypropylene): 1 g support was mixed with 3 ml ethanol and then used for immobilization. For the pre-treatment with albumin: to 2 g Accurel treated with 6 ml of ethanol, were added 250 ml of phosphate buffer 10 mM, pH 7.0 and 516 mg bovine serum albumin. The mixture was stirred gently at room temperature for 24 hours, then filtered and treated with the lipase solution.

- **$SiO_2$** and **$Al_2O_3$**: no pre-treatment
- Matrices for anion exchange chromatography (**DEAE**, **SuperQ-C**, **SuperQ-M**): the supports were washed three times with distilled water and then used for immobilization.

The immobilized lipase was collected by vacuum filtration, washed three times with phosphate buffer (pH 7.0, 20 mM) and then dried for 4 hours in a freeze dryer. It was then stored at 4 °C until use.

### *2.8.11.2 Covalent attachment*

- **With glutardialdehyde in two steps**: the support was left overnight in an atmosphere saturated with glutardialdehyde vapors. Afterwards it was incubated overnight at 10 °C with the lipase solution for the immobilization. The covalent attachment with glutardialdehyde was used for Trisopor and active carbon.
- **With Accurel and glutardialdehyde**: 0.5 g Accurel (pretreated with albumin) was incubated at 4 °C in 25 ml sodium acetate buffer 0.2 M, pH 6.3 with the lipase solution. After 12 hours, 2.5% glutardialdehyde in 25 ml of the same buffer was added to the mixture and allowed to react for 2 hours at RT. The powder was then filtered, washed and dried.
- **With glycidol**

  To 1 g of support material 25 ml of 2 M glycidol in 0.16 M NaOH were added and 150 mg of sodium borohydride. The mixture was magnetically stirred for 24 hours at 25 °C, then filtered and washed with distilled water until the pH of the filtrate returned to 7.0. The solid was resuspended in 10 ml of distilled water and 2.5 ml of 0.11 M NaIO4 was added. It was stirred for 4 h at 25 °C, and then washed with 20 ml distilled water.

  1 g of glycidol-activated solid was gently stirred for 3 h at 25 °C with the lipase solution (400 LU) in Tris-HCl buffer (0.1 M, pH 8.0). This method was used for $Al_2O_3$ and $SiO_2$. The schematic reaction which occurs is reported in Fig. 5.

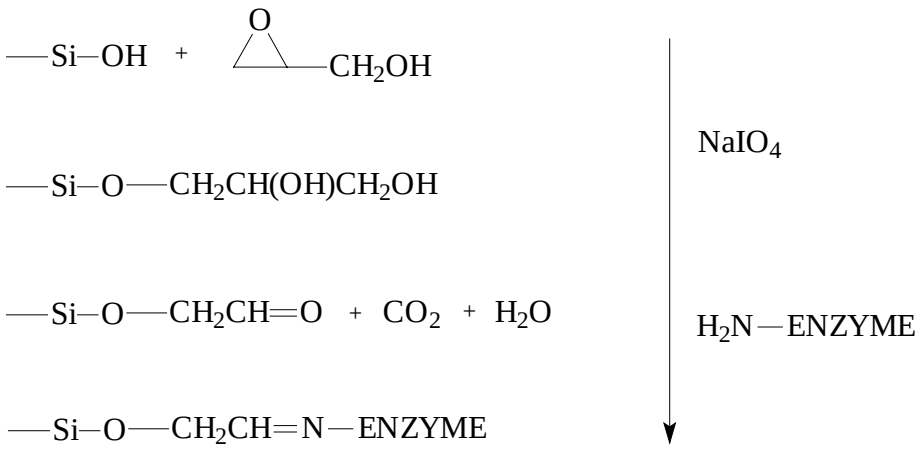



Fig. 5. Immobilization of an enzyme using glycidol.

### 2.8.12 Determination of the stability of the immobilized lipase

A certain amount of the immobilized lipase powder was incubated at 50 °C in 600 µl Tris-HCl buffer, 0.1 M, pH 8.0, for the determination of its stability. The residual activity was measured at different times with the pH-Stat on triolein at 37 °C, pH 8.0 and was related to the activity of the solid before incubation.

# 3 RESULTS

## 3.1 Cloning and expression in Saccharomyces cerevisiae

For the expression in *Saccharomyces cerevisiae* of GCL A, B and of a hybrid lipase A/B, which could be interesting to check which fragment of the lipase gene is responsible for the peculiar substrate specificity of lipase B, the vector pYES2 was used. It contains a GAL1 promoter for the regulation of transcription initiation, a URA3 gene for selection in yeast, the ampicillin gene, the *E. coli* origin of replication (ColE1), the T7 RNA Polymerase promoter and F1 origin to allow maintenance, selection and transcription in *Escherichia coli*. Plasmids pGL2.1 and pGL3.4, kindly obtained from Unilever Research, Colworth Laboratory, Colworth House, Sharnbrook, England, containing the genes, preceded by the natural leader sequences, for the precursors of lipase A and B, respectively, were *Eco*RI/*Xho*I digested and the resulting 640 and 1143 bp fragments ligated into pYES2 cleaved with the same enzymes, giving pYprelipA and pYprelipB. The ligation occurred between three fragments at the same time, as *Xho*I cuts the lipase genes in two sites (Figs. 6-7).

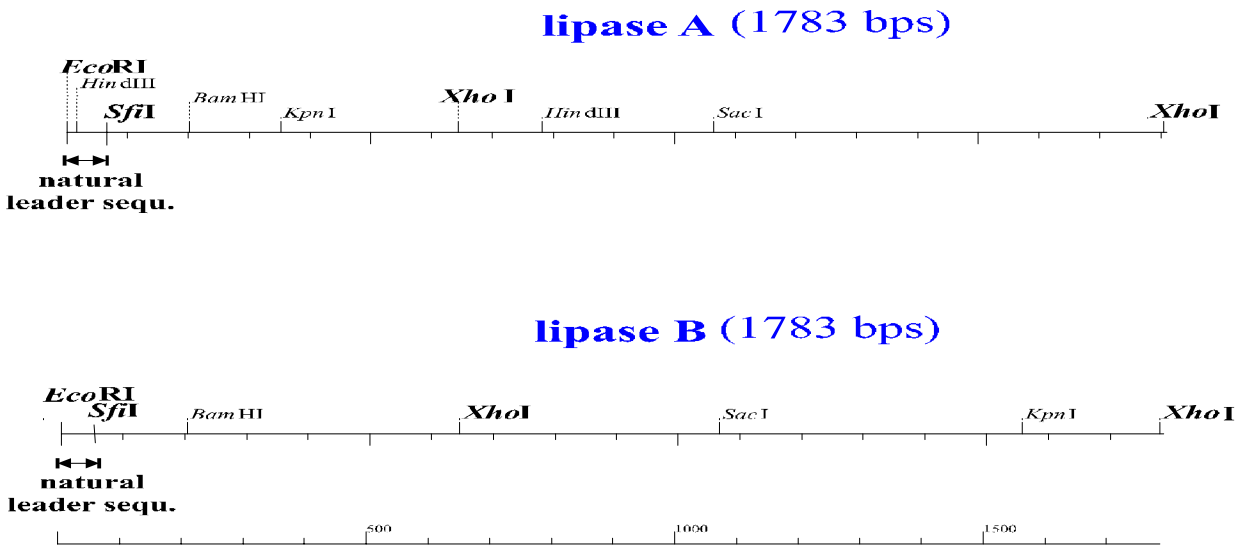



Fig. 6. Schematic representation of the GCL genes with some restriction sites.

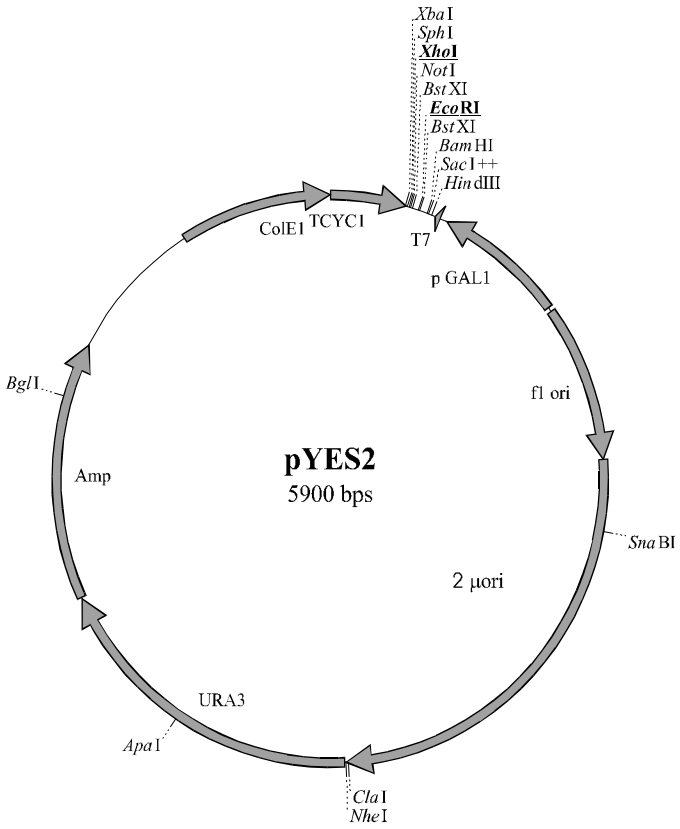



Fig. 7. Schematic representation of the vector pYES2 with some restriction sites.

In a third cloning a hybrid lipase was obtained ligating the first 640 bp of lipase B (till the first *Xho*I site) with the remaining 1143 bp of lipase A. From the ligation of the hybrid lipase gene in pYES2, the vector pYpreLipA/B was obtained.

The three plasmids were transformed in *Saccharomyces cerevisiae*, the transformants were grown and the lipase production was induced with galactose. After two days of induction, the cells were harvested through centrifugation, and cell extracts were made. The activity measurements, neither with the culture supernatant nor with the cell extracts, showed any lipolytic activity.

An SDS-PAGE followed by Western Blotting with the polyclonal antibodies against the lipase from *Geotrichum candidum* was made with samples of the culture supernatants and on the cell extracts. The membrane corresponding to the supernatant showed no bands (not shown), whereas the one for the cell extracts showed clear bands in relation to the samples taken after induction with galactose (Fig. 8).


Fig. 8. Nitrocellulose membrane after Western-blotting with cell extracts from *S. cerevisiae* transformed with pYprelipA and pYprelipB. 1. pYprelipA, not induced; 2. pYprelipA induced; 3. pYprelipB, not induced; 4. pYprelipB, induced.

This means that the lipases are expressed, but in *Saccharomyces cerevisiae* the natural leader sequence of the lipase genes does not allow the secretion in the medium. The proteins remain inactive inside the cells.

### *3.2 Substituting the original signal sequence of Geotrichum candidum with the α-factor leader sequence from Saccharomyces cerevisiae*

The next step was then to substitute the natural leader sequence of *Geotrichum candidum* lipases A and B by the α-factor leader sequence from *S. cerevisiae*. Another host organism was also used for the expression: the yeast *Pichia pastoris*. This methylotrophic yeast was initially developed by Phillips Petroleum Company for the production of single-cell protein for feed stock, as it is a GRAS organism and after high cell density fermentation it is possible to obtain up to 130 g/l dry-cell weight. Furthermore, *Pichia pastoris* is a particularly valued expression system for its ability to secrete expressed proteins with high efficiency, whereas only very low levels of native proteins are secreted (Tschopp, Sverlow *et al.* 1987). It possesses a highly inducible methanol utilization pathway; the methanol oxidase, the first enzyme of the pathway, accounts for up to 35% of the total protein in cells grown on limited amount of methanol. The enzyme is undetectable in cells grown on glucose or glycerol (Wegner and Harder 1986). Its promoter (AOX1) can be used for the expression of heterologous proteins, leading to high expression yields. Furthermore during the passage of the heterologous proteins through the secretory pathway, they are subjected to post-translational modifications such as glycosylation (Cregg, Vedvick *et al.* 1993), that may be important for the correct protein folding. Also the disulfide bonds are likely to be properly formed in proteins passing through the secretory pathway.

For the expression of GCL A, B and A/B in *Pichia pastoris* the vector pPICZαC was used. It contains the AOX1 inducible promoter for the regulation of the expression and the Zeocin resistance gene to allow selection.

pYpreLipA, pYpreLipB and pYpreLipA/B were cut with *Xba*I and *EcoR*I and the obtained fragments ligated into the *Pichia pastoris* expression vector pPICZαC, giving pPpreLipA, pPpreLipB and pPpreLipA/B (Fig. 9), containing the lipase genes with the natural leader sequence.

A PCR was performed for each of the plasmids in order to fuse the sequences encoding the mature lipases directly in frame with the α-factor signal sequence, deleting the natural leader sequence and the few bases from the multiple cloning site between the 3' end of the α-factor leader sequence and the 5' end of the lipase gene. One of the oligonucleotides used in the PCR (Pme) was complementary to the pPICZαC sequence including the *Pme*I site, located in the AOX promoter, and the second (Prepro) was complementary to the 3' end of the α-factor signal sequence and included the 5' sequence of the mature lipases along with a *Sfi*I site, which comprises the first amino acids of the mature lipase.

In the same way, a second PCR was carried out using the oligo (Pre) instead of the prepro complementary to the 3' end of the pre-α-factor leader sequence (composed only of the first 19 residues out of 89 total) and to the 5' sequence of the mature lipase, including the *Sfi*I site.

The pre-α-factor leader sequence was already successfully used for the secretion of proteins in *S. cerevisiae* (Bertolini, Schrag *et al.* 1995). In this work it was used to compare its secretion efficiency with the complete α-factor leader sequence.

The fragments obtained were digested with *Pme*I and *Sfi*I and ligated into the *Pme*I/*Sfi*I linearised vectors pPpreLipA, pPpreLipB and pPpreLipA/B to create pPαLipA, pPαLipB and pPαLipA/B with the complete leader sequence, and pPα'LipA, pPα'LipB and pPα'LipA/B with the pre-leader sequence (Fig. 9).

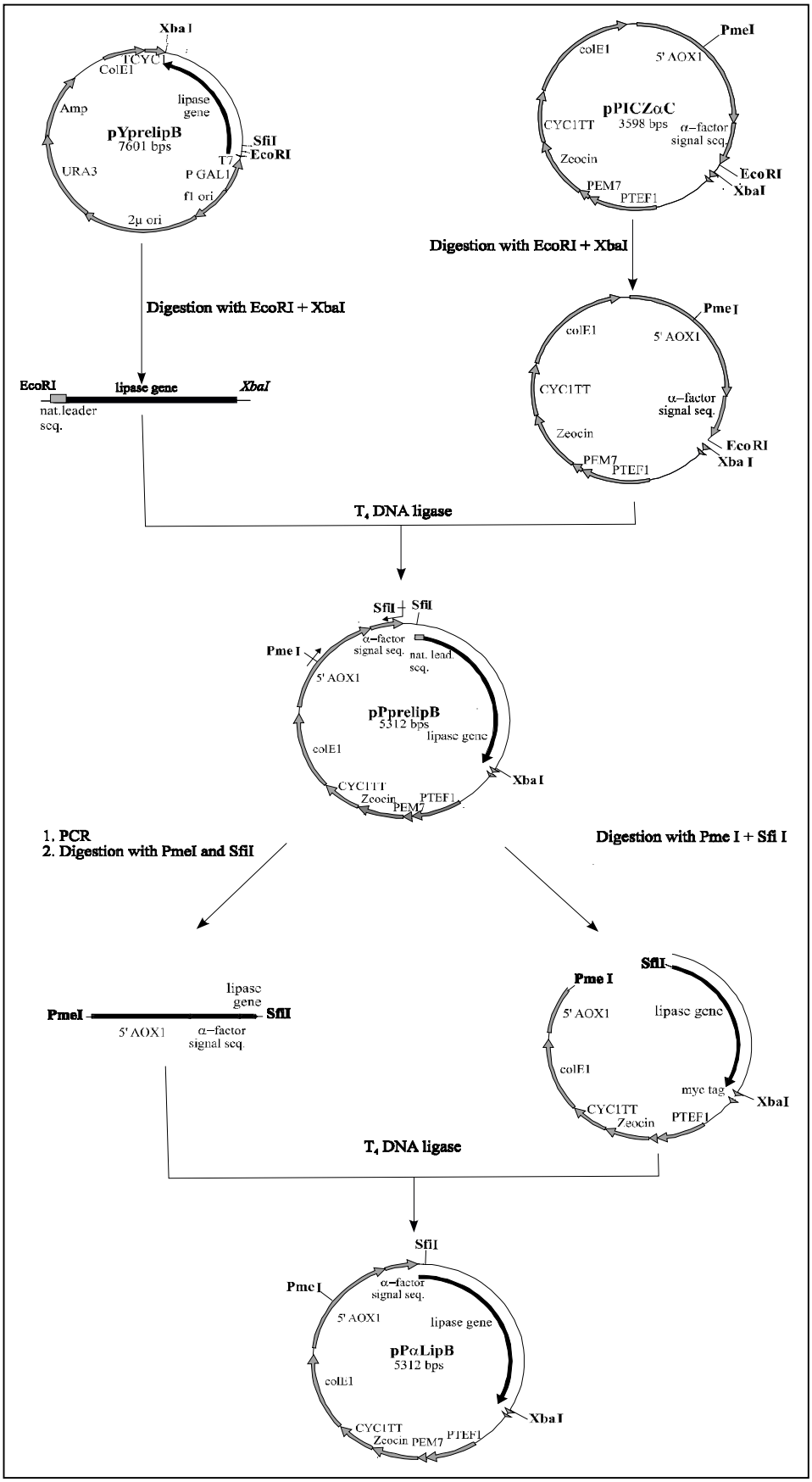



Fig. 9. Cloning strategy for the insertion of lipase B gene into pPICZαC. The cloning strategy for insertion of lipase A and lipase A/B into pPICZαC was done in a similar way, resulting in plasmid pPαLipA and pPαLipA/B.

At this point, the lipase genes preceded by the α-factor leader sequence could be transferred again into a pYES2 vector for the expression in *Saccharomyces cerevisiae*. pPαLipA, pPαLipB and pPαLipA/B were cut with *Sfu*I, treated with the Klenow fragment DNA-polymerase to form blunt ends (*Sfu*I/blunt), followed by digestion with *Xba*I, after thermal inactivation of the polymerase. Fragments of about 2000 bp were extracted from a gel and ligated into pYES2 previously cut with *Eco*RI/blunt-ended and then with *Xba*I.

The plasmids, named pYαLipA, pYαLipB and pYαLipA/B, were transformed in *Saccharomyces cerevisiae*. The transformants were grown in synthetic medium with glucose until they reached an $OD_{600}$ = 2 and then transferred to synthetic medium with galactose as carbon source for induction of lipase expression.

After 2 days of induction, the presence of active lipase was tested in the culture supernatant.

An activity of 6-8 U/ml for the three lipases was detectable in the medium, clearly due to the expressed lipases, as the clone transformed with pYES2 showed no activity.

### *3.3 Cloning and expression in Pichia pastoris*

*Pichia pastoris* GS115 cells were transformed with pPαLipA, pPαLipB, pPαLipA/B, pPα'LipA, pPα'LipB and pPα'LipA/B by electroporation, and plated on YEPDS plates containing zeocin. Positive transformants (four for each expression vector) were selected, grown in shaking flasks until they reached an $OD_{600}$ = 2-6 and then transferred to BMMY medium for induction of lipase expression.

After 48 h of induction, activity of lipase A, B and of the hybrid lipase A/B was detected in the supernatant using triolein as substrate in a pH-Stat. The lipases were successfully expressed and secreted from *Pichia pastoris*, however, the lipase production for the different clones varied significantly from 1 U/ml to 50 U/ml (Table 2).

Table 2. Lipolytic activity of the transformed *Pichia pastoris* culture supernatant after 2 days of induction.

| Clone | Activity on triolein, pH 7.5, T=37 °C [U/ml] |
|---|---|
| pPα'LipA, 1 | 18 |
| pPα'LipA, 2 | 1 |
| pPα'LipA, 3 | 1 |
| pPα'LipA, 4 | <1 |
| pPα'LipB, 1 | <1 |
| pPα'LipB, 2 | <1 |
| pPα'LipB, 3 | <1 |
| pPα'LipB, 4 | <1 |
| pPα'LipA/B, 1 | <1 |
| pPα'LipA/B, 2 | 10 |
| pPα'LipA/B, 3 | <1 |
| pPα'LipA/B, 4 | 3 |
| pPαLipA, 1 | 23 |
| pPαLipA, 2 | <1 |
| pPαLipA, 3 | 1 |
| pPαLipA, 4 | 1 |
| pPαLipB, 1 | 16 |
| pPαLipB, 2 | 48 |
| pPαLipB, 3 | 17 |
| pPαLipB, 4 | <1 |
| pPαLipA/B, 1 | 17 |
| pPαLipA/B, 2 | 10 |
| pPαLipA/B, 3 | 5 |
| pPαLipA/B, 4 | <1 |

For further investigations, the best clone (pPαLipB, 2) was chosen for the production of the highly substrate specific lipase B and cultivated in 2 l Erlenmeyer flasks containing 400 ml BMGY medium to an $OD_{600}$ = 4. The cells were then transferred to the BMMY medium and after 6 days of induction the activity of the supernatant, measured at the pH-Stat on triolein at 37 °C, pH 7.5, was 130 U/ml. Supernatants were separated by an SDS-PAGE, stained with Coomassie. Fig. 10 shows a band of approximately 60 kDa, corresponding to the molecular weight of the GC lipases, as reported in literature (Vernet, Ziomek *et al.* 1993).

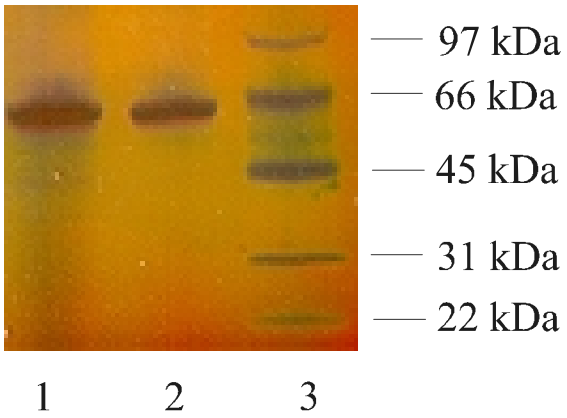



Fig. 10. SDS-PAGE of the supernatant of *Pichia pastoris* transformed with the genes of lipase A and B from *Geotrichum candidum*. 1. pPαLipA; 2. pPαLipB; 3. Molecular weight standard.

### *3.4 Cloning and expression using pGAPZαC*

The genes of lipase A and B from *Geotrichum candidum* preceded by the α-factor leader sequence were cloned also in the vector pGAPZαC for expression in *Pichia pastoris*, which has the constitutive promoter for the glyceraldehyde-3-phosphate dehydrogenase (GAP) and often leads to higher expression yields in comparison to the pPICαC vector.

The lipase genes were cut out from pPαLipA and pPαLipB with *Sfu*I and *Xba*I and ligated in pGAPZαC digested with the same enzymes, resulting in the vectors pGαLipA and pGαLipB, respectively (Fig. 11).

The obtained plasmids were transformed in *Pichia pastoris*. The transformants were picked and grown in YEPD, and the lipolytic activity of the supernatants was checked. After two days, when the corresponding pPαLipB cultures were transferred to YEPM medium for induction, the lipase activity in supernatants of cells harboring pGαLipB was already 20 U/ml. If the cultivation was prolonged for up to one week, the activity detected with the pPαLipB plasmid reached 130 U/ml, but the one with the pGαLipB plasmid remained constant at around 30 U/ml.

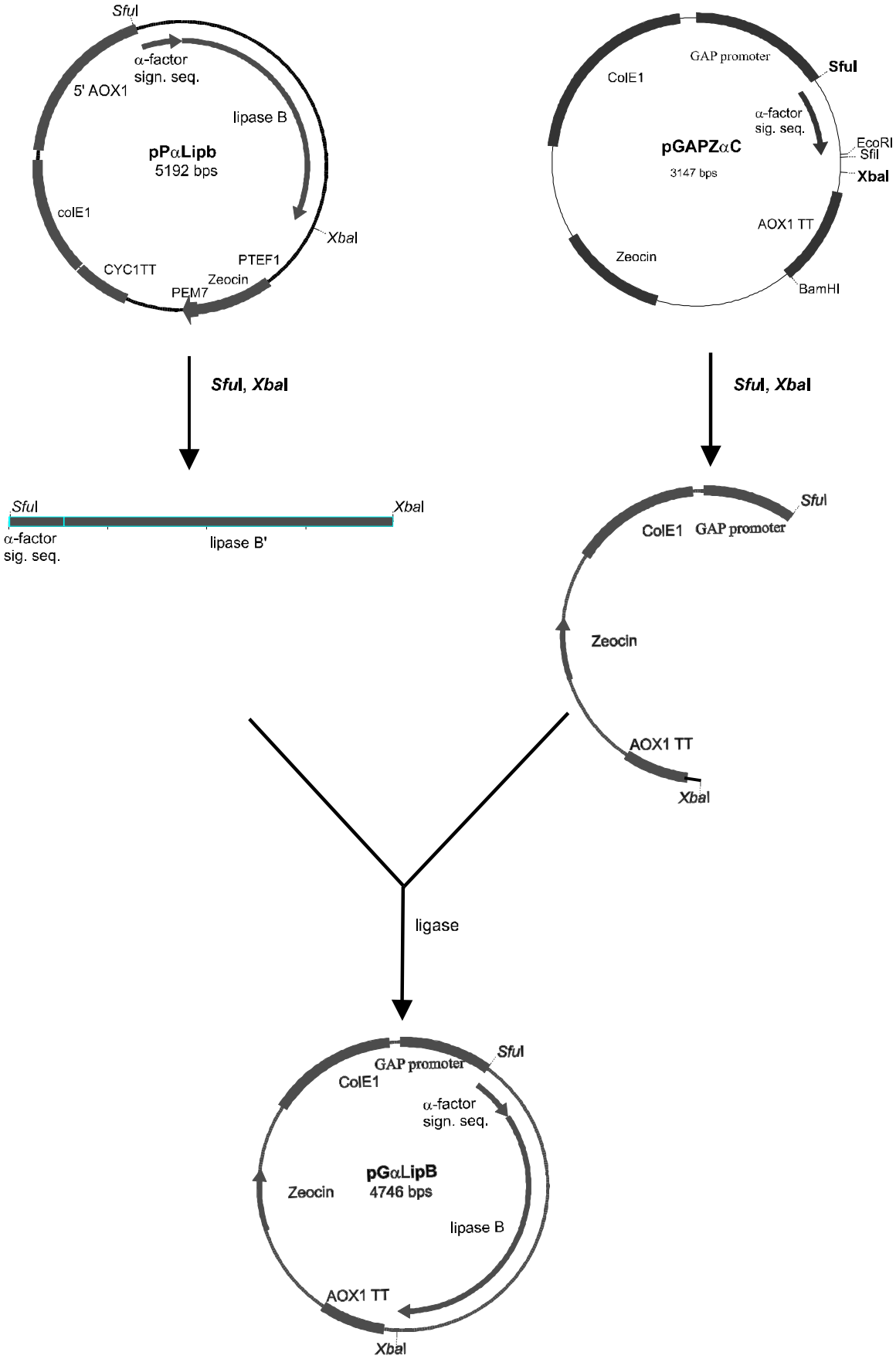



Fig. 11. Cloning of the lipase B gene in pGAPZαC. For lipase A the same strategy was used.

### *3.5 N-terminal sequencing of the lipase B*

The recombinant lipases A and B expressed from pPαLipA and pPαLipB were blotted on a PVDF membrane which was used for the N-terminal sequencing. The following sequences were obtained:

for lipase A: -Glu-Ala-Glu-Ala--Ala-Pro-Thr-Ala-Val-Leu-Asn-Gly-Asn-Glu-Val-Ile-Ser-Gly-Val-Val-Glu

for lipase B: -Glu-Ala-Glu-Ala--Ala-Pro-Thr-Ala-Val-Leu-Asn-Gly-Asn-Glu.

The sequences correspond to the correct ones for the mature lipases, and it was pointed out that the leader sequence was processed, but its last four amino acids (Glu-Ala-Glu-Ala) remained attached to the proteins.

### *3.6 Fermentations of Pichia pastoris in 1 l scale*

To improve the recombinant lipase production in a large scale, optimal cultivation conditions for *Pichia pastoris* harbouring pPαLipB were investigated using different media and two different pH values. The bioreactor (1 l) was inoculated with 50 ml of a flask culture grown overnight to an $OD_{600}$ = 2-10, to have an initial $OD_{600}$ of almost 0.1 in the fermentor. 5 ml of methanol (= 0.5% (v/v)) were added daily to the medium in order to keep its concentration almost constant during the cultivation time. The agitation rate was 350 rpm and the aeration rate 1 l/min. The lipolytic activity of the supernatants and the cell wet weight were monitored throughout the cultivation.

The conditions used are reported in Table 3.

Table 3. Different fermentation conditions.
T = 30 °C, agitation = 350 rpm, volume = 1 l.

| | **Medium composition** | **pH** |
|---|---|---|
| Medium A | 1% yeast extract, 2% peptone, **1.34% YNB, 4*10$^{-5}$% biotin**, 0.5% methanol | 6.0 |
| Medium B | 1% yeast extract, 2% peptone, 0.5% methanol | 6.0 |
| Medium C | 1% yeast extract, 2% peptone, 0.5% methanol | **7.5** |
| Medium D | 0.3% yeast extract, 0.5% peptone, 0.5% **malt extract**, 0.5% methanol | 6.0 |

The results obtained for the different cultivations, in terms of lipolytic activity of the supernatant and cell wet weight are reported in Figures 12 and 13.

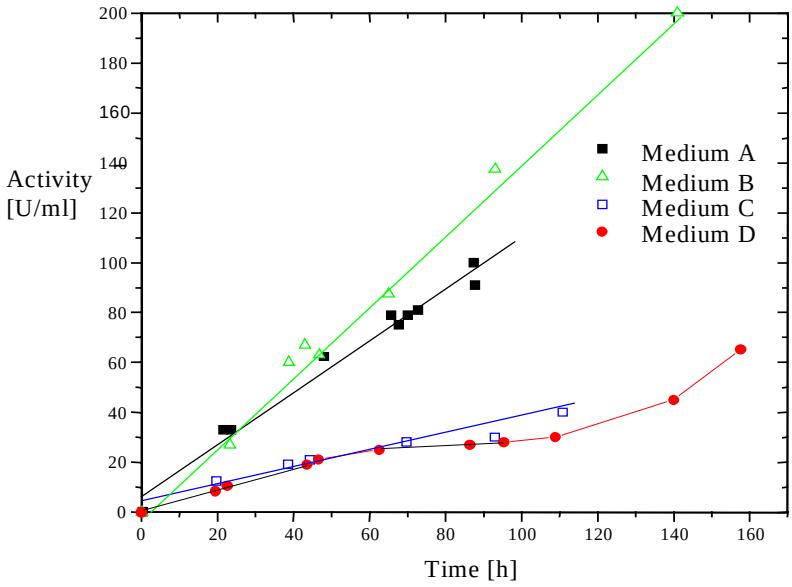



Fig. 12. Lipase B production during cultivation with different media and pH. Medium containing: (■) 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB, 4∗10$^{-5}$% (w/v) biotin, 0.5% (v/v) methanol (pH 6.0); (Δ) 1% (w/v) yeast extract, 2% (w/v) peptone, 0.5% (v/v) methanol (pH 6.0); (□) 1% (w/v) yeast extract, 2% (w/v) peptone, 0.5% (v/v) methanol (pH 7.5); (●) 0.3% (w/v) yeast extract, 0.5% (w/v) peptone, 0.5% (w/v) malt extract, 0.5% (v/v) methanol (pH 6.0).

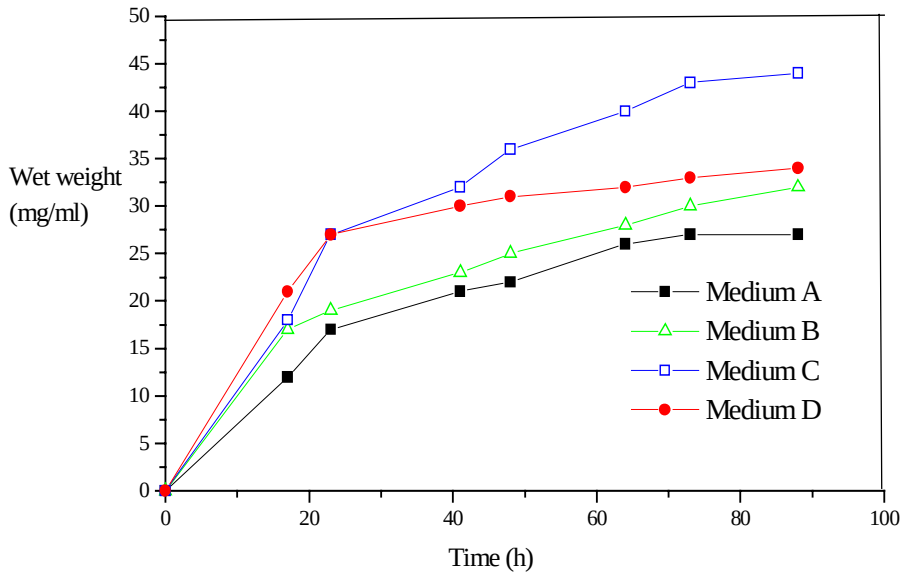



Fig. 13. Cell wet weight during cultivation with different media and pH. Medium containing: (■) 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB, $4*10^{-5}$% (w/v) biotin, 0.5% (v/v) methanol (pH 6.0); (Δ) 1% (w/v) yeast extract, 2% (w/v) peptone, 0.5% (v/v) methanol (pH 6.0); (□) 1% (w/v) yeast extract, 2% (w/v) peptone, 0.5% (v/v) methanol (pH 7.5); (●) 0.3% (w/v) yeast extract, 0.5% (w/v) peptone, 0.5% (w/v) malt extract, 0.5% (v/v) methanol (pH 6.0).

After 100 h of cultivation in medium A, the lipolytic activity in the supernatant was 100 U/ml. A less expensive medium without yeast nitrogen base and biotin (medium B) was used in a second cultivation. The lipase production was comparable or even higher than in the rich medium, reaching 200 U/ml after 140 h of cultivation (Fig. 12).

Increasing the pH of this inexpensive medium to 7.5 (medium C), did not increase the lipase activity in the supernatant as it was expected from the pH-stability profile of lipase B (Sugihara, Shimada *et al.* 1990). Although the lipase activity was lower in this cultivation (40 U/ml after 100 h) when compared to the previous ones, the amount of lipase in the supernatant was the same as determined by qualitative and quantitative protein determinations (SDS-PAGE, protein concentration, data not shown).

The further simplification of the medium in a fourth cultivation at pH 6.0 (medium D), resulted in only a low lipase activity of 30 U/ml after 100 h.

The cell growth was comparable for fermentations in medium A, B and D and was higher in medium C, despite the lower lipase production.

## *3.7 Fermentation of Pichia pastoris in 6 l scale*

### 3.7.1 1st fermentation

*Pichia pastoris*, transformed with pPαLipB, was fermented in 6 liters BMMY.

The temperature was kept at 30 °C, the pH at 6.00, the air flow at 1 l/min, the stirrer speed at 350 rpm.

100 ml of a preculture, grown in YEPG to an $OD_{600}$ = 10.0, were inoculated in the fermentor. The off-gas was continuously analyzed with a mass-spectrometer, so that the content of dissolved oxygen ($DO_2$), the produced carbon dioxide ($RCO_2$), the consumed oxygen ($RO_2$) and the methanol content were followed during the fermentation. The peak of the methanol corresponds to the one of the ethanol, in the trends of the off-gas reported in Fig. 14.

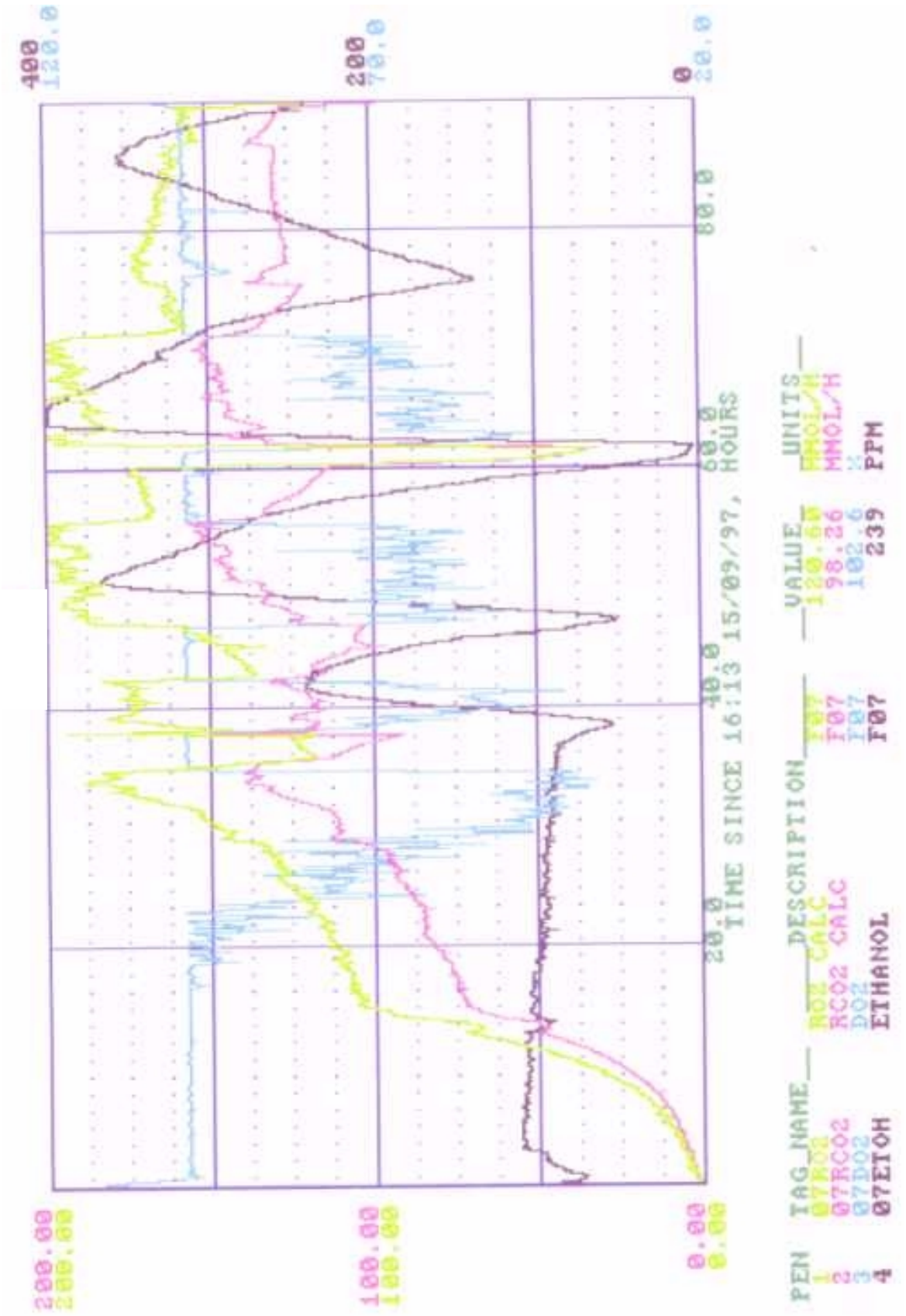
The methanol was added when, from the off-gas, it was seen that its level has dropped down. In particular, 60 ml of methanol were added at the beginning of the fermentation, after 29 hours and 47 hours, and 30 ml were added after 55 hours and 71 hours.

As the level of dissolved oxygen decreased rapidly, as shown in Fig. 14, after 23 hours of fermentation the stirrer speed was increased to 500 rpm, and after 26 hours the air flow was set at 3 l/min, and the level of the oxygen raised again. The $RO_2$ and $RCO_2$ increased in the first 20-30 hours and then decreased, showing that the cells did not grow anymore.

Samples were taken automatically every three hours, and the lipolytic activity, the cell dry-weight and the $OD_{600}$ were measured.

All the results obtained are reported in Table 4. It can be seen that already after 30 hours the cell growth almost discontinued ($OD_{600}$ and cell dry-weight remain almost constant), as shown also from the off-gas profile, but the lipolytic activity increased continuously till the end of the fermentation, reaching, after 75 hours of fermentation, 373 U/ml.

Fig. 14. Off-gas profile for the fermentation of *Pichia pastoris* transformed with pPαLipB. Medium: 1% yeast extract, 2% peptone, methanol.



Tab. 4. Fermentation of *Pichia pastoris* transformed with pPαLipB.
Medium: 1% yeast extract, 2% peptone, methanol.

| Time of fermentation (h) | $OD_{600\ nm}$ | Dry-weight (mg/ml) | Activity (U/ml) |
|---|---|---|---|
| 0 | | | -- |
| 3 | 1 | 0.3 | -- |
| 6 | 2 | 0.5 | -- |
| 9 | 3 | 0.8 | 3 |
| 12 | 5 | 1.5 | 13 |
| 15 | 9 | 2.3 | 34 |
| 18 | 15 | 3.3 | 49 |
| 21 | 16 | 3.8 | 78 |
| 24 | 19 | 4.9 | 101 |
| 27 | 14 | 5.2 | 122 |
| 30 | 15 | 5.8 | 130 |
| 33 | 16 | 5.4 | 144 |
| 36 | 16 | 6.1 | 166 |
| 39 | 16 | 6.1 | 179 |
| 42 | 16 | 5.9 | 193 |
| 45 | 17 | 6.1 | 206 |
| 48 | 18 | 6.0 | 204 |
| 51 | 14 | 5.5 | 232 |
| 54 | 13 | 5.8 | 269 |
| 57 | 21 | 5.8 | 275 |
| 60 | 21 | 6.0 | 285 |
| 63 | 21 | 5.9 | 299 |
| 66 | 25 | 5.9 | 330 |
| 69 | 22 | 6.1 | 339 |
| 72 | 23 | 6.0 | 352 |
| 75 | 24 | 6.3 | 373 |

### 3.7.2 2nd fermentation

*Pichia pastoris*, transformed with pPαLipA from *Geotrichum candidum*, was fermented using the same conditions as in the first fermentation for lipase B, apart from the fact that the air flow was set at 3 l/min from the beginning of the fermentation.

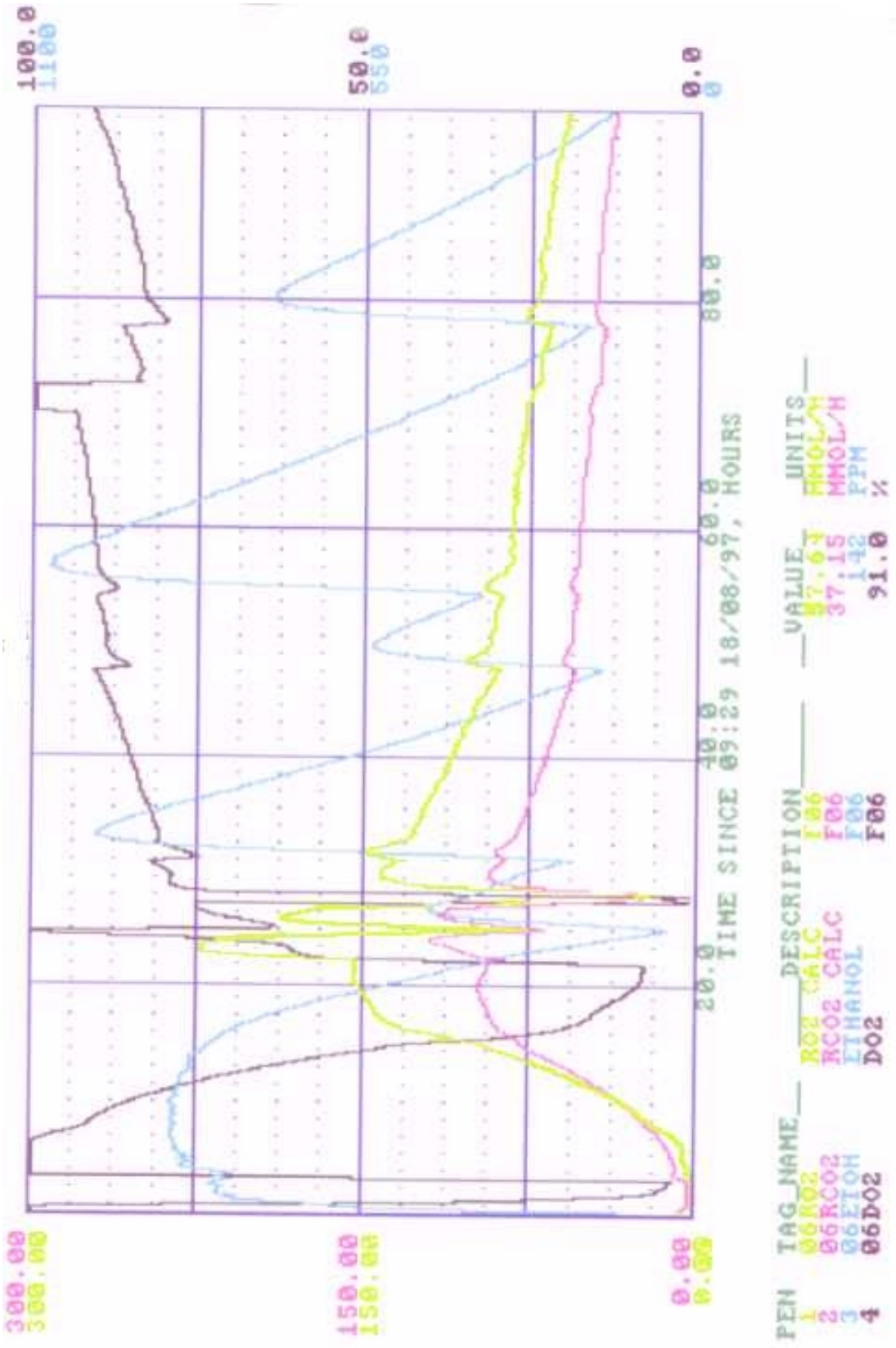
100 ml of a preculture, grown in YEPG to an $OD_{600}$ = 14.0, were inoculated in the fermentor.

As the level of dissolved oxygen decreased rapidly, as shown in the trend of the off-gas in Fig. 15, after 22 hours of fermentation the stirrer speed was increased to 500 rpm and the $DO_2$ increased again. After 70 hours the stirrer speed was decreased again to 400 rpm.

The methanol was added when its level, determined from the off-gas profile, decreased. In particular, 60 ml of methanol were added at the beginning of the fermentation, 30 ml after 25 hours, 60 ml after 31 hours, 30 ml after 48 hours, 50 ml after 54 hours, 40 ml after 79 hours.

All the results obtained from the analysis of the samples are reported in Table 4. Also in this fermentation, the dry-weight and the $OD_{600}$ increased in the first 30 hours and then remained constant till the end of the fermentation (96 hours). The lipase activity in the supernatant increased throughout the whole fermentation reaching 74 U/ml, significantly lower than the 373 U/ml reached in the fermentation of lipase B carried out in the same conditions.

Fig. 15. Off-gas profile for the fermentation of *Pichia pastoris* transformed with pPαLipA. Medium: 1% yeast extract, 2% peptone, methanol.



Tab. 5. Fermentation of *Pichia pastoris* transformed with pPαLipA.
Medium: 1% yeast extract, 2% peptone, methanol.

| Time of fermentation (h) | $OD_{600\ nm}$ | Dry-weight (mg/ml) | Activity (U/ml) |
|---|---|---|---|
| 24 | 13 | 3.7 | 40 |
| 30 | 18 | 5.4 | 47 |
| 33 | 17 | 5.2 | 35 |
| 36 | 19 | 5.9 | 37 |
| 39 | 17 | 5.6 | 38 |
| 42 | 19 | 5.7 | 44 |
| 45 | 18 | 5.9 | 48 |
| 48 | 19 | 6.0 | 51 |
| 51 | 19 | 5.9 | 47 |
| 54 | 22 | 6.5 | 47 |
| 57 | 20 | 6.3 | 47 |
| 60 | 20 | 6.1 | 49 |
| 66 | 19 | 6.0 | 53 |
| 69 | 19 | 6.0 | 53 |
| 72 | 19 | 6.1 | 58 |
| 75 | 19 | 6.0 | 56 |
| 78 | 20 | 6.0 | 60 |
| 81 | 21 | 6.0 | 65 |
| 84 | 21 | 6.0 | 68 |
| 87 | 20 | 6.0 | 69 |
| 90 | 22 | 6.0 | 72 |
| 93 | 22 | 6.0 | 72 |
| 96 | 22 | 6.0 | 74 |

### 3.7.3 3rd fermentation

*Pichia pastoris*, transformed with pPαLipB, was fermented in 6 litres of synthetic medium, keeping the temperature at 30 °C, the pH at 5.00.

The fermentor was inoculated with 200 ml of a preculture grown in the same synthetic medium to an $OD_{600}$ = 28. For the first hours, in the batch phase, the air flow was kept at 4.0 l/min, the stirrer speed at 400 rpm. When the methanol reached a level lower than 70 ppm (after 38 hours), the feed was started automatically.

The feed rate was calculated according to the following initial settings:

starting biomass: 75 g;

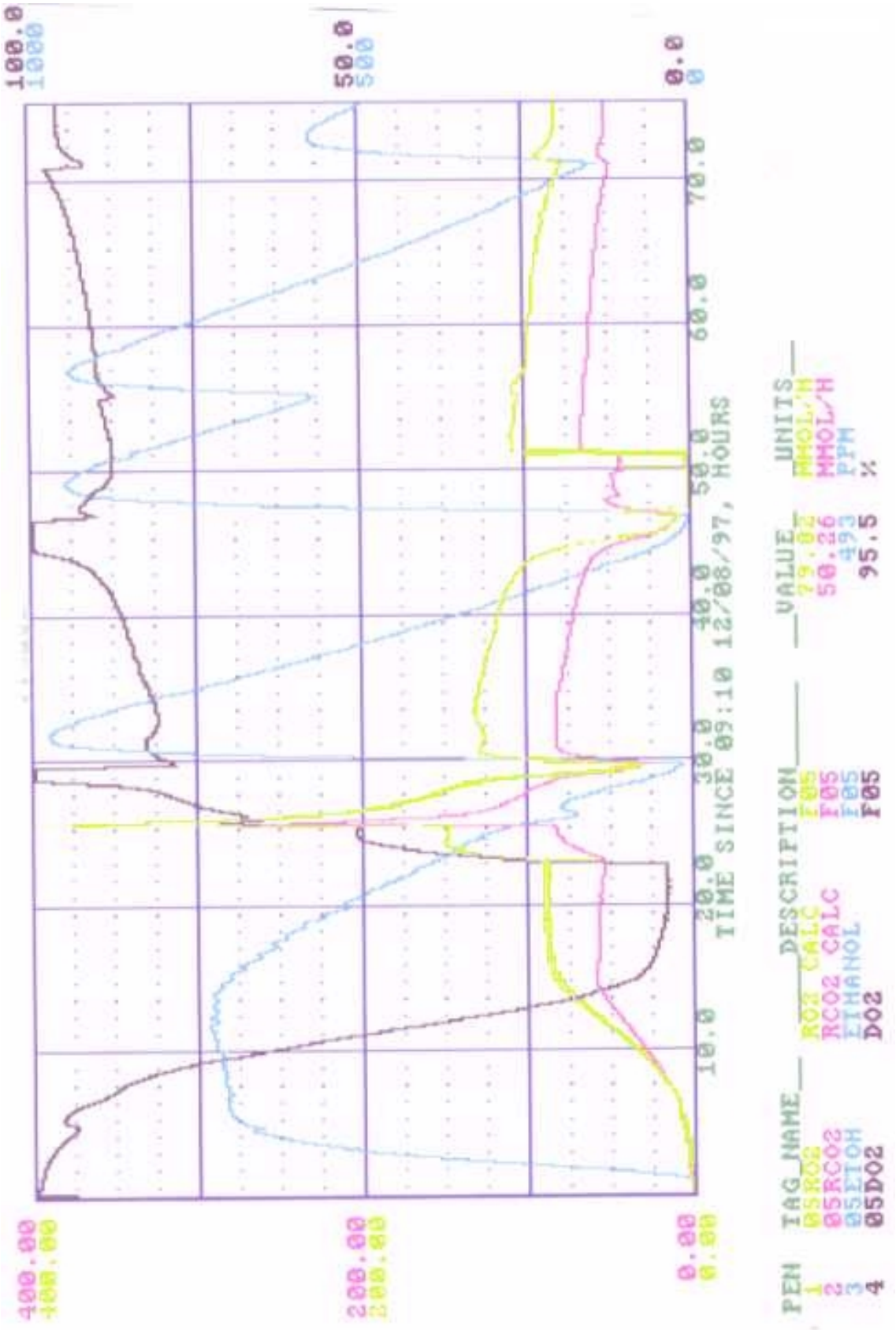
growth rate: 0.150 $h^{-1}$, derived by previous fermentations of *P. pastoris* carried out at Unilever Research Laboratories, Vlaardingen, The Netherlands.

The feed was not continuously added, as the methanol concentration should not reach a level which is too high and could be toxic for the cells. The methanol is used to induce the lipase production, but the main carbon source is glycerol. For this reason, the feed was stopped again after 3 hours, and when the methanol dropped down (47 hours of fermentation), as shown in the trend of the off-gas in Fig. 16, it was added again, with a rate of 0.65 g/min for another 3 hours. After 62 hours of fermentation, it was started with 1.27 g/min, then soon changed to 2.0 g/min for 2 hours. It then began again after 74 hours of fermentation with a rate of 0.40 g/min and stopped after 9 hours.

From the trend of $RCO_2$ and $RO_2$ (Fig. 16), whose values increased constantly, it can be seen that the cells grew very well in the first 70 hours of fermentation.

The results obtained from the samples analysis are reported in Table 6. It can be confirmed that, in this fermentation, the cell growth is observed throughout the whole fermentation, reaching, after 87 hours an $OD_{600}$ of 92 and a dry-weight of 36 mg/ml. The lipolytic activity, which was detectable only after starting the feed, as in the batch phase the methanol concentration was not high enough to induce the lipase production, raised immediately to 134 U/ml. After 40 hours from the starting of the feed, the activity in the medium was 625 U/ml.

Fig. 16. Trend of the off-gas from the fermentation of *Pichia pastoris* transformed with pPαLipB in synthetic medium.



Tab. 6. Fermentation of *Pichia pastoris* transformed with pPαLipB.
Synthetic medium.

| Time of fermentation (h) | $OD_{600\ nm}$ | Dry-weight (mg/ml) | Activity (U/ml) | |
|---|---|---|---|---|
| 0 | 1 | 0.1 | -- | |
| 3 | 1 | 0.3 | -- | |
| 6 | 2 | 0.6 | -- | |
| 9 | 3 | 0.9 | -- | |
| 12 | 5 | 1.6 | -- | |
| 15 | 12 | 3.4 | -- | |
| 18 | 20 | 5.6 | -- | |
| 21 | 34 | 6.9 | -- | |
| 24 | 22 | 8.9 | -- | |
| 27 | 38 | 11.2 | -- | |
| 30 | 42 | 11.5 | -- | |
| 33 | 55 | 16.0 | -- | |
| 36 | 50 | 16.4 | -- | |
| 39 | 49 | 15.6 | -- | |
| 42 | 56 | 19.7 | -- | ← Started feed |
| 45 | 53 | 19.4 | 134 | |
| 48 | 60 | 19.9 | 182 | |
| 51 | 65 | 21.8 | 223 | |
| 54 | 71 | 20.3 | 204 | |
| 57 | 73 | 25.7 | 252 | |
| 60 | 74 | 24.6 | 395 | |
| 63 | 76 | 24.7 | 443 | |
| 66 | 84 | 26.2 | 448 | |
| 69 | 91 | 29.8 | 413 | |
| 72 | 98 | 32.4 | 410 | |
| 75 | 94 | 31.4 | 490 | |
| 78 | 89 | 32.3 | 620 | |
| 81 | 99 | 33.7 | 625 | |
| 87 | 92 | 36.3 | 625 | |

The comparison of the three different fermentations in terms of lipolytic activity (measured on triolein at the pH-Stat) and cell dry-weight is reported in Figs. 17-18.

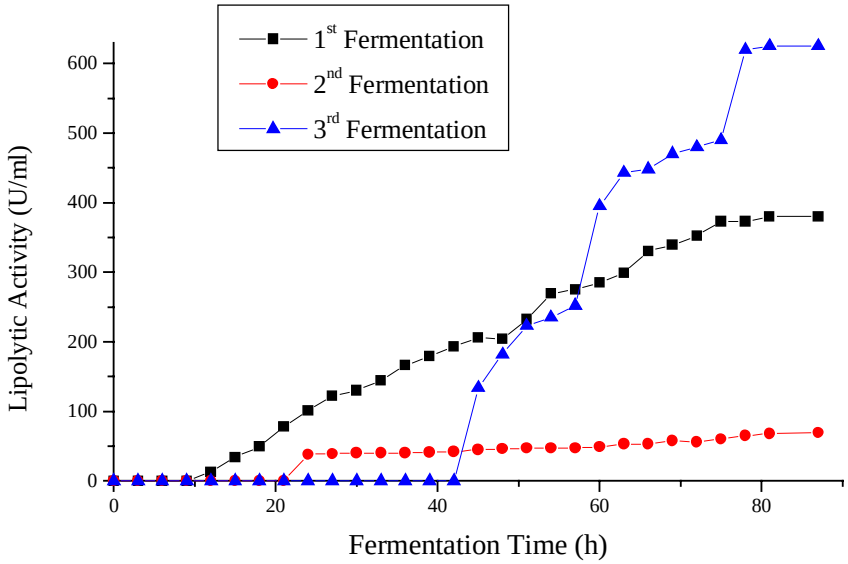



Fig. 17. Lipolytic activity of lipases A and B of the culture broth for the three different fermentations in function of the fermentation time.

In the first fermentation there is, with the time, a linear increase of the activity, that reaches up to 400 U/ml after 70 hours of fermentation. Clearly better results are obtained for lipase B in the synthetic medium, which shows a stepwise increase in the activity, reaching 600 U/ml in 80 hours. It must be also considered, that the lipase expression in the synthetic medium starts after 40 hours (when the feeding starts) and not from the beginning of the cultivation as in the first fermentation.

The activity detected in the broth from the second fermentation (lipase A) is distinctly lower than in the fermentation of lipase B in the same conditions. A difference in the expression levels of lipase A and B in *Pichia pastoris* was already observed from the activity measurements carried out to select the best clone for each transformation (Tab. 2, Section 3.3). In fact, the best clone for lipase B (pPαLipB, 2) reached, after 2 days of induction, 48 U/ml and the one for lipase A (pPαLipA,1) 23 U/ml.

The dry-weight profile (Fig. 18) for the two fermentations in complex medium is almost the same, increasing in the first 20-30 hours and then remaining constant at 6.0 mg/ml. In the third fermentation the cells grew much more, reaching up to 36 mg/ml, due to the fact that they could use glycerol as carbon source, on which they grow better than on methanol, the only carbon source for the cultivations in complex medium.

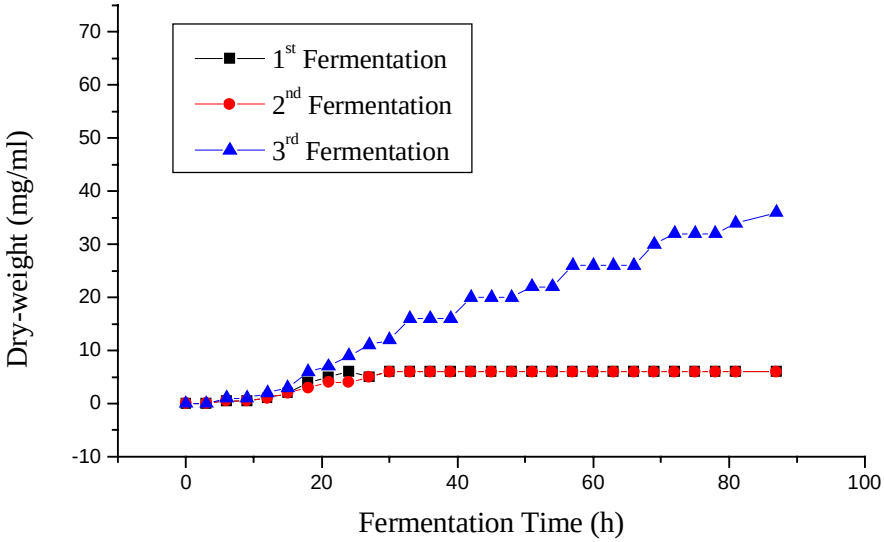



Fig. 18. Cell dry-weight for the three different fermentations in function of the fermentation time.

## *3.8 Purification*

Although the lipase was secreted as almost the only protein in the medium (see Fig. 10), a purification was carried out, principally to eliminate all the peptides present in the medium.

### 3.8.1 Two step purification of lipase B

#### *1. Hydrophobic Interaction Chromatography (HIC)*

The hydrophobic interaction chromatography is based on the interaction, in aqueous solvents, of the hydrophobic patches on proteins with the hydrophobic surface of the matrix used. These interactions are strengthened by high salt concentrations, while the proteins can be released from the matrices by a decrease of the concentration of salts, or by addition of organic solvents or detergents to the elution buffer.

To 6 liters of culture broth from the first fermentation, 30 mM Tris pH 9.0 and 2 M NaCl were added. The pH was in this way increased from 6.0 to 9.0. 225 ml of Butyl Sepharose 4 Fast Flow (Pharmacia), a matrix for hydrophobic interaction chromatography, was incubated overnight, under mild stirring, in the culture broth to let the lipase bind to the support. The matrix was filtered, washed with 1.5 l of Tris buffer 30 mM pH 9.0 containing 2 M NaCl, and packed in a column. The column was washed with the same buffer until a stable baseline was obtained. 7% of the initially loaded lipase activity was found in the flow-through solution.

The elution was carried out with Tris 30 mM pH 9.0, and 10 ml fractions were collected.

The 11 fractions with lipolytic activity were pooled, and gave a total of 110 ml with an activity of 7500 U/ml. As the total initial activity was $198 \cdot 10^4$ U, corresponding to 5500 ml with 360 U/ml, 42% of the initial activity was recovered. The column was then treated with Tris-HCl 30 mM, pH 9.0 + 20% ethanol to elute all the proteins still bound to the matrix, but no more lipolytic activity was obtained. From an SDS-gel the presence of the lipase was detected also in these last fractions, but the protein was not active, as it was denatured by the 20% ethanol.

### *2. Ion Exchange Chromatography (IEC)*

To further purify the lipase, IEC was carried out on the pool from HIC.

In the ion exchange chromatography proteins bind to the ion exchangers by electrostatic interactions between the proteins' surface charges and the dense cluster of charged groups on the matrix. The positively charged Q-Sepharose is a matrix for anion exchange, that means that negatively charged proteins will adsorb on it. The elution of the bound protein is favored by lowering the pH or increasing the ionic strength, thereby weakening the electrostatic interactions between protein and adsorbent.

100 µl of the pool obtained from HIC were loaded on a Q-Sepharose column using Tris-HCl, 0.05 M, pH 7.0 as loading buffer. The lipase was eluted with a gradient in Tris-HCl 0.05 M, pH 7.0 and NaCl 1M from 0% to 50% in 40 minutes and then from 50% to 100% in 20 minutes. The elution profile is reported in Fig. 19.

Pooling the first peak (fraction 2) 5 ml eluate with 24 U/ml lipase activity (16% of the loaded amount) were obtained; the second peak (fractions 22-25) consisted of 2 ml with 83 U/ml (20% of the loaded amount).

Only 15% of the total initial activity were obtained after the two purification steps.

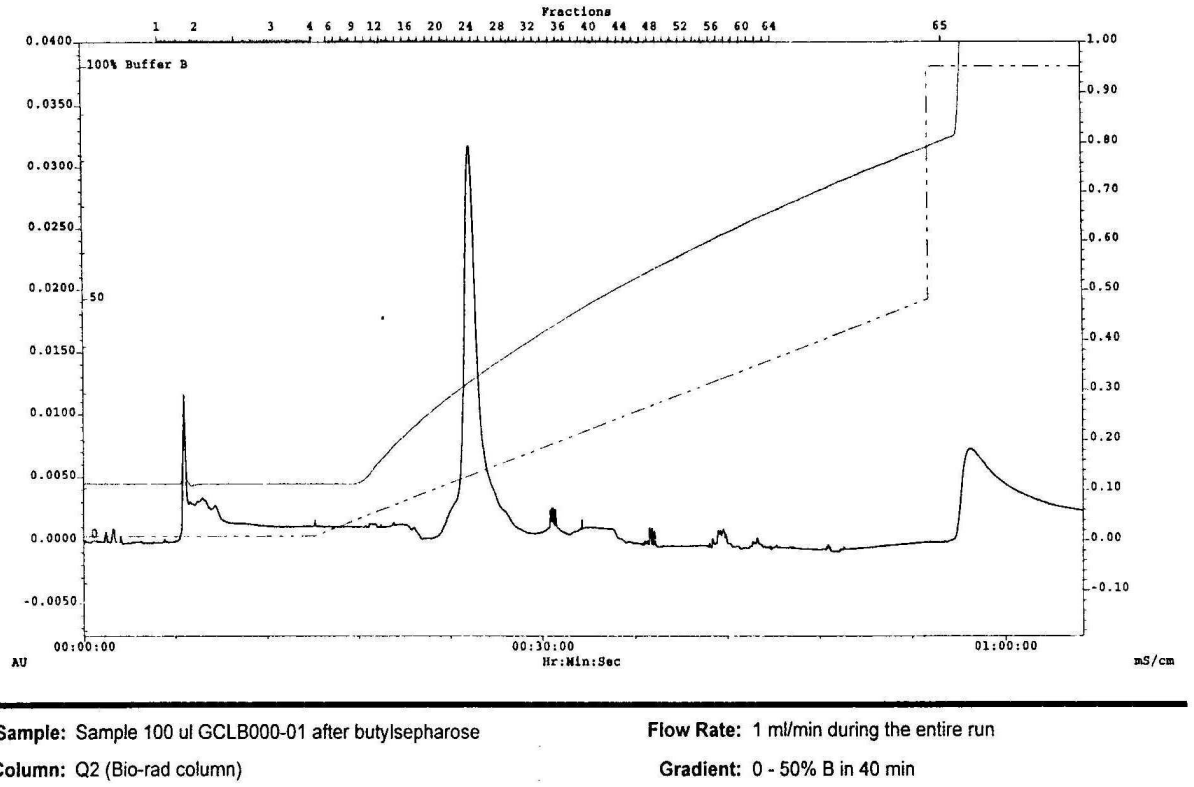



Fig. 19. Two steps purification of GCL B by HIC and IEC. Elution profile of the IEC on the pool from the HIC.

## 3.8.2 One step purification of lipase B and A

### *3.8.2.1 Purification of lipase B by Ion Exchange Chromatography*

As the two-step procedure resulted in only 15% yield, the anion exchange chromatography was carried out directly on the broth from the 1$^{st}$ fermentation, without the previous hydrophobic interaction step in order to increase the purification yield. In this way 2 ml of the fermentation broth (760 U), dialysed against Tris-HCl buffer, 0.05 M, pH 7.0, were loaded on a Q-sepharose column using Tris-HCl, 0.05 M, pH 7.0 as loading buffer. The lipase was eluted with the same gradient as reported above.

The elution profile is reported in Fig. 20.

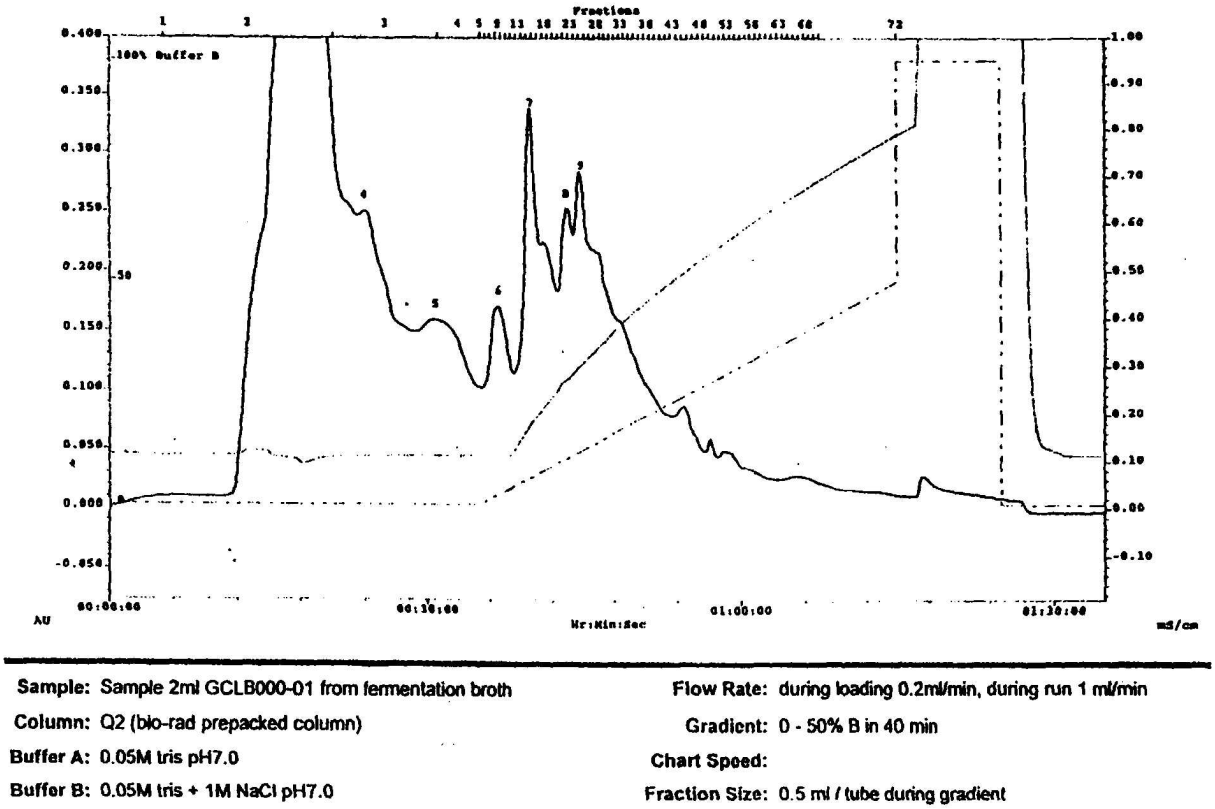



Fig. 20. One step purification of GCL B. Elution profile of the IEC on the broth from the 1st fermentation.

The fraction 25 had an activity of 139 U (19% of the loaded activity). The Q-sepharose chromatography, carried out directly on the fermentation broth, thus represents a better way to purify the lipase compared to the butyl Sepharose. In fact, the first purification step was eliminated, leading to a general higher yield, providing, anyway, the removal of the peptides as well as the clarification of the lipase solution. Therefore this method was chosen also for the purification of lipase A.

### *3.8.2.2 Purification of lipase A by Ion Exchange Chromatography*

The 5.5 l culture broth from the 2nd fermentation (8799 U) were concentrated to 1 l and dialysed against water using an hemofilter until the conductivity was 164 µS/cm, like the one of the loading buffer (Tris-HCl 0.05 M, pH7.0). After this first step, 79% of the initial activity were recovered. The sample, containing 6941 U, was loaded on a Q-Sepharose column, and, after the washing step, the lipase was eluted with a gradient in Tris-HCl 0.05 M, pH 7.0 and NaCl 1M from 0% to 50% in 80 minutes.

The elution profile and the activity of the different fractions are reported in Figs. 21 and 22.

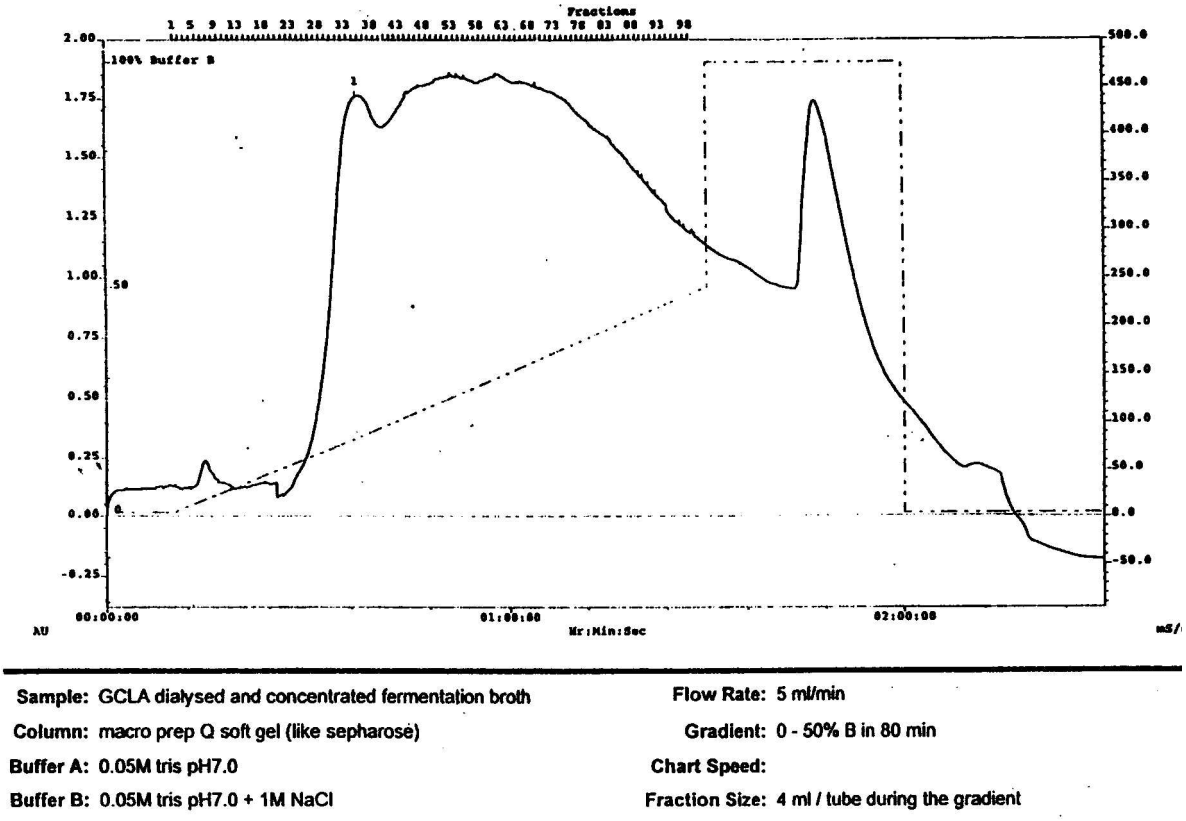



Fig. 21. One step purification of lipase A. Elution profile of the IEC on the broth from the 2nd fermentation.

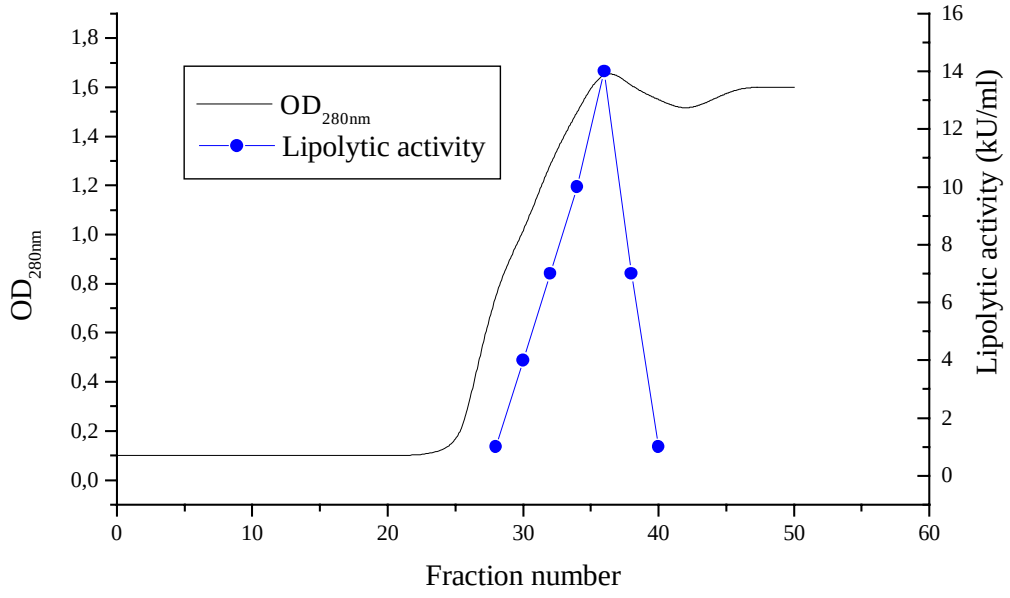



Fig. 22. One step purification of lipase A. Absorbance (280 nm) and lipolytic activity of the different fractions from the IEC on the broth from the 2nd fermentation.

The fractions 30-39 were pooled, giving 50 ml with 6247 U/ml, corresponding to 71% of the starting activity, 90% of the loaded activity.

### 3.8.3 SDS-PAGE on purified samples

Samples from the first fermentation (lipase B), from the second (lipase A) and from the purification steps were separated on SDS-Phast gels and stained with Coomassie (Fig. 23). Some samples were concentrated with TCA before separation. The gels are shown in Fig. 23.

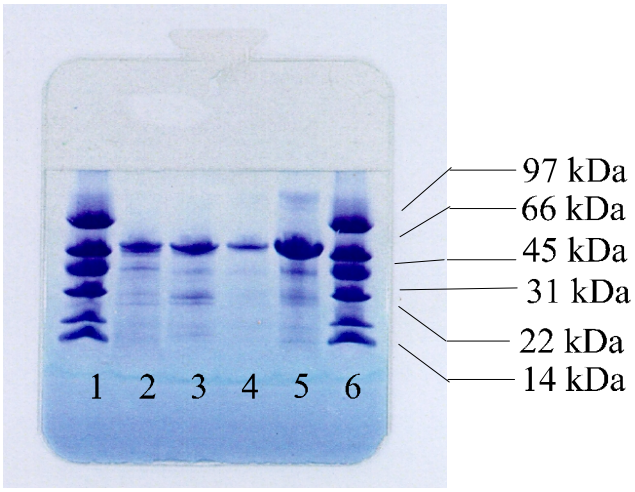



Fig. 23a. SDS-Phast gel with samples from the two steps purification of lipase B.
1. Molecular weight standard
2. Fermentation supernatant (4µl, 10 times concentrated)
3. Pool HIC (4µl)
4. Peak eluted with Tris-HCl 30 mM, pH 9.0+20% EtOH (4µl)
5. Peak eluted with Tris-HCl 30 mM, pH 9.0+20% EtOH (10 times concentrated, 4µl)
6. Molecular weight standard

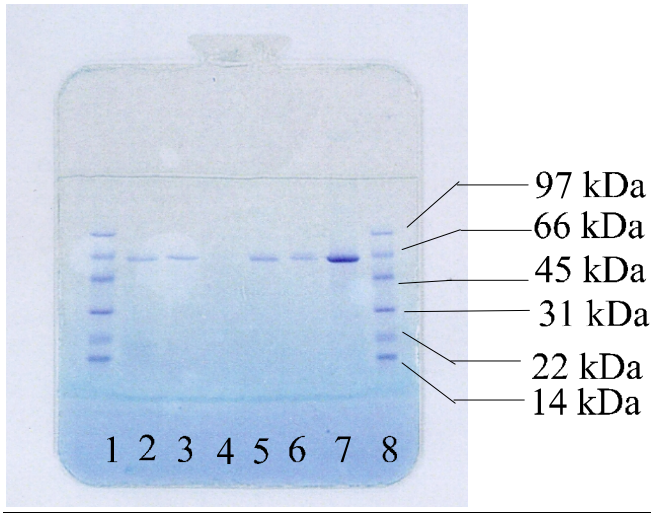



Fig. 23b. SDS-Phast gel with samples from the one step purification of lipase B and from the fermentation of lipase A.
1. Molecular weight standard
2. Fermentation supernatant lipase B(4µl)
3. Fermentation supernatant lipase A (4µl)
4. Q-sepharose after butyl sepharose, peak before gradient (10 times concentrated, 4µl)
5. Q-sepharose after butyl sepharose, peak at 13% elution buffer (10 times concentrated, 4µl)
6. Fermentation supernatant (lipase B) on Q-sepharose, peak at 13% elution buffer (4 µl)
7. Fermentation supernatant (lipase B) on Q-sepharose, peak at 13% elution buffer (10 times concentrated, 4 µl)
8. Molecular weight standard

### *3.9 Deglycosylation of the lipase B*

The polypeptide of lipase B contains two potential N-linked glycosylation consensus sequences: $Asn^{283}$-Asp-Thr and $Asn^{364}$-Ala-Thr. In *Geotrichum candidum*, the expressed lipases are glycosylated to an extent around 6% (w/w). To check if the glycosylation occurred also in the recombinant lipases, the lipase B, expressed in *Pichia pastoris*, was deglycosylated under native and denaturing conditions, using the Endo-ß-N-acetylglycosaminidase H. After separation of the samples on an SDS-PAGE, a decrease in the molecular weight of almost 3.5 kDa was observed, showing that the recombinant protein has a carbohydrate content of 5%. No difference was observed between deglycosylation under native and denaturing conditions (Fig. 24), indicating that the glycosylation sites are externally available.

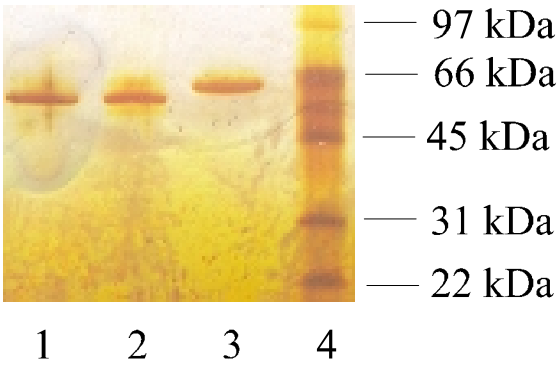



Fig. 24. SDS-PAGE analysis of lipase B samples before and after deglycosylation with Endo H. 1. Lipase B deglycosylated under native conditions; 2. Lipase B deglycosylated under denaturing conditions; 3. Lipase B directly from culture supernatant; 4. Molecular weight standard.

### *3.10 Isoelectric focusing*

To determine the isoelectrical point (pI) of the recombinant lipase B, in order to be able to compare it with the value for the homologous protein, an isoelectric focusing was performed. Two bands corresponding to proteins with slightly different pIs of 4.55 and 4.4 were seen on the gel, possibly due to different glycosylation forms (Fig. 25, lane 2). In fact, after deglycosylation only one band with a pI of 4.55 remained (Fig. 25, lane 3).

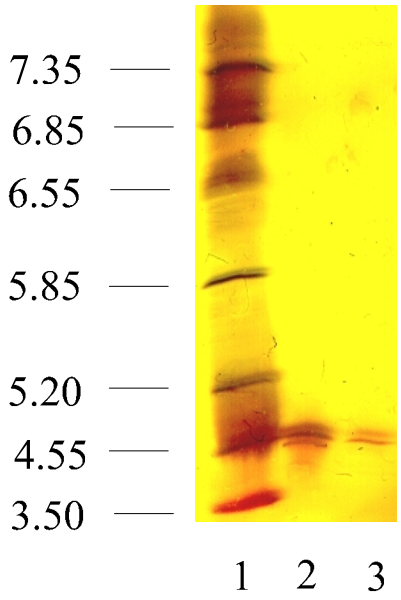



Fig. 25: Isoelectric focusing of lipase B. 1. IEF-standards; 2. Lipase B before deglycosylation; 3. Lipase B after deglycosylation.

### *3.11 Characterization of the recombinant proteins*

The pH-, temperature- stability as well as activity and substrate specificity were determined for the recombinant lipases A and B, using the purified enzymes obtained from the anion

exchange chromatography purification with Q-Sepharose.

### 3.11.1 Substrate specificity assay

A triglyceride mixture was hydrolyzed in the pH-Stat by purified lipases A and B and from the hybrid lipase A/B, consisting of the first 640 bp from lipase B and the last 1143 from lipase A. The percentage of released fatty acids with different chain lengths was measured with a GC. Table 7 shows the results.

Table 7. Substrate specificity assay for the recombinant lipases A, B and A/B from *Geotrichum candidum*.

| FA chain length | FFA released by **lipase A (%)** | FFA released by **lipase B (%)** | FFA released by **lipase A/B (%)** |
|---|---|---|---|
| C8:0 | 11.16 | 1.76 | 11.19 |
| C10:0 | 4.43 | 0.59 | 3.98 |
| C12:0 | 11.16 | 0.29 | 5.97 |
| C14:0 | 2.58 | 0.15 | 1.99 |
| C16:0 | 16.17 | 0.88 | 13.68 |
| C16:1 | 0.29 | 0.15 | 0.25 |
| C18:0 | 3.43 | 0.88 | 2.99 |
| C18:1 | 20.89 | 50.59 | 22.39 |
| C18:2 | 27.18 | 40.88 | 34.33 |
| C18:3 | 2.72 | 3.82 | 3.23 |
| C20:0 | 0.00 | 0.00 | 0.00 |
| | | | |
| SAFA (total) | 48.93 | 4.56 | 39.80 |

The assay showed, as expected, a high specificity of lipase B for substrates with a double bond in position 9 (especially C18:1, C18:2), which represents more than 95% of the total released fatty acids. In contrast lipase A showed no preference for unsatured FA, as it hydrolyzed also 50% SAFA. The hybrid lipase had a similar behavior to lipase A, hydrolyzing almost 40% SAFA.

As the hybrid lipase did not show any difference in specificity in comparison to lipase A, it

was not further investigated.

### 3.11.2 pH-activity

The activity assays were carried out in the pH-Stat at 30 °C at different pH values, using 1 ml of the Sigma lipase substrate with 29 ml of water containing 40 mM NaCl and 20 mM $CaCl_2$. The relative activity for the two lipases at different pHs is shown in Fig. 26.

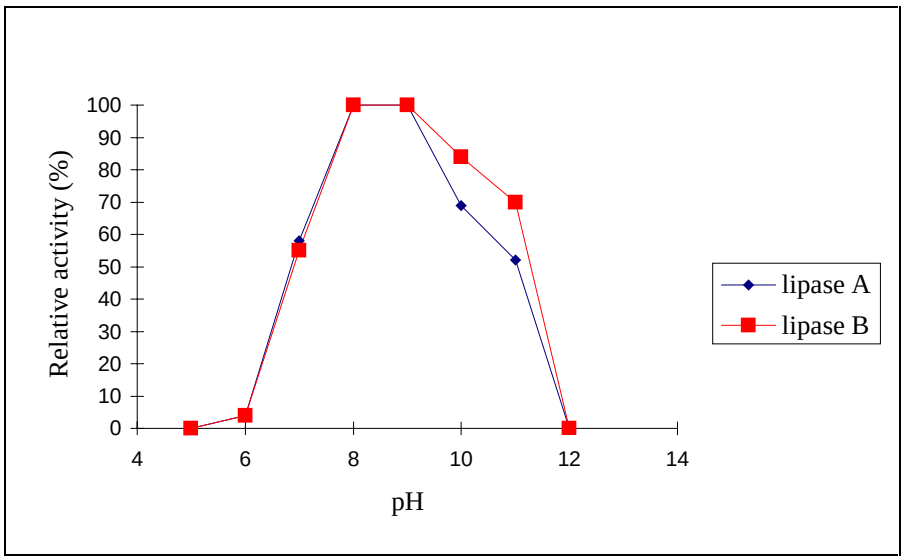



Fig. 26. pH-activity curve for *Geotrichum candidum* lipases A and B. Relative activities were determined by pH-Stat assay at 30 °C using the Sigma lipase substrate.

Fig. 26 shows that the pH/activity profile is almost the same for the two lipases, having the pH optimum between 8.0 and 9.0; around 60% of the activity was conserved at pH 7.0. The lipases are not active below pH 6.0 or at pH values above 12.

### 3.11.3 pH-stability

The different lipase preparations were incubated at a concentration of 0.1 mg/ml at 30 °C in Britton-Robinson buffers at different pH values. The residual activity was measured after 2, 4, 6 and 24 hours of incubation. The results are reported in Figs. 27-28.

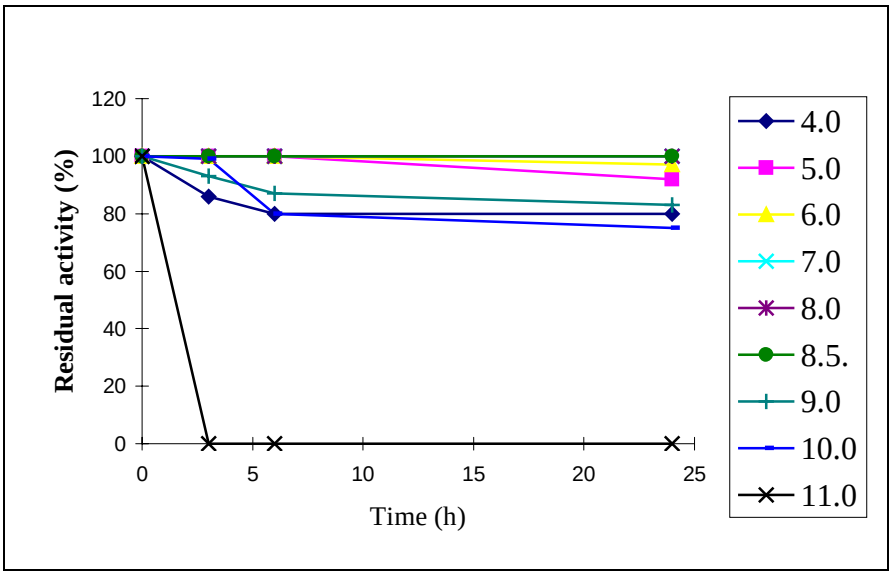



Fig. 27. Stability of lipase A from *Geotrichum candidum* incubated in Britton-Robinson buffers at different pHs, at 30 °C. Residual activities were determined by pH-Stat assay at 30 °C using the Sigma lipase substrate.

Fig. 27 shows no loss of activity for an incubation of 24 hours at 30 °C for pH values between 5.0 and 8.5 while a residual activity of about 80% remained at pH values of 4.0, 9.0 and 10.0. At pH 11.0 the lipase was rapidly inactivated after 3 hours of incubation.

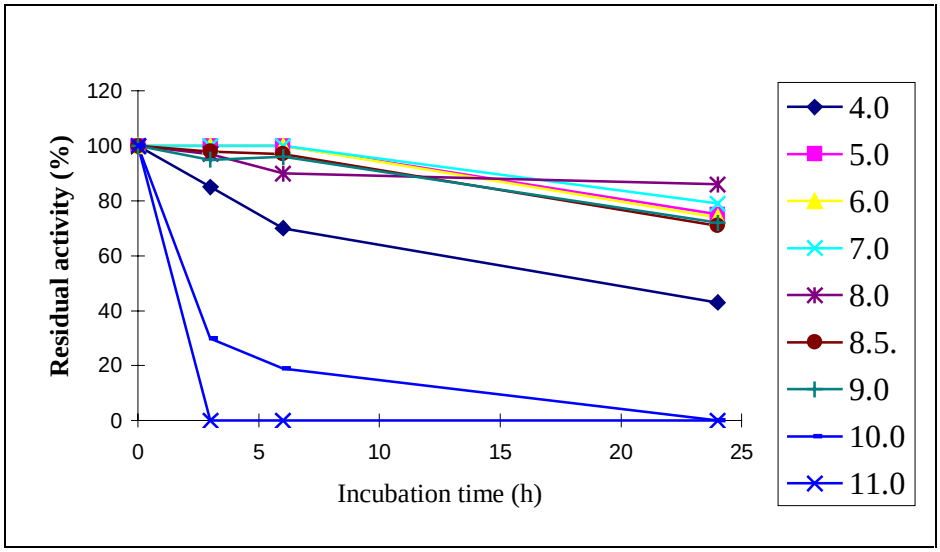



Fig. 28. Stability of the lipase B from *Geotrichum candidum* incubated in buffers at different pHs. Residual activities were determined by pH-Stat assay at 30 °C using the Sigma lipase substrate.

Fig. 28 shows that the lipase B was less stable than lipase A at almost every investigated pH value: it retains 50% activity at pH 4.0, retained no activity after 24 hours incubation at pH

10.0 as well as after 3 hours at pH 11.0. At the other pH values a residual activity of about 80-90% was shown after 24 hours of incubation.

In Fig. 29 the residual activity of the two lipases after incubation in Britton-Robinson buffers for 24 hours of at different pH values is depicted.

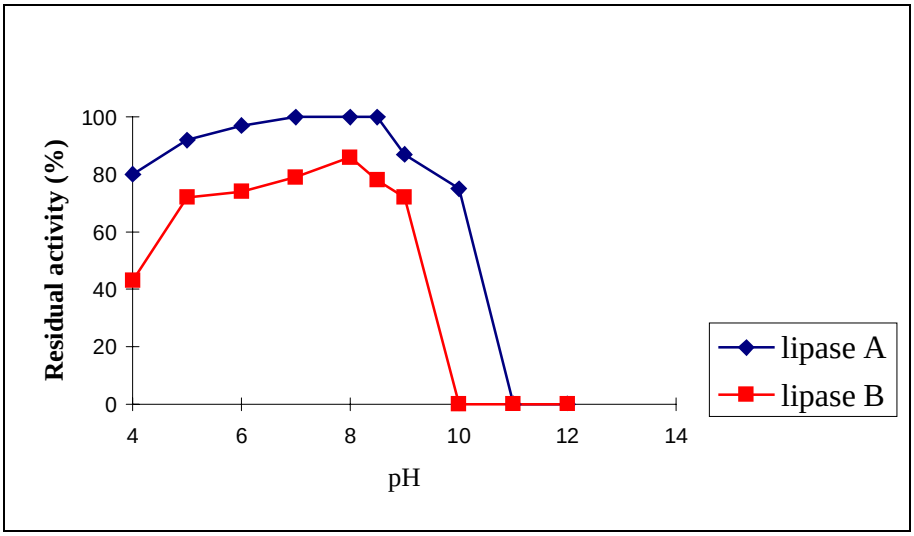



Fig. 29. pH-stability curve for the lipases A and B from *Geotrichum candidum* incubated for 24 hours in Britton-Robinsons buffers at different pH values. Residual activities were determined by pH-Stat assay at 30 °C using the Sigma lipase substrate.

The two lipases are stable over a wide pH range, starting to be denatured around pH 10.

### 3.11.4 Temperature activity

The activity assays were carried out at pH 9.0 at different temperatures.

The relative activity for the two lipases at different temperatures is reported in Fig. 30.

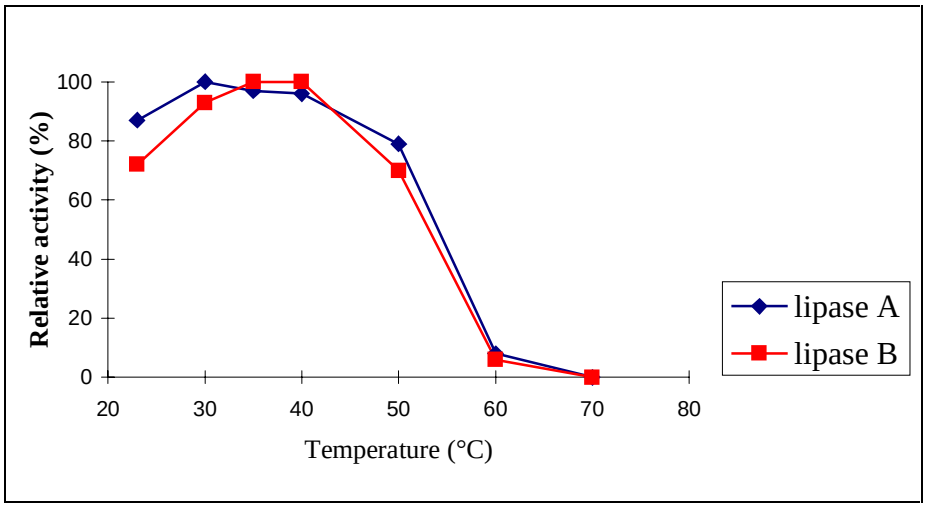



Fig. 30. Temperature-activity curve for *Geotrichum candidum* lipase A and B. Relative activities were determined by pH-Stat assay at pH 9.0 using the Sigma lipase substrate.

The temperature/activity profile is almost the same for the two lipases, having the T optimum at 40 °C. Above 50 °C the activity decreased rapidly, reaching only 5% at 60 °C, and no more residual activity at 70 °C.

### 3.11.5 Temperature stability

0.1 mg/ml of the purified lipases were incubated at different temperatures in Britton-Robinson buffer at pH 8.0, in which the lipase showed to be stable for at least 24 hours. The residual activity was measured after 2, 4, 6 and 24 hours of incubation. The results are reported in Figs. 31 and 32.

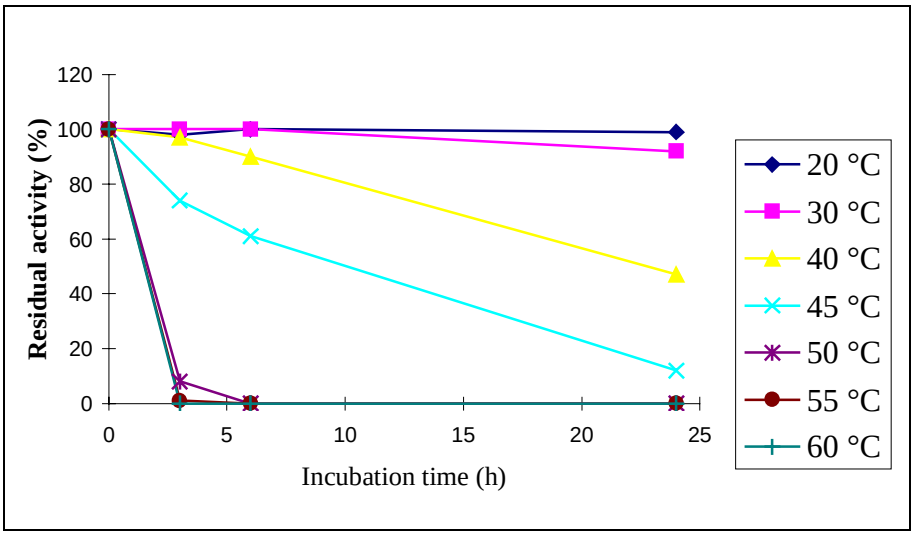



Fig. 31. Stability of the lipase A from *Geotrichum candidum* incubated at different temperatures. Residual activities were determined by pH-Stat assay at pH 9.0 using the Sigma lipase substrate.

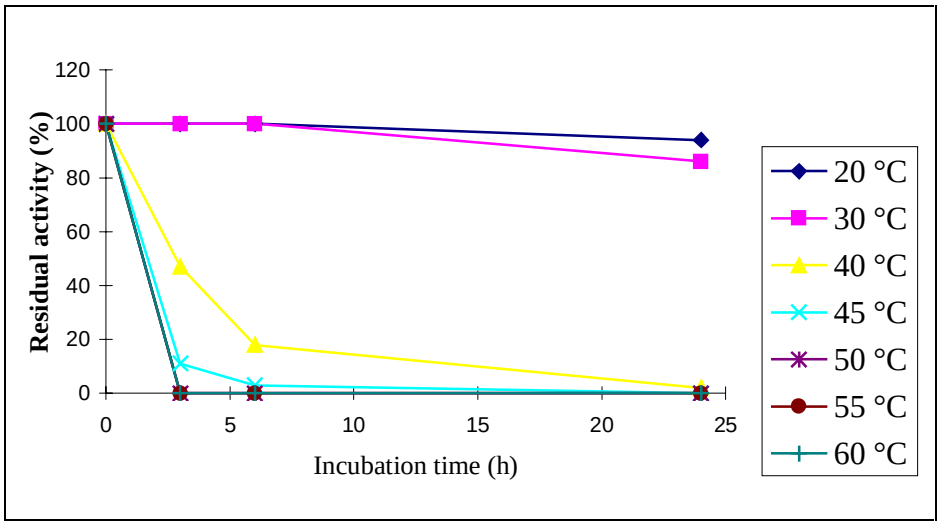



Fig. 32. Stability of the lipase B from *Geotrichum candidum* incubated at different temperatures. Residual activities were determined by pH-Stat assay at pH 9.0 using the Sigma lipase substrate.

Figures 31 and 32 show that the two lipases were stable for 24 hours at 20-30 °C; lipase B was less stable than lipase A at temperatures higher than 40 °C, its residual activity after 6 hours of incubation at 40-45 °C was less than 20%, whereas for lipase A it was still 90% at 40 °C and 60% at 45 °C. Both lipases were deactivated after 6 hours incubation at temperatures higher than 50°C.

In Fig. 33 the residual activity of the two lipases after 24 hours of incubation at different temperatures is shown.

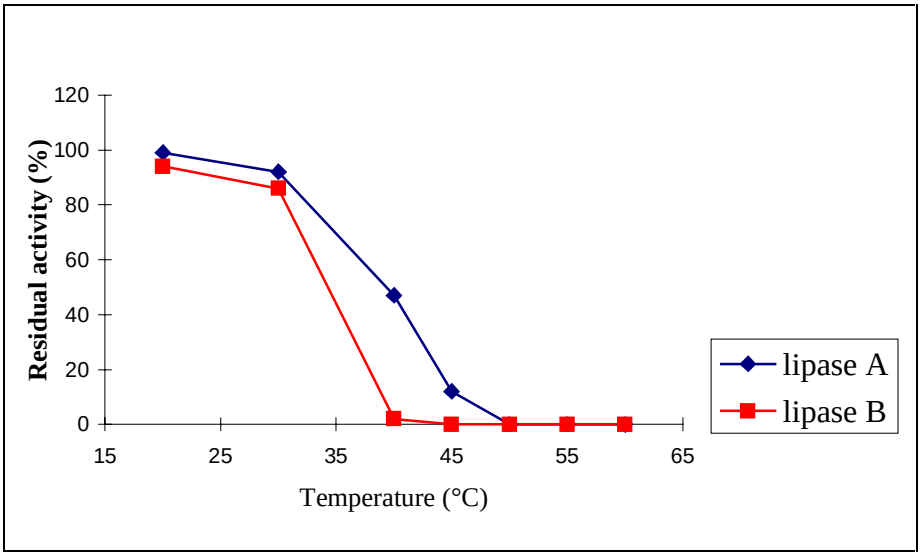



Fig. 33. Temperature-stability curve for the lipases from *Geotrichum candidum* after 24 hours incubation at different temperatures. Residual activities were determined by pH-Stat assay at pH 9.0 using the Sigma lipase substrate.

It can be seen that the two lipases at 40 °C are already rapidly denatured, although lipase A is more stable than the lipase B.

## *3.12 Increasing the thermostability of the lipase B*

The characterization of the lipase B from *Geotrichum candidum* expressed in *Pichia pastoris* revealed that the protein is not very thermostable, as it was already denatured after a few hours at 45 °C.

For industrial applications of the enzyme, an increase of its thermostability would be of economical advantage, mainly because the lipase denaturation would be slackened, allowing the reduction of catalyst to be employed and, in this way, the overall process costs.

Furthermore, studies on protein thermostability are interesting from a scientific point of view, as they can lead to understand more about the factors influencing this parameter.

Three different strategies were carried out aiming to obtain a biocatalyst with higher thermostability: in the first two, random and site-directed mutagenesis, the amino acid sequence of the lipase was modified by genetic engineering, in order to get positive changes of the desired characteristic. The third one was based on the immobilization of the enzyme on different supports, which could protect the protein from denaturation.

## *3.13 Directed evolution through random mutagenesis*

A random mutagenesis project consists of different parts which have to be independently optimized. The first step is to get random mutations to create a library of mutants of a certain gene: a technique has to be employed which leads to a number of mutations that permit the evolution without compromising the enzyme functions. The mutated gene has to be cloned and transformed in a host organism, to get a significant number of mutant clones. The mutant library has to be screened for an improved property of the desired protein. The screening procedure is clearly the rate-limiting step, and must be optimized to get a reproducible assay, capable of high throughput.

### 3.13.1 Optimization of *Saccharomyces cerevisiae* transformation

Instead of *Pichia pastoris*, which has been shown to give a higher lipase expression, *Saccharomyces cerevisiae* was used as a host organism. In contrast to *Saccharomyces cerevisiae*, *Pichia pastoris* integrates the transformed plasmids into the genomic DNA, the re-isolation of mutant genes resulting, in this way, more difficult. From *S. cerevisiae* the plasmid DNA can be isolated and the lipase gene can be sequenced to characterize the mutations occurred.

As a great number of mutants must be screened, the transformation of the host organism has to be optimized, to get as many transformants as possible, to increase the probability of finding a positive mutation.

For that reason, different transformation protocols for *Saccharomyces cerevisiae* were tested. The best results in term of number of transformants/µg DNA, were obtained with the transformation method using lithium acetate (Schiestl and Gietz 1989) (Gietz, Schiestl *et al.* 1995), where around 8000 transformants/µg DNA were obtained. The other methods (with lithium acetate and dimethyl sulfoxide, electro-transformation or with the Easy-Comp Kit from Invitrogen) resulted in a 5 to 10 times lower transformation rate.

A substantial difference was also observed between the 2 different host strains used (INVSC2 and WCG4a): transformation of the two strains with the same amount of DNA, resulted in 10 times fewer colonies for INVSC2 than for WCG4a. The influence of the medium was also tested. Plating the cells directly on tributyrin plates resulted in no colony growth. So it was not possible to screen directly for lipase activity, as tributyrin seems to be somehow toxic for the freshly transformed cells, even if they were able to grow on such plates, if the colonies were transferred on replica plates.

Bad results were obtained also using plates with galactose instead of glucose, in order to induce an early lipase production, as 2 to 4 times less transformants were obtained on galactose.

No difference in the number of colonies was observed between the transformations plated on complex medium and minimal medium.

### 3.13.2 Error prone PCR

Mutagenic PCR, carried out in error-prone conditions (Leung, Chen *et al.* 1989), was used on the vector pYαLipB, using the oligos “beginning” and “end”, homologous to the 5’ and to the 3’ ends of the lipase B gene, respectively. During the PCR cycles, mutations are generated throughout the whole gene.

The conditions for the error-prone PCR are adjusted to 0.1 mutations every 100 amplified bases. This means, that after the amplification, the lipase B gene (1700 bp) should contain 1-2 mutations, which is reasonable within the protein evolution and with the maintaining of a reasonable amount of active mutants (Rellos and Scopes 1994).

### 3.13.3 Transformation of the PCR product in yeast and analysis on the transformants

As reported in literature (Ma, Kunes *et al.* 1987) two fragments of a plasmid with overlapping extremities can be used to transform *Saccharomyces cerevisiae*. *In vivo* recombination between the homologous regions generates the complete plasmid. In this way, the ligation step, which usually leads to a limitation of the transformation yield, and the amplification of the DNA in *E. coli* can be avoided.

The DNA fragment from the error-prone PCR can be directly transformed in *S. cerevisiae* together with a 6000 bp fragment from the pYαLipB vector cut with *Bam*HI and *Kpn*I. These two fragments can recombine *in vivo*, as the they have at the extremities two homologous regions of 130 and 200 bp (Fig. 34).

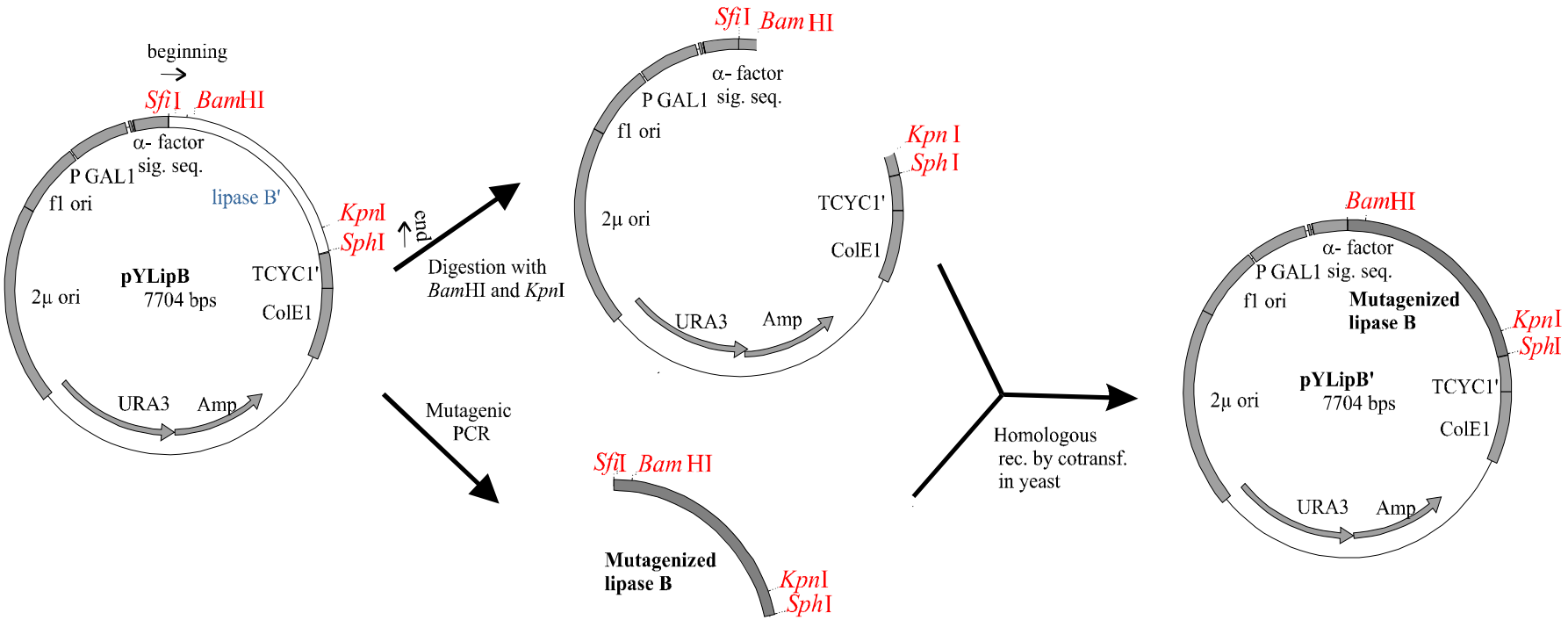
The transformation was carried out with the lithium acetate method, using different total amounts of insert and vector, and different ratios between them. A test transformation using only the gapped vector was also done to check if religation of the vector can occur. The number of colonies obtained are reported in Table 8.

Table 8. Influence on the transformation efficiency of different amounts of PCR-product and gapped vector used to transform *Saccharomyces cerevisiae*.

| **Conditions used** | **Number of transformants** |
|---|---|
| ~70 ng gapped vector | 100-400 |
| ~70 ng gapped vector + ~300 ng PCR product | 4000-8000 |
| ~70 ng gapped vector + ~150 ng PCR product | 4000-8000 |
| ~35 ng gapped vector + ~300 ng PCR product | 4000-8000 |

As can be seen, in terms of transformation efficiency, no effect could be observed varying the ratio between insert and vector in the tested range. The *in vivo* recombination seems to be very efficient, and the cotransformation of the mutagenized PCR product with the gapped plasmid led to 10-80 times more colonies when compared to the transformation of the vector alone.

Fig. 34. Strategy used to create the lipase B mutant library in *Saccharomyces cerevisiae*, using error-prone PCR and *in vivo* recombination of the mutagenized gene with the yeast vector.



### 3.13.4 Analysis of transformants

In order to test the occurred *in vivo* recombination and the presence of the plasmid with the lipase gene in the yeast transformants, some of them were grown in selective medium and afterwards cell extracts and plasmid DNA extractions were made. A PCR was performed using the plasmid DNA as template and oligonucleotides homologous to the 3' end of the lipase gene and to the 5'end of the α-factor leader sequence (beginning/end).
At the end of the reaction, the PCR mixture, separated on an agarose gel, showed a clear band corresponding to the size of the lipase gene (1700 bp), showing the presence of the lipase gene in the yeast cells extracts for 6 clones out of the 7 tested (not shown).

### 3.13.5 Activity assays on supernatants of transformant cultures

Some transformants were inoculated in 50 ml of synthetic medium. After 3 days of growth, the cells were harvested, and the lipase activity in the culture supernatant was tested with the pH-Stat, using triolein as substrate. The same was repeated using a synthetic medium to which 1% casein hydrolysate, a protease substrate, was added. The results are reported in Table 9.

Table 9. Lipolytic activity measured in the culture supernatant for three different random mutants after three days of cultivation in synthetic medium and in synthetic medium with 1% casein hydrolysate. The activity was measured on triolein at pH 9.00, at 37 °C.

| **Clone** | **Activity [U/ml] in synthetic medium** | **Activity [U/ml] in synthetic medium with 1% casein hydrolysate** |
|---|---|---|
| **A** | 1-2 | 7-8 |
| **B** | 1-2 | 1-2 |
| **C** | 1-2 | 7-8 |
| clone transformed only with pYES2, without the lipase gene | 1-2 | 1-2 |

The activity of the supernatants from the clones grown in synthetic medium showed no difference in activity in comparison to the negative control. The addition of 1% casein hydrolysate to the medium increased the activity of the culture supernatant, indicating the presence of proteases in the medium used for the first trial, that destroyed the lipase.

### 3.13.6 Mutant library

In this way, a library of random mutants of lipase B was created. 10.000 mutants were picked from the transformation plates and inoculated in microtiter plates in 200 µl of synthetic medium. The cells were grown for 48 hours, and then, if not used immediately, glycerin cultures were made, and stored at –70 °C.

### 3.13.7 Lipase screening with brilliant green plates

To screen the lipase mutant library for enhanced thermostability, a rapid assay had to be set up.

Brilliant green plates according to Svendsen *et al.* (Svendsen, Clausen *et al.* 1995) were used.

The screening assay was carried out laying two filters on the transformed yeast cells growing on a plate containing galactose: the first filter was a low protein binding filter (cellulose acetate) and the second a protein binding filter (nitrocellulose or nylon). The plates were incubated for 2-3 days at 30 °C, to let the cells grow and produce the lipase, that will be secreted and will bind to the second filter. The upper filter was then transferred in an empty plate and a mixture of agarose (1%), Tris-HCl buffer pH 9.0, brilliant green and triolein (1%) was poured on it. The colonies expressing an active lipase were singled out in the form of blue-green spots.

To screen for enhanced thermostability, the protein binding filter was incubated at a certain temperature for a specified number of minutes (conditions in which the native lipase is deactivated) and then transferred to brilliant green plates, to check the residual activity.

Different buffer and brilliant green concentrations were tried, checking the validity of this assay with different lipase solutions. 2 µl of the lipase solutions were pipetted on a nylon filter, and the assay solution with brilliant green was poured on the filter. The results are shown in Table 10. An increase up to 80 mg/l in the amount of brilliant green used, leads to an increase in the intensity of the signal; at higher brilliant green concentrations, a further increase in the signal cannot be detected. The decreasing of the buffer concentration also favours the appearance of the green spots on the filter.

Table 10. Lipolytic activity assay on different lipase solutions, using plates with triolein and brilliant green and different concentrations of Tris-HCl buffer pH 9.0 and different amounts of brilliant green. (-: no green spots were detectable after 24 hs of incubation of the plate at 30 °C; +: a green spot is visible after 12 hours incubation; ++: a green spot is visible after 10 minutes of incubation; +++: a very intense green spot is visible after 10 minutes of incubation).

| Lipase solution on the filter | [Tris-HCl]= 0.1 M [brilliant green]= 40 mg/l | [Tris-HCl]= 0.05 M [brilliant green]= 40 mg/l | [Tris-HCl]= 0.01 M [brilliant green]= 40 mg/l | [Tris-HCl]= 0.01 M [brilliant green]= 80 mg/l | [Tris-HCl]= 0.01 M [brilliant green]= 160 mg/l |
|---|---|---|---|---|---|
| 2 µl concentrated supernatant containing lipase B expressed in ***P. pastoris*** (~1000 U/ml) | - | + | + | +++ | +++ |
| 2 µl concentrated supernatant containing lipase B expressed in *S. cerevisiae*, clone **A** (~63 U/ml) | - | + | + | ++ | ++ |
| 2 µl concentrated supernatant containing lipase B expressed in *S. cerevisiae*, clone **1** (~78 U/ml) | - | + | + | ++ | ++ |
| 2 µl concentrated supernatant from **pYES2** transformed *S. cerevisiae* cells (negative control) | - | - | - | - | - |

The best results, in term of intensity of the signal per amount of lipase used, were obtained with a substrate solution composed of 0.01 M Tris-HCl pH 9.0, 1% triolein and 80 mg/l brilliant green.

The same assay was then performed in a microtiter plate, mixing 100 µl of the substrate solution with 2-4 µl of the supernatant of different mutant cultures. The microtiter plate after 20 minutes incubation at 30 °C is shown in Fig. 35.

1 2 3 4 5 6 7 8

Fig. 35. Colorimetric activity assay in a microtiter plate using different amounts of *S. cerevisiae* supernatants expressing different random mutants of GCL B.
1. 2 µl clone transformed only with pYES2 without insert
2. 2 µl clone C
3. 2 µl clone B
4. 2 µl clone A
5. 4 µl clone transformed only with pYES2 without insert
6. 4 µl clone C
7. 4 µl clone B
8. 4 µl clone A

As can be seen, the assay worked very well, and rather low amounts of lipase can also be detected, as in the case of the *S. cerevisiae* culture supernatant from the clones C and A, with a lipolytic activity of only 7-8 U/ml.

### 3.13.8 Determination of the percentage of active mutants

To check how many transformed clones were producing an active, mutagenized lipase, the activity of 300 clones was checked in microtiter plates with triolein and brilliant green.

The results showed that 20-30% of the clones were not active, whereas the others were producing active lipases. This means that the mutagenesis occurred and that the mutation yield is acceptable.

It was also pointed out that the cell growth was not homogeneous in each well, perhaps depending on the amount of cells used for inoculation, and that the lipolytic activity varied extremely from well to well. Therefore, for the development of a thermostability screening

assay, the activity after incubation of the culture supernatant must be correlated to the activity of the same supernatant before incubation. In this way, the measurements are independent from the amount of lipase in the starting solution.

### 3.13.9 A fluorescent assay

The assay method with brilliant green and triolein can be used only for a qualitative but not a quantitative measurement of the activity, as the turbidity of the solution does not permit an optical density measurement. So the residual activity after incubation cannot be correlated to the amount of lipase present originally in each culture supernatant.

For this reason, a test which permits also a quantitative determination of the activity should be preferred. To this purpose an activity assay using 4-methylumbelliferyl oleate as substrate was developed.

4-methylumbelliferyl oleate (4-MUO) can be hydrolyzed, as shown in Fig. 36, by the lipase to oleic acid and 4-methylumbelliferyl, which is intensely fluorescent. In this way, the hydrolysis reaction leads to an increase in the fluorescence signal. Esters of 4-methylumbelliferol allow the detection of very small lipase amounts (till $20X10^{-6}$ U/ml) with a very high precision (Guilbault, Sadar *et al.* 1968).

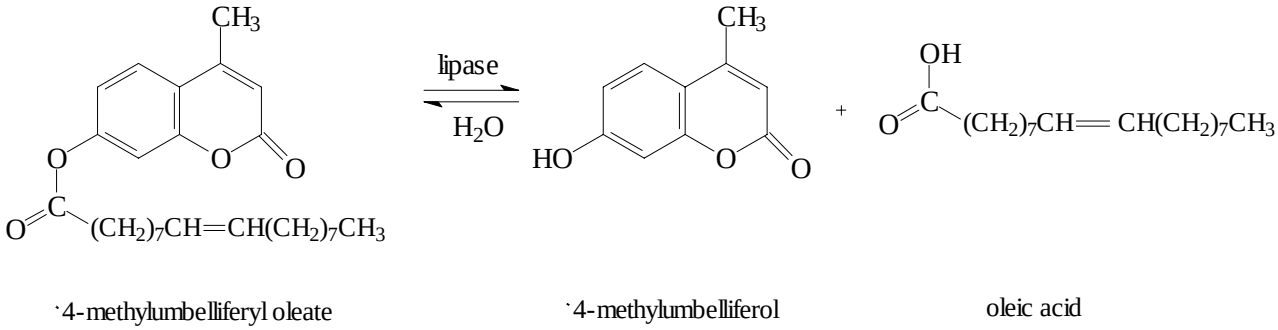



Fig. 36. Lipase catalyzed hydrolysis of 4-methylumbelliferyl oleate.

The high-throughput screening for increased thermostability of the lipase mutants was performed in the following way: the mutants were grown in 200 µl medium in microtiter plates for two days. The plates were then centrifuged, and with a pipetting robot, some microliters of the culture supernatants were transferred in two new microtiter plates. One plate was checked immediately for activity and the other one was incubated for a certain time at a defined temperature and finally the residual activity was measured. A culture producing the

wild type lipase B was present in every plate as reference for the mutants. The ratio between the activity after incubation and the one before (thermostability ratio) was calculated for the different mutants and compared to the one of the wild type. The mutants which raised a higher thermostability ratio than the wild type were considered as more thermostable.

### 3.13.10 Developing the assay

Some contrivances were developed in order to create a more reproducible assay, to find out the best measuring conditions.

#### *3.13.10.1 Choice of wave length for the measurement and gain factor of the fluorimeter*

First of all the blank of the measurement had to be reduced to decrease the relative error. Two filters (emission/excitation) had to be chosen for the fluorescence measurement among the ones available. The signal from the hydrolysis of the 4-MUO should be as high as possible with respect to the blank of the measurement, due to the plate, to the medium and to the auto-hydrolysis of the 4-MUO. In literature (Lin, Yu *et al.* 1986) (Eklund, Vainio *et al.* 1991) the use of different wave lengths for the fluorescence measurement of the hydrolysis of 4-MUO is reported. All combinations of the 2 filters in the wave length range of interest were used, and the signal of the blank and of the 4-MUO with $10^{-3}$ U of lipase (corresponding to 20 µl of culture supernatant) was tested.
The data are reported in Table 11.

Table 11. Fluorescence signal for 4-MUO at different excitation and emission wave lengths.
Microtiterplate: Greiner 96
Gain: 10
[4-methylumbelliferol oleate] = 0.01mM

| Wave lengths **(nm)** | **Signal for the plate + 4-MUO** | **Signal for the plate + 4-MUO + $10^{-3}$U lipase** |
|---|---|---|
| 355/405 | 17000 | 23000 |
| **355/460** | **2400** | **12000** |
| 355/538 | 400 | 1400 |
| 355/590 | 300 | 100 |
| 390/460 | 1800 | 4900 |
| 390/536 | 300 | 600 |

It can be pointed out that the wave lengths 355 nm for the excitation and 460 nm for the

emission are the most suitable for the measurement, as a high difference between the signal for the sample with the lipase and the one for the blank is obtained.
The gain is the amplification factor of the fluorimeter. It can be set from 1 to 255. The aim was to get a high signal with a low gain, as the amplification of the signal leads to a higher signal but at the same time also to an amplification of the absolute error. For the following measurements a gain factor = 1 was used.

#### *3.13.10.2 Plates to be used*

In trying to reduce the blank, black microtiter plates instead of the transparent ones were used. The fluorescence signal of the empty plate decreased in this way from 1500-2000 to 40.

#### *3.13.10.3 pH of the culture supernatant*

The pH of the culture supernatant which was incubated at a certain temperature, was buffered to pH 8.0 (at which the lipase showed the highest stability) with 0.1 M phosphate buffer. In this way the possible difference in pH of the various mutant cultures, which should influence also the denaturing process, was leveled.

#### *3.13.10.4 Amount of 4-methylumbelliferol oleate to be used*

In literature (Guilbalt, Sadar *et al.* 1968) a concentration of 4-MUO up to 1 mM was used, but with our measuring conditions this amount was saturating the system. The suitable concentration to be used was found to be 0.25 mM. In this way, the substrate was dissolved in 2-methoxy-ethanol to a concentration of 10 mM, and then diluted to 1 mM in Tris-HCl 0.1 M, pH 7.5. Although the lipase B from *Geotrichum candidum* shows a higher activity at more alkaline pH values, pH 7.5 was used for the activity measurements with 4-MUO to avoid self-hydrolysis of the substrate, occurring at higher pH values.
Thus 50 µl of this solution were added to the 200 µl of lipase buffered solution to start the hydrolysis reaction.

#### *3.13.10.5 Amount of supernatant to be used*

The first trials to measure the lipase activity were done using 5 µl of culture supernatant. The accuracy of the dispensing was checked, pipetting with the robot 5 µl of a pYαLipB *S. cerevisiae* supernatant in each well of a microtiter plate, adding 0.25 mM 4-MUO in 200 µl phosphate buffer (0.1 M, pH 7.5), and measuring the fluorescence signal 10 minutes after the addition of the substrate. The results are reported in Table 12.

Table 12. Fluorescence signal ($\cdot 10^{-3}$) from the different wells of 11 columns, filled with the same amount of the same solutions of WT lipase B.

| Column | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 49 | 49 | 50 | 51 | 49 | 49 | 48 | 44 | 44 | 36 | 42 | |
| | 50 | 49 | 48 | 47 | 49 | 47 | 47 | 44 | 42 | 35 | 36 | |
| | 48 | 46 | 47 | 46 | 48 | 47 | 47 | 43 | 45 | 45 | 44 | |
| | 51 | 51 | 50 | 52 | 49 | 52 | 48 | 50 | 46 | 46 | 44 | |
| | 48 | 49 | 49 | 53 | 48 | 49 | 49 | 37 | 45 | 38 | 34 | |
| | 52 | 49 | 48 | 51 | 47 | 47 | 45 | 42 | 43 | 39 | 37 | |
| | 49 | 50 | 49 | 49 | 48 | 48 | 49 | 46 | 38 | 45 | 36 | |
| | | | | | | | | | | | | Average value (whole plate) |
| **Column average value** | **50** | **49** | **49** | **50** | **48** | **48** | **48** | **44** | **43** | **41** | **39** | **46** |
| **Standard deviation** | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 4 | 2 | 4 | 4 | **4** |
| **%Error** | 3 | 3 | 2 | 5 | 1 | 3 | 3 | 8 | 6 | 11 | 10 | **10** |

It was shown that the pipetting error was very high, around 10%, and calculating the average value of every column, it can be easily seen that it increased, depending on the proceeding of the reaction during the time needed to pipette all the 12 lanes. In this way, the reaction starts 1 minute (time needed to pipette the whole plate) before for the first column with respect to the last one.

Pipetting 60 µl of a lipase solution in each well, which means a higher volume, the error on the average value of the whole plate decreased to 4%. The problem was, however, that 60 µl of the transformed *Saccharomyces cerevisiae* culture supernatant were saturating the measurement, because the lipase amounts were too high. The supernatant should be therefore diluted, but this would add another step to the screening, which should be avoided to have a high-throughput. For this reason, a compromise had to be reached between the amount of solution necessary to have a low pipetting error, and the maximal amount that could be used without saturating the measurement.

For the following measurements, 20 µl of the culture supernatant were used for the direct measurement of activity. For the determination of the residual activity 120 µl of the culture supernatant were incubated and then tested. Phosphate buffer pH 8.0 was then added to the 120 µl supernatant to a total volume of 200 µl and to a final concentration of 0.1 M.

### *3.13.10.6 Incubation time*

To decide at which temperature and how long the culture supernatant had to be incubated to check the denaturation of the different mutants, the residual activity for the wild type lipase incubated at different temperatures and different times was measured, as shown in Fig. 37. The measurement conditions (amount of supernatant and of 4-MUO used, incubation pH) are the ones reported above.

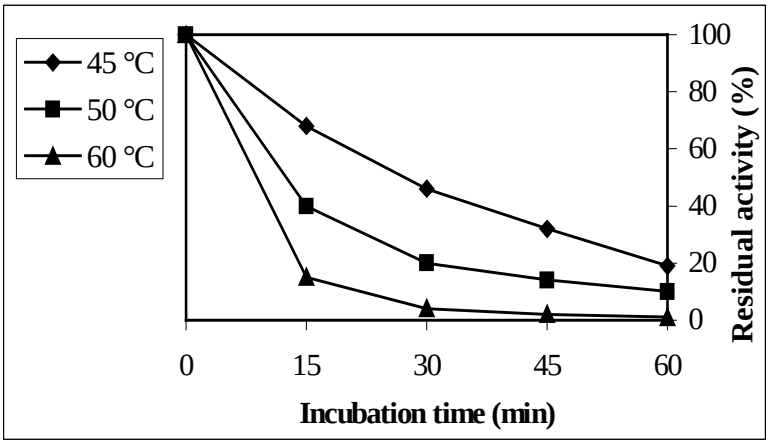



Fig. 37. Residual activity of the WT lipase B incubated for different times at different temperatures. The residual activity was determined using 0.25 mM 4-MUO, at pH 7.5.

The initial trials were carried out at 60 °C for one hour because, as the wild type in these conditions had almost no more activity, it should be easier to single out the clones which still showed some activity. The problem, in this way, was that the relative error on the measurement was too high, and small positive increases in the thermostability could not be detected, because the differences in activity of the mutants after the incubation fell within the deviations of the blank.

For this reason, for the standard assay 120 µl of the supernatants containing the lipase mutant were incubated for 30 minutes at 50 °C, resulting in about 20% of the initial activity after the incubation.

### *3.13.10.7 Reaction time*

After the addition of 4-MUO to the different samples to measure the lipolytic activity, the fluorescence signal increases with time, as the hydrolysis proceeds. A time had to be determined at which the reaction can be stopped and the fluorescence be measured. In Fig. 38 the increase of the signal with the time for different mutants, grown together in the same microtiter plate is shown.

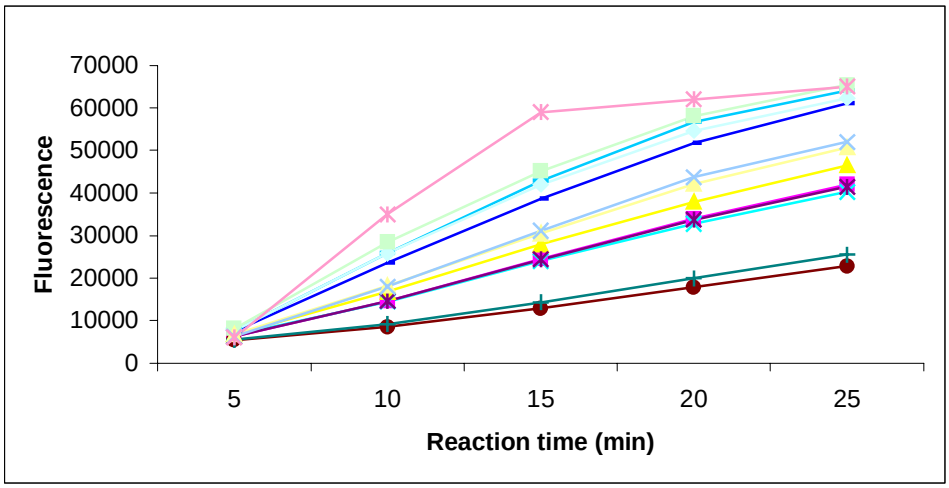



Fig. 38. Increasing of the fluorescence signal, due to the 4-MUO hydrolysis catalyzed by culture supernatants of different random mutants of lipase B.

As can be seen, the kinetic is different for each mutant, depending on the specific activity of the mutant lipase and on the lipases concentration in the medium. As the profile changes from plate to plate, the reaction time cannot be set as standard parameter, but has to be determined for each plate. Indeed, stopping the reaction after few minutes in every case, leads to very low fluorescence values with a high error rate. On the other side, waiting too long would allow some samples of the plate to reach the plateau, misrepresenting the obtained values for the thermostability. Looking at the profile each time and deciding the optimal reaction time for the particular plate is the compromise, which brings the lowest error in the measurement.
Reported in Table 13 are the kinetics for some mutants; the fluorescence signal is measured every 5 minutes for 20 minutes.

Table 13. Fluorescence signal for different wells containing culture supernatants of different random mutants of lipase B, at different reaction times. The correlation coefficient is calculated between the different columns.

| **Correlation coefficient between two columns:** | | | **A/B: 0,97995** | **B/C: 0,96179** | **C/D: 0,94501** |
|---|---|---|---|---|---|
| | | **A** | **B** | **C** | **D** |
| **Well number** | **Reaction time:** | **10 min** | **15 min** | **20 min** | **25 min** |
| **A01** | | 24682 | 32238 | 38807 | 44284 |
| **A02** | | 35328 | 45262 | 52284 | 57964 |
| **A03** | | 22611 | 29606 | 35936 | 41067 |
| **A04** | | 36976 | 46358 | 53205 | 58131 |
| **B01** | | 17946 | 23664 | 28810 | 33897 |
| **B02** | | 21828 | 28406 | 34485 | 39823 |
| **B03** | | 43944 | 49033 | 50789 | 50919 |
| **B04** | | 45605 | 49231 | 49563 | 49303 |
| **C01** | | 41766 | 46042 | 46561 | 46296 |
| **C02** | | 53226 | 56150 | 55720 | 55190 |
| **C03** | | 33531 | 38734 | 40798 | 41356 |
| **C04** | | 36947 | 40446 | 41580 | 41446 |
| **E01** | | 13008 | 17700 | 22835 | 28127 |
| **E02** | | 10615 | 14068 | 18139 | 22119 |
| **E03** | | 11829 | 16100 | 20766 | 25724 |
| **E04** | | 12329 | 16848 | 21804 | 26812 |
| **F01** | | 8762 | 11374 | 14512 | 17633 |
| **F02** | | 9275 | 11906 | 15019 | 18276 |
| **F03** | | 31180 | 42455 | 52353 | 60924 |
| **F04** | | 32860 | 45178 | 55485 | 63858 |
| **G01** | | 32794 | 44888 | 55358 | 63873 |
| **G02** | | 36054 | 48279 | 59234 | 65535 |
| **G03** | | 18475 | 25559 | 32821 | 39519 |
| **G04** | | 19568 | 27146 | 34665 | 41158 |

The correlation coefficient can be calculated between the different columns to check if the signal increases linearly with the time. The correlation factor ($\rho_{x,y}$) shows the extent of how two series of data are correlated to each other.

It is calculated using the following formula:

$$\rho_{x,y} = \frac{\mathrm{cov}(X, Y)}{\sigma_x \times \sigma_y}$$

with :

$$\mathrm{cov}(X, Y) = \frac{1}{n}\sum_i (x_i - \overline{x})(y_i - \overline{y})$$

$$\sigma_x^2 = \frac{1}{n}\sum_{x_i} (x_i - \overline{x})^2$$

where $\sigma_x$ is the standard deviation and cov(X,Y) is the covariance.

The coefficients obtained were almost 1, that means that the four columns are highly correlated. Anyway, the correlation decreases with the time, because some samples reach the plateau after 15 minutes of reaction. In this case the columns A and B, with the highest correlation coefficient, can be used to calculate the thermostability ratio.

#### *3.13.10.8 Final assay*

After the evolution of the assay procedure, the standard screening was carried out in the following way: 96 mutants were picked in a sterile microtiter plate containing 200 µl of synthetic medium in each well. The plates were well sealed in order to avoid evaporation of the medium, and were then shaken for 48 hours at 30 °C. Afterwards the plates were centrifuged in order to precipitate the cells and 20 µl of the supernatant were drawn from each well and transferred to a new microtiter plate with the help of the pipetting robot. To the 20 µl were added 130 µl of phosphate buffer (0.1 M, pH 8.0), and immediately before measuring the fluorescence, 50 µl of the standard 4-MUO solution. The plate was inserted in the fluorimeter and the fluorescence was measured.

The optimal measuring time was chosen, and the corresponding data column was saved for further elaborations.

In the same way, 120 µl supernatant were transferred to a new microtiter plate, 30 µl phosphate buffer (0.5 M, pH 8.0) were added and the plate was sealed with an aluminum sticky sheet. The plate was incubated at 50 °C for 30 minutes and was then put on ice for 5 minutes. 50 µl of the standard 4-MUO solution were added and the fluorescence was measured immediately with the method reported above. One data column was chosen; the fluorescence value of each well after the incubation was divided by the corresponding value obtained before the incubation. The resulting value is the thermostability ratio, which is an index of the thermostability of the mutant lipase expressed by the corresponding yeast clone.

If this ratio is divided per 6 (corresponding to that between the amounts of supernatant used for the two measurements = 120 µl/20 µl) it represents the residual activity (from 1 to 0) after the incubation.

As in every plate a clone expressing the wild type lipase is present, all the ratios obtained can be compared with this reference, and the higher ones can be considered as more thermostable.

### 3.13.11 Results

1000 mutant clones were screened. Three clones, that were more thermostable than the WT, were identified (Table 14), but after isolation and sequencing of the plasmids, no mutations were found, their sequence corresponding exactly to the WT.

Table 14. Results from the activity measurements on the wild type and three mutants before and after incubation at 50 °C for 30 min. The thermostability ratio and its average value were determined. The measurements were repeated twice for each sample. The activity was measured with 4-MUO, as reported in section 3.13.10.8.

| Sample | Fluorescence signal before incubation | Fluorescence signal after incubation | Thermostability ratio (=TR) | TR average value |
|---|---|---|---|---|
| **Wild Type** | 24477 | 11070 | 0.45 | 0.43 |
| **“** | 37989 | 15213 | 0.40 | |
| **Mutant 1** | 22334 | 11983 | 0.54 | 0.54 |
| **“** | 22236 | 11778 | 0.53 | |
| **Mutant 2** | 5425 | 3315 | 0.61 | 0.59 |
| **“** | 5448 | 3027 | 0.56 | |
| **Mutant 3** | 7750 | 3843 | 0.50 | 0.49 |
| **“** | 7721 | 3718 | 0.48 | |

This means that the error on a single measurement is still too large to determine small increases in the thermostability. The average value on more determinations would offer a value of a higher accuracy, but of course this would require more effort, in contrast to the concept of high-throughput screening. The assay can be used for measuring the thermostability for point mutants, as the number of clones to be tested is limited and allows also a multiple determination.

### 3.13.12 Influence of the protein content

Many different parameters were taken into account, but, as the error of the measurements was always rather high, it was thought that the different protein content in the culture supernatants,

was responsible for the different thermal denaturation of the same samples. In fact, the growth of the cells could not be homogeneous in each well, as well as the lipase concentration. The protein concentration in the solution can influence the inactivation process at 50 °C, altering the results obtained.

This could explain the observation that repeated measurements of the same supernatant are affected by an error of around 10%, while inoculating the same clone in different tubes and comparing the thermostability values obtained, an error of 16-18% affected the measurement (not shown). This means that the extent to which the cells grow influences in some way the apparent thermostability of the lipases produced.

To check this hypothesis, it was decided to incubate two clones in six different tubes and test the error on the average stability value obtained, in the first case carrying out the standard assay, in the second case adding an excess of another protein to the lipase solution to be incubated. The protein added was bovine serum albumin (BSA), in a concentration of 1 and 10%. The results with 10% BSA are reported in Table 15.

Table 15. Thermostability ratios for the wild type lipase B and for a mutant, incubated with and without albumin.

| **WITHOUT BSA** | | |
|---|---|---|
| **Samples** | **Thermostability ratios** | **Calculated parameters** |
| Wild type | 2.14 | Average value: 1,9<br>Standard deviation: 0,4<br>**Error: 18%**<br>Error on 6 measurements: 7% |
| " | 1.37 | |
| " | 2.49 | |
| " | 1.67 | |
| " | 2.07 | |
| " | 1.84 | |
| Mutant | 0.25 | Average value: 0,2<br>Standard deviation: 0,04<br>**Error: 16%**<br>Error on 6 measurements: 7% |
| " | 0.22 | |
| " | 0.24 | |
| " | 0.19 | |
| " | 0.31 | |
| " | 0.27 | |
| **WITH 10% BSA** | | |
| **Samples** | **Thermostability ratios** | **Calculated parameters** |
| Wild type | 3.32 | Average value: 3,04<br>Standard deviation: 0,56<br>**Error: 18%**<br>Error on 6 measurements: 7% |
| " | 3.97 | |
| " | 3.02 | |
| " | 3.19 | |
| " | 2.30 | |
| " | 2.45 | |
| Mutant | 0.86 | Average value: 0,67<br>Standard deviation: 0,11<br>**Error: 17%**<br>Error on 6 measurements: 7% |
| " | 0.58 | |
| " | 0.60 | |
| " | 0.59 | |
| " | 0.78 | |
| " | 0.58 | |

It can be pointed out that the error on the determined thermostability ratios, is not reduced if the protein is incubated in the presence of BSA. This shows that the protein content in the supernatants is not the main reason for the unreproducibility of the determination.

## *3.14 Site-directed mutagenesis*

### 3.14.1 Engineering a disulfide bridge

Two different strategies were used in this work to increase the thermostability of the lipase B from *Geotrichum candidum* through site-directed mutagenesis: the introduction of a disulfide bridge and the improvement of the salt bridges pattern on the surface of the protein.

To find out which positions of the GCL B could be mutated to increase its stability, its X-ray structure was compared with that of the *Torpedo californica* acetylcholinesterase (AChE) and of the lipase from *Candida rugosa* (CRL), homologous proteins, in order to point out the structural differences.

The AChE and the lipase from *Geotrichum candidum* show 30% homology, and in the central region up to 70% homology. As can be seen in Fig. 39, whereas two disulfide bridges are well conserved in both proteins and in the homologous lipase from *Candida rugosa*, an additional disulfide bridge is present in the region near the C-terminus of the acetylcholinesterase.

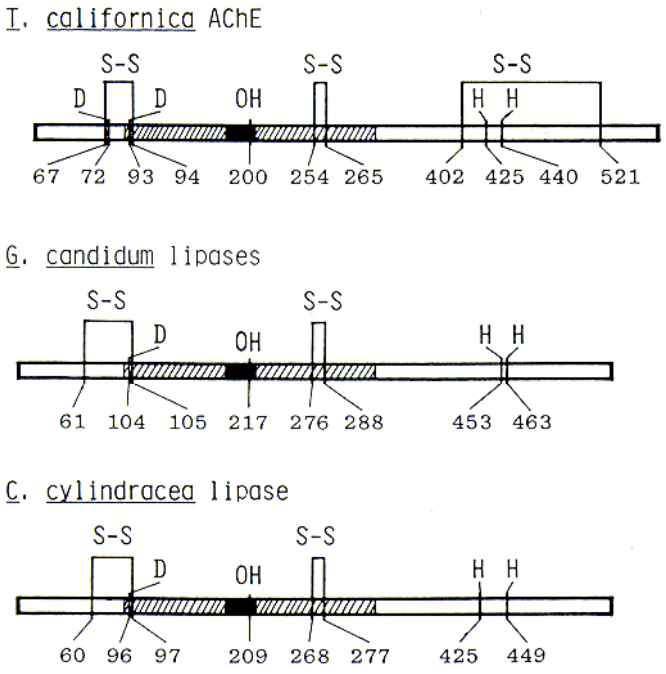



Fig. 39. Schematic diagrams of *T. californica* AChE, *G. candidum* lipase, *C. cylindracea* (now renamed *C. rugosa*) lipase. The position of the disulfide bonds is indicated by S-S. Closed and hatched boxes indicate the regions showing about 70% and 30% homology, respectively (Shimada, Sugihara *et al.* 1990). Ser: OH; Asp: D and His: H.

A detailed comparison of the structures of the AChE near the Cys402-Cys521 disulfide bond with the homologous region of the GCL B, pointed out that, although the sequence homology in this region is not very high, the structural homology is remarkable. This means that concerning the amino acid distances and structure in the GCL B a third S-S bridge could be introduced which could have a stabilizing effect. Besides, a disulfide bridge in this position would fix the flexible helix at the C-terminus, and therefore could also contribute to an increase in the stability (Fig. 40).

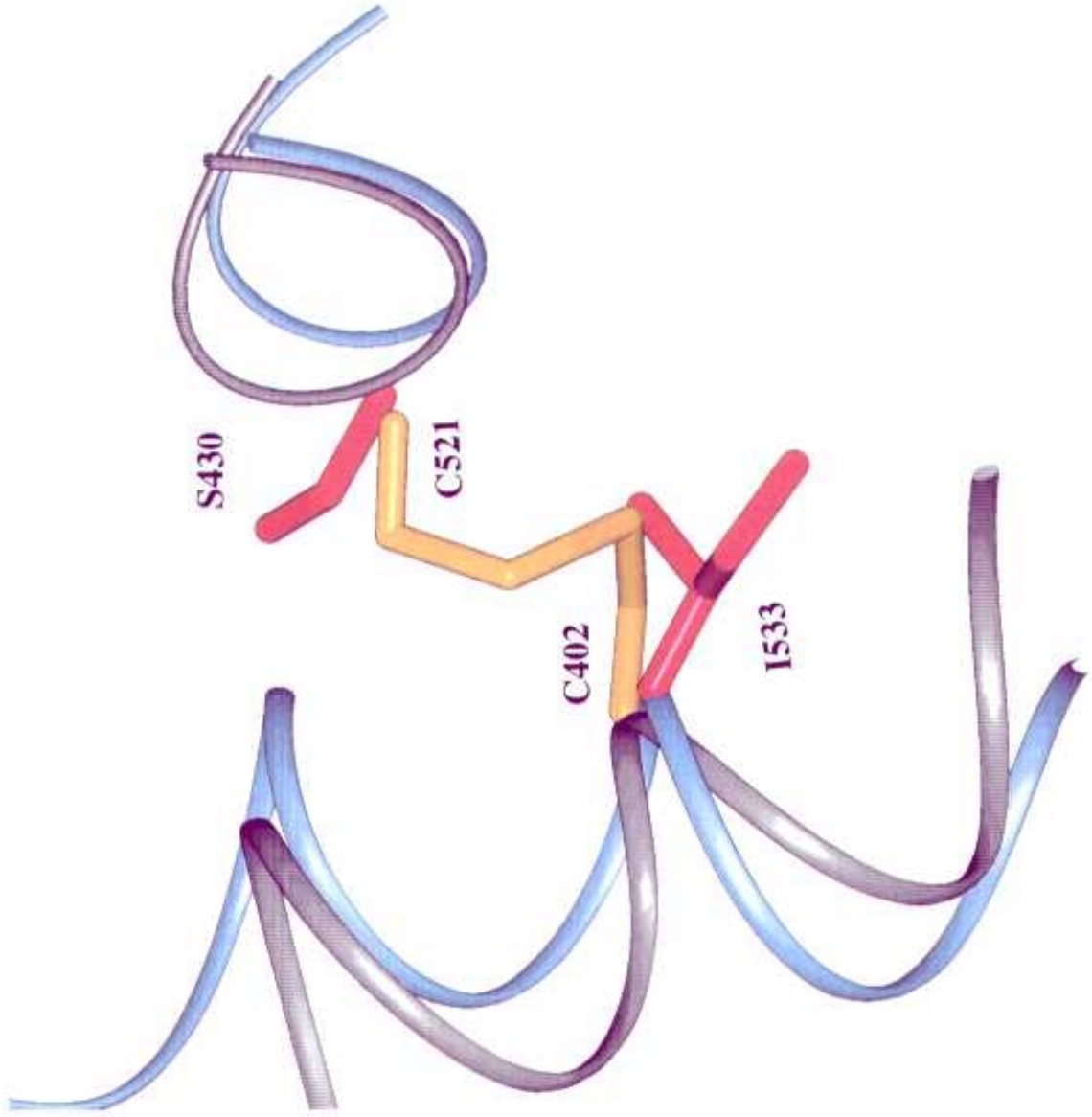



Fig. 40. Disulfide bridge C402/C521 in the AChE (in yellow) and corresponding region in the GCL (in red). In gray are the helices from the AChE, in blue those from GCL.

The introduction of an additional disulfide bond through site-directed mutagenesis has been one of the most popular ways to stabilize a protein, though the results obtained in the past years have been contradictory. The stabilization of proteins by the introduction of S-S bonds is thought to be a consequence of decreasing the conformational entropy of the backbone in the denatured state, restricting the degree of freedom for the unfolded state and thereby stabilizing the folded state (Matsumura, Signor *et al.* 1989).

The sites planking to the two cysteines building the bridge in the acetylcholinesterase and the corresponding region of GCL B are:

| | | | | |
|---|---|---|---|---|
| **AChE** | 399 | NVI**C**PLM | 518 | VQM**C**VFW |
| **GCLB** | 427 | LFQ**S**PRR | 530 | I EG **I** SNF |

In this way, to introduce the S-S bridge in the corresponding position of the lipase the two mutations, S430C and I533C, were introduced separately in the lipase gene. The mutations were introduced with the Quik-change kit using the vector pGαLipB as template and the primers S430C, S430Crev, I533C and I533Crev.

The PCR was carried out in the standard conditions, and after transformation of the mutated plasmid in *Escherichia coli*, the positive clones, containing the mutated plasmids, could be easily detected by restriction analysis, as a restriction site was introduced or deleted together with the mutation. In particular, a *Bst*BI site is introduced with the oligo I533C, and a *Bse*RI site is destroyed after PCR with the oligo S430C.

To generate a mutant containing both mutations, the S430C mutant was taken as template, and a second round of Quik-change was performed using the primers I533C and I533Crev.

The mutated lipase genes were recloned in a pGAPZαC vector in order to eliminate secondary mutations randomly occurred during the amplification of the whole plasmid. The gene was cut out from the plasmid with *Xba*I and *Sfi*I and ligated in pGAPZαC cut in the same way.

The mutated lipase genes were also sequenced to exclude the presence of additional, undesired mutations.

The obtained mutant plasmids and the WT lipase B were transformed in *Pichia pastoris* and at least four clones of each transformation were inoculated in 20 ml YEPD. After 3 days growth, the culture supernatant, containing the expressed and secreted lipase, was separated from the cells by centrifugation and used for further analysis.

To verify that the presence of two additional cysteines led to the building of an additional

disulfide bridge, a Phast-gel under native conditions was carried out with 1 µl of the culture supernatant for the wild type lipase and for the mutant S430C/I533C.

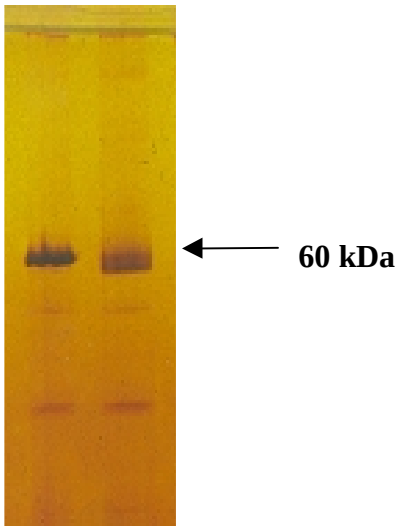



Fig. 41. Native Phast-gel of the culture supernatant after silver staining.
1: GCL B wild type expressed in *Pichia pastoris*
2: GCL B S430C/I533C expressed in *Pichia pastoris*

As shown in Fig. 41, the mutant S430C/I533C shows a slightly higher mobility in the gel. It was concluded in previous studies (Pollitt and Zalkin 1983), that proteins containing a disulfide linkage migrate further in the gel, due to shorter gyration radii, leading to more compact molecules.

### 3.14.2 Effect of the additional disulfide bridge on the thermostability

The activity of the different supernatants was measured directly and after incubation at 50 °C for 30 minutes through fluorescence measurement with 4-methylumbelliferol oleate (Table 16). The assay developed for the random approach was used to determine the thermostability ratio of the site-directed mutagenesis mutants. 4 to 6 clones were used for the measurement of each mutant in order to reduce the error on the average value. The results are reported in Fig. 42.

Table 16. Results of the thermostability measurements of the wild type lipase B and of the three mutants containing one or two additional cysteines. For each mutant four differtent clones were examined by 4-MUO assay. The error is calculated as standard deviation.

| **Sample** | **Fluorescence signal before incubation** | **Fluorescence signal after 30 min. at 50 °C** | **Thermostability ratio (=TR)** | **Average value between the TR of different clones** |
|---|---|---|---|---|
| **S430C/I533C, 1** | 42207 | 4497 | 0.11 | |
| **S430C/I533C, 2** | 42889 | 6161 | 0.14 | |
| **S430C/I533C, 3** | 19796 | 2015 | 0.10 | |
| **S430C/I533C, 4** | 19943 | 2087 | 0.10 | **0.11±0.014** |
| **S430C/I533C, 5** | 20225 | 2190 | 0.11 | |
| **S430C/I533C, 6** | 20689 | 2101 | 0.10 | |
| **S430C,1** | 39394 | 9266 | 0.24 | |
| **S430C,2** | 41081 | 9174 | 0.22 | |
| **S430C,3** | 53533 | 10590 | 0.20 | **0.21±0.019** |
| **S430C,4** | 55502 | 10297 | 0.19 | |
| **I533C,1** | 29386 | 7121 | 0.24 | |
| **I533C,2** | 29073 | 7031 | 0.24 | |
| **I533C,3** | 26092 | 6007 | 0.23 | **0.23±0.008** |
| **I533C,4** | 27084 | 5876 | 0.22 | |
| **Wild type,1** | 26108 | 6748 | 0.26 | |
| **Wild type,2** | 26793 | 6402 | 0.24 | |
| **Wild type,3** | 27411 | 7155 | 0.26 | **0.26±0.010** |
| **Wild type,4** | 27781 | 7526 | 0.27 | |

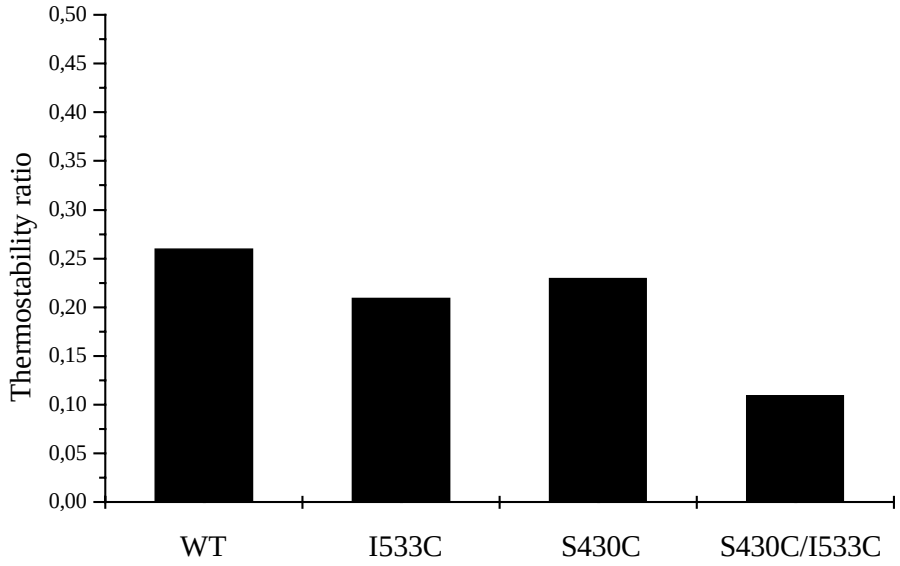



Fig. 42. Comparison of the thermostability ratios of the cysteine-mutants and of the wild type lipase B. The activity before and after incubation was measured with the 4-MUO assay.

Table 16 and Fig. 42 show that the activity of the mutants, and then their expression level, is comparable to the one of the wild type, but as can be easily seen, the mutant with the additional disulfide bridge shows an opposite behavior as expected: it is less thermostable than the wild type. In contrast, the two single mutants show almost no change in the stability. Thus, it can be concluded, that the instability of the double mutant arises from the building of the bond between the two cysteines.

### 3.14.3 Computer Modeling of the region near the additional disulfide bridge

After the unexpected results with the cysteine mutants, it was tried to understand, through careful comparison of the structures of GCL and AChE, which is the effect/factor for the greater instability due to the introduced disulfide bridge in GCL with respect to the AChE.
It was supposed, that, due to the binding of the two cysteines, there is a movement of the helix I530-T538 which comes in contact with the helix E384-L393. This could give rise to a clash between the residues N535/F536 and L393. The analogous residues in the AChE are farther away, as can be seen in Fig. 43.

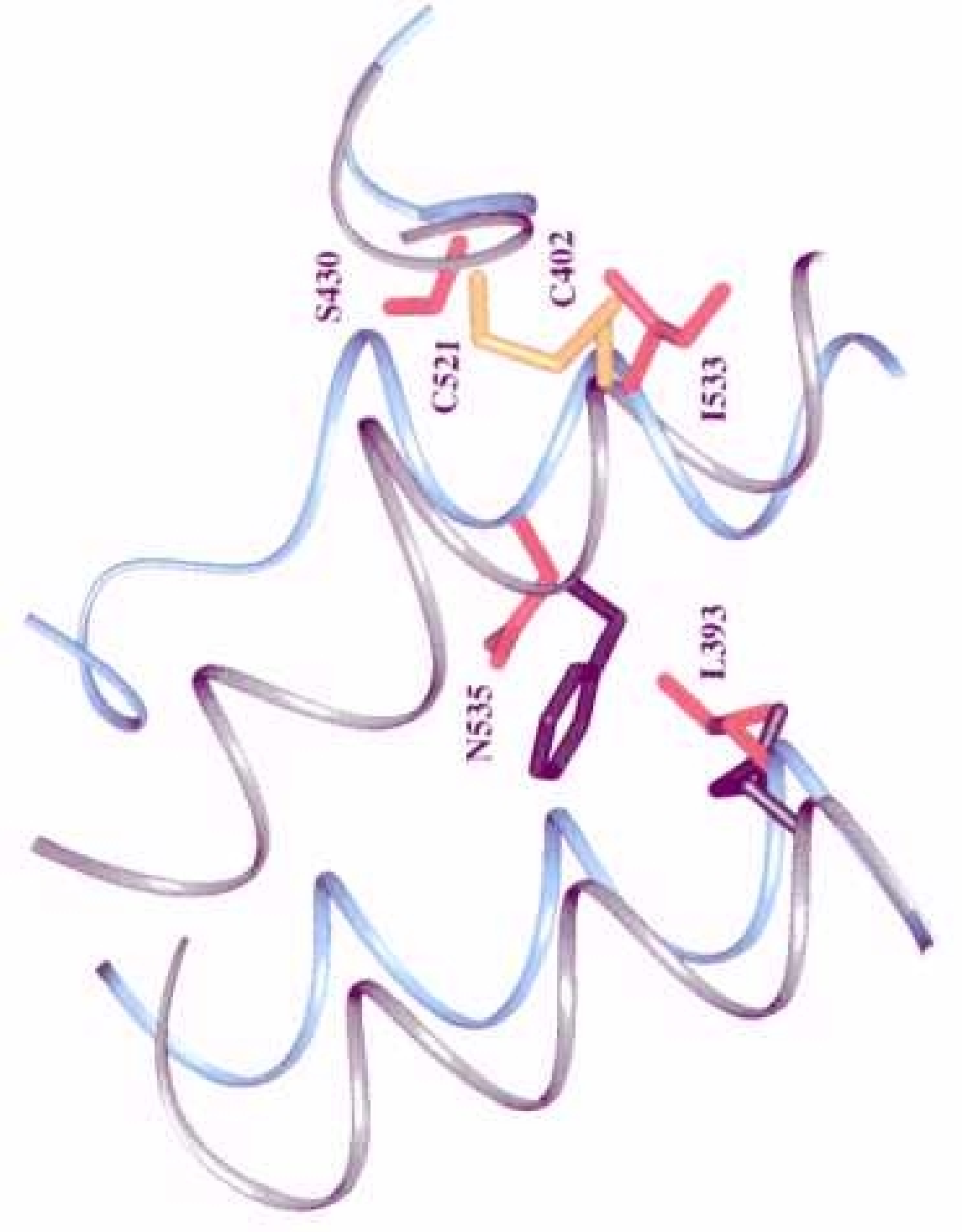



Fig. 43. Region near the additional disulfide bridge of GCL, compared to the corresponding region of AChE. In gray are the helices from the AChE, in blue those from GCL; in yellow the S-S bridge in the AChE, in red the residues in GCL which were mutated compared to the analogous from AChE (in black).

After these considerations, the second round of mutants, based on the mutant S430C/I533C was generated. In order to minimize the interaction between the two helices, the residue L393 and N535 should be replaced by smaller amino acids.

### 3.14.4 2nd round of mutations

- The residue N535 was replaced by smaller residues, alanine and valine.
  The oligos necessary to introduce these mutations through Quik-change were N535A,V and N535A,Vrev. The introduction of the mutation could be easily confirmed by *Bst*BI digestion, as this site will be destroyed in the mutant.
- In the mutants N535A/V, the residue L393 was substituted in the same way by smaller residues, alanine and valine. As this residue is hydrophobic and located on the surface of the protein, it could be of advantage to make it hydrophilic, like the corresponding amino acid present in the AChE. Which is why it was also mutated to glutamine.
  The oligos necessary to introduce these mutations through Quick-change were L393A,V, L393A,Vrev, L393Q and L393Qrev.

The mutated lipase genes after the Quik-change, were recloned in the original vector, as described previously, and sequenced in order to confirm the introduction of the desired mutations and to exclude the presence of additional ones. The four following combinations were done:

N535V
N535A
N535V/L393V
N535A/L393A
N535V/L393A
N535V/L393Q

The mutated plasmids were transformed in *Pichia pastoris* and the supernatants of the different mutants, separated from the cells by centrifugation, were used for further analysis.

### 3.14.5 Effect of the 2nd round of mutations on the thermostability

The activity of the supernatant of the different mutants was measured directly and after incubation at 50 °C for 30 minutes, by fluorescence measurement using 4-MUO.
The thermostability assay was repeated on 10 clones for each mutant, and the average value was calculated, to have more reliable results. The data are reported in Table 17 and in Fig. 44.

Table 17. Results of the thermostability measurements of the wild type lipase B and of the different mutants. For each mutant 10 different clones were examined by 4-MUO assay.

| Mutant | Fluorescence signal before incubation | Fluorescence signal after 30 min at 50 °C | Average value TR |
|---|---|---|---|
| Wild type | 78840 | 31785 | |
| " | 104806 | 38903 | |
| " | 88896 | 31168 | |
| " | 94540 | 29800 | |
| " | 70150 | 23478 | **0.37±0.06** |
| " | 76568 | 21764 | |
| " | 87134 | 39185 | |
| " | 106108 | 47772 | |
| | | | |
| S430C/I533C | 50783 | 32649 | |
| " | 49942 | 41025 | |
| " | 47082 | 47984 | |
| " | 53060 | 53182 | |
| " | 47580 | 46152 | **0.15±0.02** |
| " | 47158 | 47545 | |
| " | 47280 | 44626 | |
| " | 51522 | 46676 | |
| " | 47483 | 39092 | |
| " | 47965 | 32954 | |
| | | | |
| S430C/I533C/**N535V** | 55826 | 36938 | |
| " | 54764 | 40869 | |
| " | 48031 | 33443 | |
| " | 52311 | 36156 | |
| " | 31191 | 25481 | **0.13±0.02** |
| " | 30373 | 26820 | |
| " | 29028 | 25580 | |
| " | 30309 | 25405 | |
| " | 30086 | 21559 | |
| " | 32868 | 21320 | |
| | | | |
| S430C/I533C/**N535A** | 32577 | 25051 | |
| " | 35953 | 28036 | |
| " | 31024 | 34092 | |
| " | 30973 | 34421 | |
| " | 22520 | 26458 | **0.18±0.03** |
| " | 23389 | 26139 | |
| " | 24776 | 32739 | |
| " | 24392 | 31266 | |

| | | | |
|---|---|---|---|
| “ | 35991 | 39725 | |
| “ | 39050 | 37599 | |
| S430C/I533C/ **N535V/L393V** | 46784 | 37442 | |
| “ | 49630 | 43895 | |
| “ | 36973 | 39197 | |
| “ | 39889 | 40289 | |
| “ | 31983 | 45953 | **0.18±0.03** |
| “ | 39231 | 43754 | |
| “ | 49404 | 62919 | |
| “ | 50168 | 59483 | |
| “ | 42950 | 46450 | |
| “ | 42000 | 40913 | |
| | | | |
| S430C/I533C/ **N535A/L393A** | 32122 | 29693 | |
| “ | 31642 | 32017 | |
| “ | 20535 | 21450 | |
| “ | 19613 | 21829 | |
| “ | 33853 | 38102 | **0.18±0.02** |
| “ | 35446 | 38522 | |
| “ | 23803 | 28674 | |
| “ | 24453 | 28856 | |
| “ | 41999 | 40990 | |
| “ | 41629 | 37103 | |
| | | | |
| S430C/I533C/ **N535V/L393Q** | 50672 | 34382 | |
| “ | 49930 | 39780 | |
| “ | 28052 | 26576 | |
| “ | 29830 | 28836 | |
| “ | 25705 | 21821 | **0.15±0.02** |
| “ | 25798 | 21963 | |
| “ | 31732 | 29052 | |
| “ | 31310 | 31374 | |
| “ | 19302 | 20658 | |
| “ | 24329 | 18584 | |
| | | | |
| S430C/I533C/ **N535V/L393A** | 31625 | 20547 | |
| “ | 32814 | 22447 | |
| “ | 34220 | 27534 | |
| “ | 37117 | 29470 | |
| “ | 33227 | 27941 | **0.13±0.01** |
| “ | 34898 | 28884 | |
| “ | 48102 | 43719 | |
| “ | 50301 | 43943 | |

| | | | |
|---|---|---|---|
| “ | 36627 | 29751 | |
| “ | 38565 | 26736 | |

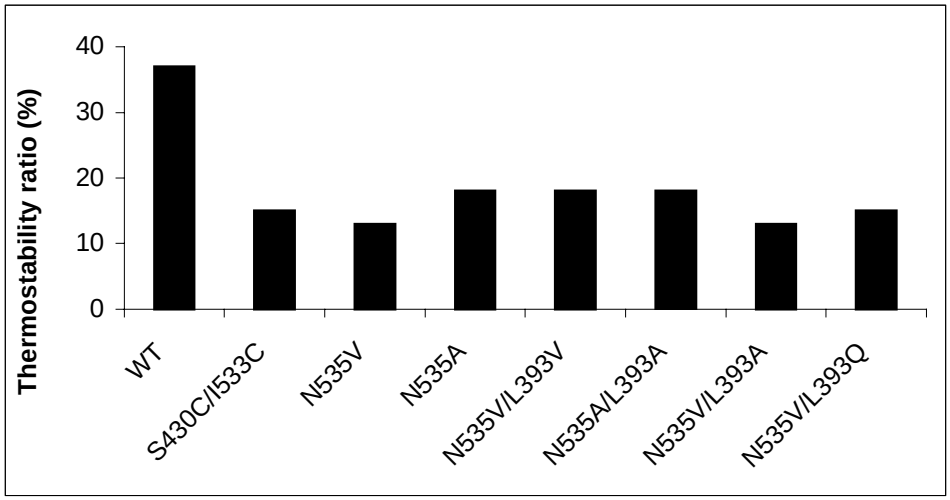



Fig. 44. Average values of the thermostability ratio of the different mutants in comparison to the WT lipase B. The activity before and after incubation was measured with the 4-MUO assay.

It can be seen that the residual activity of the six mutants after incubation at 50 °C for 30 minutes is still around only 42% of the one of the wild type B incubated in the same conditions.

### 3.14.6 Mutating lysines in arginines

Increasing the number of H-bonds on a protein surface provides a higher general stability (Wilkinson, Fersht *et al.* 1983; Alber, Sun *et al.* 1987), as in the case of the other electrostatic interactions, as additional salt bridges (Perutz and Raidt 1975), or the interaction of charged groups with the so- called “α-helix dipole” (Mitchinson and Baldwin 1986; Sali, Bycroft *et al.* 1988; Nicholson, Becktel *et al.* 1988) and of polar amino acid side chains with α-helix ends (Serrano and Fersht 1989).

The number of salt bridges on the surface of a protein can be increased by the substitution of selected lysines by arginines, in positions where a bigger group does not lead to steric hindrances.

The additional H-bonds formed by the arginines with other polar groups (for example with carboxylate) in the neighborhood, is a consequence of the capacity of the longer arginine side chain (Fig. 45) to reach out further in space, and of the higher hydrogen-bonding potential of the guanidinium group in which the positive charge can be delocalized providing an extended

H-bond pattern (Riordan 1979).

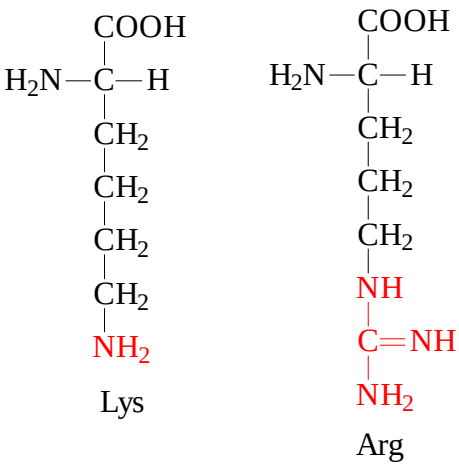



Fig. 45. Structures of lysine and arginine.

Stability can also be gained by replacing water-mediated hydrogen bonds of the lysine side chain by direct H-bonds of the guanidinium group, since reduced contact with the solvent is expected to strengthen the corresponding electrostatic interactions.

A sequence alignment between the GCL and the homologous and more stable proteins *Torpedo californica* AChE and the lipase from *Candida rugosa* was carried out.

The positions in which a lysine in GCL corresponds to an arginine in the other proteins were identified. All of these positions were analyzed with the X-ray structures of the GCL to check at which position the lysines could be replaced by arginine without requiring significant structural rearrangements or resulting in steric hindrances, but inducing an increase of the number of salt bridges.

For three of these lysines (K40, K239 and K372), shown in Fig. 46, the mutation to arginine could be of advantage, while for the others the mutation seems to cause no benefits.

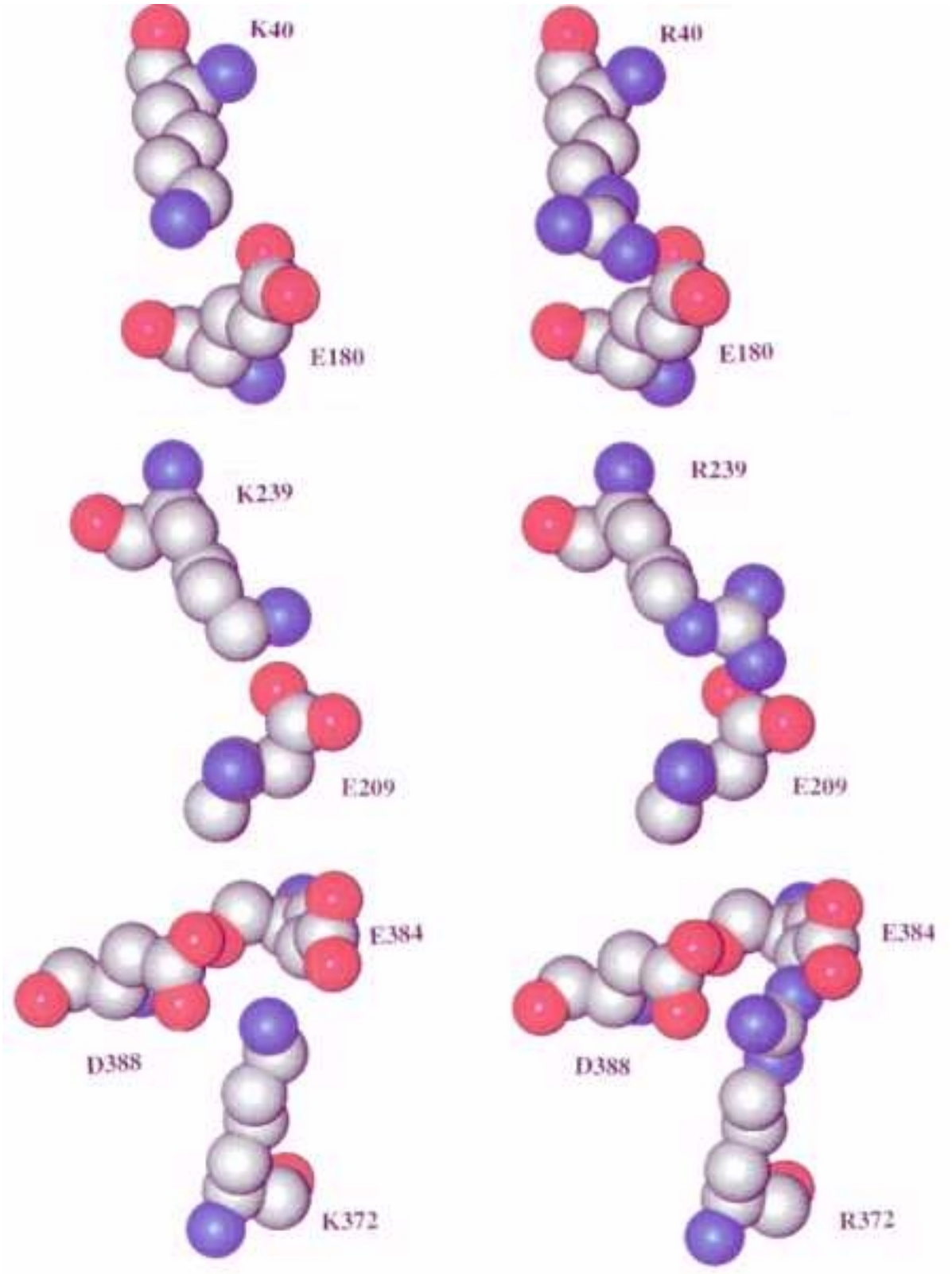



Fig. 46. Comparison of the interaction of the mutated lysines and arginines with the acidic residues in their neighborhood. Nitrogen is represented in blue, oxygen in red and carbon in grey.

In the mutants K40R and K239R, the formation of the salt bridges K40/E180 and K239/E209, respectively, will be favored by the longer side chain of the arginine.
In K372R the delocalization of the positive charge of the side chain of the arginine raises the possibility of interaction with 2 residues: D388 and E384.
These mutations were applied on the GCL B using the primers K40R, K40Rrev, K239R, K239Rrev, K372R and K372Rrev and pGαLipB as a template.
The mutant K40R was made only as double mutant on the K372R. After PCR using the standard conditions for the Quik-change kit, the mutated lipase genes were sequenced and recloned in the original pGAPZαC vector. The new plasmids were transformed in *Pichia pastoris* and for every mutant 10 clones were picked, grown for 3 days in YEPD, and then the culture supernatant was used for further assays.

### 3.14.7 Effect of the Lys-Arg mutations on the thermostability of GCL B

The thermostability assay was carried out as reported for the other mutants. The results are reported in the Table 18.

Table 18. Results of the thermostability measurements on the wild type lipase B and of the different mutants. The activity before and after incubation (fluorescence signal) was measured with the 4-MUO assay.

| Mutant | Fluorescence signal before incubation | Fluorescence signal after 30 min. incubation at 50 °C | TR average value |
|---|---|---|---|
| Wild type | 78840 | 31785 | |
| “ | 104806 | 38903 | |
| “ | 88896 | 31168 | |
| “ | 94540 | 29800 | |
| “ | 70150 | 23478 | **0.37±0.057** |
| “ | 76568 | 21764 | |
| “ | 87134 | 39185 | |
| “ | 106108 | 47772 | |
| | | | |
| K239R | 49086 | 28356 | |
| “ | 73498 | 27461 | |
| “ | 42932 | 12191 | |
| “ | 40656 | 15012 | |
| “ | 60086 | 23827 | **0.38±0.03** |
| “ | 63666 | 26548 | |
| “ | 54714 | 20697 | |
| “ | 59184 | 20497 | |
| “ | 71128 | 24102 | |
| “ | 72732 | 27641 | |
| | | | |
| K372R | 82542 | 33107 | |
| “ | 82574 | 33364 | |
| “ | 79320 | 33682 | |
| “ | 84210 | 30070 | |
| “ | 91018 | 30896 | **0.31±0.04** |
| “ | 88936 | 32919 | |
| “ | 69722 | 28193 | |
| “ | 72706 | 28601 | |
| “ | 79100 | 32665 | |
| “ | 89862 | 28008 | |
| | | | |
| K40R/K372R | 31772 | 8838 | |
| “ | 31856 | 9770 | |
| “ | 61430 | 22280 | |
| “ | 67616 | 23613 | |
| “ | 82924 | 28465 | **0.35±0.02** |
| “ | 90854 | 30162 | |
| “ | 98694 | 29139 | |
| “ | 99358 | 31389 | |
| “ | 81182 | 27687 | |
| “ | 86064 | 30258 | |

The average values of the 10 measurements for each mutant are reported in Fig. 47.

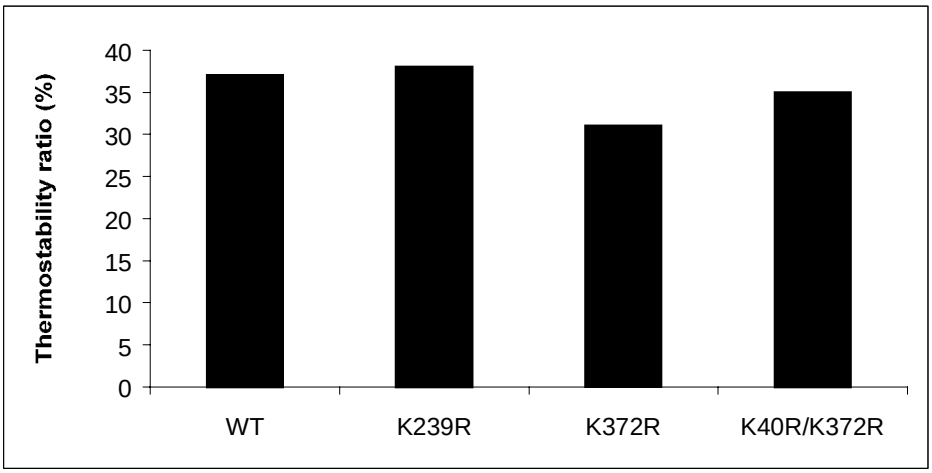



Fig. 47. Average values of the thermostability ratios for the mutants K239R, K372R and K40R/K372R in comparison to the WT GCL B. The activity before and after incubation was measured with the 4-MUO assay.

The mutants K372R and K40R/K372R were less stable than the wild type and were not investigated further.

For the mutant K239R, which seems to be slightly more stable than the wild type, further tests were carried out. The mutant K239R and the wild type GCL B were incubated at 45 °C and 60 °C, and the residual activity was measured at different times (Fig. 48).

The results show that incubation at different temperatures and for different times revealed no substantial differences between the mutant and the wild type GCL B.

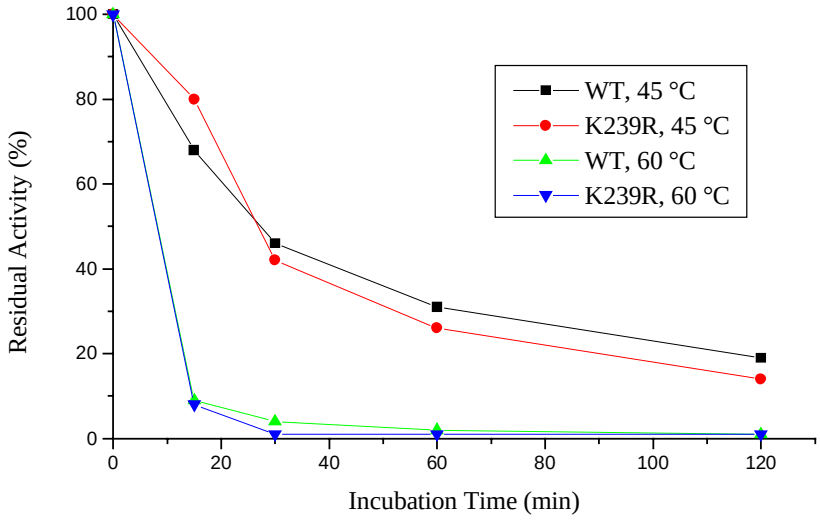



Fig. 48. Residual activity for the wild type GCL B and for the mutant K239R incubated at 45 °C and at 60 °C for different times. The activity was measured with the 4-MUO assay.

## *3.15 Immobilization as a 3rd method to enhance thermostability*

### 3.15.1 Immobilization yields and stability of the immobilized protein

Different supports and different protocols were tested for the immobilization of the lipase B from *Geotrichum candidum* in order to get a higher thermostability. 500 µl of a lipase solution corresponding to 1800 U were used for 1 g of each support.

The immobilization on accurel, accurel + albumin, celite, $Al_2O_{3ads}$, $SiO_{2\ ads}$ are based on hydrophobic adsorption, the one on DEAE, SuperQ-C, SuperQ-M, which are actually resins for the anion exchange chromatography, are based on ionic interactions. On active carbon and Trisopor the lipase was immobilized through covalent attachment mediated by glutardialdehyde. The $–NH_2$ functional groups of the protein (from lysine and arginine) are involved in the binding to the matrix. On accurel + albumin + glutardialdehyde the lipase was first absorbed on the polypropylene support and cross-linked by the addition of glutardialdehyde. For $Al_2O_{3cov}$, the support was first activated with glycidol to form an aldehyde group which can react with the free $–NH_2$ of the enzyme to form a Shiff base.

After every immobilization procedure, the samples were filtrated and the activity of the filtrate and of the solid was tested at the pH-stat, on triolein at pH 8.0, 37 °C. The yields are reported in Table 19.

The immobilization yield was higher than 10% only in the case of $Al_2O_{3ads}$, $Al_2O_{3cov}$ and $SiO_{2ads}$. A significant activity remained in the filtrate when accurel + albumin or celite were used, showing that these immobilization procedures could be optimized to allow the binding of a higher lipase amount to the matrix. In the other cases, all the lipase seemed to bind to the support (no activity in the filtrate) but the most of it bound probably not in an active way.

Table 19. Immobilization yields of the lipase B from *Geotrichum candidum* on different supports. The activity was measured at the pH-stat, at pH 8.0, 37 °C.

| Support used | Activity in the filtrate (%) | Immobilization yield (%) |
|---|---|---|
| **Accurel** | 3 | 3 |
| **Accurel + albumin** | 74 | 3 |
| **Accurel + albumin + glutardialdehyde** | 5 | 2 |
| **Active carbon$_{cov}$** | 9 | <1 |
| **$Al_2O_{3ads}$** | 2 | 18 |
| **$Al_2O_{3cov}$** | 16 | 14 |
| **Celite** | 85 | <1 |
| **DEAE** | 1 | 1 |
| **$SiO_{2\ ads}$** | 8 | 25 |
| **SuperQ-C** | 2 | 6 |
| **SuperQ-M** | 3 | 7 |
| **Trisopor$_{cov}$** | 25 | 3 |

For the lipase immobilized on accurel, accurel + albumin, accurel + albumin + glutaraldehyde, DEAE, SuperQ-C, SuperQ-M, Trisopor the stability at 50 °C was tested at different times. For $SiO_2$ and $Al_2O_3$ the measurements could not be carried out, because of the interference of the support with the activity measurement at the pH-Stat, which increased in comparison as the lipase activity decreased as a consequence of the incubation at 50 °C. For the other supports the amount of immobilized lipase was too low to permit such a determination.

The results for the tested matrices are shown in Fig. 49 with the data also relative to the soluble lipase.

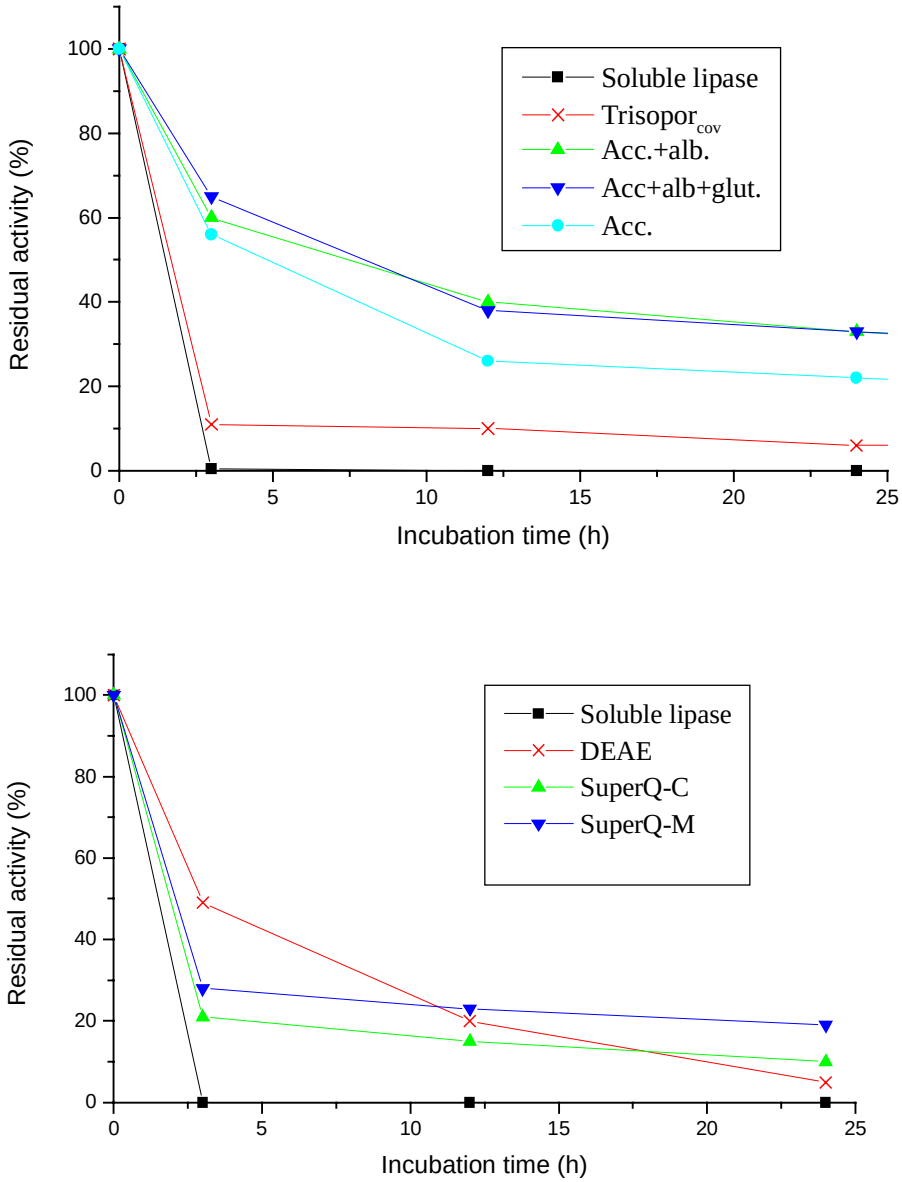



Fig. 49. Residual activity of the GCL B immobilized on different matrices after incubation at 50 °C in comparison to the soluble GCL B. The residual activity was measured at the pH-Stat on triolein, at pH 8.0, 37 °C.

It appears evident that the immobilized lipase retains remarkably more activity at 50 °C than the soluble lipase, independently from the tested support. In particular, the protein immobilized on accurel + albumin with and without cross-linking with glutardialdehyde retained almost 40% residual activity after 24 hours at 50 °C, whereas the soluble lipase is completely inactivated after 3 hours at 50 °C.

For Trisopor, stability tests in smaller time intervals were also carried out, as reported in Fig. 50, in order to characterize the rapid inactivation within the first 3 hours.

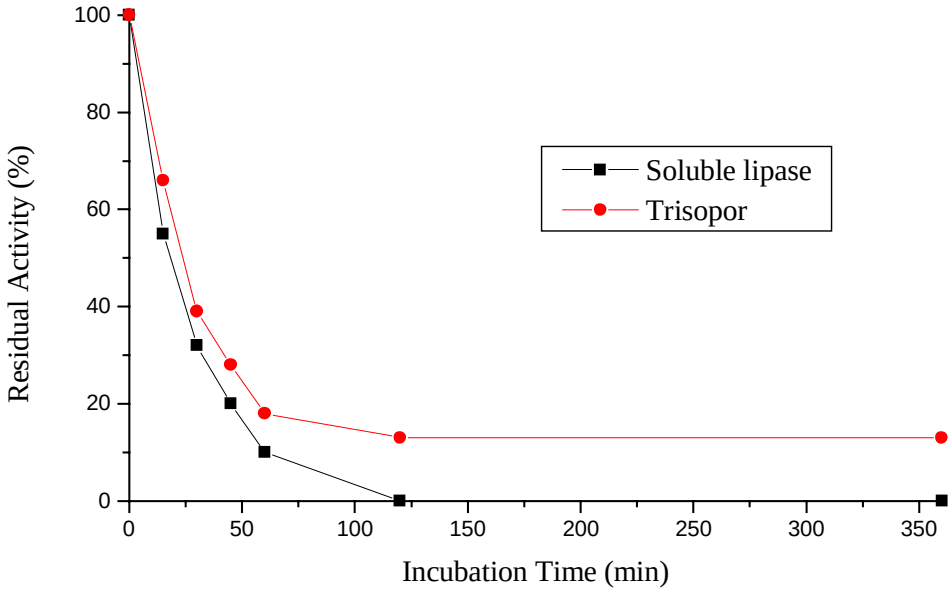



Fig. 50. Residual activity of GCL B, soluble and immobilized on Trisopor. The residual activity was measured at the pH-Stat on triolein, at pH 8.0, 37 °C.

As accurel + albumin gave the best results, the next step was to optimize the immobilization yield on this support. In the filtrate obtained after filtration of the immobilized GCL B on accurel + albumin still 74% of the initial activity was found; the yield of immobilized lipase of only 3% could be enhanced, by modification of the immobilization conditions. Therefore the influence of the lipase amount, of the temperature and of the presence of a lipase substrate (triolein) on the immobilization efficiency was investigated. The results obtained are reported in Table 20.

It can be seen that decreasing the amount of lipase used, the immobilization yield increased remarkably. Also the presence of triolein in the immobilization mixture had a positive effect on the process, resulting in yields up to 24%. Indeed, the presence of the substrate increased the amount of protein which binds to the matrix in the active form.

The immobilization on accurel + albumin carried out at 4 °C, with 450 U/g support and 1% triolein, gave rise to the highest immobilization yield and therefore was employed for further assays.

Table 20. Yields of immobilized lipase B using different conditions of immobilization on accurel + albumin. The activity was measured at the pH-Stat on triolein, at pH 8.0, 37 °C.

| **Conditions used for lipase immobilization** | **Activity in the filtrate (%)** | **Immobilization yield (%)** |
|---|---|---|
| **450 U, 4 °C** | 1 | 14 |
| **450 U, 4 °C, 1% triolein** | 1 | 18 |
| **450 U, RT, 1% triolein** | 1 | 24 |
| **900 U, 4 °C** | 1 | 4 |
| **900 U in 10ml buffer pH 7, 4°C** | 7 | 2 |
| **1800 U, 4 °C** | 1 | 2 |
| **1800 U, RT** | 74 | 3 |
| **3600 U, 4 °C** | 1 | 2 |

### 3.15.2 Characterization of GCL B immobilized on accurel + albumin

The residual activity of the immobilized lipase was measured at different incubation times, at 45, 50 and 60 °C. The results, compared to the stability of the soluble lipase, are reported in Fig. 51.

The lipase B immobilized on accurel + albumin is at each temperature much more stable than the soluble one. The half life of the protein at 50 °C increased from 35 minutes to 300 minutes, that means almost a ten fold increase.

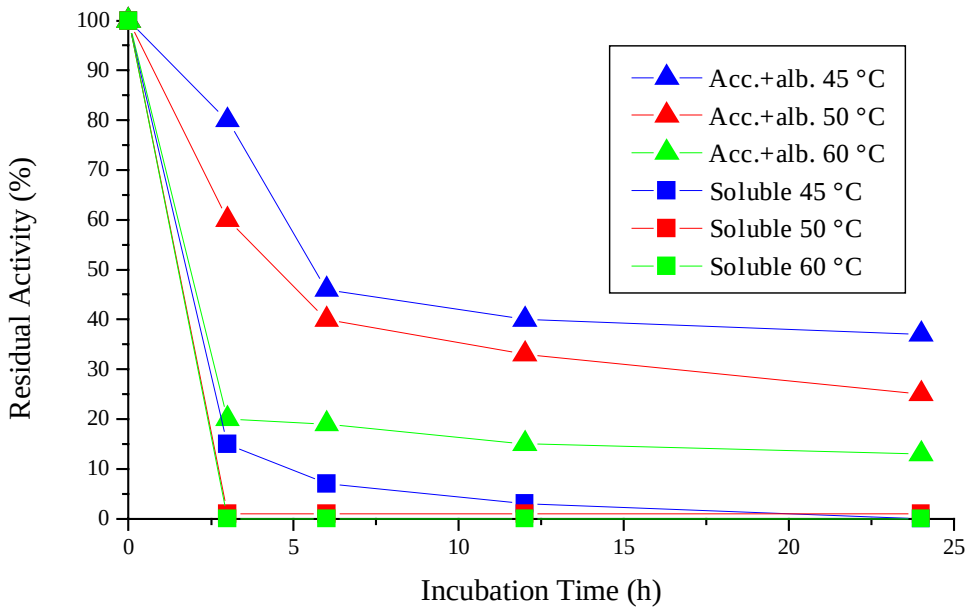



Fig. 51. Residual activity of the lipase B, soluble and immobilized on accurel + albumin in the presence of triolein at 4 °C, incubated at different temperatures for different times. The activity was measured at the pH-Stat on triolein, at pH 8.0, 37 °C.

For the immobilized lipase the temperature-activity and the pH-activity was determined and compared with the activities of the soluble ones. The data are shown in Figs. 52-53.

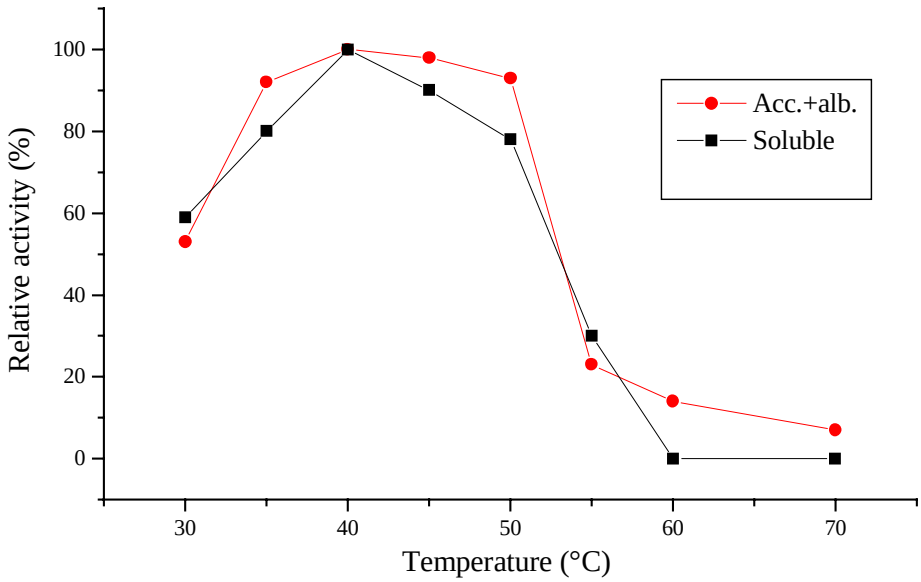



Fig. 52. Temperature-activity profile for the lipase B, soluble and immobilized on accurel + albumin in the presence of triolein at 4 °C. The activity was measured at the pH-Stat on triolein, at pH 8.0, 37 °C.

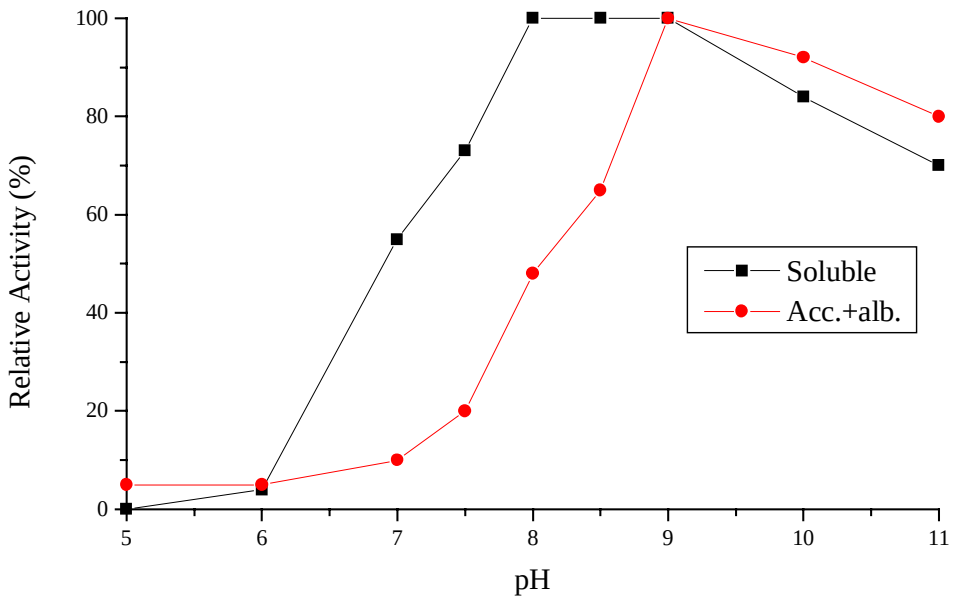



Fig. 53. pH-activity profile for the lipase B, soluble and immobilized on accurel + albumin in the presence of triolein at 4 °C. The activity was measured at the pH-Stat on triolein, at 37 °C.

There is almost no difference between the temperature-activity of the soluble and of the immobilized GCL B, and the temperature optimum remains at 40 °C. However the immobilized one shows a wider range of 100% relative activity, with respect to the soluble one.

In the pH profile of the immobilized lipase there is a shift of the optimal pH toward more alkaline pH values. In particular, for the immobilized lipase the pH optimum resulted pH 9.0, whereas for the soluble one there was a broader range (from pH 8.0 to 9.0) of pH optimum, at which the lipase conserved 100% relative activity.

# 4 Discussion

It was realized more than three decades ago that the lipases from the fungus *Geotrichum candidum* have an unusually high substrate preference for neutral lipids containing *cis* (*Δ*-9) unsaturated fatty acid residues such as oleic acid (Jensen, Sampugna *et al.* 1965). The identification of two lipase genes in *G. candidum* CMICC 335426 revealed that GCL B has the unique substrate preference, whereas the GCL A accepts a broader range of substrates despite the fact that 84% of the 544 amino acid residues are identical. To this day, the basis for the different substrate specificity of the two isoenzymes remains elusive. The high-level expression of the single isoenzymes allows not only the production of the pure lipases to be used in industrial applications, such as the restructuring of lipids and oils into products with defined fatty acid composition (Macrae 1985), but also mutational and structural studies of the two enzymes.

## 4.1 *Cloning and expression of lipase A and B from* G. candidum *in* S. cerevisiae *and* P. pastoris

The choice of the expression organisms was due to the fact that trials to express the lipases from *G. candidum* in *Escherichia coli* failed as the proteins accumulated as inclusion bodies in the cells (Unilever, personal communication). On the opposite side, the lipases I and II from *G. candidum*, homologous to A and B were already expressed, but at low levels (1 mg/l), in the yeast *S. cerevisiae*, so that the use of a yeast system seemed appropriate.

Furthermore, the methylotrophic yeast *Pichia pastoris* shows several advantages that support its employment. It can be grown to high cell density, with pellets routinely exceeding 100 g wet cell weight per liter of culture. If an appropriate secretion signal is used, this yeast secretes heterologous proteins through the secretory pathway; this permits post-translational modifications such as proteolytic maturation, glycosylation, native folding and disulfide bond formation. Purification is made easier by the low levels of native proteins secreted to the media.

Expression in *Pichia pastoris* is usually regulated by the alcohol oxidase (AOX1) promoter; the AOX1 gene is repressed when the cells are grown on glucose, whereas, when grown on methanol, the alcohol oxidase will accumulate to levels approximating 30% of total soluble protein in the cells.

The lipases A and B from *Geotrichum candidum* were first cloned with their original leader sequence in pYES2, a vector for the expression in *Saccharomyces cerevisiae*; in several

instances such as human serum albumin (Barr, Hopkins *et al.* 1992) or invertase (Tschopp, Svelow *et al.* 1987) the native signal peptide has been shown to be adequate for the secretion also in a yeast host.

In the treated case, the native signal sequence did not lead to the secretion of the lipases in *S. cerevisiae*, but only an accumulation of inactive proteins inside the cells was detectable.

A signal sequence based on that found in the *S. cerevisiae* α-mating factor can be used. It is composed of 89 amino acids, and proved to work in many cases also for the expression of heterologous proteins in *Pichia pastoris*, as for the blood factor XII (White, Kempi *et al.* 1994), antibody single chain fragment (Ridder, Schmitz *et al.* 1995), and others.

The lipase genes were then fused to the signal sequence of the α-factor and were cloned again in a pYES2 vector and at the same time in a pPICZαC vector, for the expression in *Pichia pastoris*. At the end of the signal sequence a KEX2 protease cleavage site is present and then a spacer peptide (Glu-Ala-Glu-Ala), which should help to alleviate the steric interference by the fused protein resulting in an efficient cleavage of the pro-sequence by the *P. pastoris* KEX2 protease. The Glu-Ala spacer sequence can be subsequently cleared by a diamino peptidase (DAP), the product of the STE13 gene.

In both of the transformed yeasts, the lipases were successfully expressed and secreted in the culture supernatant, yielding up to 7 U/ml (about 2 mg/l culture) with *Saccharomyces cerevisiae* and up to 200 U/ml (80 mg/l culture) with *Pichia pastoris*. As expected, the recombinant proteins represented almost the only ones expressed from the yeast, and can be gained at a high purity grade in the culture supernatant.

From the N-terminal sequencing of the secreted proteins, it was shown that whereas the KEX2 protease reacts properly, cutting out the signal sequence, the diamino peptidase did not react, remaining the four residues of the spacer (Glu-Ala-Glu-Ala) attached to the protein. In other cases the fusions made with the native spacer peptide have resulted in the secretion of a heterologous protein mixture, thought to be the result of incomplete diamino peptidase processing (Bitter, Chen *et al.* 1984; Brake, Merryweather *et al.* 1984). As the lipases with the 4 additional residues at the N-terminal were in any case active, the spacer was not deleted.

### *4.2 Fermentations*

To look for the optimal cultivation conditions for *Pichia pastoris*, different fermentations were carried out, and the cell growth and the lipase production were checked in each case. It was pointed out that the presence of the expensive biotin and yeast nitrogen base in a medium

composed of yeast extract, peptone and methanol had no effects on the cell growth and on the lipase production. The substitution of a part of the yeast extract with the cheaper malt extract led to a clear lower lipase production, although the cell growth was not influenced. The possible reason of this result remained unclear.

A next fermentation was carried out increasing the pH from 6.0 to 7.5, at which the lipase B shows a higher stability. Although the optimal pH for *Pichia*'s growth is reported to be 6.0 (Cregg and Madden 1988), in this case the cells grew better at the higher pH. The lipase activity of the supernatant from the culture grown at pH 7.5 was, anyway, lower than the one from the culture in the same medium at pH 6.0. This seems to be due either to a different protein folding or to a different glycosylation pattern, as the amount of protein expressed is the same in the two cases, that means that the difference is in the specific activity.

*Pichia pastoris* transformed with the genes of lipase A and B from *Geotrichum candidum* was fermented also in a 6 liters scale in a complex medium, with methanol as carbon source. For the fermentations of the two lipases the cell growth was the same, reaching a dry weight of 6.0 mg/ml after about 40 hours of fermentation and remaining constant for the rest of the process, although the cells were feeded every day with methanol. The lipolytic activity reached 373 U/ml for lipase B after 80 hours of cultivation, whereas only 70 U/ml were reached for lipase A; the difference in activity depends on the different expression levels, noticed already for the different clones isolated from a single transformation (section 3.3).

Very good results in terms of lipase production were obtained fermenting *Pichia pastoris* expressing the lipase B in a synthetic medium. Up to 600 U/ml were reached after 80 hours of fermentation. The process consisted of a first batch phase, in which the glycerol was used as only carbon source (except 0.1% methanol that was also added to let the cells already activate the methanol metabolic pathway), and a feed phase. The feed contained, apart from salts, metals and vitamins, glycerol as carbon source and a higher amount of methanol (8.5%) in order to induce the lipase production.

Glycerol, unlike glucose, does not inhibit the AOX promoter, so that, at the same time, the cells can grow on glycerol (in which the growth is four times faster than in methanol as the only carbon source) and the presence of methanol allows also the induction of the alcohol oxidase promoter, regulating the lipase expression. In this way, the higher lipase production is due to the better growth of the cells in such a medium; a dry weight of 36 mg/ml was reached after 87 hours of fermentation against the 6 mg/ml reached in a medium with only methanol as carbon source.

### *4.3 Characterization of the recombinant lipases*

The molecular mass of the lipases A and B from *G. candidum* expressed in *Pichia pastoris*, estimated by SDS-PAGE, was about 60 kDa for both lipases, in agreement with the calculated values of 61 kDa for the WT [ExPASy Molecular Biology Server, http://www.expasy.ch].

After treatment of the recombinant lipase B with endo-*β-N*-acetylglycosaminidase under native and denaturing conditions, a decrease in the molecular weight of around 4 kDa was observed, showing a carbohydrate content of almost 5%. The extent of N-linked high mannose glycosylation of the lipase at the two potential consensus sequences, $Asn^{283}$-Asp-Thr and $Asn^{364}$-Ala-Thr, was then in a range similar to that seen with the native lipases from *G. candidum* (Sugihara, Shimada *et al.* 1990) or to the recombinant lipases I and II produced in *S. cerevisiae* (Bertolini, Schrag *et al.* 1995). The fact that an identical electrophoretic shift to lower molecular mass was observed following endo-H treatment both under native and denaturing conditions, means that the glycosylation sites are externally available, as concluded also for the homologous lipase (Sidebottom, Charton *et al.* 1991).

The effect of the deglycosylation on the pI of the recombinant lipase B was also checked. For the native lipases A and B from *G. candidum* CMICC 335426 and for lipase I from ATCC 34614, the determined pI values were 4.71, 4.50 and 4.61 respectively, and these values were not altered by treatment with endo H. In contrast, the pI of the lipase II from ATCC 34614 shifts from 4.47 before endo H treatment to 4.61 after the treatment, showing that the deglycosylation exposes residues normally concealed by carbohydrates or removes charged carbohydrates. In the case of the recombinant lipase B expressed in *Pichia pastoris*, making an isoelectric focusing before deglycosylation, two bands with a pI of 4.55 and 4.40 were observed, whereas, after endo H treatment, only the band at pI 4.55 can be seen. This shows that a microheterogeneity in the glycosylation is present, being that the lipases with the lower pI are more glycosylated than the one with the pI 4.55.

Very similar pI values have been reported by other workers for extracellular lipases from ATCC 34614 (Sugihara, Shimada *et al.* 1990; Veeraragavan, Colpitts *et al.* 1990; Jacobsen, Olsen *et al.* 1990).

Concerning the pH/temperature activity, the recombinant lipases A and B showed similar behavior, with a temperature optimum of 40 °C and a pH optimum between 8.0 and 9.0. No data from literature is available for the native lipases A and B, but for I and II a temperature optimum of 40 °C was found (Sugihara, Shimada *et al.* 1990). The pH optimum has been

reported to be 8.0 for lipase I and 6.0 for lipase II, differing in this way with the values obtained in this work for the recombinant A and B.

These retained between 70% and 90% activity when incubated for 24 hours at 30 °C at pH values from 5.0 to 9.0, being quickly inactivated at higher or lower pH values, as also reported for lipases I and II (Veeraragavan, Colpitts *et al.* 1990).

In regard to temperature stability, the two lipases are stable for 24 hours at 20-30 °C; at higher temperatures lipase B is rapidly inactivated, whereas lipase A retains still 50% activity after 24 hours at 40 °C and 10% at 45 °C, showing a higher thermostability. In a previous report (Sugihara, Shimada *et al.* 1990), a higher stability was observed for lipase I, homologous to B, in comparison to II, homologous to A. This could reflect a difference in primary and tertiary structure between the isoenzymes.

The specificity assay on recombinant lipases A, B and the hybrid A/B, composed fusing the N-terminal part (194 amino acids) of lipase B with the C-terminal (349 amino acids) of lipase A, confirmed the unique substrate preference of lipase B for lipids containing long-chain unsaturated fatty acids with a *cis* double bond in position 9. The total amount of saturated fatty acids hydrolyzed by lipase B is only 4.6%, whereas it is 48.9% for lipase A. The hybrid lipase behaves like the A, not being specific for unsaturated fatty acid, and showing in this way, that the peculiar substrate specificity of lipase B is due to the residues present in the second part of the molecule. While this work was carried out, Holmquist *et al.* (Holmquist, Tessier *et al.* 1997) identified, through creation of a set of lipase hybrids, some residues essential for differential fatty acyl specificity of *Geotrichum candidum* lipases I and II. Crucial elements were found to be located completely in the C-terminal part of the lipase: at the active site entrance (Ile357/Leu358) and at the bottom (Cys379/Ser380) in the active site cavity of GCL I. The replacement in GCL I of these residues by their equivalent amino acids from GCL II (Ala357/Phe358/Phe379/Phe380) led to a specificity profile very similar to that of GCL II. The mutations at the entrance of the GCL I active site primarily increased the specific activity towards trioctanoin, whereas amino acid replacements at the end of the acyl binding tunnel effected activities towards both triolein and trioctanoin. Introducing specificity into the non-specific GCL II resulted more challenging. The reverse mutations in GCL II recovered only a fraction of the specificity observed in GCL I. It became obvious that there are residues in other parts of the enzyme also contributing to the peculiar substrate specificity.

## *4.4 Increasing the thermostability of lipase B*

Increasing of thermostability of proteins for application in industrial processes has always garnered much interest, as it would permit a reduction of the overall process costs, by diminishing the amount of enzyme to be used, and would allow the use of higher reaction temperatures, connected to some benefits like increased substrate solubility.

Furthermore, studies on protein stability are interesting from a scientific point of view, because still so little is known about the factors influencing this parameter, and still no general rules, applicable to every protein, have been discovered.

Thermostability can be achieved through genetic engineering techniques (random and site-directed mutagenesis), modifying the amino acid sequence of the protein influencing in this way its properties, or through immobilization, acting on the already expressed protein.

### 4.4.1 Random mutagenesis

Directed evolution, involving mutation, recombination and screening or selection to accumulate the mutations required to achieve significant changes in protein function, does not require any structural information or knowledge of the principles governing the property to be improved, and that is why it is very useful to improve the thermostability, whose determining factors are mainly still unknown.

In the literature, in a large number of random-mutagenesis experiments, single beneficial mutations typically increase the melting temperature ($T_m$) of the enzyme by 1-2 °C. Further improvements are usually obtained by the repetition of the cycle mutagenesis-screening.

In an early directed-evolution experiment, Liao *et al.* (Liao, McKenzie *et al.* 1986) found two amino acid substitutions in the antibiotic resistance enzyme kanamycin nucleotidyltransferase that resulted in a 15 °C increase in $T_m$. Through several mutagenesis steps Pjura *et al.* (Pjura and Matthews 1993) identified 11 single mutations that increased the thermostability of T4 lysozyme by 0.8-1.4 °C; Haruki *et al.* (Haruki, Noguchi *et al.* 1994) found eight mutations in the ribonuclease HI, of which the best one yielded up to 7.8 °C increase in the $T_m$. Joyet *et al.* (Joyet, Declerck *et al.* 1992) combined a stabilizing mutation, which they had identified using sequence comparison between mesophilic and thermophilic versions of α-amylase, with a new mutation generated by random mutagenesis to raise the $T_m$ of barley α-amylase by 11 °C.

Random mutagenesis was employed in this work as one approach to increase the thermostability of the lipase B from *Geotrichum candidum*.

The lipase gene was mutagenized through error-prone PCR and was transformed with high transformation yield in *S. cerevisiae*, creating a library of 10000 mutants. For a successful directed evolution experiment, the screening assay has to be reproducible and with high-throughput, in order to allow a quick and sensitive screening of a large number of mutants. An early developed thermostability assay was based on growth of the mutant colonies under a nitrocellulose filter, to which the secreted lipase can bind, following incubation of the filter in conditions that inactivate the wild type lipase, whereas the more thermostable mutants will retain some activity; the filter can then be moved to a plate with an indicator able to point out the eventual residual activity of some mutants. This assay was rapidly abandoned as it could not take in consideration the different amounts of protein expressed from each clone and the different specific activity of single mutants.

This same screening procedure was successfully employed in different cases, as for the barley β-amylase for which an increase of 2.3 °C in the thermostability was obtained (Okada, Yoshigi *et al.* 1995) or for two selected mutants of the subtilisin Carlsberg, which showed significantly enhanced residual activity at 68 °C (Saettler, Kanka *et al.* 1996).

An assay was then developed in which the lipolytic activity of the culture supernatant was checked before and after incubation at high temperatures through a fluorescence test, and the ratio between residual and initial activity was taken as an index of the thermostability. In this way, the response will be independent from the amount of lipase expressed by the mutants and from its specific activity. The assay was improved, controlling different parameters which could have an effect on its reproducibility, such as the temperature and time of incubation, the pH and the volume of the supernatant to be used for the activity test, and the conditions of the activity measurements.

The screening of 1000 mutants led to the identification of three clones with a higher thermostability ratio in comparison to the wild type, but their sequencing showed a wild type pattern. A big disadvantage was that the error affecting the measurement was too high to allow the detection of small thermostability increases. In particular, looking at the measurement error it was pointed out, that it was less than 10% on multiple determination on the same culture supernatant, but it increased up to about 20% for determinations on supernatants from different cultivations of the same clone. This was associated with the different amount of protein in the medium, which could have an influence on the denaturation process. For this reason, the same assay was carried out in the presence of an excess of another protein (BSA), in order to level the protein content in the solution to incubate for the

stability determination. The results show no error-variation in the two assay conditions, meaning that the error must derive from other parameters, such as the amount of lipase in the single incubation wells.

In a recent report, a 174 times improvement of the thermostability of a fungal peroxidase subjected to multiple rounds of directed evolution is reported (Cherry, Lamsa *et al.* 1999). In another case, directed evolution led to an evolved *Bacillus subtilis* subtilisin E, whose temperature optimum was 17 °C higher and its half-life at 65 °C more than 200 times that of wild type subtilisin (Zhao and Arnold 1999). In both of these examples, a screening procedure, similar to that used in this work was applied, consisting of an initial activity assay, an enzyme inactivation step and a residual activity assay. In these reports the measurement errors or the influence of the amount of expressed protein on the assay reproducibility, were not taken in account or mentioned.

### 4.4.2 Site-directed mutagenesis

Concerning the approach using site-directed mutagenesis, the thermostability of enzymes can be enhanced by replacing single residues at a specific position, identified after molecular modeling of a structure obtained from X-ray diffraction studies of the crystallized protein.

Factors which direct the choice of the residues to mutate, because they can be considered to have a stabilizing effect on proteins, are:

- Interaction or bonds (disulfide bond, hydrogen bond, electrostatic interaction, hydrophobic interaction).
- Conformational factors (stability of tertiary structure, compact packing, conformational flexibility, internal hydrophobicity, entropic stability)
- Protection (deamination of carboxyamide, oxidation of sulfhydryl groups, intramolecular S-H/S-S exchange, oxidation of tryptophan or methionine).

Different methodologies were employed in this field. For example in some cases (Russel and Taylor 1995; Menendez-Arias and Argos 1989; Tanner, Hecht *et al.* 1996; Kiefer, Mao *et al.* 1997; Chan, Mukund *et al.* 1995) comparative approaches, based on the comparison of the sequences of proteins from mesophilic and thermophilic organisms were carried out to point out the features which might be responsible for the difference in thermostability. In many cases this approach proved to be successful, leading to a dramatic enhancement of the thermostability of the mesophilic enzyme (Kimura, Nakamura *et al.* 1992; Toma, Campagnoli

*et al.* 1991; Imanaka, Shibazaki *et al.* 1986; Suzuki, Ito *et al.* 1989; Zulli, Weber *et al.* 1990; Zulli, Schneiter *et al.* 1991).

Another approach to increase the thermostability consisted of the mutation of some residues to proline at β-turn structures (Li, Reilly *et al.* 1997; Masui, Fujiwara *et al.* 1994) but also in α-helices, despite the fact that proline is a helix-breaker (Nakamura, Tanaka *et al.* 1997; Herning, Yutani *et al.* 1992). It was proposed that in general, an increased number of proline residues correlates with increased protein thermostability (Suzuki 1989; Suzuki, Hatagaki *et al.* 1991), as proline restricts backbone bond rotation because of its pyrrolidine ring, decreasing in this way the entropy during protein unfolding by reducing the numbers of unfolded conformations that can be sampled by the protein (Matthews, Nicholson *et al.* 1987). In other cases, the filling of cavities with amino acid side chains that replace buried water molecules, resulted in some positive effects (Vriend, Berendsen *et al.* 1991;Eijsink, Dijkstra *et al.* 1992).

In different proteins, the thermostability was increased by replacing positively charged amino acids in the N-terminal turn of α-helices (Watanabe, Masuda *et al.* 1994; Eijsink, Van der Zee *et al.* 1992; Sali, Bycroft *et al.* 1988; Nicholson, Beckel *et al.* 1988; Serrano and Fersht 1989), considered unfavorable because they can give rise to repulsive interactions with the helix dipole.

Also other single amino acid substitutions based on different principles, (Eijsink, Van der Zee *et al.* 1991; Igarasaki, Hatada *et al.* 1998; Matsumura, Wozniak *et al.* 1989; Cha, Cho *et al.* 1994; Yaoi, Hayashi-Iwasaki *et al.* 1996; Akanuma, Qu *et al.* 1997; Chen, Bakir *et al.* 1994) were not always successful.

### 4.4.3 Engineering an extra disulfide bridge

Another method, often applied to improve thermostability by site-directed mutagenesis, is the introduction of an additional disulfide bridge, although it was not always possible to predict *a priori* its effect in one protein for the still reductive knowledge available on all the phenomenons responsible for the proteins' thermostability.

This idea was first applied with success by Villafranca *et al.* (Villafranca, Howell *et al.* 1983) with the dihydrofolate reductase. Since then, other trials have been carried out for instance on the *Bacillus circulans* xylanase (Wakarchuk, Sung *et al.* 1994), in which the introduction of both intra- and intermolecular disulfide bridges led to an increase of the thermostability of 15

°C compared to the wild type enzyme.

On subtilisins the results are discordant: engineering a S-S bridge in the subtilisin E (Tagaki, Takahashi *et al.* 1990) on the basis of structural comparison with a thermophilic serine protease, led to a 2-3 times longer half life at 55 °C than that of the wild type; Pantoliano *et al.* (Pantoliano, Ladner *et al.* 1987) reported that such a bridge in the subtilisin BPN' has a significant effect on the stability, whereas Wells (Wells and Powers 1986) and Mitchinson (Mitchinson and Wells 1989) with other subtilisins did not reach a greater stability against irreversible inactivation compared to the wild type enzyme.

In the phage λ repressor (Sauer, Hehir *et al.* 1986), the formation of an intramolecular disulfide-bonded dimer also increased the thermostability.

The thermostability of the ribonuclease H (Kanaya, Katsuda *et al.* 1991) was also increased with an extra S-S bond, but the protein almost totally lost its activity.

Different additional disulfide bonds introduced in the *Bacillus subtilis* protease decreased its stability (Van den Burg, Dijkstra *et al.* 1993), as well as those engineered in other proteases (Mitchinson and Wells 1989; Wetzel, Perry *et al.* 1988; Wells and Powers 1986).

In some cases the negative results have been attributed to the negative effect of cysteines (Volkin and Klibanov 1987), retained to undergo significant β-elimination in the relevant pH range (from 4 to 8) (Florence 1980; Whitaker and Feeney 1983) or to the introduction of steric clashes or to the disruption of favorable interactions present in the folded wild type protein.

In T4 lysozyme, the engineering of a disulfide bridge showed a stabilizing effect on the irreversible thermal inactivation (Wetzel, Perry *et al.* 1988). In the same protein Matsumura *et al.* investigated the effect of multiple disulfide bonds (Matsumura, Signor *et al.* 1989) obtaining different effects on the protein thermostability. They concluded that the factors which seem to be helpful in the design of the stabilizing disulfide bridges include the use of large loops to maximize the entropic effect on the unfolded state and the choice of flexible sites to avoid the introduction of strain into the folded protein.

Only one application is reported for lipases, in which an extra disulfide bond has been introduced in the *Penicillium camembertii* lipase (Yamaguchi, Takeuchi *et al.* 1996), which is homologous to the lipases from *Rhizomucor miehei*, *Humicola lanuginosa* and *Rhizopus delemar*, but lacks the S-S bridge conserved in the other three. The extra bridge was found to increase the melting temperature of the protein from 51 °C to 63 °C, although the temperature

optimum decreased by 10 °C.

The lipase B from *Geotrichum candidum* has four cysteines which build two disulfide bridges, conserved also in the homologous *Candida rugosa* lipase and *Torpedo californica* acetylcholinesterase. In the latter protein a third disulfide bridge is also present at the C-terminus (Shimada, Sugihara *et al.* 1990) and the protein is more stable than the lipase. With structural studies through molecular modeling, it was found that the structural homology between AChE and GCL B in this region is very high, despite the differences in the sequence, and that the residues of GCL B corresponding to the two cysteines in the AChE are at the correct distance to be able to form a disulfide bridge. It was thought that an extra bridge for the GCL B in this position could have a positive effect on the thermostability, by generally decreasing the conformational entropy of the backbone in the denatured state and, furthermore, fixing the flexible helix at the C-terminus. Unfortunately, the results from the thermostability measurement on the GCL B mutant with the engineered disulfide bridge (S430C/I533C), showed a clear destabilization of the mutant, maintaining only 11% residual activity after 30 minutes of incubation at 50 °C in comparison to the 26% of the wild type lipase. The destabilization appeared to be due to the formation of the bridge, and not to the replacement of the two amino acids with two cysteines, as the single mutants, in which only one residue was mutated to cysteine, showed almost the same stability as the wild type lipase. Responsible for the destabilization could be the interaction of the residues L393 and N535, following a movement of the respective helices due to the binding of the two additional cysteines. The respective residues in the AChE are further away. The mutations of these two amino acids in smaller ones (alanine and valine) led to a slightly increment in the thermostability of the mutant with the disulfide bridge, but the residual activity after incubation of 30 minutes at 50 °C was only 42% that of the wild type, against 35% retained by the S430C/I533C mutant incubated in the same conditions.

The destabilization must then therefore result from some effect not predictable by computer modeling.

### 4.4.4 Mutating a lysine in arginine

It has been suggested that certain amino acid residues or certain amino acid replacements are used preferentially in thermophilic proteins. For example Menendez-Arias *et al.* (Menendez-Arias and Argos 1989; Dekker, Yamagata *et al.* 1991) found that the ratio Arg/Lys is larger in

proteins from thermophilic organisms than in those from mesophiles, providing an indication of the stabilizing role of arginines.

In fact, the longer side chain of the arginines that can also reach further residues, and the possibility of the guanidinium group to delocalize the positive charge, lead generally to a more extended H-bond pattern, in comparison to lysines.

In the case of a glucose isomerase (Quax, Mrabet *et al.* 1991), one single arginine substitution for lysine provided a big gain in stability, with a nearly three-fold increase in half-life.

The same substitution of a surface exposed lysine by arginine in the human copper, zinc-superoxide dismutase as well as in the D-xylose isomerase and of a D-glyceraldehyde-3-phosphate-dehydrogenase also resulted in a significant increase in thermostability (Mrabet, Van der Broeck *et al.* 1992).

In this work, a sequence alignment between the homologous *T. californica* AChE, *C. rugosa* lipase, *G. candidum* lipase B was carried out. The positions in which an arginine in the AChE and CRL corresponded to a lysine in GCL B were analyzed through computer modeling, in order to figure out in which positions the mutation Lys/Arg could possibly lead to a more favorable salt bridges pattern, without introducing steric hindrances.

Three lysines were identified for which the longer chain of the Arg could be more suitable to reach the adjacent acid groups (Glu, Asp) to form salt bridges, and in this way could contribute to increase the thermostability of the protein.

The mutations K40R, K239R and K372R were introduced in the GCL B gene and the corresponding proteins were expressed in *P. pastoris*. Their thermostability was checked, but it seemed not to be influenced by these mutations. In fact, the differences in thermostability were comprised in the error of the measurement and the three mutants were inactivated in the same way as the wild type. The introduced arginines, hence, did not have the expected stabilizing effect, indicating that the unfolding of the protein with the temperature starts from some other not identified regions.

### 4.4.5 Immobilization

As an alternative way to increase the thermostability, GCL B was immobilized on different matrices through adsorption, ionic binding, covalent binding and cross-linking.

Only a few reports exist about the thermostabilization of lipases by immobilization. Some examples are the immobilization of the lipoprotein lipase on chitosan beads which, after incubation at 60 °C for 1 hour, maintained 55% of its initial activity, compared to only 10%

residual activity of the soluble lipase (Itoyama, Tokura *et al.* 1994); the lipases from *Pseudomonas fluorescens, Aspergillus niger*, *Candida rugosa* immobilized on duolite and celite did not show any altered thermostability (Mustranta, Forssell *et al.* 1993), whereas the immobilization of the *Candida rugosa* lipase on several inorganic supports both covalently and by adsorption (Moreno and Sinisterra 1994; Sinisterra 1997a; Sinisterra 1997b), led to enhanced thermostability, especially by covalent immobilization. For the use in organic solvents, a remarkable thermal stabilization was obtained for the same lipase by sol-gel entrapment in organically modified silicates (Kawakami 1996; Kawakami and Yoshida 1996).
In the present work the thermostability of GCL B was enhanced by immobilization, with each of the different matrices used. The half life of the enzyme at 50 °C increased from 30 minutes for the soluble lipase to some hours for the immobilized one. The best results were obtained with the immobilization on a polypropylene support, accurel, pretreated with albumin; in this case the half life increased to 5 hours, that means a ten-fold increase with respect to the soluble lipase B. The immobilization yield on accurel + albumin could be enhanced decreasing the amount of protein immobilized per gram of support and adding triolein, the lipase substrate, in the mixture. In this way, the triolein, binding in the active site of the protein, could assist the protein in maintaining its active conformation during the immobilization process.
The characterization of the lipase immobilized on accurel + albumin showed that, whereas the temperature optimum remained unchanged through immobilization, the pH optimum shifted to more alkaline values. Furthermore, whereas the soluble lipase conserved 100% relative activity from pH 8.0 to 9.0, the immobilized one showed 100% activity only at pH 9.0.
The possibility of a pH shift towards basic values is also reported in the literature (Ruttloff 1994), but for the case of strong charged matrices, that is not the case of polypropylene. In fact, the charged groups of the support provide that in the immobilized enzyme "micro-environment" there is a higher $H^+$ concentration than in the whole solution, and in this way the enzyme shows an apparent higher activity at alkaline pH values.

## *4.5 Conclusions*

The lipase B from *Geotrichum candidum*, interesting for its peculiar specificity for substrates with a *cis*-Δ9 insaturation, and its isoenzyme lipase A were successfully expressed in *Saccharomyces cerevisiae* and in high yields in *Pichia pastoris*. The recombinant proteins, being almost the only ones secreted in the culture medium, resulted already pure.

A fermentation protocol in a synthetic medium was applied, to increase the lipase production, reaching at the end around 240 mg/l fermentation broth.

Attempts to enhance the lipase B thermostability through random and site-directed mutagenesis failed, showing the complexity of the stability phenomenon, which is a combination of different stabilizing and destabilizing interactions.

Probably, future directed evolution efforts will overcome the complexity of the thermostability problem, using a combination of the available tools: random mutagenesis, site-directed mutagenesis and DNA shuffling, as already successfully done in two recent reports (Cherry, Lamsa *et al.* 1999; Zhao and Arnold 1999). Using rational enzyme design and random mutagenesis a number of individual point mutations, contributing to the thermal stability, can be identified and then combined by DNA shuffling, to find an optimal mutation combination.

The third method used in this work to increase the thermostability of GCL B was the immobilization on different supports. The immobilized lipase was shown to be more stable than the soluble lipase for each matrix used. In particular, in the best case, when the protein was immobilized on accurel pretreated with albumin, the half life of the enzyme at 50 °C showed a ten-fold increase.

The overexpression of lipase B in *Pichia pastoris* together with the developed immobilization procedure allow the utilization of the enzyme, at low costs, in industrial processes. For example, the use of a reactor with the immobilized lipase in which the substrates are allowed to flow continuously through, can be reasonably applied for the modification of oils to vary their content in saturated/unsaturated fatty acids.

# 5 Summary

The interest for the lipase B from *Geotrichum candidum* CMICC 335426 arises from its high specificity for substrates with an insaturation in position *cis*-Δ9, so that it can find an application in the selective modification of the fatty acid profile of an oil. The homologous lipase A from the same strain does not show this high specificity. Cloning the two isoforms would allow to get the two proteins for separate structural studies or applications.

In this work, the genes encoding for lipase A and B from *Geotrichum candidum* were successfully expressed in *Saccharomyces cerevisiae*. The transformants expressed and secreted the lipases at low levels (6-8 U/ml after 2 days of culture).

Therefore, the lipase genes were also cloned in a vector suitable for expression in the yeast *Pichia pastoris*. In this expression system, the proteins were functionally expressed and secreted in the culture medium at high levels (50 U/ml after 2 days of culture). The protein of interest was almost the only protein secreted into the medium by *Pichia pastoris*, thus it was already obtained pure.

Fermentations of the *Pichia pastoris* clone producing lipase B were carried out using different media and conditions. In a medium containing only yeast extract and peptone, up to 300.000 U of lipase B per liter culture were obtained, which is comparable to the production level of the mixture of native lipase isoforms by *Geotrichum candidum*. In a synthetic medium, also economically favored, were reached nearly 600.000 U of pure lipase B per liter culture.

The recombinant lipase B was characterized in terms of substrate specificity, pH and temperature stability, activity at different pH values and temperatures. The investigated enzyme properties were comparable to those reported for the native enzyme.

The next step was to increase the thermostability of lipase B from *Geotrichum candidum*. Three different approaches were used: random mutagenesis, site-directed mutagenesis and protein immobilization.

The random mutagenesis approach consisted of creating random mutants of the lipase B from *Geotrichum candidum* CMICC 335426, to be expressed in *Saccharomyces cerevisiae* and then screened for increased thermostability.

*S. cerevisiae* transformation efficiency was optimized, in order to get a high number of transformants. The library of mutagenized lipase genes was created through error-prone PCR, and then transformed in yeast. A high-throughput screening procedure based on a fluorescence assay was developed in order to check the thermostability of the different lipase mutants. The screening of 1000 mutants led to the selection of false positives due to the still

high error rate of the assay.

The site-directed mutagenesis approach consisted first on the introduction of an additional disulfide bridge in a position where it is present in the homologous *Torpedo californica* AChE. The mutant (S430C/I533C) with the additional S-S bridge was faster deactivated than the wild type lipase, and also further mutants of S430C/I533C did not lead to enhanced thermostability.

The exchange of three lysines in arginines located on the surface of GCL B, which could lead to a more favorable salt bridges pattern with the adjacent acid groups, were introduced in the lipase B gene. Unfortunately, the thermostability of the mutants did not differ from that of the wild type, showing that the unfolding of the protein with the temperature starts from some other not identified region, and is not influenced by the introduced mutations.

Another technique used to enhance the lipase B thermostability was immobilization. Lipase B was immobilized on different supports and the thermal deactivation of the immobilized enzyme was tested and compared to the one of the soluble lipase.

The thermostability of GCL B was enhanced with each of the different matrices used. The half life of the enzyme at 50 °C increased from 30 minutes for the soluble lipase to some hours for the immobilized one. The best results were obtained with the immobilization on a polypropylene support, accurel, pretreated with albumin; in this case the half life increased to 5 hours, that means a ten-fold increase with respect to the soluble enzyme. The immobilization yield on accurel + albumin was enhanced from 3% to 24%, decreasing the amount of protein immobilized per gram of support and adding triolein in the mixture.

This work raises the possibility to develop an industrial application of the specific lipase B from *Geotrichum candidum*, reducing the costs, due to the high expression levels in *Pichia pastoris* and increased thermostability by immobilization.

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# 7 Curriculum Vitae

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| Surname: | **Catoni** |
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## Education History

**1984-‘89** High school **Liceo Scientifico** “I. Vian”, Rome, Italy.

**1989/’90-1993/’94** Attendance at the **University courses**, at the University of Rome “La Sapienza”, Italy.

**1994-’95** Experimental work on the **degree thesis** in the lab. of Prof. Enrico Cernia at the **University of Rome “La Sapienza”**. Title of the thesis: “Biocatalysis in supercritical fluids: importance of the reaction media and of the substrate structure”.

**11/4/’95** **Chemistry degree** with the score **110/110 summa cum laude**, at the University of Rome “La Sapienza”, Italy.

**1995-’96** **Experimental research** in the laboratory of Prof. Enrico Cernia, **University of Rome “La Sapienza”**, Italy, concerning the development of systematic strategies of different kinds of chromatography to purify the lipase from *Candida rugosa*.

**7/1996- 7/1999** **Ph.D. work** in the lab. of Prof. Rolf D. Schmid at the **Institute of Technical Biochemistry, University of Stuttgart**, Germany.

**8/’97-10/’97** Part of the **fermentation work** for the Ph.D. was carried out at **Unilever Research Laboratories, Vlaardingen**, The Netherlands.

Hiermit erkläre ich, daß ich die Arbeit selbstständig verfaßt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt wurden.

Stuttgart, den 20.8.1999