Cloning And Directed Evolution Of Hydrolytic Enzymes

For Deracemisation Of Enol Esters

Thesis submitted in accordance with the requirements of

The University of Liverpool

For the Degree of Doctor of Philosophy

by

Céline Parsy

December 2004

THE UNIVERSITY
of LIVERPOOL

Abstract

Cloning and directed evolution of hydrolytic enzymes for deracemisation of enol esters

Céline Parsy

The resolution of esters using hydrolytic enzymes such as lipases, proteases and esterases is well established for the production of enantiomerically enriched esters, acids and alcohols. These enzymes can be used in organic solvents to catalyse the reverse reaction, formation of esters, in which case it is advisable to use an irreversible acyl donor such as isopropenyl acetate to drive the reaction. Dynamic kinetic resolution has also been employed successfully where the substrate is amenable to *in situ* racemisation, resulting in high yields of single enantiomer products. The use of a commercially available lipase enzyme, from *Pseudomonas fluorescens*, for the effective desymmetrisation of prochiral ketone 4-cyano-4-phenylcyclohexanone was previously reported.

This project describes the cloning, expression and refolding of *P. fluorescens* lipase in preparation for directed evolution for the improvement of activity and selectivity in this industrially important reaction. The lipase gene, from a non-commercial strain of a *P. fluorescens*, was cloned and expressed in an *Escherichia coli* expression system. However, the lipase was produced in an inactive form as inclusion bodies and could only be recovered using denaturing conditions such as high urea concentrations. Unfortunately, there is no information currently available concerning relevant chaperones for this particular protein to assist folding. Therefore an alternative approach was adopted to generate soluble active lipase and the cloning, expression in *E. coli* and refolding of a histidine tagged, biocatalytically active *P. fluorescens* lipase were achieved.

In order to realise the directed evolution as planned, an alternative enzyme was studied. An esterase from *P. fluorescens* was selected and used to develop mutation steps and high-throughput screening. A screen was developed to test the activity of mutant esterases. This consisted of two steps. The first involved a tributyrin assay to test the general esterase activity of each mutant and to allow the isolation of recombinant enzymes showing no alteration in such activity. The second screen involved a colourimetric assay using 2,4-dinitrophenyl hydrazone (2,4-DNPH) to react with the ketone produced. This reaction led to the formation of a coloured complex which was monitored by spectrophotometric assay. The use of an organic solvent in this screen (dimethyl sulfoxide: DMSO) improved the accuracy and reproducibility by allowing the use of higher concentrations of substrate. Reactions were performed with the racemic enol acetate and the corresponding single enantiomer in parallel with a selection of esterase mutants. Enantioselective esterase mutants were identified.

Acknowledgements

I would like to thank Dr Andrew Carnell and Dr Lesley Iwanejko for giving me the opportunity to realise this PhD, for their supervision and advice throughout the three years of my practical work, for their encouragements, help and useful comments during the writing of this thesis. I also would like to thank Alan Pettman from Pfizer for the funding of this project.

Thanks to the people in the previous Lab 210, Austin, Lynne and the current Lab G of the Biosciences Centre, Hayeit, Igor for their good company and a special thank to Jean and Annie for their everyday help and unconditional happiness. Thanks to everyone (past and present) in the Chemistry Department and Lab 185 especially Jo, Andy and Maria for the lively atmosphere. Thanks to all members of AJC's and LI's group Gareth, Rob, Pedro, Jenny, Ian for really good moments, Jon for his precious help to reach the final line and especially Mark for his help and all these chats about work and everything during the whole three years of this experience.

Thanks to all my friends, including flatmates and colleagues, most of them members of the French connection that I had the pleasure to meet during my PhD: Hélène, Nadia, Mathieu, Ella, Hélène, Dominique, Carine, Pierrick, Vincent and the members of the volleyball club but also older friends, even if far away, they are always presents: Gaëlle and Caroline.

I would also like to thank Adaś, for his unconditional support and help during the work in the lab and during the writing of this thesis. Since I met you, you have always been there for me, during the good and bad moments, I am so thankful for that.

Finally, I want to thank my family, my brother Christophe and Valérie for their precious advice and for all these wonderful moments in Saffron Walden, as well as my nephews Quentin and Adrien. But most importantly, I would like to thank my parents, you are always present and you support me whatever the circumstances. You always find a way to cheer me up. This would have not been possible without you, so this is for you.

Abbreviations

δ_H	chemical proton shift
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometre
µmol	micromole
amp	ampicillin
dATP	deoxyadenosine triphosphate
b	base
bp	base pair
nBuOH	n-butanol
^{t}BuOK	potassium tert-butoxide
c	conversion
°C	degree Celsius
$CaCl_2$	calcium chloride
carb	carbenicillin
Cl	chlorine
$CDCl_3$	deuterochloroform
dCTP	deoxycytidine triphosphate
Da	daltons
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double strand DNA
ssDNA	single strand DNA
2,4-DNPH	dinitrophenyl hydrazone
DTT	dithiothreitol
E	enantiomeric ratio
EDTA	ethylene diamine tetra-acetic acid
e.e.	enantiomeric excess
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
ep-PCR	error prone PCR
eq	equivalent
ESI	electrospray ionisation
Ficoll	Poly(sucrose-co-epichlorhydrin)
g	gram
g	centrifugal force
gDNA	genomic DNA
dGTP	deoxyguanosine triphosphate
h	hour
H_2O	water
HLADH	horse liver alcohol dehydrogenase
HPLC	high pressure liquid chromatography
HRMS	high resolution mass spectrometry
IPTG	isopropyl-β-D-thiogalactopyranoside
IR	infrared

KAc	potassium acetate
kb	kilo base
kbp	kilo base pair
kDa	kilodaltons
kg	kilogram
KOH	potassium hydroxide
l	litre
LB	Luria-Bertani
LiCl	lithium chloride
M	molar (mol/l)
MCS	multiple cloning site
mg	milligram
$MgCl_2$	magnesium chloride
MHz	mega Hertz
min	minute
ml	millilitre
mM	millimolar
mmol	millimole
$MnCl_2$	magnesium chloride
MOPS	3-(N-Morpholino)propanesulfonic acid
m.p.	melting point
MS	mass spectrometry
MTP	microtitre plate
MW	molecular weight
NaCl	sodium chloride
NAD/NADH	nicotinamide adenine dinucleotide
NaH_2PO_4	sodium phosphate monobasic
ng	nanogram
Ni	nickel
Ni-NTA	nickel nitrilotriacetic acid
nm	nanometre
NMR	nuclear magnetic resonance
dNTP	deoxy nucleotides tri-phosphate
OD	optical density
PCR	polymerase chain reaction
pg	picogram
PFL	*Pseudomonas fluorescens* lipase
PFE	*Pseudomonas fluorescens* esterase
pmol	pico mole
RbCl	rubidium chloride
RNA	ribo nucleic acid
rpm	rotation per minute
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sec	second
TBE	Tris-Bore-EDTA buffer
TEMED	NNN'N'- tetramethylethylenediamine
tet	tetracycline
TFB	transformation buffer

TGS	Tris-Glycine-SDS buffer
THF	tetrahydrofurane
TLC	thin layer chromatography
Tm	melting temperature
t_R	retention time
Triton X-100	polyoxyethylene(10) isooctylphenyl ether
Tris	tris(Hydroxymethyl)aminomethane
dTTP	deoxythymidine triphosphate
Tween	polyoxyethylene-sorbitan monolaurate
U	unit (restriction enzyme units)
UV	ultraviolet
V	volts
Vis	visible
v/v	volume over volume
w/v	weight over volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YADH	yeast alcohol dehydrogenase
>	greater than
<	less than

Contents

Chapter 1

Introduction

Chapter 1

Introduction

1.1 Catalysis: definitions and characteristics

1.1.1 Catalysis

A catalyst is a substance that initiates or accelerates a chemical reaction without itself being permanently affected by the reaction. Catalysts provide an alternative reaction pathway that requires less energy than an uncatalysed reaction. They decrease the activation reaction energy required for the chemical reaction to proceed. They also provide a surface on which reactants are absorbed and accelerate the rate at which equilibrium is attained. Further advantages of some catalysts are their capacity to introduce regioselectivity (stereoselectivity and enantioselectivity) into the reaction. A non chiral reactant can be transformed into a single enantiomer or an enantiomerically enriched mixture.

1.1.2 Enzymes - general characteristics

1.1.2.1 Definitions

A biocatalyst is a biochemical catalyst, such as an enzyme. Enzymes are complex structures responsible for sophisticated functions in reactions which occur in the mild environments of living organisms. The majority of biocatalysts are proteins, although some nucleic acids fragments and antibodies (abzymes) possess catalytic activities (Kulminskaya *et al.*, 2004; Shchurov, 1997; Wang *et al.*, 2002). Enzymes are named and classified in six categories according to the type of reaction they catalyse by the

Nomenclature Commitee of the International Union of Biochemistry and Molecular Biology (IUBMB):

Oxidoreductases: Involved in oxidation-reduction reactions

Transferases: Transfer groups from one molecule to another

Hydrolases: Add water to break a bond

Lyases: Addition/Elimination reactions between two or more molecules

Isomerases: Operate intramolecular rearrangements

Ligases: Form bonds between two substrate molecules

(Bairoch, 2000; SwissProt, 2004).

1.1.2.2 Structure and mechanism

Two models of enzyme-substrate binding exist: the lock-and-key model and the induced-fit model (Faber, 2004). The lock-and-key model assumes that the enzyme active site has a rigid structure that is complementary to the substrate. In the induced-fit model, the shape of the enzyme active site conforms to the shape of the substrate. These models lead to the specificity observed in enzyme catalysis.

Enzymes are specific, this is a unique feature of these proteins as catalysts. Usually a given enzyme will catalyse only one type of reaction for one class of compound, in some cases for one specific compound. The area directly responsible for catalysis is the active site. The reactant molecules (substrates) bind to this unique, intricately shaped binding surface, which is a small cleft or pocket in an otherwise large protein molecule. Amino acids side chains often participate in the catalytic process. Several factors contribute to enzyme catalysis, such as proximity (ensuring correct orientation of the substrate in the active site in relation to catalytic groups), accommodation of

the enzyme-substrate complex and stabilisation of the transition state. Electrostatic effects can also be involved as the charge distribution in the active site influences the reactivity of the substrate (Faber, 2004). Chemical groups can often be made more reactive by the addition or removal of a proton. Moreover, covalent catalysis can occur when a nucleophilic side chain group forms a temporary covalent bond with the substrate. Several amino acids are known to play this role, for example the CH_2OH group of serine, the imidazole group of histidine and the carboxylate groups of glutamate are involved in the catalytic triad of lipase like the lipase from *Candida rugosa* ,CRL, (Bornscheuer & Kazlauskas, 1999), whereas the sulfhydryl group of cysteine is involved in the active site of cysteine protease like the endopeptidase IdeS from *Streptococcus pyogenes* (Wenig *et al.*, 2004).

Non-protein cofactors (metal cations and complex organic molecules: coenzymes) can also aid catalysis. The metal ions Fe^{+2}, Cu^{+2}, Zn^{+2}, Mg^{2+} and Mn^{2+} are most often involved in catalysis. They are useful due to their high concentration of positive charge. They can also act as Lewis acids or bind to two or more ligands at the same time and facilitate the proper orientation of the substrate within the active site. Coenzymes are derived from organic molecules known as vitamins. Major coenzymes are NAD (Nicotinamide Adenine Dinucleotide) and NADP (Nicotinamide Adenine Dinucleotide Phosphate), involved in redox reactions; thiamine pyrophosphate, involved in decarboxylation and aldehyde transfer; FAD (Flavin Adenine Dinucleotide) and FMN (Flavin MonoNucleotide), involved in redox reactions; tetrahydrofolate, involved in one carbon group transfer reactions and deoxyadenosylcobalamin and methylcobalamin, involved in intramolecular rearrangements. For example, NAD/NADP, serve as carriers of electrons for several

enzymes in a group known as the dehydrogenases that catalyse oxidation-reduction and hydrogen removal reactions. Enzymes that require NADH to catalyse reactions are involved in energy generation. Enzymes that require NADPH catalyse biosynthetic reactions.

1.1.2.3 Kinetics

Most enzyme reactions either display Michaelis-Menten (MM) kinetics or approximate to MM kinetics. When a substrate (S) binds to the enzyme active site (E), an enzyme-substrate (ES) complex is formed. The substrate is converted into product via the formation of a transition state. After a finite amount of time, the product dissociates from the enzyme. This process can be explained through different equations.

Michaelis-Menten equation:

$$V = (V_{max}\,[S]) \;/\; ([S] + K_m)$$

V_{max} is the maximal velocity that the reaction can attain; K_m is a rate constant and [S] is the concentration of the substrate (S). When [S] is equal to K_m, the denominator is equal to 2[S], and V equals half of V_{max}.

The MM kinetic model explains several aspects of the behaviour of most enzymes. Each enzyme has a K_m value that is characteristic of that enzyme and substrate under specified conditions. It reflects the affinity of the enzyme for its substrate; the lower the K_m value, the greater the affinity. However, some problems appear in the determination of V_{max} and K_m when the MM plot is used. Determinations of K_m and V_{max} are also made with Lineweaver-Burk double-reciprocal plots. Rearrangement of

the MM equation (equation for a hyperbola) is made by taking reciprocal plots of the initial velocities as functions of reciprocals of the substrate concentrations. This leads to a straight line generated: $y = mx + b$, where:

$y = 1/v$

$x = 1/[S]$

$m = K_m / V_{max}$

$b = 1 / V_{max}$

Intercept on y (vertical axis) is $1 / V_{max}$

Intercept on x (horizontal axis) is $-1 / K_m$

The influence of activators or inhibitors can be studied through kinetics as they involve changes in substrate binding or specificity and even activity. Differences can therefore be observed in the K_m and V_{max} of the enzyme, highlighting the importance of such studies and calculations.

1.2 Lipases and esterases

1.2.1 Introduction

The employment of biocatalysts for organic synthesis has become an increasingly attractive alternative to conventional methods. As previously mentioned, enzymes often display high chemo-, regio-, and enantioselectivity, which makes these catalysts especially attractive for the pharmaceutical and agrochemical areas, where the demand for enantiomerically pure and specifically functionalised compounds is continuously growing. Another advantage is the normally mild conditions, under which enzyme-catalysed reactions are performed, such as room temperature and

neutral or almost neutral pH. These conditions minimise problems of product isomerisation, racemisation or epimerisation. Enzymes can be highly efficient catalysts, showing tremendous rate enhancements. These processes are also potentially less hazardous, polluting, and energy consuming than conventional chemistry.

Of all the potential enzyme catalysts, specific attention has been given to lipases and esterases, part of the hydrolase family. This group of enzymes catalyses ester bond cleavage by reaction with water. The natural function of most hydrolases is digestive, to break down nutrients into smaller units for metabolism. Since numerous different nutrients exist, hydrolases have a broad substrate specificity. Because of their broad substrate specificity, hydrolases often accept as substrates various synthetic intermediates. Hydrolases often show high stereoselectivity, even towards unnatural substrates. Besides hydrolysis, hydrolases also catalyse other related reactions, condensations (reversal of hydrolysis) (Isowa *et al.*, 1979) and alcoholysis (a cleavage using an alcohol in place of water) (Morgan *et al.*, 1997). One other advantage of hydrolases is that many of them are commercially available. Also they do not require co-factors and they tolerate the addition of water-miscible solvents (e.g. DMSO, DMF). Lipases and esterases can also be stable and active in neat organic solvents.

1.2.2 Characteristics of lipases

1.2.2.1 General reactions

Lipases and esterases catalyse the hydrolysis of esters but lipases preferentially catalyse hydrolysis of water-insoluble esters such as triglycerides (e.g. triolein or

diolein). They also hydrolyse unnatural esters often displaying high enantio- or regioselectivity, which explains their use as catalysts for organic synthesis. Lipases are used in a variety of biotransformations to prepare enantiomerically pure pharmaceuticals and synthetic intermediates (Berglund, 2001; Turner, 2004), to protect and deprotect synthetic intermediates, to modify natural lipids and for more specific reactions (Bornscheuer & Kazlauskas, 1999).

One of the other advantages of lipases in industry, as previously mentioned, is their reactivity with water-insoluble substrates, as a result of their ability to catalyse hydrolysis at water-organic interface, using interfacial activation. Lipases are also used in the food industry to hydrolyse milk-fat and enhance flavours or to accelerate cheese ripening. Mixtures containing lipases, like mixture from the calf pregastric gland, are added to impart characteristic flavour in cheese (Bornscheuer & Kazlauskas, 1999). Another important use of lipases includes the detergent industry where microbial lipases are used to aid the removal of fat stains and prevent the redeposition of fats on textiles.

1.2.2.2 Secretion and folding

A significant number of bacterial lipase genes have been cloned. However, the molecular mechanisms regulating their expression and secretion are relatively unknown. As lipases are extracellular enzymes, they need to be transported through the bacterial membrane to reach their final destination. Three secretion pathways have been identified for proteins and lipases follow the same process. However, the second and third pathways are associated for some gram-negative bacteria as it first allow the

secretion of protein through the inner membrane and then through the outer membrane.

The first pathway is through an ABC exporter (Jaeger *et al.*, 1999). Lipases from *P. fluorescens* or *S. marcescens* lack a typical N-terminal signal peptide and are secreted by an ABC exporter consisting of three proteins. In this case, the inner membrane protein LipB containing an ATP-binding cassette (ABC protein) confers the substrate specificity to the system. The second component is LipC protein, a membrane fusion protein (MFP). This protein is an inner membrane protein with a short N-terminal hydrophobic domain, anchoring it in the inner membrane, a large hydrophilic domain, presumably located in the periplasm and a C-terminal domain which could interact with the outer membrane. The third protein is LipD, an outer membrane protein. Lipases and metalloproteases are secreted through this ABC transporter.

However, many lipases, from Gram-positive and Gram-negative bacteria, possess and N-terminal signal sequence, which mediates their secretion through the inner membrane, with the help of the Sec translocase. This characterises the second pathway which allows the passage of proteins across the inner membrane. The lipases are secreted by the Sec translocase, which is a multisubunit protein complex with a soluble part, SecA and a membrane complex, SecY, E, D, G and F. Transport is performed through the activation of an ATPase (Jaeger *et al.*, 1999).

After being secreted through the inner membrane of Gram-negative bacteria, lipases need to be secreted across the outer membrane, which corresponds to the third secretion pathway. Lipases fold in an active conformation when they have been

secreted through the inner membrane. The transport through the outer membrane is then performed by another protein complex called the secreton, which can consist of up to 14 proteins (Jaeger *et al.*, 1999).

In order for the lipase to correctly fold, specific proteins, called intramolecular chaperones or Lif for Lipase specific foldases, are required in the periplasm. These proteins are often expressed with the lipase itself (Frenken *et al.*, 1993a; Rosenau *et al.*, 2004). These proteins are anchored to the membrane through a hydrophobic N-terminal fragment but they still possess foldase activity when this fragment is removed. For some Gram-positive bacteria, lipases are expressed as a pre-proenzyme with an N-terminal pro-region of about 260 amino acids which acts as a foldase (Jaeger *et al.*, 1999). Figure 1.1 summarises an example of a lipase secretion pathway for *P. aeruginosa*.

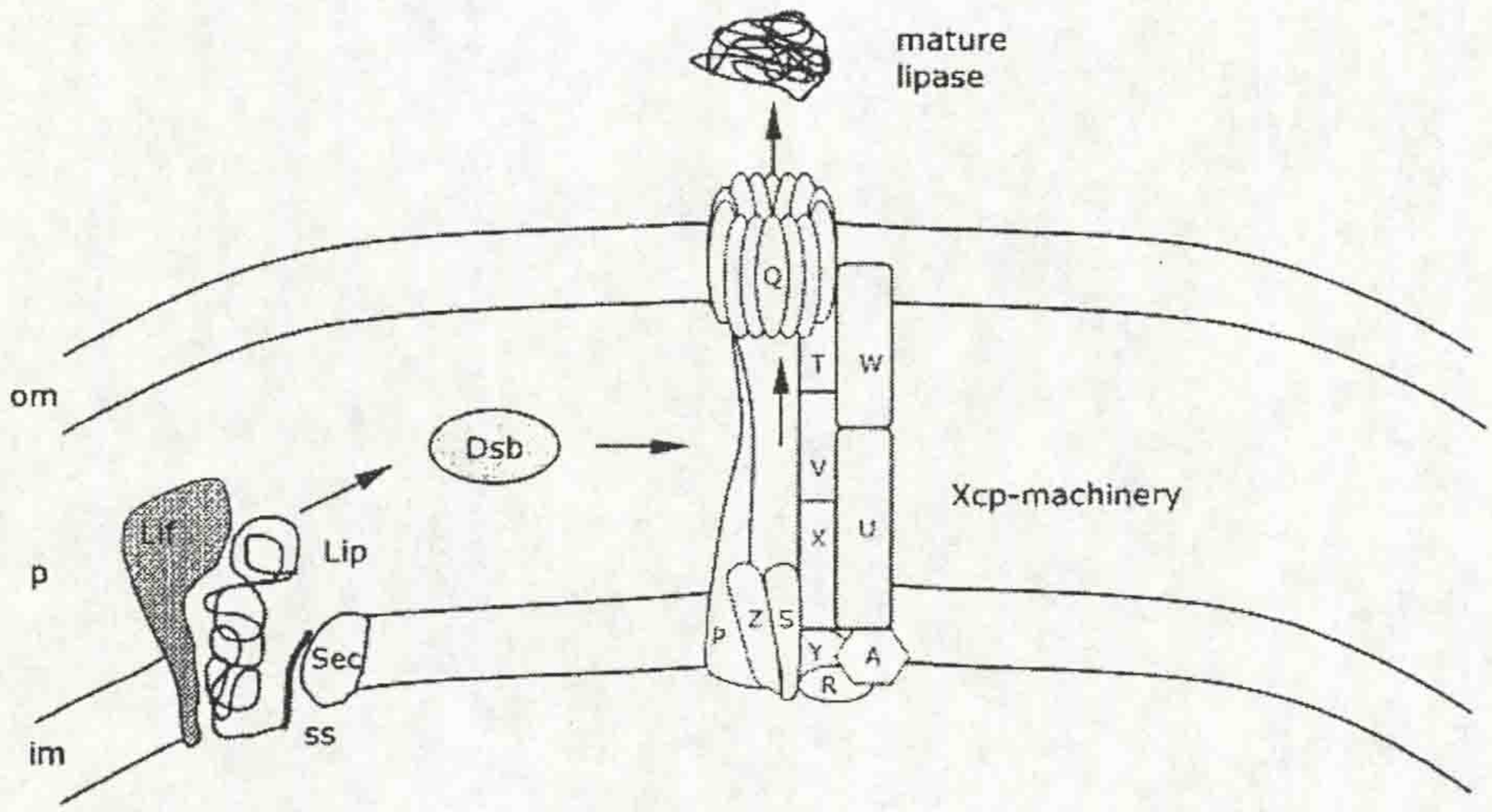
1.2.3 Characteristics of esterases

1.2.3.1 General reactions

Like lipases, esterases catalyse the hydrolysis of carboxylic acid esters. They can be isolated from the same organisms and share characteristics with lipases (see Section 1.2.4). The role of esterases for bacterial or eukaryotic cells is generally not well known, with the exceptions of acetyl- or butyryl choline esterases which are involved in the hydrolysis of neurotransmitters. Other esterases are also present in the metabolic degradation pathways of hemicellulose (Dalrymple & Swadling, 1997). It has also been shown that some insecticide resistance is linked to amplification of esterase genes (Field & Blackman, 2003). Esterases can be used in the conversion of heroin into morphine (Rathbone *et al.*, 1997) or in the formation of ω-hydroxy acids

Figure 1.1: Model for the *P. aeruginosa* pathway

Lipase (Lip) is secreted as prelipase including a signal sequence (ss) through the bacterial inner membrane (im) by a Sec-dependent mechanism. Interaction with the lipase-specific foldase Lif and Dsb proteins assists folding and formation of disulfide bonds in the periplasm (p). The final secretion through the outer membrane (om) is mediated by the Xcp machinery (Jaeger *et al.*, 1999).



from lactones (Khalameyzer & Bornscheuer, 1999). These reactions are performed *in vivo*, following an enzymatic Baeyer-Villeger oxidation. Hence, they can be used to enable growth on carbon sources such as cyclic alkanes.

The use of esterases in organic synthesis is not as wide as the use of lipases, one of the limiting factors being the commercial availability of these enzymes. The most widely used mammalian esterase is the pig liver esterase. This enzyme has been employed for its selectivity properties and is still used in the separation of enantiomers or in immobilisation studies (Heilmann *et al.*, 2004; Tanyeli & Turkut, 2004). However, the use of new bacterial esterases, from *P. fluorescens* or *Humicola insolens*, has recently been investigated (Hatzakis & Smonou, 2004; Kim *et al.*, 2004).

1.2.3.2 Secretion and folding

Excretion pathways for esterases follow the same pathway as other proteins. For Gram positive bacteria like *Streptomyces*, proteins targeted for secretion are released directly into the culture medium. These proteins are typically synthesised as a precursor which is processed during the secretion. This process involves the removal of a signal peptide from the amino terminus (Hale *et al.*, 1992; Hale & Schottel, 1996). In contrast, proteins that are secreted from Gram-negative bacteria such as *E. coli* are localised to the periplasmic space and are then excreted through the outer membrane but they also have a signal peptide and these pathways are similar to the one previously described for lipases. However, for the esterases to be expressed in an enzymatically active form, no foldase is required. It seems that this family does not need the presence of chaperone proteins to adopt correct folding.

1.2.4 Common features

1.2.4.1 α/β-hydrolase fold

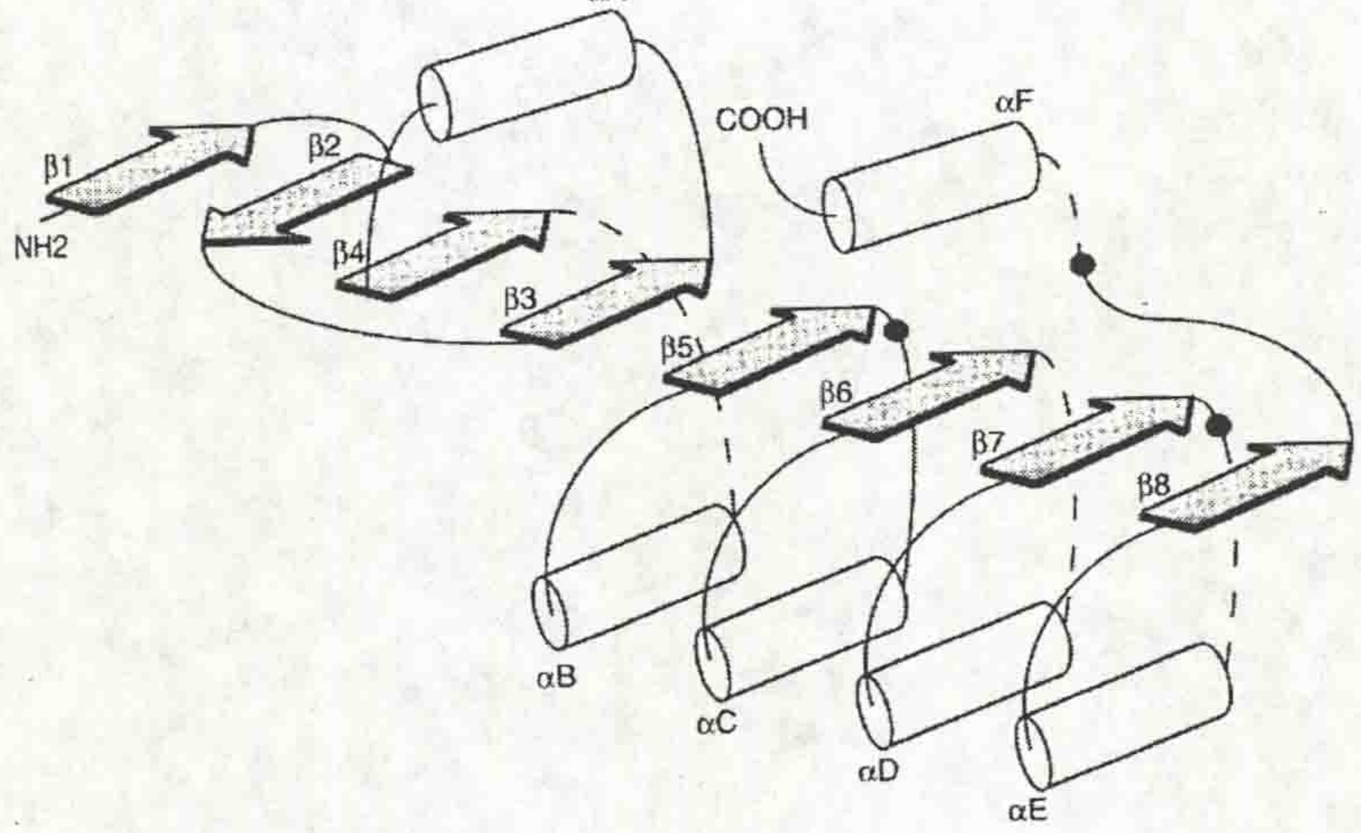
Comparison of the X-ray structures for hydrolases as different as haloalkane dehalogenase, acetylcholinesterase and serine carboxypeptidase revealed that these enzymes all share the same folding pattern and show similar 3D-structures. As these enzymes all catalyse hydrolysis reactions, the pattern was named the α/β-hydrolase fold (Ollis *et al.*, 1992). This model consists of a core of a mostly parallel β sheets of eight strands with the second strand antiparallel. The parallel strands β3 to β8 are connected by α-helices (Figure 1.2). The bacterial lipase structures obey the α/β-hydrolase fold though some variations may be observed in the number of β strands. A similar structural pattern was found for an esterase from *P. fluorescens* (Kim *et al.*, 1997), with seven strands. However, even if primary sequence homology is obvious, large differences can be observed in activity and selectivity.

1.2.4.2 Catalytic residues and mechanism

The active site of the α/β-hydrolase fold enzyme consists of a catalytic triad: a nucleophilic residue (serine, cysteine, or aspartate), a catalytic acid residue (aspartate or glutamate), a histidine residue and several oxyanion-stabilizing residues (Figure 1.2). These residues occur in the same order in all lipase amino acid sequences and orientate in the same three-dimensional way in all the structures. Two positions before and two positions behind the nucleophile, one or both residues at these positions are glycines. They are in some cases substituted for other small residues such as alanine, valine, serine or threonine. The nucleophile is present in the middle of an arrangement with a strand and a helix, which was named the "nucleophile elbow" (Jaeger *et al.*, 1999). It allows easy access of the substrate to the active site

Figure 1.2: Canonical fold of α/β-hydrolases

The α helices are indicated by cylinders and the β strands are indicated by shaded arrows. The topological positions of the active-site residues are shown by solid circles; the nucleophile is the residue after β strand 5, the Asp/Glu residue is after β strand 7, and the histidine residue is in the loop between β8 and αF (Jaeger *et al.*, 1999).



and positions the nucleophile at the N-terminal end of the following helix. This structure helps to stabilise the tetrahedral intermediate and the ionised form of the nucleophile. The catalytic acidic residue (Asp or Glu) is present in a reverse turn after strand 7 of the central β sheet. It is bonded to the active site histidine by a hydrogen atom. However, the position of this acid differs from one hydrolase to the other. The third catalytic residue is the catalytic histidine, it is situated in a loop after strand 8 but with variations (Figure 1.2) (Jaeger *et al.*, 1999).

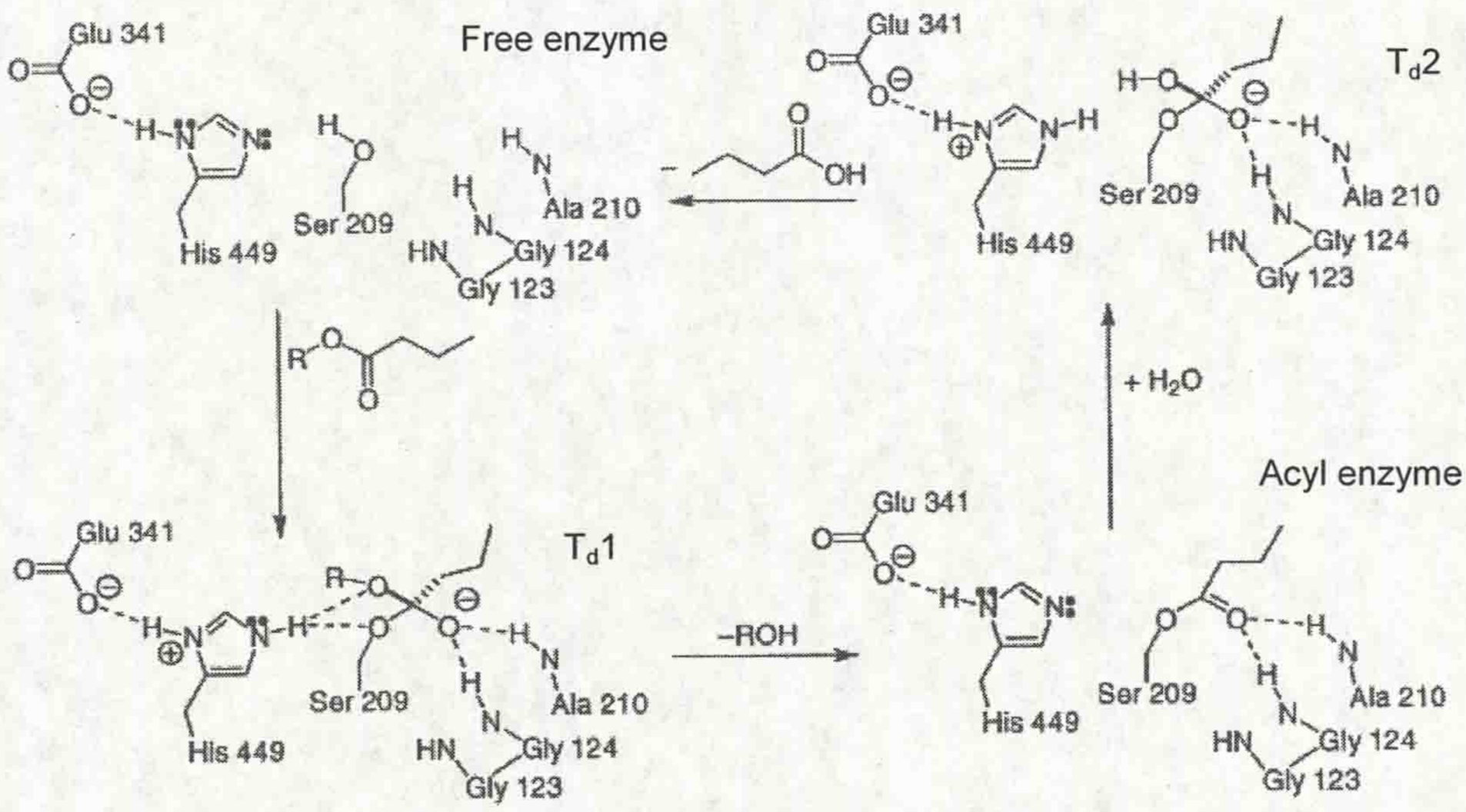
Lipases are serine esterases and share the same reactivity. The catalytic triad consists in this case of Ser-His-Asp/Glu. It is similar to the triad of serine proteases and adopts the same mechanism (Figure 1.3). First, the ester binds to the lipase and the catalytic serine attacks the carbonyl group forming a tetrahedral intermediate. The rearrangement of this intermediate releases the alcohol and leaves an acyl enzyme intermediate. Rearrangement of this intermediate releases the acid. Another nucleophile, such as an alcohol, can then attack the acyl enzyme and lead to a new ester (transesterification reaction). The formation of the acyl enzyme is the fast step whereas the deacylation is the rate-determining step.

1.2.4.3 Substrate binding

X-ray crystal structures of hydrolases have allowed the identification of binding sites for the alcohol and the acid portions of the esters. The alcohol binding site is similar in all hydrolases, in fact, it consists of a hole with two regions, a large hydrophobic pocket opened to the solvent and a small pocket that faces the floor of the hole. The shape of this last pocket sets the stereoselectivity of lipases toward secondary alcohols.

Figure 1.3: Hydrolysis of a butyric acid ester catalysed by lipase or esterase

This mechanism involves an acyl enzyme intermediate and two different tetrahedral intermediates. Formation of the acyl enzyme involves the first tetrahedral intermediate, T_d1. Alcohol is released in this step and this step determines the selectivity of lipases towards alcohols. Release of the acyl enzyme involves the second tetrahedral intermediate, T_d2. When deacylation limits the rate, the selectivity of the lipase towards its target acid is selected. The amino acid numbering corresponds to the active site of lipase from *Candida rugosa*, CRL (Bornscheuer & Kazlauskas, 1999).



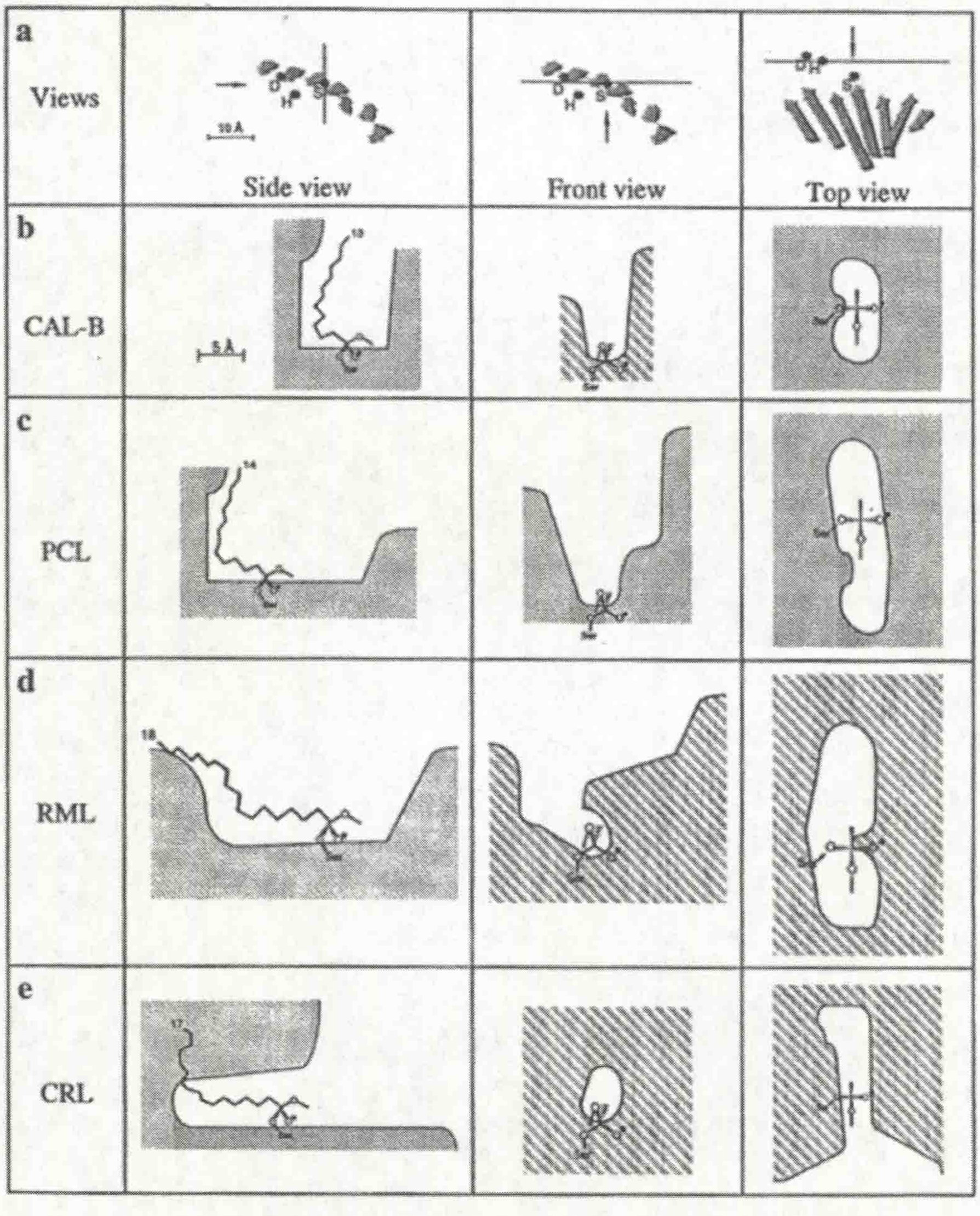
The binding site for the acid portion of the ester varies considerably among the hydrolases, it can be a tunnel long enough to incorporate at least an eighteen carbon chain or it can be a small hole at the surface of the active site (Figure 1.4) (Bornscheuer & Kazlauskas, 1999). However, in most cases, the α-carbon of an acyl chain binds just below the large hydrophobic region of the alcohol binding site and the other groups at the α-carbon extend into the hydrophobic pocket (Figure 1.4).

1.2.5 Interfacial activation

The activity of lipases is characterised by an important increase in activity when the enzyme is at the lipid-water interface, termed the interfacial activation. This phenomenon is due to a certain conformation and rearrangement of the active-site region, determined from crystal structures (Derewenda *et al.*, 1992). For lipases, a lid or flap, composed of a single helix, two helices or a loop region, blocks the active site. However, when the lipase is in the presence of hydrophobic substances the lid is opened, making the catalytic residues accessible to the substrate and exposing a large hydrophobic surface, causing interfacial activation. When lipases are present in aqueous solution, the reactivity of the enzyme towards a soluble substrate is poor because the lid is closed. Esterases lack a lid and contain a pre-formed oxyanion hole and therefore do not show interfacial activation. However, certain lipases do not show this activation even though they have a small lid, showing that the mechanism of interfacial activation is more complex (Bornscheuer & Kazlauskas, 1999).

Figure 1.4: Shapes of the binding sites from four lipases (CAL-B, PCL, RML, CRL)

CAL-B: *Candida antartica* B lipase, PCL: *Pseudomonas cepacia* lipase, RML: *Rhizopus oryzae* lipase, CRL: *Candida rugosa* lipase



a: Orientation of the cross-sections which are planes perpendicular to the paper plane and indicated by a straight line (D, Asp; H, His; S, Ser). The direction of the view is indicated by an arrow.
b-e: Shape of the binding sites in side, front and top view; the number indicates the length of the longest fatty acid which completely binds inside the binding pockets (Pleiss *et al.*, 1998).

1.2.6 Use in synthetic organic chemistry and improvement of existing enzymes

1.2.6.1 Lipases and esterases as catalysts

The number of reports concerning the use of lipases and esterases as catalysts in synthetic organic chemistry continues to increase (Emgenbroich & Wulff, 2003; Rahman *et al.*, 2004; Snellman & Colwell, 2004; Topakas *et al.*, 2004). In addition to regioselective hydrolysis, acylation or transesterification, an incredibly wide variety of enantioselective processes have been reported (Carrea & Riva, 2000). These include kinetic resolutions and desymmetrisation of prochiral substrates, normally involving carboxylic esters. However, the last fifteen years have seen the extension of lipase substrates to include molecules like cyanohydrins, chlorohydrins, diols, α- and β-hydroxy acids, amines, diamines and amino alcohols. Reactions can be performed in aqueous mediums for hydrolysis, or in organic solvents for acylation and transesterification. The most common bacterial lipases used for these catalytic reactions are from *Pseudomonas aeruginosa*, *P. fluorescens*, and other *P. spp.*, *Burkholderia cepacia*, *Chromobacterium viscosum*, *Bacillus subtilis*, *Achromobacter spp.*, *Alcaligenes spp.* and *Streptococcus marescens*.

One of the disadvantages of choosing esterases was the poor commercial availability of these enzymes, except for the pig liver esterase. The only sources for esterases are therefore the organisms itself, with purification of the proteins from the organisms or the study, cloning and expression of the protein of interest as a recombinant enzyme. However, the development and study of new bacterial esterases and their easy expression and purification have allowed an extension of the use of esterases as catalysts in organic synthesis (most of them similar to the ones cited for lipases) and

fungi (Bornscheuer, 2002; Topakas *et al.*, 2004). The potential substrate range is, as for the lipases, wider than was originally believed.

1.2.6.2 Kinetic resolutions

In kinetic resolutions, the enantiomeric purity of the product and starting material varies as the reaction proceeds. The comparison of enantiomeric purity for substrate and product therefore needs to be done at the same extent of conversion. To compare kinetic resolutions, equations were developed to calculate their inherent enantioselectivity (Chen & Sih, 1989; Sih *et al.*, 1986; Sih & Wu, 1989). The parameter used to measure this selectivity is the enantiomeric ratio E, which measures the ability of the enzyme to distinguish between enantiomers. A non-selective reaction has an E of 1, while resolutions with E's above 20 are useful for synthesis. The E value of an enantioselective biotransformation, where A and B are the fast and slow reacting enantiomers, is the ratio of the specificity constants, V/K for each enantiomer, V being the maximal velocity and K the Michaelis constant for the biotransformation. The E value relates the extent of conversion, c, and the enantiomeric purity of the starting material (e.e.) or product. The higher the enantiomeric ratio E, the more selective the enzyme (Figure 1.5). These equations include assumptions such as an irreversible reaction, one substrate and product and no product inhibition. However, even given these limitations, they are reliable in the vast majority of cases.

One disadvantage of using a kinetic resolution is the fact that only 50% of the total material is used. However, methods have been developed to allow a conversion higher than 50%. One method is called a dynamic kinetic resolution (Stecher &

Figure 1.5: Enantiomeric ratio (E), extent of conversion (c) and enantiomeric excess (e.e.)

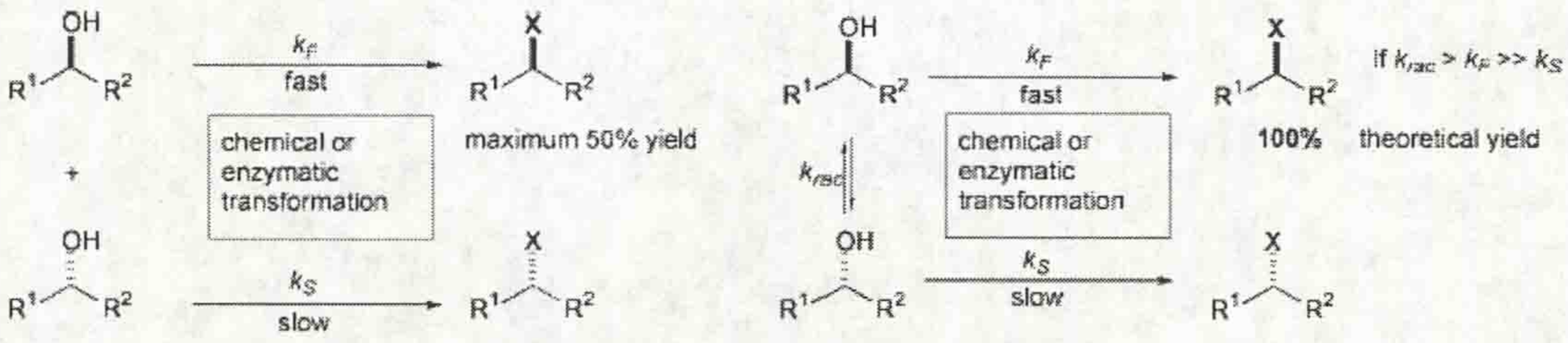
$$E = \frac{V_A/K_A}{V_B/K_B} = \frac{\ln[(1 - c)(1 - e.e.(S))]}{\ln[(1 - c)(1 + e.e.(S))]}$$

where

$$c = 1 - \frac{A + B}{A_o + B_o} \qquad e.e.(S) = \frac{B - A}{A + B}$$

Figure 1.6: Comparison of kinetic *vs.* dynamic kinetic resolution using alcohols as examples.

(Huerta *et al.*, 2001)



Faber, 1997). In this case, the principal enzyme, that allows the kinetic resolution, is mixed with a second catalyst which causes the rapid racemisation of the substrate. As the product is not racemised under the reaction conditions, 100% conversion to a single enantiomeric product is theoretically possible (Figure 1.6) (Huerta *et al.*, 2001). To enhance the enantiomeric purity, the enriched material can be isolated and resolved again. This double resolution is called recycling (Allan *et al.*, 2000; Carnell *et al.*, 2000). Equations to predict the optimum degree of conversion in recycling reactions were established and many researchers have used this strategy (Brown *et al.*, 1993; Vanttinen & Kanerva, 1997). A computer program for these calculations and calculations of enantiomeric ratio E are available (Faber & Kleewein). To minimise the work in recycling reactions, several groups used *in situ* recycling where the two resolutions are carried out at the same time but without isolation of the intermediate products (Chen & Liu, 1991; Majeric & Sunjic, 1996).

1.2.6.3 Screening and directed evolution of hydrolases

Microorganisms are a valuable source of enzymes for biocatalysis. Characterisation of an organism's phenotype and genotype allows the identification of new enzymes with novel activity. Most of the commercially valuable enzymes have been discovered in this way. Enzymes have evolved to be specific for certain substrates and in some cases have enantioselectivity. However, these modifications have occurred as a consequence of environmental pressures and may not be suitable for use in organic synthesis applications. Natural enzymes are therefore often unstable at high temperatures and/or in organic solvents and have very low selectivity for a given substrate. However, when a given enzyme shows a low enantioselectivity towards a substrate of interest, this can be improved by changing the reaction conditions, such

as varying the solvent or temperature, but this is an empirical strategy and limits the application of these enzymes in an industrial environment.

Over the last fifteen years, approaches for improving the biocatalytic activity and specificity of industrially relevant enzymes have been developed. These methods include site-directed mutagenesis and the use of computer modelling to predict the three-dimensional structure and mechanisms of action of the protein of interest. However, the need to perform several rounds of mutagenesis slows down the process and computer modelling cannot currently help predict the effects of pH or solvent stability on catalytic activity or substrate specificity.

A relatively new approach to increase the enantioselectivity of enzymes is based on directed evolution, this method has been the source of numerous studies and reviews (Alexeeva *et al.*, 2003; Fernandez-Gacio *et al.*, 2003; Turner, 2003; Valetti & Gilardi, 2004). Directed evolution is the use of random mutagenesis or recombination combined with an efficient assay to identify and select enzymes with improved characteristics. Contrary to classical random mutagenesis, directed evolution restricts mutations to the gene encoding the protein of interest or even specific regions of the gene. In the directed evolution of biocatalysts, mutations are introduced into genes of interest by error-prone PCR or recombinative methods such as DNA shuffling (Section 1.4). After the expression of these mutated genes in suitable microbial hosts, functional biocatalysts are selected or screened in order to identify the "best" mutant enzyme in often large libraries (10^4 to more than 10^6 variants) of potential candidates (Kuchner & Arnold, 1997). The procedure is then repeated until the desired catalytic features have been achieved. In principle, this technique could be applied to create

biocatalysts with increased enantioselectivity. However, this is not a simple parameter to measure and the development of rapid assay systems, capable of identifying improved enantioselective biocatalysts from large libraries, is crucial to enable this technology to be adopted more widely.

1.3. Methods of directed evolution

1.3.1. Introduction

As previously described, catalyst activity and selectivity in naturally occurring enzymes are often too low for numerous substrates of interest. Sometimes the selectivity may be high but the use of solvents and other conditions used in organic synthesis could lead to low enzyme stability, activity or selectivity. Protein engineering has therefore been used to improve enzymes.

From 1980, molecular biologists developed practical techniques for random mutagenesis (Cadwell & Joyce, 1994; Eckert & Kunkel, 1991; Stemmer, 1994a; Stemmer, 1994b.). When mutations are randomly introduced into a gene and expression is performed in a suitable bacterial system, mutant enzymes are produced. It is therefore possible to create libraries of mutant enzymes in which amino acids have been exchanged randomly. Mutations can be induced by various methods which will be described in the next sections. The frequency of mutation can also be controlled (Cadwell & Joyce, 1994; Eckert & Kunkel, 1991). After realising it would be possible to produce large numbers of mutant proteins, the problem of identifying the best mutant of the library needed to be solved. The major characteristic beyond the combinatorial chemistry aspect was the evolutive process. The probability of

obtaining the optimal mutant after one round is low. However, a few improved mutants can be identified and used as a starting point for the next cycle of mutagenesis and selection or screening. This process can then be repeated until the desired activity and selectivity is achieved for the enzyme. This summarises the Nature's evolution system. However, the time is considerably reduced from million of years for Nature to months for scientists to create an optimal catalyst for a reaction of interest.

1.3.2. Production of mutants

1.3.2.1 Site-directed mutagenesis

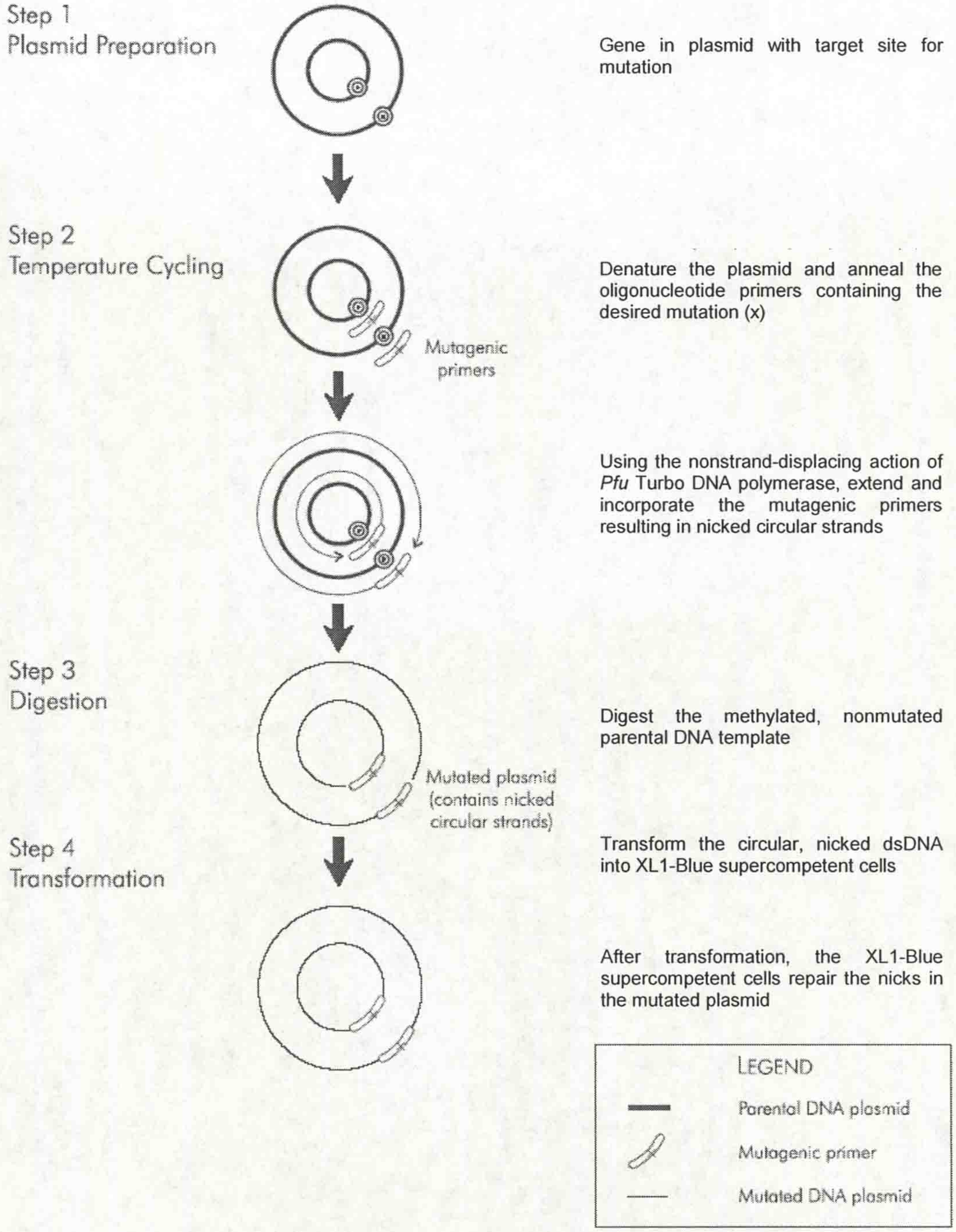
Site-directed mutagenesis involves exchanging, inserting or deleting one or more defined nucleotides of a given gene, resulting in the desired amino acid substitutions. This is usually achieved using a short oligonucleotide primer that is annealed to a single-stranded DNA template or a circular plasmid which contains the gene of interest. This oligonucleotide is complementary to a region of the template except for the mismatch needed to insert the mutation. The primer is then extended by DNA polymerase and the DNA fragment is cloned and expressed in *E. coli* (Figure 1.7). This technique is widely used (Cadwell & Joyce, 1994; Eckert & Kunkel, 1991).

1.3.2.2 Saturation mutagenesis

Saturation mutagenesis refers to the substitution or insertion of codons encoding all possible amino acids at any predetermined position in a gene. The most frequently used method is the application of a site-directed mutagenesis to introduce the nucleotide exchanges necessary to obtain all desired codons. However, a set of mutagenic oligonucleotide primers is needed and all of the resulting mutagenised

Figure 1.7: Overview of the site-directed mutagenesis method

Example of a site-directed mutagenesis protocol (Stratagene).



DNAs have to be sequenced in order to confirm the presence of the mutations. Another method involves the use of PCR to introduce site-directed mutations. Oligonucleotide primers, degenerate at one codon position, are used. They are synthesised employing concentrations of the four nucleoside phosphoramidites (dA, dC, dG, dT) (Airaksinen & Hovi, 1998). All of the 64 codons are therefore formed. However, the redundancy of the genetic code means that six different codons can sometimes produce the same amino acid, *e.g.* arginine. Nonsense codons also occur and result in the termination of the translation which leads to a truncated protein.

1.3.2.3 Cassette mutagenesis

Cassette mutagenesis requires that the three-dimensional structure of the protein of interest is known as an entire gene segment will be replaced by combinatorial cassette mutagenesis (CCM) (Reidhaar-Olson & Sauer, 1988). A cassette is a DNA fragment consisting of from three to several hundred nucleotides, encoding one to several hundred amino acids for a sequence of the enzyme known to have an importance in the activity for best results. CCM uses oligonucleotides containing randomised codons as mutagenic cassettes. They are introduced into the gene of interest by PCR-methods. The desired variant is then selected by screening. This method has been used in a wide range of studies such as antibiotic synthesis (Zirkle *et al.*, 2004) and antitumor drug synthesis (Remsing *et al.*, 2003). An extension of the method is called combinatorial multiple cassette mutagenesis (Reetz *et al.*, 2001). This allows the creation of a library of mutants in which each of the multiple cassettes contains a mixture of all wild-type and randomised sequences, resulting in all permutations of all wild-type and mutant cassettes.

1.3.2.4 Directed enzyme evolution by non recombinative methods

Directed evolution mimics natural evolution with two major differences. In directed evolution, it is the researchers who define the properties to be optimised and the process of evolution takes place in a much shorter period. The whole process involves three steps: creation of a library consisting of mutated genes, functional expression of the genes in suitable hosts and identification of enzyme variants with improved properties by selection or screening.

1.3.2.4.1 Error-prone PCR

The Polymerase Chain reaction (PCR) is a technique which employs a DNA template, oligonucleotide primers and thermostable DNA polymerase to amplify any given DNA with high fidelity. The intrinsic 3'→5' exonuclease activity (proof-reading activity) of DNA polymerases ensures that DNA amplification proceeds in an accurate manner. Error prone PCR (ep-PCR) involves modification of the proof-reading activity of the polymerase by generally increasing the concentration of $MgCl_2$, dCTP, dTTP and Taq polymerase and by adding $MnCl_2$. In such a case, the very small error rate of the Taq DNA polymerase can be increased from $0.1\text{-}2\text{x}10^{-4}$ per nucleotide to $7\text{x}10^{-3}$ per nucleotide. For a whole gene, the desired frequency is around one to two nucleotide mutations per gene, which corresponds to a change of one amino acid per mutant enzyme (Zhou *et al.*, 1991).

1.3.2.4.2 Bacterial mutator strain

Wild-type *E. coli* possesses a spontaneous mutation frequency of about $2.5\text{x}10^{-4}$ mutations per 1000 nucleotides of DNA inserted into a pBlue-script-like plasmid after 30 generations of growth. When the DNA repair pathways of these bacteria contain

mutations, a considerable increase in the spontaneous mutation rate can be observed, from 5- to 100-fold higher than the wild-type strain. Some strains such as *E. coli* XL1-Red (Stratagene) (Greener *et al.*, 1997) have been specifically modified to have high mutation frequencies. In order to generate a mutant library, a gene encoding a biocatalyst protein can be cloned into an appropriate plasmid, transformed into *E. coli* XL1-Red and the strain grown overnight. However, if the target DNA is of small size (<100bp) or if multiple mutations are required, the number of generations needed for propagation of the plasmid DNA will become high but as the strain lacks DNA repair pathway, the strain accumulates mutations and is therefore unstable and cannot be propagated for prolonged periods.

1.3.2.5 Directed enzyme evolution by recombinative methods

The genetic definition of recombination is the breaking and rejoining of DNA in new combinations and is the process by which the order of genetic information on a DNA molecule is altered. In living species, this process speeds up the process of evolution. Recombination *in vivo* can either be homologous recombination, which occurs between identical or very similar sequences, or non homologous recombination, which occurs between different DNA sequences. Both require specific enzymes to break and rejoin the DNA molecules. Attempts have been made to mimic these processes *in vitro* as explained in the next paragraphs.

1.3.2.5.1 DNA-shuffling

This technique leads to the recombination of one or more related genes into one full-length gene after enzymic digestion and PCR. The original gene(s) are digested by DnaseI in double-stranded fragments, which are then purified and used in semi-PCR

reactions. Cycles of strand separation and reannealing with DNA polymerase and a final cycle of amplification lead to the production of a whole gene. By this method, fragments of DNA can be switched, introducing mutations into the original gene. Often these techniques are not used in isolation, e.g. single mutations from ep-PCR can be used in large blocks of combination with DNA shuffling which can lead to the use of DNA-shuffling on a pool of mutant genes, which are homologous but with different point mutations (Figure 1.8) (Stemmer, 1994a; Stemmer, 1994b).

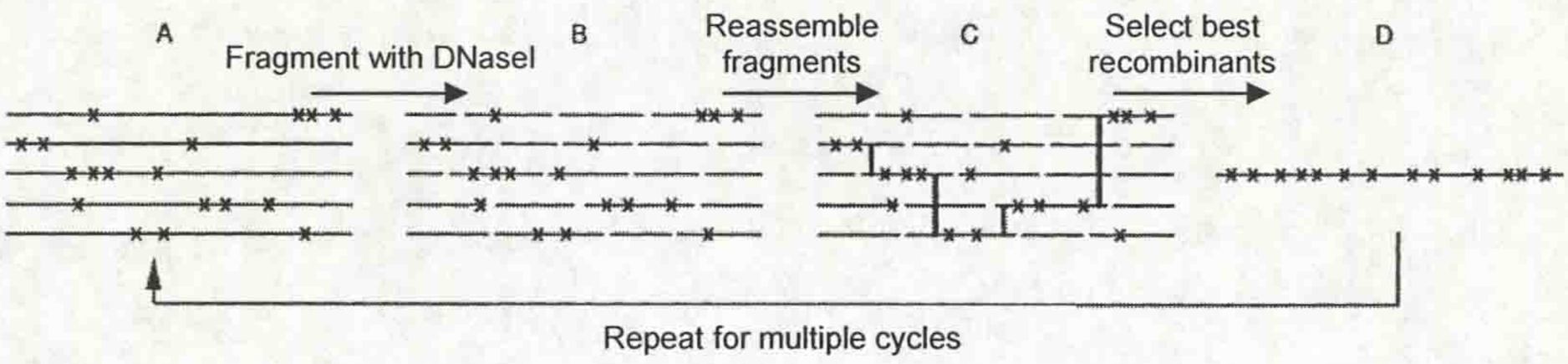
1.3.2.5.2 Staggered extension process

An efficient method for *in vitro* mutagenesis and recombination is based on a staggered PCR-like reaction (Zhao *et al.*, 1998). A specific primer is added to the template DNA which may consist of two or more genes. A really short primer extension reaction, catalysed by DNA polymerase, produces short DNA fragments which can anneal to different templates and are further extended during the next short cycle of primer extension. This process is repeated until full length genes are formed. These genes are finally amplified by performing a classic PCR using external primers.

A similar method is called random-priming recombination (RPR) (Shao *et al.*, 1998). Random primers are used to generate short DNA fragments complementary to different segments of a target DNA. These fragments, which carry mutations due to mispriming and base misincorporation, can prime one another and result in recombination during reassembly to full length genes, performed by repeated thermocycling in the presence of a thermostable DNA polymerase.

Figure 1.8: Overview of the gene-shuffling method

(A) A pool of homologous genes with different point mutations is fragmented with DNase I.
(B) For simplicity, all mutations shown are considered beneficial and additive.
(C) Reassembly of the random fragments into full-length genes results in frequent template switching and recombination. A recombinant gene containing the four crossovers (thick lines) can be selected from the library of recombinants based on its improved function.
(D) Selected pool of improved recombinants provides the starting point for another round of mutation and recombination. The recombination process alone causes a low level of point mutations but, if desired, additional mutations could be introduced by error-prone PCR or UV mutagenesis of the pool of genes.
(Stemmer, 1994a)



1.3.3 Gene expression systems

1.3.3.1 Gene expression

After the introduction of mutations in a gene coding for a specific biocatalyst, the next step consists in the functional expression of the recombinant gene in a suitable host organism. This step is essential for the successful identification of an enzyme variant with improved properties.

Most bacterial genes are expressed in *E. coli*, the most frequently used prokaryotic expression host for heterologous proteins. The gene is cloned into an appropriate plasmid behind a promoter which allows a tight control of gene expression (induction as well as repression of transcription). Promoters like *lac*, *tac* and T7-*lac* can be induced by the addition of molecules such as isopropyl-β-D-thiogalactopyranoside (IPTG) and regulated by an appropriate repressor ($lacI^Q$). Expression of the gene coding for the biocatalyst can therefore be performed. However, in some cases, expression of a gene is not sufficient to obtain enzymatically active protein (see Section 1.2.3.2). The choice of the correct expression system is therefore an important prerequisite in the design of a successful strategy for directed evolution of a biocatalyst.

1.3.3.2. Purification of the recombinant protein

The recombinant enzymes need to be purified to facilitate detailed analyses of their biocatalytic activities. The easiest method is to use affinity chromatography such as the use of a "His" tag which is inserted at the N-terminal or the C-terminal of the recombinant protein. Usually, this tag, often a 6-histidine tag, does not interfere with the activity of the protein. It allows easy purification of the recombinant protein,

through binding to a Nickel column (Ni-NTA column) and elution with increasing imidazole concentrations.

1.3.4. Selection and screening strategy

By using directed evolution, large libraries of up to 10^{10} mutant genes can be created. The development of appropriate systems for screening the library is therefore essential. The method should be easy to set up, efficient and produce rapid results that can be readily interpreted. A difference exists between selection and screening. Selection is the process of identifying a desired member of a library in the presence of all other members. In this case, only the desired mutant appears as a viable microbial clone. This represents an *in vivo* selection which is usually very efficient as only the micro organisms which express a gene encoding for a particular enzyme are able to survive. However, it can be difficult to develop such a system as microbial cells are extremely sensitive to their environment and the conditions in which they can grow. On the other hand, screening methods have to handle every single member of a given library and usually require automation.

Novel strategies of selection and screening have recently been reviewed (Goddard & Reymond, 2004a; Goddard & Reymond, 2004b; Reetz, 2004; Turner, 2004). Identification of mutants of interest can be performed by using many analytical techniques such as UV/Vis spectroscopy, pH indicator colorimetry, fluorescence spectroscopy, HPLC-CD spectroscopy, IR thermography, ESI-MS. No screening method is universal, therefore, for each experiment, a high throughput screening strategy needs to be devised. Two techniques can be also combined, e.g. a first screen which leads to a preselection of positive and interesting mutants for a specific activity and a second one to give a more detailed analysis (e.g.: e.e. and E values).

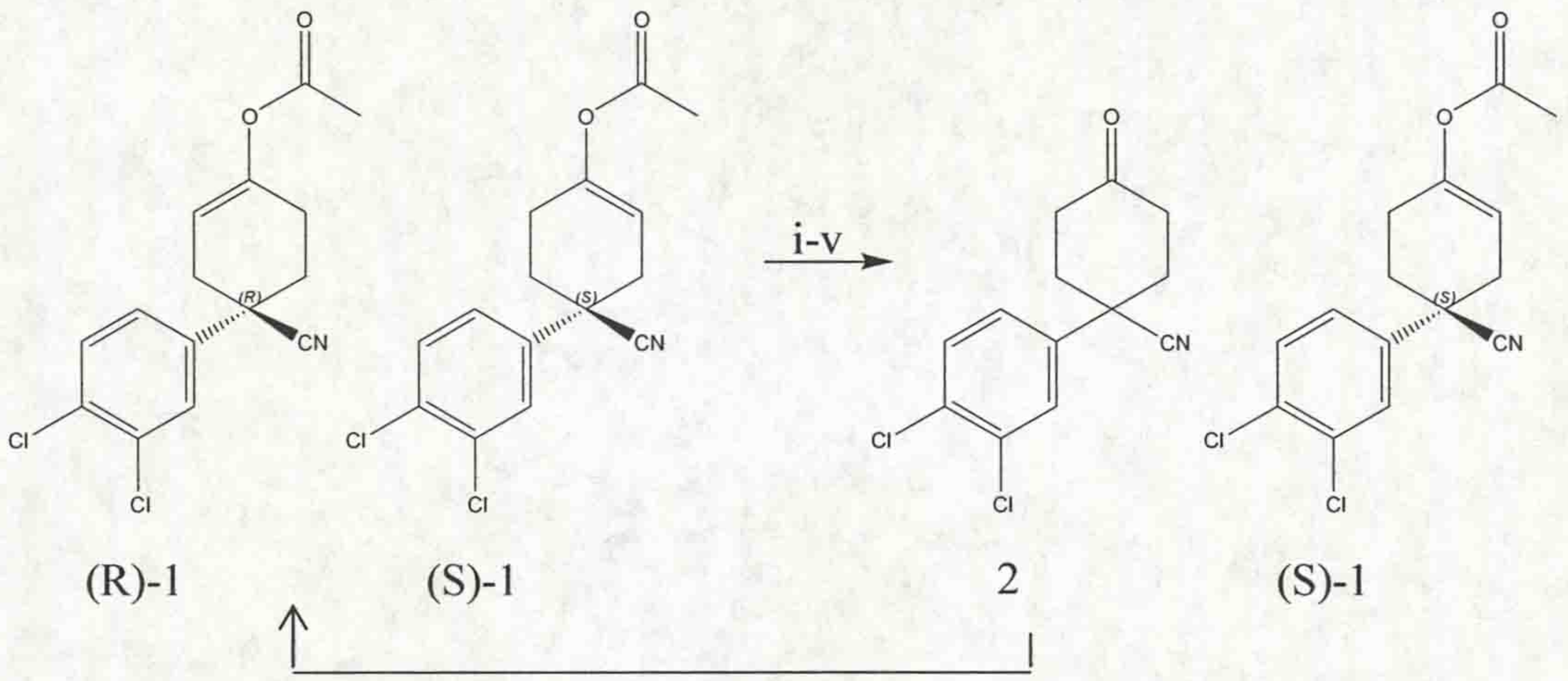
1.4 Aim of the project

A novel biotransformation has been developed for the synthesis of chiral enol acetates derived from prochiral ketones and has been successfully employed for the synthesis of chiral enol ester (1) that is used for the synthesis of tachykinin NK-2 antagonist (Allan *et al.*, 2001a; Carnell *et al.*, 1997; Carnell *et al.*, 2000) (Figure 1.9). In this method, the 4-Cyano-4-aryl substituted cyclohex-1-enyl acetate (1) is resolved with *P. fluorescens* lipase (PFL) by transesterification with n-butanol in THF. The approach gives good to excellent enantiomeric purities (e.e.'s) for the chiral enol acetate (Allan *et al.*, 2001a; Carnell *et al.*, 1997; Carnell *et al.*, 2000). The success of this method comes from the ability to recycle the product prochiral ketone. When there is efficient recycling, the overall transformation (resolution plus ketone recycle) constitutes a desymmetrisation of the prochiral ketone or a deracemisation of the enol acetate. Eventually, even with a relatively low E value of 13, corresponding to the selectivity of the enzyme, it is possible, using this approach, to isolate enantiomerically pure enol acetate (1) in 82% yield after 4 cycles (Figure 1.9).

The aim of this project was to investigate whether a directed evolution strategy could be employed to alter the enantioselectivity of *P. fluorescens* lipase (PFL) to such an extent that the number of cycles needed for the deracemisation of enol ester (1) could be significantly reduced (Figure 1.9). In order to achieve this, it was also first necessary to develop appropriate screening protocols to enable the identification of mutant enzymes with improved enantioselectivity. Preliminary studies with the PFL were disappointing as it was not possible to express and recover a correctly folded, catalytically active version of the wild type PFL. This difficulty prohibited the use of

Figure 1.9: Deracemisation of enol acetate (1) by biotransformation using PFL AK20

i: *Pseudomonas fluorescens* lipase (PFL), nBuOH, THF
ii: Remove PFL
iii: Add isopropenyl acetate and ^{t}BuOK
iv: Add Dowex H^{+} resin
v: Remove resin and add PFL and nBuOH



PFL in a directed evolution strategy because it could have been very difficult to screen the large numbers of mutant enzymes necessary to successfully isolate an improved enzyme. Initial studies with a *P. fluorescens* esterase (EstF, PFE) indicated that it could substitute for the PFL in the deracemisation scheme, and in fact might be a better choice of enzyme. A screening strategy was developed to enable the high-throughput screening of EstF mutant libraries. Preliminary data with a limited number of mutant enzymes demonstrated that directed evolution of *estF* could be used to alter the enantioselectivity of the enzyme for use in the deracemisation of chiral enol esters.

Chapter 2

Materials and Methods

Chapter 2

Materials and Methods

2.1 General cell culture techniques

2.1.1 Bacteria characteristics

2.1.1.1 General media

LB and LA media were routinely used for cell culture (Sambrook *et al.*, 1989).

2.1.1.2 Bacteria strains

The following strains were used for the expression of the lipase.

Strains and culture conditions:

E. coli strains:

BLR(DE3)	F$^-$ *ompT hsd* $S_B(r_B\text{-}m_B\text{-})$*gal dcm* Δ*(srl-recA)306*::Tn *10*(tetR)(DE3) (Novagen)
DH5α	F- Φ80*lac*ZΔM15 Δ(lacZYA-argF), U169, *end* A1, *rec*A1, hsdR17 (r_k^-, m_k^+), *sup*E44, *thi*-1, *gyr*A96, *rel*A1, *pho*A (Invitrogen)
JM109	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, λ^-, Δ*(lac-proAB)*, [F', *tra*D36, *pro*A^+B^+, *laq*$I^qZ\Delta$ M15] (Promega)
TOP10	F$^-$, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L, *end*A1, *nup*G (Invitrogen)
XL1-Blue	*end*A1, *rec*A1, *gyr*A96, *thi-1, hsd*R17, *rel*A1, *sup*E44 *lac* [F′, *pro*AB, *lac*IqZΔM15, Tn*10* (Tetr)] (Stratagene)

XL10-Gold *end*A1, *rec*A1, *gyr*A96, *thi-1*, *rel*A1, *sup*E44, Tetr, Δ(*mcrA*)*183*, Δ(*mcrCB-hsdSMR-mrr*)*173*, *lac* Hte [F′ *proAB lacI*qZΔ*M15* Tn*10* (Tetr) Amy Camr] (Stratagene)

E. coli broth cultures were grown at 37°C in LB medium in a shaking incubator (220rpm). LA plates were incubated overnight at 37°C. Antibiotics were supplemented when needed.

<u>*P. fluorescens* strains:</u>

Migula 1895 (ATCC 13525, NCTC 10038)

C9 (Dieckelmann *et al.*, 1998) (GenBank AF031226)

Pseudomonas strains broth cultures were grown at 30°C in LB medium in a shaking incubator (220rpm). LA plates were incubated overnight at 30°C. Ampicillin was supplemented for the C9 strain.

2.1.1.3 Strain storage

Long-term storage of plasmid bearing and non-plasmid bearing bacterial strains was achieved by mixing one half volume of bacterial broth culture with one half volume of sterile glycerol. The mixtures were then instantaneously snap frozen in liquid nitrogen and stored at -80°C.

2.1.2 Antibiotic selection

When appropriate, bacterial growth media were supplemented with sterile antibiotic solutions as follows.

Ampicillin (amp) and carbenicillin (carb) were added to a final concentration of 100μg/ml from a stock of 100mg/ml. Tetracycline (tet) was added to a final concentration of 12.5μg/ml from a stock of 12.5mg/ml.

Ampicillin and carbenicillin stocks were made up in H_2O and stored at -20°C. Tetracycline stock was made up in 100% ethanol and stored at -20°C. Antibiotic stock solutions were filter sterilised with a filter syringe of 0.45μm pore size (Micropore) prior to first use. Antibiotics were added to molten LA after cooling to approximately 50°C or to LB.

2.1.3 Plasmid characteristics

All plasmids constructed in this project are referenced in Appendix I.

2.2 DNA manipulations

2.2.1 *P. fluorescens* genomic DNA extraction

P. fluorescens Migula 1895 strain was used to inoculate 10ml LB which was incubated overnight. Genomic DNA (gDNA) was extracted using QIAGEN's Genomic-tip 100/G, following the manufacturer's protocol for bacterial genomic DNA extractions. gDNA was finally dissolved in 1.5ml of 10mM Tris-Cl, pH8.8 buffer. The same method was used to purify genomic DNA from *P. fluorescens* C9 strain. Purified DNA was stored at -20°C

2.2.2 Plasmid DNA extractions

Plasmid DNA was purified from *E. coli* cultured in selective medium, using Qiagen's QIAprep Spin Miniprep Kit following the manufacturer's protocol.

2.2.3 Determination of DNA concentration and purity

The concentrations and purity of DNA solutions was determined by spectrophotometry using a Helios spectrophotometer (Unicam). Samples, diluted in distilled water, were placed in quartz cuvettes and OD readings taken at 260nm and 280nm against a blank containing only distilled water. DNA concentrations were calculated on the basis that 1 OD unit at 260nm=50µg/ml dsDNA or 33µg/ml ssDNA. Purity was determined on the basis that, for a pure DNA preparation, the OD_{260nm}:OD_{280nm} ratio is 1.8.

2.2.4 PCR protocol

The same protocol was used in all the PCR reactions of this project. All PCR primers used in this project are detailed in Appendix II. The final volume used for PCR was 50µl. Taq DNA polymerase enzyme (MBI or Invitrogen) was used for routine experiments and development of optimum PCR conditions, with the corresponding manufacturer's supplied reaction buffer (MBI or Invitrogen). Primers solutions were added to a final concentration of 0.5pmol/µl from a stock solution of 10pmol/µl. A mix of the four dNTP (Roche) was added to a final concentration of 0.2mM from a stock solution of 10mM. $MgCl_2$ was added to a final concentration of 1.5mM from a stock solution of 25mM or 50mM depending on the manufacturer. Taq polymerase was added to a final concentration of 0.05U/µl from a stock solution of 5U/µl. gDNA or other DNA templates were always added last to a final concentration which will be

specified for each experiment. Ultrapure water was added to a final volume of 50µl. Negative controls were performed by replacing the volume of DNA template by the same volume of ultrapure water. A typical PCR programme can be seen below, the annealing temperature will be specified for each primer set.

94°C for 4min → (94°C for 30s, x°C for 1min, 72°C for 1min) x 30 → 72°C for 10min → 4°C. Details are given if other programmes were used.

2.2.5 Agarose gel electrophoresis

PCR products or DNA fragments were routinely analysed on agarose gel (Sambrook *et al.*, 1989). The concentration of agarose gels prepared depended on the expected size of the DNA fragments but 0.8% and 1% were the only concentrations used. Agarose was melted in 1X TBE buffer (108g Tris base, 55g orthoboric acid, 9.3g EDTA in 1l H_2O), prepared from a 10X stock. Gels were immersed in 1X TBE during electrophoresis. Ethidium bromide solution (1µl from a 10mg/ml stock solution) was added to the molten gel to allow visualisation of the DNA under UV light.

Samples were loaded into the gel with either 4µl 6X Orange G loading buffer (0.25% w/v Orange G, 10mM EDTA, 15% w/v ficoll (type 400) in H_2O) or 6X DNA loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 15% w/v ficoll (type 400) in H_2O). Gels were run at 120V for 30-40min.

Three types of molecular weight markers were used: Gibco 1Kb Ladder (Gibco BRL), Generuler 1Kb DNA Ladder (MBI Fermentas) or 1Kb plus DNA Ladder (Invitrogen), prepared according to the manufacturer's protocols.

2.2.6 Cloning into pGEM-T Easy vectors

PCR amplified *lipA*, *lipAHis*, *lipANoHis* and *lipAThioHis* sequences (defined in Chapter 3 and Appendix I and II) were routinely cloned into pGEM-T Easy plasmid (Promega) (Appendix III), used according to the manufacturer's instructions. Ligation was achieved due to the 3'-deoxyadenosine nucleotides added to the ends of PCR products by most DNA polymerases. Ligation reactions were transformed into *E. coli* JM109, BLR(DE3), DH5α or TOP10 depending on the experiments and transformants were selected via resistance to antibiotics (Section 2.1.2). Those transformants containing the pGEM-T plasmid with inserted DNA were distinguished via blue/ white selection, due to disruption of the β-galactosidase gene (see manufacturer's instructions) (Section 2.2.7).

2.2.7 Transformation of *E. coli* cells

Competent *E. coli*, either strain JM109 or strain DH5α, was transformed by heat shock. LB (5ml) was inoculated with a single untransformed colony and was grown at 37°C in a shaking incubator (220rpm). LB (100ml) was inoculated with 0.5ml from the overnight culture and incubated as usual. The OD_{550nm} was measured every 30-45min until it reached 0.6 (mid-log phase). The cells were harvested by centrifugation for 10min at 3000rpm at 4°C in a Jouan MR1822 centrifuge.

Cell pellets were gently resuspended in a total of 7.5ml sterile ice cold TFB1 buffer (100mM RbCl, 45mM $MnCl_2$, 35mM KAc, 10mM $CaCl_2$, 0.5Mm LiCl, 15% glycerol, pH5.8 with acetic acid), cooled on ice for 10min and re-centrifuged as previously described. Cells were then gently resuspended in 4ml sterile ice cold TFB2 (10mM MOPS, 75mM $CaCl_2$, 10mM RbCl, 15% glycerol, pH6.5 with KOH),

incubated on ice for 15min and divided into 100µl aliquots. Competent cells were snap frozen in liquid nitrogen and stored at -80°C until use.

Transformation was performed using 10-100ng plasmid DNA (typically from 5 to 20µl of ligation product or plasmid solution). Competent cells (100µl) were thawed and placed on ice for 10min. DNA was gently mixed with the cells and the cells were kept on ice for 30min. Positive (with plasmid solution) and negative (without DNA) controls were made when necessary. Cells were heat shocked by placing them in a water bath at 42°C for 2min, followed by cooling on ice for 2min. LB (1ml) was added to the tube and cells were incubated at 37°C for 1h. Cells were then plated out onto LA (amp) plates containing IPTG (40µg/ml) and X-Gal (40µg/ml) (Sigma) (Sambrook *et al.*, 1989). The plates were incubated at 37°C for 16 to 20 hours. Blue/white selection was used to detect positive clones due to the disruption of the β-galactosidase gene. The positive clones were unable to cleave X-Gal and so unable to produce a blue colorant. White colonies were picked and grown in 3ml LB (amp) for 16 to 20 hours. Plasmids from positive clones were extracted as previously described (Section 2.2.2).

2.2.8 DNA sequencing

All sequencing used a Beckman Counter CEQ2000 sequencer and was performed by Miss Hayeit Tensaout (School of Biological Science, University of Liverpool). DNA sequences were analysed using the DNAStar suite of programmes (Lasergene).

2.2.9 Gel extraction and purification

PCR products were routinely analysed by electrophoresis and the desired bands excised from the gel with a sterilised scalpel. DNA was eluted by use of a QIAquick Gel Extraction Kit (QIAGEN) following the manufacturer's protocol.

2.2.10 Restriction digests and ligation

Restriction endonucleases and reaction buffers were supplied by MBI Fermentas or Invitrogen and used in accordance with the manufacturer's instructions. Double digests were performed using the buffer with the best efficiency for the 2 enzymes according to the manufacturer. Restriction digests were typically performed in a volume of 20µl and incubated 2-3h at 37°C. The concentration of enzymes and DNA will be indicated for each experiment. Reactions were terminated by incubation at 60°C for 20min.

Ligation of digested DNA fragments was catalysed by T4 DNA ligase (MBI Fermentas). 10:1 to 3:1 molar ratios of insert:vector were used in 10µl reactions, containing 5 Weiss units of ligase and the appropriate buffer. Ratio will be given for each ligation. Reactions were incubated 2h at room temperature or overnight at 16°C and terminated by heating to 60°C for 10min.

2.3 Expression and purification of P. fluorescens lipase

2.3.1 Expression of pETLipAHis and extraction of the recombinant lipase

2.3.1.1 Expression and extraction under denaturing conditions

In order to enable expression, pETLipAHis (Chapter 3) was transformed by heat shock into competent *E. coli* BLR(DE3) and transformants selected on LA (amp, tet). Antibiotic selection was performed as previously described (Section 2.1.2). Expression was first carried out on a small scale. A single clone was used to inoculate 10ml LB (amp, tet). These cells were cultured overnight. This culture (1ml) was used to seed 10ml LB (amp, tet) expression cultures which were incubated until mid-log phase (OD_{600}=0.6), after which expression was induced by the addition of sterile IPTG (Sigma) to a final concentration of 1mM. A non-induced control was provided by removing 1ml of the expression culture immediately prior to the addition of IPTG. Occasionally further samples were taken at regular intervals following induction to provide a time course for expression. The non-induced control was submitted to the exact same steps as the other samples. After induction, incubation was continued for 4-5h. Expression cultures were harvested by centrifugation for 5 min at 5000g, using a Jouan MR1822 centrifuge. The supernatant was discarded and the cell pellet stored at –20°C before lysis.

The cell pellet was resuspended in 1ml of Qiagen denaturing lysis buffer (8M urea, 100mM NaH_2PO_4, 10mM Tris-Cl, pH8.0) vortexed and transferred to a 1.5ml microcentrifuge tube. Cells were lysed by a freeze-thaw cycle of 3x(-80°C for 20min, 37°C for 20min) with vortexing after each thaw. Cellular debris was removed by centrifugation at 13000rpm for 30min in a microcentrifuge. The supernatant was

transferred to a fresh tube. All fractions collected from extraction were analysed by SDS-PAGE (Section 2.3.6).

2.3.1.2 Expression and extraction under native conditions

The same experiment was performed under native conditions with increased volumes of culture, 500ml of LB (amp, tet) were inoculated with 10ml of a transformed BLR(DE3) overnight culture. Induction by IPTG was performed as previously described and cells were harvested by centrifugation for 15min at 10000g at 4°C. The cell pellet was resuspended in 5ml Qiagen native lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, pH8.0) by vortexing. Lysozyme was added (1mg/ml final concentration) from a 100mg/ml stock. The cell solution was incubated on ice for 30min and 3 cycles of freeze-thaw were performed as previously described. Cellular debris and insoluble protein were then removed by centrifugation at 13000rpm for 30min at 4°C. The resulting supernatant was then analysed by SDS-PAGE (Section 2.3.6) and the remaining solution stored at -20°C.

2.3.2 Purification of the recombinant lipase from pETLipAHis

2.3.2.1 Under denaturing conditions

Purification of the recombinant lipase under denaturing conditions was performed using Qiagen's Ni-NTA spin columns and the lysates described above (Section 2.3.1.1), following the manufacturer's instructions, QIA*expressionist* "Protocol 14: Batch purification under denaturing conditions", using the denaturing wash buffer (8M urea, 100mM NaH_2PO_4, 10mM Tris-Cl, pH6.3) and denaturing elution buffers (8M urea, 100mM NaH_2PO_4, 10mM Tris-Cl, pH5.9; 8M urea, 100mM NaH_2PO_4,

10mM Tris-Cl, pH4.5). All fractions collected from the purification were analysed by SDS-PAGE (Section 2.3.6) and stored at -20°C.

2.3.2.2 Under native conditions

Purification of the recombinant lipase under native conditions was performed using Qiagen Ni-NTA spin columns and the lysates described above (Section 2.3.1.2), following the manufacturer's instructions, QIA*expressionist* "Protocol 11: Batch purification under native conditions" using the native wash buffer (50mM NaH_2PO_4, 300Mm NaCl, 20mM imidazole, pH8.0) and the native elution buffer (50mM NaH_2PO_4, 300Mm NaCl, 250mM imidazole, pH8.0). All fractions collected from the purification were analysed by SDS-PAGE (Section 2.3.6) and stored at -20°C.

2.3.3 Optimisation of expression and purification conditions

2.3.3.1 Induction conditions

Induction of pETLipAHis was performed using varying concentrations of IPTG, incubation times and growth temperatures. A single colony was used to inoculate 100ml LB (amp, tet) and this was incubated overnight. The overnight culture (20ml) was used to seed 800ml LB (amp, tet) expression cultures and incubated until mid-log phase (OD_{600}=0.6). These cultures were aliquoted into 5 bottles of 200ml of LB (amp, tet), and IPTG was added to final concentrations of 0.1mM, 0.25mM, 0.5mM, 0.75mM and 1mM. Aliquots (15x10ml) were taken for each IPTG concentration and grown at different incubation temperatures, 5 of each IPTG concentration at 20°C, 5 at 30°C and 5 at 37°C with shaking (220rpm) for all the samples. After 2h, one aliquot was removed from each temperature and each IPTG concentration. Cells were harvested by centrifugation for 15min at 1000g at 4°C, the supernatant discarded and

the pellet stored at –20°C prior to extraction. The same steps were performed after 3h, 4h, 5h and after an overnight incubation.

Cell pellets were then resuspended in 1ml of native lysis buffer (Section 2.3.1.2), vortexed and 3 cycles of freeze-thaw were performed as previously described. Lysates were centrifuged 30min at 13000rpm and the supernatants were transferred to a fresh tube and stored at -20°C. All the fractions collected were analysed by SDS-PAGE (Section 2.3.6).

All the fractions collected for the 5h incubation at 37°C were centrifuged for 30min at 13000rpm. Each pellet, collected from the centrifugation, was resuspended in 1ml of phosphate buffer pH7.8. All these samples were mixed altogether to give a solution of crude insoluble proteins which was freeze-dried for 48h (Section 2.5.2.2.1).

2.3.3.2 Test of solubility and new extraction protocols

A test of solubility was performed, to determine if the recombinant protein was present with the insoluble proteins or the soluble proteins during the extraction. During this experiment, two different methods of cell lysis were tested, sonication and cycles of freeze-thaw. Growth and induction were performed as previously described, the cell pellet was resuspended in 5ml of native lysis buffer (Section 2.3.1.2). Lysozyme was added to a final concentration of 1mg/ml and incubated 30min on ice. The solution (2x1.5ml) was transferred to a 1.5ml microcentrifuge tube. Three cycles of freeze-thaw were performed on one of the tubes as previously described. For the other, a SANYO sonicator was used 6x10s with 10s pause at a power of 5 microns. The two tubes were centrifuged for 30min at 13000rpm and the supernatants decanted from each tube and saved on ice (soluble proteins). The pellets

were resuspended in 1.5ml Native lysis buffer (insoluble proteins). Each fraction from the two methods, soluble and insoluble proteins was analysed by SDS-PAGE (Section 2.3.6).

The same solubility test was performed with different buffers: Tween 20-EGTA lysis buffer, 1% Triton X-100 and 2% Triton X-100 lysis buffers (Tween-EGTA: 50mM NaH_2PO_4, pH8.0, 300mM NaCl, 10mM imidazole, 0.25% Tween 20, 0.1mM EGTA; Triton X-100 1%: 50mM NaH_2PO_4, pH8.0, 300mM NaCl, 10mM imidazole, 1% Triton X-100; Triton X-100 2%: 50mM NaH_2PO_4, pH8.0, 300mM NaCl, 10mM imidazole, 2% Triton X-100). The fractions collected were SDS-PAGE analysed (Section 2.3.6).

2.3.3.3 Test of different concentration of imidazole buffers

A new purification under native conditions was performed to test a range of imidazole buffers for the elution step. The growth, induction and extraction steps were performed as previously described (Sections 2.3.1.2 and 2.3.2.2). The Ni-NTA columns were loaded with the lysates according to the manufacturer's instructions. The column was washed with the native wash lysis buffer as previously described (Section 2.3.6). The column was then washed with two additional wash buffers, first with 5ml of a 50mM imidazole buffer (50mM NaH_2PO_4, 300Mm NaCl, 50mM imidazole, pH8.0) and then with 2ml of a 100mM imidazole buffer (50mM NaH_2PO_4, 300Mm NaCl, 100mM imidazole, pH8.0). Wash fractions were collected for future analysis. The protein was eluted by adding 500µl of each of the following buffers: 200mM imidazole, 300mM imidazole, 400mM imidazole, 500mM imidazole, 600mM imidazole, 700mM imidazole, 800mM imidazole, 900mM imidazole,

1000mM imidazole (50mM NaH_2PO_4, 300Mm NaCl, 200mM imidazole, pH8.0, same composition except for imidazole concentration for 300mM, 400mM, 500mM, 600mM, 700mM, 800mM, 900mM, 1M) and finally two 500µl volumes of 50mM EDTA. The fractions collected during the elution steps were analysed with the fractions collected during extraction, and wash steps by SDS-PAGE (Section 2.3.6).

2.3.4 Purification under denaturing conditions and renaturation

An attempt to renature the recombinant lipase on the affinity column was done (Lemercier *et al.*, 2003), using the optimised conditions of growth at 30°C and 5h induction. The samples were centrifuged for 30min at 13000rpm and the supernatant discarded. Binding buffer (6M urea, 0.5M NaCl, 20mM Tris-Cl, 1% Triton X-100, pH7.9) (1ml) was added in all tubes, the tubes were mixed together and centrifuged for 30min at 13000rpm. The supernatant was collected, mixed with 1ml of Ni-NTA resin (QIAGEN) for 1h and the pellet was resuspended in 1ml of the same buffer (insoluble extract). The resin was applied on the column and the flow-through collected. 10x1ml of Refolding buffer (4M urea, 0.5M NaCl, 20mM Tris-Cl, 1% Triton X-100, pH7.9) was applied through the column and collected. The same steps were performed with two others buffers with decreasing concentrations of urea (2M urea, 0.5M NaCl, 20mM Tris-Cl, 1% Triton X-100, pH7.9; 0M urea, 0.5M NaCl, 20mM Tris-Cl, 1% Triton X-100, pH7.9). Elution buffer (6x2ml) (1M imidazole, 0.5M NaCl, 20mM Tris-Cl, 1% Triton X-100, pH7.9) was then applied. All the fractions collected from the purification were analysed by SDS-PAGE (Section 2.3.6).

2.3.5 Dialysis

Elution samples from the purification under denaturing conditions followed by renaturation (described above) were mixed (6ml) and dialysed against 1l of 10mM Tris-Cl, pH7.5 buffer (Kojima *et al.*, 1994) for 3h at room temperature. This step was repeated in the same buffer overnight. A sample of dialysed protein was analysed by SDS-PAGE (Section 2.3.6). The same experiment was then performed at 4°C.(Sambrook *et al.*, 1989)

2.3.6 SDS-PAGE of protein samples

SDS-PAGE was performed using 40µl samples taken from the various stages of induction, extraction and purification. SDS-PAGE 5x sample buffer (5µl) (225mM Tris-HCl (pH6.8), 50% glycerol, 5% SDS, 250mM DTT, 0.05% bromophenol blue) was added to each sample and mixed in. The proteins were denatured by heating at 95°C for 5min, and the samples were then cooled to room temperature.

SDS-PAGE was performed with a mini-protean II electrophoresis kit (Bio-Rad) (Sambrook *et al.*, 1989). A 12% resolving gel (5ml), and a 5% stacking gel (2ml) were prepared as described. Electrophoresis was performed in TGS buffer (25mM Tris-HCl (pH8.3), 192mM glycine, 0.1% w/v SDS) for 1.5h at 120V, until the sample dye front had exited the bottom of the gel.

Gels were then rinsed in distilled water and stained overnight, using a protein staining solution (90ml methanol:H_2O (1:1 v/v), 10ml glacial acetic acid, 0.25g Coomassie brilliant blue R-250 (Sigma)), (Sambrook *et al.*, 1989). The gels were then destained for 3h, in a solution that was identical to the staining solution except for the

commission of Coomassie brilliant blue. Two different molecular weight marker sets were used in SDS-PAGE analysis: Dalton Mark VII-L marker (Sigma) and ProtoMetrics Recombinant Protein Markers (National Diagnostics). Gels were dried using a BioRad gel dryer Model S83, following the manufacturer's protocol.

2.3.7 Expression and purification of *P. fluorescens* lipase with pThioHis plasmid

2.3.7.1 Expression of pThioHisLipA and optimisation

A second expression and purification strategy was also employed following the protocol 'Pilot Expression' in the Instruction Manual of the His-Patch ThioFusion Expression System (Invitrogen) utilising the pThioHisLipA construct (Chapter 3, Appendix I). All the fractions collected were analysed by SDS-PAGE.

This system was then optimised for growth conditions and cell lysis, following the protocol as previously described, except that the final IPTG concentration was 0.5mM and the induction temperature was 30°C. The buffer used was identical to the one described in the instruction manual except 250mM NaCl was added to minimise the interactions of the recombinant protein with the bacterial cell membranes. All the fractions collected during these tests were analysed by SDS-PAGE.

2.3.7.2. Purification of the recombinant lipase from pThioHisLipA by osmotic shock

2.3.7.2.1 Small scale osmotic shock purification

Purification following the protocol 'Purification by Osmotic Shock' in the Instruction Manual of the His-Patch ThioFusion Expression System (Invitrogen) was performed. An overnight culture of transformed *E. coli* TOP10 (Invitrogen) (0.5ml of a 2ml

culture) was used to inoculate 10ml of LB (amp). Induction by IPTG was performed at 37°C with a final IPTG concentration of 1mM following the manual instructions. OD_{550nm} were measured for the sample before and after induction. These measures allowed the calculation of the suitable volumes of osmotic shock buffers, required to perform purification by osmotic shock and as described in the manufacturer's protocol. Samples collected during this experiment were SDS-PAGE analysed.

2.3.7.2.2 Scale-up of osmotic shock purification

From a 40ml overnight culture of transformed *E. coli* TOP10, 30ml were added to 500ml of LB (amp), protocol was performed following the manufacturer's protocols and OD_{550nm} were measured for the sample before and after induction as previously described, in order to proceed to purification by osmotic shock. Samples collected were SDS-PAGE analysed.

2.3.8 Refolding of the lipase from pETLipAHis with glycerol

2.3.8.1 Expression and purification of the recombinant lipase

Competent TOP 10 *E. coli* cells were transformed with pETLipAHis (Appendix I) and plated as previously described. A colony was picked to inoculate 10ml of LB (amp). The culture was allowed to grow overnight and 200ml of LB (amp) were inoculated with that overnight culture and cultured for 2h.

Induction with IPTG (1mM final concentration) was performed as previously described. After a 4h30 induction time, cells were harvested at 3000g for 20min and the pellet stored at -20°C.

The cell pellet was lysed in 12ml lysis buffer under denaturing conditions as previously described (Section 2.3.1.1). Purification was performed under native conditions as previously described (Section 2.3.2.2). Elution was performed with 4x1.5ml of 250mM imidazole elution buffer (Section 2.3.2.2) and 4x1.5ml of 500mM imidazole elution buffer (Section 2.3.3.3). Samples were collected and analysed by SDS-PAGE.

2.3.8.2 Refolding of denatured protein with glycerol

The first three fractions of the previous elution (250mM imidazole buffer) (Section 2.3.8.1) were diluted fifty times (150ml) in a glycerol refolding buffer (33mM phosphate buffer, pH7.8, 40% v/v glycerol, 0.1mg/ml bovine albumin) (Frenken *et al.*, 1993b) and incubated for 2h at room temperature.

2.4 Expression and purification of P. fluorescens esterase

2.4.1 Expression and purification of the wild-type esterase

pJOE2792 (Appendix I) was transformed by heat shock into competent *E. coli* JM109 or *E. coli* DH5α cells and the transformants were selected for on LA (amp). Expression of PFE was performed following the protocol provided by Pr U.W Bornscheuer who provided the pJOE2792 plasmid (Krebsfanger *et al.*, 1998). A single colony was inoculated into 3ml LB (amp) and grown overnight at 37°C, 140rpm. This culture (1ml) was used to seed 250ml LB (amp) expression culture and this culture was incubated at 37°C, 140rpm, until OD_{600}=0.5-0.6, upon which expression of PFE was induced by addition of sterile 20% w/v L-rhamnose (Sigma)

to a final concentration of 0.2% w/v. As a negative control, 1ml of culture was removed immediately prior to L-rhamnose induction.

After induction, the culture was incubated overnight at 37°C, 140rpm. Expression cultures were harvested by centrifugation for 15 min at 4500rpm and 4°C, in a Jouan MR1822 centrifuge. The supernatant was discarded, the cells washed with 20ml of ice-cold sodium phosphate buffer (50mM, pH7.5), and the solution was centrifuged again as previously described. The washing step was repeated. The cells were then resuspended in 10ml ice-cold sodium phosphate buffer as previously described and disrupted on ice with a SANYO sonicator using 50% power and 5 cycles of 1min sonication pulse and 1min rest. Alternatively, cells were lysed by freeze-thaw cycles of 3x(-80°C for 20min, 37°C for 20min) with vortexing after each thaw. After another centrifugation step, the supernatant was carefully removed and stored at -20°C. All samples were analysed by SDS-PAGE as previously described. Esterase activity was measured spectrophotometrically in sodium phosphate buffer (50mM, pH7.5) using *p*-nitrophenyl acetate (10mM in DMSO) as substrate and the amount of *p*-nitrophenol was determined at 410nm and 25°C. One unit (U) of activity was defined as the amount of enzyme releasing 1μmol *p*-nitrophenol per min under assay conditions (Krebsfanger *et al.*, 1998).

2.4.2 Microscale expression (microtitre plate)

To allow the screening of mutants in microtitre plates (MTP), a microscale expression protocol was developed first with the wild-type to determine the conditions needed, following, in some part, work previously published (Horsman *et al.*, 2003). The pJOE2792 plasmid was used to transform by heat shock competent *E. coli* DH5α

cells and transformants were selected for on LA (amp) as previously described. A single colony was inoculated into 1ml LB (amp) in deep-well MTP (Eppendorf) and grown overnight at 37°C, 325rpm. This culture (20µl) was used to seed 1ml LB (amp) expression cultures and incubated at 37°C, 325rpm for 3h, upon which expression was induced by addition of 50µl of sterile 4% w/v L-rhamnose (Sigma) to a final concentration of 0.2% w/v.

After the addition of L-rhamnose, the cultures were incubated for at least 5h or overnight at 37°C, 325rpm. The expression cultures were harvested by centrifugation for 10min at 2700g and 4°C, in a Sorvall Legend RT centrifuge (Sorvall). The supernatant was discarded, the cell pellets were resuspended in 400µl sodium phosphate buffer (50mM, pH7.5) and lysed by freeze-thaw cycles of 3x(-80°C for 20min, 37°C for 20min) with vortexing after each thaw. The MTP was centrifuged as previously described. The supernatants were carefully transferred to another MTP and stored at -20°C prior to use in the biotransformations.

2.5 Biotransformations

2.5.1 Original enol ester resolution

To a solution of 4-cyano-4-phenylcyclohex-1-eny-acetate (1) (5g, 16mmol), lipase from *P. fluorescens* (Amano AK20, 4g (20,000u/g)) and n-butanol (2.6cm^3, 32.35mmol) in THF (60cm^3) was tired at room temperature for 6h whereupon HPLC (Section 2.5.2.5) indicated 100% e.e. for the enol acetate. The solution was filtered through a glass sinter funnel, the residual enzyme washed with THF and the solvent removed *in vacuo*. The crude residue was purified by flash column chromatography

on silica (petroleum ether:diethyl ether ; 2:1) to give the ketone (3.7g) and the (S)-enol acetate (+)-1 (150mg, 3%) as a white solid (HRMS: found 309.03227. $C_{15}H_{13}Cl_2O_2N$ requires 309.03235). m.p. 148-150°C. δ_H (300MHz, $CDCl_3$): 2.23 (3H, s, $COCH_3$), 2.23-2.67 (6H, m, 2xH-3, 2xH-5 and 2xH-6), 5.46 (1H, s, H-2), 7.22-7.56 (3H,Ar) (Allan *et al.*, 2000; Allan *et al.*, 2001a; Carnell *et al.*, 2000).

2.5.2 Biotransformation using the recombinant lipase

2.5.2.1 Recombinant lipase purified under native conditions

The supernatant (crude proteins), collected after extraction under native conditions (Section 2.3.1.1), was tested with the substrate of interest, racemic 4-cyano-4(3, 4-dichlorophenyl) cyclohex-1-enylacetate (1). Two biotransformations were performed, one with enol acetate (1) (5mg), phosphate buffer (pH7.8) (200µl) and crude proteins (100µl), the final volume of his reaction is 300µl; the second with enol acetate (1) (5mg), phosphate buffer (pH7.8) (2ml) and crude proteins (100µl), the final volume of this reaction is 2.1ml. A negative control comprising enol acetate (1) (5mg) in phosphate buffer (ph 7.8) (2ml) and a positive control comprising enol acetate (1) (5mg) in phosphate buffer (ph 7.8) (2ml) with AK20 Lipase (Amano) (1.6mg) were run at the same time (2.1ml final volume). The solutions were gently stirred at room temperature, 27h for the positive control and 70h for the other reactions.

The reactions were monitored by TLC (thin layer chromatography) (1:3, ethylacetate: petroleum ether) after 3h, 27h and 70h. After 70h, the reaction mixtures involving the recombinant lipase were centrifuged for 4min at 13000rpm to remove solid particles. Ethylacetate (2x1ml) was used to extract the products from the 2.1ml reaction mix. Ethylacetate (2x300µl) was used to extract the products from the 300µl reaction mix.

Extracts were dried ($MgSO_4$) and filtered. Solvent was removed by evaporation and propan-2-ol (1ml) was added for HPLC analysis (Section 2.5.2.5).

2.5.2.2 Analysis of freeze-dried recombinant lipase from insoluble fractions

2.5.2.2.1 Freeze-drying of the lipase

Previous insoluble fractions from optimisation of induction (Section 2.3.3.1) for incubation performed at 37°C were mixed. The solution was centrifuged and the supernatant discarded. The pellet was resuspended into 2ml of phosphate buffer (pH7.8) and the crude proteins freeze-dried using a freeze-drier (Heto FDI.0).

2.5.2.2.2 Transesterification

Transesterification was performed with the freeze-dried enzyme in THF, toluene or phosphate buffer (pH7.8). Enol acetate (1) (20mg) was mixed with butanol (11.72µl, 2eq) and the previously prepared freeze-dried enzyme (7mg) in THF (2ml), toluene or phosphate buffer pH7.8. The reaction was gently stirred at room temperature for 48h and analysed by TLC as previously described. Negative controls without enzymes were run at the same time.

2.5.2.2.3 Hydrolysis

Hydrolysis was performed with the freeze-dried enzyme in THF or phosphate buffer (pH7.8). Enol acetate (1) (20mg) was mixed with freeze-dried enzyme (7mg) in THF or phosphate buffer (pH7.8) (5ml). Another reaction was performed mixing enol acetate (1) (5mg) with freeze-dried enzyme (15mg) and THF or phosphate buffer (pH7.8) (5ml). Reactions were gently stirred at room temperature for 52h and

analysed by TLC as previously described. Negative controls without enzymes were run at the same time.

2.5.2.3 Use of osmotic shock purified recombinant protein

Hydrolysis was performed with the recombinant enzyme purified by osmotic shock (Section 2.3.7.2.2) in phosphate buffer (pH7.8). Enol acetate (1) (5mg) was mixed with 100µl of the 90ml solution containing the purified protein and phosphate buffer (pH7.8) (2ml). The reaction was gently stirred at room temperature for 70h and TLC analysed regularly as previously described. A negative control with the osmotic shock buffer was run at the same time.

2.5.2.4 Use of glycerol refolded recombinant protein

Enol acetate (1) (20mg) was added to refolding solution (10ml) (Section 2.3.8.2) containing the recombinant lipase (or only the buffer for control), with or without butanol (11.72µl, 2eq), using THF or toluene (10ml) (Table 2.1). The reaction mixture was stirred gently at room temperature for 6 days and TLC analysed as previously described. All the experiments are summarised in the Table 2.1. The mixture was then filtered through a celite pad and extracted with diethyl ether (2x10ml). The organic phase was dried over $MgSO_4$ and the solvent was removed *in vacuo* prior to analysis by HPLC (Section 2.5.2.5).

	Buffer		**THF**		**Toluene**				
Enol acetate	20mg		20mg		20mg				
Refolding buffer	10ml		10ml		10ml				
Solvent			10ml		10ml				
Butanol	_	2eq	_	2eq	2eq	_	2eq	2eq	_	2eq
Enzyme	_	_	Yes	Yes	_	Yes	Yes	_	Yes	Yes

Table 2.1: Biotransformations using the glycerol refolded recombinant lipase

2.5.2.5 Analysis of biotransformation reactions

Biotransformations were analysed by HPLC, using a Waters 2690 Separation Module. The final samples of the biotransformations were resuspended in propan-2-ol and analysed by HPLC to determine conversion, yield and enantiomeric excess of the enol acetate (1) and the resulting ketone (2). The HPLC column used was a Chiralpack AD column (Allan *et al.*, 2001b). The mobile phase used was 100% ethanol at a constant flow rate of $0.35cm^3.min^{-1}$. The product mixture was monitored at λ_{obs}=220nm using a Waters 996 photodiode array detector. The retention times of the (*R*)- and (S)-enantiomers of enol ester (1) were 29.2min and 35.5min respectively and 30.5min for the ketone (2).

2.5.3 Biotransformation using the wild-type esterase

2.5.3.1 Freeze-dried PFE for transesterification and hydrolysis

Freeze-dried crude protein containing the PFE was kindly supplied by Pr U.W. Bornscheuer. Biotransformations were performed using protocol previously

published (Krebsfanger *et al.*, 1998) and using the substrate of interest (4-cyano-4(3, 4-dichlorophenyl) cyclohex-1-enylacetate) (1).

Transesterification was performed with the freeze-dried enzyme toluene dried on molecular sieves. Enol acetate (1) (20mg) was mixed with benzyl alcohol (13.2µl, 2eq), freeze-dried PFE (6.4mg) and toluene (385µl). The reaction was stirred at 200rpm at 37°C for 3 days and TLC analysed as previously described. Negative controls without enzymes were run at the same time. Every day, a sample was removed from the reaction and the mixture was then filtered through cotton wool in a Pasteur pipette and washed with ethyl acetate. The organic phase was dried over $MgSO_4$ and the solvent was removed *in vacuo* prior to analysis by HPLC (Section 2.5.2.5). The same experiment was performed with enol acetate (1) (156mg), benzyl alcohol (103.5µl, 2eq) and freeze-dried PFE (50mg) in toluene (3ml). The reaction was stirred at 200rpm at 37°C for 3 days following steps as previously described.

Hydrolysis was performed with the freeze-dried enzyme in sodium phosphate buffer (50mM, pH7.5). Enol acetate (1) (20mg) was mixed with freeze-dried enzyme (6.4mg) and sodium phosphate buffer (pH7.5) (385µl). The reaction was stirred at 200rpm at 37°C for 3 days and TLC analysed as previously described. Negative controls without enzymes were run at the same time. Every day, a sample was removed from the reaction and the mixture was then filtered through cotton wool in Pasteur pipette, washed with ethyl acetate. The organic phase was extracted in ethyl acetate dried over $MgSO_4$ and the solvent was removed *in vacuo* prior to analysis by HPLC (Section 2.5.2.5).

2.5.3.2 Use of induced whole cell extract for transesterification and hydrolysis

2.5.3.2.1 Preparation of whole cell solution

Cell culture and induction were performed in 125ml LB (amp) as previously described. Cells were washed with ice-cold sodium phosphate buffer twice as previously described and centrifuged but no lysis was performed as whole cells were wanted. The final pellet was then resuspended in 5ml of toluene or phosphate buffer depending on the reaction.

2.5.3.2.2 Transesterification and hydrolysis

For the transesterification, enol acetate (1) (78mg) was mixed with benzyl alcohol (51.5µl, 2eq), whole cells expressing PFE in toluene (1.5ml) from the 5ml preparation (Section 2.5.3.2.1) and toluene (1ml). The reaction was stirred at 150rpm at 37°C for 3 days and analysed by TLC as previously described. Negative controls without enzymes were run at the same time and analysed as previously described.

For the hydrolysis, enol acetate (1) (78mg) was mixed with whole cells expressing PFE in sodium phosphate buffer (pH7.5) (1.5ml) from the 5ml preparation (Section 2.5.3.2.1) and toluene (1ml). The reaction was stirred at 150rpm at 37°C for 3 days and analysed by TLC as previously described. Negative controls without enzymes were run at the same time and analysed as previously described.

2.5.3.3 Hydrolysis with crude protein

2.5.3.3.1 Range of substrate concentrations

The supernatant (crude proteins) collected from previous expression (Section 2.4.1.1), was tested with enol acetate (1). The biotransformations were performed, with 312µg

of enol acetate (1) (1mM final concentration), 624µg (2mM), 1.56mg (5mM) or 3.12mg (10mM), 1ml (95U) of crude protein in sodium phosphate buffer (pH7.5, 50mM). The solutions were stirred at 200rpm at 37°C for 3 days. Negative controls without enzymes were run at the same time and analysed as previously described.

2.5.3.3.2 Dimethylformamide (DMF) as a co-solvent

The biotransformations were performed, with 1.56mg of enol acetate (1) (5mM final concentration), 950µl (90U) of esterase (crude protein) in sodium phosphate buffer (pH7.5, 50mM) and 10µl (1% final concentration), 20µl (2%), 30µl (3%), 40µl (4%) or 50µl (5%) of DMF and the necessary amount of sodium phosphate buffer to reach a final volume of 1ml. The solutions were stirred at 200rpm at 37°C for 3 days. Negative controls without enzymes were run at the same time and analysed as previously described.

2.5.3.3.3 Dimethyl sulfoxide (DMSO) as a co-solvent

To allow a maximal efficiency and reproducibility of the biotransformations, the exact same amount of substrate needed to be used in each experiment. To determine the saturation concentration of the enol acetate (1) in DMSO, tests were performed. Solutions of substrate in DMSO were made from 0.1 to 0.5mM. Solutions were heated at 50°C and solutions showing the best dissolution of the substrate were chosen. The experiments were repeated until the limit concentration was found.

Hydrolysis reactions were performed first with 0.2M enol acetate (1) in DMSO. The biotransformations were performed in deep-well microtitre plate. Five identical reactions were performed in parallel. This solution (50µl) (5% DMSO final

concentration, 10mM substrate final concentration) were added to 200µl (19U) of esterase (crude protein) in sodium phosphate buffer (pH7.5, 50mM) and the necessary amount of sodium phosphate buffer to reach a final volume of 1ml. The solutions were stirred at 300rpm at 37°C for 4 days. Negative controls without enzymes but with DMSO were run at the same time and analysed as previously described.

The same reactions were performed then with 0.2M enol acetate (1) in DMSO so the final concentration of substrate was proportional to the DMSO concentration in the reaction mix. DMSO concentrations were tested 7.5, 10, 15, 20 and 30%, corresponding respectively to 15mM, 20mM, 30mM, 40mM and 60mM substrate final concentration, with esterase (crude proteins) (200µl, 19U) in sodium phosphate buffer (pH7.5, 50mM) and the necessary amount of sodium phosphate buffer to reach a final volume of 1ml were added to the DMSO solution. The solutions were stirred at 300rpm at 37°C for 5 days. Negative controls as previously described and analysed as previously described.

DMSO concentrations of 22.5, 25 and 27.5% were tested in the same conditions as previously described but the reactions were incubated at 37°C and 300rpm for 4 days. Controls and analysis were performed as previously described.

2.5.3.3.4 Time course of esterase reaction using DMSO as a co-solvent

Hydrolysis reactions were performed as previously described with 0.2M enol acetate (1) in DMSO. DMSO concentrations of 10, 20 and 30% were tested (20mM, 40mM and 60mM substrate final concentration) with esterase (crude proteins) (200µl, 19U) in sodium phosphate buffer (pH7.5, 50mM) and the necessary amount of sodium

phosphate buffer to reach a final volume of 1ml were added to the DMSO solution. The solutions were stirred at 300rpm at 37°C for 4 days. Samples were taken every day for each concentration. Negative controls and analysis were performed as previously described.

2.5.3.4 Comparison between AK20 PFL and PFE

Transesterification was performed with the freeze-dried AK20 PFL in the same conditions as previously described (Section 2.5.3.1). Enol acetate (1) (20mg) was mixed with benzyl alcohol (13.2µl, 2eq), freeze-dried AK20 PFL (6.4mg) and toluene (385µl). The reaction was stirred at 200rpm at 37°C for 3 days and analysed by TLC as previously described. Negative controls without enzymes were run at the same time. Every day, a sample was removed from the reaction, analysis as previously described. The same experiment was performed with enol acetate (1) (156mg), benzyl alcohol (103.5µl, 2eq) and freeze-dried AK20 PFL (50mg) in toluene (3ml). The reaction was stirred at 200rpm at 37°C for 3 days following steps as previously described.

2.6 Development of screening methods

2.6.1 Plate assay screenings using pH indicator

2.6.1.1 Enol acetate and acetic acid

To allow a quick and easy screen, attempts were made to screen mutants on agar plate. pH indicator, neutral red, was used to pick positive mutants possessing an esterase activity by a change of colour due to the relase of acetic acid during the biotransformation.

Cells were grown on LA (amp) plates using a protocol previously published (Bornscheuer *et al.*, 1999). pH indicator was added to minimal media agar plates and LA agar plates supplemented with ampicillin as previously described. Neutral red and crystal violet (Sigma) were made up in H_2O at 5g/l for neutral red and 1g/l for crystal violet. Indicators stock solutions were filtered sterilised with a filter syringe of 0.45µm pore size (Micropore) and stored at -20°C. To 200ml of molten minimal media (amp) or molten (LA) were added 2ml of sterile rhamnose solution (20% w/v), 200µl of sterile crystal violet (1g/l) and 400µl of sterile neutral red (5g/l). The molten agar was then poured into Petri dishes and stored at 4°C for future use.

A 1mM enol acetate solution (1) in ether (100µl) was spread on minimal media agar plates as described above. The solvent was allowed to evaporate by leaving the plates at 37°C for 2h prior the spread of 100µl of a 5ml overnight culture of *E. coli* JM109 transformed with pJOE2792. Plates were then incubated for 7 days at 37°C.

A new transformation was performed with competent *E. coli* DH5α and JM109 and pJOE2792 as previously described. A 10mM enol acetate (1) solution in ether (100µl and 200µl) was spread on minimal agar or LA plates supplemented as previously described. Transformants (50µl) were directly spread on those plates and incubated for 4 days at 37°C. Negative controls were performed on identical plates by spreading DH5α and JM109 cells transformed with pETLipAHis, which does not possess esterase or lipase activity in those conditions.

The same experiments were performed by replacing the substrate by 10mM acetic acid. The acid (100µl and 200µl) was spread on 2 minimal media agar plates

supplemented as described above and only minimal agar plates. A 5ml overnight culture of *E. coli* JM109 transformed with pJOE2792 (50µl) was spread and incubated for 7 days.

2.6.1.2 Tributyrin

Tributyrin is a specific substrate for the *P. fluorescens* esterase (Choi *et al.*, 1990). Tributyrin (10mM, Sigma) (100µl and 200µl) was spread on 2 minimal media agar plates supplemented as previously described and 2 LA agar plates supplemented as previously described. A 5ml overnight culture of *E. coli* JM109 transformed with pJOE2792 (100µl) was spread on them and incubated for 7 days.

Tributyrin (100%) (100µl and 200µl) was spread on 2 LA agar plates supplemented as previously described. A 5ml overnight culture of *E. coli* JM109 transformed with pJOE2792 (100µl) was spread on them and incubated for 7 days at 37°C.

Tests were performed by pouring directly tributyrin in the molten minimal media or LA agar supplemented as previously described for a final concentration of 1% v/v (Kugimiya *et al.*, 1986; Pelletier & Altenbuchner, 1995). Plates without pH indicators were also poured in the same conditions. A 5ml overnight culture of *E. coli* JM109 transformed with pJOE2792 (100µl) was spread on them and incubated for 6 days.

A new transformation was performed with competent *E. coli* DH5α and pJOE2792 as previously described. Transformants (50µl and 100µl) were directly spread on minimal agar plates supplemented with rhamnose, antiobiotics, tributyrin and with or without pH indicators and on LA agar plates supplemented with rhamnose,

antiobiotics, tributyrin and with or without pH indicators. The plates were incubated for 2 days for LA plates and 4 days for minimal media plates at 37°C. Negative controls were performed on identical plates by spreading DH5α cells transformed with pETLipAHis. The same experiments were repeated with JM109 cells.

2.6.1.3 Enol acetate in DMSO

In order to supply the substrate into the agar media, 1%, 2.5%, 5%, 10% and 15% of 0.2M enol acetate in DMSO were added to pH indicators, rhamnose and antiobiotics during the preparation of LA agar plates. On those plates, 100µl of DH5α transformed with pJOE2792 were spread. Plates were incubated for 2 days. They were then subjected to 2 cycles of freeze-thaw (-20°C, 37°C) to release induced esterase and incubated 3 more days.

Transformed DH5α cells were also cultivated on LA agar plates supplemented as previously described for 2 days. Three cycles of freeze-thaw were performed as previously described. A filter paper (Whatman) of the size of the Petri dish, soaked into a 0.2M enol acetate (1) in DMSO was applied onto the lysed cells and the plate was incubated 24h. The same experiment was performed by leaving the filter paper 1h and then removing it with a further 24h incubation. Controls were made with cells transformed with pETLipAHis.

2.6.2 Screening using dehydrogenase enzymes

To analyse the activity of the mutant enzymes, a secondary screen that detects the ketone produced during the reactions was performed. Yeast Alcohol Dehydrogenase

(YADH) and Horse Liver Alcohol Dehydrogenase (HLADH) (Sigma) were used following work previously published (Andersson *et al.*, 1998; Yang & Brush, 1993).

Ketone (2) (200μmol) was mixed with YADH (0.8mg), phosphate buffer (pH7.5, 50mM) (200μl), 1μmol of NADH into 2ml of heptane or toluene. The reaction was performed at room temperature and 180rpm. A control was performed without enzyme. After 3h and 24h, 1ml of the reaction mix was taken and centrifuged. The OD_{340nm} of the supernatant was determined with a Helios spectrophotometer (Unicam) and the sample added back to the reaction mix. After 24h, the samples (including controls) were analysed by HPLC, as previously described.

Ketone (2) (12.5μmol) was mixed with a 0.1g/l YADH or HLADH solution in phosphate buffer (pH7.5, 50mM) (250μl), a 30mM NADH solution in phosphate buffer (pH7.5, 50mM) (500μl) and the same phosphate buffer containing 1%, 2% or 5% DMF for a final volume of 2.5ml. The reaction was performed at 30°C and 180rpm. Controls were performed without enzyme. After 1h and 24h, 1ml of the reaction mix was taken and centrifuged. The measure of OD_{340nm} of the supernatant was performed and the sample added back to the reaction mix.

2.6.3 Secondary screen using colourimetric assay

2.6.3.1 Use of sodium nitroprusside

In order to have a colourimetric assay for the detection of the ketone, sodium nitroprusside (Sigma) was used according to previously used protocols (McLennan, 2004). The experiment was performed with of ketone (2) solution (0.1mM, 0.5mM, 1mM, 1.5mM, 2mM, 2.5mM, 3mM, 3.5mM, 4mM, 4.5mM and 5mM) in phosphate

buffer (pH7.5, 50mM). In order to have at least an equimolar reaction, a solution of 0.05% w/v of sodium nitroprusside in 0.2M glycine-NaOH buffer (pH10) was prepared. Sodium nitroprusside solution (150µl) was added to each ketone concentration (50µl) and incubated at room temperature for 10min fand then for 24h. A negative control with only buffer was performed at the same time, as well as a positive one with 5mM of acetone.

The same experiment was performed with a 3% w/v sodium nitroprusside solution under the same conditions, using each ketone solution (200µl) and the 3% w/v sodium nitroprusside solution (200µl) at room temperature for 24h. A 5mM solution of cyclohexanone (Sigma) in phosphate buffer (pH7.5, 50mM) was prepared. This solution (50µl) was mixed with 3% w/v sodium nitroprusside solution (150µl) and incubated at room temperature for 10min.

2.6.3.2 Use of 2,4-dinitrophenylhydrazine (2,4-DNPH) formation

2.6.3.2.1 Large scale

2,4-DNPH was used following work previously published (Haidle & Knight, 1960). Experiments were performed with 0, 1, 2 and 3µmole of ketone (2) in 1ml of distilled water. A solution of 0.1% 2,4-DNPH was prepared in 2M HCl (100ml). The solution was filtered and stored at 4°C prior to use. A solution of 6M NaOH in distilled water was also prepared. To each sample (1ml) was added 0.1% w/v 2,4-DNPH solution (0.5ml) and distilled water (3ml). The mix was shaken and allowed to stand exactly 20min. After the first 20min, 3ml of ethanol 95% was added. The mix was shaken and allowed to stand exactly 20min after which 6M NaOH (1ml) was added. The mix was vigorously shaken and allowed to stand exactly 10min. The OD_{540nm} was then

obtained from 1ml of the final mix for each concentration. The same steps were performed with 0, 1, 2 and 3μmole of enol acetate (1) in 1ml of distilled water to see if any interference of the ester function occured during the measure of the absorbance.

2.6.3.2.2 Microtitre plate format

The same experiment was performed at a micro scale to fit a screen in microtitre plates (MTP). Six mixes of enol acetate (1) and ketone (2) in phosphate buffer (pH7.5, 50mM) with 2% DMF were prepared (5mM enol acetate + 0mM ketone, 4mM enol acetate + 1mM ketone, 3mM enol acetate + 2mM ketone, 2mM enol acetate + 3mM ketone, 1mM enol acetate + 4mM ketone, 0mM enol acetate + 5mM ketone). A 0.1% w/v 2,4-DNPH solution (10μl) was added to each mixed concentration (20μl), then water (60μl), 95% ethanol (60μl) and 6M NaOH (20μl). Shaking steps and reaction time same as previously described. The OD_{540nm} was measured for each concentration.

A biotransformation in deep-well MTP was performed in 8 wells with PFE in phosphate buffer (pH7.5, 50mM) (200μl, 19U) (Section 2.5.3.3.1) and 2mM enol acetate (1) in the same phosphate buffer (final volume 1ml). The MTP was incubated at 37°C and 200rpm for 2 and a half days for hydrolysis. From this biotransformation, 8x20μl samples were taken and submitted to the ketone assay described before in normal MTP. Standards were made with ketone (0mM, 0.5mM, 1mM, 1.5mM, 2mM) in phosphate buffer as previously described and submitted to the same reaction as the samples. The OD_{540nm} was measured using a Spectra MAX 340 plate reader spectrophotometer (Molecular Devices) using its proprietary software, Soft Max Pro 4.3.

2.6.3.2.3 Optimisation of the screen in microtitre plates

To increase the concentration of ketone reacting in the assay, the 60µl of distilled water added in the previous protocol (Section 2.6.3.2.2) were replaced by the same amount of reaction mixture, increasing the volume of reaction mixture tested from 20µl to 80µl. Each reaction from the biotransformation (2x80µl) (Section 2.5.3.3.3) was analysed using the 2,4-DNPH assay as previously described. Standards with enol acetate (1) and ketone (2) in phosphate buffer as previously described and 5% DMSO were performed at the same time (0.88µmole enol acetate + 0µmole ketone, 0.7µmole enol acetate + 0.18µmole ketone, 0.53µmole enol acetate + 0.35µmole ketone, 0.35µmole enol acetate + 0.53µmole ketone, 0.18µmole enol acetate + 0.7µmole ketone, 0µmole enol acetate + 0.88µmole ketone) to match in the assay the same conditions of the biotransformation (80µl final volume). The OD_{540nm} for samples and standards were measured.

The biotransformation was repeated with the same conditions in 8 wells but the MTP was centrifuged 10min at 4°C at 2700g in a Sorvall Legend RT centrifuge (Sorvall). The standards were performed on the same MTP. Volume (80µl) was taken from each well and the 2,4-DNPH assay was performed on samples and standards as previously described. The OD_{540nm} was measured.

To improve the reproducibility and the accuracy of the ketone assay, standards composed of the enol acetate (1) and the ketone (2) were made in 312.5µl DMSO (5µmole enol acetate + 0µmole ketone, 4µmole enol acetate + 1µmole ketone, 3µmole enol acetate + 2µmole ketone, 2µmole enol acetate + 3µmole ketone, 1µmole enol acetate + 4µmole ketone, 0µmole enol acetate + 5µmole ketone). Phosphate

buffer (pH7.5, 50mM) was added to a final volume of 1ml. The standards were centrifuged 1min at 13000rpm and 2x80µl of each mixed concentration were taken and submitted to the 2,4-DNPH assay. This time 10µl or 20µl of 0.1% w/v 2,4-DNPH solution were added to the samples, following steps as previously described. The OD_{540nm} for each sample was measured as previously described.

2.6.3.2.4 Confirmation of screening capabilities

A 96 MTP of freeze-dried mutant *P. fluorescens* esterase were received from Prof. Bornscheuer. These proteins were extracted from microtitre plate culture as previously described (Section 2.4.1.2). Fourty seven proteins were resuspended into 400µl of phosphate buffer (pH7.5, 50mM). Fourty seven hydrolysis reactions in deep-well microtitre plate were performed using a 0.2M solution of racemic enol ester (1) in DMSO and a 0.1M solution of pure (S)-enantiomer (1) in DMSO, in parallel with the same enzyme. Ninety four reactions were therefore performed with reactions testing the same enzyme in 2 consecutive wells with racemic or pure enantiomer. The substrate solution (125µl) (25% DMSO final concentration, 50mM substrate final concentration for the racemic solution and 25mM for the single enantiomer solution) was added to 100µl of the previously resuspended esterase (crude proteins) in sodium phosphate buffer (pH7.5, 50mM) and the necessary amount of sodium phosphate buffer to reach a final volume of 500µl. The solutions were stirred at 300rpm at 37°C for 4 days. MTP was centrifuged 10min at 4°C at 2700g as previously described prior to analysis.

To analyse the biotransformation reactions, a screen using the 2,4-DNPH was performed in the same conditions as previously described (Section 2.6.3.2.1). Each

reaction (80µl) from the biotransformation described above was taken. Standards with racemic enol acetate (1) or pure (S)-enantiomer (1) and ketone (2) in phosphate buffer in the same conditions as the reaction (25% DMSO final concentration and phosphate buffer) were submitted to the same treatment and analysed at the same time. The initial amount of racemic enol acetate (1) in the screen assay was 4µmole and 2µmole for the pure single enantiomer, the maximum amount of ketone which could be produced was therefore 4µmole when reaction was performed with the racemic and 2µmole when performed with the single enantiomer. The following mixes were therefore used as standards (4µmole racemic enol acetate + 0µmole ketone, 3µmole racemic enol acetate + 1µmole ketone, 2µmole racemic enol acetate + 2µmole ketone, 1µmole racemic enol acetate + 3µmole ketone, 0µmole racemic enol acetate + 4µmole ketone and 2µmole pure enantiomer enol acetate + 0µmole ketone, 1µmole pure enantiomer racemic enol acetate + 1µmole ketone, 0µmole pure enantiomer racemic enol acetate + 2µmole ketone). The standards were centrifuged as well 1min at 13000rpm before collection and 80µl were taken. A solution of 0.1% w/v 2,4-DNPH (10µl) was added to the samples, the following steps were performed as described before. The OD_{540nm}, for each sample and standard, was measured. Differences between OD_{540nm} for reactions with racemic or single enantiomer were then compared.

Chapter 3

Pseudomonas fluorescens Lipase Studies

Chapter 3

Pseudomonas fluorescens Lipase Studies

3.0 Summary

As previously described in the Chapter 1, hydrolytic enzymes are versatile biocatalysts with increasing applications in organic chemistry. Industrial processes have been developed to benefit from their activities and selectivities. However, even if lipases and esterases can utilise a broad range of non-natural substrate, high activity and high selectivity for a specific substrate are not often found. Therefore, scientists are more likely to deal with low reaction rates and low enantioselectivity.

The use of a *P. fluorescens* lipase (PFL) was previously mentioned (Section 1.4) in the resolution of enol acetate (1). This resolution is important in the synthesis of NK-2 antagonist for the development of new molecules to control pain and inflammation by Pfizer. The (S)-enol acetate (1) is used in a chain of reactions and the means of obtaining a high yield of the pure molecule were investigated in the studies mentioned before (Allan *et al.*, 2000; Carnell *et al.*, 2000). A series of enzymes were tested in the resolution of the enol acetate (1) and the PFL AK20 from Amano was the best candidate. However, the E value of this enzyme for this reaction was only 13.

In order to increase this value, the use of directed evolution to improve the enantioselectivity of this enzyme was planned as previous examples showed such results for bacterial lipases when using this process (Liebeton *et al.*, 2000; Reetz *et al.*, 2001). As the *P. fluorescens* lipase was a good candidate for the reaction of interest, we decided to choose this enzyme for our project and to find a suitable strain.

To produce a mutant recombinant protein, the gene encoding for PFL needed to be isolated and cloned from *P. fluorescens* C9. To facilitate the purification of the lipase via nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography, the insertion of an Histidine tag at the C-terminal of the protein was used.

Chapter three covers generation of various plasmid constructs used in the expression and purification of the *P. fluorescens* C9 lipase. The generation of each construct is described in detail, accompanied by selected results. Expression of the PFL using various methods and construction of new vectors in order to solubilise the protein and avoid the formation of inclusion bodies is then discussed. Purification of the lipase via Ni-NTA affinity chromatography and the attempts to obtain a soluble recombinant protein using a refolding buffer are also described.

3.1 Purification of genomic DNA from P. fluorescens

3.1.1 Purification of genomic DNA from *P. fluorescens* Migula 1865 and isolation of *lipA* gene

As this was the only commercially available strain, genomic DNA was purified from *P. fluorescens* Migula 1895 strain as previously described (Section 2.2.1). The use of the QIAGEN kit allowed RNA free DNA purification. The concentration of purified DNA, as determined by OD_{260nm} readings, was 100ng/µl.

In order to introduce mutation into the lipase gene (*lipA*), the isolation of this gene needed to be performed. Different PCR protocols were used with different annealing temperatures from 47°C to 54°C by using primers lip3 and lip4, designed in previous

work (Dieckelmann *et al.*, 1998) (Appendix II) and 100ng of gDNA. As the results were not satisfying, another PCR protocol was tried (Dieckelmann *et al.*, 1998):

94°C for 17min → (50°C for 3min, 70°C for 3min, 94°C for 11min)x35 → 50°C for 3min → 70°C for 7min → 4°C.

Despite several attempts with different protocols, none of the PCR amplifications with gDNA produced a product, implying that the sequence for the *lipA* differed in this strain. An extensive literature search reveals that the *lipA* gene might not actually be present in this strain, as the strain does not possess a lipolytic phenotype (Johnson *et al.*, 1992). We therefore decided to find another strain that did possess a lipolytic activity.

3.1.2 Purification of genomic DNA from *P. fluorescens* C9 and isolation of *lipA* gene

As mentioned before, the PFL AK20 from Amano was the best candidate in previous studies for the resolution of enol ester (1). In our search for a new strain of *P. fluorescens*, we found that the strain from which Amano extracted the lipase is *P. fluorescens* AK 102 (Kojima *et al.*, 1994). The lipase in this strain has a molecular weight of 33kDa. However, despite requesting it, the company did not wish to provide us with the strain. Only one other strain from *P. fluorescens* produces a lipase of the same molecular weight (Beven *et al.*, 2001; Dieckelmann *et al.*, 1998). This strain, called C9, is also called *P. fluorescens* Biovar V and was a kind gift of Dr Beacham (University of Queensland, Australia). Sequence analysis of this lipase showed that the 20 first amino acids were different to the Amano lipase

(Dieckelmann *et al.*, 1998; Kojima *et al.*, 1994), however, it was thought that the sequences were similar enough to support further work with the C9 lipase.

3.2 Generation of pETLipAHis construct

3.2.1. Construction of pC9LipA

New PCR primers were designed, C9 forward and C9 reverse (Appendix II), from the sequence previously published (Dieckelmann *et al.*, 1998) for the *lipA* gene in the C9 strain of *P. fluorescens*.

The analysis of the PCR product, *lipA* gene, revealed a band of the size expected 998bp (Figure 3.1). The *lipA* gene was TA cloned into pGEM-T Easy plasmid (Promega) (Appendix I and III) (Section 2.2.6) to facilitate easy sequencing of the PCR products.

The plasmid was designated pC9lipA (Figure 3.2) (Appendix I). The insert was sequenced and found to perfectly match the sequence previously reported (Dieckelmann *et al.*, 1998) for the C9 *lipA* gene.

3.2.2. Construction of pETLipAHis and pETLipANoHis

An overview of the cloning steps leading to the construction of these plasmids is presented in the diagram on Figure 3.3.

3.2.2.1 pET22b vector

To facilitate easy screening of the mutant proteins we wanted to develop a protocol that would enable their easy purification. The insertion of a Histidine tag at the C-

Figure 3.1: Isolation of *P. fluorescens* C9 *lipA* gene by high fidelity PCR

PCR was performed with primers C9 forward and C9 reverse. 12µl of each reaction were analysed by using 1% agarose gel, containing 0.6µg:µl ethidium bromide
1: Generuler 1Kb DNA Ladder (MBI Fermentas)
2: Negative control (no DNA)
3: *lipA* from *P. fluorescens* C9

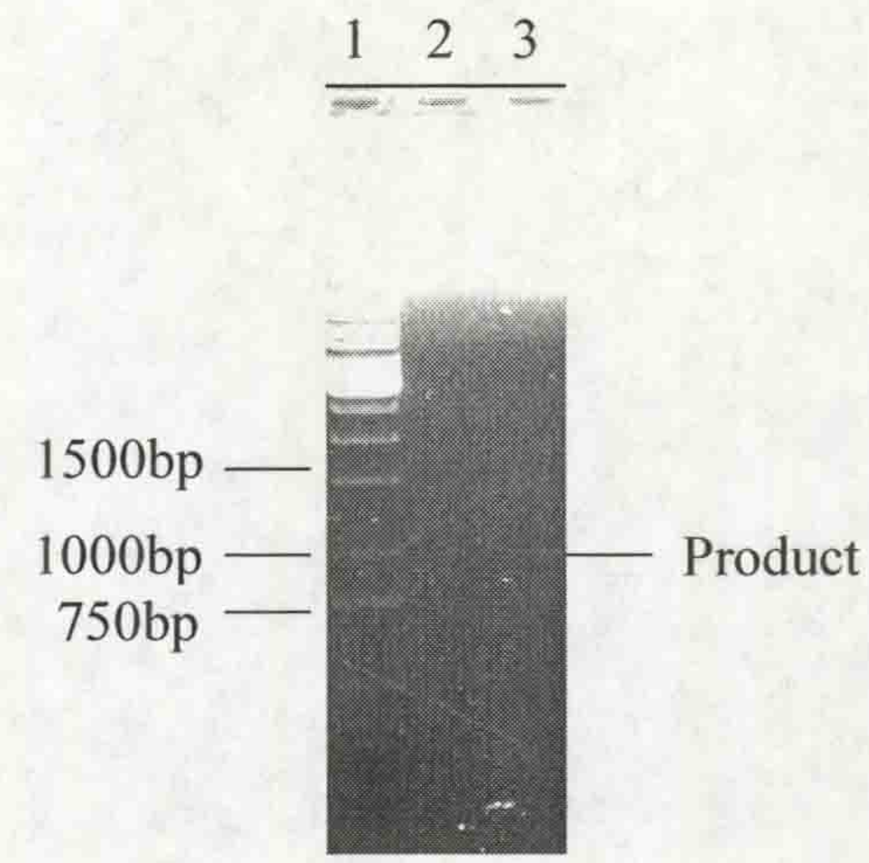
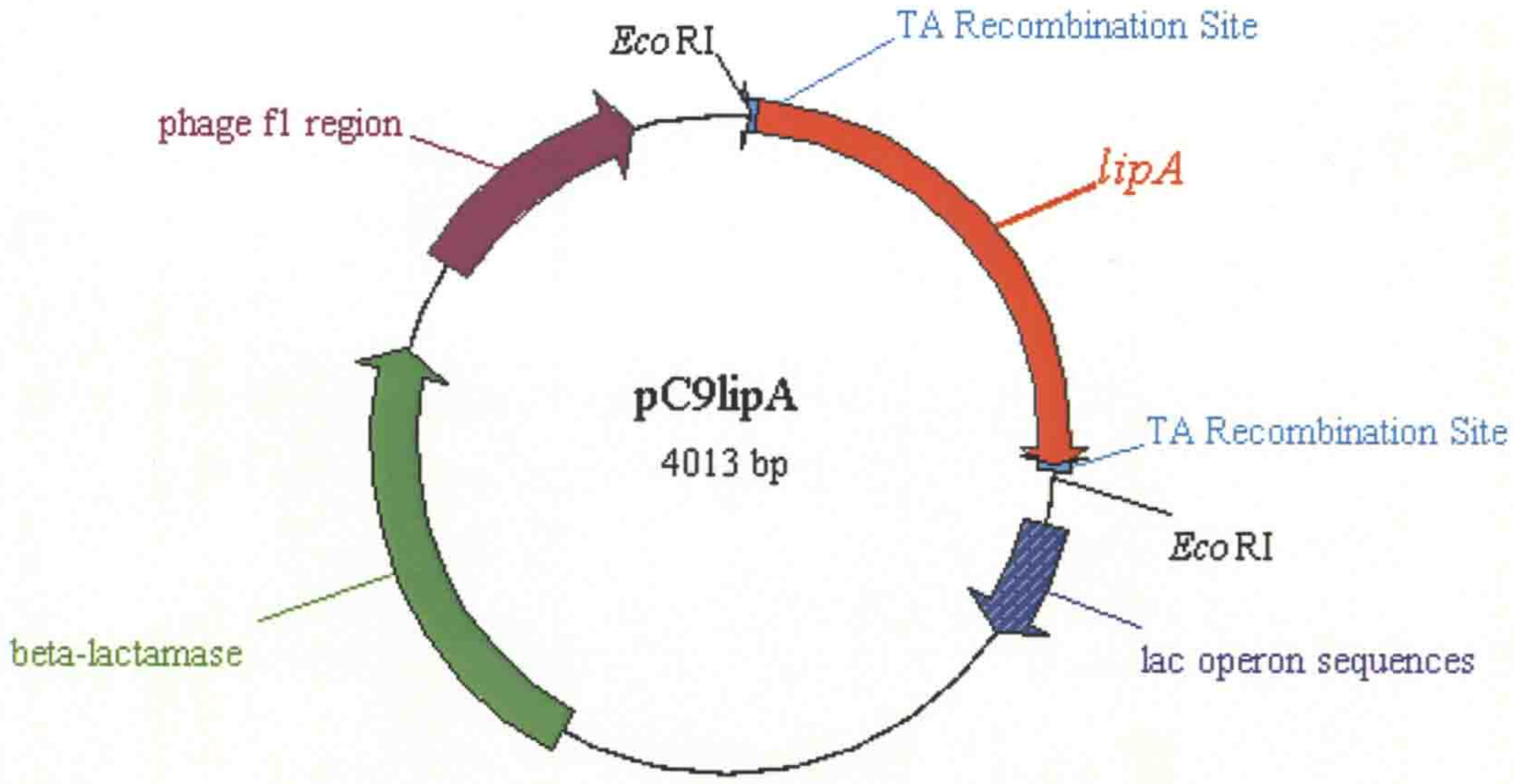


Figure 3.2: Plasmid map of pC9lipA



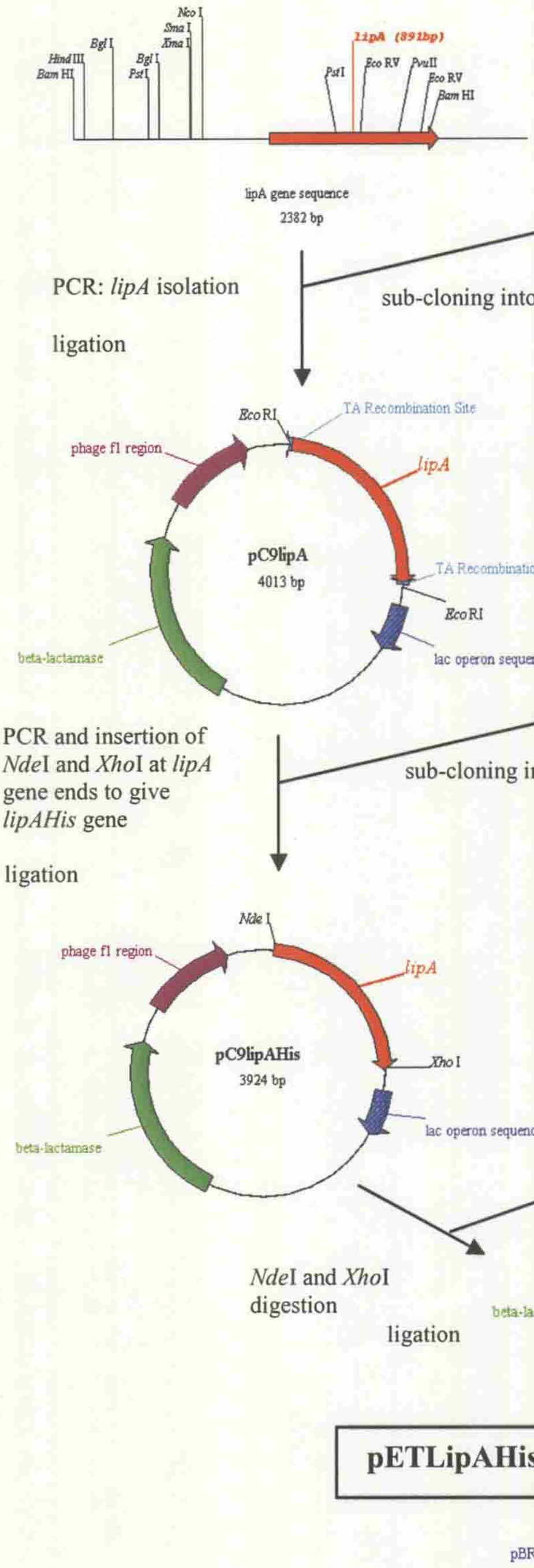
Important restriction enzymes sites:

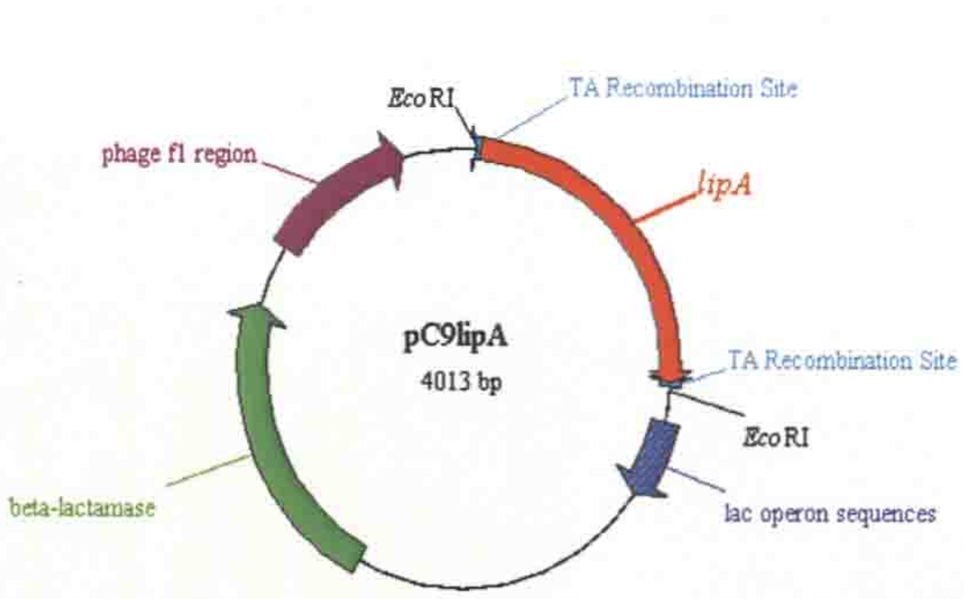
*Ava*I	139
*BamH*I	921
*EcoR*I	53, 1069
*Nco*I	38
*Pst*I	446, 1087

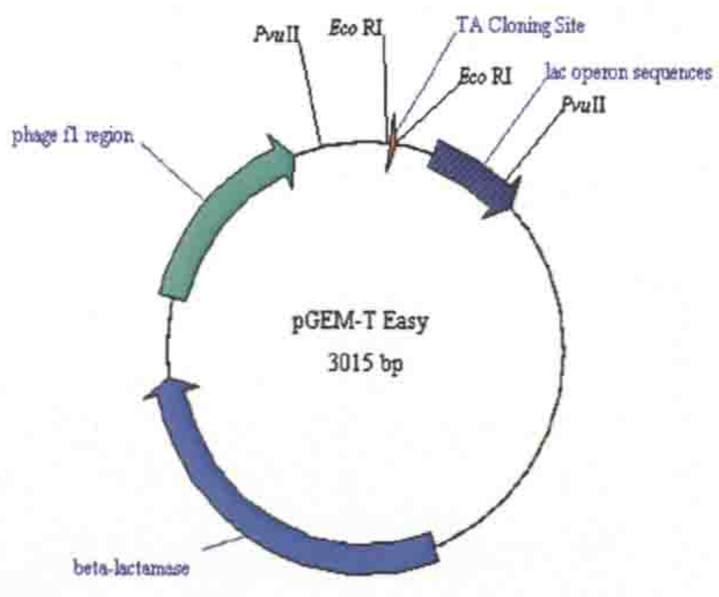
Important features:

Phage f1 region	3378-3833
Beta-lactamase gene	2335-3195
Lac operon sequence	1164-1393
lipA gene	61-1058
TA recombinant sites	60, 1059

Figure 3.3: Construction of pETLipAHis and pETLipANoHis



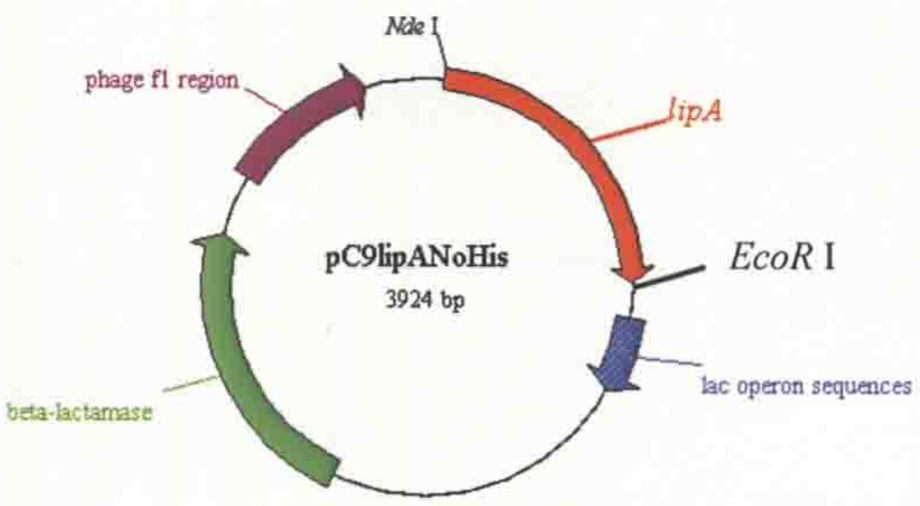


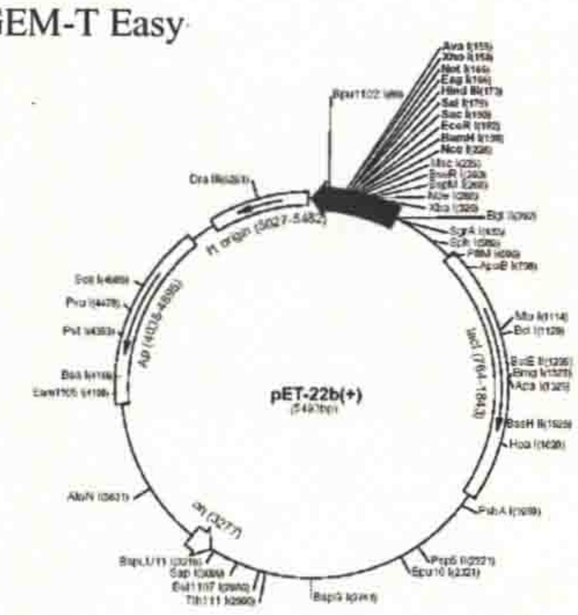


PCR and insertion of *Nde*I and *EcoR*I at *lipA* gene ends to give *lipANoHis* gene

sub-cloning into p-GEM-T Easy

ligation



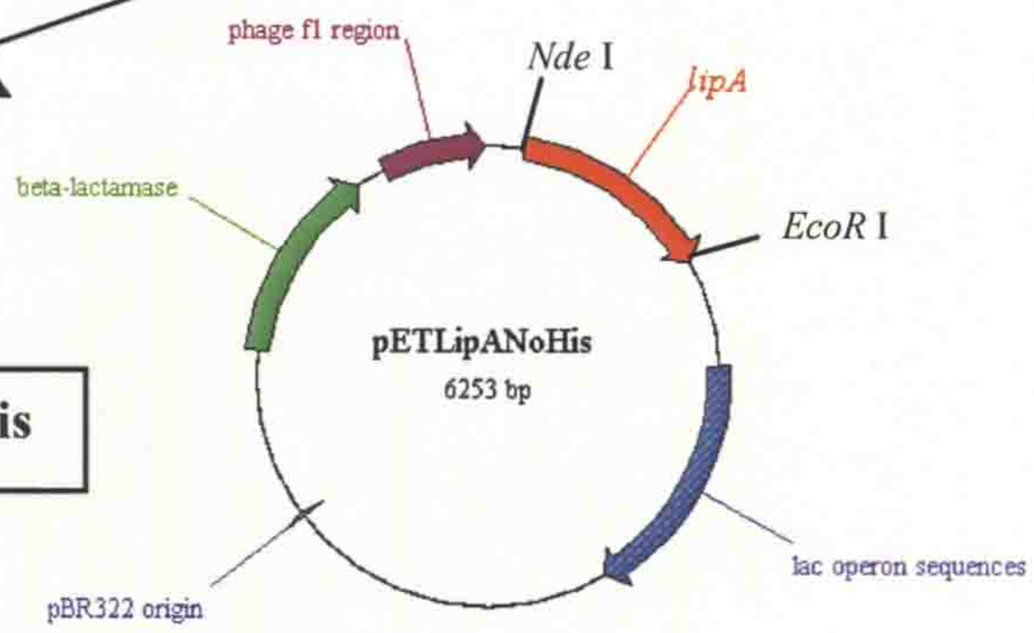


*Nde*I and *EcoR*I digestion

*Nde*I and *EcoR*I digestion

ligation

pETLipANoHis



terminus of the LipA protein would allow easy recovery of the mutant enzymes and has the potential for easy recovery using Nickel coated microtitre plates. This would greatly facilitate high-throughput screening. The pET22b (Novagen) (Appendix I and III) was chosen to express this His-tagged lipase. This expression system offers very tight control of His tagged protein expression due to use of a T7 promoter, which, unlike the T5 promoter, is not recognised by the host *E. coli* RNA polymerase. This enzyme is not present in *E. coli* JM109, resulting in extremely low basal expression levels. JM109 was therefore used for the storage and propagation of pET22b and pET-based expression constructs. For expression of C-terminally His tagged protein, pET22b with the corresponding *lipA* insert, was transformed into *E. coli* BLR(DE3). This host was lysogen of bacteriophage λDE3, and therefore possesses a chromosomal copy of the T7 RNA polymerase gene.

BLR(DE3) expresses T7 RNA polymerase from a *lacUV5* promoter. This promoter is subject to control by the *lac* repressor protein, which is also expressed in λDE3 lysogens. When IPTG is added, it binds the *lac* repressor protein blocking its repression of the *lacUV5* promoter. This causes expression of T7 RNA polymerase, which then goes on to transcribe the His tagged protein. BLR(DE3) has intrinsic tetracycline resistance and pET plasmid confers resistance to ampicillin. BLR(DE3) containing these plasmids were maintained in culture by addition of these antibiotics.

3.2.2.2 pETLipAHis cloning

For construction of the *lipAHis* gene, restriction sites were incorporated into oligonucleotides primers to enable the digested PCR product to be ligated into the similarly digested plasmid vector, in such way as to leave the lipase sequence in

frame with the ATG start codon and 6xHis tag on the pET22b plasmid (Appendix III). The insertion of the *Nde*I site corresponded with the start codon but the insertion of the *Xho*I site, for the *lipAHis* gene, involved the insertion of a leucine and a glycine residue in the original sequence of the protein just before the His tag. The stop codon was omitted from the PCR product, so that the coding sequence ran directly into the His tag coding sequence on the plasmid vector. However, those relatively small amino acids at the C-terminal end of the protein are not supposed to interfere with the activity. Restriction enzymes were also chosen on the basis that they did not cleave the lipase coding sequence internally. The advantages of using two different enzymes are that it allows directional cloning and minimises the probability of re-circularisation of the plasmid.

Primers C9 lipA-NdeI and C9 lipA-XhoI (Appendix II) were designed from the sequence previously published (Dieckelmann *et al.*, 1998) to insert *Nde*I and *Xho*I restriction sites at both ends of the *lipA* gene. A high fidelity PCR was performed (Section 2.2.4), using an annealing temperature of 63°C for 35 cycles with 1.6µg of pC9lipA (2µl) as the template. The product matched the expected size of 911bp and was named *lipAHis* (Figure 3.4). After purification, the *lipAHis* gene was TA cloned and blue and white colonies were obtained and analysed as previously described. The plasmids extracted were found to be identical and were named pC9lipAHis (Figure 3.5 and Appendix I).

The plasmids pET22b (Novagen) and pC9lipAHis were digested with *Nde*I and *Xho*I enzymes (Section 2.2.10). Each plasmid (3µg) was mixed with 10U of each enzyme. The digestion performed on the pET22b revealed one band equivalent to the expected size of 5367bp (Figure 3.6). The digestion performed on the pC9lipAHis (Section

Figure 3.4: Construction of *lipAHis* gene and *lipAHisNo* gene by high fidelity PCR

PCR was performed with primers lipA-NdeI and lipA-XhoI. 30µl of each reaction were analysed by using 1% agarose gel, containing 0.6µg:µl ethidium bromide
1: Generuler 1Kb DNA Ladder (MBI Fermentas)
2: Negative control (no DNA)
3: l*ipAHis* gene
4: *lipANoHis* gene

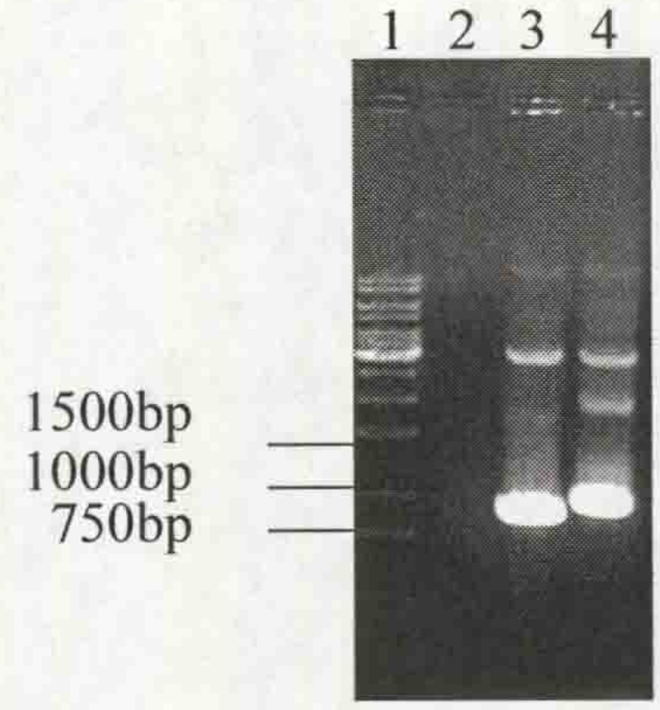
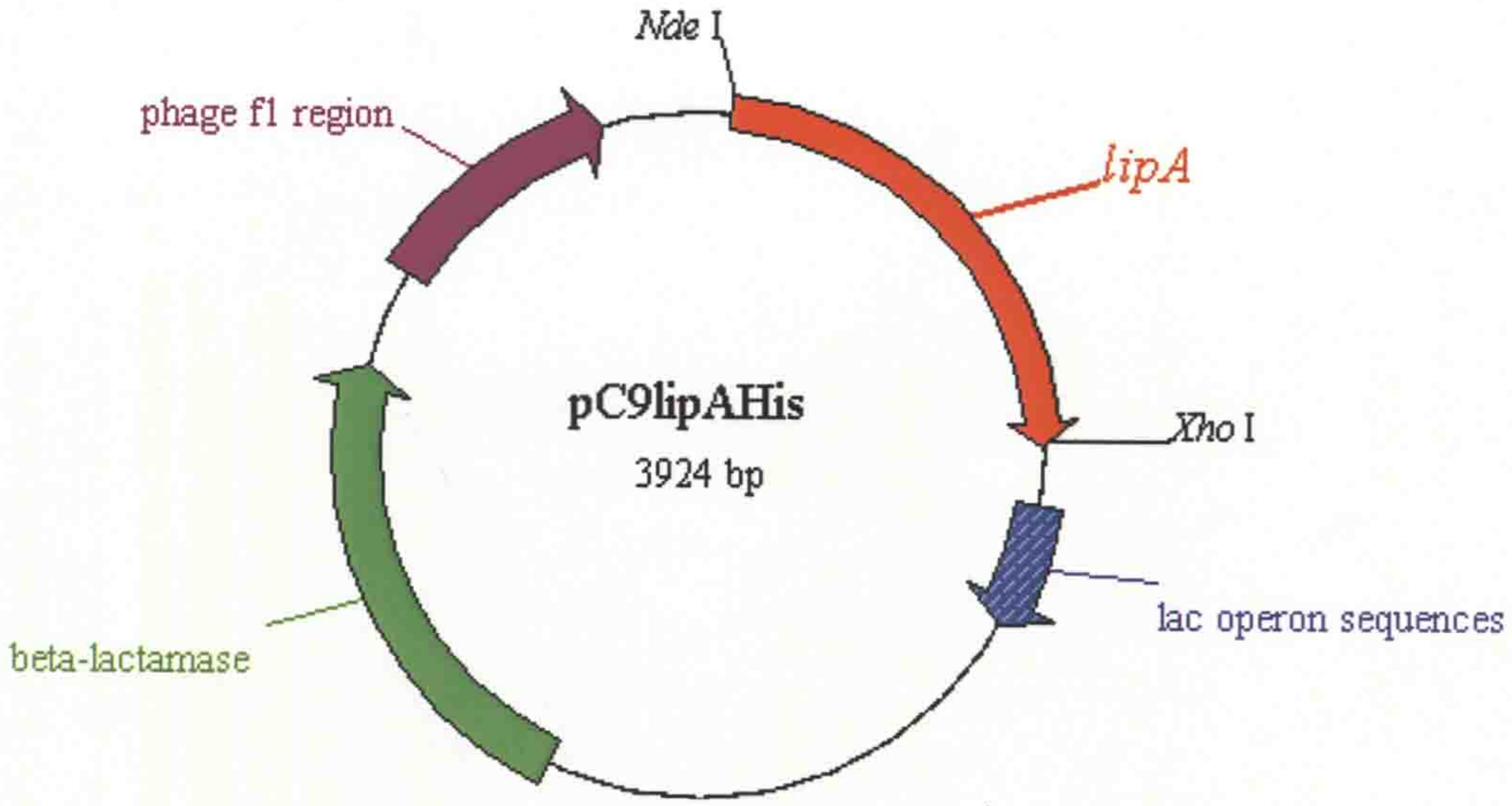


Figure 3.5: Plasmid map of pC9lipAHis



Important restriction enzymes sites:

*Ava*I	122, 959
*BamH*I	904
*EcoR*I	53, 980
*Nco*I	38
*Nde*I	69
*Xho*I	959

Important features:

Phage f1 region	3289-3744
Beta-lactamase gene	2246-3206
Lac operon sequence	1075-1304
lipA gene	60-970

2.2.10) revealed two bands close to each other between 750bp and 1000bp (Figure 3.6). These results can be explained as the pC9lipAHis was constructed from pGEM-T Easy vector which possesses an *Nde*I restriction site in its multiple cloning site. The digestion with *Nde*I can in that case cut at these two sites and lead to two fragments on the agarose gel. The two bands were extracted for ligation to check which fragments was the correct one.

The digested *lipAHis* gene was ligated into the digested pET22b by T4 DNA ligase with a 3:1 insert:vector ratio overnight at 16°C. Competent *E. coli* JM109 were heat shocked with 5µl of the ligation product using the previously described protocol. Plasmids were extracted as previously described and named pETLipAHis 1 (band close to 1000bp) and pETLipAHis 2 (band close to 750bp). PCR analyses of the colonies revealed a band at less than 1kb only for pETLipAHis 1. The plasmid extracted from this colony was sequenced and confirmed that the expressed coding sequence was identical to the published sequence and correctly aligned with the 6xHis tag and it was renamed pETLipAHis (Figure 3.7 and Appendix I).

3.2.2.3 pETLipANoHis cloning

The same experiments were performed on the *lipA* gene to insert by PCR the *Nde*I restriction site for the construction of the *lipANoHis* gene, using the primers C9 lipA-NdeI and C9 reverse (Appendix II). An *Eco*RI site was not inserted by PCR as the *lipANoHis* gene was TA cloned into pGEM-T Easy which possesses the *EcoR*I site in its MCS. The aim was to express a wild-type protein, i.e. without the 6xHis tag, to enable a comparison of the activity of the two enzymes to check if the 6xHis tag has any influence on enzyme activities.

Figure 3.6: Digestion of pET22b and pC9LipAHiS by *NdeI* and *XhoI* and digestion of pET22b and pC9LipANoHis by *NdeI* and *EcoRI*

30µl of digested pET22b and 20µl of pC9LipAHis and pC9LipANoHis were analysed by using 1% agarose gel, containing 0.6µg:µl ethidium bromide
1: Generuler 1Kb DNA Ladder (MBI Fermentas)
2: pET22b digested by *NdeI* and *XhoI*
3: pC9LipAHis digested by *NdeI* and *XhoI*
4: pET22b digested by *NdeI* and *EcoRI*
5: pC9LipANoHis digested by *NdeI* and *EcoRI*

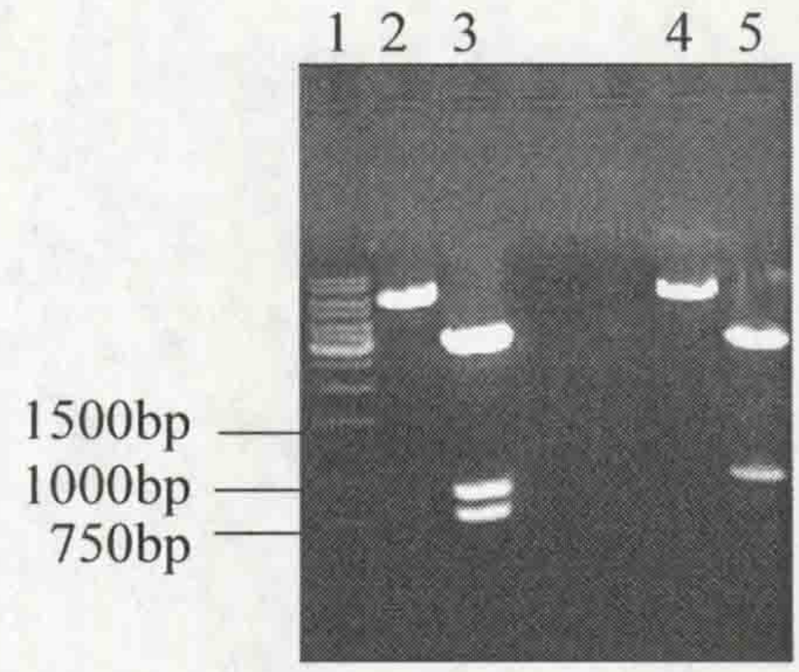
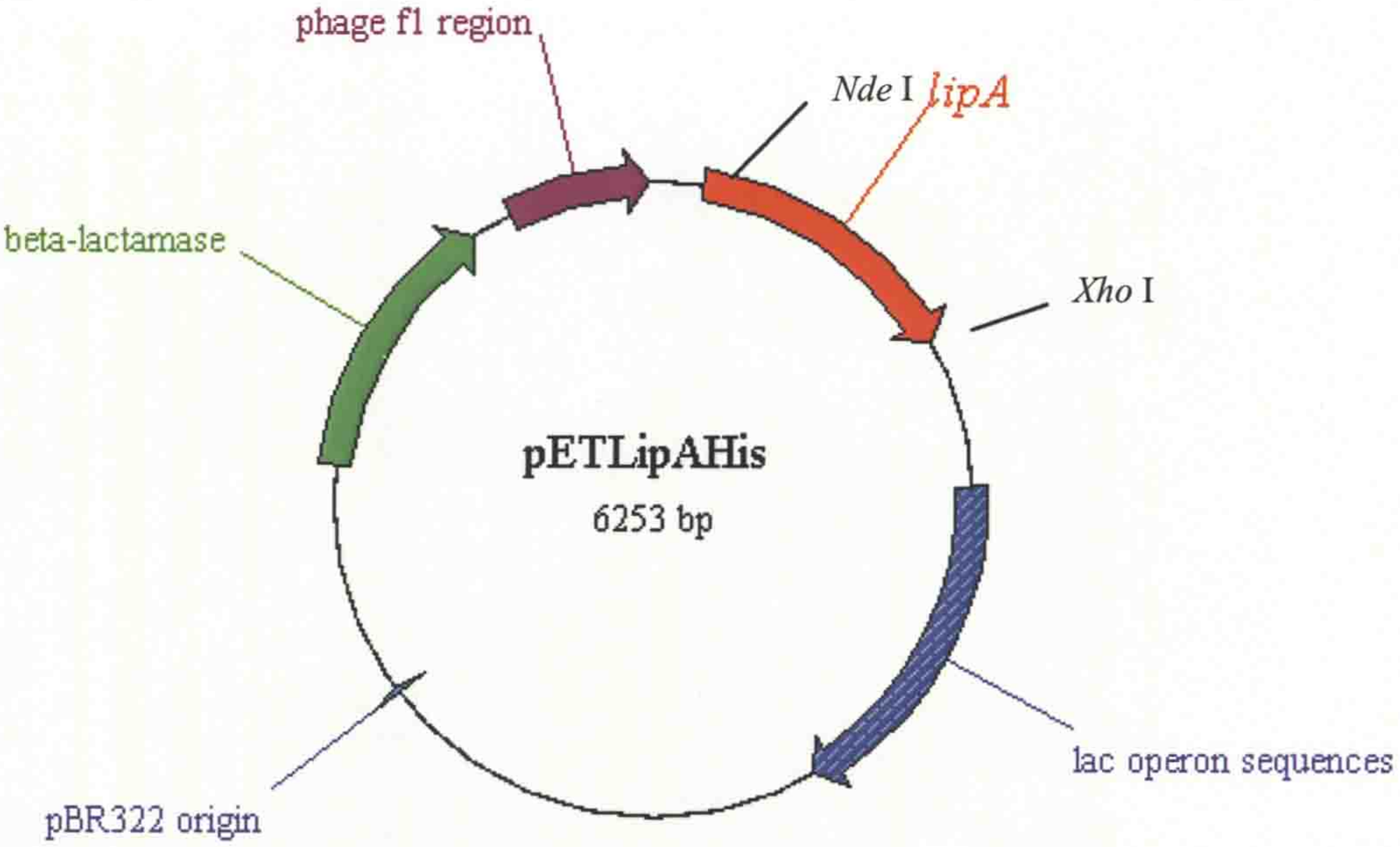


Figure 3.7: pETLipAHis plasmid map



Important restriction enzymes sites:

*Ava*I	122, 959
*BamH*I	904
*Eco*RI	53, 980
*Nco*I	38
*Nde*I	69
*Xho*I	959

Important features:

pBR322 origin	4037	
Phage f1 region		5787-6242
Beta-lactamase gene		4798-5655
Lac operon sequences		1075-1304
lipA gene		159-1048

A high fidelity PCR was performed with the primers C9 lipA-NdeI and C9 reverse (Appendix II) (Section 2.2.4), using an annealing temperature of 63°C for 35 cycles. The PCR gave a product of less than 1kbp (*lipANoHis* gene), equivalent to the size expected of 982bp (Figure 3.4). As previously described, the corresponding plasmid was extracted and was named pC9lipANoHis (Appendix I). As described for the pETLipAHis cloning, the plasmids pET22b (Novagen) and pC9lipANoHis were digested with *Nde*I and *EcoR*I enzymes (Section 2.2.10). The digestion performed on the pET22b revealed one band as expected and equivalent to the size of 5401bp (Figure 3.6). The digestion performed on the pC9lipANoHis revealed one band of the expected size (987bp) (Figure 3.6).

Unfortunately, no colonies were obtained from the ligation. As *Nde*I is also present on pGEM-T Easy after the MCS, a digestion with only this enzyme was performed on pET22b and pC9lipANoHis. However, this attempt failed. This failure is difficult to explain as well as the failure of the ligation from a single digestion with *Nde*I as it is not supposed to be due to re-circularisation of the plasmid, especially in the case of the double digestion. Another explanation can be a secondary structure at the 3' end of the *lipANoHis* after the stop codon which could limit the ligation. No further experiment was done to produce pETLipANoHis as the main interest of these experiments was to produce pETLipAHis for the expression, purification and biotransformation ability of the lipase.

3.3 Expression of pETLipAHis, extraction and purification of the recombinant lipase

3.3.1 Purification of His tagged protein

3.3.1.1 Theory

Insertion of a tag coding sequences into the previously described plasmid expression vectors enabled the expression of recombinant enzymes containing a C-terminal 6xHis tag. Histidine tags are uncharged at physiological pH and are relatively small. Therefore they do not normally interfere with folding of the nascent protein, or its function. However, the His tag does have a high affinity for nickel-nitrilotriacetic acid (Ni-NTA) (Hochuli & Dobeli, 1987; Hochuli *et al.*, 1987). This enabled the single step purification of tagged proteins from crude lysates, using the QIA*express* Ni-NTA metal-affinity chromatography system (Qiagen).

The QIA*express* system exploits the high affinity of the His tag for a Ni-NTA agarose matrix in order to trap the His-tagged protein. Non-tagged contaminating proteins either flow directly through the column or bind weakly and are easily removed by a series of washes. This allows pure His-tagged protein to be eluted separately. The histidine residues in the His tag have a pK_a of approximately 6.0 and will become protonated if the pH is reduced below this. When this happens the His tag can no longer bind to the nickel ions and will dissociate from the Ni-NTA matrix. Although for purification under denaturing conditions, elution by lowering the pH was adequate, the preferred method of elution was by an increase in imidazole concentration to avoid an acidi. Imidazole is a histidine analogue, which can outcompete the His tagged protein for binding sites on the resin. An increase in imidazole concentration to 250mM will cause rapid dissociation of tagged protein

from the Ni-NTA complex. Unlike a decrease in pH, which will denature the protein, imidazole leaves the protein intact and fully active. Imidazole elution was therefore the method of choice for purification under native conditions.

Alternatively elution can be achieved by addition of EDTA or EGTA to the elution buffer. These are chelaters of divalent cations (such as nickel ions) and will remove them from the NTA groups. Although the protein is eluted in an essentially native state, elution is a protein-metal complex. Also the eluate contains a high metal ion concentration. For these reasons it was rarely used in this project.

3.3.1.2 Purification under denaturing conditions

High level expression of foreign protein can lead to the formation of insoluble protein aggregates in host cells, known as inclusion bodies (Rudolph & Lilie, 1996). Apart from accessibility of the His tag, purification under denaturing conditions offers the further advantages that inclusion bodies will be completely solubilised. Consequently all His tagged protein will be available for binding to the Ni-NTA matrix and purification.

Under native conditions, the inclusion bodies remain insoluble and aggregated protein cannot be purified. Purification under native conditions only captures the small fraction of expressed protein that has remained soluble in the cytoplasm. The conditions during induction were adjusted to optimise the amount of His tagged protein in the soluble fraction.

The potential for non-specific binding of untagged proteins to the Ni-NTA matrix is also reduced under denaturing conditions, meaning protein can be purified to a greater extent, using fewer washes. Together, the complete solubilisation of inclusion bodies, coupled with a need for fewer wash steps, mean much larger quantities of His tagged protein could be purified. For this reason, prior to purification under native conditions, His-tagged proteins were usually purified under denaturing conditions, in order to test extraction efficiency.

3.3.1.3 Purification under native conditions

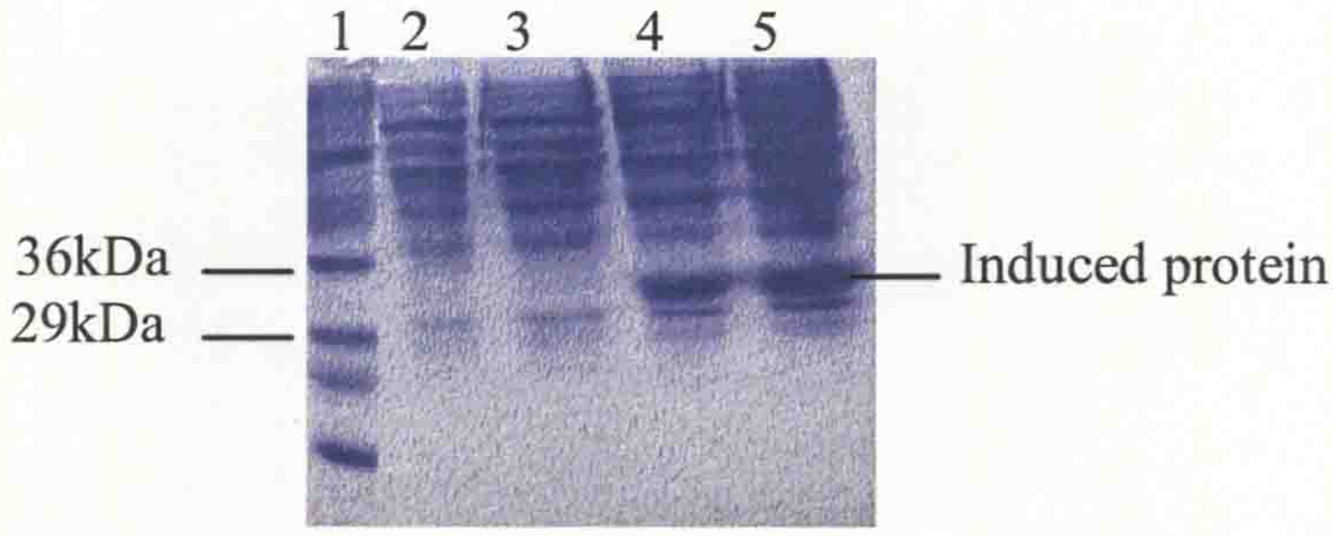
The increase in non-specific binding by contaminating protein that occurs under native conditions was countered by the addition of low imidazole concentrations (10mM) to the native lysis buffers. This was sufficient to reduce bonding to the Ni-NTA resin by non-tagged contaminants, whilst not significantly disrupting the strong affinity of the His tag. A series of washes with buffers containing increasing concentrations of imidazole, gradually removed contaminating proteins. The number of washes at each concentration was optimised to reduce loss of His-tagged protein.

3.3.2 Expression and purification under denaturing conditions of the recombinant lipase

The transformation of competent BLR(DE3) *E. coli* cells with pETLipAHis (Section 2.3.1.1) was highly effective. One clone induced by IPTG allowed the extraction of the induced lipase with a His tag in denaturing conditions. The SDS-PAGE analysis and blue staining, of the different fractions (Section 2.3.6) of the extraction process, showed that a novel band, revealed by blue staining, was present in the soluble part of the induced cell fraction, at the size expected (33kDa) (Figure 3.8). This band was not

Figure 3.8: Extraction of the recombinant lipase under denaturing conditions

Transformation performed from BLR(DE3) with pETLipAHis.
Extraction under denaturing conditions.
12% SDS-PAGE analysis with Coomassie blue staining
1: Dalton Mark VII-L Mr marker
2, 3: non induced cells samples (10µl and 20µl loaded), negative control
4, 5: induced cells samples (10µl and 20µl loaded)



present either in the soluble or insoluble fractions of the non-induced cells, meaning that expression is not detectable in the non-induced controls.

The presence of a C-terminal His tag on the protein expressed, was confirmed by purification under denaturing conditions (Section 2.3.2.1). Analysis of the fractions collected during the purification, showed a novel band, corresponding to a 33kDa protein (Figure 3.9). Its high affinity binding to the Ni-NTA matrix (still bound after washing steps), coupled with its elution at low pH, strongly suggested it was the recombinant lipase. Other proteins were also visible on the gel. They were contaminating proteins with affinity to the matrix but as the 33kDa protein is the major band and is not present in the non-induced cells, contrary to the other bands, those proteins were not considered as recombinant proteins. This contamination can occur with proteins whose sequence contains a high proportion of histidine, unfortunately, even with washing steps, purification of the recombinant lipase can not be totally efficient.

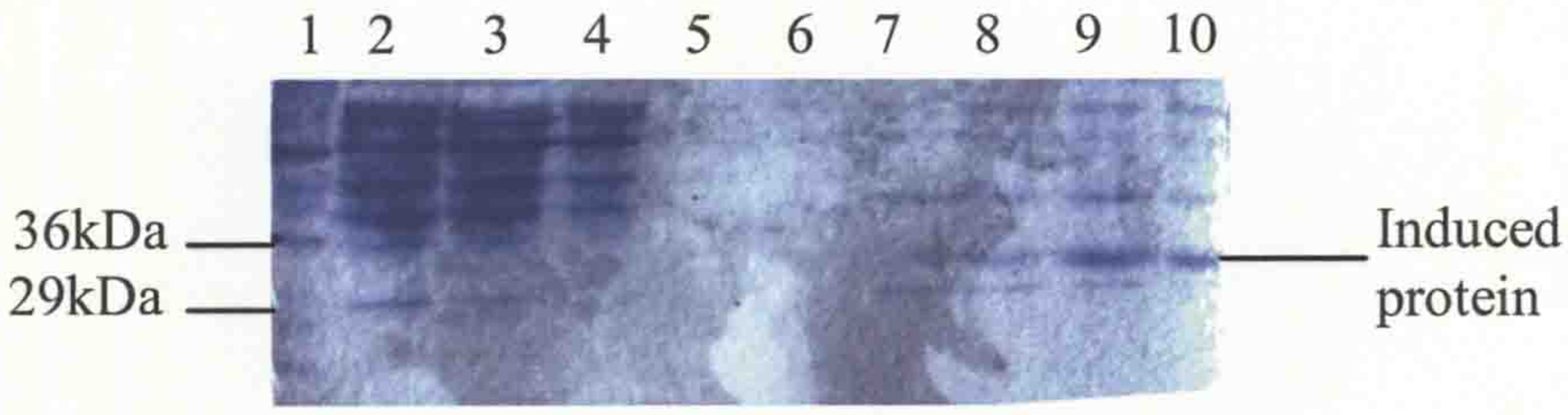
3.3.3 Expression and purification under native conditions of the recombinant lipase

The same experiments performed in native conditions (Section 2.3.1.2 and 2.3.2.2) did not reveal detectable expression by SDS-PAGE analysis. Over-expression occurred as previously described but SDS-PAGE analysis did not reveal a band at the size expected.

To determine if the conditions of purification were adequate, a range of imidazole buffers was used to purify the recombinant protein (Section 2.3.3.3). Washing steps and elution steps were performed with increasing concentrations of imidazole in the

Figure 3.9: Purification of the recombinant lipase under denaturing conditions

Transformation performed from BLR(DE3) with pETLipAHis.
Extraction under denaturing conditions and purification under denaturing conditions
12% SDS-PAGE analysis with Coomassie blue staining.
1: Dalton Mark VII-L Mr marker
2: non induced cells samples (20µl loaded), negative control
3: flow-through from column (20µl loaded)
4, 5: first and third washes of the column (20µl loaded)
6, 7, 8, 9: second, fourth, sixth and eighth elution fractions (20µl loaded)
10: second EDTA elution fraction (20µl loaded)



buffers. Unfortunately, as previously described, induction allowed the overexpression of the lipase but no protein could be purified as no band was visible following SDS-PAGE analysis, on any of the samples taken during the experiments. It was concluded that the recombinant lipase was largely insoluble in the native buffer as not even a small quantity of protein could be purified.

3.3.4 Solubility test and difference between sonication and freeze-thaw cycles

To confirm these suppositions, a solubility test was performed (Section 2.3.3.2). The test showed that expression was efficient, as the same major band as previously mentioned appeared at 33kDa, which is not present in the non-induced sample, showing high level of lipase expression has been achieved. But no overexpressed lipase band is visible in the clear lysate corresponding to the soluble proteins. This is not due to inadequate lysis as numerous bands can be seen in the soluble fractions. When the pellet, corresponding to cell debris and membranes, was collected during extraction and resuspended in buffer, the lipase was the major constituent (Figures 3.10 and 3.11). This indicates that the lipase has been pelleted with the insoluble fractions. The overexpression of the protein leads to the formation of inclusion bodies by the bacteria.

This test was also performed with two methods of extraction (sonication and cycles of freeze-thaw). But no differences could be seen for the extraction with these two methods (Figures 3.10 and 3.11). The bands, corresponding to the overexpressed lipase, are roughly similar. This indicated that both methods are efficient for lysis of the cells. As it is easier to perform, it was decided to use freeze-thaw cycles for extraction steps.

Figure 3.10: Test of solubility with sonication process

Transformation performed from BLR(DE3) with pETLipAHis. Extraction under native conditions using a sonicator. Insoluble pellet was resuspended into native buffer.
12% SDS-PAGE analysis with Coomassie blue staining
1: Dalton Mark VII-L Mr marker
2, 3: non induced cells samples (10µl and 20µl loaded), negative control
4, 5: induced cells samples (10µl and 20µl loaded)
6, 7: induced cells, soluble fraction (10µl and 20µl loaded)
8, 9: induced cells, insoluble fraction (10µl and 20µl loaded)

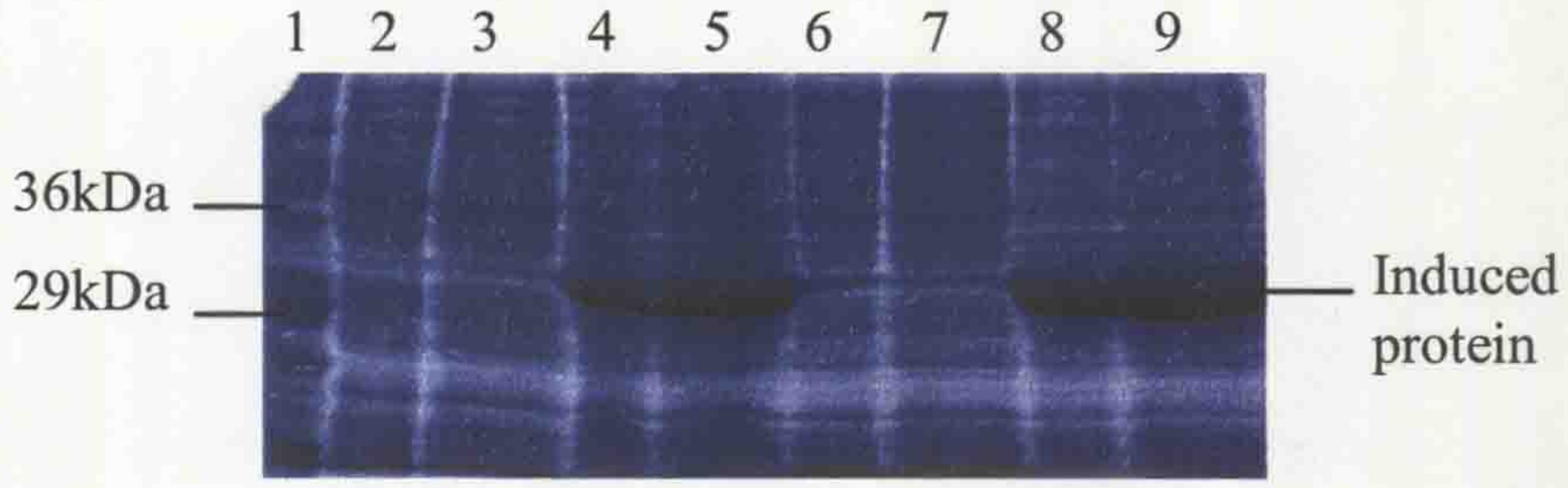
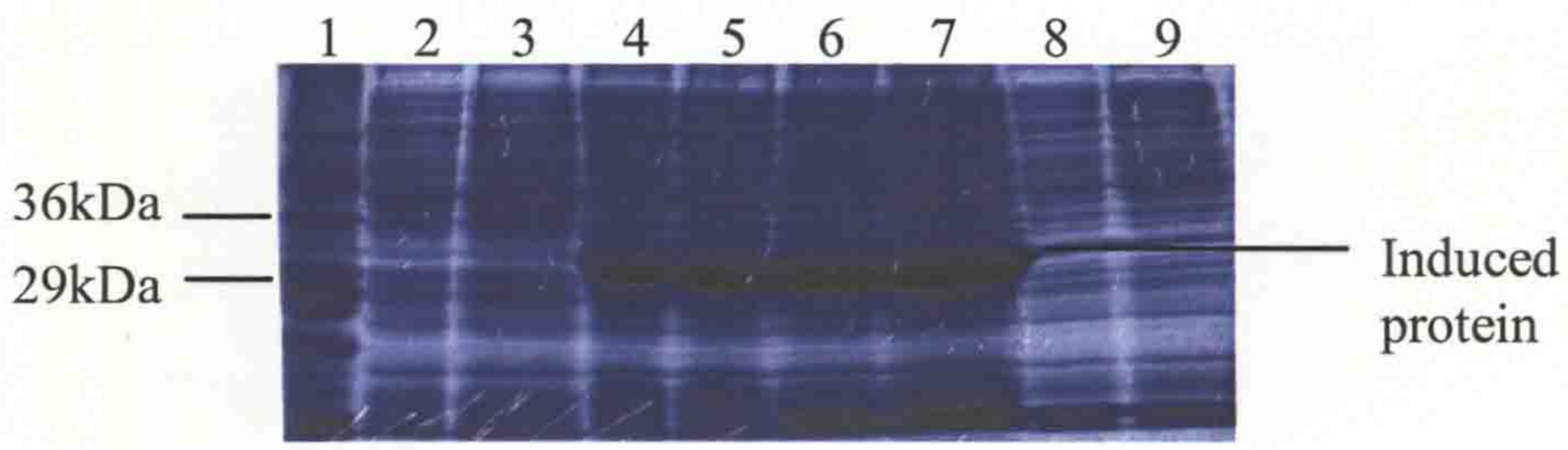


Figure 3.11: Test of solubility with freeze-thaw process

Transformation performed from BLR(DE3) with pETLipAHis. Extraction under native conditions using freeze-thaw cycles. Insoluble pellet was resuspended into native buffer.
12% SDS-PAGE analysis with Coomassie blue staining
1: Dalton Mark VII-L Mr marker
2, 3: non induced cells samples (10µl and 20µl loaded), negative control
4, 5: induced cells samples (10µl and 20µl loaded)
6, 7: induced cells, insoluble fraction (10µl and 20µl loaded)
8, 9: induced cells, soluble fraction (10µl and 20µl loaded)



3.3.5 Optimisation of expression and purification conditions

Following failure to purify lipase with the Qiagen native buffers, the solubility test was repeated using buffers containing Tween and EGTA or Triton-X100 (Section 2.3.3.2). These compounds are known to help to solubilise insoluble proteins in purification steps. Extraction was performed with these buffers, a sample from each supernatant containing soluble proteins and one from the insoluble pellet, previously resuspended in the same buffer, were analysed by SDS-PAGE (Figure 3.12). As previously described the major band corresponding to the overexpressed lipase was found in the insoluble fractions of the extraction. This indicates that the buffers tried did not change the insolubility of the lipase nor help for the solubilisation of inclusion bodies.

As the formation of inclusion bodies generally occurres due to a high level of expression, attempts to reduce this level were performed by lowering the IPTG concentration as well as the time and the temperature during the incubation period for expression (Section 2.3.3.1). A sample of each concentration of IPTG, at each temperature and for each time was taken and the soluble and insoluble fractions for these samples analysed by SDS-PAGE. Unfortunately, even with the weakest conditions of induction (2h at 20°C with 0.1mM IPTG) (Figure 3.13), the recombinant lipase was overexpressed and was visible in the insoluble fractions of the extraction. This indicates that even with low conditions for induction, the formation of inclusion bodies is inevitable.

Figure 3.12: Test of different buffer for the extraction

Transformation performed from BLR(DE3) with pETLipAHis. Extraction under native conditions with buffer containing Tween, EGTA or Triton. Insoluble pellet was resuspended into the same buffer used for extraction.
12% SDS-PAGE analysis with Coomassie blue staining
1: Dalton Mark VII-L Mr marker
2: induced cells samples (10µl loaded), negative control
3: induced cells, insoluble fraction, extraction with Tween-EGTA buffer (20µl loaded)
4: induced cells, soluble fraction, extraction with Tween-EGTA buffer (20µl loaded)
5: induced cells, insoluble fraction, extraction with 1% Triton X-100 (20µl loaded)
6: induced cells, soluble fraction, extraction with 1% Triton X-100 (20µl loaded)
7: induced cells, insoluble fraction, extraction with 2% Triton X-100 (20µl loaded)
8: induced cells, soluble fraction, extraction with 2% Triton X-100 (20µl loaded)

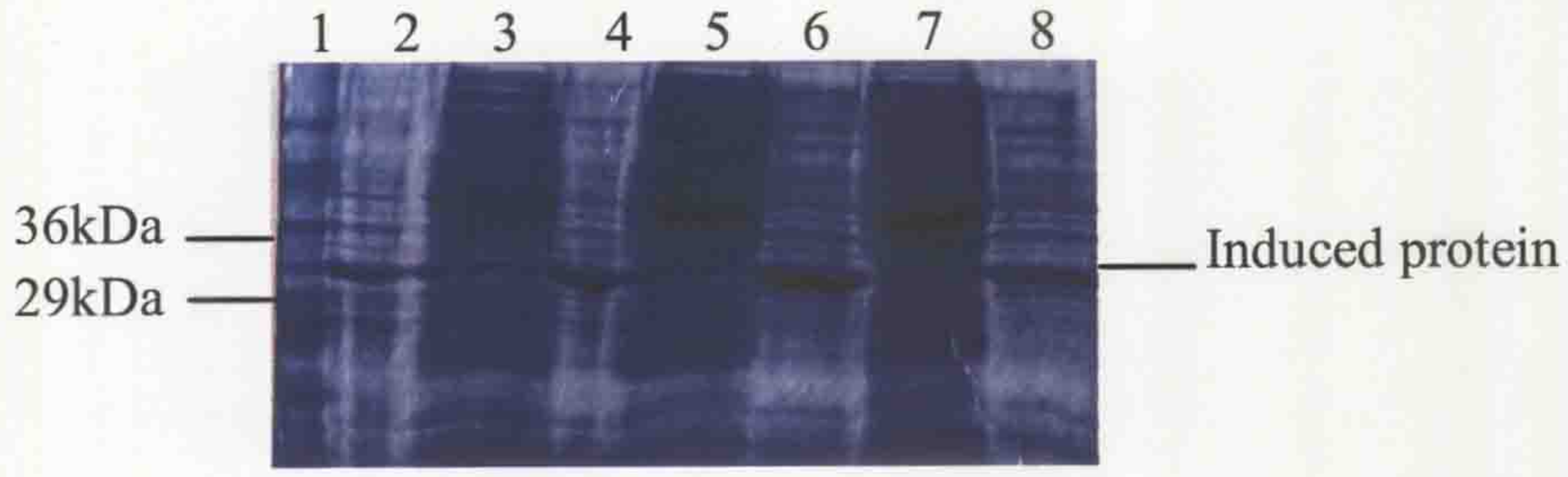
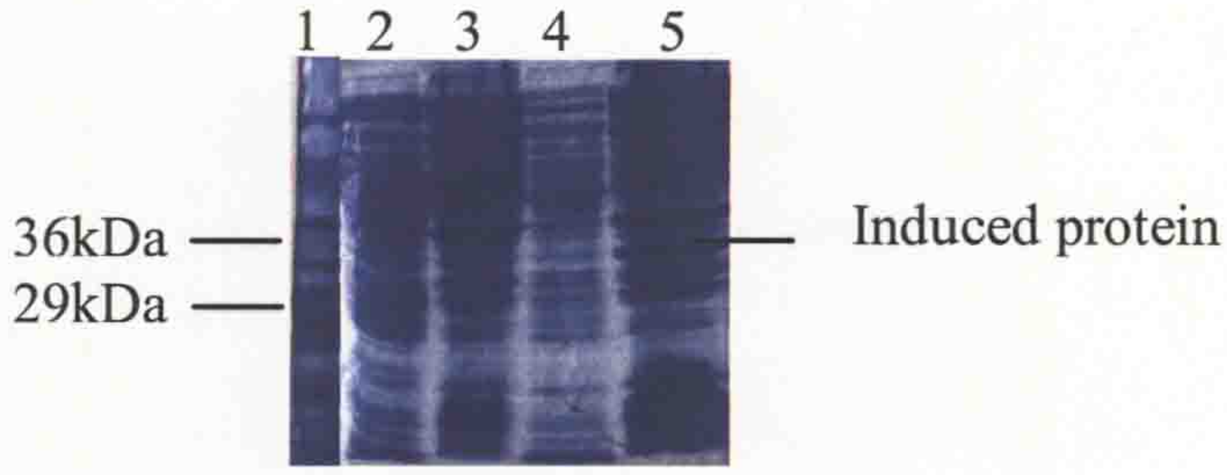


Figure 3.13: Optimisation of expression

Transformation performed from BLR(DE3) with pETLipAHis. Extraction under native conditions with after 2h incubation at 20°C with 0.1mM IPTG. Insoluble pellet was resuspended into native buffer.
12% SDS-PAGE analysis with Coomassie blue staining
1: Dalton Mark VII-L Mr marker
2: non induced cells samples, insoluble fraction (20µl loaded), negative control
3: non induced cells samples, soluble fraction (20µl loaded), negative control
4: induced cells, insoluble fraction, extraction with Tween-EGTA buffer (20µl loaded)
5: induced cells, soluble fraction, extraction with Tween-EGTA buffer (20µl loaded)



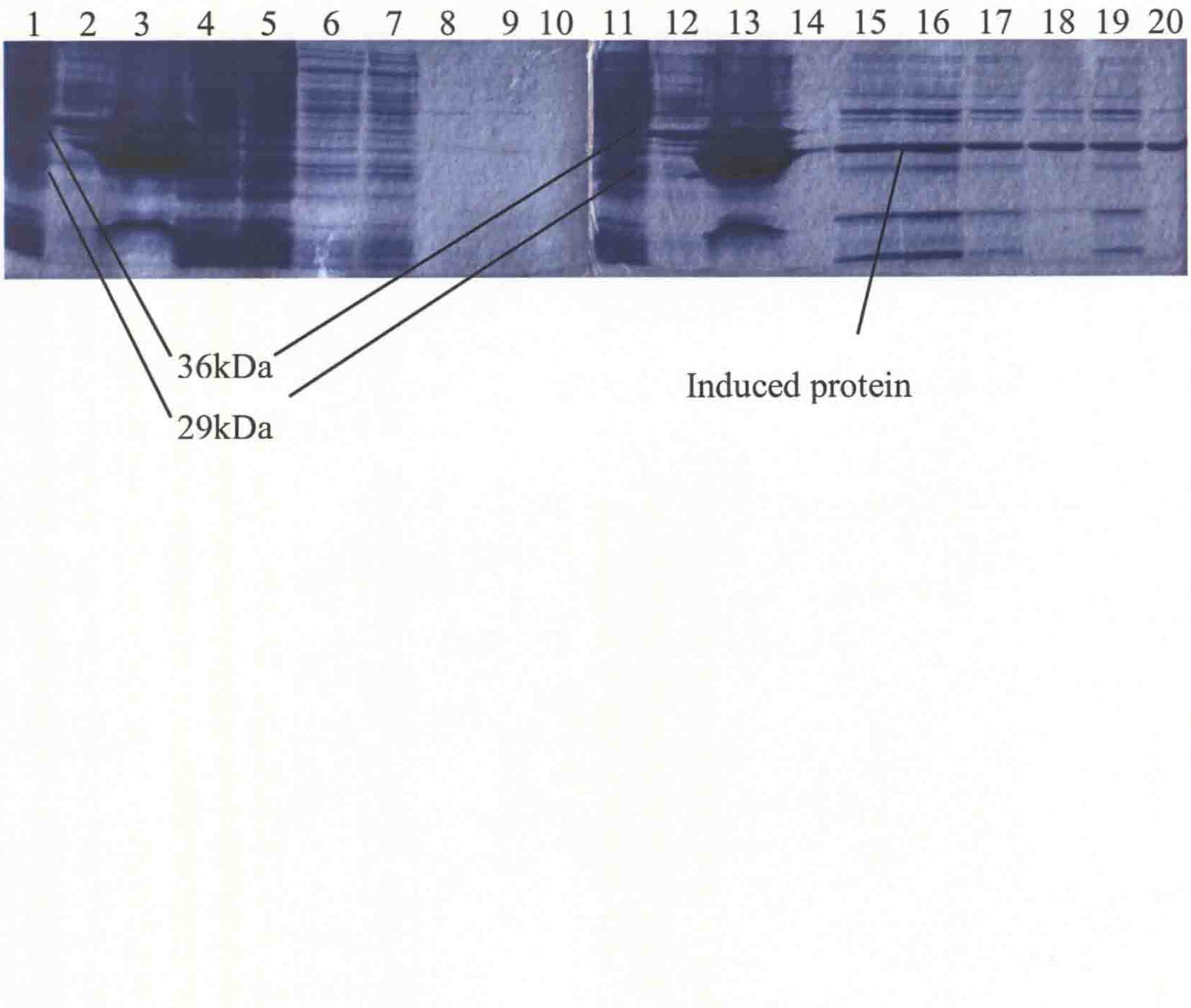
3.3.6 Renaturation on column

To allow a purification of the protein on a Ni-NTA matrix, the extraction was made under denaturing conditions. The clear lysate was loaded on the column. Attempts at renaturation were performed by using buffers with detergent (Triton-X100) and with decreasing concentrations of urea to end with an elution buffer containing 1M imidazole (Section 2.3.4). The analysis by SDS-PAGE of the fractions from every step of the process, revealed a band at a size corresponding to the one expected (33kDa), corresponding as well to the same band in the control for induced cells (Figure 3.14). Despite the several wash steps performed, contaminating proteins were present during the purification but the recombinant protein at 33kDa is the major band.

The advantage of this method is that even though it involves the denaturation of the protein, the urea is eliminated from the column during the washing steps and the elution does not involve low pH, as for normal purification under denaturing conditions. In order to use this purified protein in biotransformation, dialysis was performed (Section 2.3.5). SDS-PAGE performed from dialysed sample revealed the protein at 33kDa as expected, showing no degradation of the protein. Unfortunately, precipitation occurred during dialysis, making the protein unsuitable as it leads to the inactivity of the protein.

Figure 3.14: Renaturation on column

Transformation performed from BLR(DE3) with pETLipAHis. Extraction under denaturing conditions with buffer containing Tween and 4M urea. Renaturation was performed with decreasing concentration of urea buffers.
12% SDS-PAGE analysis with Coomassie blue staining
1, 11: Dalton Mark VII-L Mr marker
2, 12: induced cell samples, soluble fraction (20µl loaded), negative control
3, 13: induced cell samples, insoluble fraction (20µl loaded), negative control
4, 5: induced cells, flow-through fractions 1 and 2 (20µl loaded)
6, 7: induced cells, 4M urea buffer wash fractions (20µl loaded)
8, 9: induced cells, 2M urea buffer wash fractions (20µl loaded)
10, 14: induced cells, 0M urea buffer wash fraction (20µl loaded)
15, 16, 17, 18, 19, 20: induced cells, elution buffer fractions (20µl loaded)



3.4 Generation of pThioHisLipA construct

3.4.1 Construction of pThioHisLipA

In order to solubilise the recombinant lipase and allow large scale purification on Ni-NTA matrix, another expression system was chosen, the His-Patch ThiFusion Expression System (Invitrogen). Foreign genes inserted into the multiple cloning site of the expression vector, pThioHis are expressed as fusions to a modified version of the *E. coli* protein thioredoxin. The thioredoxin is an 11.7kDa protein found in yeast, plants and mammals, as well as in bacteria. When it is overexpressed in *E. coli*, thioredoxin is able to accumulate to approximately 40% of the total cellular protein. This means that high expression levels can be achieved with fusion protein. The thioredoxin moiety also appears to confer solubility to formerly insoluble heterologous proteins (Lavallie *et al.*, 1993).

The thioredoxin gene in pThioHis has been mutated to create a metal-binding domain in the thioredoxin protein. The glutamate residue at position 31 and the glutamine residue at position 63 were mutated to create histidine residues. When the resulting protein, His-Patch thioredoxin (HP-thioredoxin), folds the histidine at positions 31 and 63 interact with a native histidine at position 7 to form a "patch". This histidine patch was shown to have a high affinity for divalent cations. HP-thioredoxin proteins can therefore be purified on metal-chelating resin (Ni-NTA). High level expression of HP-thioredoxin fusion proteins is driven by the *trc* (*trp-lac*) promoter. The *trc* promoter contains the -35 region of the *trp* promoter together with the -10 region of the *lac* promoter. To regulate expression from the *trc* promoter, the gene encoding Lac repressor ($lacI^q$) is provided in the pThioHis vector. IPTG is used to induce

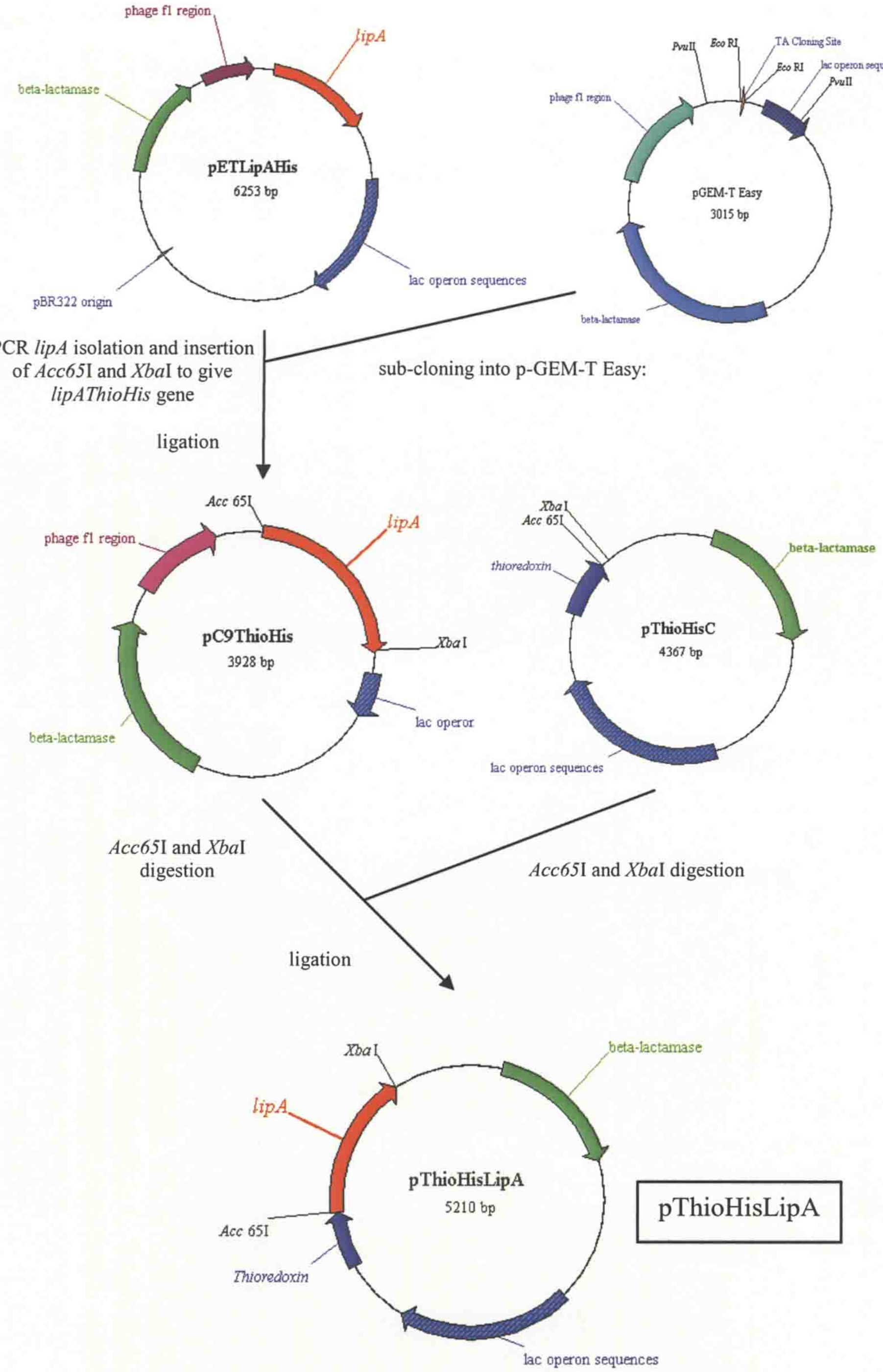
expression of the fusion protein. Translation is enhanced by the bacteriophage T7 gene 10 translation enhancer that provides a highly efficient initiation site for the translation of HP-thioredoxin.

For purification of the overexpressed protein, Ni-NTA matrix can be used. Release of recombinant proteins can also be performed due to the properties of thioredoxin. Native thioredoxin is localised at particular sites on the cytoplasmic side of the inner membrane known as adhesion zones. These sites create an "osmotically sensitive" cellular compartment. Thioredoxin is quantitatively released during osmotic shock into the shock fluid. HP-thioredoxin fusion proteins may localise to the adhesion zones, facilitating release into the medium for simplified purification. There is also an enterokinase cleavage site engineered into the pThioHis vector in a 30bp spacer between the modified *trxA* gene and the multiple cloning site. Enterokinase recognises the amino acid sequence Asp-Asp-Asp-Asp-Lys and hydrolyses the peptide bond between the lysine and the next amino acid. The activity of the native original protein can then be analysed without a potential effect from the thioredoxin.

Three different pThioHis were available. The sequences only differ from the position of the stop codon and the choice of different restriction enzymes. For our purpose, the most suitable vector was the pThioHis C vector (Appendix I and III). In this construct, the stop codon is situated just after a *Xba*I site which results in the insertion of only one amino acid, a leucine, to the sequence of the lipase.

An overview of the cloning steps leading to the construction of these plasmids is presented in the diagram on Figure 3.15. A PCR was performed, using the previous

Figure 3.15: Construction of pThioHisLipA



construct pETLipAHis as a template, to insert *Acc*65I and *Xba*I restriction sites on the *lipA* gene to produce the *lipAThioHis* gene using the oligonucleotides primers lipAThioHis 1 and 2 (Appendix II). As previously described, restriction sites were chosen to enable the digested PCR product to be ligated into the similarly digested plasmid vector, leaving the lipase sequence in frame with the promoter and the ATG start codon to express the thioredoxin and the lipase from the same open reading frame in the pThioHis C. The insertion of the *Acc*65I site corresponded to the enterokinase cleavage site, allowing the release of the lipase from the thioredoxin moiety after purification (Appendix III). For the 5' end of the gene, as explained previously, the insertion of a *Xba*I site involves the insertion of a leucine before the stop codon. As previously described, *Acc*65I and *Xba*I were chosen on the basis that they did not cleave the lipase coding sequence internally. The use of two restriction enzymes reduces the probability of re-circularisation of the plasmid.

A high fidelity PCR was performed using primers lipAThioHis 1 and lipAThioHis 2 (Appendix II) to insert *Acc65*I and *Xba*I restriction sites at both end of the *lipA* gene, annealing temperature was 66°C for 35 cycles using 0.5µg of pETLipAHis plasmid as a template. The PCR product was equivalent to the expected size of 911bp and named *lipAThioHis* gene (Figure 3.16). This gene was TA cloned in pGEM-T Easy (Promega). The recombinant plasmid was named pC9ThioHis (Figure 3.17) (Appendix I).

The pThioHis C (3µg) and pC9ThioHis (2µg) were digested with *Acc65*I and *Xba*I restriction enzymes (MBI) (15U for pThioHis C digestion and 10U for pC9ThioHis digestion). The digestion performed on the pThioHis C revealed one band on agarose

Figure 3.16: Isolation of *lipA* gene from pETLipAHis

PCR was performed with primers lipAThioHis 1 and lipAThioHis 2 from pETLipAHis. 10µl of each reaction was analysed by using 1% agarose gel, containing 0.6µg:µl ethidium bromide
1: Generuler 1Kb DNA Ladder (MBI Fermentas)
2: Negative control (no DNA)
3: *lipAThioHis* gene

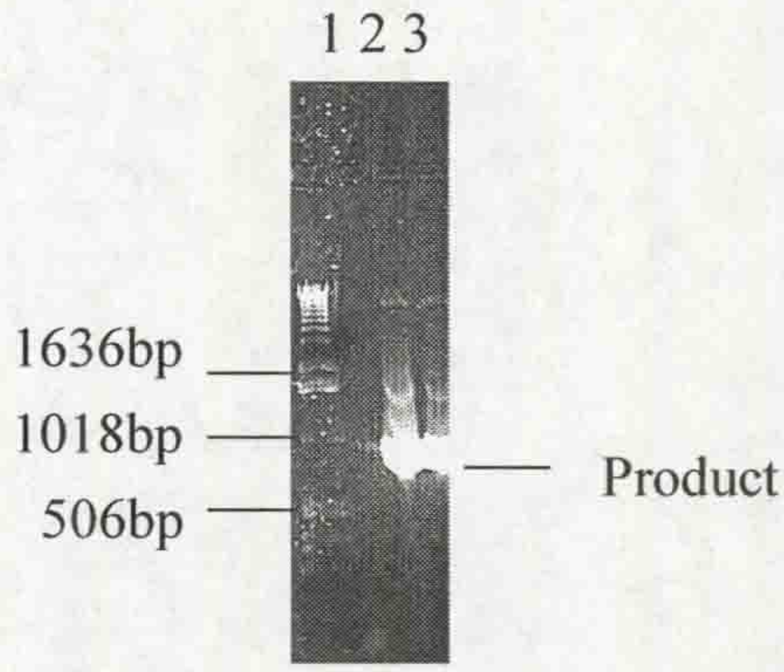
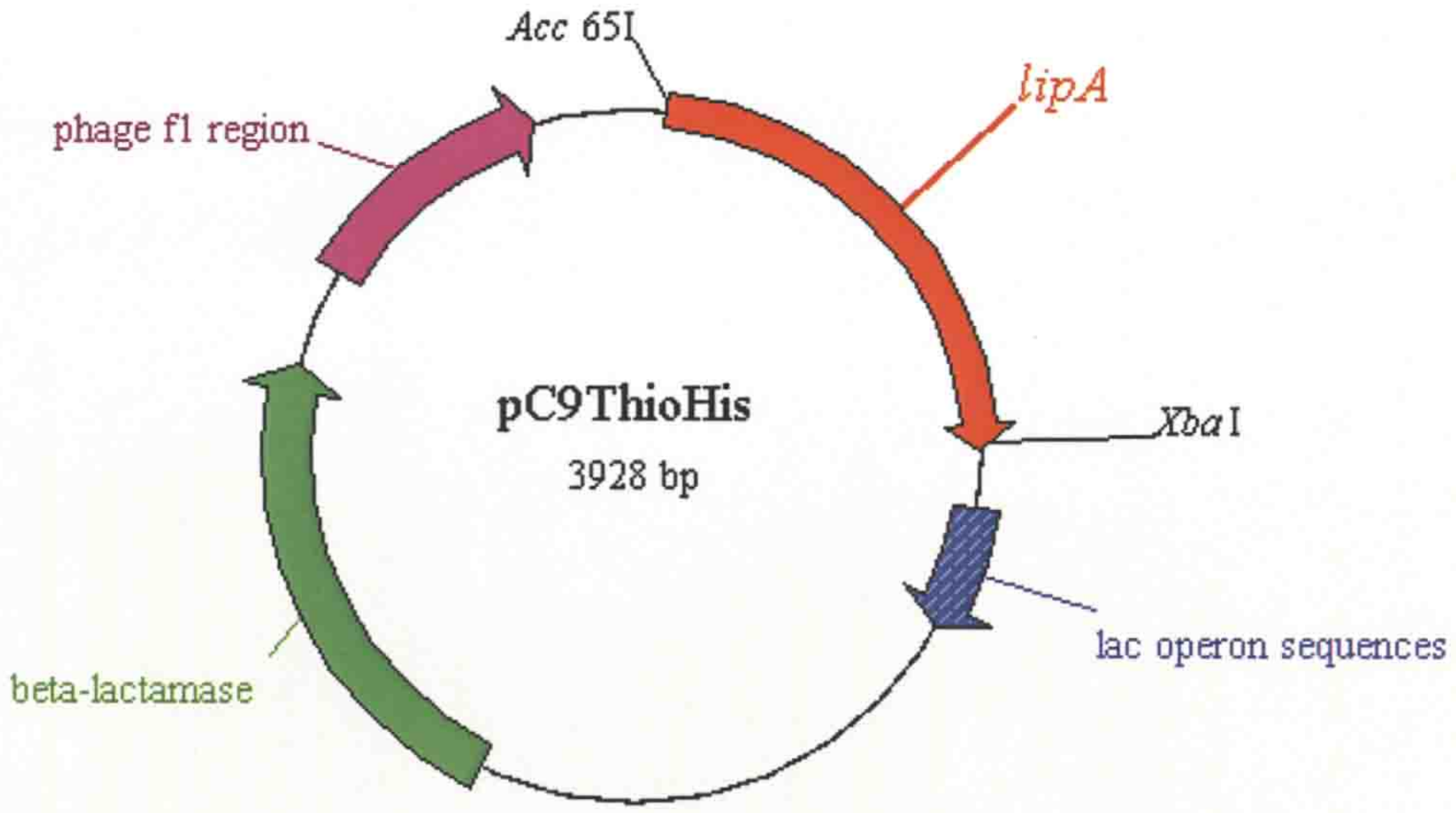


Figure 3.17: pC9ThioHis plasmid map



Important restriction enzymes sites:

*Acc65*I	69
*Ava*I	127
*BamH*I	909
*Nco*I	38
*Nde*I	1011
XbaI	962

Important features:

Phage f1 region	3293-3748
Beta-lactamase gene	2250-3110
Lac operon sequence	1079-1308
lipA gene	61-973

gel between 4kbp and 5kbp equivalent of the size expected of 4.4kbp (Figure 3.18). The digestion performed on the pC9ThioHis revealed one band of the expected 891bp (Figure 3.18).

The digested *lipAThioHis* gene was ligated into the digested pThioHis C by T4 DNA ligase. A 1:1 molar ratio of insert:vector was used (20µl final volume). The plasmid extracted from transformants was named pThioHisLipA (Appendix I). It was sequenced which confirmed that the expressed coding sequence was identical to the published one and that the reading frame had been correctly fused to the thioredoxin sequence and the stop codon (Figure 3.19).

3.4.2. Expression of pThioHisLipA and extraction of the recombinant lipase

The transformation of competent TOP10 *E. coli* cells with pThioHisLipA (Section 2.3.7.1) was highly effective. One clone induced by IPTG allowed the extraction of the induced lipase with a thioredoxin tag and a histidine patch under native conditions. The analysis by SDS-PAGE of the different fractions of the extraction process showed a novel band was revealed by blue staining in the insoluble fraction of the induced cells for all the different incubation times at the size expected, 45kDa. The HP-thioredoxin fusion protein is at least 12.8kDa (HP-thioredoxin plus the enterokinase site) larger than the fusion protein, 33kDa. This band was not present either in the soluble or insoluble fractions of the non-induced cells, meaning that overexpression has not occurred in the non-induced controls. It also revealed that the thickness of the band increased with increasing incubation times. This meant that the longest incubation time afforded the best overexpression (Figure 3.20). This pilot expression was like the solubility test performed previously during the expression of

Figure 3.18: Digestion of pThioHis C and pC9ThioHis by *Acc65I* and *XbaI*

30µl of digested pET22b and 20µl of pC9LipAHis and pC9LipANoHis were analysed by using 1% agarose gel, containing 0.6µg:µl ethidium bromide
1: Generuler 1Kb DNA Ladder (MBI Fermentas)
2: pC9ThioHis digested by *Acc65I* and *XbaI*
3: pThioHis C digested by *Acc65I* and *XbaI*

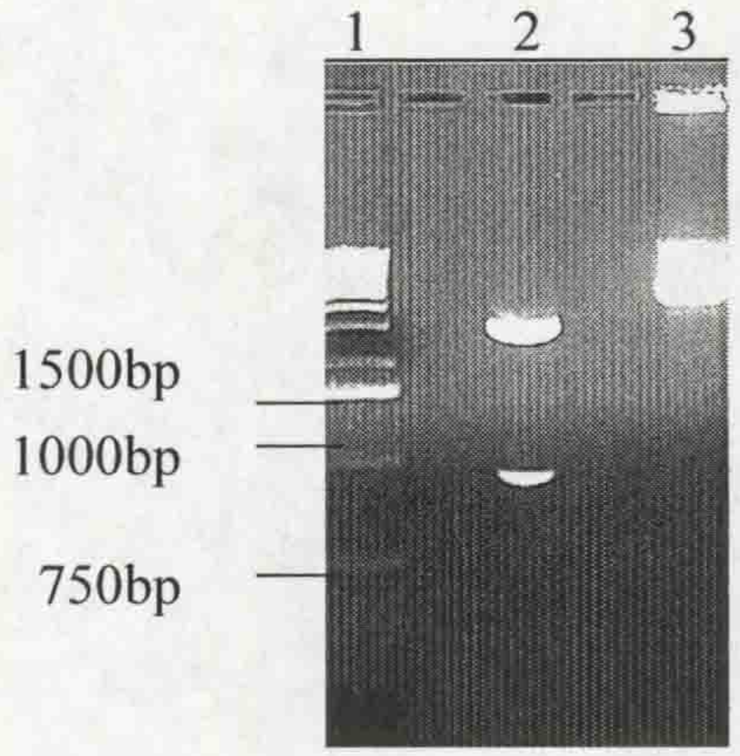
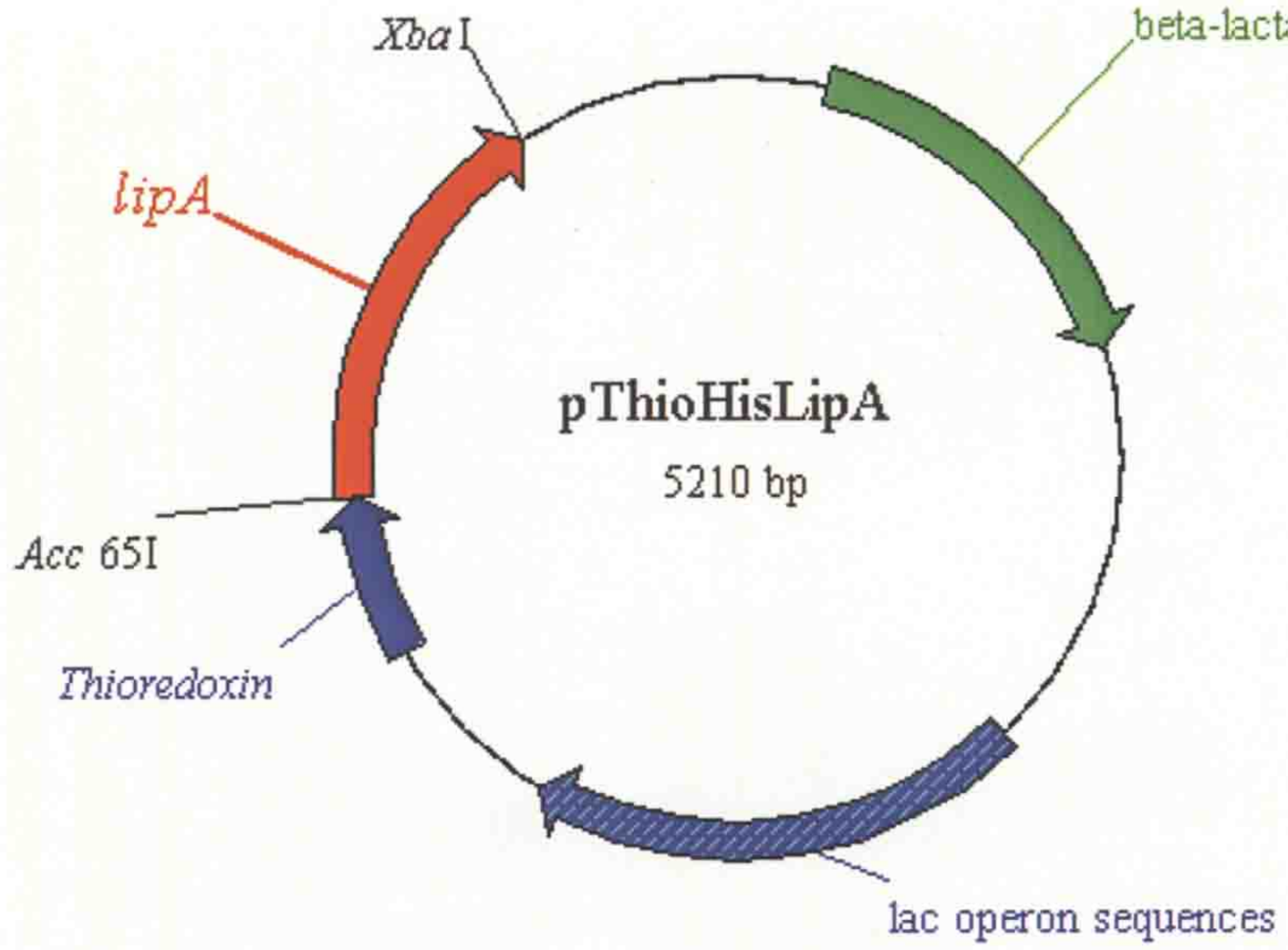


Figure 3.19: pThioHisLipA plasmid map



Important restriction enzymes sites:

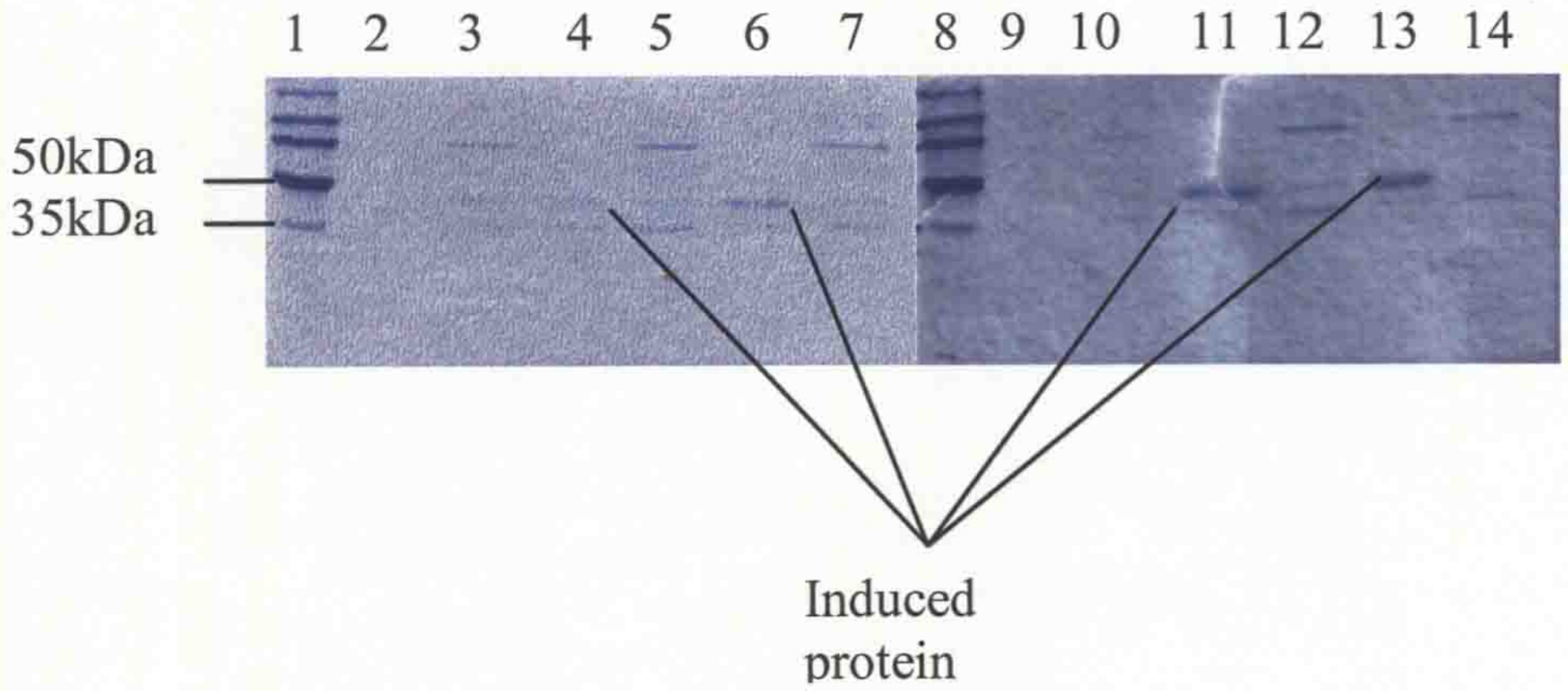
*Acc65*I	3828
*Ava*I	3663, 3886
*BamH*I	3805, 4668
*Nde*I	3470
*Xba*I	4721

Important features:

Phage f1 region	3289-3744
Beta-lactamase gene	201-1061
Lac operon sequence	1967-3049
lipA gene	3828-4720
Thioredoxin	3471-3828

Figure 3.20: Pilot expression of pThioHisLipA

Transformation performed from TOP10 *E. coli* with pThioHisLipA. Expression at 37°C with 1mM IPTG. Extraction under native conditions
12% SDS-PAGE analysis with Coomassie blue staining
1, 8: ProtoMetrics Recombinant Protein Markers (National Diagnostics)
2, 9: non induced cells samples, insoluble fraction (20µl loaded), negative control
3, 10: non induced cells samples, soluble fraction (20µl loaded), negative control
4: induced cells samples, expression for 1h, insoluble fraction (20µl loaded)
5: induced cells samples, expression for 1h, soluble fraction (20µl loaded)
6: induced cells samples, expression for 2h, insoluble fraction (20µl loaded)
7: induced cells samples, expression for 2h, soluble fraction (20µl loaded)
11: induced cells samples, expression for 3h, insoluble fraction (20µl loaded)
12: induced cells samples, expression for 3h, soluble fraction (20µl loaded)
13: induced cells samples, expression for 4h, insoluble fraction (20µl loaded)
14: induced cells samples, expression for 4h, soluble fraction (20µl loaded)



pETLipAHis and also analysed the clear lysate corresponding to the soluble proteins. Unfortunately, no overexpressed lipase band was visible in the clear lysate. This is not due to inadequate lysis as other bands, corresponding to other proteins can be seen in the soluble fractions and the recombinant lipase is the major constituent in the insoluble fractions. This indicates that even with the presence of the thioredoxin, the lipase has been pelleted with the insoluble fractions. This can be explained as described before, the overexpression of the protein leads to the formation of inclusion bodies by the bacteria.

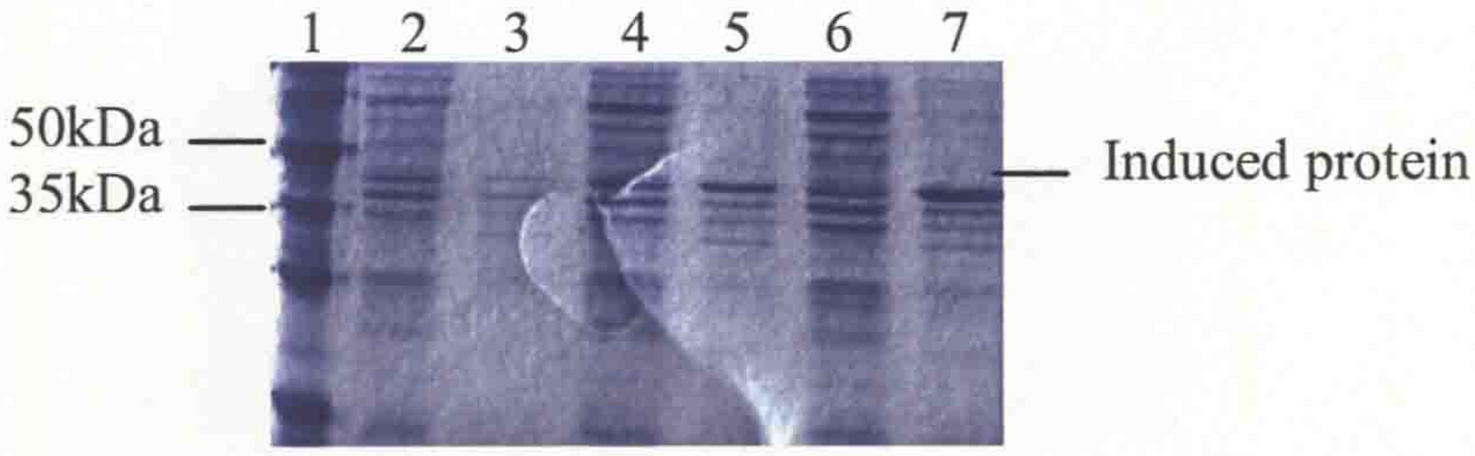
3.4.3 Optimisation of the expression

As the recombinant lipase was present only in the insoluble fractions of the extraction process, attempts to reduce the expression and the formation of inclusion bodies were performed as previously described. Reduced concentrations of IPTG, shorter times and lower temperatures were tested during the induction incubations (Section 2.3.7.1), as well as a buffer containing NaCl to minimise interactions with membranes.

A sample of each IPTG concentration, at each temperature and for each time was taken. The soluble and insoluble fractions for these samples were analysed by SDS-PAGE. Unfortunately, even with the lowest conditions of induction (2h at 30°C with 0.5mM IPTG) (Figure 3.21), the recombinant lipase was overexpressed and was visible in the insoluble fractions of the extraction. This indicates that even with low conditions for induction, the formation of inclusion bodies is inevitable.

Figure 3.21: Optimisation of expression

Transformation performed from TOP10 *E. coli* with pThioHisLipA. Extraction under native conditions after 1h and 2h incubation at 30°C with 0.5mM IPTG. Insoluble pellet was resuspended into native buffer.
12% SDS-PAGE analysis with Coomassie blue staining
1: ProtoMetrics Recombinant Protein Markers (National Diagnostics)
2: non induced cells samples, soluble fraction (20µl loaded), negative control
3: non induced cells samples, insoluble fraction (20µl loaded), negative control
4: induced cells, soluble fraction, 1h induction (20µl loaded)
5: induced cells, insoluble fraction, 1h induction (20µl loaded)
6: induced cells, soluble fraction, 2h induction (20µl loaded)
7: induced cells, insoluble fraction, 2h induction (20µl loaded)



3.4.4 Extraction by osmotic shock

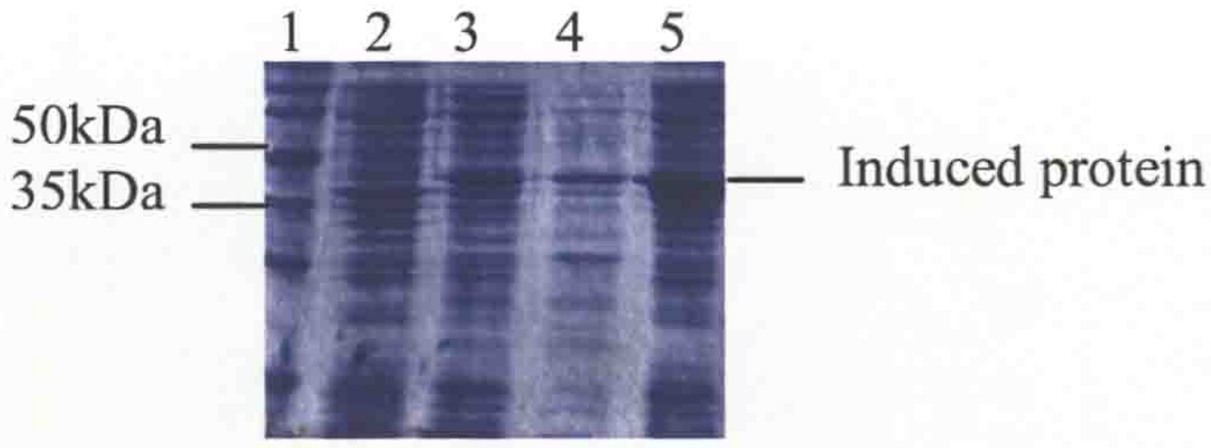
3.4.4.1 Small scale

As the previous protocol led to the formation of inclusion bodies from pThioHisLipA even with various conditions for incubation and as solubilisation of the protein of interest was not achieved, an alternative method was used, as advised in the manufacturer's protocol (Invitrogen). As thioredoxin is often localised to osmotically sensitive compartments, it can be quantitatively released by osmotic shock. Cells are osmotically shocked by transferring them from a high ionic strength buffer, containing sucrose to a low ionic strength buffer, without sucrose. The cell "shockate" is then fractionated into cells and shock fluid by centrifugation. The shock fluid corresponds to the protein released by osmotic shock where as the cell pellet still corresponds to the cell associated and insoluble proteins even with purification by osmotic shock.

Samples of shock fluid and cell pellet were taken and compared to soluble and insoluble fractions of non-induced cells submitted to the same treatment. These samples were analysed by SDS-PAGE (Figure 3.22). It was visible on the gel that compared to the non-induced cells a novel band appeared in the soluble and insoluble fractions of the extraction at the size expected (45kDa) and identical to the previous analysis. This indicates that the lipase was successfully overexpressed from pThioHisLipA and partially solubilised by osmotic shock according to its presence in the shock fluid.

Figure 3.22: Extraction of the recombinant lipase by osmotic shock

Transformation performed from TOP10 *E. coli* with pThioHisLipA. Extraction by osmotic shock. Cell pellet was resuspended into native buffer.
12% SDS-PAGE analysis with Coomassie blue staining
1: ProtoMetrics Recombinant Protein Markers (National Diagnostics)
2: non induced cells samples, shock fluid (20µl loaded), negative control
3: non induced cells samples, cell pellet fraction (20µl loaded), negative control
4: induced cells, shock fluid (20µl loaded)
5: induced cells, cell pellet fraction (20µl loaded)



3.4.4.2 Large scale

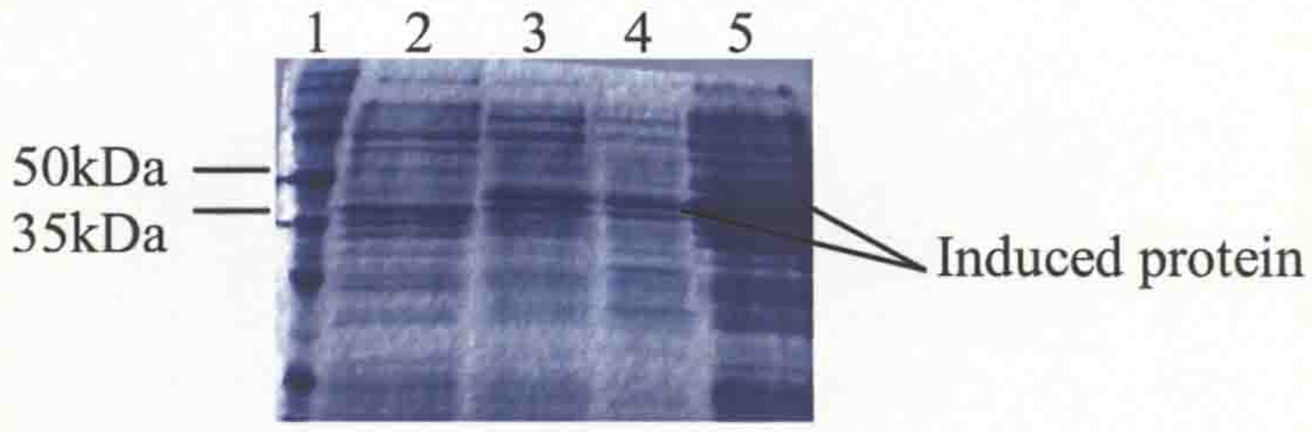
As mentioned in the manufacturer's protocol, purification by affinity chromatography cannot be performed from shock fluid. In order to test the activity of the recombinant lipase, the preparation parameters were increased to use 250ml LB inoculated with transformed bacteria. From this medium, 90ml of recombinant soluble lipase were collected, following the previous instructions. Samples from the different fractions were analysed as previously described (Figure 3.23) and gave the same results though the proportion of soluble recombinant lipase is still low and the majority of the expressed protein is associated with the cell pellet. From this shock fluid, experiments were performed with the substrate of interest to determine activity and selectivity of the recombinant lipase (Section 3.6.3).

3.5 Refolding lipase from pETLipAHis with glycerol

We were made aware of similar problems occurring with another lipase from *Pseudomonas glumae* (person contacted Pr Romas Kazlauskas) (Frenken *et al.*, 1993a; Frenken *et al.*, 1993b). During these studies, the lipase from *P. glumae*, secreted into the extracellular medium of the organism, was expressed and purified in *E. coli*. It was confirmed that to be active and correctly folded the lipase required the presence of a lipase-specific foldase (Lif) to adopt its three-dimensional structure and fold into its active and protease-resistant conformation. An attempt to refold the lipase was undertaken. The buffer used for this renaturation process contained 40% glycerol. Glycerol seems to create a non aqueous environment which enhances hydrophobic interaction within an unfolded protein (Rariy & Klibanov, 1997) and mimics the role of the Lif. By excluding water molecules, the folding intermediate of

Figure 3.23: Extraction of the recombinant lipase by osmotic shock

Transformation performed from TOP10 *E. coli* with pThioHisLipA. Extraction by osmotic shock. Cell pellet was resuspended into native buffer.
12% SDS-PAGE analysis with Coomassie blue staining
1: ProtoMetrics Recombinant Protein Markers (National Diagnostics)
2: non induced cells samples, shock fluid (20µl loaded), negative control
3: non induced cells samples, cell pellet fraction (20µl loaded), negative control
4: induced cells, shock fluid (20µl loaded)
5: induced cells, cell pellet fraction (20µl loaded)



the lipase may be more stable and the entire process may in that case lower the energy necessary to reach the final conformation.

In this experiment, expression and purification were performed as previously described (Section 2.3.8) using the pETLipAHis for expression and led to the purification of the protein as a single band (Figure 3.24) at the expected size (33kDa). The first three samples containing the eluted protein were then diluted 50 times in the glycerol buffer. To test the efficiency of the refolding step, the refolded enzyme was used in a biotransformation reaction (Section 3.6.4).

3.6 Biotransformation using the recombinant lipase

3.6.1 Use of recombinant lipase purified under native conditions

See figure 3.25 for molecules.

The biotransformations performed from the crude protein present in the soluble fraction of the first extraction under native conditions (Section 2.5.2.1) led to a conversion of the substrate 4-cyano-4(3',4'-dichlorophenyl) cyclohex-1-enylacetate (1) into ketone 4-cyano-4(3',4'-dichlorophenyl) cyclohexanone (2) (Figure 3.25) after 27h for the positive control involving the AK20 Amano lipase and for the 2.1ml final volume reaction mixture. The yield for the 2.1ml final volume reaction was 33% (t_R (R)-1 24.9min, (S)-1 29.8min), the e.e. value was 5% for the (R)-enantiomer and the E value was 1.5. From TLC, it was established that conversion started after 27h. A conversion into ketone was also observed on TLC for the 300µl final volume reaction but only after 70h and attempts to extract the product failed.

Figure 3.24: Purification of the recombinant lipase prior refolding

Transformation performed from TOP10 *E. coli* with pETLipAHis. Extraction under denaturing conditions and purification under native conditions.
12% SDS-PAGE analysis with Coomassie blue staining
1: ProtoMetrics Recombinant Protein Markers (National Diagnostics)
2: non induced cells samples, shock fluid (20µl loaded), negative control
3: induced cells, flow-through (20µl loaded)
4, 5: induced cells, wash fractions (20µl loaded)
6-13: induced cells, elution fractions (20µl loaded)

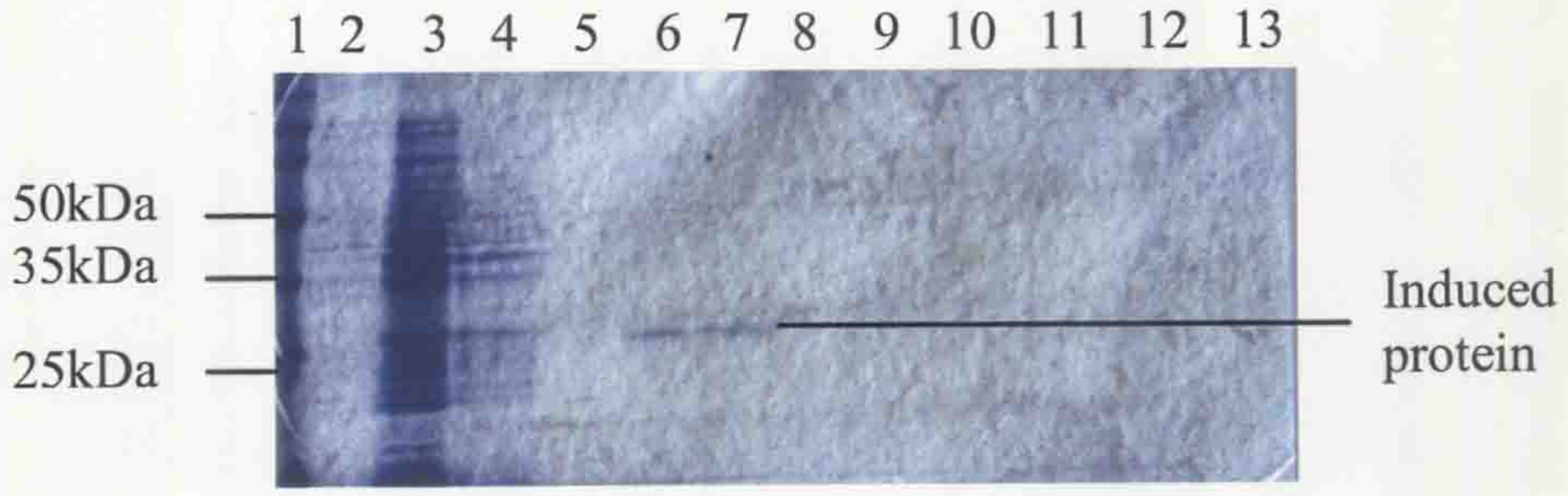
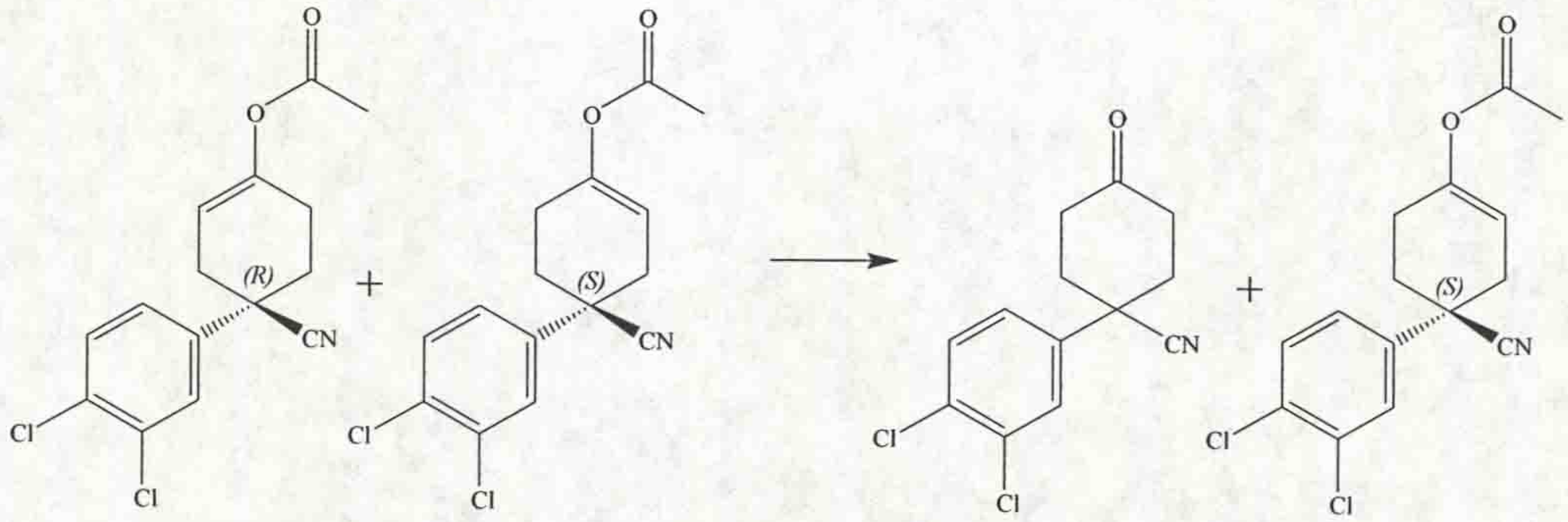


Figure 3.25: Typical biotransformation reaction

1: 4-cyano-4(3',4'-dichlorophenyl)cyclohex-1-enylacetate
2 : 4-cyano-4(3',4'-dichlorophenyl) cyclohexanone



1-(R) 1-(S) 2 1-(S)

These results showed that a conversion was achieved with the sample containing the recombinant lipase, proving the activity of the crude protein, on the biotransformation. As the reaction performed as a negative control, containing only buffer, did not lead to a reaction, the conversion of the enol acetate into ketone was only the result of the soluble crude protein activity. However, the selectivity for the (S)-enantiomer, as it was used in a larger amount than the (R)-enantiomer, was inverted to the one of interest though with a low e.e. From the SDS-PAGE gel analysis, the lipase was not even visible in this crude preparation, so the activity observed could be due to a co-action from other proteins present in this crude preparation. To be sure of the activity of the protein, it needs to be purified to remove most of the other proteins and reassayed.

3.6.2 Analysis of freeze-dried recombinant lipase from insoluble fractions

For all the biotransformations performed using the freeze-dried insoluble crude protein, collected from the insoluble fractions of the extraction (Section 2.5.2.1) no conversion of the racemic enol acetate (1) into ketone (2) could be detected, either for transesterification or hydrolysis, whatever the conditions (Sections 2.5.2.2.2, 2.5.2.2.3). TLC did not produce any spot corresponding to the ketone for any of the reactions. As the protein used was freeze-dried from insoluble protein crude, the results can be explained by the formation of inclusion bodies. In those conditions, the protein is denatured and does not possess any enzymatic activity. This indicates the importance of obtaining a soluble enzyme as the solubility seems to be linked with the activity.

3.6.3 Use of osmotic shock purified recombinant protein

The soluble protein obtained from purification by osmotic shock was tested on the same biotransformation (Section 2.5.2.3). The reaction was monitored by TLC. Unfortunately, as previously described, no conversion of the racemic enol acetate (1) into ketone (2) could be achieved whatever the conditions. This indicates that even if the lipase was soluble it did not regain its activity. Purification was performed where the recombinant lipase was the major protein in the insoluble fraction of the extraction. It is supposed that the protein was present in inclusion bodies and so denatured. Even if a minor part was released during the osmotic shock, it is highly probable that the lipase did not regain its activity through that process. This can explain the inefficiency of the recombinant protein to convert enol acetate (1) into ketone (2).

3.6.4 Use of the glycerol refolded recombinant protein

As all the attempts to solubilise the recombinant enzyme on native conditions failed and as all the other forms in which the protein was expressed lacked activity, it was necessary to solubilise the protein and refold it in an attempt to restore its activity. The lipase refolded by the glycerol buffer was tested in the reaction (Section 2.5.2.4). The results of conversion are presented below (Table 3.1). As predicted, no reactions were observed with the purified but denatured lipase or enzyme free control reactions. However, in the presence of butanol, conversion of racemic enol acetate (1) into ketone (2) did occur with the refolded enzyme. HPLC analysis showed a conversion into ketone (14 %) and the e.e. was 3% for the (R)-enantiomer (E = 1.5).

	Buffer				**THF**			**Toluene**		
Enol acetate	20mg				20mg			20mg		
Refolding buffer	10ml				10ml			10ml		
Solvent					10ml			10ml		
Butanol	_	2eq	_	2eq	2eq	_	2eq	2eq	_	2eq
Enzyme	_	_	Yes	Yes	_	Yes	Yes	_	Yes	Yes
Results (conversion into ketone, 2 spots on TLC)	No	No	No	Yes	Yes	Yes	Yes	No	No	No

Table 3.1: Biotransformations using the glycerol refolded recombinant lipase

Unfortunately, to allow refolding, the samples need to be diluted 50 times which make the whole process unsuitable for high throughput screening in microtitre plates. The high concentration of glycerol could also influence the reaction as it can be seen in samples with THF. In these experiments, without enzyme present in the mix, a conversion into ketone is occurring. The presence of glycerol is also a problem for freeze-drying as it cannot be removed by this method and dialysis lead to precipitation of the recombinant lipase as previously described.

However, these results are similar to the ones from biotransformation using the soluble crude protein (Section 3.6.1). This indicates that the refolded lipase possesses similar activity to the soluble crude extract. Unfortunately, this crude extract cannot be used in the perspective screen as it is at too low concentration to be feasible for large scale screening of libraries with any reliability and reproducibility.

3.7 Discussion

In conclusion, even if a new lipase from *P. fluorescens* was expressed and purified in *E. coli* and its activity was restored by a refolding step and it was promising for the biotransformation of interest, the whole process leading to these results demonstrated that the use of *P. fluorescens* C9 lipase is not suitable for directed evolution and high throughput screening. A decision was therefore taken to cease work on the lipase and to turn over attention to different enzyme.

Chapter 4

Pseudomonas fluorescens Esterase Studies

Chapter 4

Pseudomonas fluorescens Esterase Studies

4.0 Summary

The lipase from *P. fluorescens* proved to be an unsuitable choice of enzyme to use to create a library of recombinant enzymes and find a mutant which can improve the enantioselectivity of a hydrolase in the reaction of interest. In order to continue this, our interest then turned to the *P. fluorescens* esterase. This chapter covers the esterase expression and its use in different biotransformations involving the reaction of interest. The activity of this enzyme was optimised by testing various co-solvents of buffer and dimethylformamide (DMF) or dimethyl sulfoxide (DMSO), leading to the establishment of the best conditions for this reaction.

4.1 P. fluorescens esterase: expression and use

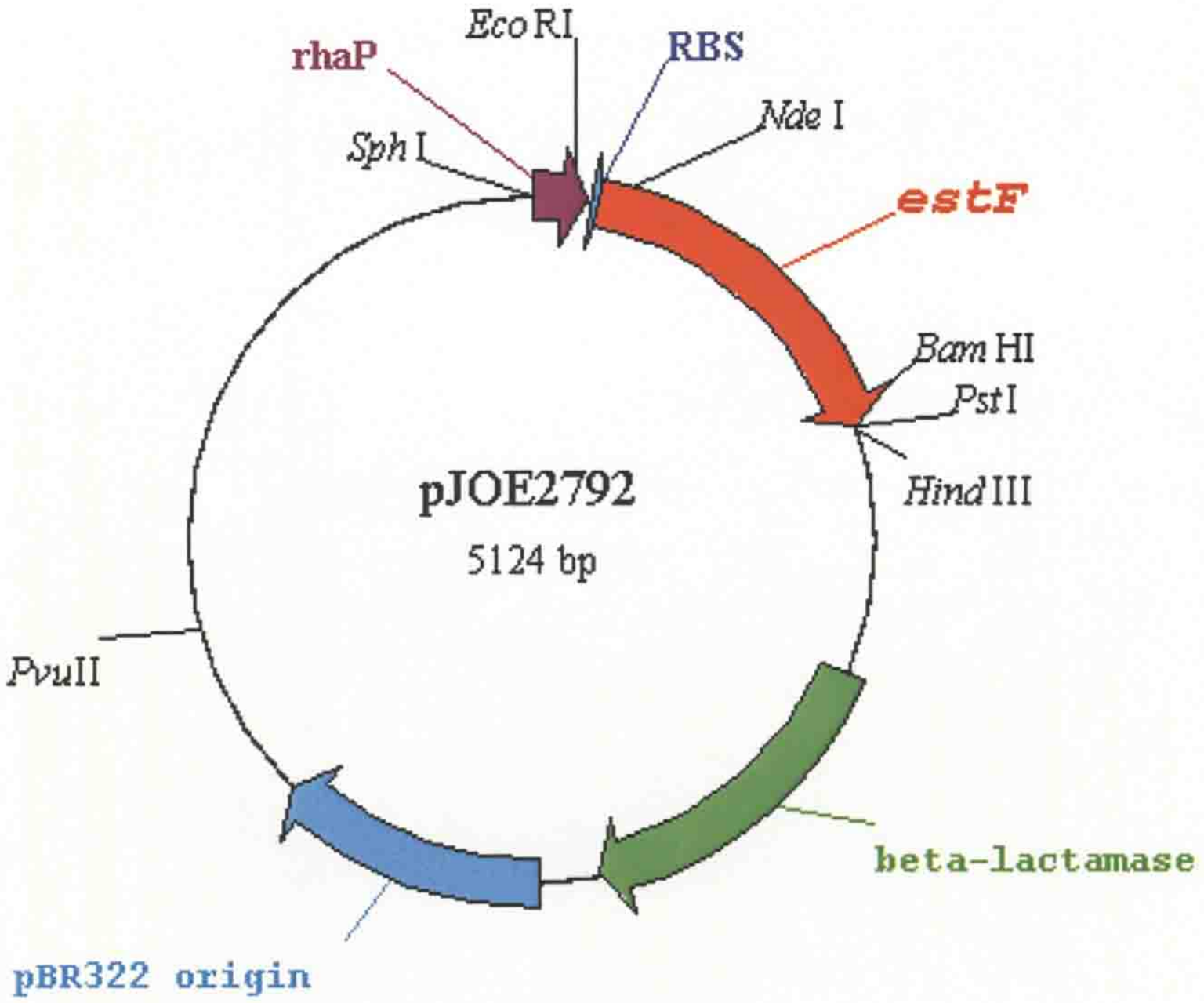
The esterase studied in this project was successfully cloned, expressed in *E. coli* and characterised (Choi *et al.*, 1990). The cloning sequence for the esterase gene was isolated from the strain *P. fluorescens* SIK WI and cloned into the plasmid pUC19 to create the plasmid pUE1251. This plasmid was used for further studies (Pelletier & Altenbuchner, 1995). This supports the expression of an esterase composed of two sub-units of 272 amino acids (homo-dimer) with a calculated molecular weight of 30kDa and an estimated molecular weight of 29.5kDa by SDS-PAGE analysis.

The plasmid pUE1251 was the source of the cloned esterase coding gene (*estF*). This was subcloned into another plasmid, pJOE2775, (Krebsfanger *et al.*, 1998). This

plasmid is a rhamnose-inducible expression vector. It possesses a sequence allowing the insertion of six his-codons (His-Tag) between the *BamH*I and *Hind*III sites, on the original plasmid used, pJOE2702 (Volff *et al.*, 1996). The esterase gene from pUE1251 was amplified by PCR, cut with *Nde*I and *BamH*I and cloned into pJOE2775 cut with the same enzymes to produce the plasmid pJOE2792 (Figure 4.1) (Krebsfanger *et al.*, 1998). An ampicillin marker gene was used to select for positive mutants. Expression of the recombinant esterase is under the control of a rhamnose inducible promoter. The insertion of a His tag coding sequence on the plasmid pJOE2775 allowed the insertion of 6 histidines at the end of the recombinant esterase for easy purification by affinity chromatography with Ni-NTA.

Previous expression of the gene by rhamnose induction had not resulted in the formation of inclusion bodies, compared to the *P. fluorescens* lipase in the previous chapter. The esterase was expressed in a soluble form in the extraction fraction containing the soluble protein. From this soluble protein crude, purification on Ni-NTA led to a homogeneous solution of recombinant esterase (Krebsfanger *et al.*, 1998). Crude protein and purified esterase were tested in different reactions in buffer and in organic solvents. Similar activities were observed for hydrolysis reactions but no activity of the purified enzyme could be observed in the presence of organic solvents (Krebsfanger *et al.*, 1998). This enzyme has been used in directed evolution experiments using error-prone PCR or mutator strain (Bornscheuer *et al.*, 1999) to increase activity and selectivity for the resolution of a sterically hindered 3-hydroxy ester.

Figure 4.1: pJOE2792 plasmid map



Important enzyme restriction sites:

*BamH*I	979
*EcoR*I	104
*Hind*III	1014
*Nde*I	161
*Pst*I	1010
*Pvu*II	3624
*Sph*I	6

Important features:

pBR322 origin	2545-3189
Beta-lactamase resistance gene	1610-2398
estF gene	162-1004
rhaP rhamnose promoter	3-133
RBS	147-154

In summary, this enzyme is easy to produce and is active in organic solvents when produced as a crude extract. It was used for directed evolution experiments and was therefore a perfect candidate for this project. As Pr. Bornscheuer is still working on this recombinant esterase, he was contacted and kindly provided the plasmid pJOE2792 (Appendix I), a sample of the wild-type freeze-dried enzyme and a protocol for expression.

The plasmid pJOE2792 provided by Pr. Bornscheuer was sequenced to confirm that the expressed coding sequence was identical to the published sequence (Pelletier & Altenbuchner, 1995) and possessed the His tag. It was sequenced with the primers pJOE2792 forward and pJOE2792 reverse (Appendix II). Sequence analysis confirmed the identity of the construct. Protein expression and purification resulted in bands by SDS-PAGE analysis of the expected size.

4.2 Testing of the wild-type esterase on the biotransformation

4.2.1 Freeze-dried PFE for transesterification and hydrolysis

The transesterification performed with the supplied freeze-dried esterase in toluene and described previously (Section 2.5.3.1) led to a conversion of the substrate 4-cyano-4(3',4'-dichlorophenyl) cyclohex-1-enylacetate (1) into 4-cyano-4(3',4'-dichlorophenyl) cyclohexanone (2). The reaction was followed over 3 days (Table 4.1).

Hours	% conversion	e.e.	E value
24	16	16 (S)	13.4
48	23	29 (S)	90
72	27	32 (S)	19

Table 4.1: % conversion, e.e. and E values for transesterification reaction with freeze-dried esterase.

Enol acetate (1) (20mg), benzyl alcohol (13.2µl), freeze-dried PFE (6.4mg), toluene (385µl). HPLC conditions (Section 2.5.2.5): (R)-1 25.3min, (S)-1 30.5min

These results showed that the conversion was achieved by using freeze-dried *P. fluorescens* esterase, indicating that the esterase is active on the substrate, 4-cyano-4(3',4'-dichlorophenyl) cyclohex-1-enylacetate (1), a new substrate for this enzyme. This reaction is only due to the presence of the esterase as the negative control did not show conversion on the biotransformation. The selectivity of the enzyme for the (S)-enantiomer was of interest and reached 32% after 3 days. This value is low but this showed a real selectivity for the (S)-enantiomer. The percentage conversion was low reaching 27% after 3 days. The anomalously high E value observed after 2 days is probably due to low conversion and e.e. values. However, from those encouraging results, further experiments were performed and the esterase was considered for improvement by directed evolution.

For the transesterification performed in 3ml toluene (Section 2.5.3.1), the results were 15% of conversion to ketone and 20% e.e. for the (S)-enantiomer, leading to an E

value of 19, after 3 days. The E values obtained were similar to the reaction in 385µl. Lower conversions and e.e.'s may be explained by the larger volume of reaction, which could limit the availability of the enzyme for the substrate.

The same experiments were performed to test hydrolysis reaction with the esterase (Section 2.5.3.1). As for the transesterification, the hydrolysis performed with the freeze-dried esterase in buffer led to the expected conversion. The reaction was followed over 2 days (Table 4.2).

Hours	% conversion	e.e.	E value
24	7	< 5 (R)	N/A
48	11	< 5 (R)	N/A

Table 4.2: % conversion, e.e. and E values for hydrolysis reaction with freeze-dried esterase.

Enol acetate (1) (20mg), freeze-dried PFE (6.4mg), sodium phosphate buffer pH7.5 (385µl). HPLC conditions (Section 2.5.2.5): (R)-1 25.2min, (S)-1 30.5min

These results showed that hydrolysis of the substrate, was achieved using freeze-dried *P. fluorescens* esterase. As mentioned previously, this reaction is only due to the presence of the esterase as the negative control did not show conversion on the biotransformation. Unfortunately, the esterase showed very poor selectivity under these conditions and the results were not reproducible.

4.2.2 Comparison between AK20 PFL and PFE

As the esterase results for transesterification were similar in terms of E value to those obtained with the AK20 PFL from Amano, the same experiments were performed with this enzyme to compare the activity of the two enzymes.

For the reactions with the lipase (Section 2.5.3.4), HPLC analysis showed 100% conversion (t_R 27.4min) compare to 7% of conversion of the racemic enol acetate into ketone after 1 day for the esterase. This indicates than even if the activity and reactivity looked similar, the difference between the AK20 PFL and the PFE is on the overall rate of the reaction. This may be explained by the structural differences between the two enzymes.

4.2.3 Use of induced whole cell extract on transesterification and hydrolysis

The transesterification was performed with induced whole cells from *E. coli* DH5α transformed with pJOE2792 (Section 2.5.3.2.1) in toluene (Section 2.5.3.2.2). The experiment was performed to show if an increase in activity and selectivity could be observed with whole cells and if the esterase could be accessible to the substrate. The experiment led to conversion of the enol acetate (1) into the ketone (2). The reaction was followed over 3 days (Table 4.3).

Hours	% conversion	e.e.	E value
24	0	0	0
48	9	< 5 (R)	N/A
72	13	< 5 (R)	N/A

Table 4.3: % conversion, e.e. and E values for transesterification reaction with induced whole cells expressing the recombinant esterase.

Enol acetate (1) (78mg), benzyl alcohol (51.5µl), induced whole cells in toluene (1.5ml) (Section 2.3.5.2.2), toluene (1ml). HPLC conditions (Section 2.5.2.5): (R)-1 25.2min, (S)-1 30.4min

These results demonstrated that the conversion of enol acetate (1) into ketone (2) occurred. This reaction is only due to the presence of the esterase in induced cells as the negative control in which non induced cells were used did not show conversion. However, the activity and selectivity were poor and the results not reproducible. This may be explained as the solution of induced whole cells in toluene was viscous and it is possible that the substrate was unable to access the enzyme. The use of whole cells in toluene was therefore not pursued.

The same experiments were performed to test for hydrolysis with induced whole cells resuspended in a biphasic solution buffer (Section 2.5.3.2.1) and toluene (Section 2.5.3.2.2). The experiment led to a conversion of the substrate enol acetate (1) into ketone (2) over 3 days (Table 4.4).

Hours	% conversion	e.e.	E value
24	8	< 5 (R)	N/A
48	25	12 (S)	2.4
72	31	34 (S)	10

Table 4.4: % conversion, e.e. and E values for hydrolysis reaction with induced whole cells expressing the recombinant esterase.

Enol acetate (1) (78mg), induced whole cells in phosphate buffer pH7.5 (1.5ml) (Section 2.3.5.2.2), toluene (1ml). HPLC conditions (Section 2.5.2.5): (R)-1 24.7min, (S)-1 29.7min

These results showed that hydrolysis was possible with induced whole cells expressing the esterase and with the negative control showing no conversion. The efficiency of the reaction was higher than for the transesterification with conversion reaching 31% after 3 days. The reaction also showed selectivity for the (S)-enantiomer giving an e.e. of 34% after 3 days. In this case, the mobility and accessibility of the cells in the buffer was not a limiting factor and the use of a co-solvent system (buffer and toluene) may explain the improved results compared to the transesterification. Toluene was chosen according to previous work (Bornscheuer *et al.*, 1999; Krebsfanger *et al.*, 1998). The substrate showed a relatively good solubility in toluene but other solvents were tried (Sections 2.5.3.3.2 and 2.5.3.3.3) in an attempt to increase the amount of soluble substrate available for the esterase.

4.2.4 Hydrolysis with crude protein

4.2.4.1 Range of substrate concentrations

In order to improve the activity and selectivity of the esterase and optimise conditions for high-throughput screening, a range of substrate concentrations was tested. The use of crude protein (Section 2.4.1.1), in which the induced esterase is the major constituent, gave equivalent results to purified protein (Bornscheuer *et al.*, 1999) for the hydrolysis reaction. The crude protein showed an activity of 95U.ml^{-1} as determined with *p*-nitrophenyl acetate (Section 2.4.1). Substrate solutions (1, 2, 5 and 10mM) were tested (Section 2.5.3.3.1) and the reactions analysed after 3 days of reaction (Table 4.5).

Substrate concentrations	% conversion	e.e.	E value
1mM substrate	8	0	N/A
2mM substrate	8	< 5 (R)	N/A
5mM substrate	16	9 (S)	3
10mM substrate	7	< 5 (R)	N/A

Table 4.5: % conversion, e.e. and E values for hydrolysis reactions with a range of substrate concentration and with crude recombinant esterase protein.

Crude protein in phosphate buffer pH7.5 (1ml, 95U) (Section 2.5.3.3.1). HPLC conditions (Section 2.5.2.5): (R)-1 24.7min, (S)-1 30.1min

Conversions and selectivities were low under these conditions. Even for the best reaction with 5mM substrate, the results were difficult to reproduce. The limited substrate solubility in buffer may explain these results.

4.2.4.2 Dimethylformamide (DMF) as a co-solvent

To improve the solubility of the substrate for the biotransformation, DMF was included as a co-solvent and range of concentrations were tested (1 to 5%) (Section 2.5.3.3.2). The reactions were analysed after 3 days (Table 4.6).

DMF Concentration	% conversion	e.e.	E value
1% DMF	9	10 (S)	200
2% DMF	22	29 (S)	95
3% DMF	8	10 (S)	16
4% DMF	38	9 (S)	1.5
5% DMF	39	9 (S)	1.4

Table 4.6: % conversion, e.e. and E values for hydrolysis reactions with a range of DMF concentrations and with crude recombinant esterase protein.

Enol acetate (1) (5mM), crude protein in phosphate buffer pH7.5 (950µl, 90U) (Section 2.5.3.3.2), phosphate buffer pH7.5 (to reach 1ml). HPLC conditions (Section 2.5.2.5): (R)-1 24.7min, (S)-1 30.1min

The results were more impressive with DMF than with buffer only although the result for 3% DMF was anomalous. However, the substrate was still largely insoluble in DMF and the results were difficult to reproduce reliably, as significant changes in selectivity were observed. The conversion improved at higher DMF concentrations at the expensed of selectivity.

4.2.4.3 Dimethyl sulfoxide (DMSO) as a co-solvent

As the reaction involving DMF (Section 4.2.4.2) showed improvements in the conversion, the same experiments were tried with DMSO, to improve the solubility of the substrate. Tests were performed to determine the saturation limit of the substrate in DMSO. When heated at 50°C, the racemic enol acetate (1) is soluble at a concentration of 0.2M in DMSO. Biotransformations were performed with 5% DMSO and 10mM final substrate concentration as previously described (Section 2.5.3.3.3). Five identical reactions were analysed after 4 days (Table 4.7).

	% conversion	e.e.	E value
Reaction 1	75	< 5 (S)	N/A
Reaction 2	76	< 5 (S)	N/A
Reaction 3	78	< 5 (S)	N/A
Reaction 4	93	< 5 (R)	N/A
Reaction 5	82	< 5 (S)	N/A

Table 4.7: % conversion, e.e. and E values for hydrolysis reactions with 5% DMF concentration and with crude recombinant esterase protein.

Enol acetate (1) (10mM), crude protein in phosphate buffer pH7.5 (200µl, 19U) (Section 2.5.3.3.3), DMSO (5%), phosphate buffer pH7.5 (to reach 1ml). HPLC conditions (Section 2.5.2.5): (R)-1 25.0min, (S)-1 30.2min

The reaction with DMSO resulted in conversions reaching 93%. These results were also reproducible compared to previous experiments in buffer and DMF. Unfortunately, no selectivity could be achieved under these conditions. However, the

overall aim was to improve the selectivity of the enzyme. The reproducibility indicates that the substrate was distributed more efficiently than before. As the substrate was soluble in DMSO, the amount introduced in each reaction was the same, contrary to previous experiments were the relative insolubility of the substrate may have led to variations in substrate concentration and therefore less reproducibility.

The same experiments were then reproduced with different DMSO concentrations, 7.5%, 10%, 15%, 20% and 30% (Section 2.5.3.3.3). HPLC analyses were performed after 5 days (Table 4.8).

DMSO concentration	% conversion	e.e.	E value
7.5% DMSO (15mM substrate)	96	< 5 (R)	N/A
10% DMSO (20mM substrate)	87	42 (R)	1.5
15% DMSO (30mM substrate)	86	51 (R)	1.7
20% DMSO (40mM substrate)	85	48 (R)	1.7
30% DMSO (60mM substrate)	43	< 5 (S)	N/A

Table 4.8: % conversion, e.e. and E values for hydrolysis reactions with different concentrations of DMSO and with crude recombinant esterase protein.

Crude protein in phosphate buffer pH7.5 (200µl, 19U) (Section 2.5.3.3.3), phosphate buffer pH7.5 (to reach 1ml). HPLC conditions (Section 2.5.2.5): (R)-1 25.1min, (S)-1 30.3min

Results indicate that the conversion of enol acetate (1) into ketone was higher and similar over the concentration range 10-20%. At 30% DMSO, the conversion dropped to 43%. These results were encouraging and reproducible. Regarding selectivity, results were variable with almost no selectivity observed for the 30% DMSO reaction. As the conversion was high for DMSO concentrations below 20%, the selectivity (E) could not be accurately determined. For 30% DMSO concentrations, conversion was high enough for these conditions to be considered for further experiments.

To optimise the concentration of DMSO needed in the biotransformation reaction, DMSO concentrations between 20% and 30% were tested (Section 2.5.3.3.3). The reaction time was also decreased to 4 days to limit conversion and allow accurate determination of selectivity. HPLC analyses were performed after 4 days (Table 4.9).

DMSO concentration	% conversion	e.e.	E value
22.5% DMSO (45mM substrate)	26	6 (R)	1.5
25% DMSO (50mM substrate)	44	< 5 (R)	N/A
27.5% DMSO (55mM substrate)	42	< 5 (R)	N/A

Table 4.9: % conversion, e.e. and E values for hydrolysis reactions with different concentrations of DMSO and with crude recombinant esterase protein.

Crude protein in phosphate buffer pH7.5 (200µl, 19U) (Section 2.5.3.3.3), phosphate buffer pH7.5 (to reach 1ml). HPLC conditions (Section 2.5.2.5): (R)-1 24.1min, (S)-1 28.8min

The four day incubation period resulted in conversions between 26% and 44%. This time period is better for application in high-throughput screening. The conversion reached 44% with a DMSO concentration of 25% with selectivity similar to that observed with 27.5% DMSO. The fact the conversion and selectivity were slightly lower for 27.5% DMSO, was expected by comparison with previous results where both were decreasing with increase in DMSO concentration. However, the conversion for 22.5% DMSO was surprisingly low with slightly improved selectivity. By comparison with previous results, the loss of products during extraction was a more likely explanation than a real difference in the kinetics of the reaction.

As mentioned previously, the selectivity of the enzyme was directed towards the (S)-enantiomer of enol ester (1). The e.e. was low and the aim was to use directed evolution to increase and reverse the selectivity towards the (R)-enantiomer. Following these results, a concentration of 25% DMSO was used in further biotransformations as conversion and selectivity for this concentration gave the best compromise.

4.2.5 Time course of esterase reaction using DMSO as a co-solvent

In order to study more precisely the biotransformation using the esterase, the reaction was followed during 4 days with different concentrations of DMSO (Section 2.5.3.3.4). HPLC analyses were performed during the 4 days (Table 4.10).

DMSO	Days	% conversion	e.e.	E value
10%	1	39	5 (R)	1.2
	2	54	26 (R)	2
	3	66	30 (R)	2
	4	69	4 (R)	1
20%	1	24	<5 (R)	N/A
	2	27	11 (R)	2
	3	36	<5 (R)	N/A
	4	36	<5 (R)	N/A
30%	1	12	<5 (R)	N/A
	2	20	<5 (R)	N/A
	3	27	<5 (R)	N/A
	4	43	5 (R)	1

Table 4.10: Kinetics for hydrolysis reactions with recombinant esterase protein crude (% conversion, e.e. and E values).

Enol acetate (1) (20mM, 40mM, 60mM), crude protein in phosphate buffer pH7.5 (200µl, 19U) (Section 2.5.3.3.4), phosphate buffer pH7.5 (to reach 1ml). HPLC conditions (Section 2.5.2.5): (R)-1 27.3min, (S)-1 33.5min

The results showed that the fastest reaction occurred with a concentration of 10% DMSO as expected and as observed previously. These results showed an increase of conversion with the time of reaction and an increase of the selectivity until the conversion reached 60%. For 20% DMSO, the reaction rate was lower and the

selectivity much reduced. The decrease in selectivity may be explained by the increase of DMSO concentration. For 30%, overall conversion increased with time and selectivity was again lower than with 10% or 20% as expected.

All these results show that the higher the concentration of DMSO, the slower is the rate of reaction and selectivity. However, the higher the concentration of DMSO, the higher the concentration of substrate in the reaction mixture. Even if good conversions and e.e.'s could be achieved with 10% DMSO, the amount of substrate present needs to be increased for accuracy and analysis during high-throughput screening in microtitre plates. Reactions performed previously with 25% DMSO showed promising results after 4 days, close to those observed with 30% DMSO. From these results, the DMSO concentration chosen for screening was 25%.

4.3 Discussion

The excellent yield of protein expression and its solubility in buffer confirmed that the *P. fluorescens* esterase is a good candidate for alteration of enantioselectivity. In order to decide if this enzyme was the perfect candidate for the directed evolution process, tests on the reaction of interest, involving the substrate enol acetate (1), needed to be performed. These tests and all the reactions performed allowed establishing the optimal conditions to carry out this reaction with the esterase. The use of DMSO was proven to highly increase the reproducibility of the results by helping to solubilise the enol acetate (1) and the availability of this substrate towards the esterase. From these results, the *P. fluorescens* esterase was considered to be an ideal candidate in this project. Before creating a mutant library, it was necessary to develop a protocol to facilitate screening of the mutant esterase library.

Chapter 5

Development of High-Throughput Screening

Chapter 5

Development of High-Throughput Screening

5.0 Summary

The intention was to use a mutation strategy that would involve the generation of a large number of mutants. To be able to analyse all these mutants a fast and accurate protocol to allow the screening of thousands of mutants was needed. This chapter describes the development of the screening protocol.

5.1 Plate assay screen

As the *P. fluorescens* esterase was a good candidate to be used in directed evolution, a high-throughput screening procedure, allowing the identification of interesting mutants needed to be developed. This screening needed to be easy to perform to screen thousands of mutants quickly. Various methods of screening had been developed for PFE (Bornscheuer *et al.*, 1998; Henke & Bornscheuer, 1999; Horsman *et al.*, 2003; Janes *et al.*, 1998). Unfortunately, screening methods are often substrate specific and cannot be applied to other reactions.

5.1.1 Enol acetate (1) and acetic acid

For experiments involving hydrolysis of racemic enol acetate (1), the use of pH indicators to reveal colour was envisaged (Bornscheuer *et al.*, 1999). As the reaction (Figure 3.1) releases acetic acid, pH indicator neutral red, present in the media, should colour the cells red if they possess esterase activity. In our hands, use of

different volumes and concentrations of substrate spread on agar plates (Section 2.6.1.1) produced no change in colour in the presence of indicators. For all experiments, colonies grew on the plate after 4 days, indicating that the substrate was not toxic to the cells. However, apparently no reaction occurred as no colour change was observed. This may be explained by the natural insolubility of the substrate. In this reaction, the substrate needs to diffuse through the bacterial membrane to the cytoplasm where the esterase is produced in response to the inducer (rhamnose) in the media. Even if the substrate could potentially diffuse through the membrane, due to its insolubility its presence in the cytoplasm is improbable. Although the reaction occurred with whole cell extracts (Section 4.2.3), this was performed in liquid, which allows a better mix of the reactants.

Following the results of biotransformation using DMSO to solubilise the substrate, attempts to use DMSO to provide a more soluble substrate in the plate test were made. Racemic enol acetate (1) solubilised in DMSO was poured directly into molten agar (Section 2.4.1.3). The substrate was well distributed in the agar but precipitated as white needles. After 2 days of incubation, cells grew on all the plates even with 15% DMSO present in the media, but no change in colour could be observed. In order to release the induced protein, cells were lysed by freeze-thaw cycles but, as before, even after 3 more days of incubation no colour change could be observed.

Experiments performed on LA plates with rhamnose and pH indicator and using filter paper soaked into substrate solution, in order to improve the accessibility of the substrate to the enzyme (Section 2.6.1.3), did not show a colour change whatever the incubation time. These results confirmed that the substrate was too insoluble to react

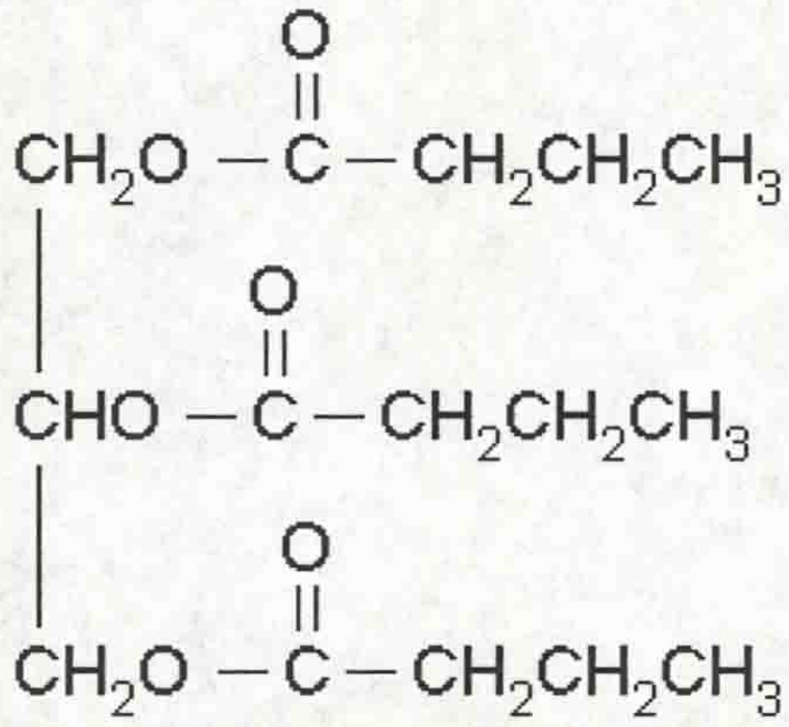
on agar plates. Even with DMSO present to increase solubility, no reaction could be observed. Therefore, no agar plate assay directly involving the substrate of interest could be developed.

Experiments were performed to check if the cells could survive using acetic acid as the sole carbon source. From previous studies (Bornscheuer *et al.*, 1999), the release of glycerol from an esterification reaction was used as the carbon source to select for positive mutants. The release of acetic acid from active clones, possessing an esterase activity on the substrate, would allow positive colonies to be selected. Colonies indeed appeared on minimal media with acetic acid (Section 2.6.1.1), indicating that *E. coli* utilise this acetic acid as a carbon source. Unfortunately, as described above, since hydrolysis of the enol ester did not occur, this plate screen was not suitable.

5.1.2 Tributyrin

Several approaches were taken using tributyrin (Figure 5.1) to characterise the *P. fluorescens* esterase activity (Choi *et al.*, 1990; Pelletier & Altenbuchner, 1995). Spreading tributyrin solution in LB or minimal media or even the direct addition of tributyrin in the molten agar, which led to the dispersion of small drops of this compound in the media (Section 2.6.1.2) did not support bacterial growth after 7 days. Another attempt was performed by directly spreading the transformed bacteria on these plates. After 4 days, colonies appeared on the plates for bacteria transformed with pJOE2792 and pETLipAHis. However, no consumption of tributyrin could be observed from the plate as no clear halo appeared around these colonies. The growth was not specific to the expression of esterase activity. Therefore minimal media could

Figure 5.1: Tributyrin molecule



not be used for further experiments as no difference could be observed between growth of bacteria transformed with pJOE2792 or with pETLipAHis.

When the tributyrin solution or pure tributyrin (Section 2.6.1.2) was spread on LA plates both led to the growth of bacteria but no obvious effect of the use of tributyrin could be observed around colonies, making the process unsuitable for a screening purpose. The same experiment made with minimal media and tributyrin poured in agar were performed with LA (Section 2.6.1.2). When bacteria were spread from liquid culture, growth was observed after 6 days. However, no difference could be observed between bacteria transformed with pJOE2792 or pETLipAHis. When transformed bacteria were directly spread on LA plates, growth could be observed as well for minimal media and LA in which tributyrin was poured.

When transformants containing the pJOE2792 were spread on this media, a clear halo was observed after 2 days, around the resultant colonies, this corresponds to a zone of tributyrin clearing. These halos could not be observed for bacteria transformed with pETLipAHis, indicating the protein was induced by rhamnose and allowed the expression of an active esterase, which consumed the tributyrin added to the media. Identical results were observed with *E. coli* JM109. This represented a primary screening strategy allowing the identification of mutants, which did not lose the esterase activity during the mutation process and therefore decrease the number of mutants to further characterisation against the racemic enol acetate (1).

5.2 Screen using dehydrogenase enzymes

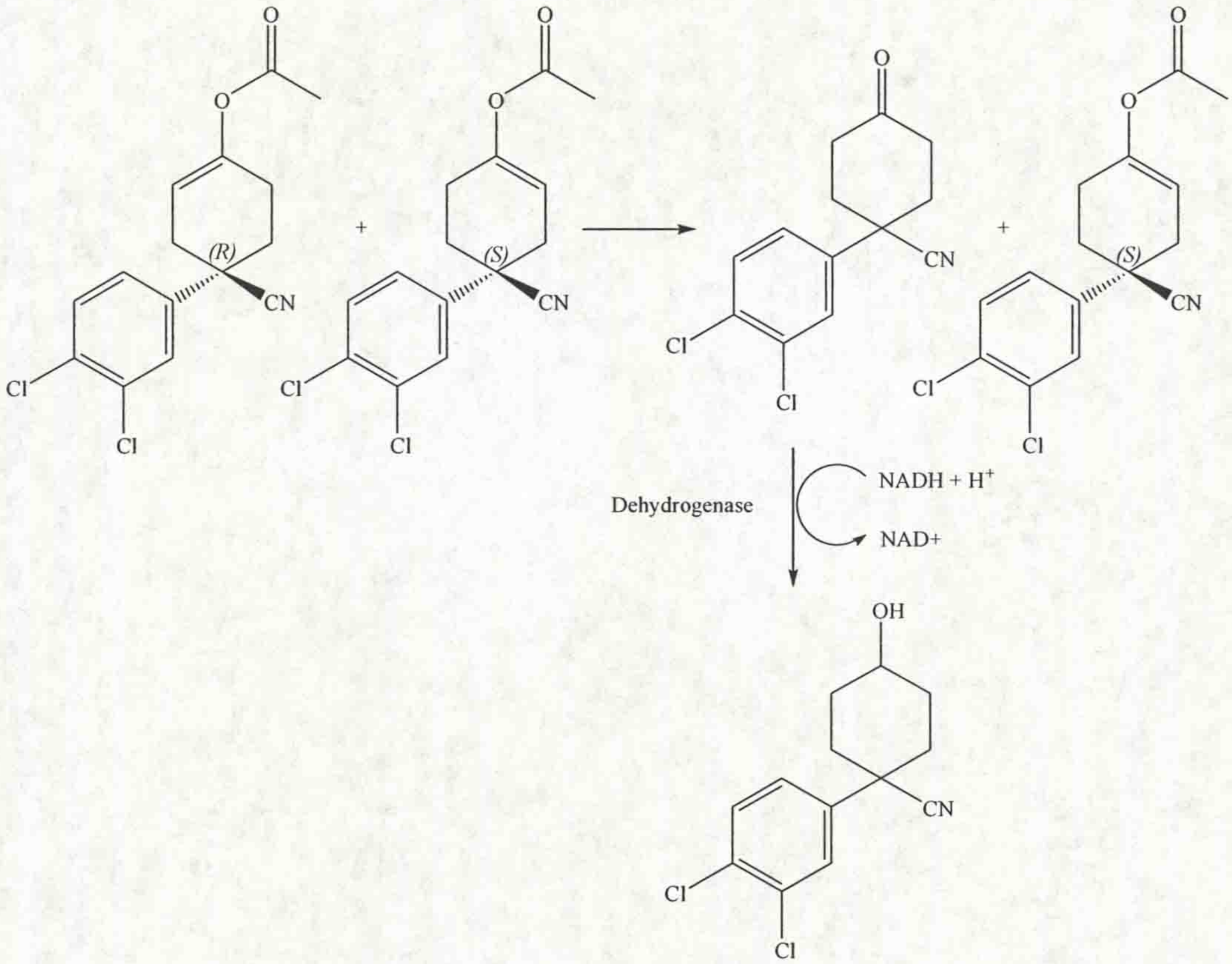
In order to find a screen suitable for the analysis of mutants reacting with the racemic enol acetate (1), reactions involving the ketone produced during the biotransformation were investigated. The first idea was to use a dehydrogenase to reduce the ketone product to the corresponding alcohol (Figure 5.2). The advantage of this method would be to follow, quantitatively, the ketone (2) produced according to the decrease in absorbance upon oxidation of NADH to NAD at 340nm. Parallel screening with the racemic and enantiomerically pure (S)-enol acetate available from the biotransformation would allow us to screen for improved enantioselectivity against the (R)-enantiomer.

To test this approach, biotransformation of the ketone (2) with Yeast Alcohol Dehydrogenase (YADH) and Horse Liver Alcohol Dehydrogenase (HLADH) were attempted (Section 2.-.2). Since the substrate was hydrophobic, co-solvents were used. Use of toluene or heptane with YADH or DMF with HLADH as co-solvents resulted in no conversion under a variety of conditions. Accordingly the use of a secondary screen involving a dehydrogenase was abandoned.

5.3 Secondary screen using a colourimetric assay

As the previous screen was unsuccessful, other methods to react with the ketone produced during the biotransformation were investigated. The use of a colourimetric assay was particularly attractive in its simplicity. Various colourimetric assays for

Figure 5.2: Theoretical reaction involving dehydrogenase



ketones, which could be adapted to microtitre format, had already been developed and will be described in the following sections.

5.3.1 Use of sodium nitroprusside

Sodium nitroprusside was first used in a reaction with acetone (Rothera, 1908). It was then used for medical assay to measure ketone bodies in urine or blood of patients with diabetes as an adjunct to the diagnosis of diabetic ketoacidosis. Various tests have been commercialised to detect the ketone bodies acetoacetate, acetone and β-hydroxybutyric acid which are catabolic products of free fatty acids as well as to detect ketosis (high concentration of β-hydroxybutyric acid in blood) in dairy cows (Geishauser *et al.*, 1998). These tests use the colorimetric reaction that occurs between the ketone and nitroprusside (sodium nitroferricyanide), $Na_2[Fe(CN)_5NO]$, resulting in a red-purple colour (Rothera, 1908).

Experiments were performed to observe a reaction between the ketone (2) and sodium nitroprusside (Section 2.6.3.1), following the available protocol (McLennan, 2004). Reactions were performed with 0.05% w/v sodium nitroprusside and different concentrations of ketone (2). The colour of the mixture was observed after 10min and 24h and compared to a control without ketone as well as a positive control where the ketone was acetone. Unfortunately, no change of colour could be observed for the samples involving the ketone (2) compared to the negative control. The positive control with acetone gave rise to a deep purple colour which appeared immediately after the sodium nitroprusside solution was added. The same experiment was performed with 3% w/v sodium nitroprusside and the colour analysed after 24h. As

before, no change could be observed when compared to the negative control, whereas the positive control gave a deep purple colour.

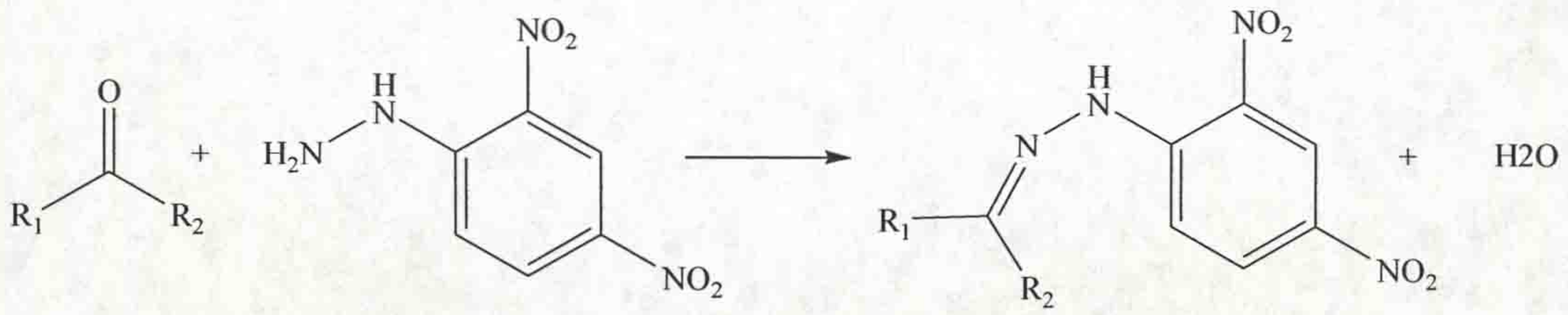
To confirm that the results observed in previous tests were not only efficient with biogenic ketones, the experiment was performed between cyclohexanone and sodium nitroprusside (Section 2.6.3.1). In this case, immediately after the addition of sodium nitroprusside solution, the solution of cyclohexanone showed a dark red colour. This indicates that even for cyclohexanone, the reaction with sodium nitroprusside occurs. These results may be explained by the steric differences between cyclohexanone and the ketone (2). These may prevent the sodium nitroprusside reacting with the ketone to give the red-purple complex, observed in the experiments involving other ketones.

5.3.2 Use of 2,4-dinitrophenylhydrazine (2,4-DNPH) formation

5.3.2.1 Large scale

As the previous colorimetric assay did not give the expected results, other assays were investigated. Another well-known analysis for ketones is 2,4-dinitrophenylhydrazine (2,4-DNPH) formation. This reaction is commonly used in synthetic chemistry to characterise aldehyde and ketone functions. The carbonyl group reacts with the 2,4-DNPH to form an orange precipitate (Figure 5.3). Previous assays were developed with 2,4-DNPH and 2-heptanone and other ketones (Haidle & Knight, 1960). In this assay, 2-heptanone reacted with 2,4-DNPH and led to the formation of a yellow coloured complex with NaOH. First, the ketone was mixed with 2,4-DNPH in an acidic solution. As the 2-heptanone was only slightly soluble in NaOH, it was necessary to dissolve it in an organic solvent. The solvent chosen was ethanol due to its miscibility with water in which the ketone had been dissolved. The

Figure 5.3: Reaction of 2,4-DNPH with carbonyl group



advantage of ethyl alcohol was also to intensify the colour formed upon the addition of NaOH and so increase its stability. Although the reaction between the ketone and the 2,4-DNPH was not quite complete, the time of 20min was found to be satisfactory for accurate determinations in this study.

Due to the slight solubility of ketone (2) in water and as ethanol increased the stability of the complex, it was decided to use ethanol as described in the original assay. The same conditions described in the study with the heptanone were used (Section 2.6.3.2.1). Analysis of the samples was performed using a spectrophotometer and the OD measured at 540nm (Table 5.1).

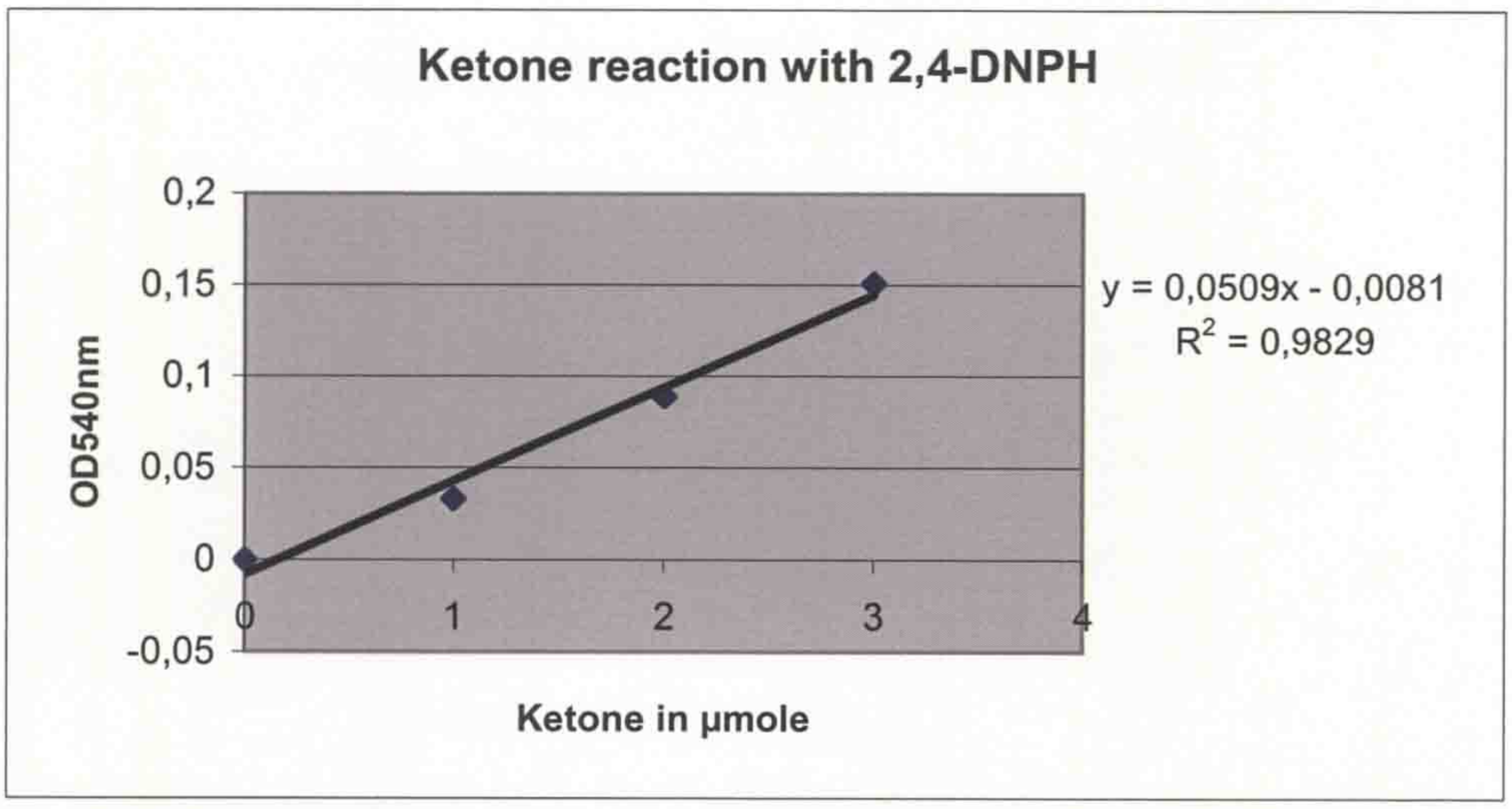
Ketone in µmole	**OD_{540nm}**
0	0
1	0.033
2	0.089
3	0.151

Table 5.1: Results of absorbance measurements for a large scale experiment

The amount of ketone was then plotted against the OD_{540nm} (Figure 5.4). Linear regression analysis showed the linear response of the OD with the concentration of ketone possessing a correlation coefficient of 0.98. This indicates that the OD was proportional to the ketone concentration and that the assay follows the Beer-Lambert's law with the ketone (2). The same experiment was performed with the same range of concentrations for the racemic enol acetate (1). This was performed to

Figure 5.4: Large scale reaction of 2,4-DNPH with ketone (2)

Measurements were made 10min after the addition of NaOH.



check if the presence of the enol acetate would interfere with the reaction involving the ketone and the 2,4-DNPH or with the measure of the absorbance. Samples were analysed as before and the OD was measured and compared to the negative control (buffer). As before the control was set up to be the zero. Measurement of the samples containing the enol acetate (1) all gave a value of zero, indicating that the enol acetate had no effect on the reaction or the absorbance measurement.

According to the accuracy of these results, the rapidity of this assay and the fact that no interference could be observed with the enol acetate, more tests were carried out to adapt this assay for high-throughput screening and to use it as on a micro scale.

5.3.2.2 Adoption of the assay for microtitre plate format

The experiments performed above were repeated in microtitre plates (Section 2.6.3.2.2). Mixes of ketone (2) and enol acetate (1) were subjected to the assay with a proportional reduction of the volume used. DMF was added in order to increase the solubility of ketone in buffer. A range of concentrations were tested up to a maximum amount of 0.1μmole of ketone (2) in the assay. Measurements of absorbance were performed as before (Table 5.2).

Ketone in µmole	Enol acetate in µmole	OD_{540nm}
0	0.1	0.001
0.02	0.08	0.021
0.04	0.06	0.031
0.06	0.04	0.035
0.08	0.02	0.039
0.1	0	0.050

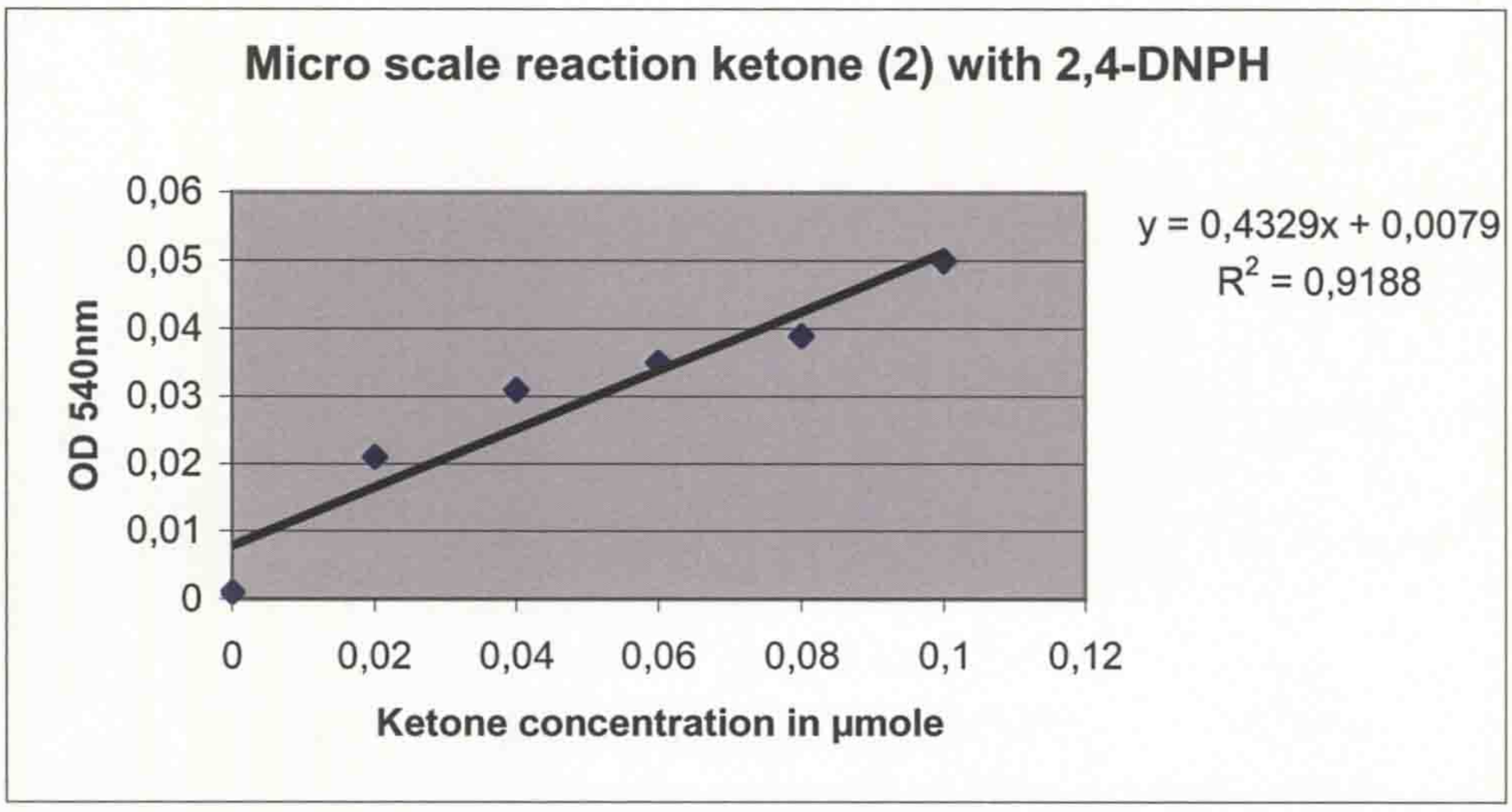
Table 5.2: Results of absorbance measurements for micro scale experiment in presence of buffer and DMF

The ketone concentration was plotted against the OD (Figure 5.5). A linear regression showed a linear response of the OD with the concentration of ketone. In this case, the correlation coefficient was only 0.92, indicating less proportional response with ketone concentration possibly resulting from the insolubility of the ketone in the buffer even in the presence of DMF.

The absorbance response was then testing using biotransformations with the wild-type enzyme (Section 2.6.3.2.2). A range of standards containing mixtures of ketone and enol acetate was tested. The reactions were done in duplicate to test the reproducibility of the assay and the absorbances were measured as previously described (Table 5.3).

Figure 5.5: Microscale reaction of 2,4-DNPH with ketone (2) using DMF as co-solvent

Measurements were made 10min after the addition of NaOH.



Ketone in µmole	**Enol acetate in µmole**	**OD_{540nm}**
0	0.04	0.120-0.107
0.01	0.03	0.190-0.283
0.02	0.02	0.167-0.147
0.03	0.01	0.156-0.194
0.04	0	0.154-0.191

Table 5.3: Results of absorbance measurements for standards to determine biotransformation yields

The results in Table 5.3 showed that the OD measurements were not accurate or reproducible. Results of the 16 biotransformations performed in parallel (Section 2.6.3.2.2) gave OD readings between 0.107 and 0.366 and with variation up to 0.1 units for the same sample for reaction supposed to possess the same activity and selectivity on the substrate. These measurements did not follow Beer-Lambert's law, as even in the case of complete conversion of the enol acetate into ketone, the amount of ketone could not be present more than 0.04µmole in the assay, which corresponded to a ketone assay concentration of 2mM. It was surmised that the insolubility of the substrate was still a major problem.

In order to be able to use this assay for the high-throughput screening, further optimisation was required, especially to increase the solubility of the substrate and the reproducibility of the measurements.

5.3.2.3 Optimisation of the screen in microtitre plates

Another experiment, in which the enol acetate and the ketone were solubilised in DMSO, was performed (Section 2.6.3.2.3). To increase the volume of reaction, 80µl of the reaction was used as the sample for the colorimetric assay in place of the 60µl of water and 20µl of the reaction mix used previously. For the first experiment without centrifugation prior to collection of samples, OD measurements gave non reproducible results as before. Variations occured and results were not accurate for the concentration of reactants in the mixture. The same experiment was repeated (Section 2.6.3.2.3) but the samples and standards were centrifuged prior to collection for assay. This allowed us to avoid interference from particles in the reaction and the OD measurements and gave more accurate results (Table 5.4).

Ketone in µmole	**OD_{540nm}**	**OD_{540nm} samples**
0	0.187 S.E.M.: $\pm 9.9 \times 10^{-4}$	0.371 0.250 0.323 0.377 0.369 0.392 0.351 0.384 Mean: 0.352 S.E.M.: $\pm 5.4 \times 10^{-3}$
0.18	0.252 S.E.M.: $\pm 3.9 \times 10^{-3}$	
0.35	0.393 S.E.M.: $\pm 3.1 \times 10^{-3}$	
0.53	0.305 S.E.M.: $\pm 3.7 \times 10^{-3}$	
0.7	0.330 S.E.M.: $\pm 4.2 \times 10^{-3}$	
0.88	0.438 S.E.M.: $\pm 4.1 \times 10^{-3}$	

Table 5.4: Results of absorbance measurements for standards after centrifugation to determine biotransformation yields

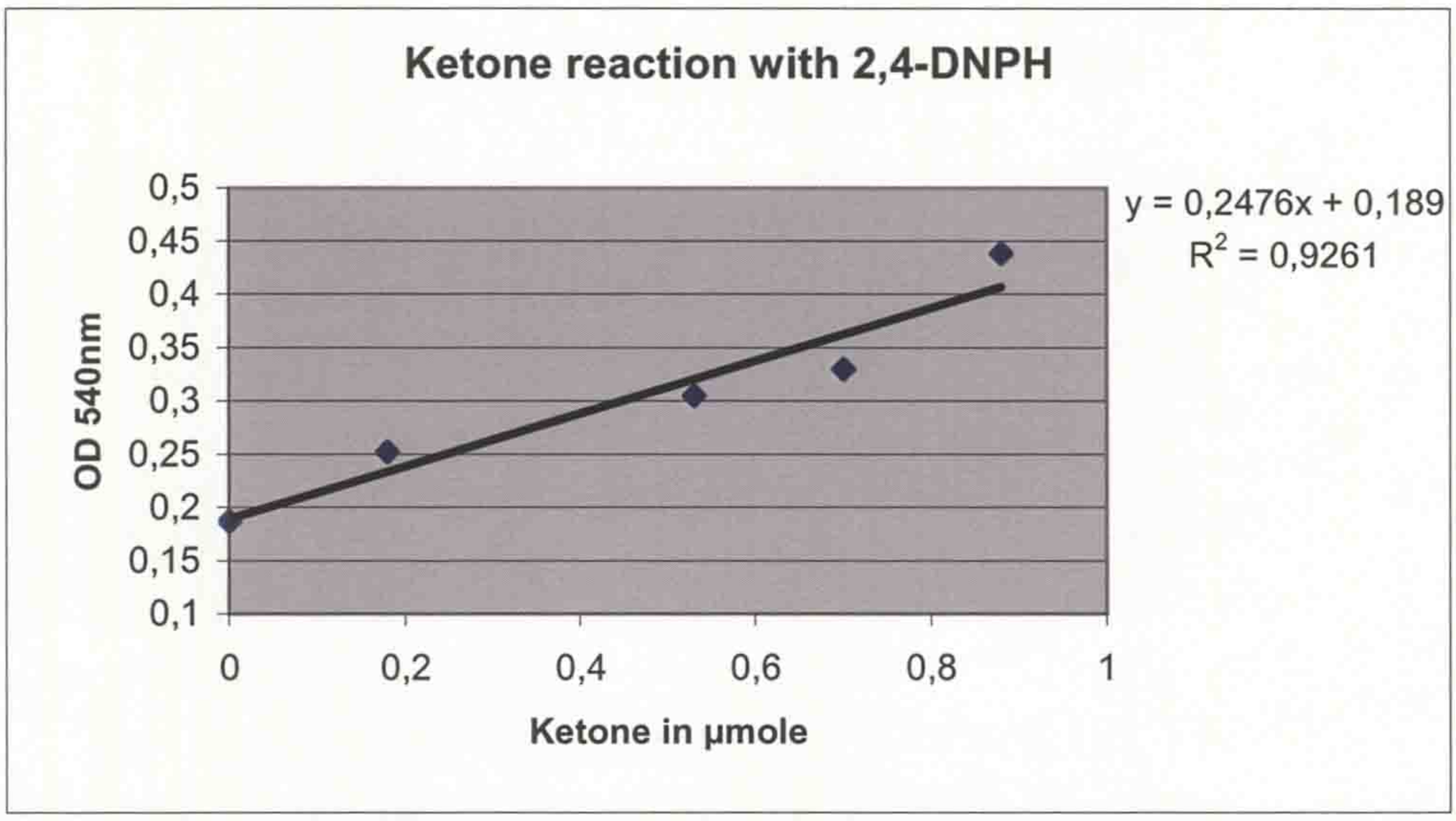
Standards were performed in duplicate and the OD represents the mean between each standard value.

As before the ketone concentration was plotted against the OD (Figure 5.6). A linear regression showed the linear response of the OD with the concentration of ketone. In this case the correlation coefficient was 0.93. The OD's of the samples were compared with HPLC results for reactions performed in the same conditions with 5% DMSO after 4 days of reaction (Section 4.2.4.3). From HPLC, the mean for the conversion of the enol acetate into ketone was 81%. The OD measures gave a mean of 0.352 for the biotransformation under the same conditions. According to the linear regression, the amount of ketone present in the biotransformation reaction was therefore 0.66µmole, which corresponds to a final ketone concentration of 8.25mM in the biotransformation. As the initial concentration of the enol acetate was 10mM, the conversion of this reaction was therefore 75% which compares to the 81% calculated from HPLC results. As for this reaction the correlation coefficient was 0.93, this indicates a percentage of error which could reach 7%, which is the case in this experiment. This showed that centrifugation of the samples and standards, prior to analysis, is important for accurate results.This also showed that this screening gave accurate results and values for the determination of the percentage of conversion and can therefore be used as a screening method for this project.

In order to increase this coefficient, new experiments were performed (Section 2.6.3.2.3). Higher concentrations of DMSO (31.25%) and ketone (from 0 to 5 µmole) were used. Different volumes of 2,4-DNPH were also tested (10 and 20µl). The first experiment involved the addition of 10µl of 2,4-DNPH as previously mentioned (Table 5.5)

Figure 5.6: Microscale reaction of 2,4-DNPH with ketone (2) using DMSO as a co-solvent

Measurements were made 10min after the addition of NaOH.



Ketone in µmole	**OD_{540nm}**
0	0.348 S.E.M.: $\pm 4.5 \times 10^{-5}$
1	0.446 S.E.M.: $\pm 4.7 \times 10^{-5}$
2	0.507 S.E.M.: $\pm 6.7 \times 10^{-5}$
3	0.705 S.E.M.: $\pm 5.2 \times 10^{-5}$
4	0.828 S.E.M.: $\pm 5.1 \times 10^{-5}$
5	0.886 S.E.M.: $\pm 4.9 \times 10^{-5}$

Table 5.5: Results of absorbance measurements for standards, microscale with use of 10µl of 2,4-DNPH.

Standards were performed in duplicate and the OD represents the mean between each standard value.

As before the ketone concentration was plotted against the OD (Figure 5.7). Linear regression analysis showed a linear response of the OD with the concentration of ketone. In this case the correlation coefficient was 0.98. This indicates that even with a high concentration of DMSO and a ketone concentration up to 5µmole, the OD was still proportional to the ketone concentration. These results were important as, at a ketone concentration of 5µmole, the amount of DNPH present in the screening reaction was 0.05µmole. This showed that the reaction occurring between the ketone and the DNPH during the first 20min of reaction was not limited by the low concentration of DNPH. This also indicates that the reaction was not complete as described before (Haidle & Knight, 1960) but that it was slow enough not to reach the

Figure 5.7: Microscale reaction of 2,4-DNPH with ketone (2) using DMSO as a co-solvent

Measurements were made 10min after the addition of NaOH.
10µl of 2,4-DNPH were added on this reaction.

limit of 0.05μmole, which would be exceeded if the reactions were complete and interfere with the determination of the ketone concentration from OD measures.

In order to test if this theory was correct, the same reaction was performed with 20μl of 2,4-DNPH. This increased the amount of DNPH present in the reaction to 0.1μmole (Table 5.6).

Ketone in μmole	**OD_{540nm}**
0	0.283 S.E.M.: $\pm 3.9 \times 10^{-5}$
1	0.442 S.E.M.: $\pm 3.8 \times 10^{-5}$
2	0.567 S.E.M.: $\pm 4.1 \times 10^{-5}$
3	0.745 S.E.M.: $\pm 4.1 \times 10^{-5}$
4	0.938 S.E.M.: $\pm 4 \times 10^{-5}$
5	1.274 S.E.M.: $\pm 4.5 \times 10^{-5}$

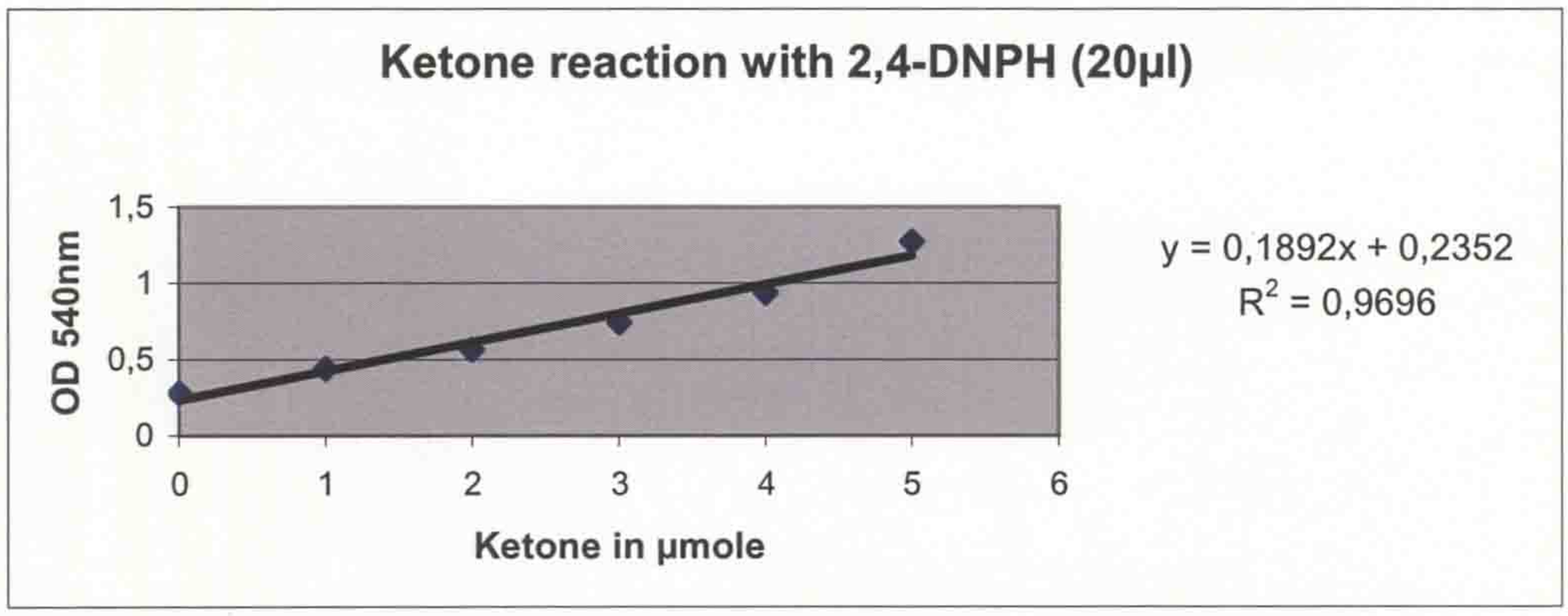
Table 5.6: Results of absorbance measurements for standards, microscale with use of 20μl of 2,4-DNPH.

Standards were performed in duplicate and the OD represents the mean between each standard value.

As before the ketone concentration was plotted against the OD (Figure 5.8). Linear regression analysis showed a linear response of the OD with the concentration of ketone. In this case the correlation coefficient was 0.97. This indicates that there was no difference in the reaction even with double the concentration of DNPH. This showed that, as mentioned before, the reaction between the ketone and the DNPH did

Figure 5.8: Microscale reaction of 2,4-DNPH with ketone (2) using DMSO as a co-solvent

Measurements were made 10min after the addition of NaOH.
20µl of 2,4-DNPH were added on this reaction.



not need to be stoichiometric to observe a linear response between the OD and the ketone concentration when stopped after 20min. The DNPH in this case was not a limiting factor. However, a difference could be observed on the broadness of the OD which reached 1.274 in this case compared to 0.886 with 10µl. This experience indicates that an increase in the ketone concentration led to more accurate results, as the correlation coefficient (0.98) was higher when the standards reached 5µmole compared to 0.88µmole (0.93). As this method allowed accuracy, reproducibility and fast reactions, it was decided to use this screen in order to test esterase mutants.

5.4 Discussion

At that stage, production of the recombinant esterase from *P. fluorescens*, optimisation of the screen using DNPH, and biotransformation in microtitre plates were achieved. It was shown that relatively high DMSO concentrations can be used in order to increase the quantity of enol acetate (1) present in the assay, allowing better accuracy and reproducibility even at a micro scale. The development of a primary screening using a tributyrin plate assay was also performed to allowing limiting the number of mutants reaching the secondary screening. Directed evolution could then be started in order to produce mutants and increase the selectivity towards the (S) enol acetate (1).

Chapter 6

Screening For Altered Enantioselectivity

Chapter 6

Screening For Altered Enantioselectivity

6.0 Summary

This chapter covers the unsuccessful attempts to produce a library of mutant *P. fluorescens* esterase by submitting the *estF* gene to ep-PCR. Forty mutant EstF proteins were generated by ep-PCR and provided by an external source. They were used in the biotransformation and submitted to the screen described in the previous chapter, in order to confirm the screen capabilities

However, most importantly, this chapter demonstrates how directed evolution of *estF* could specifically and significantly improve the original activity and enantioselectivity of the original enzyme for a specific substrate and therefore could be employed to alter the selectivity of EstF for use in the deracemisation of chiral enol esters.

6.1 Mutant library construction

Several unsuccessful attempts were made to create a mutant library. All these experiments are explained and detailed in Appendix V. Possible explanations of these unsuccessful experiments are also provided. Due to lack of time, it was decided to perform preliminary screen of 40 mutant proteins (kind gift from Pr. Bornscheuer) to test our hypothesis that enantioselectivity of the *P. fluorescens* esterase EstF, for the enol acetate (1) could be altered by mutation.

6.2 Screening of mutant EstF enzyme

Forty randomly chosen mutants of PFE, generated by ep-PCR (gift of Pr. Bornscheuer), expressed by rhamnose induction, purified as previously described and freeze-dried were assayed with the novel screen using the 2,4-DNPH previously to identify potentially interesting mutants.

DMSO (25%) was used during the biotransformation which led to a range of ketone concentration between 0 and 4 μmole for the racemic enol acetate (1) or between 0 and 2 μmole for the single enantiomer enol acetate (1). According to the results previously described (Section 5.3.2.2), a volume of 10μl of 2,4-DNPH solution was used in the screening process. Centrifugation of the samples prior to the screen was performed as previously described. OD measures of the standards were performed as previously described for the racemic and single enantiomer standards (Table 6.1).

Ketone in μmole	**OD_{540nm}**
0	0.334 S.E.M.: $\pm 3.2 \times 10^{-5}$
1	0.406 S.E.M.: $\pm 2.9 \times 10^{-5}$
2	0.459 S.E.M.: $\pm 3.1 \times 10^{-5}$
3	0.53 S.E.M.: $\pm 3.1 \times 10^{-5}$
4	0.587 S.E.M.: $\pm 3 \times 10^{-5}$

Table 6.1: Results of absorbance measurements for standards for microscale screen with use of 10μl of 2,4-DNPH.

Standards were performed in duplicate and the OD represents the mean between each standard value.

As previously described, the ketone amount was plotted against the OD (Figure 6.1). Linear regression analysis showed a linear response of the OD with the amount of ketone. In this case, the correlation coefficient was 0.998. This indicates that the amount of ketone was proportional to the OD. These results confirmed the observation and theory described in the previous chapter (Section 5.3.2.2) where it was shown that the kinetics of the reaction during the first 20min was not limited by the concentration of DNPH.

The OD measures were performed at the same time for the samples taken from the biotransformation experiments involving the racemic and the single enantiomer enol acetate (1) and using mutant proteins (Section 2.6.3.2.4). OD measures were performed as previously described for the 80 reactions (Figure 6.2). From the 40 proteins tested, 35 showed no difference for the OD measures between the reactions with racemic or single enantiomer enol acetate (1). The mean of OD for these proteins was 0.443, which corresponded to a ketone amount of 1.68 mole according to the equation provided from the linear regression (Figure 6.1). This indicates a yield of 42% for the reaction which was the yield expected for a concentration of 25% DMSO (Section 4.2.4.3). These proteins are associated with a wild-type phenotype. This indicates that the mutations made on the gene sequence did not lead to a change in the amino acids sequence or change with minor effect on the activity or selectivity of the esterase.

Figure 6.1: Microscale reaction of 2,4-DNPH with ketone (2)

Measurements were made 10min after the addition of NaOH.
10µl of 2,4-DNPH were added on this reaction.

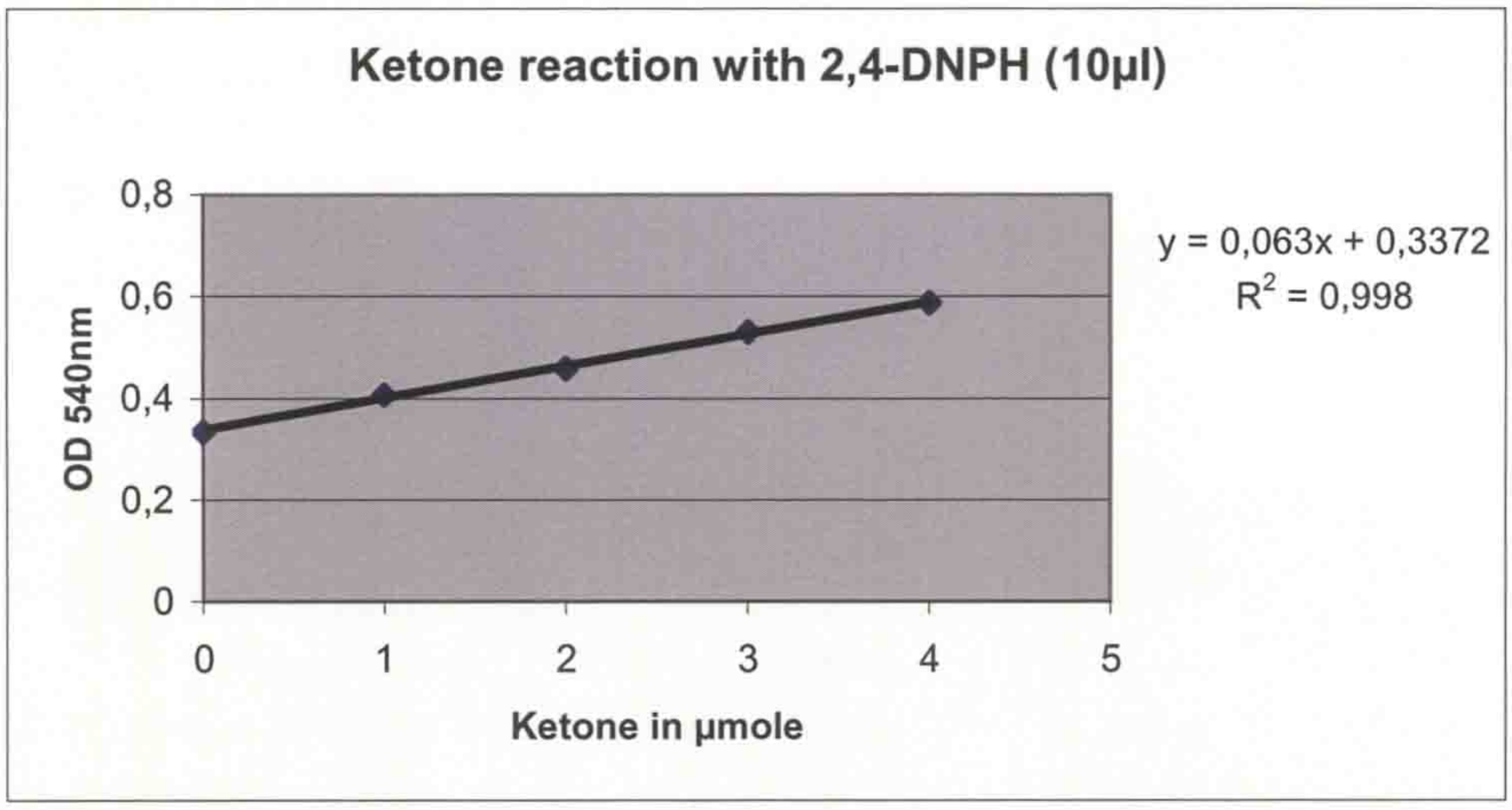
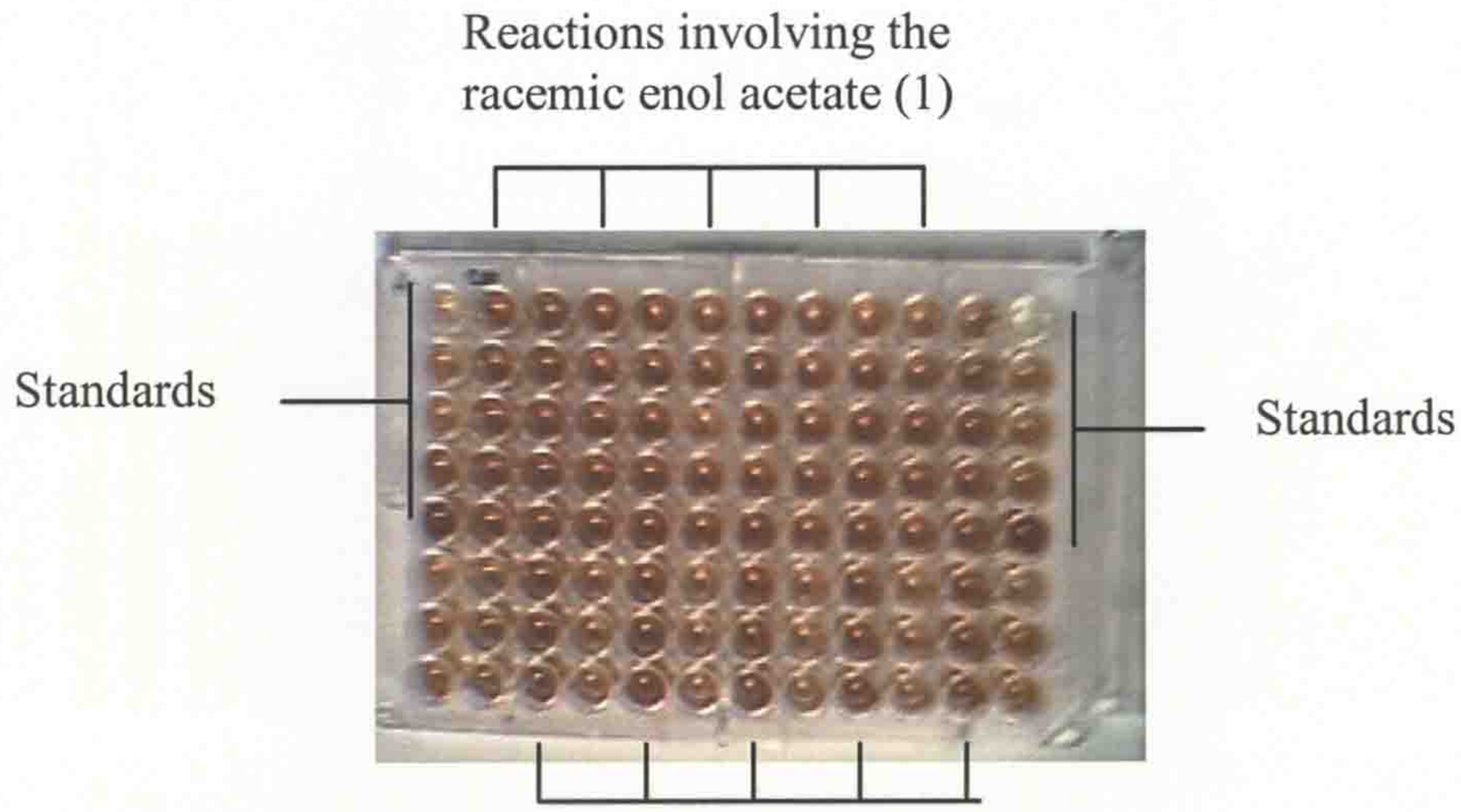


Figure 6.2: Microscale reaction of mutant esterase with racemic or single enantiomer enol acetate (1) and screening with 2,4-DNPH



For the 5 remaining proteins, the OD measures showed difference from 0.094 to 0.155 OD units (Table 6.2).

Clone	OD_{540nm} (racemic)	OD_{540nm} (single enantiomer)
1	0.579 (3.8µmole)	0.437 (1.6µmole)
2	0.564 (3.6µmole)	0.409 (1.1µmole)
3	0.532 (3.1µmole)	0.431 (1.5µmole)
4	0.481 (2.3µmole)	0.374 (0.6µmole)
5	0.468 (2.1µmole)	0.374 (0.6µmole)

Table 6.2: Results of absorbance measurements for biotransformation performed with mutant proteins using racemic or single enantiomer enol acetate (1).

For the first three proteins, a difference between the conversions into ketone from the reaction with racemic or single enantiomer enol acetate (1) could be observed. For these cases, the mutations allow the reaction to occur faster with a higher yield (from 78% to 95%) for the same reaction times compared to the other reactions using mutant proteins or wild-type protein. However, the yields of reactions, involving the single enantiomer, were similar to the other ones, showing the mutant esterase did not show a selectivity towards the (S)-enantiomer as both enantiomers reacted and the (S)-enantiomer was used as well in the reaction where it was the only substrate. These mutants were therefore not the ones of interest as it increased the activity of the enzyme but not its selectivity.

For the last two proteins, yield of conversion into ketone (2) from reaction using the racemic enol acetate (1) as the substrate were respectively 53% and 58%. This indicates that the activity of the enzyme was increased as the average yield for the other proteins was 42% of conversion. However, the most interesting results were obtained with the conversion into ketone (2) from the reaction using the single enantiomer. Only 30% of conversion into ketone (2) could be observed with these two mutant proteins, compared to an average of 84% obtained with the rest of mutant esterases. In these cases, the mutation seemed to have an effect on the activity but also on the selectivity as the (S)-enantiomer was not used as much as in other reactions. This indicates that the conversion made with the racemic enol acetate (1) was, for its major part, due to reaction with the (R)-enantiomer. It was only this kind of mutant which was interesting as they showed a difference of activity and selectivity, with a higher selectivity to use the (R)-enantiomer compared to the original wild-type enzyme.

6.3 Discussion

These results showed the efficiency of the colourimetric screen using the 2,4-DNPH to discover interesting mutants as mutant esterases showed a significant difference in their enantioselectivity compared to the wild-type protein. They also confirm our hypothesis which was to prove that directed evolution strategy could be employed to alter the enantioselectivity of an enzyme towards a substrate of interest. We showed that mutations introduced by ep-PCR into the *estF* gene coding for the *P. fluorescens* esterase, EstF, can lead to the observation of altered enantioselectivity towards the enol acetate (1) and the (S)-enol acetate (1). Two proteins were found to show an increased conversion into ketone (2) from the racemic enol acetate while they showed

a decrease in selectivity towards the (S) enantiomer of the enol acetate, which are the results expected for an interesting mutant protein. Such proteins can then be produced in a larger quantity and used in a larger scale biotransformation. HPLC analysis can be performed to establish a more precise yield and enantiomeric excess for this mutant. Mutant protein structure analysis can therefore link the observed change of activity and selectivity with the mutations observed by comparison with the wild-type EstF, and allow high-lighting region of interest, responsible for the change in activity observed, for further rounds of mutagenesis.

Chapter 7

General Discussion

Chapter 7

General Discussion

The *Pseudomonas fluorescens* lipase C9

The initial aim of this project was to clone a lipase gene from *P. fluorescens* then express and purify the corresponding lipase. This enzyme was then supposed to be subjected to directed evolution to investigate if this process could alter the enantioselectivity of the original protein towards a substrate of interest, the (S)-enantiomer enol acetate (1). Histidine-tagged *lipA* was over-expressed in *E. coli* in different vectors. However, the protein was produced as insoluble inclusion bodies when purified under native conditions. This problem is often encountered when attempting heterologous expression of lipases or other proteins (Clark, 1998; Hang *et al.*, 1999; Lavallie *et al.*, 1993; Lee *et al.*, 1993; Lemercier *et al.*, 2003; Reetz & Jaeger, 1998; Rudolph & Lilie, 1996; Sunitha *et al.*, 2000). These structures are present in the bacterial cytoplasm and lead to storage of the over-expressed protein, in an enzymatically inactive form. To reduce the overexpression, the IPTG concentration during the induction, or induction time or induction temperature were decreased. Detergents were also introduced into extraction buffers to allow the solubilisation of the inclusion bodies. Several different plasmids were constructed, in order to solubilise the recombinant lipase. However, none of these attempts were successful in allowing purification of the protein under native conditions.

The only way to achieve lipase solubilisation was to perform the extraction under denaturing conditions using urea. However, this leads to an enzymatically inactive

form. First attempts to recover the correct folding and activity of the enzyme failed. However, use of a glycerol-based refolding buffer allowed recovery of activity against enol acetate (1), through renaturation of the protein. The effect of glycerol seems to mimic the activity of foldase proteins associated with the expression of lipase. Lipases are extracellular enzymes and need to be translocated through the bacterial membranes. For *P. fluorescens*, the lack of a typical N-terminal signal peptide involved the use of another secretion pathway compared to other bacteria (Jaeger & Reetz, 2000). Lipases are secreted in this case by an ABC exporter which involves three proteins, an inner membrane protein, a membrane fusion protein and an outer membrane protein. However, correct folding of the lipase is necessary to ensure proper secretion. This correct folding involves chaperones proteins or foldases (Lif, lipase-specific foldase) (Frenken *et al.*, 1993a; Frenken *et al.*, 1993b; Reetz & Jaeger, 1998) which allow the lipase to adopt the three dimensional structure necessary for their secretion and activity. As previously described, the glycerol, by creating a non-aqueous environment, mimics the role of the Lif. The absence of water seems to allow a specific configuration, which corresponds to the one created by the foldase, in a normal cellular environment leading to lipase expression and secretion. The folding of the lipase therefore occurred without the presence of the chaperone protein and the activity of the lipase was regained.

Unfortunately, the ultimate planned screen for this project involved culture in microtitre plates. The expression levels obtained were not sufficient to allow extraction and purification on a small scale. For the renaturation of the protein, a high degree of dilution was also needed, this involved another unsuitable step for high-throughput screening of mutant proteins on a micro scale. However, these results

demonstrate that refolded heterologously expressed *P. fluorescens* lipase has potential enantioselective biocatalytic activity.

The *P. fluorescens* esterase and production of mutants

The *P. fluorescens* lipase was not a suitable enzyme for improvement of the enantioselectivity towards the enol acetate (1) using a mutation strategy. Therefore our attention turned to another enzyme that previously had been studied, cloned, expressed and purified (Choi *et al.*, 1990; Pelletier & Altenbuchner, 1995). The enzyme was the *P. fluorescens* esterase (PFE) from the SIK WI strain that had been used in directed evolution project to increase the selectivity towards different esters (Bornscheuer *et al.*, 1998; Bornscheuer *et al.*, 1999; Bornscheuer & Pohl, 2001; Henke & Bornscheuer, 1999). The advantage of using this enzyme was that it had the potential to be altered in its enantioselectivity with respect to enol acetate (1). The plasmid carrying the *estF* gene encoding the esterase of interest was kindly provided by Pr. Bornscheuer, whose group studied the directed evolution of this enzyme.

The wild-type enzyme was expressed, purified and used in the biotransformation of the racemic enol acetate (1). It was also demonstrated that PFE could be used with high DMSO concentrations. It has been shown only recently that *Mucor miehei* esterase (MME) can be used with unusual high concentrations of organic solvents (up to 75%) (Humphrey *et al.*, 2004). In our case, a concentration of up to 30% DMSO was used with re-producible efficiency. It was shown that the higher the DMSO concentration, the slower the reaction with concomitant loss of enantioselectivity. The advantage in using DMSO was that the concentration of the substrate could be

increased ensuring homogeneity of the reaction mixture and facilitating the microscale assay. Attempts to generate a mutant *estF* library of sufficient size were unsuccessful in the time available. Only five mutants were produced and only one could be entirely sequenced. However, according to this sequencing, the mutation strategy applied by the use of ep-PCR was efficient and lead to the medium mutation rate expected. In this case, the failure was mainly attributed to the ligation step.

The most important step in directed evolution experiments is the screening procedure. The screen needs to be specifically designed for the reaction and substrate of interest. To have an efficient screen, it has to be fast and accurate. A wide diversity of methods has been employed on agar media plates (Bornscheuer *et al.*, 1998); microtitre plates; colourimetric assays (Henke & Bornscheuer, 1999); pH indicators (Bornscheuer *et al.*, 1999); and UV/Vis, fluorescence (Henke & Bornscheuer, 2003) and mass spectrometry (Reetz, 2001).

In this project, the major problem was the low solubility of the substrate in most of the suitable solvents. Attempts at using plates for the single step selection of positive mutants were tried. Unfortunately, insertion of the substrate in the media did not allow the development of a suitable screen. In this case, to observe a reaction, the substrate needed to reach the cytoplasm of the bacteria. However, the recombinant protein was not excreted as no signal peptide was expressed in *E. coli* which is required for the excretion of protein. Additionally, in the experiments performed, the substrate precipitated at the surface of the media or in the media. The plate was freeze-thawed to allow the lysis of the cells in order for the esterase to be in contact with the substrate and react with it. The presence of pH indicators were supposed to

enhance the selection of positive mutants, by the observation of a colour change, due to the release of acetic acid from the enol acetate (1) during the conversion into ketone (2). Unfortunately, none of these methods allowed the observation of a colour change. The insolubility of the substrate on the plate, even when previously dissolved in organic solvent, seems to be the main reason for this failure.

In order to limit the number of mutants which needed to be screened, methods involving two screenings were employed, one to test the general esterase activity and the second to test the esterase activity on the substrate of interest. A tributyrin assay was therefore used to select mutants, with esterase activity. This allowed elimination of all mutants which expressed inactive esterase or mutants which could not express the esterase at all. Positive mutants for esterase activity could then be selected and submitted to another specific screen. This secondary screen needed to involve the enol acetate (1). First attempts at the development of this screen were made using dehydrogenase enzymes. Dehydrogenases were supposed to react with the ketone (2), leading to the corresponding alcohol by conversion of co-enzyme NADH into NAD^+, a reaction which could be followed by spectrophotometry (Andersson *et al.*, 1998; Tkachenko & Winston, 2000). However, no reaction occurred with these enzymes and no decrease of NADH concentration could be observed. In this experiment, the low solubility of the substrate may have prevented the reaction occurring and the ketone (2) may have been an unsuitable substrate for the dehydrogenase. The use of dehydrogenase was therefore abandoned and the development of a colourimetric assay was investigated.

The first colourimetric assay involved the use of sodium nitroprusside. This assay was efficient with acetone and even with cyclohexanone, with a clear colour change from yellow to dark red or deep violet. However, this assay did not show a colour change with the ketone (2). In this case, the phenyl and cyano groups of the ketone may have prevented formation of the correct complex. As this attempt was unsuccessful another colourimetric assay was performed using 2,4-DNPH (Haidle & Knight, 1960). This assay allowed the formation of a complex between the DNPH and the ketone (2) which could be monitored by spectrophotometry. Standards made with known concentrations of enol acetate (1) and ketone (2) showed linear OD response and followed the Beer-Lambert law. It was also shown that even if the concentration of DNPH was lower than the ketone concentration, the limiting effect on the assay which would have occurred with a complete reaction could not be observed for a reaction in 20min. From these standards, it was possible to determine the yield of conversion into ketone (2) in the biotransformation reactions, by submitting the reaction samples to the same assay. The presence of DMSO in the biotransformation did not interfere with the measure of absorbance and this assay was still efficient when applied at the micro scale in microtitre plates.

Biotransformations performed with racemic enol acetate (1) and (S)-enantiomer were carried out in parallel with mutant esterases, provided by Pr. Bornscheuer to confirm the capabilities of this screen. After four days of reaction, samples were taken from each well to study the reaction of the 40 mutant esterases on the racemic and single enantiomer. From these analyses, two mutants showed the characteristics expected for mutants of interest. These mutant proteins showed a slightly higher conversion of the racemic enol acetate (1) into ketone (2) compared to other mutants but overall, a

major difference could be observed with a much lower conversion to ketone (2) from a reaction with the (S)-enantiomer. The selectivity of the mutant esterase for the (S)-enantiomer had in these cases decreased compared to the wild-type esterase. However, as the yield remained almost the same for reaction involving the racemic, this showed the selectivity of the mutant esterase had been directed towards the (R)-enantiomer which was the aim of this project. This showed the screen was accurate and efficient to allow observation of these differences, confirming it as the method of choice in this project. The major advantage of this screen is that it can be applied to any reaction which involves the production of a ketone-containing molecule.

The selected mutants may then be used to produce mutant esterases in larger quantities for biotransformation and analysis by HPLC, to determine the exact enantiomeric excess. The mutant *estF* gene from these clones could then be purified and sequenced. After identification of the mutations on the gene, the corresponding mutations could be identified on the proteins. Site-directed mutagenesis may be applied to study the potential increase in activity and selectivity of the corresponding mutant esterase. The gene may also be submitted to gene-shuffling with other identified mutant genes. By submitting the selected mutant gene to further rounds of mutagenesis, the probability of finding further beneficial mutations is increased.

Future work

From all the studies performed in this project, the most important point to highlight is the necessity in directed evolution of an efficient high-throughput screening to allow for the selection of positive clones. This was the major problem in this project as the

substrate was highly insoluble in conditions normally used to carry out simple screens. A mutation strategy needs to be chosen with due regard to the characteristics of the gene, plasmids and conditions of expression and purification previously developed. The choice of a soluble protein or a protein which can be easily solubilised is however essential to facilitate the downstream experiments. Some suggestions are therefore proposed, in order to improve the methods and the protocols for further experiments.

For the directed evolution process of *P. fluorescens* esterase, the use of a mutator strain would be strongly recommended. Even if the mutation rate cannot be as tightly controlled as it could be by using ep-PCR, the process is much easier. The only step is the transformation of the mutator strain with the plasmid, in this case pJOE2792. One of the disadvantages of this method is the insertion of mutations on the whole plasmid rather than on the gene of interest. This can lead to the production of truncated proteins, or proteins which have lost their activity increasing the number of non-interesting mutants. However, in this study, the development and use of a tributyrin plate assay limit this inconvenience, as a large number of mutants can be screened by using this method. Only mutants, showing an esterase activity on tributyrin, are therefore selected and submitted to the secondary screen, which determines the real activity of the enzyme towards the substrate of interest.

For the use of the 2,4-DNPH screen, handling samples may introduce a loss in accuracy. The reaction time is short (50min) and to add the reacting solution (2,4-DNPH, ethanol and NaOH solutions) at the exact same time in the microtitre plate wells, automation with the use of robot would eliminate potential inaccuracy. The

investigation of other solvents, which could be used in the biotransformation reaction to improve selectivity of the esterase might also be investigated. Toluene gave encouraging results and could be an interesting solvent in which the screen may be performed.

Conclusion

This project has allowed the expression of a new lipase from *P. fluorescens* without the need of a Lipase-specific foldase (Lif). The denaturation involved during the purification step was reversed by the use of a renaturation step using glycerol. This allows the lipase to regain its activity. This method is interesting as lipases are essential compounds used as catalysts in bioorganic chemistry. However, the difficulties encountered in cloning lipase genes, expressing and purifying them, limit the willingness of scientists to use such powerful enzymes. By using the renaturation step without the need of cloning associated foldase, this could lead to more studies where the role of lipase could be highlighted.

This project also described the difficulty encountered in developing an accurate and efficient screen towards a specific substrate, as no universal screening method exists. Directed evolution projects often use a reverse angle in the study by choosing an enzyme which can be easily cloned and expressed. A suitable substrate is then chosen, substrate which can be incorporated using a previously developed screening method. Improvement in activity and selectivity can therefore lead to further rounds of mutagenesis and result in high mutation efficiency. In this study, problems of solubility and expression encountered with the lipase were solved by switching to an

esterase from the same organism and in the same family of hydrolase enzymes. Directed evolution was then performed but further failures occurred and no library of mutants could be produced.

However, the principal achievement was the development of a suitable screen for the small selection of interesting mutants available to us. The solubility of the substrate and its bulky structure led to several failures and the continuous search for an ideal screen for this particular substrate. This was achieved by using a colourimetric assay which involved dinitrophenylhydrazone. This screen was validated by showing the difference in reaction rate of a mutant esterase with the racemic substrate and one of the single enantiomers. This method is of potential general interest for other studies with different substrates, producing a ketone-containing product.

References

References

Airaksinen, A. & Hovi, T. (1998). Modified base compositions at degenerate positions of a mutagenic oligonucleotide enhance randomness in site-saturation mutagenesis. *Nucleic Acids Res* 26, 576-81.

Alexeeva, M., Carr, R. & Turner, N. J. (2003). Directed evolution of enzymes: new biocatalysts for asymmetric synthesis. *Organic & Biomolecular Chemistry* 1, 4133-4137.

Allan, G., Carnell, A. J., Hernandez, M. L. E. & Pettman, A. (2000). Desymmetrisation of 4,4-disubstituted cyclohexanones by enzyme- catalysed resolution of their enol acetates. *Journal of the Chemical Society-Perkin Transactions 1*, 3382-3388.

Allan, G., Carnell, A. J., Hernandez, M. L. E. & Pettman, A. (2001a). Chemoenzymatic synthesis of a tachykinin NK-2 antagonist. *Tetrahedron* 57, 8193-8202.

Allan, G. R., Carnell, A. J. & Kroutil, W. (2001b). One-pot deracemisation of an enol acetate derived from a prochiral cyclohexanone. *Tetrahedron Letters* 42, 5959-5962.

Andersson, M., Holmberg, H. & Adlercreutz, P. (1998). Evaluation of Alcaligenes eutrophus cells as an NADH regenerating catalyst in organic-aqueous two-phase system. *Biotechnology and Bioengineering* 57, 79-86.

Bairoch, A. (2000). The ENZYME database in 2000. *Nucleic Acids Research* 28, 304-305.

Berglund, P. (2001). Controlling lipase enantioselectivity for organic synthesis. *Biomolecular Engineering* 18, 13-22.

Beven, C. A., Dieckelmann, M. & Beacham, I. R. (2001). A strain of *Pseudomonas fluorescens* with two lipase-encoding genes, one of which possibly encodes cytoplasmic lipolytic activity. *J Appl Microbiol* 90, 979-87.

Bornscheuer, U. T. (2002). Microbial carboxyl esterases: classification, properties and application in biocatalysis. *Fems Microbiology Reviews* 26, 73-81.

Bornscheuer, U. T., Altenbuchner, J. & Meyer, H. H. (1998). Directed evolution of an esterase for the stereoselective resolution of a key intermediate in the synthesis of epothilones. *Biotechnology and Bioengineering* 58, 554-559.

Bornscheuer, U. T., Altenbuchner, J. & Meyer, H. H. (1999). Directed evolution of an esterase: Screening of enzyme libraries based on pH-Indicators and a growth assay. *Bioorganic & Medicinal Chemistry* 7, 2169-2173.

Bornscheuer, U. T. & Kazlauskas, R. J. (1999). *Hydrolases in organic Synthesis: Regio- or Stereoselective Biotransformations*, John Wiley & Sons.

Bornscheuer, U. T. & Pohl, M. (2001). Improved biocatalysts by directed evolution and rational protein design. *Current Opinion in Chemical Biology* 5, 137-143.

Brown, S. M., Davies, S. G. & Desousa, J. A. A. (1993). Kinetic Resolution Strategies .2. Enhanced Enantiomeric Excesses and Yields for the Faster Reacting Enantiomer in Lipase-Mediated Kinetic Resolutions. *Tetrahedron-Asymmetry* 4, 813-822.

Cadwell, R. C. & Joyce, G. F. (1994). Mutagenic PCR. *PCR Methods Appl* 3, S136-40.

Carnell, A. J., Barkley, J. & Singh, A. (1997). Desymmetrisation of prochiral ketones by catalytic enantioselective hydrolysis of their enol esters using enzymes. *Tetrahedron Letters* 38, 7781-7784.

Carnell, A. J., Hernandez, M. L. E., Pettman, A. & Bickley, J. F. (2000). Chemoenzymatic synthesis of a non-peptide tachykinin NK-2 antagonist. *Tetrahedron Letters* 41, 6929-6933.

Carrea, G. & Riva, S. (2000). Properties and synthetic applications of enzymes in organic solvents. *Angewandte Chemie-International Edition* 39, 2226-2254.

Chen, C. S. & Liu, Y. C. (1991). Amplification of Enantioselectivity in Biocatalyzed Kinetic Resolution of Racemic Alcohols. *Journal of Organic Chemistry* 56, 1966-1968.

Chen, C. S. & Sih, C. J. (1989). General-Aspects and Optimization of Enantioselective Biocatalysis in Organic-Solvents - the Use of Lipases. *Angewandte Chemie-International Edition in English* 28, 695-707.

Choi, K. D., Jeohn, G. H., Rhee, J. S. & Yoo, O. J. (1990). Cloning and Nucleotide-Sequence of an Esterase Gene from *Pseudomonas-Fluorescens* and Expression of the Gene in *Escherichia-Coli*. *Agricultural and Biological Chemistry* 54, 2039-2045.

Clark, E. D. B. (1998). Refolding of recombinant proteins. *Current Opinion in Biotechnology* 9, 157-163.

Dalrymple, B. P. & Swadling, Y. (1997). Expression of a *Butyrivibrio fibrisolvens* E14 gene (cinB) encoding an enzyme with cinnamoyl ester hydrolase activity is negatively regulated by the product of an adjacent gene (cinR). *Microbiology-Uk* 143, 1203-1210.

Derewenda, U., Brzozowski, A. M., Lawson, D. M. & Derewenda, Z. S. (1992). Catalysis at the interface: the anatomy of a conformational change in a triglyceride lipase. *Biochemistry* 31, 1532-41.

Dieckelmann, M., Johnson, L. A. & Beacham, I. R. (1998). The diversity of lipases from psychrotrophic strains of *Pseudomonas*: a novel lipase from a highly lipolytic strain of *Pseudomonas fluorescens*. *J Appl Microbiol* 85, 527-36.

Eckert, K. A. & Kunkel, T. A. (1991). DNA polymerase fidelity and the polymerase chain reaction. *PCR Methods Appl* 1, 17-24.

Elion, E. A. (1997). Constructing Recombinant DNA Molecules by the Polymerase Chain Reaction.

Emgenbroich, M. & Wulff, G. (2003). A new enzyme model for enantioselective esterases based on molecularly imprinted polymers. *Chemistry* 9, 4106-17.

Faber, K. (2004). *Biotransformaions in Organic Chemistry: A textbook*. 4th edit, Springer.

Faber, K. & Kleewein, A. Selectivity. Institute of Organic Chemistry, Graz Univeristy of Technology.

Fernandez-Gacio, A., Uguen, M. & Fastrez, J. (2003). Phage display as a tool for the directed evolution of enzymes. *Trends in Biotechnology* 21, 408-414.

Field, L. M. & Blackman, R. L. (2003). Insecticide resistance in the aphid *Myzus persicae* (Sulzer): chromosome location and epigenetic effects on esterase gene expression in clonal lineages. *Biological Journal of the Linnean Society* 79, 107-113.

Frenken, L. G. J., Bos, J. W., Visser, C., Muller, W., Tommassen, J. & Verrips, C. T. (1993a). An Accessory Gene, Lipb, Required for the Production of Active *Pseudomonas-Glumae* Lipase. *Molecular Microbiology* 9, 579-589.

Frenken, L. G. J., Degroot, A., Tommassen, J. & Verrips, C. T. (1993b). Role of the Lipb Gene-Product in the Folding of the Secreted Lipase of *Pseudomonas-Glumae*. *Molecular Microbiology* 9, 591-599.

Geishauser, T., Leslie, K., Kelton, D. & Duffield, T. (1998). Evaluation of five cowside tests for use with milk to detect subclinical ketosis in dairy cows. *Journal of Dairy Science* 81, 438-443.

Goddard, J. P. & Reymond, J. L. (2004a). Enzyme assays for high-throughput screening. *Curr Opin Biotechnol* 15, 314-22.

Goddard, J. P. & Reymond, J. L. (2004b). Recent advances in enzyme assays. *Trends Biotechnol* 22, 363-70.

Greener, A., Callahan, M. & Jerpseth, B. (1997). An efficient random mutagenesis technique using an *E. coli* mutator strain. *Mol Biotechnol* 7, 189-95.

Haidle, C. W. & Knight, S. G. (1960). A Colorimetric Assay for 2-Heptanone and Other Ketones. *Biochimica Et Biophysica Acta* 39, 536-537.

Hale, V., McGrew, M., Carlson, B. & Schottel, J. L. (1992). Heterologous expression and secretion of a *Streptomyces scabies* esterase in *Streptomyces lividans* and *Escherichia coli*. *J Bacteriol* 174, 2431-9.

Hale, V. A. & Schottel, J. L. (1996). Mutational analysis of the *Streptomyces scabies* esterase signal peptide. *Applied Microbiology and Biotechnology* 45, 189-198.

Hang, Q., Woods, L., Feiss, M. & Catalano, C. E. (1999). Cloning, expression, and biochemical characterization of hexahistidine-tagged terminase proteins. *Journal of Biological Chemistry* 274, 15305-15314.

Hatzakis, N. S. & Smonou, I. (2004). Enantioselectivity and diastereoselectivity in the transesterification of secondary alcohols mediated by feruloyl esterase from *Humicola insolens*. *Tetrahedron Letters* 45, 2755-2757.

Heilmann, S. M., Drtina, G. J., Haddad, L. C., Rasmussen, J. K., Gaddam, B. N., Liu, J. J., Fitzsimons, R. T., Fansler, D. D., Vyvyan, J. R., Yang, Y. N. & Beauchamp, T. J. (2004). Azlactone-reactive polymer supports for immobilizing synthetically useful enzymes - Part I. Pig liver esterase on dispersion polymer supports. *Journal of Molecular Catalysis B-Enzymatic* 30, 33-42.

Henke, E. & Bornscheuer, U. T. (1999). Directed evolution of an esterase from *Pseudomonas fluorescens*. Random mutagenesis by error-prone PCR or a mutator strain and identification of mutants showing enhanced enantioselectivity by a resorufin-based fluorescence assay. *Biological Chemistry* 380, 1029-1033.

Henke, E. & Bornscheuer, U. T. (2003). Fluorophoric assay for the high-throughput determination of amidase activity. *Anal Chem* 75, 255-60.

Hochuli, E. & Dobeli, H. (1987). Alpha-Alkyl Nitrilotriacetic Acid Adsorbents, a New Type of Metal Chelating Gel Selective for Proteins with Neighboring Histidine-Residues. *Biological Chemistry Hoppe-Seyler* 368, 748-748.

Hochuli, E., Dobeli, H. & Schacher, A. (1987). New Metal Chelate Adsorbent Selective for Proteins and Peptides Containing Neighboring Histidine-Residues. *Journal of Chromatography* 411, 177-184.

Horsman, G. P., Liu, A. M. F., Henke, E., Bornscheuer, U. T. & Kazlauskas, R. J. (2003). Mutations in distant residues moderately increase the enantioselectivity of *Pseudomonas fluorescens* esterase towards methyl 3-bromo-2-methylpropanoate and ethyl 3-phenylbutyrate. *Chemistry-a European Journal* 9, 1933-1939.

Huerta, F. F., Minidis, A. B. E. & Backvall, J. E. (2001). Racemisation in asymmetric synthesis. Dynamic kinetic resolution and related processes in enzyme and metal catalysis. *Chemical Society Reviews* 30, 321-331.

Humphrey, C. E., Easson, M. A. M. & Turner, N. J. (2004). Dimedone esters as novel hydrolase substrates and their application in the colorimetric detection of lipase and esterase activity. *Chembiochem* 5, 1144-1148.

Isowa, Y., Ohmori, M., Ichikawa, T., Mori, K., Nonaka, Y., Kihara, K., Oyama, K., Satoh, H. & Nishimura, S. (1979). Thermolysin-Catalyzed Condensation-Reactions of N-Substituted Aspartic and Glutamic Acids with Phenylalanine Alkyl Esters. *Tetrahedron Letters*, 2611-2612.

Jaeger, K. E., Dijkstra, B. W. & Reetz, M. T. (1999). Bacterial biocatalysts: Molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annual Review of Microbiology* 53, 315-+.

Jaeger, K. E. & Reetz, M. T. (2000). Directed evolution of enantioselective enzymes for organic chemistry. *Current Opinion in Chemical Biology* 4, 68-73.

Janes, L. E., Lowendahl, A. C. & Kazlauskas, R. J. (1998). Quantitative screening of hydrolase libraries using pH indicators: Identifying active and enantioselective hydrolases. *Chemistry-a European Journal* 4, 2324-2331.

Johnson, L. A., Beacham, I. R., Macrae, I. C. & Free, M. L. (1992). Degradation of Triglycerides by a *Pseudomonas* Isolated from Milk - Molecular Analysis of a Lipase-Encoding Gene and Its Expression in *Escherichia-Coli*. *Applied and Environmental Microbiology* 58, 1776-1779.

Khalameyzer, V. & Bornscheuer, U. T. (1999). Overexpression and characterization of an esterase from *Streptomyces diastatochromogenes*. *Biotechnology Letters* 21, 101-104.

Kim, J. H., Choi, G. S., Kim, S. B., Kim, W. H., Lee, J. Y., Ryu, Y. W. & Kim, G. J. (2004). Enhanced thermostability and tolerance of high substrate concentration of an esterase by directed evolution. *Journal of Molecular Catalysis B-Enzymatic* 27, 169-175.

Kim, K. K., Song, H. K., Shin, D. H., Hwang, K. Y., Choe, S., Yoo, O. J. & Suh, S. W. (1997). Crystal structure of carboxylesterase from *Pseudomonas fluorescens*, an alpha/beta hydrolase with broad substrate specificity. *Structure* 5, 1571-84.

Kojima, Y., Yokoe, M. & Mase, T. (1994). Purification and Characterization of an Alkaline Lipase from *Pseudomonas-Fluorescens* Ak102. *Bioscience Biotechnology and Biochemistry* 58, 1564-1568.

Krebsfanger, N., Schierholz, K. & Bornscheuer, U. T. (1998). Enantioselectivity of a recombinant esterase from *Pseudomonas fluorescens* towards alcohols and carboxylic acids. *Journal of Biotechnology* 60, 105-111.

Kuchner, O. & Arnold, F. H. (1997). Directed evolution of enzyme catalysts. *Trends in Biotechnology* 15, 523-530.

Kugimiya, W., Otani, Y., Hashimoto, Y. & Takagi, Y. (1986). Molecular-Cloning and Nucleotide-Sequence of the Lipase Gene from *Pseudomonas-Fragi*. *Biochemical and Biophysical Research Communications* 141, 185-190.

Kulminskaya, A. A., Saveliev, A. N. & Neustroev, K. N. (2004). Human abzymes with amylolytic activity. *Trends in Glycoscience and Glycotechnology* 16, 17-31.

Lavallie, E. R., Diblasio, E. A., Kovacic, S., Grant, K. L., Schendel, P. F. & McCoy, J. M. (1993). A Thioredoxin Gene Fusion Expression System That Circumvents Inclusion Body Formation in the *Escherichia-Coli* Cytoplasm. *Bio-Technology* 11, 187-193.

Lee, Y. P., Chung, G. H. & Rhee, J. S. (1993). Purification and Characterization of *Pseudomonas-Fluorescens* Sik-W1 Lipase Expressed in *Escherichia-Coli*. *Biochimica Et Biophysica Acta* 1169, 156-164.

Lemercier, G., Bakalara, N. & Santarelli, X. (2003). On-column refolding of an insoluble histidine tag recombinant exopolyphosphatase from Trypanosoma brucei overexpressed in *Escherichia coli*. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 786, 305-309.

Liebeton, K., Zonta, A., Schimossek, K., Nardini, M., Lang, D., Dijkstra, B. W., Reetz, M. T. & Jaeger, K. E. (2000). Directed evolution of an enantioselective lipase. *Chemistry & Biology* 7, 709-718.

Majeric, M. & Sunjic, V. (1996). Preparation of S-2-ethylhexyl-para-methoxycinnamate by lipase catalyzed sequential kinetic resolution. *Tetrahedron-Asymmetry* 7, 815-824.

McLennan, P. A. (2004). Rapid analytical techniques for diagnosing and managing diabetes: Methods.

Morgan, B., Dodds, D. R., Zaks, A., Andrews, D. R. & Klesse, R. (1997). Enzymatic desymmetrization of prochiral 2-substituted-1,3- propanediols: A practical chemoenzymatic synthesis of a key precursor of SCH51048, a broad-spectrum orally active antifungal agent. *Journal of Organic Chemistry* 62, 7736-7743.

Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I. & Schrag, J. (1992). The alpha/beta hydrolase fold. *Protein Eng* 5, 197-211.

Pelletier, I. & Altenbuchner, J. (1995). A Bacterial Esterase Is Homologous with Nonheme Haloperoxidases and Displays Brominating Activity. *Microbiology-Uk* 141, 459-468.

Pleiss, J., Fischer, M. & Schmid, R. D. (1998). Anatomy of lipase binding sites: the scissile fatty acid binding site. *Chem Phys Lipids* 93, 67-80.

Rahman, M. B., Basri, M., Hussein, M. Z., Rahman, R. N., Zainol, D. H. & Salleh, A. B. (2004). Immobilization of lipase from *Candida rugosa* on layered double hydroxides for esterification reaction. *Appl Biochem Biotechnol* 118, 313-20.

Rariy, R. V. & Klibanov, A. M. (1997). Correct protein folding in glycerol. *Proceedings of the National Academy of Sciences of the United States of America* 94, 13520-13523.

Rathbone, D. A., Holt, P. J., Lowe, C. R. & Bruce, N. C. (1997). Molecular analysis of the *Rhodococcus sp.* strain H1 her gene and characterization of its product, a heroin esterase, expressed in *Escherichia coli*. *Applied and Environmental Microbiology* 63, 2062-2066.

Reetz, M. T. (2001). Combinatorial and evolution-based methods in the creation of enantioselective catalysts. *Angewandte Chemie-International Edition* 40, 284-310.

Reetz, M. T. (2004). Controlling the enantioselectivity of enzymes by directed evolution: Practical and theoretical ramifications. *Proceedings of the National Academy of Sciences of the United States of America* 101, 5716-5722.

Reetz, M. T. & Jaeger, K. E. (1998). Overexpression, immobilization and biotechnological application of *Pseudomonas* lipases. *Chemistry and Physics of Lipids* 93, 3-14.

Reetz, M. T., Wilensek, S., Zha, D. X. & Jaeger, K. E. (2001). Directed evolution of an enantioselective enzyme through combinatorial multiple-cassette mutagenesis. *Angewandte Chemie-International Edition* 40, 3589-+.

Reidhaar-Olson, J. F. & Sauer, R. T. (1988). Combinatorial cassette mutagenesis as a probe of the informational content of protein sequences. *Science* 241, 53-7.

Remsing, L. L., Gonzalez, A. M., Nur-e-Alam, M., Fernandez-Lozano, M. J., Brana, A. F., Rix, U., Oliveira, M. A., Mendez, C., Salas, J. A. & Rohr, J. (2003). Mithramycin SK, a novel antitumor drug with improved therapeutic index, mithramycin SA, and demycarosyl-mithramycin SK: three new products generated in the mithramycin producer *Streptomyces argillaceus* through combinatorial biosynthesis. *J Am Chem Soc* 125, 5745-53.

Rosenau, F., Tommassen, J. & Jaeger, K. E. (2004). Lipase-specific foldases. *Chembiochem* 5, 153-161.

Rothera, A. C. H. (1908). Note on the sodium nitro-prusside reaction for acetone. *Journal of Physiology* 37, 491-494.

Rudolph, R. & Lilie, H. (1996). In vitro folding of inclusion body proteins. *Faseb Journal* 10, 49-56.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning - A laboratory manual*. Second edit, Cold Spring Habor Laboratory Press.

Shao, Z., Zhao, H., Giver, L. & Arnold, F. H. (1998). Random-priming in vitro recombination: an effective tool for directed evolution. *Nucleic Acids Res* 26, 681-3.

Shchurov, D. V. (1997). Catalytic antibodies. *Molecular Biology* 31, 1-9.

Sih, C. J., Shieh, W. R., Chen, C. S., Wu, S. H. & Girdaukas, G. (1986). Biochemical asymmetric catalysis. *Ann N Y Acad Sci* 471, 239-54.

Sih, C. J. & Wu, S. H. (1989). Resolution of Enantiomers Via Biocatalysis. *Topics in Stereochemistry* 19, 63-125.

Snellman, E. A. & Colwell, R. R. (2004). Acinetobacter lipases: molecular biology, biochemical properties and biotechnological potential. *J Ind Microbiol Biotechnol*.

Stecher, H. & Faber, K. (1997). Biocatalytic deracemization techniques: Dynamic resolutions and stereoinversions. *Synthesis-Stuttgart*, 1-&.

Stemmer, W. P. (1994a). DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. *Proc Natl Acad Sci U S A* 91, 10747-51.

Stemmer, W. P. (1994b). Rapid evolution of a protein in vitro by DNA shuffling. *Nature* 370, 389-91.

Stratagene. QuikChange® Site-Directed Mutagenesis Kit. Stratagene.

Sunitha, K., Chung, B. H., Jang, K. H., Song, K. B., Kim, C. H. & Rhee, S. K. (2000). Refolding and purification of *Zymomonas mobilis* levansucrase produced as inclusion bodies in fed-batch culture of recombinant *Escherichia coli*. *Protein Expression and Purification* 18, 388-393.

SwissProt. (2004). Enzyme classification.

Tanyeli, C. & Turkut, E. (2004). Enzyme catalyzed reverse enantiomeric separation of methyl (+/-)-3-cyclohexene-1-carboxylate. *Tetrahedron-Asymmetry* 15, 2057-2060.

Tkachenko, A. G. & Winston, G. W. (2000). Interaction of alcohol dehydrogenase with tert- butylhydroperoxide: Stimulation of the horse liver and inhibition of the yeast enzymes. *Archives of Biochemistry and Biophysics* 380, 165-173.

Topakas, E., Stamatis, H., Biely, P. & Christakopoulos, P. (2004). Purification and characterization of a type B feruloyl esterase (StFAE-A) from the thermophilic fungus *Sporotrichum thermophile*. *Appl Microbiol Biotechnol* 63, 686-90.

Turner, N. J. (2003). Directed evolution of enzymes for applied biocatalysis. *Trends in Biotechnology* 21, 474-478.

Turner, N. J. (2004). Enzyme catalysed deracemisation and dynamic kinetic resolution reactions. *Current Opinion in Chemical Biology* 8, 114-119.

Valetti, F. & Gilardi, G. (2004). Directed evolution of enzymes for product chemistry. *Natural Product Reports* 21, 490-511.

Vanttinen, E. & Kanerva, L. T. (1997). Optimized double kinetic resolution for the preparation of (S)- solketal. *Tetrahedron-Asymmetry* 8, 923-933.

Volff, J. N., Eichenseer, C., Viell, P., Piendl, W. & Altenbuchner, J. (1996). Nucleotide sequence and role in DNA amplification of the direct repeats composing the amplifiable element AUD1 of *Streptomyces lividans* 66. *Molecular Microbiology* 21, 1037-1047.

Wang, D. Y., Lai, B. H. Y., Feldman, A. R. & Sen, D. (2002). A general approach for the use of oligonucleotide effectors to regulate the catalysis of RNA-cleaving ribozymes and DNAzymes. *Nucleic Acids Research* 30, 1735-1742.

Wenig, K., Chatwell, L., von Pawel-Rammingen, U., Bjorck, L., Huber, R. & Sondermann, P. (2004). Structure of the streptococcal endopeptidase IdeS, a cysteine proteinase with strict specificity for IgG. *Proceedings of the National Academy of Sciences of the United States of America* 101, 17371-17376.

Yang, C. F. & Brush, E. J. (1993). A Spectrophotometric Assay for Alpha-Ketoaldehydes Using Horse Liver Alcohol-Dehydrogenase. *Analytical Biochemistry* 214, 124-127.

Zhao, H., Giver, L., Shao, Z., Affholter, J. A. & Arnold, F. H. (1998). Molecular evolution by staggered extension process (StEP) in vitro recombination. *Nat Biotechnol* 16, 258-61.

Zhou, Y. H., Zhang, X. P. & Ebright, R. H. (1991). Random mutagenesis of gene-sized DNA molecules by use of PCR with Taq DNA polymerase. *Nucleic Acids Res* 19, 6052.

Zirkle, R., Ligon, J. M. & Molnar, I. (2004). Heterologous production of the antifungal polyketide antibiotic soraphen A of *Sorangium cellulosum* So ce26 in *Streptomyces lividans*. *Microbiology* 150, 2761-74.

Appendices

Appendix I

Overview of Plasmids:

Designation:	**Selection:**	**Description:**
pGEM-T Easy	Ampicillin	Promega
pC9lipA	Ampicillin	C9 *lipA* gene in pGEM-T Easy
pC9lipAHis	Ampicillin	C9 *lipA* with 5′ *Nde*I and 3′ *Xho*I sites in pGEM-T Easy
pC9lipANoHis	Ampicillin	C9 *lipA* with 5′ *Nde*I sites in pGEM-T Easy
pET22b(+)	Ampicillin	C-terminal histidine tagged to inserts. Expression vector (Novagen)
pETLipAHis	Ampicillin	C9 *lipA* with 5′ *Nde*I and 3′ *Xho*I sites in pET22b(+). Expression vector.
pETLipANoHis	Ampicillin	C9 *lipA* with 5′ *Nde*I and 3′ *EcoR*I sites in pET22b(+). Expression vector
pThioHis C	Ampicillin	Invitrogen
pC9ThioHis	Ampicillin	C9 *lipA* with 5′ *Acc65*I and 3′ *Xba*I sites in pGEM-T Easy
pThioHisLipA	Ampicillin	C9 *lipA* with 5′ *Acc65*I and 3′ *Xba*I sites in pThioHisC
pJOE2792	Ampicillin	(Krebsfanger *et al.*, 1998)
pPCR-Script Amp SK(+)	Ampicillin	Stratagene

Appendix II

PCR primer sequences:

MWG-Biotech

lip3 5'-TGTCAGGATCCATGGGTGTATTCGAC-3' Tm: 64.8°C

lip4 5'- TGTCAAAGCTTCCACCGTTTTAACTG-3' Tm: 61.6°C

C9 forward 5'-A(996)CTCACGACCACAAGGATGTTTGC(1020)-3'

Target: lipA sequence Tm: 62.7°C

C9 reverse 5'-C(994)GCAGCAGCCCTTGTTTTGAGATA(1970)-3'

Target: lipA sequence Tm: 62.7°C

C9 lipA-NdeI 5'-CCCCCCCATA(93)TGTCACAAAGTACGGCGAC(107)-3'

Target: pC9lipA Tm: 72.2°C

C9 lipA-XhoI 5'-CCCCCCCCTCGAGC(975)AATCCCGCAGCCTTC(959)-3'

Target: pC9lipA Tm: > 75°C

lipAThioHis 1 5'-CCCCCCCGGTACC(158)TATGTCACAAAGTACGG(171)-3'

Target: pETLipAHis Tm: 72.2°C

lipAThioHis 2 5'-CCCCCCCTCTAGAGT(1058)CCCGCAGCCTTCAGC'1029)-3'

Target: pETLipAHis Tm: > 75°C

pJOE2792 forward 5'-C(669)AGGTCGTCTCCCAAGGC(687)-3'

Target: pJOE2792 Tm: 60.5°C

pJOE2792 reverse 5'-G(359)TCCAGGGTTGGTCCGAG(341)-3'

Target: pJOE2792 Tm: 66.7°C

pJOE2792-NdeI 5'-CCCCCCCCA(160)TATGAGCACATTTGTTGC(179)-3'

Target: pJOE2792 Tm: 68.0°C

pJOE2792-BclI 5’-CCCCCCCCCT(879)GATCAACTCCGCCGC(863)-3’

Target: pJOE2792 Tm: 73.0°C

pJOE2792-HindIII 5’-CCCCCC(1021)CCAAGCTTGGCTGCAGTCAATG(999)-3’

Target: pJOE2792 Tm: 72.4°C

TAGN

pET22b forward 5’-A(376)TACGACTCACTATAGGG(358)-3’

Target: pET22b Tm: 51.4°C

pET22b reverse 5’-C(70)TAGTTATTGCTCAGCGG(88)-3’

Target: pET22b Tm: 53.7°C

Universal primers

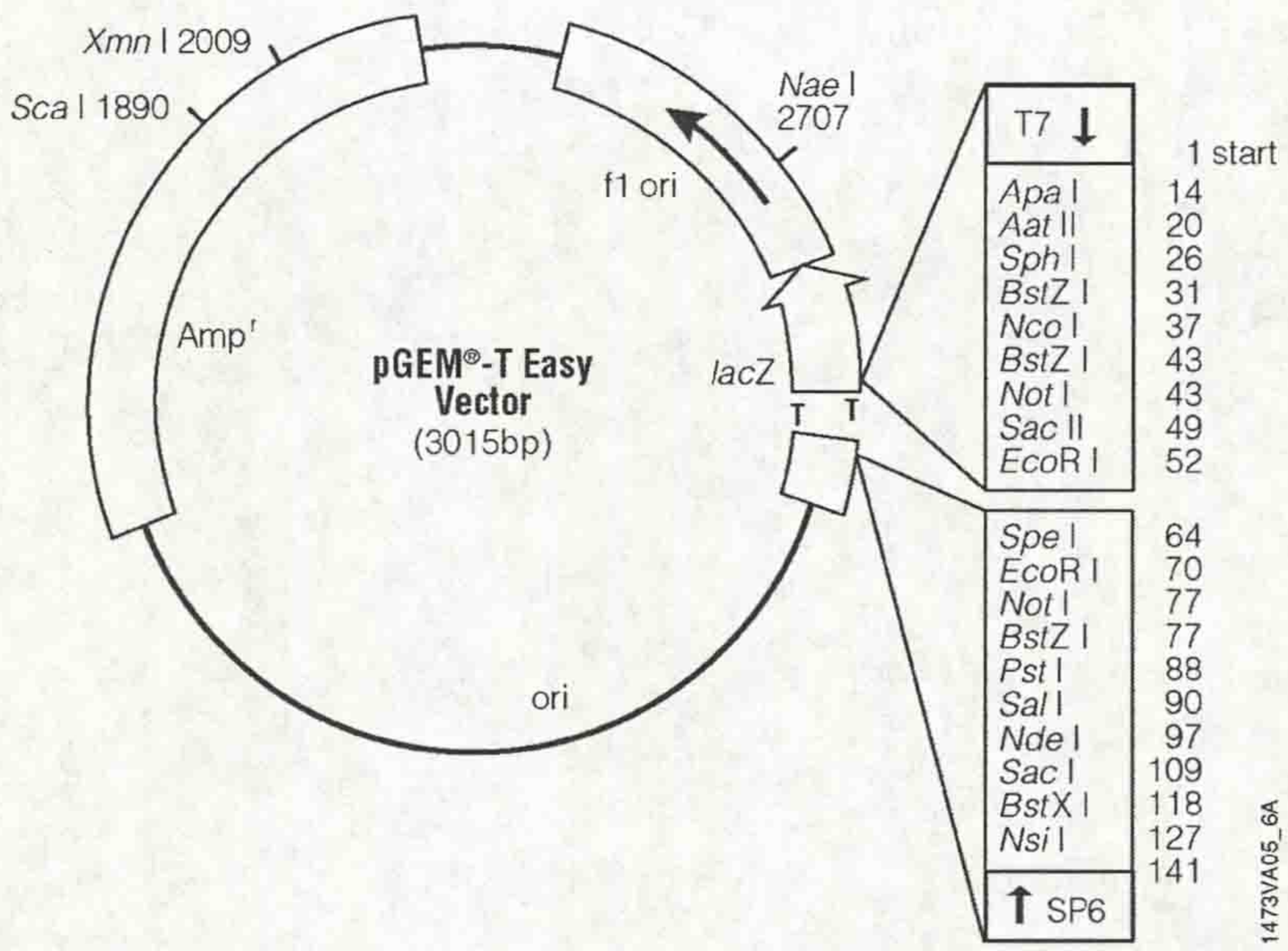
M13 forward 5’-GTAAAACGACGGCCAG-3’

M13 reverse 5’-CAGGAAACAGCTATGAC-3’

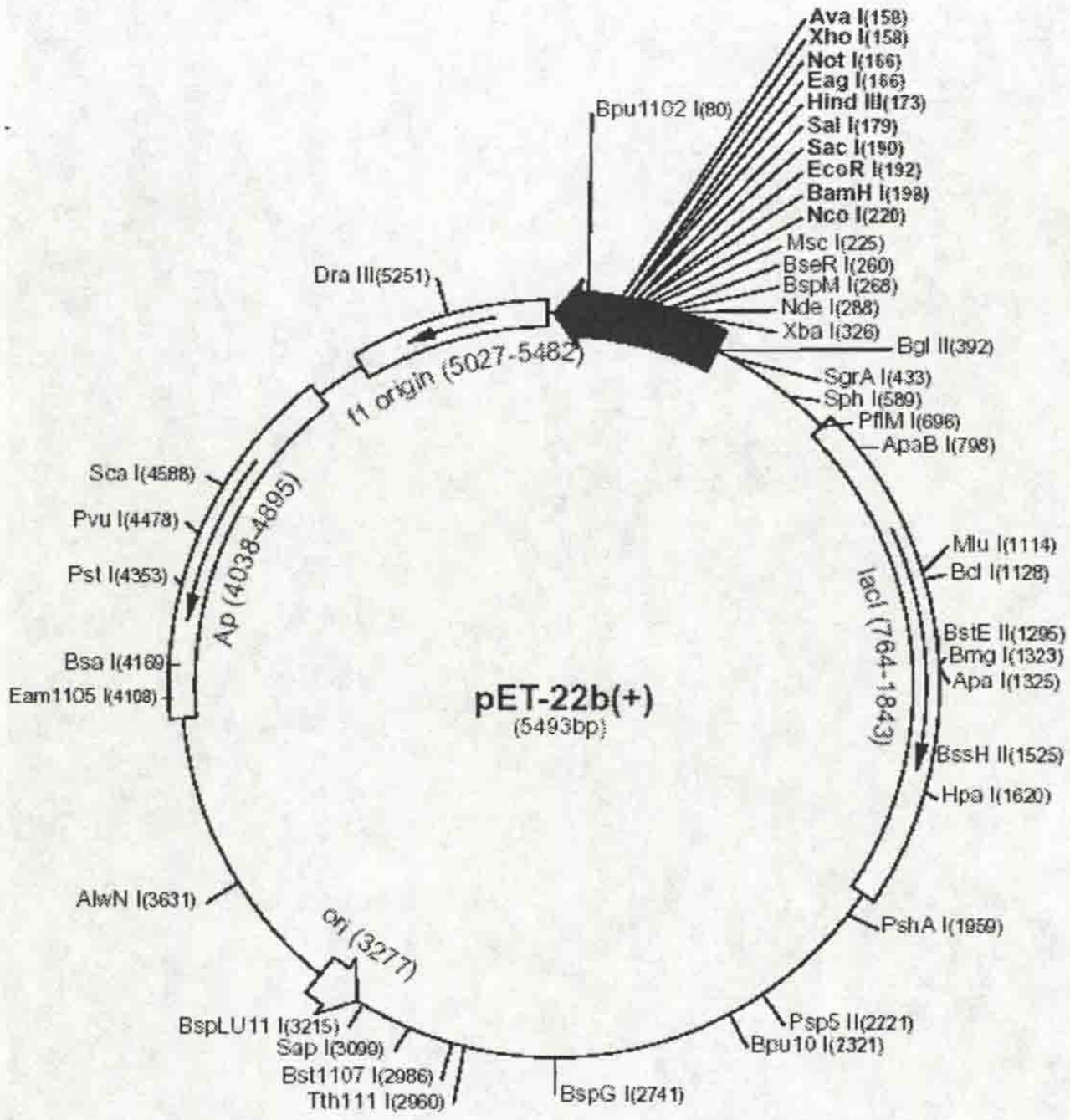
Appendix III

Vector maps:

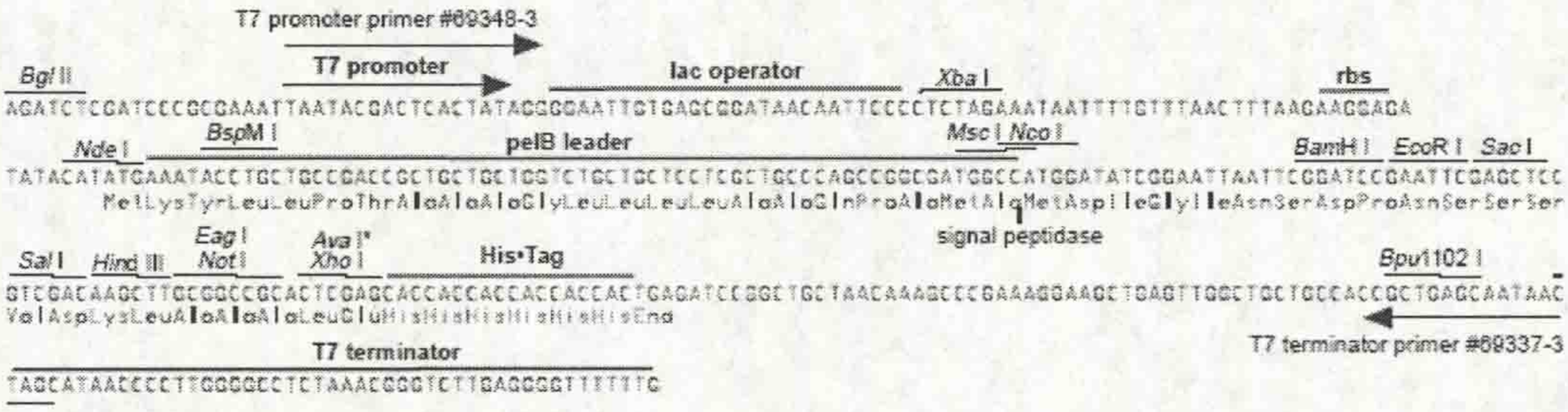
pGEM®-T Easy (Promega) Vector Map



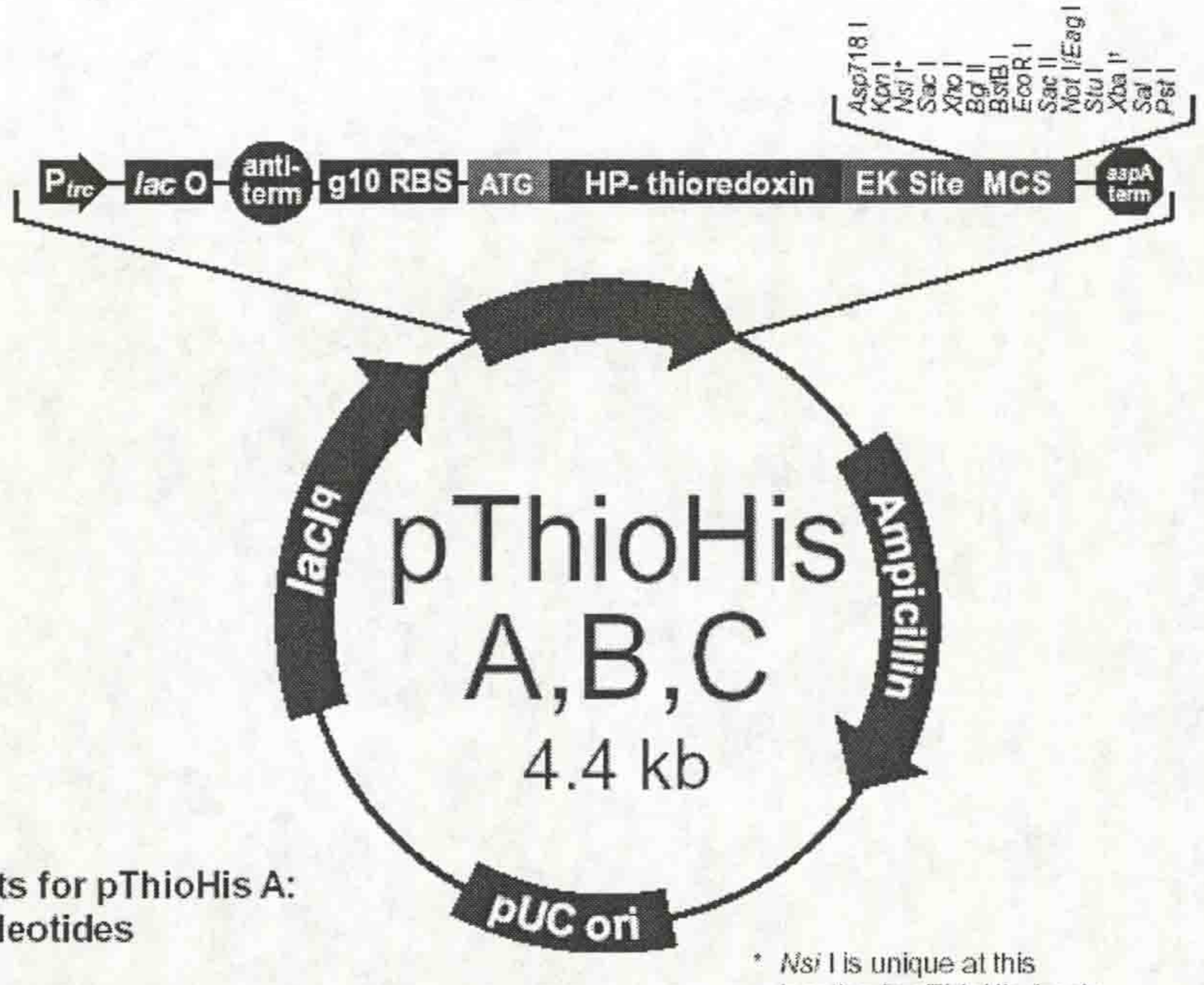
pET22b(+) (Novagen) Vector Map



pET22b(+) cloning/expression region



pThioHis C (Invitrogen) Vector Map



Comments for pThioHis A:
4365 nucleotides

Ampicillin resistance ORF: bases 201-1061
pUC origin: bases 1206-1879
Lac Repressor (*lacI*^{q}) ORF: bases 1967-3049
Trc Promotor Region: bases 3261-3470
-35 region: bases 3281-3292
-10 region: bases 3304-3309
lac operator (*lac*O): bases 3316-3336
*rrn*B antitermination: bases 3352-3421
gene 10 region: bases 3434-3442
Ribosome binding site: bases 3457-3461
Thioredoxin ATG: bases 3471-3473
Trx forward priming site: bases 3777-3794
Enterokinase site: bases 3813-3827
Multiple cloning site: bases 3827-3892
*asp*A Termination: bases 3893-3959
Trx reverse priming site: bases 3967-3986

* *Nsi* I is unique at this location in pThioHis A only.

Nco I is unique at this location in pThioHis B only.

Sph I is unique at this location in pThioHis C only.

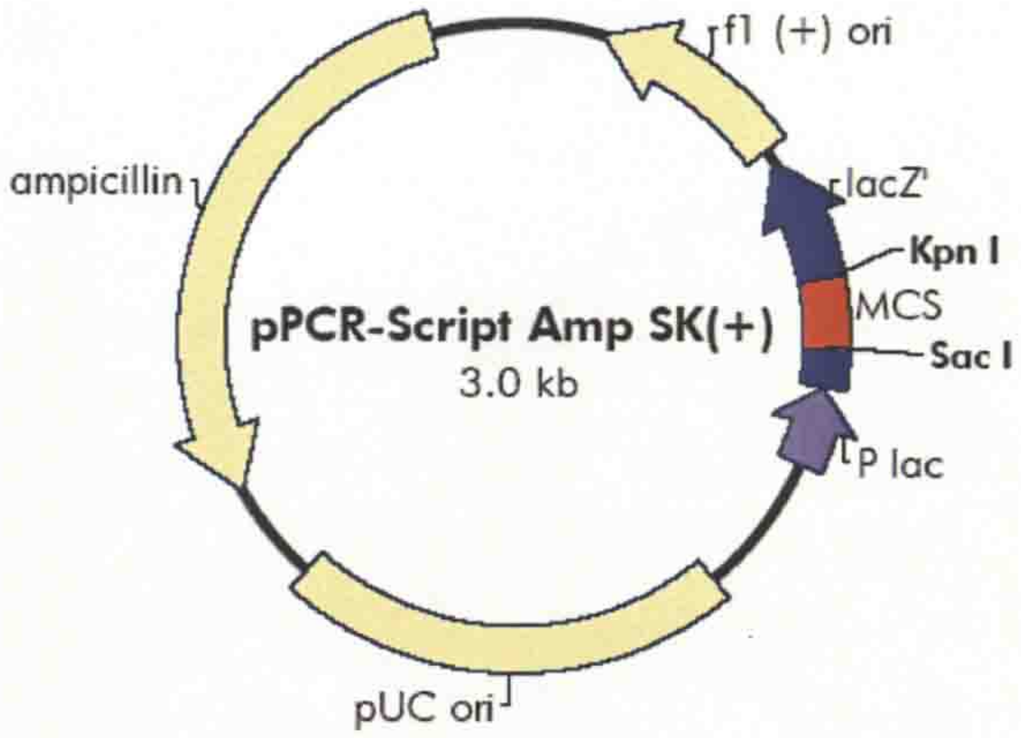
† There is a stop codon following the *Xba* I site in pThioHis C

Multiple Cloning Site of pThioHis C (Invitrogen)

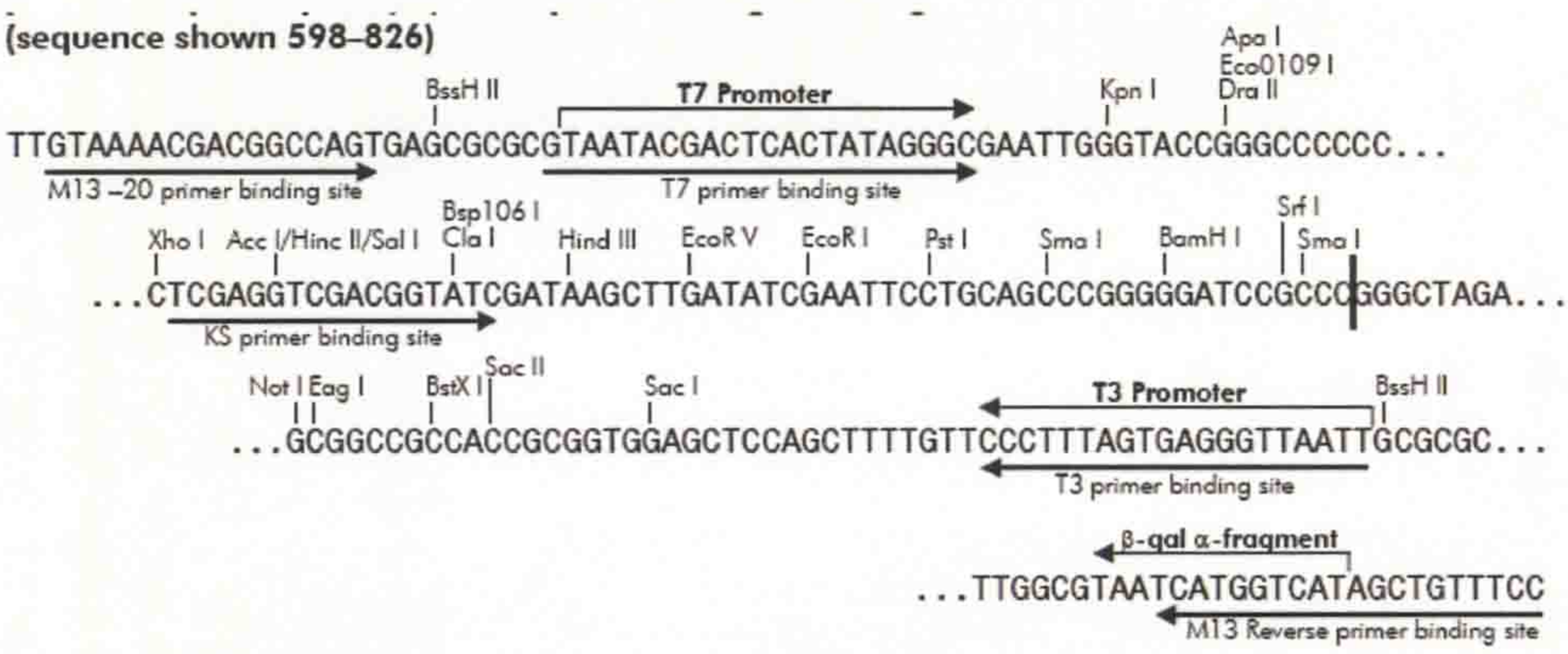
HP-Thioredoxin translational start site
HP-Thioredoxin open reading frame (ORF)

```
3469  AT ATG TCT GAT AAA ATT ATT CAT CTG ACT GAT GAT TCT TTT GAT ACT
         Met Ser Asp Lys Ile Ile His Leu Thr Asp Asp Ser Phe Asp Thr

3516  GAT GTA CTT AAG GCA GAT GGT GCA ATC CTG GTT GAT TTC TGG GCA CAC
      Asp Val Leu Lys Ala Asp Gly Ala Ile Leu Val Asp Phe Trp Ala His
                 RsrII
3564  TGG TGC GGT CCG TGC AAA ATG ATC GCT CCG ATT CTG GAT GAA ATC GCT
      Trp Cys Gly Pro Cys Lys Met Ile Ala Pro Ile Leu Asp Glu Ile Ala

3612  GAC GAA TAT CAG GGC AAA CTG ACC GTT GCA AAA CTG AAC ATC GAT CAC
      Asp Glu Tyr Gln Gly Lys Leu Thr Val Ala Lys Leu Asn Ile Asp His

3660  AAC CCG GGC ACT GCG CCG AAA TAT GGC ATC CGT GGT ATC CCG ACT CTG
      Asn Pro Gly Thr Ala Pro Lys Tyr Gly Ile Arg Gly Ile Pro Thr Leu

3708  CTG CTG TTC AAA AAC GGT GAA GTG GCG GCA ACC AAA GTG GGT GCA CTG
      Leu Leu Phe Lys Asn Gly Glu Val Ala Ala Thr Lys Val Gly Ala Leu
                                  Trx forward sequencing priming site
3756  TCT AAA GGT CAG TTG AAA GAG TTC CTC GAC GCT AAC CTG GCC GGC TCT
      Ser Lys Gly Gln Leu Lys Glu Phe Leu Asp Ala Asn Leu Ala Gly Ser
            Enterokinase recognition sequence  Asp718 I  KpnI      SphI      XhoI SacI BglII
3804  GGA TCC GGT GAT GAC GAT GAC AAG GTA CCT GGC ATG CTG AGC TCG AGA
      Gly Ser Gly Asp Asp Asp Asp Lys Val Pro Gly Met Leu Ser Ser Arg
                                     ▲ Enterokinase cleavage site
      BstB I EcoR I      Sac II Not I      Stu I  Xba I      Sal I          Pst I
3852  TCT TCG AAT TCC GCG GCC GCA GGC CTC TAG AGTCGACCTG CAGTAATCGT
      Ser Ser Asn Ser Ala Ala Ala Gly Leu ***
                                  aspA transcriptional terminator
3902  ACAGGGTAGT ACAAATAAAA AAGGCACGTC AGATGACGTG CCTTTTTTCT TGTGAGCAGT
                        Trx reverse sequencing priming site
3962  AAGCTTGGCA CTGGCCGTCG TTTTACAACG TCGTGACTGG GAAAA
```

pPCR-Script™ Amp SK(+) (Stratagene) Vector Map



pPCR-Script Amp SK(+) (Stratagene) Multiple Cloning Site Region



Appendix IV

PCR-Script™ Amp cloning method (Stratagene):

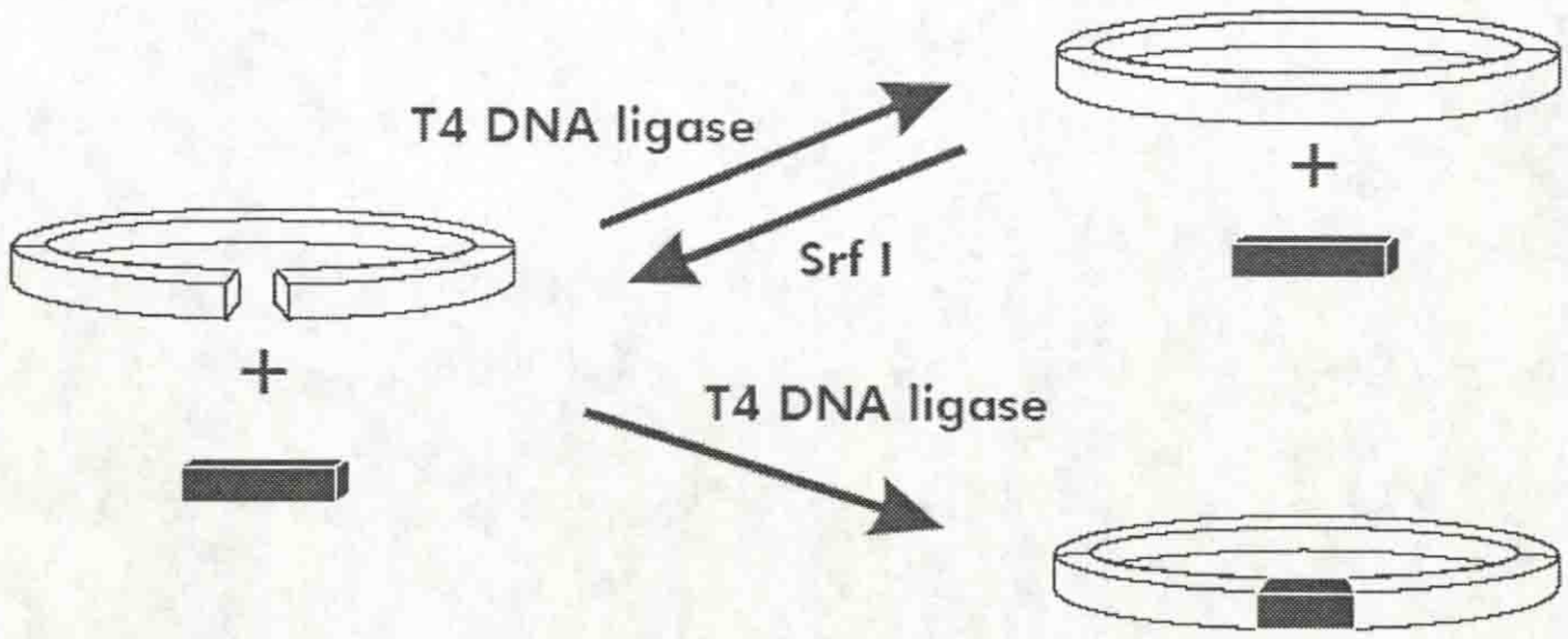


FIGURE 1 The PCR-Script™ Amp cloning method. An aliquot of a PCR product is added to 10 µl of a ligation reaction containing PCR-Script 1 × reaction buffer, 0.5 mM rATP, and 10 ng of *Srf* I-digested pPCR-Script Amp SK(+) cloning vector. The enzymes *Srf* I and T4 DNA ligase are added. The reaction is allowed to proceed at room temperature for 1 hour before heat treating at 65°C for 10 minutes. A 2-µl aliquot of the reaction is then used to transform 40 µl of XL10-Gold Kan ultracompetent cells.

Appendix V

Mutant library construction:

In order to construct a library of mutants, a mutation strategy needed to be chosen. As the substrate of interest, 4-Cyano-4-aryl substituted cyclohex-1-enyl acetate (1), was a new one compared to previous ones tested with the *P. fluorescens* esterase, random mutagenesis was thought to be the best method to insert mutations on the whole *estF* gene.

For random mutagenesis, two methods could be used, ep-PCR or a mutator strain (Section 1.3.2.4). However, work was previously carried out for directed evolution with this esterase in which these two methods were compared (Henke & Bornscheuer, 1999). It was shown that even if both methods are different, the increase in enantioselectivity was similar. Both methods have advantages and disadvantages. The use of a mutator strain led to mutations not only on the gene of interest but also on the entire plasmid, which decreases the number of beneficial mutations. But this method is much simpler compared to ep-PCR where problems can occur in finding appropriate PCR conditions and involve ligation steps which can be a cause of troubles.

However, the use of the GeneMorph PCR Mutagenesis Kit (Stratagene), in ep-PCR, allows more control of the mutation compared to the use of a mutator strain. This kit involves the Mutazyme DNA polymerase. This enzyme has a high intrinsic error rate, which allows more frequent insertion of errors compared to *Taq* DNA polymerase.

Errors are randomly introduced and the enzyme produces a unique mutational spectrum. Mutazyme and *Taq* DNA polymerase generate all possible nucleotides substitutions but the frequency of incorporating each type of mutation differs. Mutazyme DNA polymerase preferentially replaces G's or C's with A's or T's contrary to *Taq* DNA polymerase which tends to replace A's or T's by G's or C's. Mutazyme also makes fewer insertion and deletion mutations, which may create frame shifts that result in inactive proteins. Low, medium and high mutation frequencies can also be achieved by varying the initial amount of target DNA in the reaction. Targets amplified from low amounts of target DNA undergo more duplications than targets amplified from high concentrations of DNA. The more times a target is replicated, the more errors accumulate. Therefore, higher mutation frequencies are achieved by lowering input DNA template concentration. On the other hand, lower PCR mutation frequencies can be achieved by using higher DNA template concentrations to limit the number of target duplications. According to the advantages offered by this kit, it was decided to use ep-PCR as the mutation strategy in this project.

1 Mutation strategy: ep-PCR

Ep-PCR was performed on the wild-type *estF* gene using a Gene Morph PCR Mutagenesis Kit (Stratagene) and following the manufacturer's protocol. As a medium mutation frequency was targeted, the initial amount of the *estF* gene needed was between 10pg and 10ng. For 5ng of the gene, 30ng of the plasmid was needed and the final volume used for PCR was 50µl. The specific Mutazyme DNA polymerase (from Stratagene kit) was used with the 10X Mutazyme reaction buffer. This buffer was added to ultrapure water as well as a provided mix of the four dNTP

for a final concentration of 0.2mM from a stock solution of 40mM following the manufacturer's protocol. A mix of primer was made containing 250ng/µl of each primer and 0.5µl of this solution was added to the previous reaction mixture. Mutazyme DNA polymerase was added from a stock solution of 2.5U/µl followed by pJOE2792 solution (30ng of plasmid). Primers pJOE2792-NdeI and pJOE2792-HindIII (Appendix II) were used for this PCR reaction (typical protocol as previously described), annealing temperature: 65°C. The PCR product was purified from agarose gel.

The PCR produced a product of the expected size (861bp) (Figure 1). The total amount of product loaded could be estimated at 1µg. Following the equations given in the instruction manual (Stratagene), d, the number of duplication during the PCR was:

$d = \log_{10}$ (PCR yield/initial target)/log 2

$d = \log_{10}$ (1000/5)/log2

$d = 7.64$

The mutation frequency was:

Mutation frequency = 0.31d + 0.41

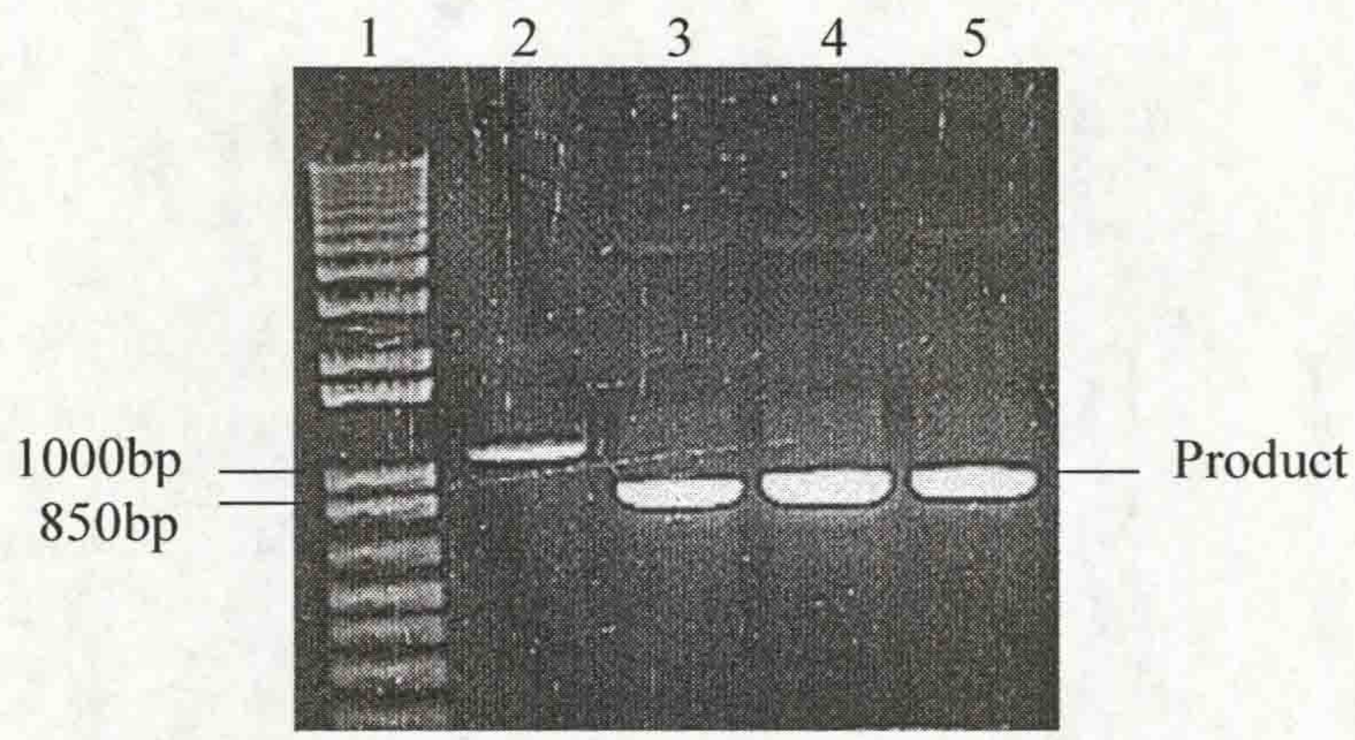
Mutation frequency = 0.31 x 7.64 + 0.41

Mutation frequency = 2.8

A mutation frequency of 3 mutations/kb could then be expected in this mutant library which corresponded to the limit between a low range and a medium range mutation frequency as expected.

Figure 1: Library of mutant *estF* performed by ep-PCR

PCR was performed with primers primers pJOE2792-NdeI and pJOE2792-HindIII. The whole reaction volume was loaded on 1% agarose gel, containing 0.6µg:µl ethidium bromide.
1: 1Kb plus DNA Ladder (Invitrogen)
2: 1.1kb gel standard (20ng/µl) from GeneMorph Random mutagenesis Kit (Stratagene)
3: *estF* ep-PCR products (10µl)
4,5: *estF* ep-PCR products (20µl)



2 ep-PCR products cloning

2.1 Cloning of the mutated gene in the original plasmid

In order to clone the product from ep-PCR, digestion of the mutated gene and the original plasmid (pJOE2792) needed to be performed. The insert (mutated gene) and pJOE2792 (Appendix III) were digested with restriction enzymes *Nde*I and *Hind*III. The wild-type plasmid (1µg) and the mutated gene (100ng) were mixed with 15U of each enzyme. The digestion revealed two bands of the expected size (4271bp and 853bp respectively) (Figure 2). These DNA fragments were extracted for the ligation. This experiment was also carried out with *Nde*I and *BamH*I restriction enzymes followed by a ligation step (Figure 3).

The digested insert was cloned into the matching digested pJOE2792 (the same restriction enzymes were used for both insert and plasmid) with T4 DNA ligase (MBI) using various insert:vector ratios, incubation times and incubation temperatures. Ligation products were transformed into DH5α competent cells and into JM109 competent cells. Unfortunately, none of the plasmids extracted from these potential clones showed the expected plasmid size, this indicates that possible contamination of the DH5α strain occurred. Therefore, all experiments were performed again with *E. coli* JM109.

In order to test the efficiency of the restriction enzymes, single digestions of the wild-type pJOE2792 were performed with *Nde*I, *Hind*III and *BamH*I (Figure 4). These results showed that these enzymes were efficient and active. Different ligases were used as well: T4 DNA ligase (MBI) and T4 DNA ligase from the pGEM-T Easy ligation kit (Promega) in order to test the efficiency of the ligases. Products of

Figure 2: Digestion of wild-type pJOE2792 and mutant *estF* by *Nde*I and *Hind*III

30µl of digested pJOE2792 and 50µl of digested *estF* were analysed by using 1% agarose gel, containing 0.6µg:µl ethidium bromide
1, 5: 1Kb plus DNA Ladder (Invitrogen)
2: Non digested wild-type pJOE2792
3, 4: Digested pJOE2792 by *Nde*I and *Hind*III
6, 7: Digested mutant *estF* by *Nde*I and *Hind*III

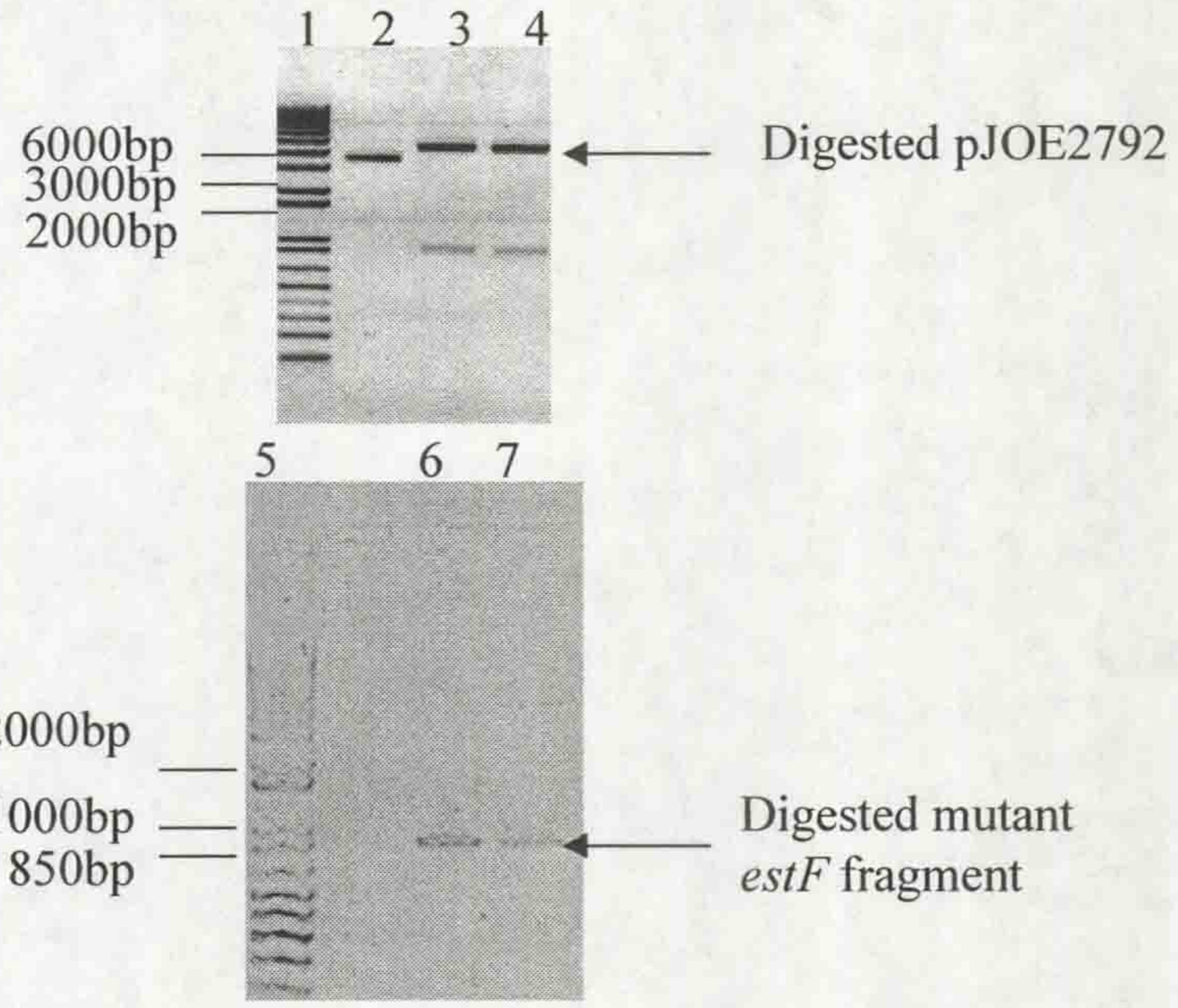


Figure 3: Digestion of wild-type pJOE2792 and mutant *estF* by *Nde*I and *BamH*I

50µl of digested pJOE2792 and 50µl of digested *estF* were analysed by using 1% agarose gel, containing 0.6µg:µl ethidium bromide
1: 1Kb plus DNA Ladder (Invitrogen)
2, 3: Digested pJOE2792 by *Nde*I and *BamH*I
4, 5, 6: Digested mutant *estF* by *Nde*I and *BamH*I

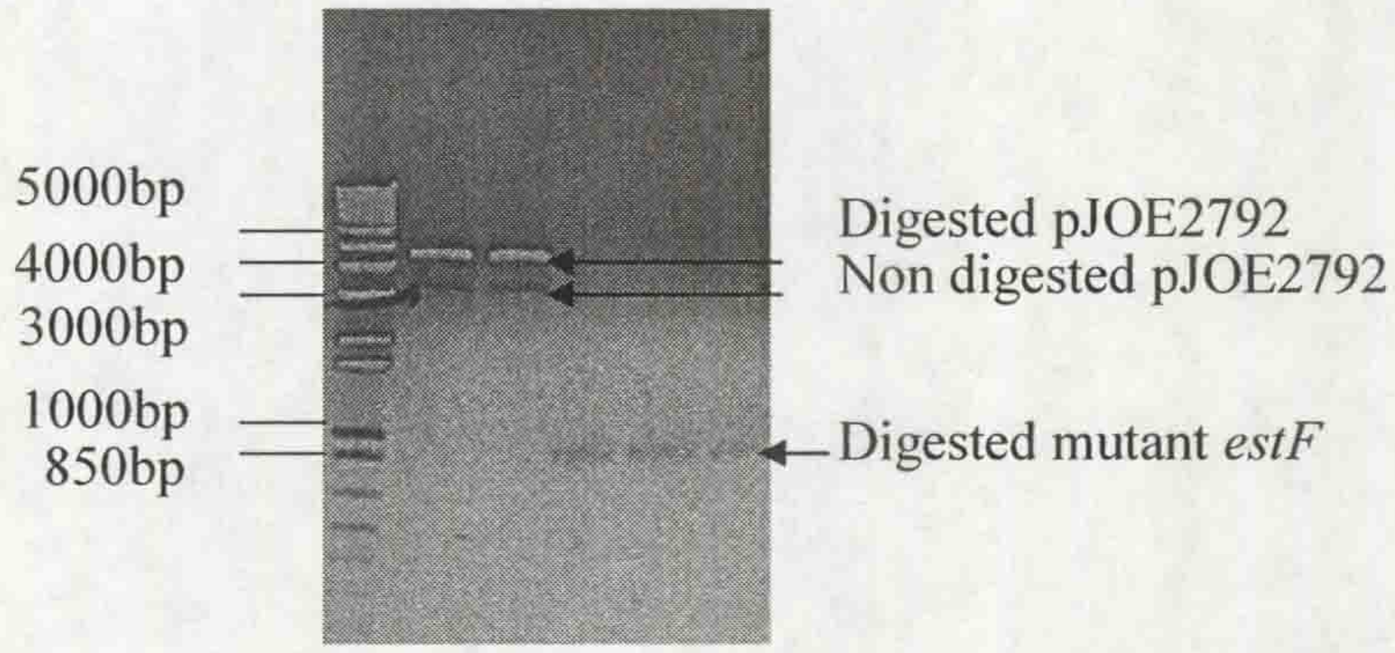
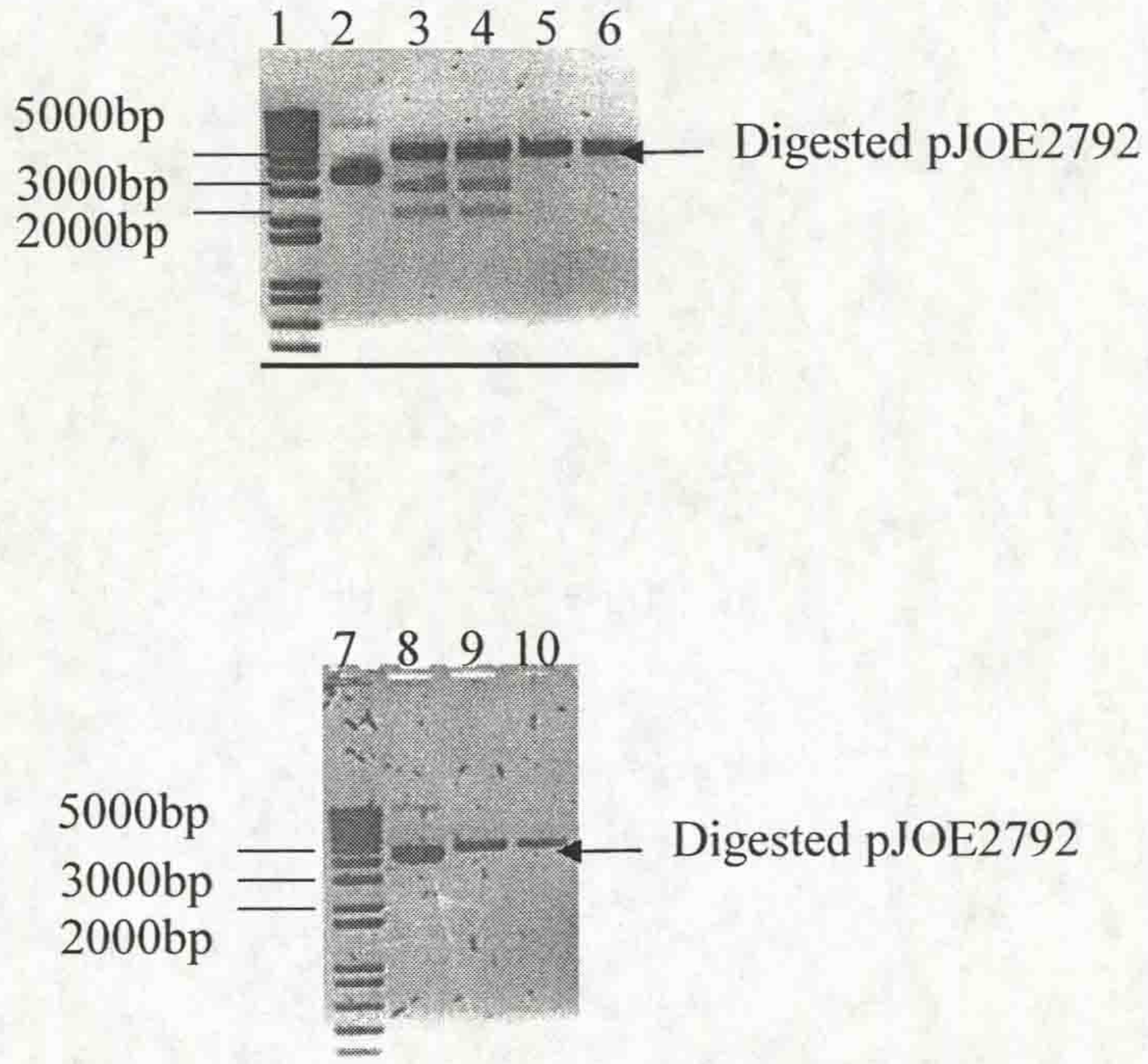


Figure 4: Digestion of wild-type pJOE2792 by NdeI, BamHI and *Hind*III

20µl of digested pJOE2792 were analysed by using 1% agarose gel, containing 0.6µg:µl ethidium bromide
1, 7: 1Kb plus DNA Ladder (Invitrogen)
2, 8: Non digested wild-type pJOE2792
3, 4: Digested pJOE2792 by *BamH*I
5, 6: Digested pJOE2792 by *Nde*I
9, 10: Digested pJOE2792 by *Hind*III



ligation were transformed only into JM109 competent cells. All ligation products allowed the observation of colonies on incubated Petri dishes, showing the efficiency of the different ligases by allowing the re-ligation of single digested plasmids.

However, even if the restriction enzymes and ligase were efficient, the ligation of the mutant *estF* and pJOE2792 could not be achieved. The most probable explanation could be a problem during the digestion of the mutant *estF*. Even though eight additional C bases were inserted at the end of the gene sequence, to allow the restriction enzymes to cut the mutant gene, digestion of PCR products is a common source of problem for ligation (Elion, 1997). To solve this problem, it was decided to clone the mutant gene into a plasmid in which, digestion was not necessary.

2.2 Cloning of the mutated gene into PCR-Script plasmid (Stratagene)

To improve the digestion of the mutated gene by *Nde*I and *Hind*III restriction enzymes, the mutated gene was cloned into another plasmid. As ep-PCR products using the Gene Morph PCR Mutagenesis Kit (Stratagene) are blunt-ended, a specific plasmid allowing ligation of blunt-ended products was needed as the PCR-Script plasmid. Most of the commercially available plasmids only allow the cloning fragments ending with As. However, the PCR-Script plasmid (Stratagene) allows the cloning of blunt-ended fragment. PCR products are incubated with the pre-digested pPCR-Scrip Amp SK(+) cloning vector (Appendix I and III), *Srf*I, and T4 DNA ligase. Using the restriction enzyme in the ligation reaction maintains a high-steady-state concentration of digested vector DNA and allows the use of non phosphorylated, unmodified PCR primers. The ligation efficiency of blunt-ended DNA fragments is increased by the simultaneous, opposite reactions of the *Srf*I restriction enzyme and

T4 DNA ligase (Appendix IV) on non recombinant vector DNA. *Sfr*I is a novel rare-cleavage restriction enzyme that recognises the oligonucleotide sequence 5'-GCCCGGGC-3'. It was therefore decided to use this plasmid for the cloning of the mutant *estF* gene.

Ligation was performed between the mutated *estF* and the pPCR-Script Amp SK(+) following the manufacturer's manual. The ligation product was transformed into *E. coli* XL10-Gold competent cells provided in the kit. After extraction of the plasmids, named pPCR-Script-estF, only one clone was a true positive according to the size obtained and its sequencing. In this sequence, three mutations could be observed on the sequence L(CTA)163L(TTA), Q(CAG)260stop(TAG) and L(TTG)270F(TTC). This mutation range was in the range expected of 2-3 mutation in the gene. However, in this case, the expression of the protein corresponding to this gene would have led to a 10 amino acid-long truncated protein, as a glutamine was replaced by a stop codon.

2.3 Cloning of the mutated gene into pGEM-T Easy (Promega)

In parallel with the use of the pPCR-Script Amp SK(+) plasmid, an attempt to allow the ligation of the mutated *estF* for further digestion was performed with pGEM-T Easy. As previously described, this plasmid allows TA cloning, and as the mutant *estF* purified from ep-PCR is blunt-ended, TA ends, needed to be added to the mutant gene sequence. To allow this addition, a normal PCR using DNA Taq polymerase was used with the mutant *estF* as a template. The insertion of mutation by the Taq polymerase was not in this case considered as a potential problem as the gene was

already mutated during the ep-PCR and the principal aim of these experiments was to introduce mutations in the *estF* gene.

A normal PCR using usual Taq DNA polymerase was performed in the same conditions used for the ep-PCR, using 20ng of the mutated gene library, concentrations and protocols as previously described. The PCR product was purified using a High Pure PCR Purification Kit (Roche), following the manufacturer's protocol. After analysis, a band could be observed between 850bp and 1000bp corresponding to the size expected (Figure 5). Ligation of the ep-PCR product and pGEM-T Easy plasmid was performed. Blue and white colonies were observed but unfortunately, analysis of white colonies showed only the presence of the original pGEM-T Easy plasmid.

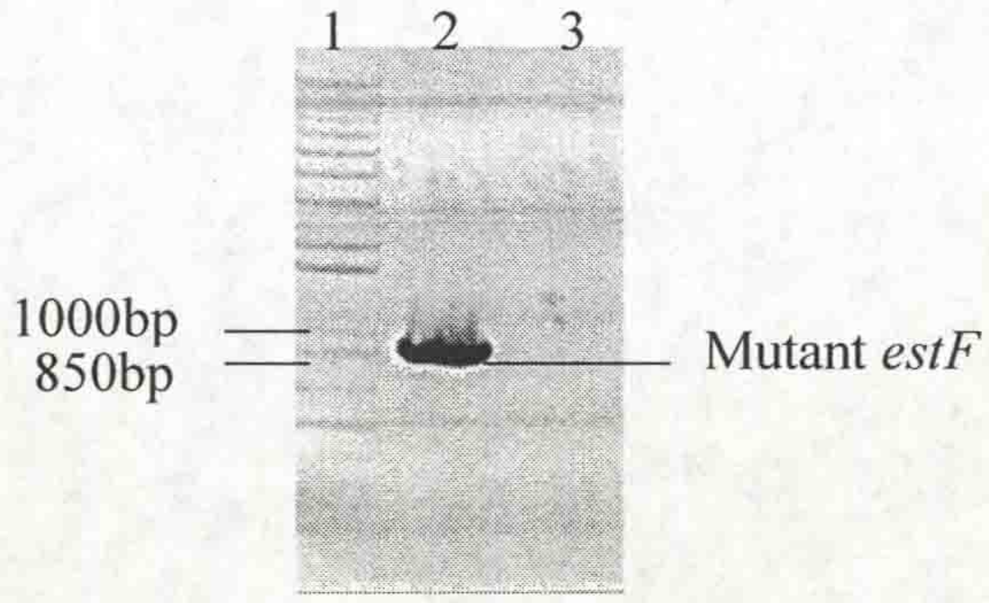
3 Analysis of unsuccessful attempts to produce a library of mutant pJOE2792

From the previous results, it was shown that no ligation could be achieved from digested mutant *estF* and digested wild-type pJOE2792. Investigations discovered problems of contamination of competent DH5α cells. Though the use of other competent cells did not produce mutants, this indicates that the problematic step was the ligation. Problems for the efficient ligation of PCR products occurred in previous experiments (Henke & Bornscheuer, 1999). These problems may also come from the digestion step of the different fragments or the ligase.

Investigations to test the different enzymes were made and showed the three different restriction enzymes *Nde*I, *Hind*III and *BamH*I were all efficient on the pJOE2792 plasmid as well as the ligases. As previously described, the digestion of PCR products may allow troubleshooting, this was the reason why supplementary bases were

Figure 5: PCR of ep-PCR product (*estF*) with DNA Taq plymerase

PCR was performed with primers pJOE2792-NdeI and pJOE2792-HindIII. 10µl of each reaction were analysed by using 1% agarose gel, containing 0.6µg:µl ethidium bromide
1: 1Kb plus DNA Ladder (Invitrogen)
2: Mutant *estF*
3: Negative control (no DNA)



inserted on both ends of primers used during the ep-PCR, eight cytosine bases were inserted. This number was superior to the addition of the seven bases commonly recommended when using *Nde*I and cytosine was used as it also involved an increase in the primers stability. However, the digestion of PCR products could not be accurately checked on agarose gel.

In order to allow an effective ligation, attempts to clone the mutant gene in a common cloning vector were performed. This was achieved by using the pPCR-Script Amp SK(+) (Stratagene) and the pGEM-T Easy (Promega). Attempts using the pGEM-T Easy were unsuccessful. The use of pPCR-Script Amp SK(+) allowed the ligation of the mutant *estF* and the sequencing of one of the five positive mutants, isolated from this experiment, in which three mutations could be observed. This indicates that the mutation process administered to the gene, using the GeneMorph PCR Mutagenesis Kit (Stratagene), was efficient and in the range expected. From the three mutations, two led as well to a change in the protein sequence and one with a dramatic effect as it inserted a stop codon, 10 amino acids before the original length of the esterase. However, ligation and transformation into two different strains of competent cells, using ligases tested before, did not allow the production of mutant into pJOE2792, which could have led to the expression of mutant protein.