
بسم الله الرحمن الرحيم
يؤتي الحكمة من يشاء ومن يؤت الحكمة فقد أوتي خيرا كثيرا
١٣٧٧

Characterisation of esterase genes in the genomes of *Streptomyces coelicolor* A3(2) and *Streptomyces avermitilis*

Dem Fachbereich Biologie der Universität Kaiserslautern zur Erlangung des akademischen Grades „Doktor der Naturwissenshaften“ eingereichte Dissertation

Vorgelegt von

Sameh Soror

Vorsitzender der Prüfungskommission: Prof. Dr. J. Herrmann

Erster Berichterstatter: Prof. Dr. J. Cullum

Zweiter Berichterstatter: Prof. Dr. R. Hackenbeck

Lehrbereich Genetik der Universität Kaiserslautern, März 2007

D386

Abbreviations

ACE	Angiotensin Converting Enzyme
A.d	Aqua Distillata
Amp	Ampicillin
APS	Ammonium Persulphate
ATP	Adenosine Triphosphate
AUDs	Amplifiable Units of DNA
bp	Base Pair
B.C.	Before Christ
BSA	Bovine Serum Albumin
ca.	Circa
ccc	"Covalently Closed Circular"
CIAP	Calf intestinal alkaline phosphatase
DFP	DIisopropylfluorophosphate
DMF	N, N-Dimethylformamide
DMSO	Dimethylsulphoxide
dNTP	Deoxyribonucleotide Triphosphate
DTT	1,4-Dithiotheritol
E.coli	*Escherchia coli*
EDTA	Ethylene Diamine Tetra-Acetic Acid
EIA	Enzyme Immunoassay
EP-PCR	Error Prone PCR
EtBr	Ethidium Bromide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HMMER	Hidden Markov Model, software for protein sequence analysis
HSL	Hormone Sensitive Lipase
IPTG	Isopropylthio-β-D-galactoside
IS	Insertion Sequence
IUBMB	International Union of Biochemistry and Molecular Biology
IUPAC	International Union of Pure and Applied Chemistry
NSAIDs	Non Steroidal Anti-Inflammatory Drugs
kb	Kilobase (1000bp)

KDa	kilodalton
KV	Kilovolt
LB	Luria Broth
Mb	Megabase
MBP	maltose binding protein
NBT	4-nitro blue tetrazolium chloride
NSAIDS	Non-steroidal antiinflammatory drugs
NusA	N-utilizing substance A
OD	Optical Denisty
PAGE	Polyacrylamid Gel Electrophoresis
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PFGE	Pulsed Field Gel Electrophoresis
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
TIRs	Terminal Inverted repeats
RNase	Ribonucliease
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
Sol	Solution
Tab	Table
TAE	Tris-acetate- EDTA buffer
TEMED	N,N,N`,N`-tetramethyl-ethylenediamine
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YEME	Yeast Extract Malt Extract
Ω	Ohm

Table of contents

1. Introduction

1.1. Enzymes

The German physiologist Wilhelm Kühne was the first to use the term “Enzymes” in 1876 to describe the molecules responsible for fermentation of sugars to alcohols. The word enzyme comes from Greek “in leaven” (Friedmann 1997). An enzyme is a protein that speeds up (catalyzes) a chemical reaction. Some RNA molecules also have catalytic activity, and they are referred to as RNA enzymes or ribozymes. The first enzyme was obtained in pure form in 1926 by James Sumner. He was able to isolate and crystallize a urease enzyme from the jack beans and he postulated that all enzymes are proteins. He earned the 1947 Nobel Prize for that work (Sumner 1946; Nelson and Cox 2005).

1.1.1. Why are enzymes of interest?

Enzymes are central to every biochemical process within living cells. They are responsible for nutrient degradation, synthesis of biological macromolecules from simple precursors, DNA repair and replication etc. Simply, enzymes catalyze nearly all the metabolic reactions and in their absence the reactions will proceed at very slow rate, incompatible with living dynamics i.e. their activities are necessary to sustain life (Whitford 2005). Enzymes are interesting not only because of the aforementioned physiological roles, but also for their use in several other commercial applications. Enzymes have extraordinary catalytic power, often greater than that of synthetic or inorganic catalysts. They have a high degree of specificity for their substrates. They accelerate chemical reactions tremendously, and they function in aqueous solutions under very mild conditions of temperature and pH**.** All of the previous had encouraged the employment of enzymes in various commercial applications such as therapeutic, diagnostic and analytical reagents and as catalysts in different industries e.g. dairy, paper, cosmetics, etc (Schmid *et al*., 2001).

Various enzymes have been used as diagnostic tools for many years ago (e.g. alkaline phosphatase and peroxidase). They are used for the detection and quantification of some medically significant metabolites in biological samples. Enzymes are also largely used as labels in enzyme immunoassay (EIA). Enzymatic preparations are ideal diagnostic reagents, because they are highly selective and it possesses catalytic efficiency.

Although enzymes were first recognized scientifically only 130 years ago, humans have used the enzymes commercially since thousands of years. Enzymes have been used in ancient societies for preparing dairy products e.g. cheese, bread and meat tenderizing. In these cases people unknowingly used microorganisms as a source of the enzymes required for fermentation. Currently a large group of enzymes is used for catalysis in industry, they are called industrial enzymes or bulk enzymes. This group includes amylases, cellulases, cyclodextrin glycosyltransferase, esterases, lipases, lignocellulose degrading enzymes, pectinases, pencillin acylases, phytases and proteases (Copeland 2000; Walsh 2004).

Genetic engineering has had a great influence on the industrial enzyme sector. Production of industrial enzymes by recombinant technology is more favourable than the traditional approaches for the following reasons: recombinant proteins can be expressed in high levels, the products are of higher relative purity, recombinant enzymes are economically attractive, heterlogous expression allows commercial production of enzymes normally produced by pathogenic strains and the most important reason is that recombinant technology facilitates the alteration of enzyme's characters through protein engineering. Protein engineering may be used to tailor selected enzymes in order to make them more suitable for industrial applications (e.g. enhance thermal stability, alteration of substrate specificity, enhanced stability in the presence of detergents and organic solvents, etc (Walsh 2004).

In late 1990s the annual worldwide sales value of industrial enzymes was US $ 1.5 billion. The market value is expected to exceed US $ 2.4 billions in 2009 (source bcc research, Enzymes for industrial applications, Dec. 2004). The continual growth of enzymes' market can be attributed to the technical advances; the impact of genetic engineering on enzyme production and the development of new enzyme applications.

1.1.2. Enzyme mechanism

Many common reactions in the biochemistry require chemical events that are unfavourable in the cellular environment, such as the transient formation of unstably charged intermediate or the collision of two molecules in a precise orientation required for the reaction. An enzyme solves these problems through providing a special environment within which a reaction can occur more rapidly. Enzymes are usually specific as to the reaction they catalyze and the substrate they act upon. Shape, charge complementarities and hydrophilic/hydrophobic characters of the enzymes and substrates are responsible for this specificity (Nelson and Cox 2005).

Enzymes show different levels of specificity: absolute specificity where the enzyme catalyzes only one reaction, group specificity where the enzyme acts on specific functional groups e.g. amino groups, phosphate groups, etc, linkage specificity where the enzyme acts on particular chemical bond regardless of the rest of the molecule structure and stereochemical specificity when the enzyme acts on a particular optical isomer. The enzyme-specificity led to the development of "**lock and key theory**" in 1894 by Emil Fischer. Fischer suggested that the enzymes are very specific because both of the enzyme and the substrate possess specific complementary geometric shapes that fit exactly into one another. An enzyme fits to its substrates to form a short lived complex. This model explains the enzyme specificity, but it fails to explain the transition state stabilisation. In 1958 Daniel Koshland suggested a modification of the lock and key model, "**Induced fit theory**". Enzymes have flexible structures rather than a rigid geometry. The active site of an enzyme can be modified as the substrate interacts with the enzyme. The amino acids side chains which make up the active site are moulded into a precise shape which enables the enzyme to perform its catalytic function. In some cases the substrate molecule changes the shape slightly as it enters the active site. This model, in contrast to the lock and key theory, explains the occurrence of enzyme specificity and stabilisation of the transition state e.g. reaction of hexokinase with D-glucose (Fig 1.1) (Koshland 1994).

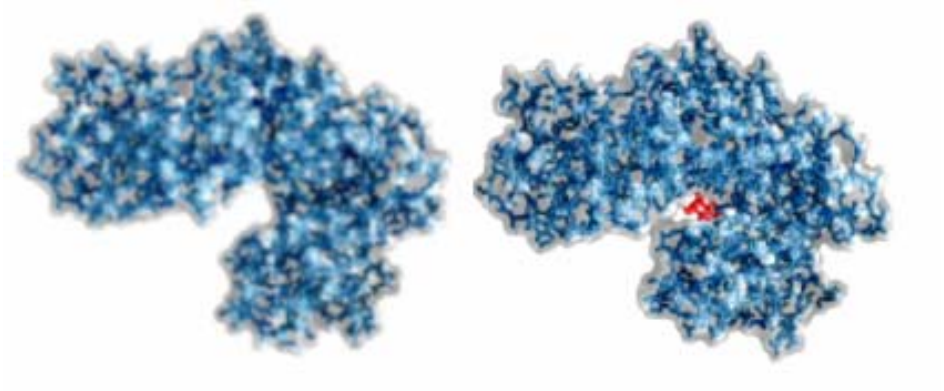


Fig. 1.1. Induced fit in hexokinase. (1) Hexokinase has a U-shaped structure (PDB ID 2YHX). (2) The ends pinch toward each other in a conformational change induced by binding of D-glucose (red) (derived from PDB ID 1HKG and PDB ID 1GLK). (Nelson and Cox 2005)

1.2. Esterases/lipases

The enzymes (EC 3.1.1.x), according to the NC-IUBMB/IUPAC classification and nomenaclature system, represent a group of hydrolases acting specifically on carboxylic esters. The major two subclasses within this group of enzymes are esterases (EC 3.1.1.1 carboxylester hydrolases) and lipases (E.C. 3.1.1.3 triacylglycerol hydrolases) (Bornscheuer 2002).

1.2.1. Structure of esterases and lipases

All of the known esterases and lipases are proteins. The polypeptide chains with any post-translational modification constitute the primary structure of the protein. The local conformation that the polypeptide chain attains to keep itself unstrained is called the secondary structure. α-helices and the β-sheets are the two common secondary structural compenents for all the lipolytic enzymes. The polypeptide chain folds in a particular fashion to produce a three-dimensional product with a tertiary structure. Individual protein chains may sometimes group together to form a complex of two or more monomers, which are the quaternary structure (Nelson and Cox 2005), e.g. an extracellular carboxylesterase from the basidiomycete *Pleurotus sapidus* is composed of eight identical subunits (Zorn *et al*., 2005).

The determination of the 3D structure of both esterases and lipases indicates that the bacterial esterases/lipases contain the characteristic α/β hydrolase fold. The α/β hydrolase fold is characteristic for the largest group of structurally related enzymes (esterases, lipases, hydrolases, proteases, etc) with diverse catalytic functions (the α/β hydrolase fold family)**.** The central enzyme core is formed by β-sheets of eight strands (Fig 1.2).

An enzyme catalyzed reaction is distinguished from other reactions by taking place within a definite pocket on the enzyme called the active site, which is a very small portion of the enzyme around 10 amino acid residues. The catalytic site of esterases/lipases is a serine protease-like catalytic triad consisting of the amino acids serine (nucleophile), histidine and aspartate or glutamate (acid); the nucleophilic serine is located in a highly conserved pentapeptide Gly-X-Ser-X-Gly and the aspartate or the glutamate residue is bounded through a hydrogen bond to the histidine (Fig 1.2).

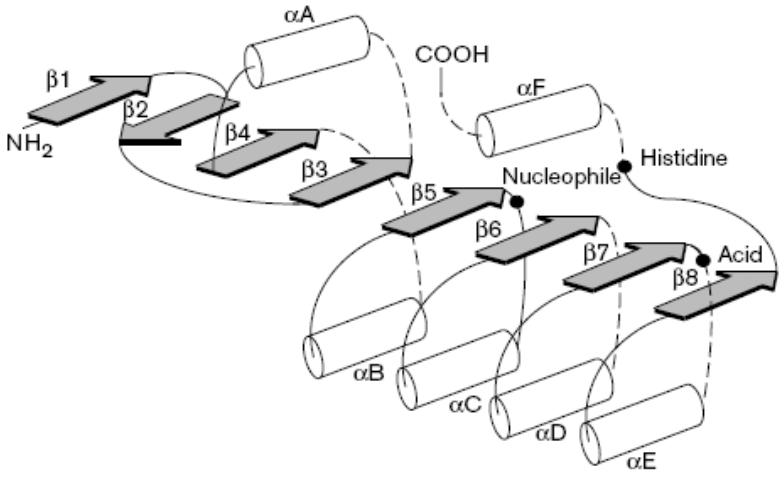


Fig. 1.2. Secondary structure diagram of the 'canonical' α/β hydrolase fold. α Helices and β strands are represented by white cylinders and grey arrows, respectively. The location of the catalytic triad is indicated by black dots. Dashed lines indicate the location of possible insertions. (Nardini and Dijkstra 1999)

1.2.2. Catalytic activity of lipolytic enzymes

Esterases and lipases show various catalytic activities with different specificties. Some lipases show different rates against mono-, di- and triglycerides. Some esters act against either primary or secondary esters while others act nonspesifically. Some lipolytic enzymes show stereospecificity and/or regioselectivity (Jensen et al., 1983).

Some lipolytic enzymes require other substances called cofactors to exert catalytic activity. Cofactors may be essential inorganic metal ions (e.g. Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, etc) or coenzymes, which are complex organic or metallo-organic molecule (e.g. coenzyme A). The metal ion or the coenzyme is called prosthetic group, when it bounds tightly or covalently to the enzyme protein (Whitford 2005). There are several reports about dependence of esterases and lipases on metal ions e.g the activity of nine lipases from six different *Staphylococcus* species are Ca^{2+} dependent (Rosenstein and Gotz 2000).

Also some esterases/lipases have binding sites for small molecules, which are often direct or indirect products or substrates of the reaction catalyzed. This binding can serve to increase or decrease the enzyme's activity (depending on the molecule and enzyme), providing a means for feedback regulation, e.g. long chain acyl coenzyme A has an inhibitory effect on the activity of HSL in adipocytes (Hu *et al*., 2005).

1.2.3. Thermodynamics of Esterases and lipases

Esterases and lipases are catalysts; they increase the speed of a chemical reaction without themselves undergoing any permanent chemical changes. In order that most chemical reactions to proceed, they need some amount of energy as a driving force, which is called activation energy. As a catalyst an esterase or lipase lowers the activation energy of a reaction. The catalyst increases the reaction rate without affecting the equilibrium state; the forward and reverse reactions are affected to the same degree by the catalyst. The chemical reaction rate is depending on the rate of formation of the transition state complex, hence lowering the activation energy leads to an increase in the reaction rate. An esterase or lipase lowers the activation energy and increase the reaction rate mostly through stabilisation of the transition state (Fig 1.3) (Marangoni 2003).

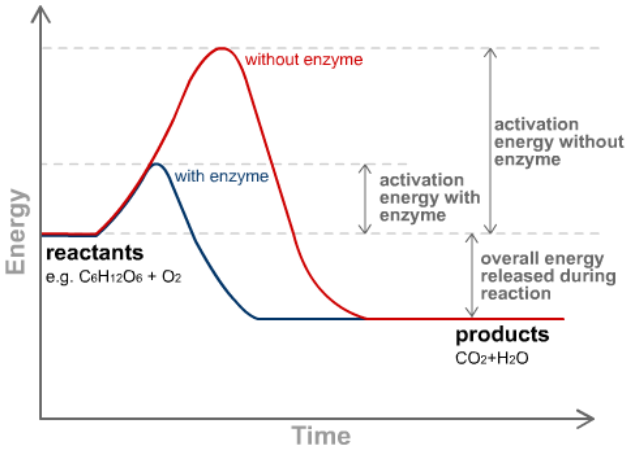
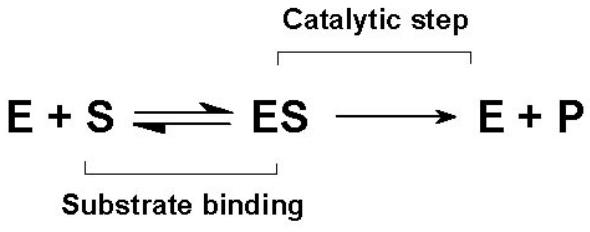


Fig. 1.3. Diagram of a catalytic reaction, showing the energy niveau at each stage of the reaction. The substrates usually need a large amount of energy to reach the transition state, which then decays into the end product. The enzyme stabilizes the transition state, reducing the energy needed to form this species and thus reducing the energy required to form products. (Voet and Voet 1995)

A simple enzymatic reaction can be considered as a two step process: substrate (S) binding to enzyme (E) and formation of an enzyme-substrate complex (ES), followed by irreversible breakdown of the enzyme-substrate complex into free enzyme and product (P).



A key factor influencing an enzymatic reaction is the substrate concentration. Michaelis and Menten postulated that the dissociation of the ES complex is the rate limiting step for the enzymatic reactions and defined two parameters characterizing enzyme kinetics: V_{max} and the Michaelis' constant "K_m". V_{max} is the maximum velocity of the reaction rate and K_m is a constant represent the substrate affinity towards the enzyme, K_m is measured as the substrate concentration which drives the reaction at a rate equal to ½ V_{max}. The Michaelis-Menten equation can be applied for a large number of enzymes (all such enzymes exhibit a hyperbolic dependence of V_0 on [S]). Generally the esterases follow Michaelis-Menten kinetics, whereas lipases do not follow this law (see 1.2.5).

1.2.4. Inhibition of esterases and lipases

Some molecular agents interfere with the enzymatic catalysis; either they slow down the catalysis rate or they prevent the catalytic activity completely. Thus enzyme inhibitors have been used extensively as pharmaceutical drugs or as toxic agents e.g. Aspirin "Acetylsalicylate" inhibits the Cox-1 and Cox-2 enzymes which are involved in synthesis of inflammation messengers "prostaglandins", and hence aspirin is used as anti-inflammatory agent. The toxicity of organophosphate DFP, wich is used in nerve gases e.g. Soman and Sarin, occurs due to irreversible inhibition of cholinesterases with DFP, the formed enzyme-phosphate ester bond is stable and does not hydrolyse spontaneously however reactivation of the enzyme can be accomplished by a strong nucleophile such as hydroxylamine [organophsphate toxicity can be treated with hydroxylamine analogues pralidoxime, obidoxime or scopolamine plus donpezil (Janowsky *et al*., 2005)].

Esterases/lipases inhibitors are either reversible or irreversible inhibitors. Irreversible inhibitors modify the enzymes covalently e.g. the reaction of the potent serine active site inhibitors PMSF (by sulphonation) and DFP (by phsophorylation) of the serine residue (Banerjee *et al*., 1991) (Fig 1.4).

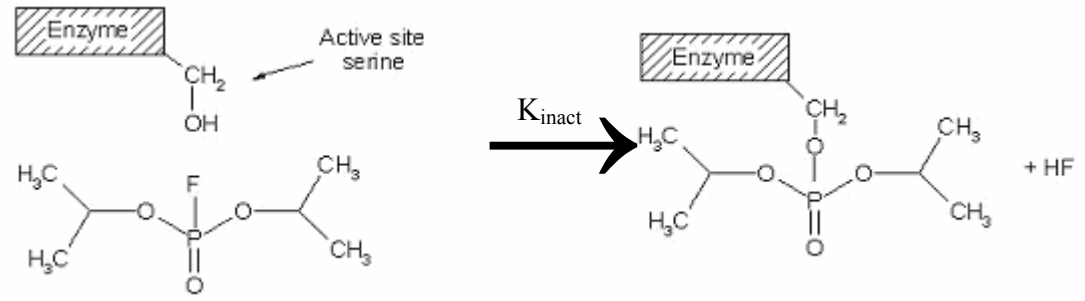


Fig. 1.4. Reaction of the irreversible inhibitor diisopropylfluorophosphate (DFP) with an esterase enzyme.

There are three types of reversible inhibition; competitive, uncompetitive and mixed inhibition (noncompetitive).

A competitive inhibitor is usually a compound that resembles the substrate in geometry. It competes with the substrate for the active site of an enzyme; it binds preferably to the active site and prevents the substrate binding (Fig 1.5a) e.g esterastin, ebelactone A and B are competitive inhibitors for esterases and lipases (Umezawa 1982), HEPES is a competitive inhibitor of the esterase EST2 from *Alicyclobacillus acidocaldarius* (Manco *et al*., 2001) and the 6-chloro-2-pyrones is a competitive inhibitior of yeast lipase (CRL1) (Stoddard-Hatch *et al*., 2002).

An uncompetitive inhibitor binds to a site other than the active site of an enzyme; it binds only to the enzyme-substrate complex. Binding of such inhibitors alters the conformation of the enzyme, thus it can not turnover its substrate anymore (Fig 1.5b) e.g. ATP is an uncompetitive inhibitor of bile salt dependent lipase (BSDL) (Pasqualini *et al*., 1997).

A noncompetitive inhibitor binds to a site other than the active site and it can bind either to the enzyme or the enzyme-substrate complex (Fig 1.5c) e.g heparin acted as noncompetitive inhibitor for esterase and lipase activities of the bovine milk lipoprotein lipase (Posner and Desanctis 1987).

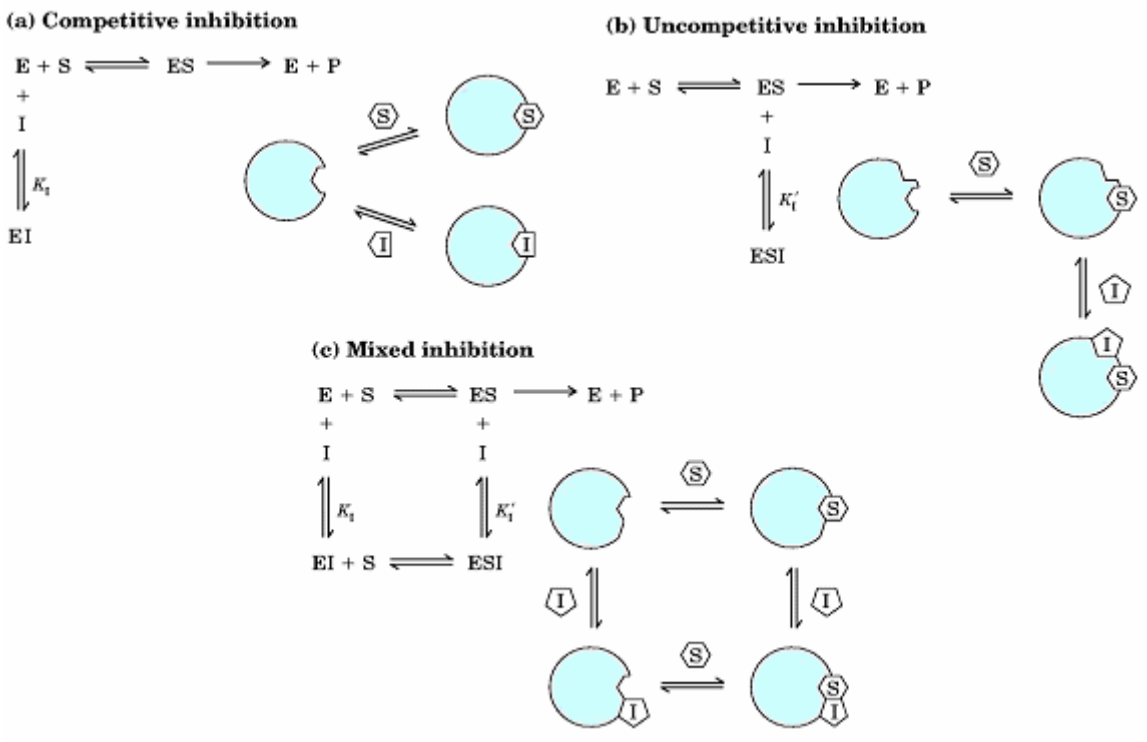


Fig. 1.5. Diagram shows the different types of enzyme inhibitors. Substrate (S) and inhibitor (I). a) A competitive inhibitor binds reversibly to the enzyme, preventing the binding of substrate. On the other hand, binding of substrate prevents binding of the inhibitor. Substrate and inhibitor compete for the enzyme. b) Uncompetitive inhibitors do not bind at the same site as the substrate. Substrate and inhibitor do not compete. c) Non-competitive inhibitor (mixed inhibitor) binds at separate site but may bind to either E or ES. (Nelson and Cox 2005)

1.2.5. Differences between esterases and lipases

Esterases can be distinguished from lipases by the phenomenon of "Interfacial activation". Lipases act at the interface generated by a hydrophobic substrate in a hydrophilic aqueous medium, a sharp increase in lipase activity observed, when the substrate starts to form an emulsion thereby presenting to the enzyme an interfacial area (i.e. minimum substrate concentration is required for lipases to achieve high level of activity) (Fig 1.6).

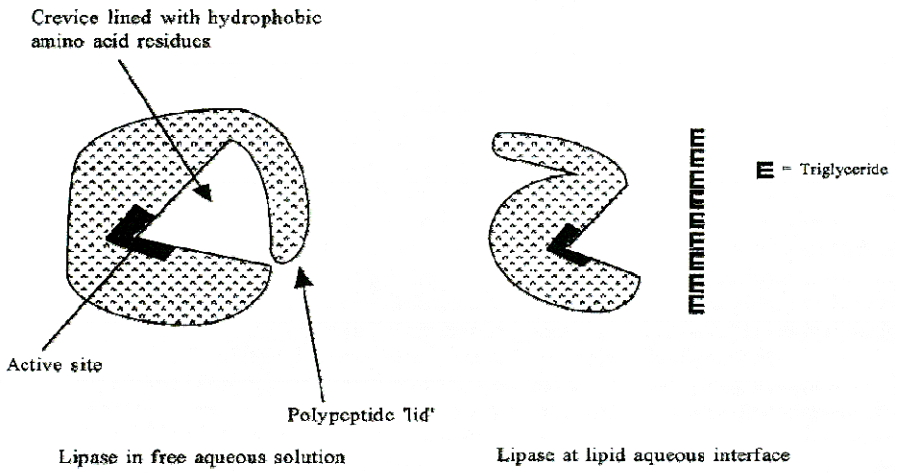


Fig. 1.6. Binding of lipase to the lipid-aqueous interface promotes a conformational change in enzyme which makes the active site accessible to the substrate. (Walsh 2004)

As a consequence the kinetics of a lipase reaction do not follow the classical Michaelis-Menten model, whereas esterases exhibit Michaelis-Menten kinetics. Structure elucidation revealed that the active site in the case of lipases is covered by a lid-like α-helical structure, which moves away upon contact of the lipase with its substrate exposing a hydrophobic residue at the surface of the protein, and mediates the contact between the protein and the substrate; this movable lid-like α-helix explains at a molecular level the lipase specific phenomena of interfacial activation. Furthermore lipases prefer water insoluble substrates e.g. triglycerides of long chain fatty acids whereas esterases prefer simple esters with short to moderate chain fatty acids (with C_2-C_8) (Tab 1.1) (Jäger *et al*., 1992; jaeger *et al*., 1999; Bornscheuer 2002).

Table 1.1 differences between esterases and lipases. (Bornscheuer 2002)

Property	Esterases	Lipases
Substrate	Simple esters and Trigylcerides (short chain)	Trigylcerides (long chain)
Interfacial activation/lid	No	Yes
Kinetics	Obey Michaelis-Menten	Don't obey Michael-Menten
Solvent stability	High to low	High
Enantioselectivity	High to low to zero	Usually high

1.2.6. Classification of esterases and lipases

In 1999 Arpigny and Jaeger have compared 53 sequences of esterases and lipases and classified them into 8 different families according to the conserved sequence motifs and the biological properties of the enzymes (Tabl 1.2).

Table 2.1: Classification of esterases and lipases (Arpigny and Jaeger 1999)

Family	Subfamily	Enzyme producing strain	Accession number
I	1	*Pseudomonas aeruginosa*	D50587
	2	*Burkholderia glumae*	X70354
	3	*Pseudomonas fluorescens*	D11455
	4	*Bacillus subtilis*	M74010
	5	*Bacillus stearothermophilus*	U78785
	6	*Propionibacterium acnes*	X99255
II (GDSL)		*Aeromonas hydrophila*	P10480
III		*Streptomyces exfoliatus*	M86351
IV (HSL)		*Alicyclobacillus acidocaldarius*	X62835
V		*Pseudomonas oleovorans*	M58445
VI		*Synechocystis sp.*	D90904
VII		*Arthrobacter oxydans*	Q01470
VIII		*Arthrobacter globiformis*	AAA99492

Family I (originally *Pseudomonas* lipases) is divided into 6 subfamilies. Subfamily I.1 showed molecular weights around 30kD whereas subfamily I.2 is slightly larger 33kD. Both depend on chaperon proteins called lipase-specific foldases "Lif". Subfamilies I.1 and I.2 possess two conserved cystein residues forming a disulphide bridge and two aspartate residues involved in a Ca^{2+} binding site. The subfamily I.3 is of higher molecular weight (50-65kDa). The secretion of these enzymes occur in one step through a three-component ATP binding cassette transporter system. Subfamilies I.4 and I.5 are *Bacillus* lipases, which are characterized by an alanine residue replacing the first glycine in the conserved pentapeptide (A-X-S-X-G). Subfamily I.4 are the smallest known true lipases (20kDa), whereas subfamily I.5 are around 45kDa and have optimal temperatures of around 65°C. Subfamily I.6 (originally Staphylococcal lipases) are large enzymes 75kDa. They are secreted into the medium and cleaved with a specific protease. The propeptide may act as interamolecular chaperone.

The GDSL family is characterized with a GDS(L) motif instead of the traditional pentapeptide GXSXG. The serine residue lies much closer to the N-terminal than the other

lipolytic enzymes. Family III possess a typical triad and shows 20% identity with the human PAF-AH.

The HSL family contains the bacterial esterases and lipases which show striking homology to the human HSL. The family members are widely spreaded within prokaryotes (psychrophilic, mesophylic and thermophilic organisms). Family V shows around 20-25% sequence similarity to non-lipolytic enzymes e.g. epoxide hydrolase. Family VI represents small esterases 23-26kDa and has a classical triad, whereas family VII is larger esterases around 55kDa and shows 40% similarity to eukaryotic choline esterases. Family VIII is esterases with striking sequence similarity to β-lactamases.

1.2.7. Applications of esterases and lipases

Esterases and lipases are widely used as industrial enzymes, the industrial demand for both increased constantly over the last 20 years. In 2000 the market value of lipoyltic enzymes was US $ 90 millions which represent around 7% of the whole enzymes market value (Walsh 2004). Esterases are employed in reactions where chemo- or regioselectivity is required. Ferulic, sinapic, caffeic and coumaric acids, which are widely used in food, beverage and perfume industries, are produced from their esters with the help of esterases. Esterases are used in dairies and for production of fruit juices, wine, beer and alcohol. Polyurethanases and cholesterol esterases are widely used for the degradation of some man made pollutants, plastics, polyurethane, polyesters, etc (Bornscheuer 2002; Panda and Gowrishankar 2005).

Lipases are used in fat hydrolysis or as a catalyst in synthetic organic chemistry where their regioselectivity and enantioselectivity are desired characteristics (Philip *et al*., 2002). Lipases can be widely used in organic chemicals processing, detergent formulations, synthesis of biosurfactants, the oleochemical industry, the dairy industry, the agrochemical industry, paper and pulp manufacture, nutrition, cosmetics and pharmaceutical processing**.** The major commercial application for hydrolytic lipases is their use in laundry detergents. In 1913 was the first trial to add a pancreatic extract to a detergent preparation, but the surfactants inactivated the pancreatic enzymes. Later in the 1970s suitable lipases for incorporation in detergents were identified. Detergent enzymes make about 32% of the total lipase sales. An estimated 1000 tons of lipases are added to approximately 13 billion tons of detergents produced each year (Sharma *et al*., 2001; Walsh 2004; Lorenz and Eck 2005).

1.2.8. Esterases/ lipases and chiral drugs

The world market for chiral fine chemicals and pharmaceuticals is expanding rapidly. In the year 2000 the world sales of chiral drugs exceeded the US $ 130 billion barrier and the average anuual growth rate was 13%. It is expected that the figure will hit US $ 200 billions in 2008. 40% of all sold dosage-form drug sales in 2000 were of single enantiomer, however in 1999 the share was 33% only, and this ratio is expected to reach 60% in 2010 (Stinson 2001; Lorenz and Eck 2005). The high demand for chiral drugs is caused by the fact that the cell surface receptors are biological molecules, which are chiral themselves, hence the effective drug molecule must match the asymmetry of the receptors. One of the most famous tragedies caused due to the admistration of a racemic drug was that of thalidomide which had been used as an anti-emetic for pregnant women in the 1950s and 1960s. Around 10000 childern were born with malformations and later research revealed that one enantiomer had the desired pharmacological properties while the other isomer is a teratogen.

Esterases and lipase are widely used for the production of various important chiral drugs. A carboxylesterase from *Bacillus subtilis* is used for the production of naproxen, and an esterase from *Trichosporon brassicae* has been used extensively for synthesis of ketoprofen (both are NSAIDs). Esterases from *Pseudomonas* sp. produce commercially anti-inflammatory drugs (NSAIDs) such as ibuprofen. A novel carboxyl esterase from *Burkholderia gladeria* is capable of hydrolysis of bulky esters of tertiary alcohols and has been used industrially for the production of semi-synthetic cephalosporin derivatives. Other stereospecific conversions in the production of pharmaceutical intermediates were reported e.g. taxol synthesis, throumboxane-A2-antagonist, acetylcholine esterase inhibitors, anti-cholesterol drugs, anti-infective drugs, Ca channel blocker drugs, K channel blocking drugs, anti-arrhythmic agents and antiviral agents (Bornscheuer 2002; Panda and Gowrishankar 2005). A lipase from *Serratia marcescens* catalyzes the synthesis of a key intermediate for “Diltiazem”, a major coronary vasodilator. Lipases are used in synthesis of anti-hypertensive agents such as angiotensin converting enzyme (ACE) inhibitors (e.g. captopril, enalapril, ceranopril, zofenopril and lisinopril (Sharma *et al*., 2001; Jaeger and Eggert 2002).

1.2.9. Directed evolution of esterases and lipases

Enzymes have been used successfully for many industrial purposes over the last two decades and biocatalysis has been the method of choice for the synthesis of different valuable chemicals, which increased the demand for new enzymes with special characteristics to be employed in other industrial applications. Often naturally occurring enzymes are not optimal for the applications of interest. Three main different but complementary approaches have been used to develop new enzymes of the desired optimal catalytic performance:

- Rational design
- Directed evolution
- Bioprospecting

However, directed evolution is considered as the most effective method in filling the gap between naturally occurring enzymes and the commercially required enzymes in terms of cost and time.

Directed evolution involves repeated cycles of random mutagenesis and/or DNA shuffling to develop a library of mutants, followed by high-throughput screening or selection of the improved mutants (Cherry and Fidantsef 2003; Rubin-Pitel and Zhao 2006). The major tasks of directed evolution in esterases and lipases was to improve the enantioselectivity, and alteration of substrate specificity, in addition comes also the improvement of thermostability and stability in the presence of organic solvents. There are several reports of successful directed evolution by esterases and lipases. An example of successful directed evolution within lipolytic enzymes is the directed evolution of an esterase from *B. subtilis*. The directed evolution improved the stability and activity of the esterase from *B. subtilis*, which can cleave the *p*-nitrobenzyl ester of Loracarbef (cephalosporin antibiotic). The wild type enzyme showed only weak activity in the presence of DMF which must be added to dissolve the substrate. Directed evolution through combination of EP-PCR and DNA shuffling produced a mutant with 150 times higher activity than the wild type in the presence of 15% DMF. The directed evolution also increased the thermostability by 14ºC (Bornscheuer 2002).

1.2.10. Esterases and lipases within *Streptomyces*

Streptomyces are characterized by their rich secondary metabolism. Therefore, *Streptomyces* species offer a relatively untapped source of interesting enzymes like esterases, lipases, hydrolases, etc. Over the last two decades few studies were carried out to investigate the esterase/lipase activity within *Streptomyces.* Sztajer *et al.* (1988) tested 15 *Streptomyces* strains and showed that three had a high extracellular lipase activity on tributyrin and olive oil. Bormann *et al*., 1993 reported that 51% out of 243 Streptomycetes screened through a plate and well method showed lipolytic activity. Gandolfi *et al.* (2000) screened 420 newly isolated *Streptomyces* strains using agar plates with three different substrates and showed that 44% of them possessed a lipolytic activity. Among the positive strains, 81% were only active on a single substrate and very few strains (2%) acted on all three substrates. In addition to these *in vivo* studies over the last few years the genome projects of *S. coelicolor* A3(2) (Bentley *et al*., 2002) and *S. avermitilis* (Ikeda *et al*., 2003) were completed and *in silico* analysis of both genomes confirmed the fact that *Streptomyces* are rich in lipolytic enzymes, as the genomes annotation revealed that *S. coelicolor* and *S. avermitilis* possess 31 and 20 putative esterases and lipases respectively in addition to the other genes annotated as cholesterol esterases, phospholipases and the genes annotated as hypothetical proteins that may exhibit esterase or lipase activity.

However, all the above evidences that *Streptomyces* are rich in esterases and lipases, there are only few reports about the lipolytic genes in *Streptomyces*. An esterase gene from *Streptomyces scabies* was cloned and sequenced in 1990 (Raymer *et al*.) An extracellular lipase from *Streptomyces* sp. M11 was cloned characterized and expressed in *Streptomyces Lividans* (Pérez *et al*., 1993). Cruz *et al* have sequenced a gene coding for a lipase from *Streptomyces albus* G in 1994. In 1996 an extracellular esterase from *Streptomyces diastatochromogenes* was characterized (Tesch *et al*.). Sommer *et al* (1997) have characterized a lipase from *Streptomyces cinnamomeus* both genetically and biochemically. An operon (*lip*AR), which encodes for an extracellular lipase and its transcriptional regulator, was found in *Streptomyces coelicolor* (Valdez *et al*., 1999). In 1999 Abramić *et al* have isolated an extracellular lipase from *Streptomyces rimosus* and completed its biochemical characterization, and later on Vujaklija *et al* (2002) have cloned and sequenced this gene.

1.3. Characteristics of *Streptomyces*

1.3.1. Morphology and taxonomy

The Streptomycetes are filamentous, aerobic, spore forming, multicellular and Gram-positive soil bacteria which belong to the order Actinomycetales (Paradkar *et al*., 2003). The soil habitat is very challenging for micoorganisms, and *Streptomyces* as an important member of this ecosystem has evolved complex morphological and physiological adaptations to survive in this environment (Claessen *et al*., 2006).

The numerical classification of 475 different strains of *Streptomyces* based on examination of 139 unit characters under standard growth conditions, classified the examined strains into 19 major, 40 minor and 18 single member clusters (Williams *et al*., 1983). These taxonomic studies were based on the morphological characters (e.g. shape, spore form, colony form or colony surface). Several studies tried to reduce the subjectivity in examining the strains by using serological, physiological, biochemical methods, etc (Christova *et al*., 1995). More recent classification used molecular biological methods and phylogenetic analysis based on 16S rRNA e.g. RFLP and RAPD (Patel *et al*., 2004; Pathom-aree *et al*., 2006; Zhang *et al*., 2006)

Streptomycetes are characterised by a complex life cycle (Fig 1.7). Under favourable conditions one or two germ tubes emerge from the spore and grow by tip extension and branch formation to give rise to a feeding substrate mycelium. As the colony grows and the nutrients become limited, further changes take place and Streptomycetes produce a second filamentous cell type. The changes are at least of five general types; increased production of some extra-cellular proteins, onset of secondary metabolites synthesis, initiation of lysis in some compartments of the substrate mycelia, onset of metabolite storage in the substrate hyphae and initiation of aerial hyphal growth. The apical compartment of an individual aerial hypha forms a spiral syncitium that contains many tens of genomes. When the aerial growth stops, multiple septa subdivide the apical compartment into unigenomic pre-spore compartments (Fig 1.8) (Ryding *et al*., 1998; Chater 2001; Claessen *et al*., 2006; Willey *et al*., 2006).

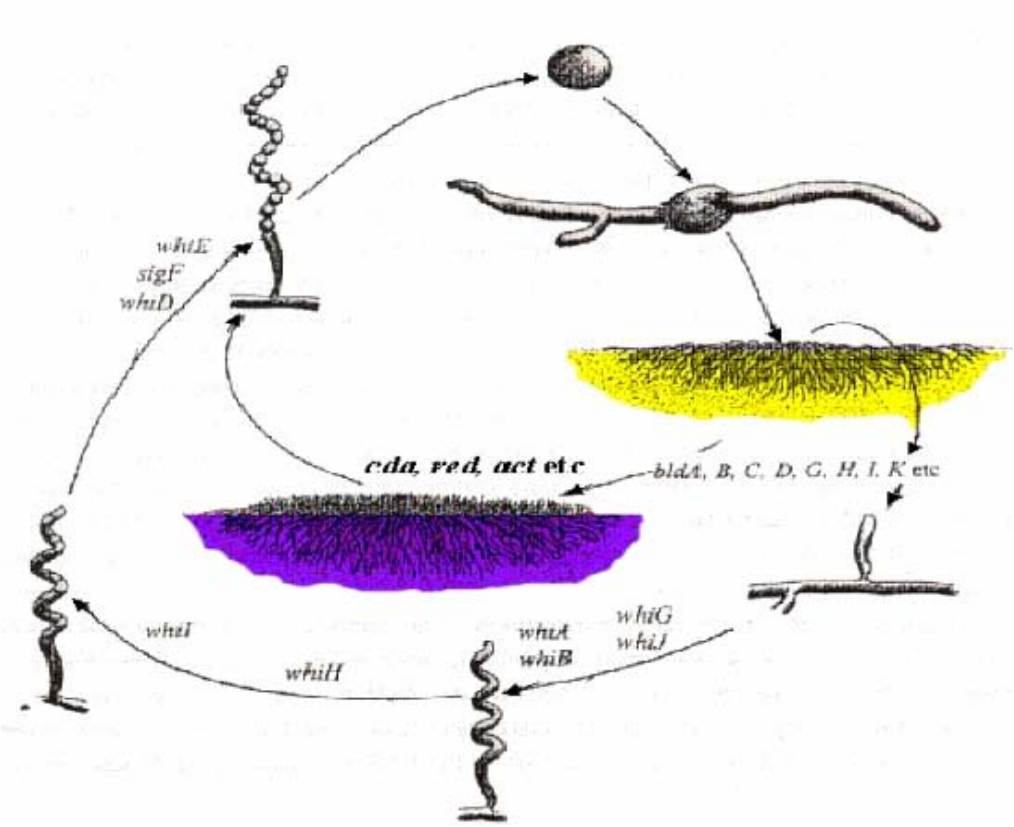


Fig. 1.7. The life cycle of *Streptomyces coelicolor* A3(2). Modified from Kieser et al (2000).

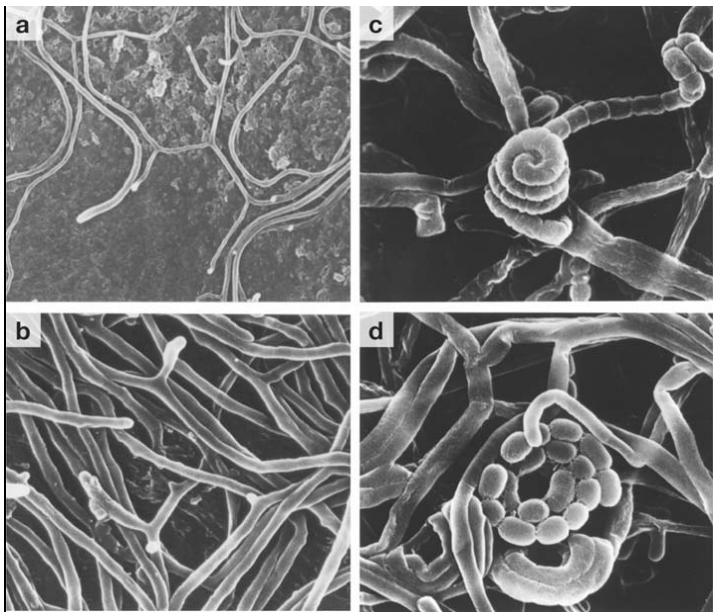


Fig. 1.8. Steps of Streptomycetes growth and sporulation. Scanning electron micrographs showing four stages in colony development in *S. lividans*. a) young substrate mycelium b) mature substrate mycelium producing aerial branches c) aerial hyphae developing into pre-spore compartment d) chain of mature spores (Hopwood 2006)

Streptomyces coelicolor A3(2) has been considered as the model organism for Streptomycetes genetics for its early development as genetic system (Hopwood 1999).

Actinomycetales produce 45% of the known bioactive microbial compounds (more than 10000 compounds) *Streptomyces* alone produces 34% (74% of all actinomycetales, 7500 compounds). Streptomycetes produce a large number of secondary metabolites like antibiotics, hydrolytic enzymes, enzyme inhibitors, herbicides and antitumour medicaments (Berdy 2005). One of the secondary metabolites produced by *Streptomyces* is geosmin, which is the etheric gas gives the characterstic smell of soil (Cane and Watt 2002). Examples of *Streptomyces* antibiotics are streptomycin (*S. griseus*), tetracycline (*S. aureofaciens*), chloramphenicol (*S. venezuelae*), oxytetracycline (*S. rimosus*), daunorubicin (*S. peucetius*), tetracenomycine (*S. glaucescens*) (Berdy 2005). The antibiotic synthesis occurs at a certain step of life cycle, which concides with the formation of the aerial mycelium i.e. the morphological differentiation and biosynthetic factor of the secondary metabolites are genetically coupled. The synthesis of an antibiotic substance requires a resistance mechanism against the antibiotic in the producing organism, the resistance encoding gene exists usually inside the gene cluster for the antibiotic synthesis (Hutchinson and Fujii 1995).

1.3.2. Genomic organisation of *Streptomyces*

DNA of *Streptomyces* is marked by its high G+C content of about 72%, where, the G+C content is ranging between 61% and 79% in each gene (Bibb *et al*., 1984). It was believed for long time that the classical circular structure of chromosomal DNA applied for all prokaryotes, but after introduction of PFGE (Schwarz and Cantor 1984) and the possibility of physical mapping, it was discovered that some bacteria have linear chromosome e.g. *Rhodococcus fascians*, *Borellia burgdorferi* and *Agrobacterium tumefaciens* (Crespi *et al*., 1992; Davidson *et al*., 1992; Allardet-Servant *et al*., 1993). All investigated *Streptomyces* species also have linear chromosomes, e.g. *S. coelicolor* A3(2), *S. lividans* , *S. griseus* , *S. ambofaciens* and *S. rimosus* (Kieser *et al*., 1992; Lin *et al*., 1993; Lezhava *et al*., 1995; Leblond *et al*., 1996; Pandaza *et al*., 1998).

The extraordinary character of the *Streptomyces* genome is its large size, the size of different *streptomyces* genomes were detrmined through their sequencing genome projects; *Streptomyces coelicolor* A3(2) was determined to be 8.66 Mb (Bentley *et al*., 2002), *Streptomyces avermitilis* was 9Mb (Ikeda *et al*., 2003) and *Streptomyces scabies* 10.148Mb (Sanger institute, incomplete).

These linear chromosomes are characterised by presence of terminal inverted repeats (TIRs) at the ends, which may be over 6 Mb in size. The longest recorded TIR was 6.5Mb in a variant of *Streptomyces ambofaciens* (Hopwood 2006). Many short palindromic repeats are present between the last few hundred base pairs of the TIRs. At the 5′ end of the chromosome there is a covalently attached protein (Bao and Cohen 2003; Yamasaki and Kinashi 2004). *Streptomyces* also possess linear plasmids. The size of such plasmids ranges between 12 kb and several hundreds of kilo-bases These plasmids show the same genetic structure as the linear chromosome (Bentley *et al*., 2004) with terminal inverted repeats, which range between 614 bp in the case of PSLA2 in *S. rochei* (Sakaguchi *et al*., 1985) and 95 kb in case of pPZG101in *S. rimosus* (Gravius *et al*., 1994). At the 5′ end of the plasmids there are covalently attached terminal proteins (Sakaguchi 1990). The first linear plasmid to be isolated from *Streptomyces rochei* was pSLA2 (Hayakawa *et al*., 1979). In addition to the linear plasmids *Streptomyces* has a large number of circular plasmids, which are either high-copy number plasmids e.g. pIJ101 (Kieser *et al*., 1982; Kendall and Cohen 1988) or low-copy number plasmids e.g. SCP2 (Schrempf *et al*., 1975; Freemann *et al*., 1977). In addition to the plasmids there are other extrachromosomal elements "phages" e.g. SF8 or SF9 from *S. fradiae* (Chung 1982) and the 42 kb phage ϕC31 in *S. lividans* (Lomovaskaya *et al*., 1972). Transposable elements, insertion sequences (IS) and transposons, have also been found in *Streptomyces* e.g. IS 466 plays a role in integration of the plasmid SCP1 into the chromosome of *S. coelicolor* A3(2) (Kendall and Cullum 1986).

Streptomyces linear plasmids and chromosomes start the replication at the central replication origin (*oriC*), and propagate bidirectionally towards the termini. Replication in the leading strand is completed at the 3′ end, but the lagging strand does not reach the 5′ end and leaves a 280bp gap, there are several suggested mechanisms involving a DNA polymerase and terminal protein through which this gap can be filled (Chen 1996; Chen *et al*., 2002; Hopwood 2006).

1.4. Objectives

Esterases and lipases are widely used in industrial applications. They are used in organic chemicals processing, detergent formulations, synthesis of biosurfactants, the oleochemical industry, the dairy industry, the agrochemical industry, paper and pulp manufacture, nutrition, cosmetics and pharmaceutical processing. One of the most important applications of esterases and lipases is their use for the production of chiral drugs. In 2000 the market value of these enzymes was US $ 90 millions. The demand for esterases and lipases for industrial applications is increasing; therefore we need to find new enzymes with interesting catalytic properties.

In many cases micro-organisms possess different enzymes having similar function and the presence of several enzymes in one pool may mask the interesting properties of some of them. Also the interesting enzymes may only be expressed under certain growth conditions or during certain growth phases of the micro-organism and hence may not be expressed under the laboratory cultural conditions used. Therefore, the traditional method of enzyme screening may miss many interesting enzymes. An alternative strategy is to identify potential genes in the available data bases. Cloning and overexpression will allow characterisation of the enzyme properties. This project was an initial survey of two *Streptomyces* genomes to see if this approach can identify interesting enzymes and to define strategies for a comprehensive use of genome data.

Despite the fact that *Streptomyces* strains are very rich in esterases and lipases, only a few lipolytic enzymes from *Streptomyces* have been studied in detail; esterases and lipases from *Streptomyces scabies* (Raymer *et al*., 1990), *Streptomyces exfoliatus* M11 (Pérez *et al*., 1993), *Streptomyces albus* G (Cruz *et al*., 1994), *Streptomyces diastatochromogenes* (Tesch *et al*., 1996), *Streptomyces cinnamomeus* (Sommer *et al*., 1997), *Streptomyces coelicolor* (Valdez *et al*., 1999) and *Streptomyces rimosus* (Vujaklija *et al*., 2002).

The analysis of the *Streptomyces coelicolor* genome revealed that from 7769 protein coding regions there are 31 different ORFs (0.4%) annotated as putative lipolytic enzymes. 19 are possible carboxylesterases, 10 are putative lipases and two are annotated as esterase/lipase. Ther *Streptomyces avermitilis* genome contains 20 putative lipolytic enzymes out of 7577 protein coding regions (0.26%). 12 are esterases, five are lipases and three are esterases/lipases.

Genes were selected that were amenable to a simple common cloning strategy, with the aim of cloning and overexpressing them in *E. coli*. The enzymatic properties of the expressed proteins were examined and one gene was selected for studies of using site-directed mutagenesis and random mutagenesis.

2. Materials and Methods

2.1. Bacterial strains

2.1.1. *Escherichia coli*

XL-1-Blue: *endA*1, *lac*I^{q}, F$^{'}$, *lacZΔ*M15, *recA*1, *tet*^{r}, *hds*R^{-}, *hsd*M^{+} (Bullock *et al*., 1987).

BL21: (DE3) F^{-}, *ompT*, $r^{-}_{B}m^{-}_{B}$ (Weiner *et al*., 1994).

Topo10 : F^{-}, *mcr*A *Δ*(*mrr-hsd*RMS-*mcr*BC), *Φ80lacZΔ*M15, *ΔlacX*74, *rec*A1, *deo*R, *ara*D139, *Δ*(*ara-leu*)7697, *gal*U, *gal*K, *rps*L, (Str^{r}), *end*A1, *nup*G (Invitogen).

2.1.2. *Streptomyces*

S. coelicolor A3(2) strain 1147 (Kieser *et al*., 2000).

S. avermitilis strani ATCC 31267 (Burg *et al*., 1979)

2.2. Vectors

2.2.1. PCR® 4-TOPO

The plasmid vector PCR® 4-TOPO (Shuman 1994) is a pUC18 derivative *lacZ α-ccdB* P_{lac} kan^{r} amp^{r} 3957bp. The plasmid is supplied linearized with

- Single 3´ thymidine (T) overhangs for TA cloning
- Topoisomerase covalently bound to the vector (activated vector)

2.2.2. pET-16b

The pET-16b (Weiner *et al*., 1994) vector carries an N-terminal His• Tag sequence, which is followed by a factor Xa site, and three cloning sites. Its size is 5711bp, the cloning/expression region of the coding strand is transcribed by T7 RNA polymerase. pET-16b is derived from the plasmid pBR322

2.2.3. pET-23b

It is similar to pET-16b, but it carries C-terminal His• Tag sequence, and N-terminal T7• Tag.

2.3. Reagents and Media

2.3.1. Buffers and other general solutions

Unless otherwise indicated, solutions were prepared as described in (Sambrook *et al*., 1989) and (Kieser *et al*., 2000).

2.3.2. Chemicals

The chemicals of general use mentioned in methods were supplied by either Sigma or Roth (Germany) PA quality.

2.3.3. Antibiotics

Table 2.1. List of the antibiotics used in this study

Stock solution	Preparation	Concentration of stock	End concentration
Ampicillin	-sterile filtration -in aliquots of 1ml - stored at –20°C	100mg/ml A.d.	50µg/ml LB
Tetracycline	-sterile filtration -in aliquots of 1ml - stored at –20°C	50mg/ml A.d.	50µg/ml LB
Kanamycin	-sterile filtration -in aliquots of 1ml - stored at –20°C	50mg/ml A.d.	50µg/ml LB

2.3.4. Other materials and kits

Agarose (Biozyme).

Restriction enzymes and DNA ladder mix marker (Fermentas)

Nucleobond AX-100 for plasmid isolation (Machery-Nagel).

GFX plasmid isolation kit (Amersham).

PCR purification kit (Qiagen).

Topo cloning kit (Invitrogen).

Protino Ni-resin for protein purification (Machery-Nagel).

Ni-NTA spin kit for protein purification (Qiagen).

Factor Xa (Novagen).

High fidelity Taq polymerase (Amersham).

protein molecular weight marker (Fermentas)

Prestained protein molecular weight marker (Fermentas)

2.4. Media and growth conditions

All the used media were autoclaved at 120°C for 20min. Also the solutions, which were added to the media after autoclaving, were autoclaved before addition to media.

2.4.1. *Streptomyces* strains

2.4.1.1. Liquid medium

YEME medium (Kieser *et al*., 2000)

Yeast extract 3g, Bacto-peptone 5g, Malt extract 3g, Glucose10g, Sucrose 340g and to 1l A.d.

After autoclaving add: $MgCl_2$ 5mM, 2ml $MgCl_2$ 2.5M and Glycine 20% 25ml. One cell colony or 100ul spore suspension was added to 100ml YEME media in a flask with spiral, and incubated for 3-4 days in a shaker at 30°C.

2.4.1.2. Solid medium

Soja media (sporulation media) (Kieser et al., 2000)

Soja flour10g, Mannitol10g, Bactoagar 8g and to 500ml A.d. One cell colony was picked from the plate or a diluted spore suspension was used to streak the soja plate, and then incubated at 30°C for 3-4 days.

2.4.2. *E.coli* strains

2.4.2.1. Liquid Medium

LB medium (Sambrook *et al*., 1989): NaCl 10g, Yeast extract 5g, Trypton 10g and to 1l A.d. One cell colony was picked in 5ml LB medium in test tube or 25-50 ml in Erlenmeyer flask,

and then incubated in a shaker at 37°C. The liquid culture can be used for isolation of plasmid.

2.4.2.2. Solid Medium

LB solid medium was used. Along with the ingredients of LB broth mentioned above, 16g/L Bacto-agar was added. The plate was streaked to get one cell colony on the LB plate, then incubated overnight at 37°C.

2.5. Strains preservation

0.765 ml of *E. coli* overnight culture was mixed with 0.325ml of 86% glycerol solution, and stored at –70°C (Sambrook *et al*., 1989).

2.6. DNA Isolation

2.6.1. Isolation of total *Streptomyces* DNA

A culture volume 50ml was used for maxi preparations. A four days YEME culture was centrifuged for 10min, 5000rpm, at RT. The mycelium was resuspended in 5ml SucTE buffer. 100ul lysozyme stock solution [→2mg/ml] was added, and the mycelium suspension incubated 60min at 30°C. 5ml TE buffer were added, followed by addition of 10ml phenol. After vortex mixing 15 sec the phases were separated by centrifugation 10min, 5000rpm at RT. The upper phase was removed by a cut tip micropipette into a new tube, and 10ml phenol/chloroform were added, followed by vortex mixing and centrifugation (as in the previous step). The upper layer was removed again in a new tube, and 1 volume chloroform/isoamyl alcohol was added, vortex mixed 15sec and centrifugation as before. The upper layer was taken into a new tube and used for precipitation of DNA. 1 volume isopropanol was added at RT and the aggregated DNA ball was removed using a Pasteur pipette to a new tube. 70% ethanol was used for washing the pellet, followed by centrifugation. The pellet was dissolved in 500-1000ul TE (Kieser *et al*., 2000).

2.6.2. Isolation of cosmid/plasmid DNA (alkali lyses)

The principle of this isolation method is the separation of cccDNA plasmids from chromosomal DNA, which consists of linear molecules due to shearing during isolation. By raising the pH to a suitable value, the linear becomes ssDNA, whereas the cccDNA remains

as dsDNA. On a rapid reduction of pH, much of ssDNA forms an aggregate that can be separated by centrifugation. Plasmids and cosmids can be separated either as maxiprep (culture volume is 50ml) or miniprep (culture volume is 5ml).

50ml -(1.5ml)- of overnight culture were centrifuged for 2min -(10sec), 10000 -(13000)-rpm at RT, the supernatant was discarded, and the pellet was resuspended in 5ml -(100µl)- Sol I (50mM glucose, 10mM EDTA, pH8 and 25mM Tris pH8). The solution was vortex mixed for a few seconds, and was incubated for 5min at RT. 250µl lysozyme stock solution [→5mg/ml] were added, then vortex mixed and incubate 10min at RT (this step is optional in case of miniprep). 10ml -(200µl)- Sol II (0.2N NaOH and 1%SDS) was added and the tube inverted to mix and after10min -(5min)- incubation on ice, 7.5ml -(150µl)- Sol III (100ml contain 11.5ml acetic acid, 28.5ml A.d. and 60ml potassium acetate) was added. After mixing and incubation 10min on ice, a white precipitate of protein, cell debris, chromosomal DNA and SDS was formed, which may sediment by centrifugation for 10min, 13000rpm at 4°C (5min , 13000rpm at RT). The supernatant contains the plasmid, and the DNA was precipitated as in 2.10. The pellet was redissolved in 500µl - (20-50µl) TE buffer (Sambrook *et al*., 1989).

2.6.3. Purification of cosmid -/plasmid DNA

2.6.3.1. Purification using nucleobond®AX, Macherey-Nagel

The purification of plasmids and cosmids from maxi-preparations was done with a "plasmid purification kit" which uses anion exchange columns made of kieselgel. DNA binds to the macroporous anion exchange column, in the presence of low salt concentrations, and the salt concentration is increased during washing and elution. Impurities are removed during washing, and the bound DNA is finally eluted from the column with the help of 1M KCl.

DNA was purified according to the protocol provided by the manufacturer.

2.6.3.2. Purification of DNA using GFX™ microplasmid prep kit, Instructions, Amersham Pharmacia Biotech Inc

Purification of plasmid and cosmid DNA from minipreps was done using "GFX™ microplasmid prep kit" from Amersham Pharmacia Biotech. This kit uses an ion exchange column, which is formed of a glass fiber matrix. At high salt concentrations the DNA binds selectively to the column at the glass matrix, while the proteins are denatured. The denatured

proteins are removed during washing and the purified plasmid or cosmid is eluted using TE buffer pH8.

DNA was purified according to the protocol provided by the manufacturer.

2.7. DNA gel electrophoresis

2.7.1. Agarose gel electrophoresis

Agarose gel electrophoresis is the standard method for separation and purification of a mixture of DNA fragments. It depends on the migration of the negatively charged DNA molecule in a constant field. The velocity of migration is proportional to the electric voltage, also the DNA migration depends on its size, conformation, the buffer composition (1xTAE or 0.5xTBE) and the nature of gel matrix.

The choice of agarose concentration (0.4%-1.2%) enables the separation of fragments in the range from 0.2-50kb, larger fragments can be separated with the help of PFGE. The DNA can be coloured on the gel using a fluorescent colouring agent like ethidium bromide. The bands can be visualized on the gel through exposure to UV light of wave length 302nm.

The agarose (0.8-1%), Seakem® GTGLE from FMC Biozyme, was added to 1x TAE buffer (1L 50x TAE contains 242g Tris, 57.1ml acetic acid and 100ml EDTA 0.5M, pH8), and boiled until the solution was clear. When the solution had cooled, it was poured inside the gel chamber, and the comb was inserted to produce suitable slots. When the gel had solidified, this comb was removed (Sambrook *et al*., 1989).

Loading dye GEB was added to the sample, and then the sample was loaded into the gel slots. The running buffer is 1x TAE. The gel loading dye GEB is composed of 0.2% bromophenol blue, 0.2M EDTA and 50% glycerol.

2.7.2. Colouring and evaluation of the gel

The gel was left after electrophoresis for 15-30 min in an ethidium bromide bath (stock solution of EtBr 10mg/ml, the colouring bath concentration 0.5µg/ml; i.e. 50µl stock solution /1l A.d.). After washing in a water bath for 20-30 min. the bands were detected with a transilluminator (Bachhofer l- 305M, 302nm), and photographed through an orange filter. A Polaroid instant photo camera, a single lens reflex camera or an INTAS gel documentation system (Göttingen) was used.

2.8. DNA manipulation

2.8.1. Phenol/Chloroform extraction

This method is used for purification of DNA e.g. removal of enzymes after restriction digest, the proteins accumulate during centrifugation at the interphase and DNA goes inside the aqueous phase. Finally the remaining phenol was removed by shaking with chloroform /isoamyl alcohol (24:1).

One volume phenol/chloroform/isoamyl alcohol (25:24:1) was added to DNA solution, vortex mixed for a few seconds and centrifuged for 5min, 5000rpm at RT. The upper aqueous layer was removed into new Eppendorf tube, this step was repeated until the upper layer was clear. 1volume chloroform/isoamyl alcohol (24:1) was added, with vortex mixing and centrifugation as described before. DNA was precipitated (see 2.8.2) form the upper aqueous layer (Sambrook *et al*., 1989).

2.8.2. DNA precipitation

DNA precipitation is required either for purification or concentration of DNA solutions. Precipitation is achieved by ethanol or isopropanol and the DNA is usually resuspended in a suitable amount of TE or A.d.

Either 100% ethanol or isopropanol was used, when isopropanol was used, 0.1 volume 3M sodium acetate and 0.6 volume isopropanol were added and left for 20min at RT. When 100% ethanol was used, 1 volume 100% ethanol was added, and then left at least 30min at –70°C. Centrifugation was carried out for 30 min, 13000rpm at RT. After centrifugation the supernatant was discarded and the pellet was washed with 70% ethanol, centrifuged as before then allowed to dry in a dissector or heating block. At the end the pellet was dissolved in suitable amount of water or TE (Sambrook *et al*., 1989).

2.8.3. DNA restriction

The digestion of DNA with restriction enzymes was according to the provider

2.8.4. Determination of DNA concentration

DNA concentration was determined using the nanodrop technique, The NanoDrop® ND-1000 Spectrophotometer, developed by NanoDrop Technologies (www. nanodrop.com), fills this

need by requiring only 1 µL of sample to generate a full wavelength spectrum without the use of cuvettes or capillaries. The sample is pipetted directly onto the measurement surface where it is retained between two optical fibers by surface tension. This retention system utilizes two different path lengths (1 mm and 0.2 mm) during each measurement cycle to achieve an extensive dynamic range that eliminates the need to perform dilutions for RNA/DNA or proteins.

2.8.5. RNase treatment

The treatment of DNA solution with RNase to remove the RNA.

RNase (stock solution concentration 10mg/ml containing 10mM Tris HCl, pH7.5 and 15mM NaCl) was added to an end concentration of 40µg/ml, then the DNA solution was incubated 30min at 37°C. Phenol/chloroform extraction was carried out as in 2.9, and finally DNA was precipitated, washed as in 2.10 and dissolved in TE (Sambrook et al., 1989).

It is also possible to remove the RNA during mini- and maxipreparation, through addition of RNase directly into SolI to an end concentration of 40µg/ml. After isolation phenol/chloroform extraction was carried out.

2.8.6. Ligation

Ligation (Revie *et al* ., 1988; Sambrook *et al*., 1989) is carried out using T_4 DNA ligase (Fermentas), which catalyzes the ligation between the two adjacent 3` OH end and 5` phosphate end of double stranded DNA, ATP is required in this reaction. One of the important characters of T_4 ligase is its ability to ligate not only cohesive ends but also blunt ends.

The efficiency of ligation depends on incubation time, temperature and concentration and the length of DNA fragments. At constant DNA concentration the smaller the DNA size, the more is the intramolecular reaction i.e. circularization, however at constant DNA length the circularization tendency increases with lower concentration.

2.8.6.1. Ligation of cohesive ends

To ligate fragments of cohesive ends, the proportion of insert to vector should be 1:1 to 2:1.

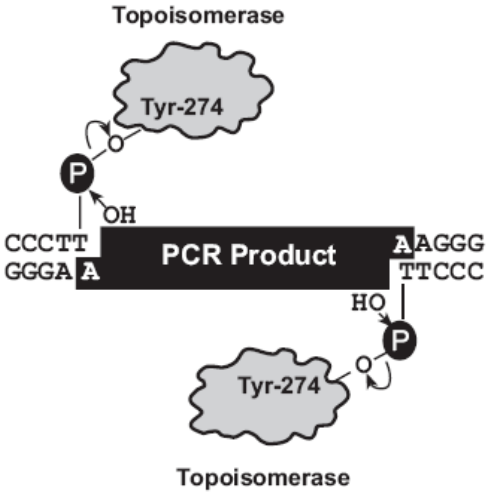
DNA was used in the ratio of 1:1 to 2:1 insert : vector, and 5U T4 ligase (Fermentas MBI) and 2µl ATP (5mM) in 15-20µl total volume containing 1x ligation buffer, followed by incubation at 14°C for 12-16h.

DNA was precipitated using 0.1volume LiCl 4M and 2.5 volume ethanol 100% and the pellet were redissolved in distilled water.

2.9. "TOPO TA-cloning"

Taq polymerase has a non template-dependent terminal transferase activity, which adds a single dATP to the 3′ ends of PCR products. Therefore ligation in a linearized vector with 3′ T overhang occurs easily.

Topoisomerase I from *Vaccinia* virus binds to dsDNA at specific sites and cleaves the phosphodiester backbone after 5′ CCCTT in one strand. The energy from the broken phosphodiester backbone is converted by formation of a covalent bond between the 3′ phosphate of the cleaved strand and the (Tyr-274) residue of Topoisomerase I. the phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of a PCR product and releasing the Topoisomerase (Shuman 1994).



Cloning was performed according to the protocol provided by the manufacturer.

2.10. Transformation

2.10.1. Production of electrocompetent cells

All the steps were carried out on ice and in sterile conditions.

2ml of E. coli [Xl-1-blue and BL21(DE3)] overnight culture were added to 200 ml LB medium, and incubated at 37°C till OD_{600} 0.6-0.8. Then the culture was incubated on ice 15-30min, followed by centrifugation 15min, 5000upm at 4°C. The pellet was washed first with 200ml ice-cold distilled water and centrifuged as before, the washing step was repeated with 100ml ice-cold water, and then the pellet was washed with 30ml 10% ice-cold glycerol, centrifuged as before. The glycerol was decanted and the pellet was resuspended in 500µl 10% ice-cold glycerol, and then divided into aliquots of 40ul, finally shock freezing was carried out in liquid nitrogen and stored at –70°C (Sambrook *et al*., 1989).

2.10.2. Transformation through electroporation

A high membrane potential is produced through an electric pulse, which increases the cellular membrane permeability. This produces "electropores" which are big enough for DNA and RNA molecules to diffuse through. A high efficiency of transporation can be achieved through optimizing different parameters like the strength of the electric field, electroporation buffer and regeneration medium.

2-5µl DNA was added to 40µl competent cells, and was incubated on ice for 1min, the whole volume was removed into a cold pulse cuvette. A Biorad E.coli Pulser™ was used for transformation, the conditions of transformation were 200Ω, 2.5KV and 25µF. 200µl LB medium were added after pulsing to the cuvette, and all transferred into a glass tube containing 800µl LB, and incubated 1h at 37°C in a shaker. At the end plates were streaked, and incubated at 37°C for 16h (Dower *et al*., 1988).

2.11. PCR

The PCR (Polymerase Chain Reaction) is an *invitro* method for the synthesis of defined sequences of DNA with an enzyme (Scharf *et al*., 1986). The reaction uses two oligonucleotide primers that hybridize to opposite strands and flank the target DNA sequence that is to be amplified. A heat-stable polymerase, such as *Taq* polymerase, catalyses the elongation of primers. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by the polymerase results in the exponential accumulation of the specific DNA fragment.

The primers were designed by eye from the sequence of *S. coelicolor* and evaluated with the primer primer 5 (primer Biosoft company) software. The primers were synthesized by the

ROTH Company, Germany. In a total reaction mixture of 50µl add 0.05-1 µg template DNA, 1pM of each primer, 1x PCR buffer, 2.5mM $MgCl_2$, 5% DMSO, 200µM of each dNTP and 5U *Taq* DNA polymerase (Fermentas). 18µl of mineral oil overlayed on the reaction mixture. PCR was performed in an Eppendorf Thermo Cycler under the following conditions: 5min at 96°C, followed by 30 cycles of denaturation at 96°C for 30sec, annealing at 50°C for 70sec, extension at 72°C for 2min and at the end final incubation at 72°C for 10min followed by hold at 4°C.

2.11.1. Site directed mutagenesis PCR

The primers were designed by eye from the sequence of SCO 7131 in pET-16b and evaluated with the "primer primer 5" (primer Biosoft company) software. The primers were synthesized by the MWG Company, Germany. In a total reaction mixture of 50µl add 50-60ng template DNA (plasmid SCO7131 in pET-16b), 1pM of each primer, 1x PCR buffer, 2.5mM $MgCl_2$, 5% DMSO, 200µM of each dNTP and 5U high fidelity DNA polymerase (Amersham). PCR was performed in an Eppendorf Thermo Cycler using the following PCR program: 5min at 96°C, followed by 30 cycles of denaturation at 96°C for 1min, annealing at 60°C for 1min, extension at 72°C for 8min and at the end final incubation at 72°C for 10min followed by hold at 4°C.

2.11.2. Random mutagenesis

The random mutagenesis was carried out through error prone PCR.

2.11.2.1. Error-Prone PCR

The primers were designed by eye from the sequence of SCO 7131 in pET-16b and evaluated with the primer primer 5 (primer Biosoft company) software. The primers were synthesized by the MWG Company, Germany. In a total reaction mixture of 50µl add 5-60ng template DNA (plasmid SCO 7131 in pET-16b), 1pM of each primer, 1x PCR buffer, 7mM $MgCl_2$, 5% DMSO, 200µM of each dNTP, 1mM d(C/T)TP, 0.2mM d(A/G)TP, 0.5mM $MnCl_2$ and 5U *Taq* DNA polymerase (Fermentas). PCR was performed in an Eppendorf Thermo Cycler using the SCO7131 PCR program.

2.11.3. Purification of PCR products

PCR products were purified using *High pure PCR purification kit*, which is based on the specific binding of nucleic acids, to the surface of glass fibers or silica materials in the presence of chaotropic salts. Since the binding process is specific for nucleic acids, the bound DNA can be separated and purified from impurities, like salts, free nucleotides, and proteins by a washing step.

PCR fragments were purified according to the protocol provided by manufacturer.

2.12. DNA Sequencing

DNA sequencing depends on the synthesis of a new strand of DNA starting at a specific priming site and ending with the incorporation of a chain terminating nucleotide such as 2`,3`-dideoxynucleoside triphosphate (Sanger *et al* ., 1977), due the absence of a 3` hydroxyl group, it is impossible to form a phosphodiester bond, hence the reaction is terminated. The relative concentrations of dNTPs and ddNTPs are balanced to produce an appropriate average chain length. The resulting DNA fragments differ in their length one from the other only one base, that they can be separated on polyacrylamide gel.

2.12.1. Sequencing reaction

Sequencing was carried out using Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham LIFE SCIENCE).

Sequencing was done in the Center for Nanotsructure Technology and Biomolecular Technology University of Kaiserslautern, http://www.nbz.uni-kl.de/.

2.12.2. Documentation and evaluation of the data

Documentation and evaluation of the data were done automatically through the "BioEdit" program (Tom Hall, Ibis Therapeutics, A division of Isis Pharmaceuticals, 1891 Rutherford Road Carlsbad, CA 92008 http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.13. Primers

The sequences for the primers used during this study either for gene amplification, mutagenesis or sequencing is presented in Table 2.1

Table 2.2. The primers used during this study

Name	Primer
Genes amplification primers	
SAV469-For	5′ GGGAGGGCATATGACCACCACG 3`
SAV469-Rev	5` CTCGAGAAAACGCGGTGCTGC 3`
SAV1549-For	5` CTCGAGTACGGTAGTCGGCCG 3`
SAV1549-Rev	5` GGAGCGCAACATATGATCTTCAGG 3`
SAV3461-For	5` AGGTCGCATATGAAGGTCACC 3`
SAV3461-Rev	5` CCCTCG AGCGTCAGGAGGATG 3`
SAV7089-For	5` GGATCCGCATTGTCCCCCGTAC 3`
SAV7089-Rev	5` TTTCAGGAGGCCATATGCAACGC 3`
SCO1265-For	5` CTCGAGTCCCGATGAACGACGA 3`
SCO1265-Rev	5` GAAAGGGAGAAGCATATGAGTTTCCTCA 3`
SCO1735-For	5` CTCGAGTGACGTGCGCCGG 3`
SCO1735-Rev	5` GGAGATCACCATATGCTGCCCTG 3`
SCO2123-For	5` GAGACCGACATATGTCGGTCCTGCC 3`
SCO2123-Rev	5` CTCGAGGTCCTCGCGGTCGG 3`
SCO 3219-For	5` CGGGATCCTACTGAGACCGTCTTTCC 3`
SCO 3219-Rev	5` CGTTCGGCATATGCGCTCACTTC 3`
SCO3644-For	5` CTCGAGCTCACCTGCGGGGTAG 3`
SCO3644-Rev	5` AGAGGTTGCATATGCCGGACGC 3`
SCO4368-For	5` CTCGAGGAGCGGTTGGAGTGA 3`
SCO4368-Rev	5` CGTCCTCCATATGACCGGCAG 3`
SCO4746-For	5` CGAAGAGGACATATGCACGTGAGCG 3`
SCO4746-Rev	5` CTCGAGGTGATCTCGACGTCGGC 3`
SCO4799-For	5` GCGGATCCAATTGTTCGAGTCCGAACG 3`
SCO4799-Rev	5` GCTGCCGTGGTGACCTTCATATGCCG 3`
SCO6966-For	5` CTCGAGGACAAACGCCCGGC 3`
SCO6966-Rev	5` CGGAGGACATATGGCCGAGGC 3`
SCO7131-For	5` CGCAAGGAGCGCATATGAGCG 3`
SCO7131-Rev	5` CTCGAGAGTGGCCCTTCCGGAT 3`
SCO 7513-For	5` CG GGA TCC TGT CGT GAA GAC CTG CGC 3`
SCO 7513-Rev	5` CGG AAG AGA GAG CAT ATG CCG AAG CCT GC 3`

Continue table 2-1: The primers used during this study

Name	Primer
Walking primers	
SAV469_For_2	5´ TGGATACGCGGCTGTGACGG 3`
SAV469_Rev_2	5` GTGGAGCGGGACGGCTATCC 3
SAV1549_For_2	5` CGATCCGGGTTTTCAGCAGC 3`
SAV1549_Rev_2	5` CGTGTTCCTGACGCCGAAGC 3`
SAV7089_For_2	5` GGGAGGTCAGGAACGAGCTTC 3`
SAV7089_Rev_2	5` GCCACCGAGGTCAGGAGCG 3`
SCO1265_For_2	5` CCGACGAAGTTGTGCACCTTG 3`
SCO1265_Rev_2	5` GCCGCGCTACTACCTCAACG 3`
SCO1735_For_2	5` GGCGCTATGCCGACCAGGG 3`
SCO1735_Rev_2	5` CAAGTTCCTCGGCGGAGCC 3`
SCO3644_For_2	5` GGTCGGTCAGCCACGGGG 3`
SCO3644_Rev_2	5` GCGCGTGGTGCTCACCGG 3`
SCO4368_For_2	5` CCCGTCCAGGTTGACCCCC 3`
SCO4368_Rev_2	5` CACGCACGCGCAGGCCGAC 3`
SCO4746_For_2	5` AGGACTCCTGGCACTTCCAGCG 3`
SCO4746_Rev_2	5` CGCTCCACCAGCTCGGCC 3`
SCO7131_For_2	5` ACTCCGTCGGCGGCAACATG 3`
SCO7131_Rev_2	5` GGTGTCCTGCCCGGCGTCG 3`
SCO6966_For_2	5` GGGAGAGCGAGGCCGGGC 3`
SCO6966_Rev_2	5` TGATCGCCTCCCGCTACGCC 3
Site directed mutagenesis primers	
SCO7131M162LF	5` CC GTC GGC GGC AAC TTG AGC GCC GCC 3`
SCO7131M162LR	5` GGC GGC GCT CAA GTT GCC GCC GAC GG 3`
SCO7131S163AF	5` GGC GGC AAC ATG GCC GCC GCC CTC ACC 3`
SCO7131S163AR	5` GGT GAG GGC GGC GGC CAT GTT GCC GCC 3`
SCO7131W87FF	5` G CAC GGC GGC GGC TTT ATC CTC GGG AAC G 3`
SCO7131W87FR	5` C GTT CCC GAG GAT AAA GCC GCC GCC GTG C 3`
Error prone PCR primers	
SCO7131EPPCRF	5` GTTATGCTAGTTATTGCTCAGCGGTGG 3`
SCO7131EPPCRR	5` GATCTTCCCCATCGGTGATGTCG 3`
Standard primers for sequencing	
M13-For	5'-GTAAAACGACGGCCAGT-3'
M13-Rev	5'-CAGGAAACAGCTATGAC-3'
T7	5'-GTAATACGACTCACTATAGGGC-3'

2.14. Protein manipulations

2.14.1. Protein expression

Induction of the cloned lipase gene was carried out according to the Novagen pET manual 2002. A single colony was picked in 5ml LB medium, exponential phase cultures (OD_{600} 0.6-1.0) were induced by adding 0.25mM IPTG and harvested after 4h induction at 30°C (Novagen pET system manual, 2002).

2.14.2. Protein isolation

Lysis buffer

50mM NaH_2PO_4	7.8g
300mM NaCl	17.5g

→ 1L A.d. pH was adjusted with NaOH to 8

The cells were harvested by centrifugation and 100 ml of culture was resuspended in 4 ml of buffer (0.05M sodium phosphate/0.3M NaCl) and treated with lysozyme (1mg/ml) on ice for 30'. The lysate was centrifuged for 40min at 12000 rpm at 4°C in an Eppendorf centrifuge and the supernatant was filtered through at 0.45µ filter.

2.14.3. Protein purification

The protein was expressed as His-Tagged protein which facilitates the purification using affinity Ni columns. For small amounts we have used Ni-NTA spin kit (Qiagen) and for large amounts we have used PROTINO resin (Macherey-Nagel). Purification was done according to the protocol provided by manufacturer.

The samples were desalted, concentrated and the buffer was changed to Tris-HCl 0.02M pH 8 by ultra-filtration using ultra-flirtation spin column CO 10000 (Amicon, Germany).

2.14.4. Determination of protein concentration

The protein was determined using the Bradford method (Bradford 1976) using bovine serum albumin as a standard.

2.14.5. SDS-PAGE (polyacrylamide gel electrophoresis)

1M Tris HCl , pH8.8	Tris HCl pH was adjusted with HCl 37%	121.1g/1l A.d.
Acrylamide Mix	30% acrylamide 0.8% Bisacryamide →100ml A.d.	30g acrylamide 8g Bisacryamide
Stacking gel mix	155mM Tris HCl , pH 6.8 0.12% SDS →1l A.d.	155ml 1M Tris pH 6.8 12ml SDS 10%
Resolving gel mix	562mM Tris HCl pH8.8 0.15% SDS 25% glycerol → 1l A.d.	562ml1M Tris pH8.8 15ml SDS10% 250g100% glycerol
10% Ammonium persulphate (APS)	Ammonium persulphate (Must be freshly prepared)	100mg/1ml A.d.
Stacking gel	Acrylamide mix stacking gel mix 10%APS l TEMED	830µl 4.12ml 40µ 5µl
Resolving gel 10%	Acrylamide mix resolving mix 10% APS TEMED	5ml 10ml 60µl 5µl
10x SDS-PAGE running buffer	250mM Tris HCl 1.92M glycin 1% SDS adjust pH 8.3 with 37% HCl → 1l A.d.	250ml 1M Tris HCl 144g glycin 100ml SDS 10%

SDS-loading buffer	125mM TrisHCl pH 6.8	2.5ml 1M Tris pH 6.8
	4%SDS	8ml SDS10%
	150mM DTT	462.75mg
	20% glycerol	8ml glycerol 50%
	0.01% Bromophenol blue	2mg

The acrylamide mix and the resolving mix were mixed, then APS and TEMED were added as polymerizing agent, the solution was poured between two glass plates, (air bubbles must be avoided), and the surface was overlaid with isopropanol, the gel polymerizes in 10-30min. After polymerisation, the isopropanol overlay was removed and washed with distilled water. The stacking gel was prepared and poured over the resolving gel, then insert the comb immediately into the stacking gel, avoiding the air bubbles. The comb was removed carefully after polymerisation, and the gel was placed in the running buffer. The slots were washed with the buffer to remove any gel debris, then the gel was loaded with sample and the marker .The gel was run using voltage for 30min at 8V/cm and then 1h at 12V/cm (Laemmli 1970).

The marker was Protein "Molecular Weight Marker" from Fermentas; it is a mixture of 7 purified proteins supplied in gel loading buffer for direct application to an SDS-PAGE, it resolves into tight bands in the range of 14.4kDa-116kDa and is easily stained by Coomassie Brilliant Blue.

2.14.6. Detection of protein bands

Fixing solution	25% isopropanol 10% acetic acid → 1l A.d.	250ml 100% isopropanol 100ml acetic acid
Rapid Coomassie Blue	10% acetic acid 0.006% coomassie blue → 1l A.d.	100ml acetic acid 60mg coomassie blue
Bleaching solution	10% acetic acid → 1l A.d.	100ml acetic acid

The gel was incubated for 15 min in the fixing solution on shaker, and then incubated in rapid coomassie blue for 2h. Finally it was incubated at least 2h in bleaching solution to remove the non-bound pigment.

2.14.7. Native Gel

Native PAGE was prepared as the SDS-PAGE without SDS. The native gel was stained for activity according to (Wang *et al*., 2000). The gels were run under native conditions and stained with 50µg α-naphthylacetate and 10mg fast blue RR salt in 100ml HEPES 0,02M pH 7 and incubation at 37°C 30-60` until the bands were developed.

2.14.8. Western blot

For the protein transfer on nitrocellulose membranes a Tanque blot apparatus (Bio-Rad) was used. After SDS-PAGE, the gel and a nitrocellulose membrane of the same size of the gel were equilibrated for 5 minutes in a transfer buffer (20% methanol, 1x SDS). Four pieces of filter paper were also soaked in the same buffer, and two of them were put on the bottom of the instrument, corresponding to the anode. The membrane was then laid on these two first layers, then the gel and at the end the other two filter papers, always without air bubbles. The apparatus was closed with the cover corresponding to the cathode. 350mA for 1h were then applied at 4ºC, in order to let the bounded proteins to the SDS, move to the anode, which means to the membrane. The membrane was in shaking 5% skimmed milk (blocking reagent) for 1h at RT. At the end of the blotting, the membrane was saturated three times for 10 minutes in TBS; 1% (m/v) (0.85% NaCl, 10 mM Tris-HCl pH 7.5). The reaction of the specific proteins with the antihistidine-tag antibody against the enzyme occurred over night at 4 °C, followed by washing of the membrane three times with TBS for 10 minutes. The membrane was then incubated with 1 U of the secondary antibody (anti-mous IgG) conjugated to a peroxidase in 1% albumin/TS for 1h at RT, remove the solution and dry as much as possible The proteins which bound specifically to the antibody were evident as black spots when developed on a Roentgen film (Towbin *et al*., 1979).

2.14.9. Removal of the His-tag

The expression vector pET-16b used in this study has a recognition site for factor Xa after the His-tag, which allows to removal of the His-tag and retrieval of the protein in its native form.

Protein digestion with factor Xa was accomplished according to the provider instructions.

2.15. Detection of enzyme activity

2.15.1. Spectrophotometric assay

2.15.1.1. Using *p*-nitrophenylesters

The standard esterase assay used *p*-nitrophenylacetate as a substrate at 37°C with a pH of 7.0 slightly modified from (Tesch *et al*., 1996; Hotta *et al*., 2002). 1mM *p*-nitrophenylacetate was added to buffer (0.02M HEPES buffer pH 7.0, 1% acetonitrile). 1ml of the substrate solution was pre-incubated for 1min at 37ºC, 2-3µl esterase (0,003mg) preparation was added and the reaction mixture incubated for 10min. The release of nitrophenyl was measured at 410nm using a model 4054 UV/visible spectrophotometer (LKB, Pharmacia). Controls without enzyme showed no significant non-enzymatic hydrolysis. One unit of lipase activity was defined as the amount of activity releases 1µM *p*-nitrophenol per minute. The standard assay was modified to test the effect of different parameters on enzyme activity.

2.15.1.2. Using naphthylesters

Comparison of the activity on *α*-naphthylacetate and *β*-naphthylacetate was carried out using a method modified from (Barbier *et al*., 2000) 10µl of a 100mM solution of each substrate in DMSO was added to 985µl dye solution fast blue RR salt 1mg/ml in HEPES 0.02M pH 7.0 and 5µl enzyme was added. After incubation for 15´ at 37°C the concentration of the coupling compound was measured by absorption at 505nm; standard curves were generated using α-naphthol and *β*-naphthol.

2.15.2. Qualitative detection of activity (plate and well method)

The modified method of (Lawrence *et al*., 1967) was followed. The proteins diffuse through agar gel as through a solvent when the concentration of agar is below 1.5% (W/V) and liberated free fatty acids from triglycerides are being detected as clearance zones on the agar after a suitable incubation time.

5ml emulsion 0.2% (V/V) triglycerides and 0.02% Tween 80 are added to 95ml hot solution of 1.2% agar in suitable buffer. The emulsion was poured in Petri dish and allowed to solidify, and then holes of 5mm were bored with a borer. 20-50µl of the sample was loaded per well. The plates were incubated 1-2h at 37ºC till the clearance zones appear.

2.16. Determination of enantioselectivity

The determination of enantioselectivity was done by our collaborators in CSIR Jammu.

2.17. Soft wares and web sites

2.17.1. Web sites

http://www.ncbi.nlm.nih.gov/ National Center for Biotechnology Information; genome search and, BLAST tool.

http://www.ebi.ac.uk/ European Bioinformatics Institute; alignment and ClustalW multiple alignment.

http://www.biotech.ou.edu/ University of Oklahoma; recombinant protein solubility prediction.

http://www.expasy.org/tools/ ExPASy proteomics tools; protein primary structure analysis.

http://swissmodel.expasy.org/ Swiss model; homology modelling

2.17.2. Software

"Primer Primer5" was used for assessing possible primers.

"GenDoc" was used for alignment of DNA and protein sequences; it is available on http://www.psc.edu/biomed/genedoc/.

"Clone manger" was used to blot restriction maps of the produced clones http://www.scied.com/seshome.htm.

"BioEdit" was used for sequence results evaluation and alignment purposes; it is available on http://www.mbio.ncsu.edu/BioEdit/bioedit.html

"Swiss pdb viewer" was used for viewing and manipulation of the pdb files; spdbv is available on http://www.expasy.org/spdbv/

"UCSF Chimera" was used for pdb files viewing and manipulation and for structural alignment; chimera is available on http://www.cgl.ucsf.edu/chimera/

"HMMER" for creation of a protein profile for HSL family http://hmmer.janelia.org/

3. Results

3.1. Cloning of putative esterase and lipase genes

3.1.1. Overview

In silico screening revealed that *S. coelicolor* genome has 31 different ORFs annotated as putative esterases and/or lipases and the genome of *S. avermitilis* has 20 ORFs. Two criteria were used to choose the genes to be used in this study. Firstly; all the genes should be compatible with one cloning strategy (described below 3.1.2). Secondly; comparison of the two genomes showed that 88% of the genes in *S. coelicolor* are conserved in *S. avermitilis* (Borodina *et al*., 2005), so ORFs from *S. avermitilis* which have homology in *S. coelicolor* were excluded because it is likely that the homologous proteins will show similar properties. I decided to clone 11 ORFs from *S. coelicolor*, and four ORFs from *S. avermitilis*.

3.1.2. General cloning strategy

The DNA sequences were obtained from the data bank and I have designed suitable PCR primers (see 2.13). During the PCR a mismatch primer was used to introduce an *Nde*I site into the start codon of the gene. This required that the gene had an ATG start codon and did not contain an *Nde*I site. The down stream primer contained either a *Bam*HI or an *Xho*I site, so that genes containing both these sites were unsuitable for this cloning strategy (table 3.1).

The genes were amplified and cloned in the TOPO-PCR4 vector. Then they were sequenced to detect if any mutation occurred during the amplification. The correct inserts were subcloned in the expression vectors (pET-16b and pET-23b) as *Nde*I/*Bam*HI or *Nde*I/*Xho*I cassettes. The positive clones in the expression vectors were confirmed through restriction analysis and sequencing, finally the constructs were expressed in *E. coli* when possible and the enzymes were characterized (Fig 3.1).

Table 3.1. The size of the putative gene and the restriction site introduced at the 3` end of each gene.

Putative gene	Size in bp	Sites created downstream
SAV 469	1243	*Xho*I
SAV 1549	1181	*Xho*I
SAV 3461	861	*Xho*I
SAV 7089	785	*Bam*HI
SCO 1265	949	*Xho*I
SCO 1735	922	*Xho*I
SCO 2123	843	*Xho*I
SCO 3644	978	*Xho*I
SCO 4368	1228	*Xho*I
SCO 4746	1332	*Xho*I
SCO 6966	874	*Xho*I
SCO 7131	986	*Xho*I
SCO 4799	1038	*Bam*HI
SCO 3219	1239	*Bam*HI
SCO 7513	1028	*Bam*HI

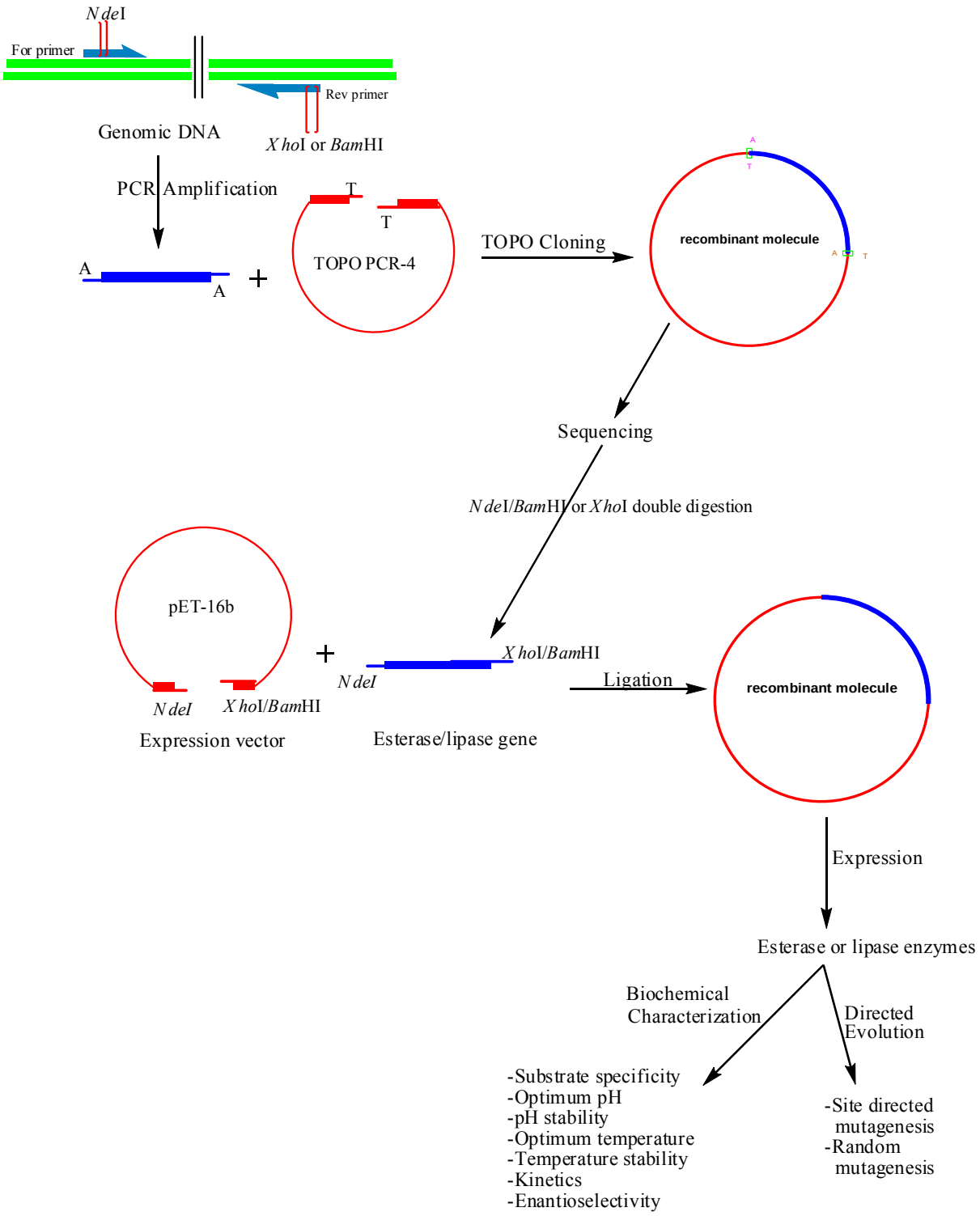


Fig. 3.1. Diagram illustrates the strategy used for the cloning of the different genes

3.1.3. Amplification of the putative esterase/lipase genes

I could amplify fragments of the expected size for all genes except one (SCO 2123). Fig 3.2 shows the obtained PCR products and their sizes.

For the putative gene SCO 2123 instead of the expected fragment size 843pb I got a fragment around 920bp. After sequencing the PCR product we found it is a fragment coding for a putative β-glucosidase (locus SCO 6604) from *S. coelicolor*, which means that the primers used made false priming on the *S. coelicolor* genome. I decided not to construct new primers for SCO2123 and continue the cloning of the other correct products. The cloning of the gene SAV 3461 was done by Zhang, Qi during his "Diplomarbeit" in the Genetics Department (May 2005).

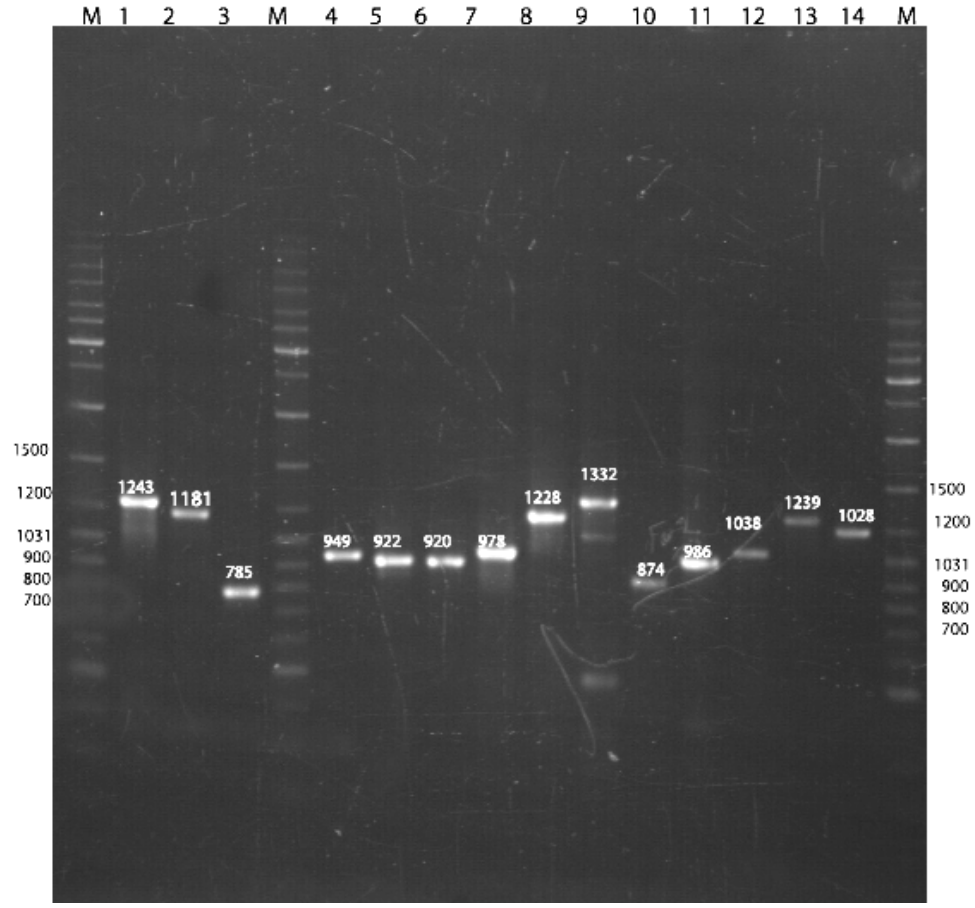


Fig. 3.2. PCR products and the size of the amplified genes. M, marker ladder mix; lane 1, SAV 469; lane 2, SAV 1549; lane 3, SAV 7089; lane 4, SCO 1265; lane 5, SCO 1735; lane 6, SCO 2123; lane 7, SCO3644; lane 8, SCO 4368; lane 9, SCO 4647; lane10, SCO 6966; lane 11, SCO 7131; lane 12, SCO 4799; lane 13, SCO 3219; lane 14, SCO 7513.

3.1.4. Cloning

All the cloning steps were the same for the 14 different esterase/lipase genes. As a representative example for the cloning is the cloning of the gene SCO 7131.

3.1.4.1. Cloning in TA vector

The DNA fragments were amplified using Taq polymerase and a characteristic property of Taq polymerase is that the enzyme introduces one or two extra deoxynucleotides on to the 3′ end of blunt double stranded DNA. In a template independent manner any nucleotide of the four deoxynucleotides can be added, but when a mixture of all deoxynucleotide triphosphates present in the reaction, a strong preference is given to incorporation of dATP. The PCR products were cloned into T-vector pCR4-TOPO, using a cloning system based on vaccinia virus DNA topoisomerase (Shuman 1994). The cloning mixtures were transformed into *E. coli* strain TOPO10 and the transformation mixtures were plated onto agar containing X-gal and kanamycin for selection. The white colonies were subjected to restriction analysis; first *Eco*RI was used to detect wether the insert was of the correct size as there are *Eco*RI sites on each side of the cloning site, second restriction analysis was carried out using the appropriate enzymes for each insert.

For SCO 7131 more than 40 white colonies were obtained. 40 clones were subjected to *Eco*RI digestion (Fig 3.3), and then the 36 positive clones were double digested with *Nde*I/ *Xho*I (Fig 3.4). In the end I found 34 clones out of 40 having the right insert. I have called the plasmid pUKG951.

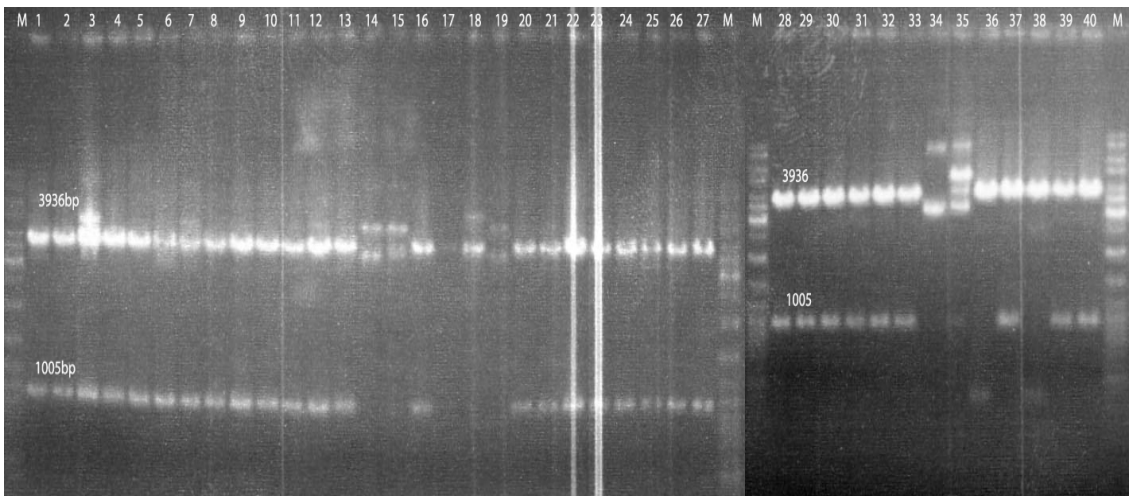


Fig. 3.3. 40 clones *Eco*RI digested, 34 clones of them have an insert of the correct size (1005bp).

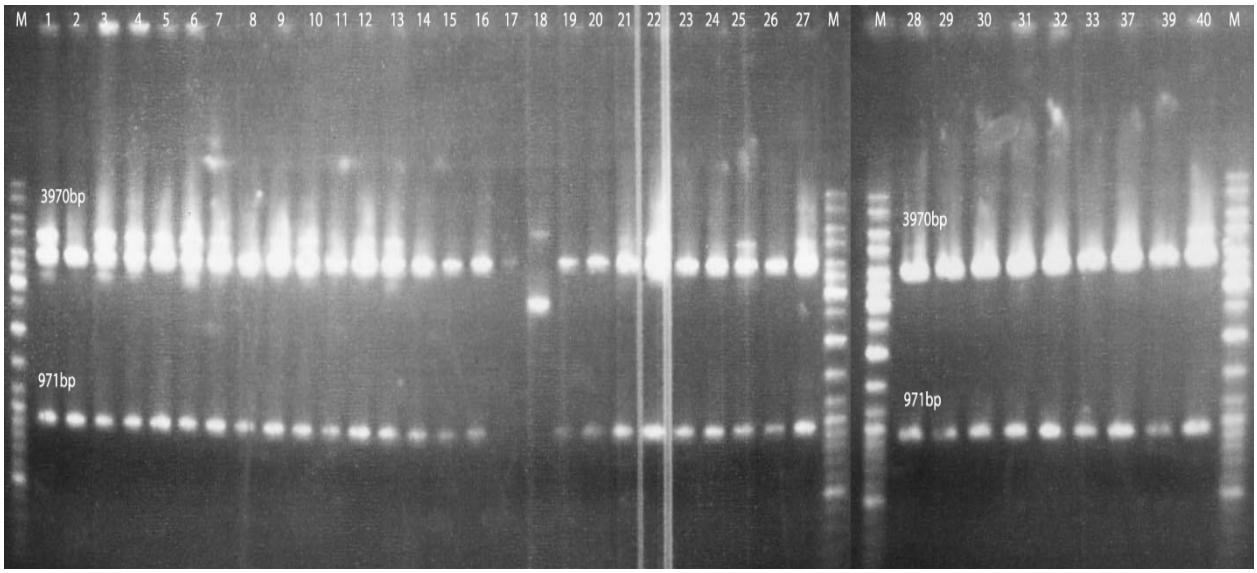


Fig. 3.4. 36 clones double *Nde*I/*Xho*I digested. Only clones 17and 18 did not have the right insert (971bp).

3.1.4.2. Sequencing

The putative esterase/lipase genes were amplified using Taq DNA polymerase, so it was necessary to sequence the cloned fragments to be sure that there were no point mutations arised during the PCR. Each insert was sequenced on both strands using M13 forward and reverse primers and the walking primers (see 2.13) the sequencing was repeated for each gene 4-5 times to exclude sequencing errors.

For SCO 7131 clone 2 was initially chosen for sequencing, but there were 3 point mutations in the sequence. However the sequence of clone 28 was 100% identical with the sequence in the database (Fig 3.5 & 3.6). In clone 2 the 3 point mutations lead to 3 substitutions in amino acids R100W, L243P and F286S.

SCO 1735 clone 1 showed 2 point mutations and Clone 2 was 100% identical to the data base entery, whereas for all the other genes only one clone was sequenced and was 100% identical to the data base entery.

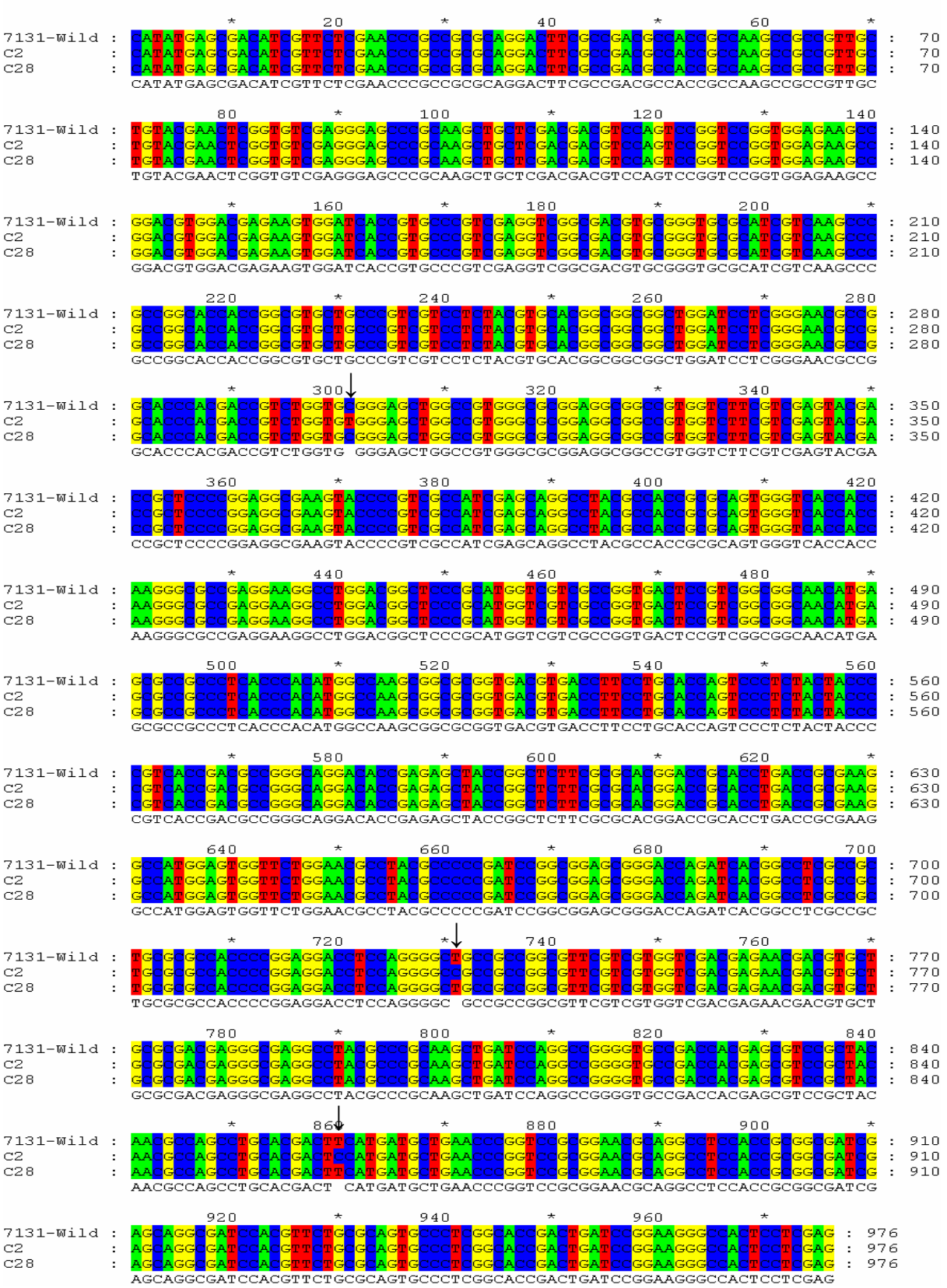


Fig. 3.5. : A comparison between the sequences obtained for C2 and C28 of SCO 7131 and the sequence in database. C28 is identical 100% with the database entery however C2 shows 3 point mutations (↓) C301T, T731C and T860C.

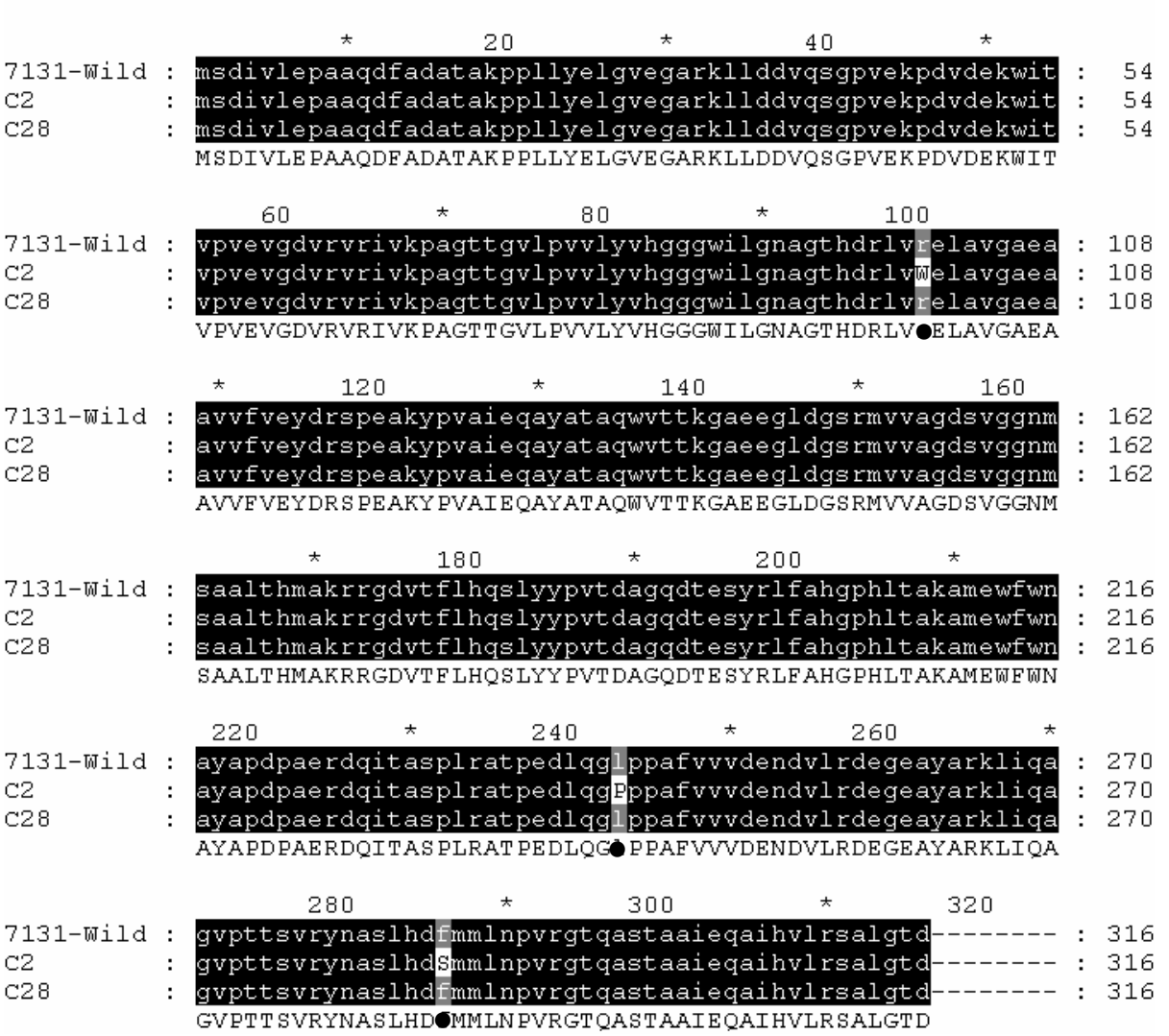


Fig. 3.6. : Clone 28 is 100% identical and Clone 2 has 3 point mutations (●) R100W, L243P and F286S.

3.1.4.3. Subcloning in the expression vector

The fragments, which were successfully cloned in the TOPO vector, were used for further subcloning in the expression vector.

In this work, pET-16b was used for the expression. In the multiple cloning site it has three restriction sites *Nde*I, *Xho*I and *Bam*HI. The fragments were cut and purified from the corresponding plasmid in TOPO vector as *Nde*I/*Bam*HI or *Nde*I/*Xho*I. From previous work I know that agarose gel elution may affect the fragment's ends or the vector's ends and hence decrease the cloning efficiency. Instead of an elution strategy to purify the fragments before the subcloning a "shotgun cloning" technique was used. TOPO-pCR4 has three *Dra*I sites whereas the high G+C content esterase/lipase fragments have no *Dra*I cutting sites. After treatment of the recombinant plasmids with either *Nde*I/*Bam*HI or *Nde*I/*Xho*I to cut the genes,

a digest with *Dra*I was carried out and finally the DNA was purified with phenol/chloroform extraction and no CIAP treatment was carried out. The pET-16b was also treated the same with either *Nde*I/*Bam*HI or *Nde*I/*Xho*I followed with phenol/chloroform extraction and no CIAP treatment. The ligation mixtures were transformed into *E.coli* strain XL-1-blue. The obtained clones were screened with a double digest either *Nde*I/*Bam*HI or *Nde*I/*Xho*I then the positive clones were confirmed with suitable restriction analysis followed by sequencing using the walking primers and T7 primer.

The SCO 7131 gene was purified as an *Nde*I/*Xho*I fragment from pUKG951 and ligated into the expression vector pET-16b. Large number of clones was obtained. 48 clones were screened through *Nde*I/*Xho*I double digestion. The positive clone should produce two bands 5704bp (vector) and 971bp (insert). Only clone one clone out of 48 screened clones possessed the correct sizes. To confirm the insert the positive was further analyzed through *Bam*HI and *Pvu*I digestion. *Bam*HI produces two bands 5962bp and 713bp and the *Pvu*I digestion produces two bands 5657bp and 1018bp (Fig 3.7). The final confirmation was carried out through sequencing using T7 primer and the walking primers SCO 7131-For-2 and SCO 7131-Rev-2. The plasmid was called pUKG 952.

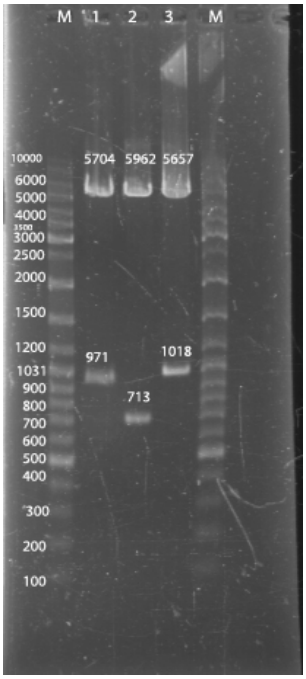


Fig. 3.7. The restriction analysis of pUKG952. M, marker ladder mix; lane 1, *Nde*I/*Xho*I double digest; lane 2, *Bam*HI digest; lane 3, *Pvu*I digest

I have also subcloned the genes in another expression vector pET-23b. Constructs in the pET-23b were designed to produce native protein: pET-23b has a C-terminal His-tag but I have cloned my genes with its natural stop codons, which means expression of my genes in this

vector will stop at the end of the gene before adding the His-tag to the C-terminal and hence the produced protein will be in native form.

The cassettes were subcloned in the expression vector pET-23b following the same strategy used for the subcloning in pET-16b.

All the cloning results in the vectors TOPO pCR4, pET-16b and pET-23b are shown in table 3.2.

Table 3.2. Summary of the cloning results and shows the plasmids created through the cloning in TOPO pCR4 and in the expression vectors pET-16b/pET-23b and their sizes.

Putative gene	Size in bp	Cloning in TOPO pCR4		Cloning in pET-16b		Cloning in pET-23b	
		Plasmid name	Size in bp	Plasmid name	Size in bp	Plasmid name	Size in bp
SAV 469	1243	pSHS1240	5196	pSHS1241	6934	pSHS1242	4816
SAV 1549	1181	pSHS1180	5129	pSHS1181	6871	pSHS1182	4753
SAV3461	861	pSQ891	4846	pSQ893	6594	pSQ892	4476
SAV 7089	785	pSHS780	4739	pSHS781	6471	pSHS782	4398
SCO 1265	949	pSHS940	4903	pSHS941	6636	pSHS942	4518
SCO 1735-C 1 [a]	922	pSHS920	4876	pSHS921	6612	pSHS922	4494
SCO 1735-C2 [b]	922	pSHS923	4876	pSHS924	6612	pSHS925	4494
SCO 3644	978	pSHS970	4932	pSHS971	6669	pSHS9712	4551
SCO 4368	1228	pSHS1220	5182	pSHS1221	6902	pSHS1222	4784
SCO 4746	1332	pSHS1330	5286	pSHS1331	7022	pSHS1332	4904
SCO 6966	874	pSHS810	4828	pSHS811	6566	pSHS812	4448
SCO 7131-C28 [c]	986	pUKG951	4941	pUKG952	6674	pUKG953	4557
SCO7131-C2 [d]	986	pUKG954	4941	pUKG955	6674	pUKG956	4557
SCO 4799	1038	pSHS1030	4996	pSHS1031	6731	pSHS1032	4658
SCO 3219	1239	pSHS1200	5194	pSHS12001	6929	pSHS12002	4856
SCO 7513	1028	pSHS1020	4972	pSHS1021	6715	pSHS1022	4642

a. SCO 1735 clone 1 which has two point mutations.
b. SCO 1735 Clone 2 which is 100% identical with the database.
c. SCO 7131 Clone 28 which is 100% identical with the database.
d. SCO 7131 Clone 2 which has three point mutations.

3.2. Protein expression

The pET system is one of the most commonly used systems for the cloning and expression of recombinant proteins in *E. coli*. The target genes in pET plasmids are under the control of the strong bacteriophage T7 transcription signal. The expression occurs when a source of T7 RNA polymerase exists within the host cells. The T7 RNA polymerase promoter is a strong one, when it is fully induced, it will use most of the host resources to synthesize the target protein. At the same time the expression level can be controlled through controlling the used amount of inducer.

The target protein expression can be initiated either by infecting the host cells with λCE6, a phage carrying the T7 RNA polymerase gene, or by transferring the plasmid into an expression host which carries a chromosomal copy of the T7 RNA polymerase gene under the control of *lacUV5*, in this case IPTG is required as inducer. The promoter in pET-16b is called T7 *lac* promoter. In this case a *lac* operator sequence exists just downstream of the T7 promoter on the plasmid, which carries the natural promoter and *lacI* (*lac* repressor). The T7 RNA polymerase gene and *lacI* are diverging. The *lac* repressor acts at the *lacUV5* promoter in the host chromosome to repress the T7 RNA polymerase gene, and at the T7*lac* promoter in the vector to prevent the transcription of the target gene by any made T7 RNA polymerase. This controls the basal expression of the gene (Novagen manual, 2002) Fig 3.8.

All the intial constructs of the esterase/lipase genes were in the *E. coli* strain XL-1-blue which is a cloning strain but not an expression strain. The plasmids must be retransferred into expression strain before the expression. We have used for this purpose the *E. coli* strain BL21 (DE3). BL21 (DE3) in addition to the production of T7 RNA polymerase is characterized by deficiency in the proteases *lon* protease and *ompT* outer membrane protease, which makes several target proteins more stable in BL21 (DE3) than in other strains which possess these proteases.

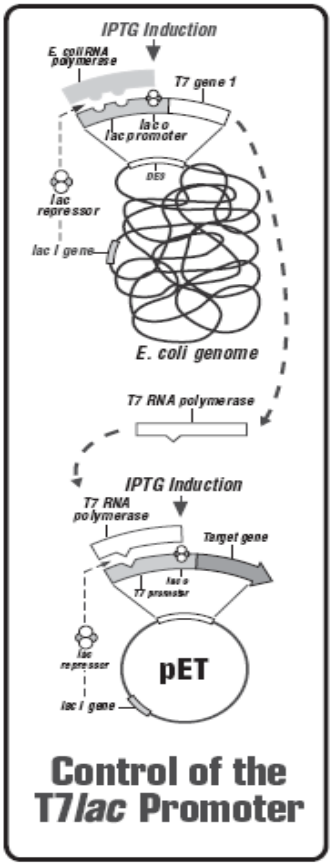


Fig. 3.8. The expression of target gene under the control of T7 *lac* promoter (pET system manual, Novagen 2002)

The 14 different putative esterase/lipase genes were cloned in pET-16b in addition to the SCO 7131 and SCO 1735 mutants. To collect more information about the expected overexpressed proteins, the protein sequences were analyzed *in silico* using the proteomics and sequence analysis tools on the ExPASy web site (http://www.expasy.org/tools/). The analysis of the primary protein structures enabled me to predict the theoretical molecular weight and the isoelectric point for each protein. Another useful tool is provided on the web site of Oklahoma University (http://www.biotech.ou.edu/). This tool predicts the solubility probability of a protein assuming it is overexpressed in *E. coli*. The determination of solubility probability was a key step for the decision which construct should be expressed first, taking in consideration that the solubility of the overexpressed proteins in *E. coli* is a bottle neck for heterologous expression. The data collected from the analysis of these sequences were gathered in table 3.3.

Table 3.3. The solubility, molecular weight and isoelectric point as obtained from the sequences and the primary structure analysis

Gene locus	Protein Acc. No	Mwt in kDa	Mwt with the His tag	Solubility probability	PI	PI With the His Tag
SAV 469	NP_821644	42.97	45.52	67% Insol	6.48	6.74
SAV 1549	NP_822725	42.07	44.71	66% Insol	7.31	7.10
SAV 7089	NP_828265	24.45	26.98	52% Insol	4.69	5.87
SAV 3461	NP_824638	30.72	33.26	74% Insol	7.73	7.82
SCO 1265	NP_625552	31.47	34.00	59% Sol	5.25	5.90
SCO 1735 (C2)	NP_626008	30.50	33.03	65% Insol	5.75	6.31
SCO 1735 (C1)		30.49	33.02	69% Insol	5.76	6.32
SCO 3644	NP_627838	31.08	33.61	74% Sol	4.76	5.50
SCO 4368	NP_628538	40.52	43.07	90% Insol	9.89	9.89
SCO 4746	NP_628904	44.03	46.56	57% Insol	5.75	6.26
SCO 6966	NP_631032	28.92	31.46	52% Sol	5.69	6.18
SCO 7131 (C28)	NP_631192	34.10	36.62	73% Sol	4.84	5.55
SCO 7131 (C2)		34.05	36.57	73% Sol	4.79	5.50
SCO 4799	NP_628956	35.14	37.69	90% Insol	9.29	9.29
SCO 3219	NP_627433	41.49	44.04	83% Insol	11.08	11.07
SCO 7513	CAC42140	30.49	33.03	50% Insol	5.28	6.05

3.2.1. Expression, characterization and directed evolution of a novel (HSL) acetylesterase from *Streptomyces coelicolor* A3(2) "gene locus SCO 7131"

3.2.1.1. Est A expression and purification

The plasmid pUKG952 contains the gene SCO 7131, which we called *estA* with product esterase A (Est A). The plasmid was transformed into the expression strain BL21 (DE3). The clone was induced using IPTG. A preliminary experiment showed that *p*- nitrophenylacetate was hydrolysed by the clone and this substrate was used to monitor the induction. The induction was carried out in several conditions; different induction temperatures (15ºC -37ºC), induction periods (1h-24h) and different IPTG concentrations (0.1-1mM IPTG). It was found that the best result was obtained when the culture was induced for 4hrs at 30ºC using 0.5mM IPTG (data not shown). After the establishment of the proper induction condition the enzyme was induced in 1l culture. The extracted total cell protein was purified by affinity chromatography on Ni-agarose gel. The purification procedure gave a 10-fold increase in enzyme specific activity with a total yield of 79% (table 3.4). Part of the purified enzyme was treated with factor Xa to remove the His-Tag. 12% SDS PAGE was run to estimate the relative molecular weight of both forms the tagged and non-tagged. The SDS PAGE gave bands of the expected Mr 38.5 kDa for the protein with the His-tag (calculated was 36.62 kDa) and 35.4 kDa after removal of the His-tag (the calculated was 34.10 kDa) Fig 3.9.

A western blot was made for Est A using anti-His tag antibody as primary antibody. In addition to the SDS PAGE a native PAGE was run, it produced strong bands after the activity staining (Fig 3.10).

Table 3.4. Purification of Est A from recombinant *E. coli*

Step	Total protein (mg)	Activity U	Specific activity U/mg	Yield (%)	Purification factor
Crude extract	128.5	13112	102	100	1
affinity column	10	10204	1020	79	10

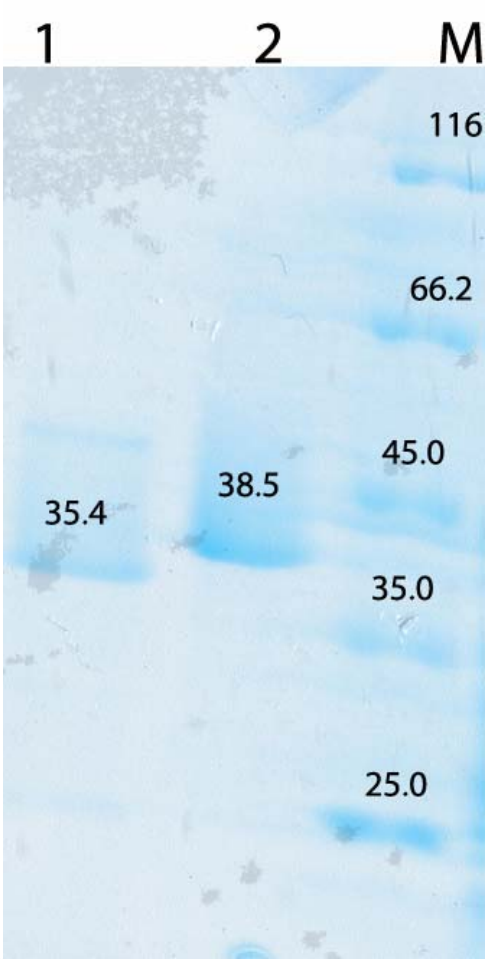


Fig. 3.9. SDS PAGE of Est A. M: protein molecular weight marker, lane 1: Est A purified on Ni column and cut with factor Xa, lane 2: Est A purified on Ni column.

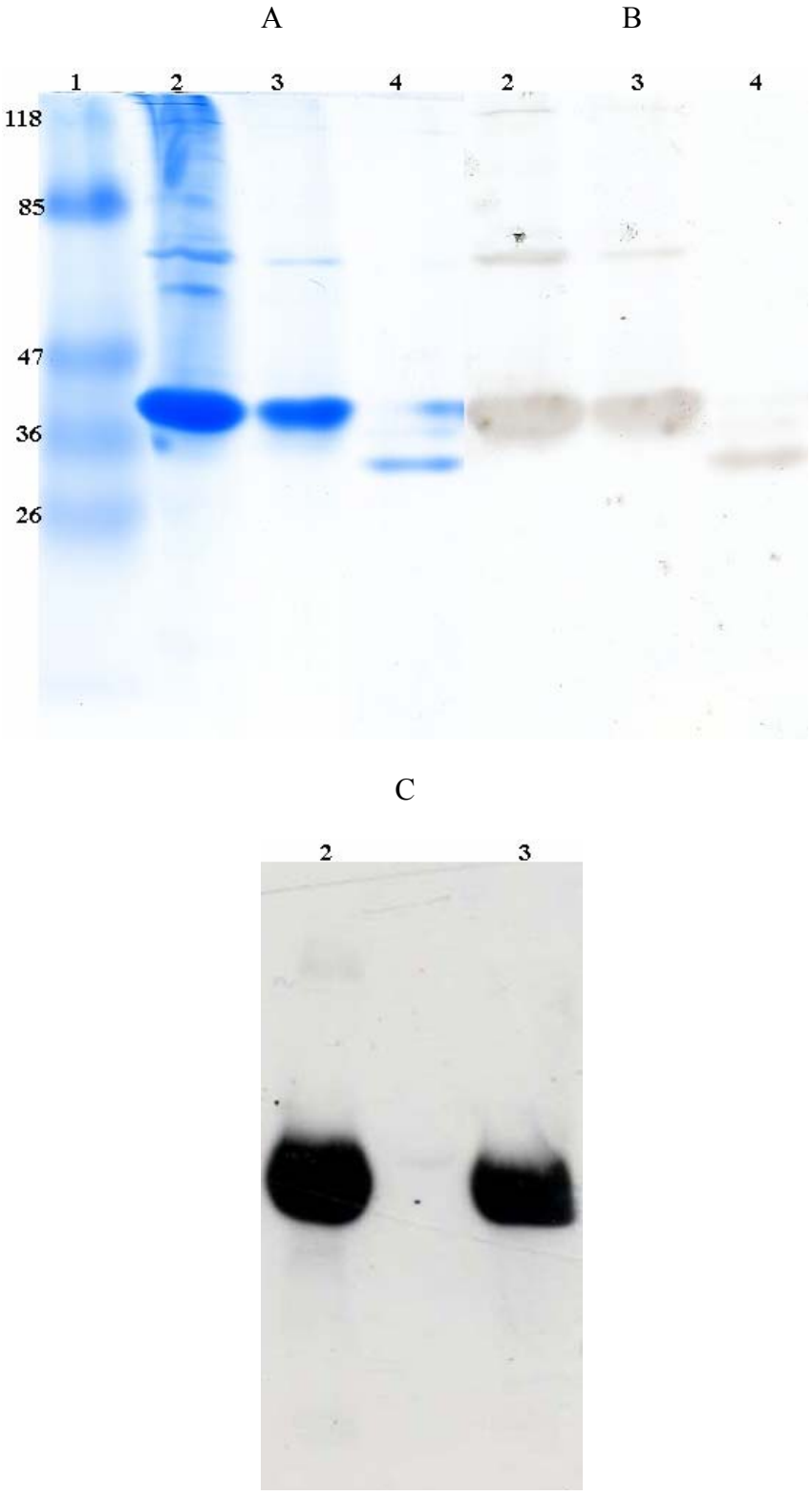


Fig. 3.10. Native PAGE of Est A, and Western Blot using anti-His tag antibody. Lane 1: The molecular weight marker, lane 2: cell extract, lane 3: affinity column purified protein, lane 4: protein after factor Xa digestion. (a) Gel stained with Coomassie brilliant blue. The molecular weights of the marker (in kDa) are indicated (b) Gel after activity staining (c) Western blot (original gel not shown).

3.2.1.2. Characterization of Est A

All the experiments were done on the His-tagged enzyme. A single replicate of the non His-tagged protein was done for substrate specificity, effect of temperature and pH on the activity and to test effect of inhibitors, they all gave results with in the SD.

3.2.1.2.1. Substrate specificity of Est A

The enzyme activity was investigated against several triglycerides using the agarose diffusion test and synthetic esters (*p*-nitrophenyl esters). Est A did not show activity against any of the tested triglycerides (tributryin, trioctanoate, tristearin, triolein, tripalmitin) or against olive oil. When Est A was tested against synthetic *p*-nitrophenyl esters, I observed activity only against C_2 acyl ester (*p*-nitrophenyl acetate), even the activity observed against C_4 and C_6 acyl esters (*p*-nitrophenyl butyrate and *p*-nitrophenyl caproate) was less than 2% of the activity recorded towards the *p*-nitrophenyl acetate.

To learn more about the activity of this enzyme, it was necessary to check if it will act against other acetate esters. Est A acted on both α- and β-naphthyl acetates, and interestingly the activity towards β-naphthyl acetate was only 27% of the activity observed with α-naphthyl acetate.

3.2.1.2.2. Effect of temperature on Est A activity and stability

The effect of temperature on enzyme activity and stability was measured spectrophotometerically using *p*-nitrophenyl acetate as substrate and HEPES pH 7 as a buffer at temperatures range 20-80ºC. The Est A activity increased with temperature reaching a plateau at 55ºC (Fig 3.11).

In order to assess the thermostability the enzyme was preincubated for 1h at temperatures range 20-80ºC before assaying the residual activity. The enzyme was fairly stable at temperatures up to 55ºC. At 55ºC Est A retained 73% of its activity after 1h incubation, whereas incubation at 60ºC or higher temperature for the same period leaded to sharp decrease in activity (Fig 3.12).

Time courses for thermostability at 37ºC and 50ºC were done up to 4.5h. The decrease in activity was gradual at both temperatures, it did not show any abrupt change at any time. At 37ºC Est A retained 67% of its activity after 270 min incubation whereas at 50ºC it had a $t_{1/2}$ 4.5h (Fig 3.13).

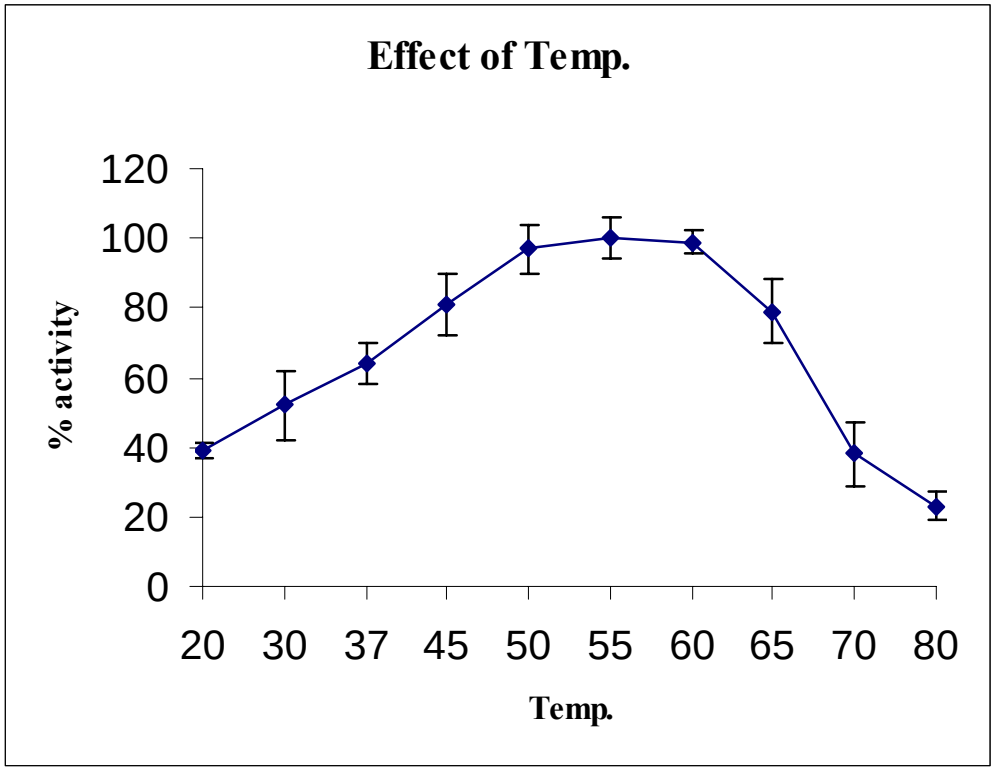


Fig. 3.11. The effect of temperature on enzyme activity. Activity is represented as percentage of the maximum activity. Standard deviations are derived from four replicates.

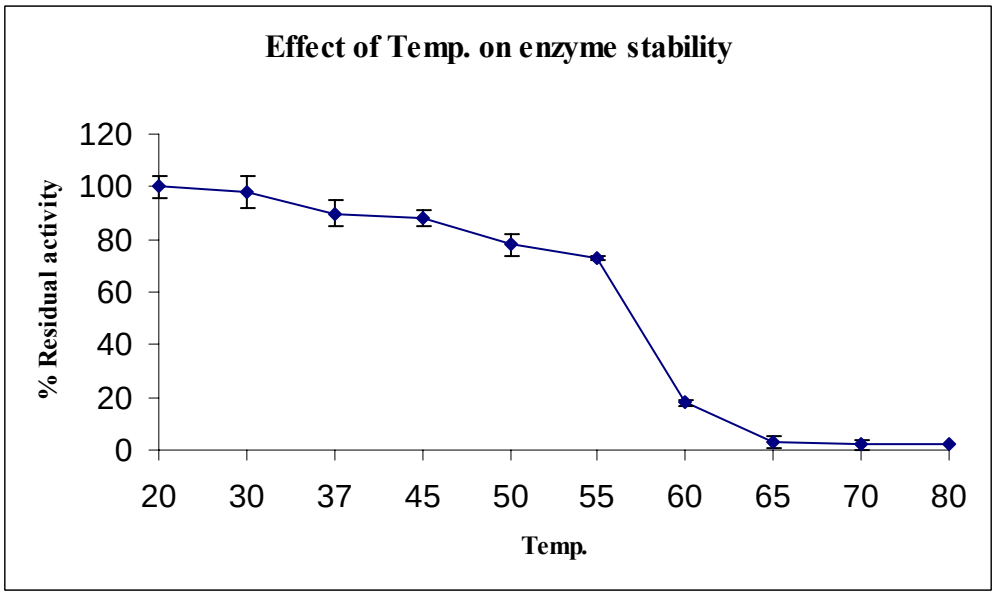


Fig. 3.12. The effect of temperature on enzyme stability. Stability was measured by incubation for 1 h at the stated temperature with determination of the residual activity at 37°C. Standard deviations are derived from four replicates

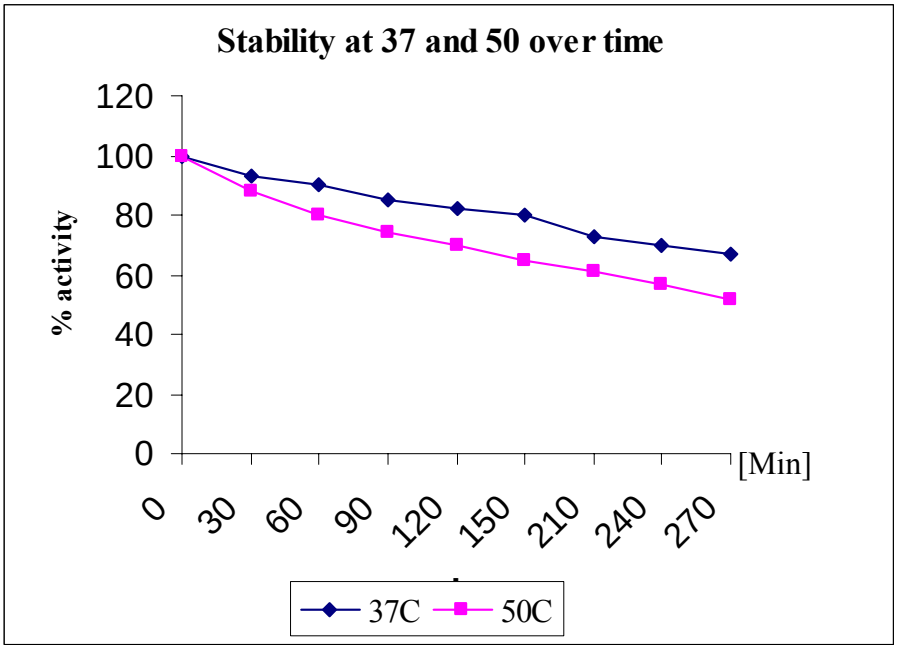


Fig. 3.13. Est A stability at 37ºC and 50ºC. Stability was measured with determination of the residual activity at 37°C.

3.2.1.2.3. Effect of pH on enzyme activity and stability

The activity and stability were measured at 37ºC at a range of pH values. The enzyme preferred slightly alkaline conditions with an optimum activity at pH 7.5 (Fig 3.14) and very little activity (21% of the maximum activity) at pH 6.5. At a pH lower than 6.5 the activity almost disappeared (less than 2% of the maximum activity). At pH 8.5 the activity decreased to 37% of the maximum activity. The enzyme was stable on storage at pH values between 5.5 and 10 for 24h at 20ºC. It retained more than 98% of its activity at pH range 6.5-8.5, at pH 9 it showed 90% of its activity and retained >86% at pH 6 and pH 9.5 (Fig 3.15).

When the buffer composition was tested, HEPES pH 7.5 and Tris-HCl pH 7.5 gave almost the same activity, whereas phosphate buffer pH 7.5 gave around 20% less activity (Fig 3.16).

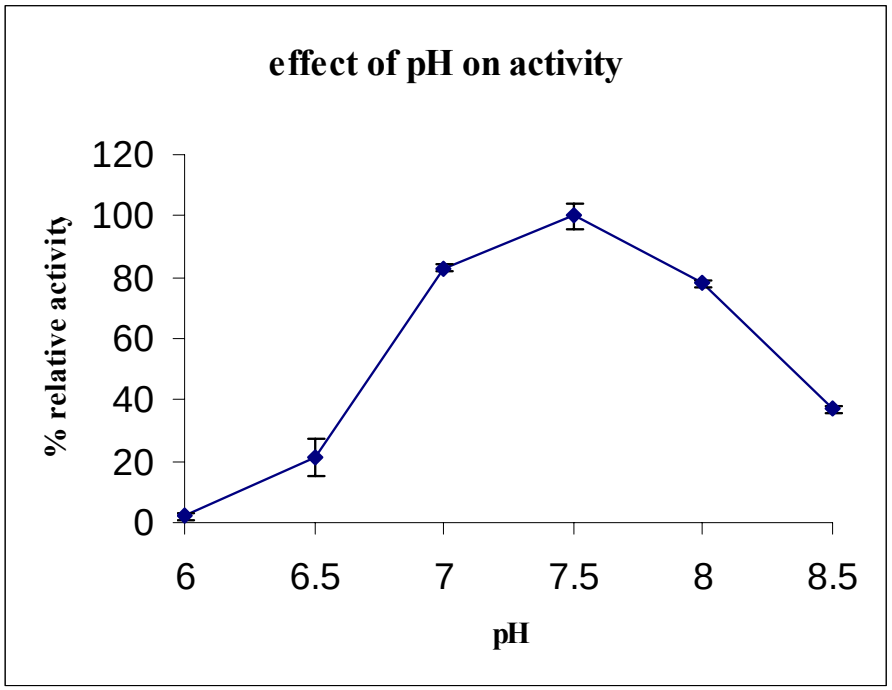


Fig. 3.14. Effect of pH on activity of Est A. Activities were shown as percentages of the maximum activity. Standard deviations were derived from four replicates. SD were indicated as error bars. The absence of an error bar indicated a deviation less than the symbol size.

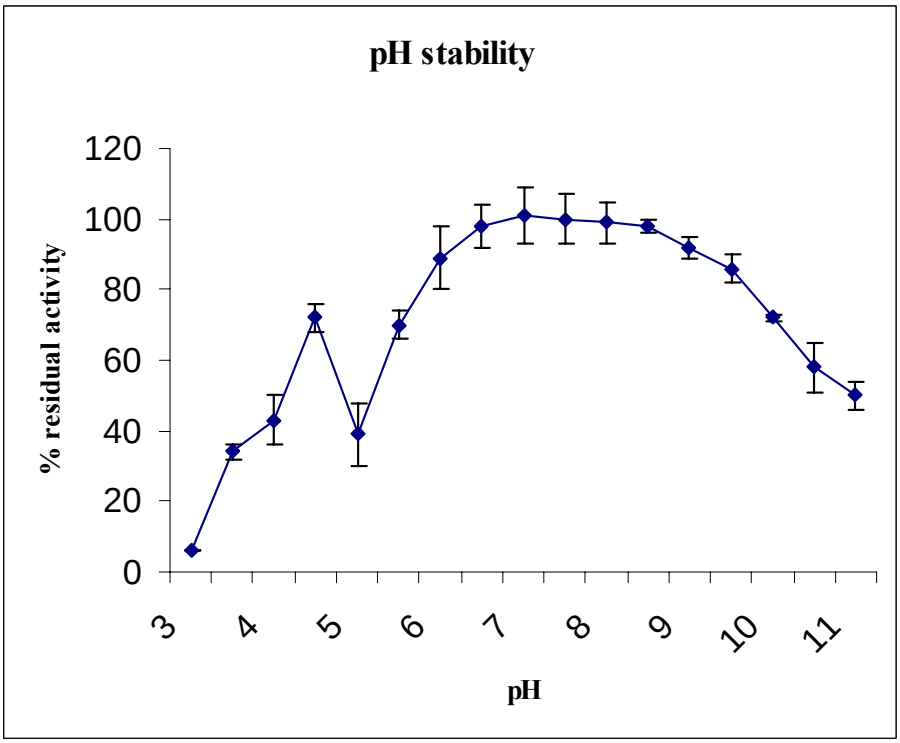


Fig. 3.15. Effect of pH on Est A stability. Stability was measured after 24h incubation in different buffers at 20˚C. Standard deviations were derived from four replicates. SD are indicated as error bars. The absence of an error bar indicates a deviation less than the symbol size.

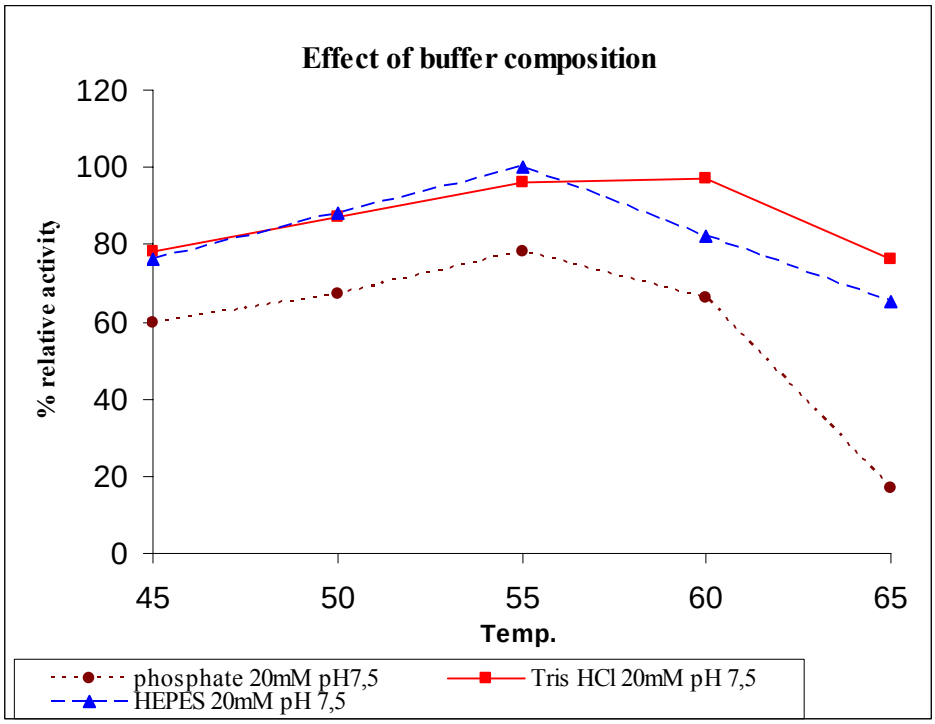


Fig. 3.16. Effect of different buffers on activity. The buffers were HEPES 20mM pH 7.5 (▲), Tris-HCl 20mM pH 7.5 (■) and Phosphate 20mM pH 7.5 (♦).

3.2.1.2.4. Effect of metal ions and inhibitors

The effect of preincubation with different metal ions and inhibitors on the activity of the enzyme was tested. The enzyme was preincubated with either 1mM or 10mM of each substance for 1h at 20ºC, then the activity was measured at 37ºC and the relative activity to the activity of untreated enzyme was calculated. There was little effect of any of the metals and chemicals at a concentration of 1mM after 1h incubation; in all cases the enzyme had more than 85% activity except PMSF decreased the activity to 82%. However, preincubation in 10mM PMSF, Cu^{2+} and Hg^{2+} decreased the activity greatly; PMSF decreased the activity to 63%, Cu^{2+} showed only 45% and Hg^{2+} 49%. Preincubation in 10mM K^{+} lead to a significant increase in activity (138%), whereas 10mM Na^{+} lead to only a small increase (118%). There was a small increase of activity (125% and 117%) in the presence of DTT and EDTA 10mM respectively (Figure 3.16).

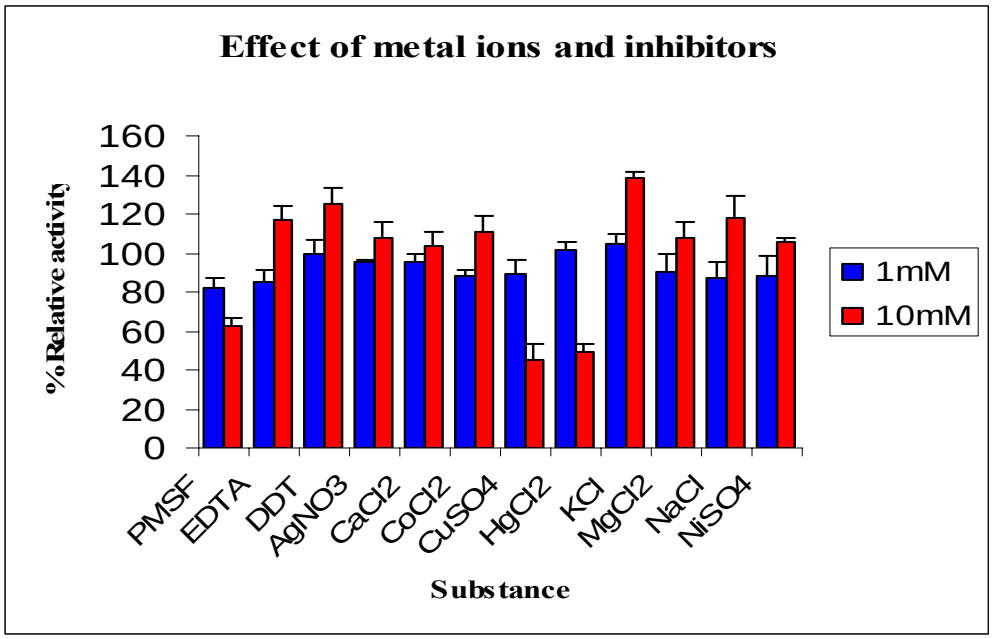


Fig. 3.17. Effects of metal ions and inhibitors on activity of the enzyme. All reagents were tested using 1 mM (blue bars) and 10 mM (red bars). The final concentration in reactions was always ≤2%. Standard deviations were derived from four replicates.

Est A was incubated with 10mm PMSF over a time course of 150min; the enzyme activity was measured at 30 min intervals. There was gradual decrease in the activity with the incubation time during the first 90min. after 90 min there was no significant change in the activity, the activity remain around 50% for 1h more (Fig 3.18)

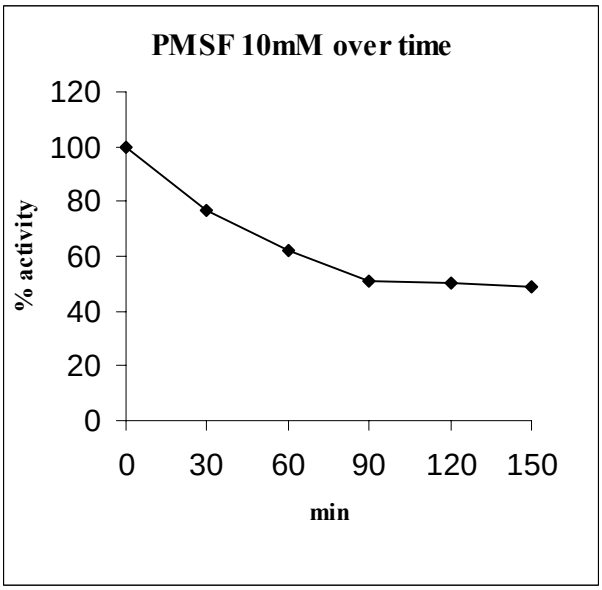


Fig. 3.18. Effect of 10mM PMSF over time course 150min

The effect of different Na^+ and K^+ concentrations in the reaction buffer was also investigated (Table 3.5) This showed optimal concentrations of about 1mM and 10mM respectively.

Table 3.5. Effect of Na^+ and K^+ on enzyme activity. Activities were percentages relative to the activity in the absence of either Na^+ or K^+. The standard deviations (SD) were derived from three replicates

Concentration	Na^+ activity (%±SD)	K^+ activity (%±SD)
0.5 mM	113±4	115±2
1 mM	116±4	123±1
10 mM	98±10	129±4
50 mM	100±2	119±8
100 mM	106±2	107±4

3.2.1.2.5. Effect of water miscible organic solvents

All water-miscible organic solvents tested showed a significant reduction in enzyme activity, when used at a concentration of 50%. However, whereas alcohols still inhibited at a concentration of 10%, dimethylformamide and DMSO retained more than 75% of the activity (Table 3.6).

Table 3.6. Effects of water-miscible organic solvents on enzyme activity. The activities were percentages relative to the activity without organic solvents. The standard deviations (SD) were derived from three replicates.

Organic solvent	10% concentration activity (%±SD)	50% concentration activity (%±SD)
Acetone	74±1	3±4
Acetonitrile	38±7	0.3±0
Dimethylformamide	77±2	3±9
DMSO	74±2	26±8
Ethanol	11±1	2±9
Methanol	19±2	7±5
2-propanol	13±5	0.7±5

The enzyme was preincubated 1h at 20ºC in different concentrations of the organic solvent (10%, 20% and 30%) and the residual activity was measured. The enzyme can moderately withstand 10% concentration of the tested organic solvents; in acetone, DMSO and dimethylformamide 10% the enzyme had more than 70% residual activity, in 10% acetonitrile, ethanol and methanol the residual activity was around 60%, whereas 10% isopropanol decreased the activity to 50%. The effect of 25% organic solvents did not differ extensively from the concentration 10%, except in case of isopropanol which decreased the activity to 18%. The maximum reduction in activity was observed after incubation in 50% isopropanol and acetonitrile (less than 15%) whereas ethanol lowered the activity to 25%. Est A has fairly good tolerance of 50% acetone, dimethylformamide and DMSO, it retained more than 50% of its activity after 1h incubation (Fig 3.19).

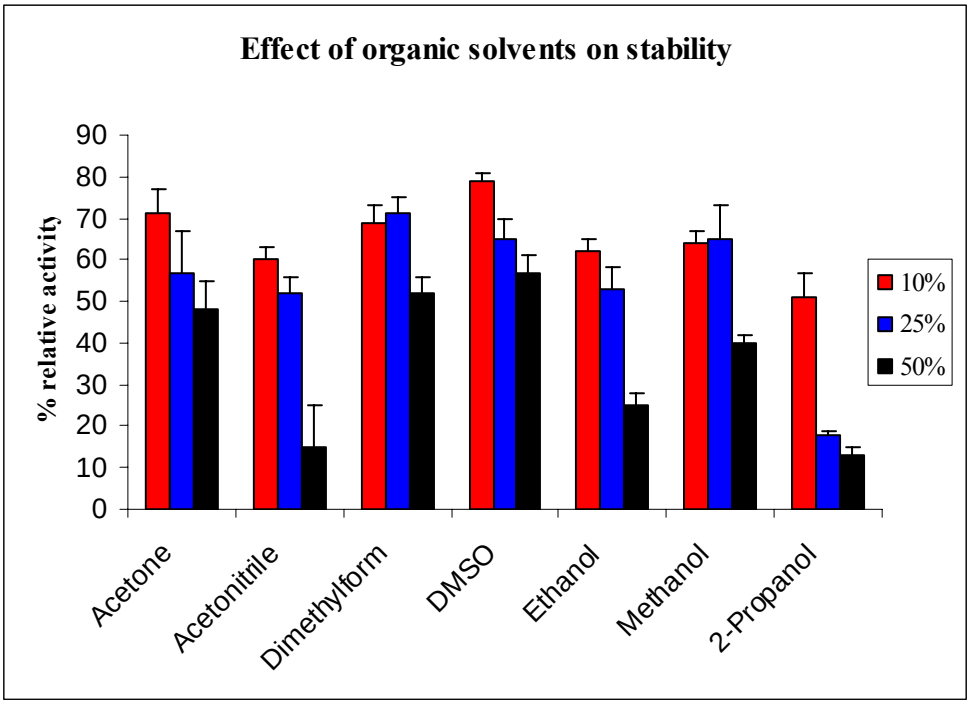


Fig. 3.19. Effect of organic solvents on stability of the enzyme. Stability was measured by incubation for 1 h at 20˚C. Different concentrations of organic solvents were used; 10% (red bars), 25% (blue bars) and 50% (solid bars). The final concentration in the reaction was always ≤1%. Standard deviations were derived from four replicates.

3.2.1.2.6. Kinetic parameters of Est A.

The K_m and V_{max} of the esterase were estimated using the activity assay with *p*-nitrophenylacetate, *α*-naphthylacetate and *β*-naphthylacetate as substrates, the Lineweaver Burk plots were plotted Figures 3.20, 3.21 and 3.22 respectively.

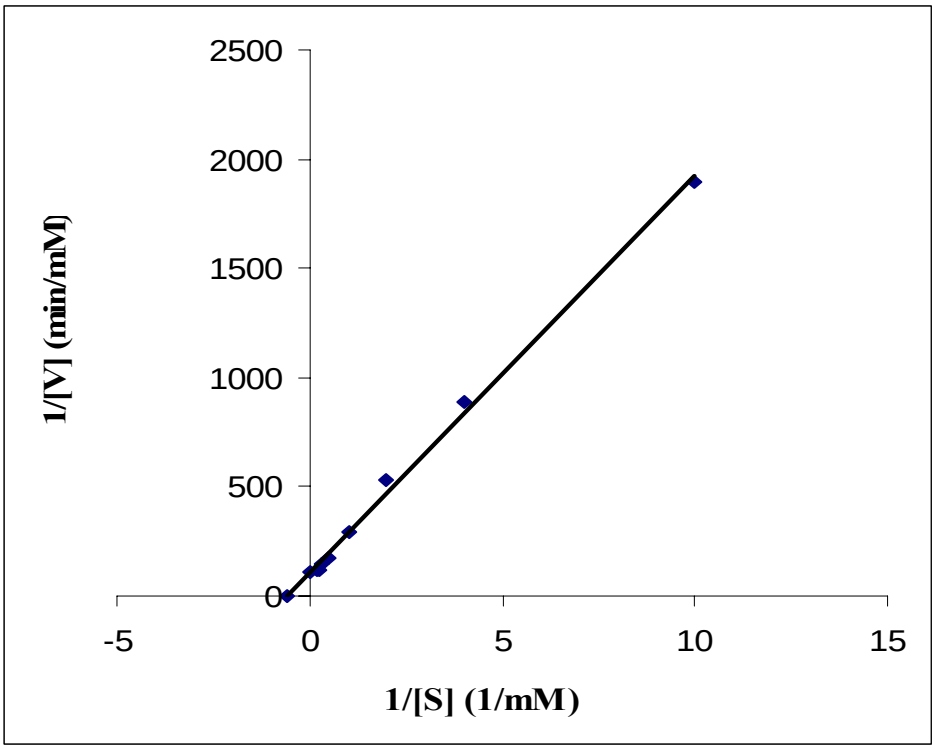


Fig. 3.20. Lineweaver Burk plot for Est A using *p*-nitrophenylacetate

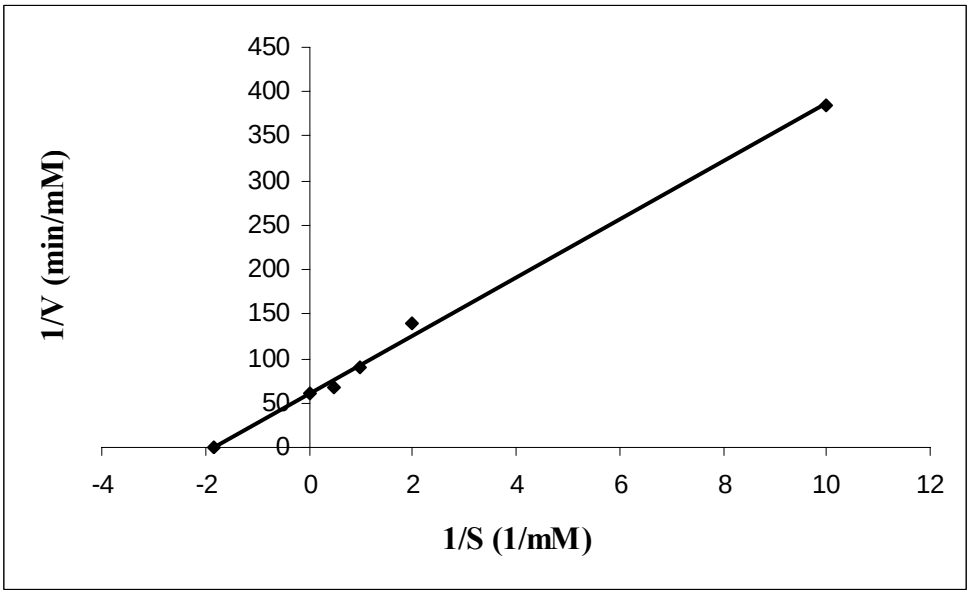


Fig. 3.21. Lineweaver Burk plot for Est A using α-naphthylacetate.

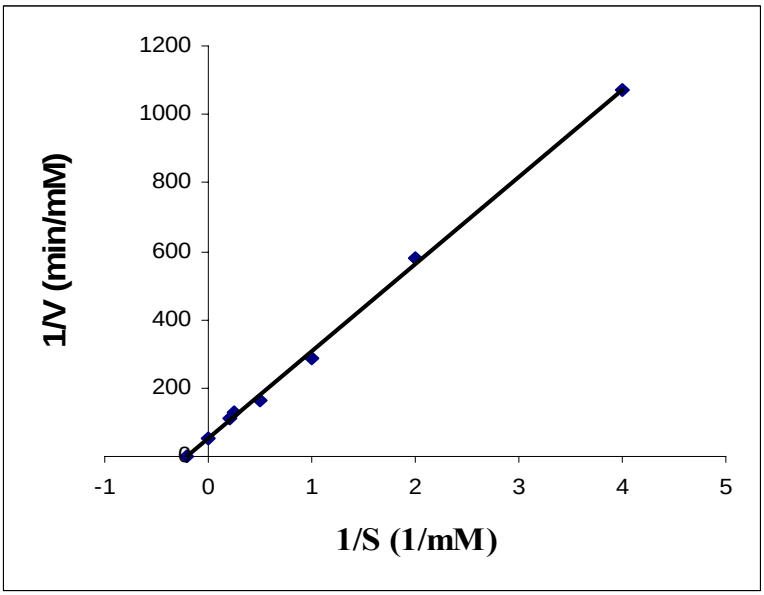


Fig. 3.22. Lineweaver Burk plot for Est A using *β* -naphthylacetate.

K_m and V_{max} were estimated from the plots (Table 3.7). The enzyme had the highest affinity towards *α*-naphthylacetate which is 10-fold its affinity towards *β*-naphthylacetate. This result correlates with the substrate specificity (3.2.1.2.1) where the activity towards *β*-naphthylacetate was only 27% of the activity towards *α*-naphthylacetate.

Table 3.7. Kinetic parameters on different substrates. Standard deviations (SD) were derived from three separate experiments.

Substrate	K_m±SD (mM)	V_{max}±SD (mM/min)
p-nitrophenylacetate	1.71±0.0217	0.0094±0.00019
α-naphthylacetate	0.5373±0.045	0.0168±0.0017
β-naphthylacetate	5.433±0.38	0.019±0.00035

3.2.1.2.7. Enantioselectivity profile of Est A

Evaluation of the enantioselectivity of Est A was carried out on a number of chiral substrates of commercial interest (enantioselectivity screening was done by our collaborators in CSIR regional research institute, Jammu, India) which included acyl esters of 1-(3,4-methylenedioxyphenyl)-ethanol, 1-(3,4-methylenedioxyphenyl)-propan-1-ol, 1–(p-chlorophenyl)-benzylalcohol, 1-(3,4-methylenedioxy-5-yl)-benzylalcohol, bisnapthol, ethyl 3-hydroxy-3-phenyl-propanoate, 1-(6-methoxy-2-naphthyl)-ethanol, 2-(6-methoxy-2-naphthyl)-propan-1-ol and alkyl esters of 2-(6-methoxy-2-naphthyl)-propanoic acid, 2-(p-(2-methyl

propyl)-2-phenyl)–propanoic acid, Ethyl- 2-hydroxy-4-phenyl butanoate, 2-bromo propanoic acid and ethyl (indol-2-yl) formate

Est A proved to be poor in stereo-selectivity as low enantiomeric excess (ee) of the hydrolysed products/starting materials was observed. Low hydrolytic activity was observed for substrates (viii, ix) and high activity for substrates (vii). 'R' alcohol was obtained as the hydrolysed product from acetyloxy and propanoyloxy esters of substrate viii and 'S' alcohol from its butyl ester. In case of alkyl esters of acids the enzyme preferred 'S' enantiomer to give enriched'R' ester and 'S' acid. (Appendix A)

3.2.1.2.8. Classification of Est A.

In 1999 Arpigny and Jaeger (Arpigny and Jaeger 1999) classified the bacterial esterases and lipases according to conserved motifs into 8 different families. Analyzing the sequence of Est A revealed that it contains all the conserved motifs of group IV (Fig 3.23) Group IV is also called the HSL family (hormone sensitive lipase), this family is a group of enzymes of bacterial origin which show a striking similarity with human HSL, all these enzymes are lipolytic enzymes from distantly related prokaryotes. Despite the marked sequence similarity they are very varied in properities; some are psychrophilic (e.g. *Moxarella* sp), some are mesophilic (e.g. *Alcaligenes eutrophus*) and other are thermophilic (e.g. *Alicyclobacillus acidocaldarius*).

```
                    ↓                   ↓                       ↓
hHSL     HGGGF..GDSAGGNLC..PVHIVACALDPMLDDSVMLARRLRNLGQ..HGFL
EstA     HGGGW..GDSVGGNMS..PAFVVVDENDVLRDEGEAYARKLIQAGV..HDFM
Ae       HGGGF..GDSAGGTLA..PAWIAVAGYDPLHDEGVAYAEKLRAAGV..HDFF
Aa       HGGGW..GDSAGGNLA..PAYIATAQYDPLRDVGKLYAEALNKAGV..HGFA
Ps       HGGGF..GDSAGGNLA..PTTLITAEFDPLRDEGEAFALRLQQAGV..HGFI
Af       HGGGF..GDSAGGNLA..PALIITAEYDPLRDEGEVFGQMLRRAGV..HGFI
Ms       HGGGF..GDSAGGCLA..PSYIVVAELDILRDEGLAYAELLQKEGV..HGFI
         *****   ****** **   *         * * * *       *   **   ****
```

Fig. 3.23. Alignment of the predicted Est A amino acid sequence with members of the hHSL family (family IV) of esterases and lipases (Arpigny and Jaeger 1999). The three critical amino acids in the active centre are shown (↓). The totally conserved amino acids are indicated (*). The four blocks characteristic of family IV correspond to amino acids 83-87, 155-163, 245-272 and 284-287 of EstA respectively. The other enzymes are from *Alcaligenes eutrophus*(Ae), *Alicyclobacillus acidocaldarius* (Aa), *Pseudomonas sp. B11-1* (Ps), *Archaeoglobus fulgidus* (Af) and *Moraxella sp.* (Ms).

The alignment of Est A with the other known (HSL) enzymes not only showed that Est A belonged to the HSL family but also some residues were conserved throughout the family except in Est A. In the first block a phenylalanine was conserved except in Est A and in an

esterase from *Alicyclobacillus acidocaldarius* where it was substituted with a larger residue (tryptophan). The alanine next to the serine active site was substituted with a larger valine in Est A. Another conserved leucine and alanine near the serine active site were substituted with methionine and serine respectively. This classification of esterases/ lipases was done in 1999, when the sequence data base was much smaller than now and the motifs are based on a very small number of sequences. I decided to look for further family members in the protein data base. Prof. Dr. Cullum kindly made a profile for the (HSL) family according to the data of Arpigny and Jaeger 1999, and then we ran HMMER against the protein data base (release 153 of genbank). The 10 best hits (Fig 3.24) were used to build a new family profile. We ran HMMER again using the new profile, it resulted in 119 significant hits (data not shown).

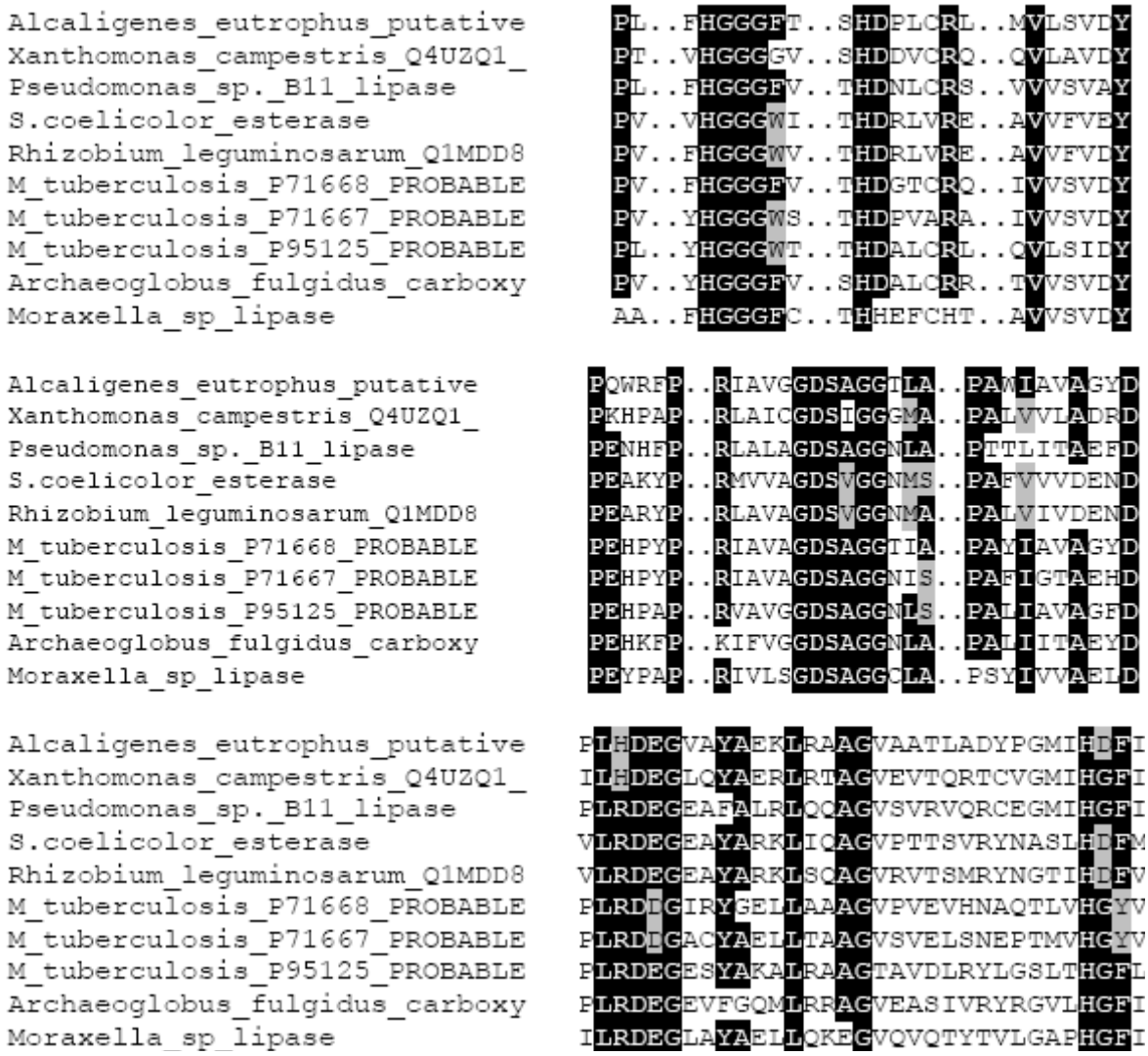


Fig. 3.24. Alignment of the best 10 hits the highly conserved residues are highlighted black and the less conserved residues are grey.

In addition to the four conserved motifs (Figure 3.23) mentioned by Arpigny and Jaeger (1999) I have found another two conserved motifs within the HSL family. There were also

some modifications of some residues in the four conserved motifs (Table 3.9). The first conserved block in the HSL family had a consensus sequence HGGG(F/W)V-G, where the H residue was 100% conserved and GGG---G were conserved more than 90% throughout the 119 sequences. The F/W were also highly conserved, both residues appeared almost equally. The conserved motif (YRLAPE--FP) was not described in 1999. The first proline residue was 100% conserved and the residues Y, E and P were highly conserved, whereas the R, L and A were moderately conserved. The serine active site conserved motif (V-GDSAGGNLA) was a little bit modified. The A residue was usually conserved. However, when the A was substituted, it was nearly always substituted with V. The motif Q-L-YP was not mentioned by Arpigny and Jaeger, the P was 100% conserved and the other three residues appeared >85%. In the HGF block the histadine active site was 100% conserved. The G residue was 26% substituted with D and the F residue was 10% substituted with Y.

Table 3.8. Shows the conserved motifs within (HSL) family and the degree of conservation for each residue. * indicate the residues belong to the catalytic triad.

Conserved motifs					
HGGG(W/F)V-G	YRLAPE---P	V-GDS(A/V)GGN(L/M)(A/S)	Q-L-YP	D-LRDEG--YA--L--AG	H(G/D)(F/Y)
H 100%	Y 98%	V 80%	Q 96%	D*1 100%	H* 100%
G1 98%	R 75%	G1 100%	L 94%	L1 93%	G 62%-D 26%
G2 91%	L 75%	D 85%	Y 85%	R 70%	F 80%-Y 10%
G3 96%	A 75%	S* 100%	P 100%	D2 94%	
W43%-F 45%	P1 100%	A 72%-V 25%		E 75%	
V 73%	E 92%	G2 100%		G 90%	
G4 91%	P2 94%	G3 94%		Y 90%	
		N 73%		A1 79%	
		L 66%-M 21%		L 95%	
		A 75%-S 19%		A 81%	
				G 98%	

3.2.1.2.9. Homology modeling of Est A.

The protein data base is expanding and the number of the proteins with the determined crystal structures is continually increasing. Now it is possible to develop a 3D model of a protein if it shows good structure alignment to a known 3D structure. I have used the service provided by expasy server, http://swissmodel.expasy.org/ "an automated comparative protein modeling server" (Peitsch 1995; Guex and Peitsch 1997; Schwede *et al*., 2003). Searching the Known 3D structures for appropriate model templates produced good hits with PDB files 2c7b (a thermophilic and thermostable carboxylesterase from metagenome library), 1jji (AFEST thermophilic carboxylesterase from the archeon *Archaeoglobus fulgidus*), 1evq (the thermophilic carboxylestrase EST2 from *Alicyclobacillus acidocaldarius*) and 1 jkm (the carboxylesterase from *Bacillus subtilis*), all of them are members of HSL family. A swiss model was produced using the previous PDBs as templates. The produced model represents the residues 35-316 of Est A sequence Fig 3.25 and 3.26 represents the alignment and the 3D model of Est A from *S. coelicolor* superimposed with the other HSL esterases. The 3D model of Est A showed the typical α/β hydrolase fold (Fig 3.27).

```
EST2          ---MPLDPVIQQVLDQLNRMPAPDYKHLSAQQFRSQQSLFPPVKKEPVAEVREFDXDLPG 57
AFEST         MLDMPIDPVYYQLAEYFDSLPKFD-QFSSAREYREAINRIYEERNRQLSQHERVER-VED 58
SCESTA        MSDIVLEPAAQDFADATAKPPLLY-----ELGVEGARKLLDDVQSGPVEKPDVDEKWITV 55
                 : ::*.  :. :     *            .   . :   :.  : :    :  :

EST2          RTLKVRX----YRPEGVEPPYPALVYYHGGGWVVGDLETHDPVCRVLAKDGRAVVFSVDY 113
AFEST         RTIKGRNGDIRVRVYQQKPDSPVLVYYHGGGFVICSIESHDALCRRIARLSNSTVVSVDY 118
SCESTA        PVEVGDVRVRIVKPAGTTGVLPVVLYVHGGGWILGNAGTHDRLVRELAVGAEAAVVFVEY 115
               .          :        *.::* ****::: .  :** : * :*  ..:.*. *:*

EST2          RLAPEHKFPAAVEDAYDALQWIAERAADFHLDPARIAVGGDSAGGNLAAVTSILAKERGG 173
AFEST         RLAPEHKFPAAVYDCYDATKWVAENAEELRIDPSKIFVGGDSAGGNLAAAVSIMARDSGE 178
SCESTA        DRSPEAKYPVAIEQAYATAQWVTTKGAEEGLDGSRMVVAGDSVGGNMSAALTHMAKRRGD 175
                :** *:*.*: :.* : :*:: .. :  :* ::: *.***.***::*. : :*:  *

EST2          PALAFQLLIYPSTGYDPAHPPASIEENAEG-YLLTGGXXLWFRDQYLNS--LEELTHPWF 230
AFEST         DFIKHQILIYPVVNFVAPTP--SLLEFGEGLWILDQKIMSWFSEQYFSR--EEDKFNPLA 234
SCESTA        VTFLHQSLYYPVTDAGQDTESYRLFAHGPH---LTAKAMEWFWNAYAPDPAERDQITASP 232
                : .* * ** ..         :   .     *      ** : *      .:   .

EST2          SPVLYPDLSGLPPAYIATAQYDPLRDVGKLYAEALNKAGVKVEIENFEDLIHGFAQFYSL 290
AFEST         S-VIFADLENLPPALIITAEYDPLRDEGEVFGQMLRRAGVEASIVRYRGVLHGFINYYPV 293
SCESTA        LRATPEDLQGLPPAFVVVDENDVLRDEGEAYARKLIQAGVPTTSVRYNASLHDFMMLNPV 292
                .   **..**** : . : * *** *: :.. * :*** .   .:.  :*.*    .:

EST2          S--PGATKALVRIAEKLRDALA-- 310
AFEST         L--KAARDAINQIAALLVFD---- 311
SCESTA        RGTQASTAAIEQAIHVLRSALGTD 316
```

Fig. 3.25. Structural alignment of S. coelicolor EstA, Alicyclobacillus acidocaldarius EST2 and Archaeogolobus fulgidus.

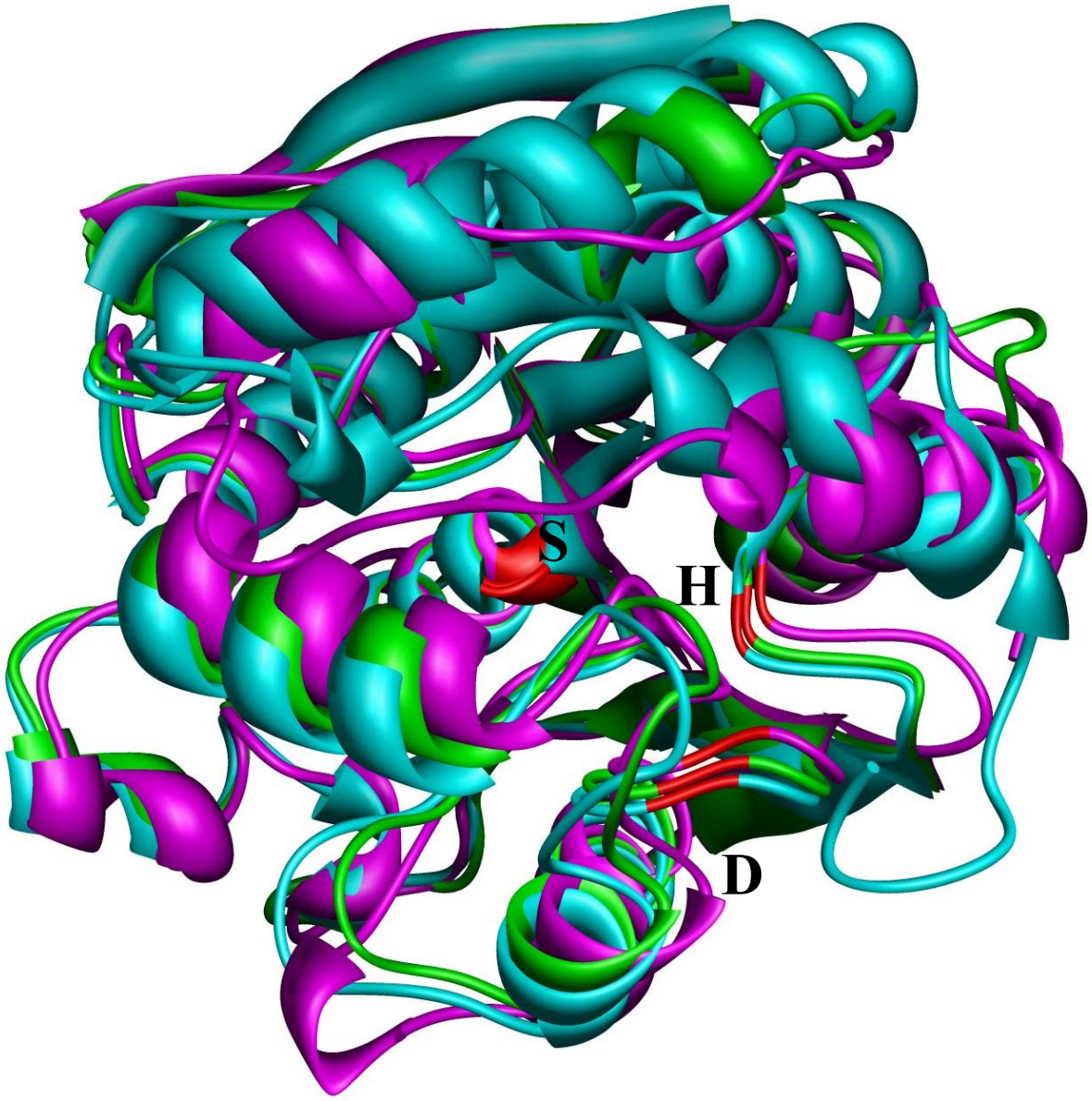


Fig. 3.26. Superimposition of the backbone traces (shown as ribbon) of the 3D model of Est A from *S. coelicolor* (green) and the known 3D structures of HSL family members EST2 of *Alicyclobacillus acidocaldarius* (magenta) and AFEST of *Archaeogolobus fulgidus* (cyan). The catalytic triad is colored red.

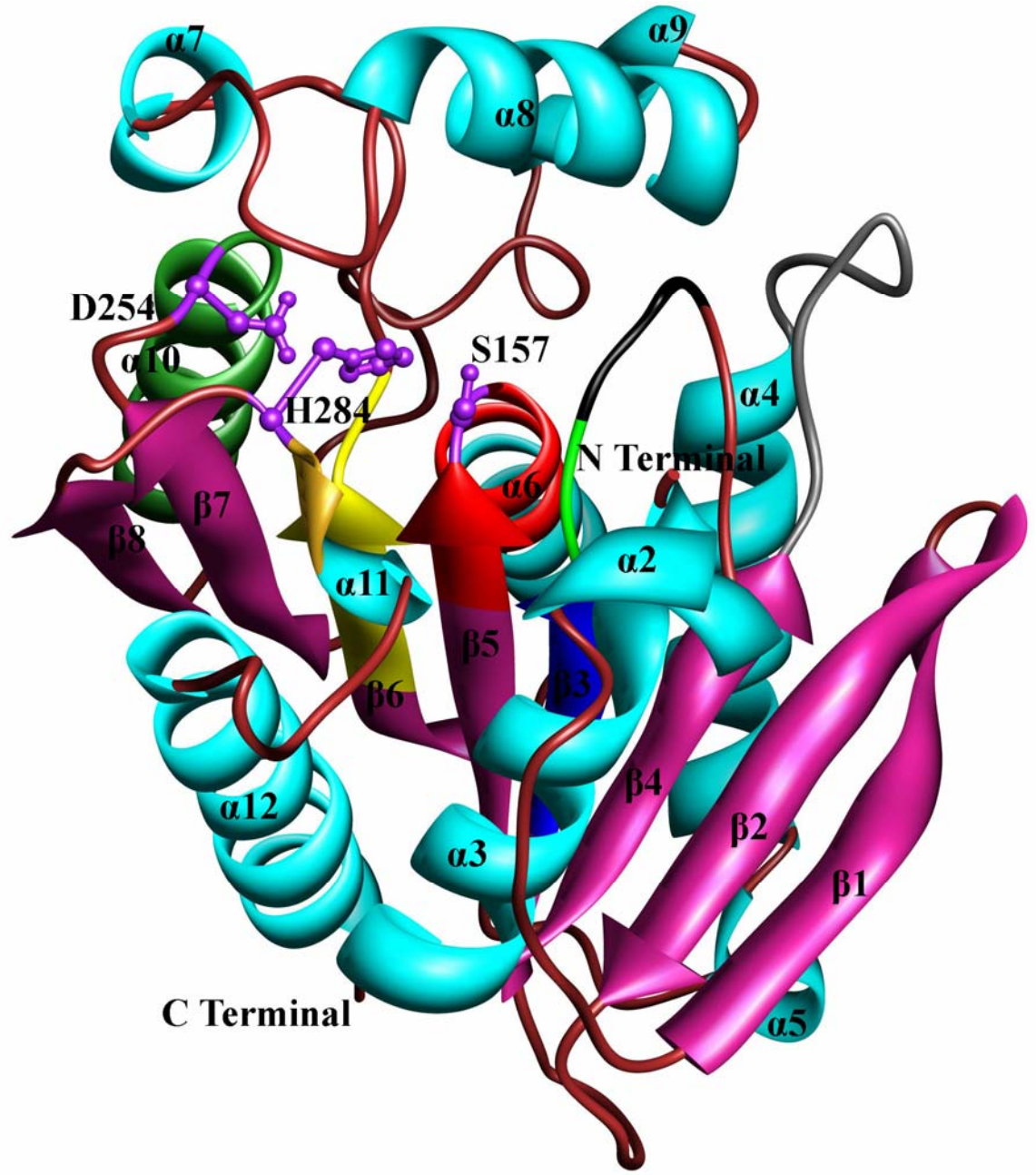


Fig. 3.27. The 3D model of Est A. The conserved motifs of HSL are indicated as follow; HGGGWI is black, YDRSPEAKYP is grey, GDSVGGNMS is red, QSLYYP is yellow, DVLRDEGEAYARRK is green and the HDF is gold. The putative oxyanion hole (VLYVHG) is represented as blue, the 2 residues HG are incorporate in both the oyanion hole and in the conserved motif (HGGGWI) is colored spring green. The catalytic triad is indicated as purple with its side chains S157, D254 and H 284. Strands are colored violet red and labeled β1– β8 pertain to the prototypic α/β hydrolase fold. α helices are colored cyan and numbered 2-12. I assumed that the missing first 34 residue will constitute α1 and the rest of α2.

The residues S157, D254 and H284 are the catalytic triad. The distances between the side chains are suitable for H bond formation (Fig 3.28)

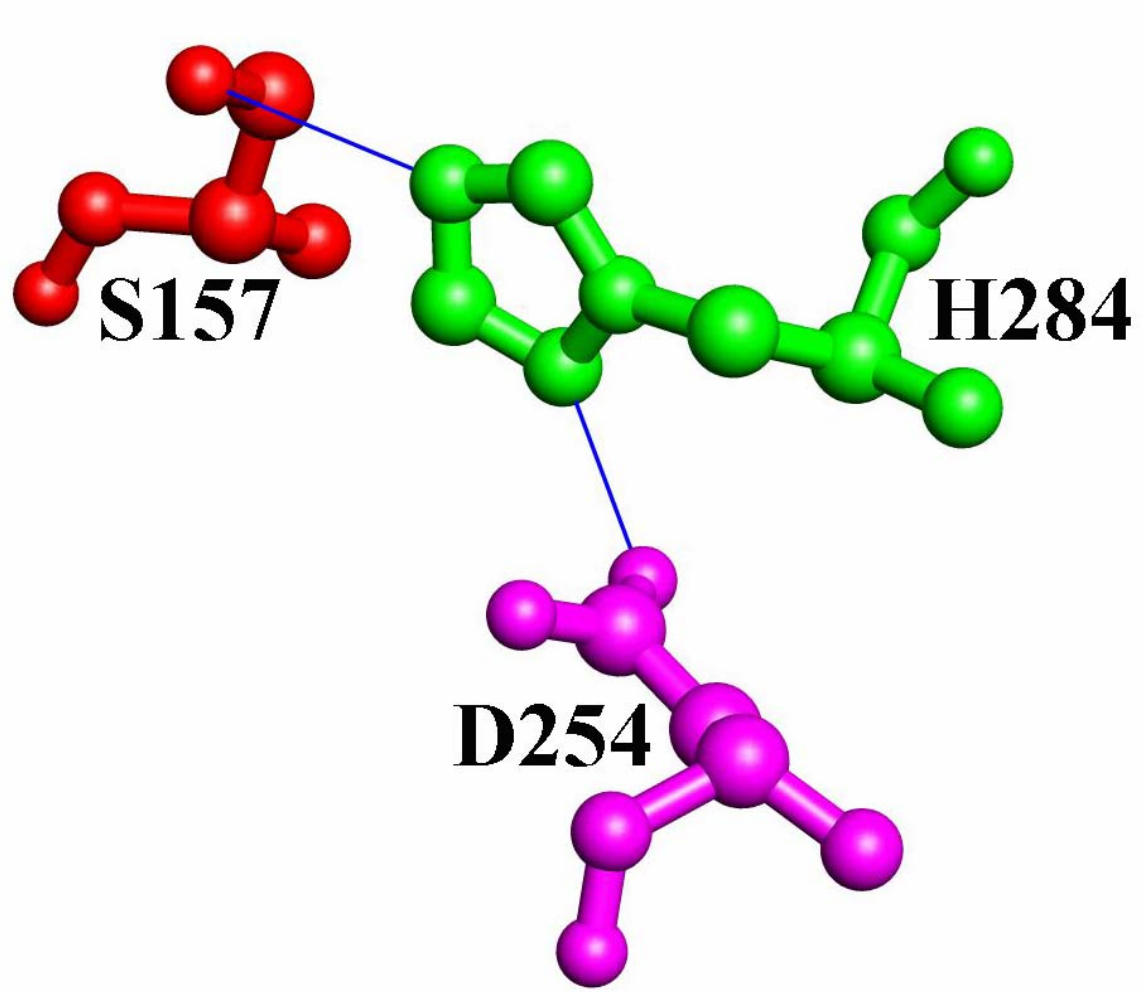


Fig. 3.28. The spatial arrangement of the catalytic triad. S157 (red), D254 (magenta) and H284 (green). The possible H bonds are indicated as blue line.

3.2.2. Est A directed evolution through site directed mutagenesis

The characterized HSL carboxylesterases act against short and middle chain esters C_2-C_{12} with best activity against C_6 as the HSL carboxylesterases from *Alicyclobacillus acidocaldarius*, *Archaeoglobus fulgidus* and the thermophilic HSL esterase from a metagenomic library (Rhee *et al*., 2005), whereas Est A acts only against the C_2 esters. When Est A was compared with the characterized HSL family, some differences were noticed. The W87 was substituted with F except that of *Alicyclobacillus acidocaldarius* and V158 was substituted with A. The M162 and S163 were L and A respectively. To verify whether these differences play a role in the substrate specificity observed in Est A or not, we changed these residues to the consensus sequences. These should enable us to understand something of the structure activity relationship and also may lead to improvement of the characters observed for Est A. Changing the residues to the consensus sequence was accomplished through site directed mutagenesis.

3.2.2.1. Site directed mutagenesis PCR

Site specific mutagenesis is an important method for directed evolution (2.11.1), analysis of gene functions and many other applications. Several approaches to this technique have been published, but these methods generally require ssDNA as a template and are labor intensive or technically difficult. So I decided to use another strategy depending on PCR amplification of the whole construct using long mismatch primers and high fidelity polymerase to avoid introducing undesired mutations. The template was removed through digestion with *Dpn*I (*Dpn*I cuts only methylated and hemimethylated DNA, so it will cut any DNA containing an original tempelate strand). The mutagenised plasmid DNA was purified and transformed into *E. coli*. This was an efficient, inexpensive and rapid method for site directed mutagenesis with no need for further cloning of the PCR product (Fig 3.29).

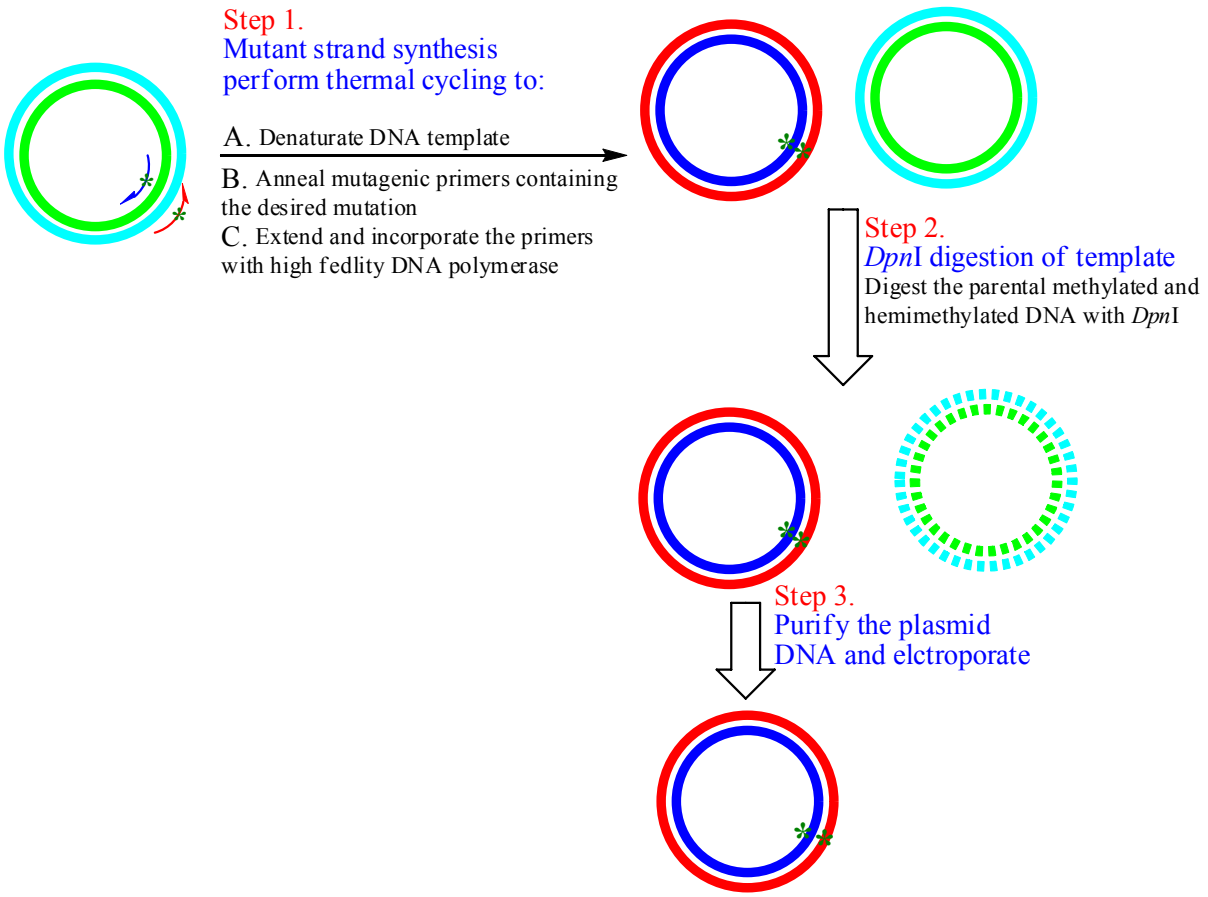


Fig. 3.29. Schematic diagram shows the strategy used for the site directed mutagenesis

Suitable long mismatch primers of around 30bp were constructed (see table 2.2). The plasmid pUKG952 was used as a template and the amplification reaction of the whole plasmid was carried out using a high fidelity Taq. The PCR product was treated with *Dpn*I then the mutagenised plasmids were transformed in *E.coli*. In case of the W87F mutant the clones were screened through *Bam*HI digest (mutagenesis removes one of two *Bam*HI sites), the other mutants through sequencing and the positive clones were sequenced on both strands to ensure that there was no other mutation except the required one. Five different mutants were produced in this way; W87F, V158A, M162L, S163A and W87F/V158A (Fig 3.30-3.31). The double mutant was produced from the W87F single mutant by a second round of mutagenesis.

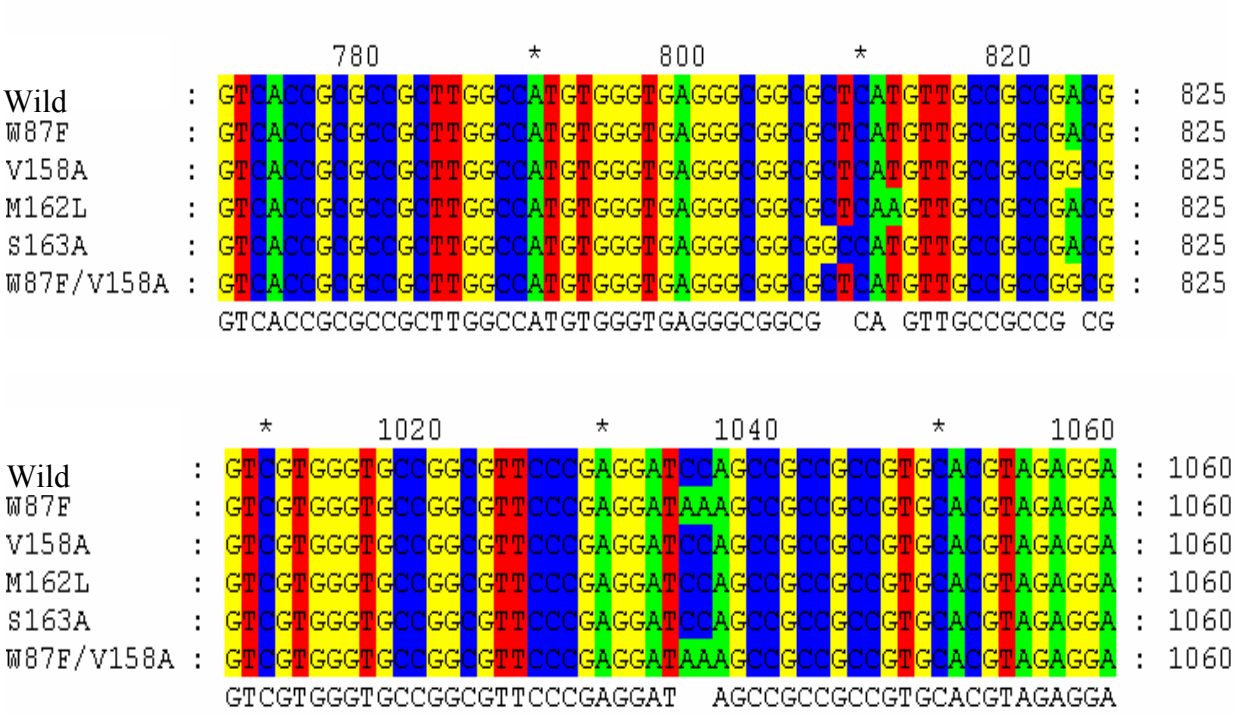


Fig. 3.30. The sequencing results of the site directed mutagenesis; the mutant W87F AA/CC, the mutant V158A G/A, the mutant M162L A/T, the mutant S163A GC/TC and the mutant W87F/V158A G/A and AA/CC.

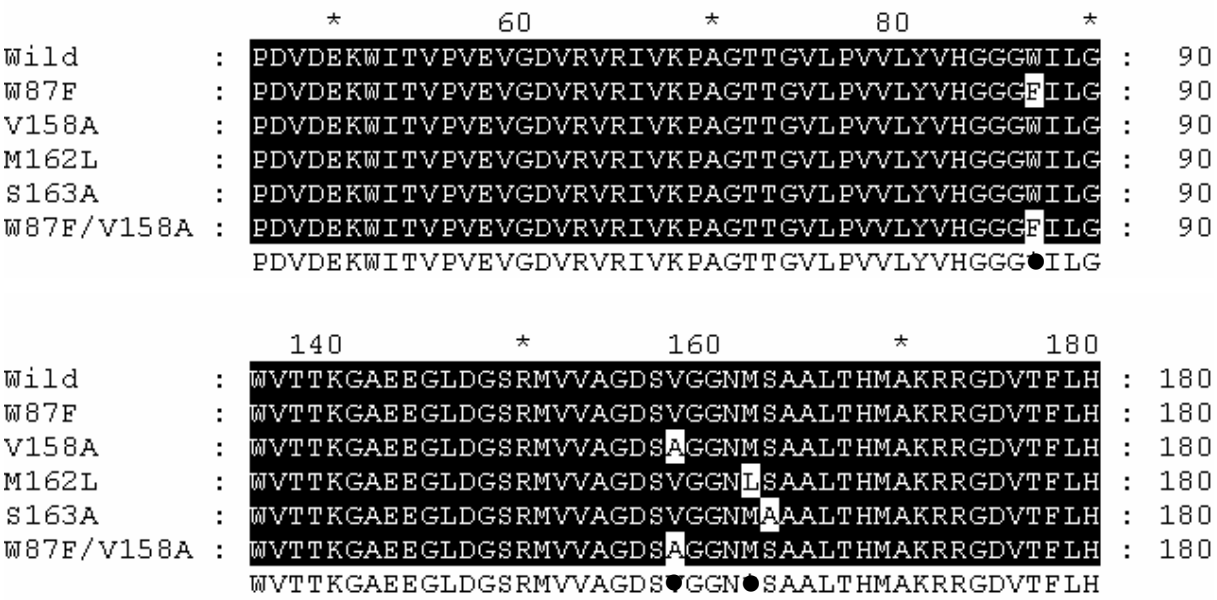


Fig. 3.31. Shows the alterations in amino acids sequence resulted from the mutagenesis (•); W87F, V158A, M162L, S163A and W87F/V158A.

3.2.2.2. Characterization of Est A mutants

3.2.2.2.1. Substrate specificity

The Est A mutants' activity against *p*-nitrophenyl esters was tested, all the mutants still showing the maximum activity against *p*-nitrophenyl acetate. However the mutants behaved differently against the other *p*-nitrophenyl esters. The mutants W87F, M162L and S163A did not show significant changes from the wild type, whereas the mutants V158A and W87F/V158A showed more activity than the wild type against both *p*-nitrophenyl butyrate and *p*-nitrophenyl caproate; the activity against the butyrate ester increased to 3% and 6% and to 9% and 9.5% against the *p*-nitrophenyl caproate respectively (Fig 3.32).

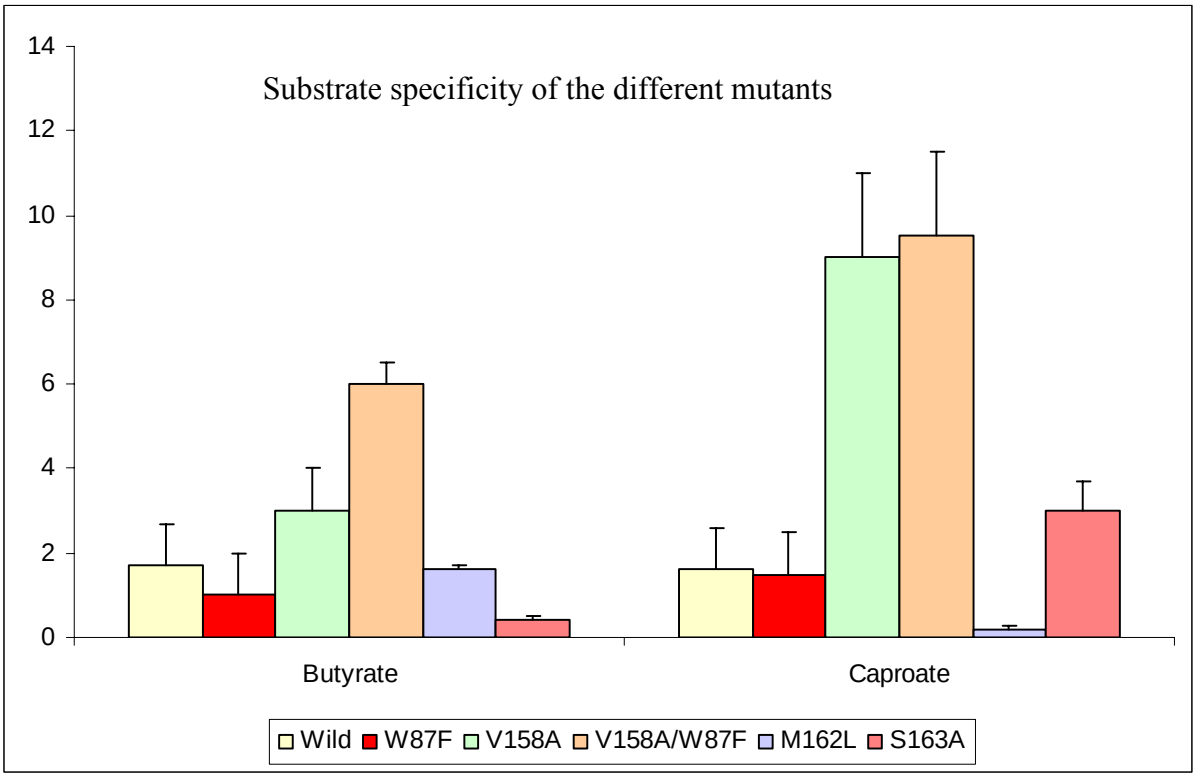


Fig. 3.32. Activity of wild type, single mutants and double mutant enzymes on esters of C_4 (butyrate) and C_6 (caproate). *p*-nitrophenyl esters were used with acetate ester as 100% activity. SD were derived from 2 separate experiments each with 4 replicates.

The Est A mutants were tested for activity against triglycerides and olive oil, which are not substrates for the wild type enzyme. V158A show activity towards tributryin but not the longer substrates. W87F/V158A showed a very faint activity also against the tributryin, whereas the other mutants were similar to the wild type (Fig 3.33).

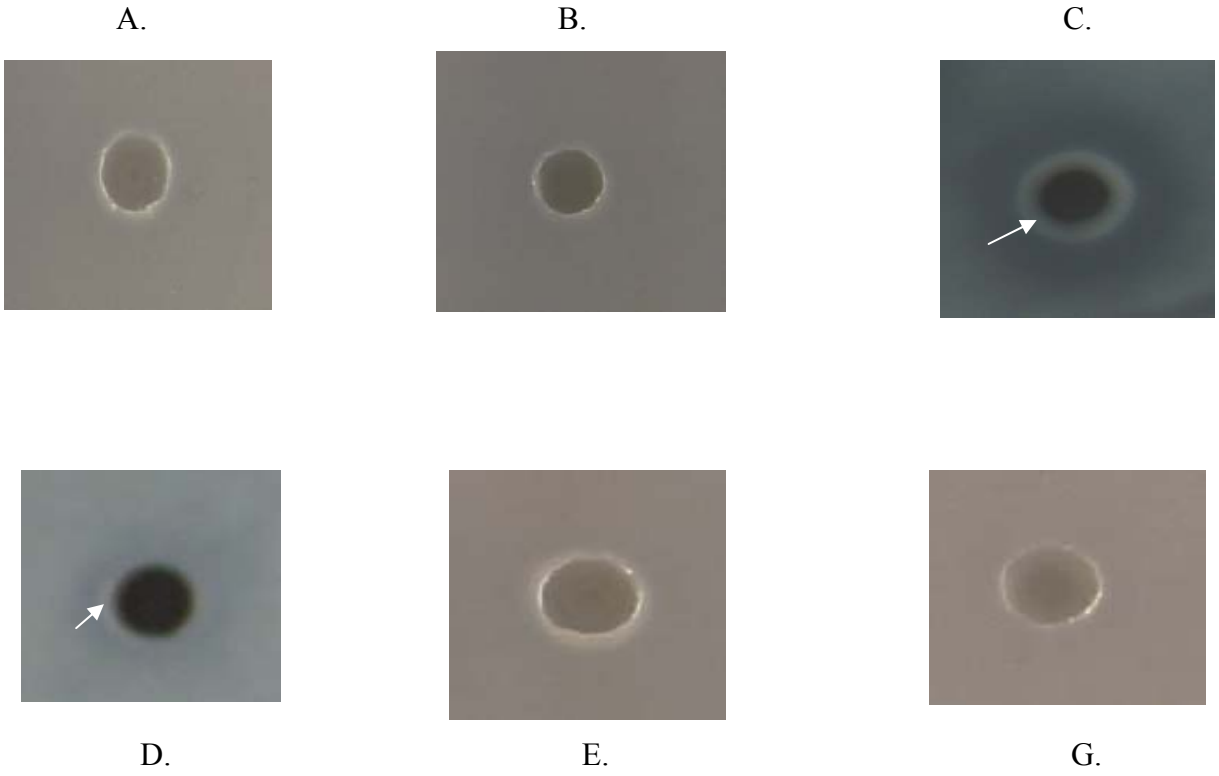


Fig. 3.33. Activity against tributyrate. Using plate and well method (A) Wild type, (B) W87F, (C) V158A, (D) W87F/V158A, (E) M162L and (F) S163A. No activity was observed except in case of V158A indicated by clearing zone around the well and very weak activity in case of W87F/V158A indicated with a very small clearing ring around the well. The clearing zones were indicated by arrows.

3.2.2.2.2. Effect of temperature on activity and stability

The mutant W87F did not show a temperature activity profile different from that produced by the wild type. The mutant V158A exhibited better activity at high temperature than the wild type and than the other mutants; it showed around 75% activity at 80ºC and 60% of its activity at 85ºC and retained more than 25% of its activity at 90ºC, and the optimum temperature was 60ºC. The double mutant W87F/V158A also showed an improvement in activity at high temperature but to a lesser extent than V158A; at 70ºC it showed 76% activity and around 40% activity at 85ºC (Fig 3.34). All the mutants except V158A showed lower temperature stability than the wild type (Fig 3.35), however V158A possessed improved temperature stability. V158A after 1h preincubation at temperatures up to 50ºC it retained more than 98% of the activity. It showed around 50% of the activity after 1h preincubation at 65ºC, whereas it had only 12% activity after 1h preincubation at 70ºC. The half life of V158A at 50ºC was measured to be 24h, which is much more stable than the wild type (half life 4.5h).

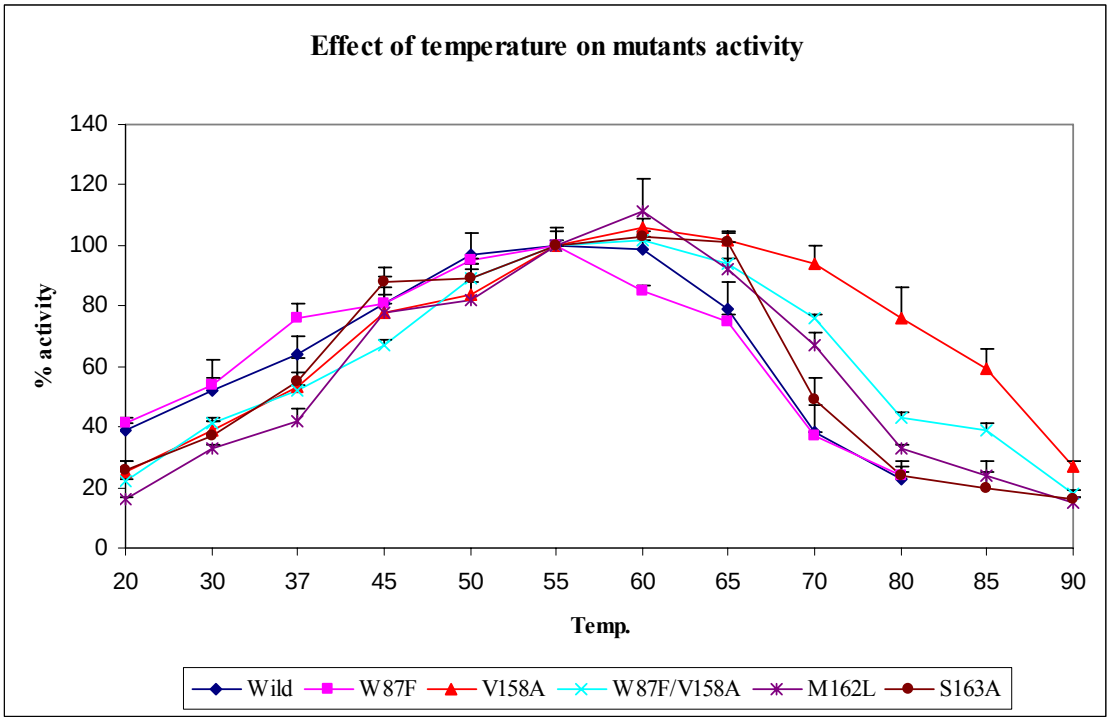


Fig. 3.34. Effect of temperature on activity for the wild type and the mutants. Activity is represented as % of the maximum activity. SD derived from 2 separate experiments each with 4 replicates.

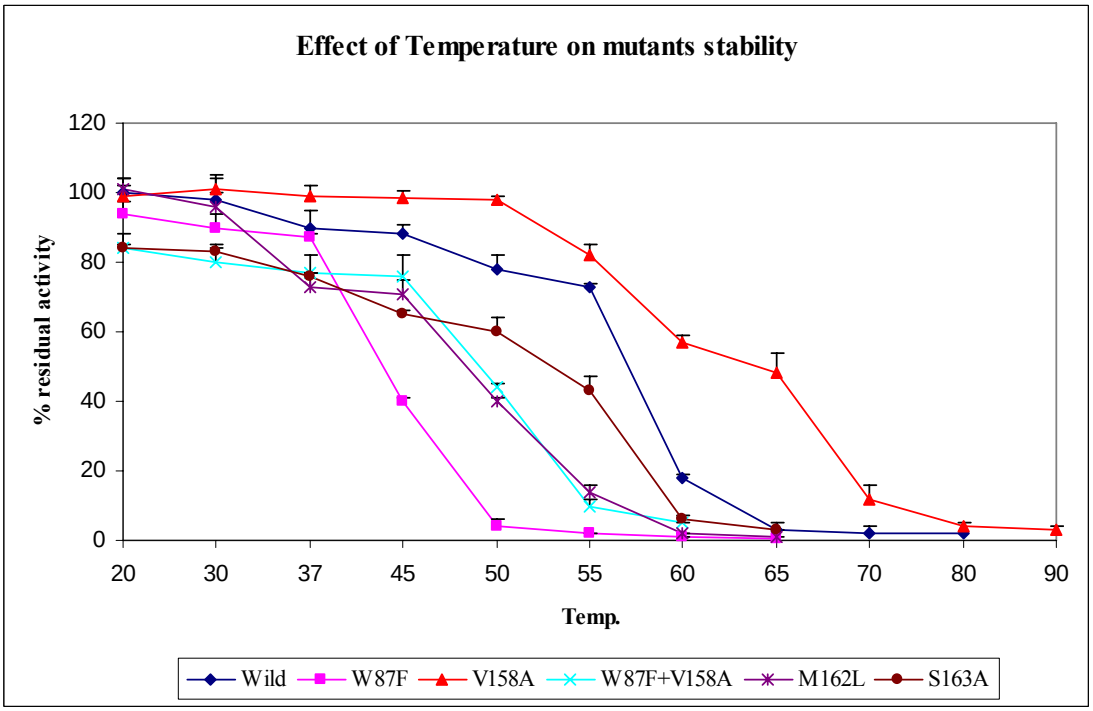


Fig. 3.35. Effect of temperature on stability for the wild type and the mutants. Stability was measured by incubation for 1 h at the stated temperature with determination of the residual activity at 37°C. SD derived from 2 separate experiments each with 4 replicates.

3.2.2.2.3. Effect of pH on activity

All the mutants showed higher activity in alkaline pH, their optimum pH was shifted to pH 8 except in M162L the optimum pH was shifted to pH 8.5. All the mutants showed in low pH 5-6.5 higher activity than the wild type; their activity at pH 6.5 was double the activity of the wild type, at pH 6 the activity ranged between 12-19% instead of 2% showed by the wild type (Fig 3.36).

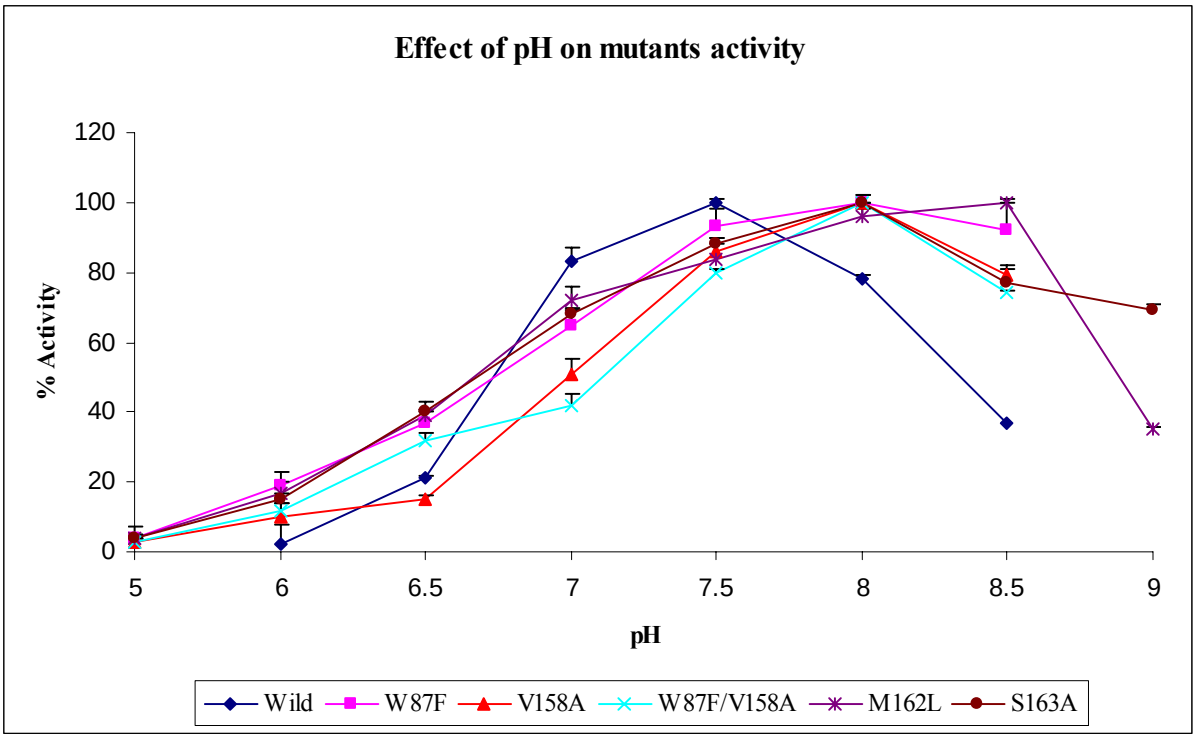


Fig. 3.36. Effect of pH on activity of Est A. Activities were shown as percentages of the maximum activity. SD were derived from 2 separate experiments each with 4 replicates.

3.2.2.2.4. Kinetic parameters

The kinetic parameters were derived from Lineweaver-Burk plots using *p*-nitrophenyl acetate as substrate. Table 3.10 presents the K_m for the wild type and the mutants. The affinity of M162L, S163A and W87F/V158A did not altered significantly from the wild type. However V158A possessed an affinity 6-fold the affinity of the wild type, and the W87F affinity decreased 4-fold.

Table 3.9. Effect of mutagenesis on Km. SD were derived from 2 separate experiments.

	K_m
Wild type	1.71±0.02mM
W87F	7.42±0,30mM
V158A	0.28±0.05mM
M162L	1.76±0.13 mM
S163A	1.60±0.13mM
W87F/V158A	1.40±0.29mM

3.2.2.2.5. Effect of inhibitors

The effect of the potent inhibitors of the wild type was investigated in the mutants. PMSF did not show different effect on S163A, however W87F, V158A, W87F/V158A and M162L showed higher resistance to PMSF they retained more than 94% of their activities. M162L resistance to $HgCl_2$ did not change, whereas V158A, W87F/V158A and S163A showed higher resistance, the same time W87F activity reduced significantly with 10mM $HgCl_2$. The sensitivity of all the mutants to $CuSO_4$ increased markedly, they showed activity less than 10% (Fig 3.37).

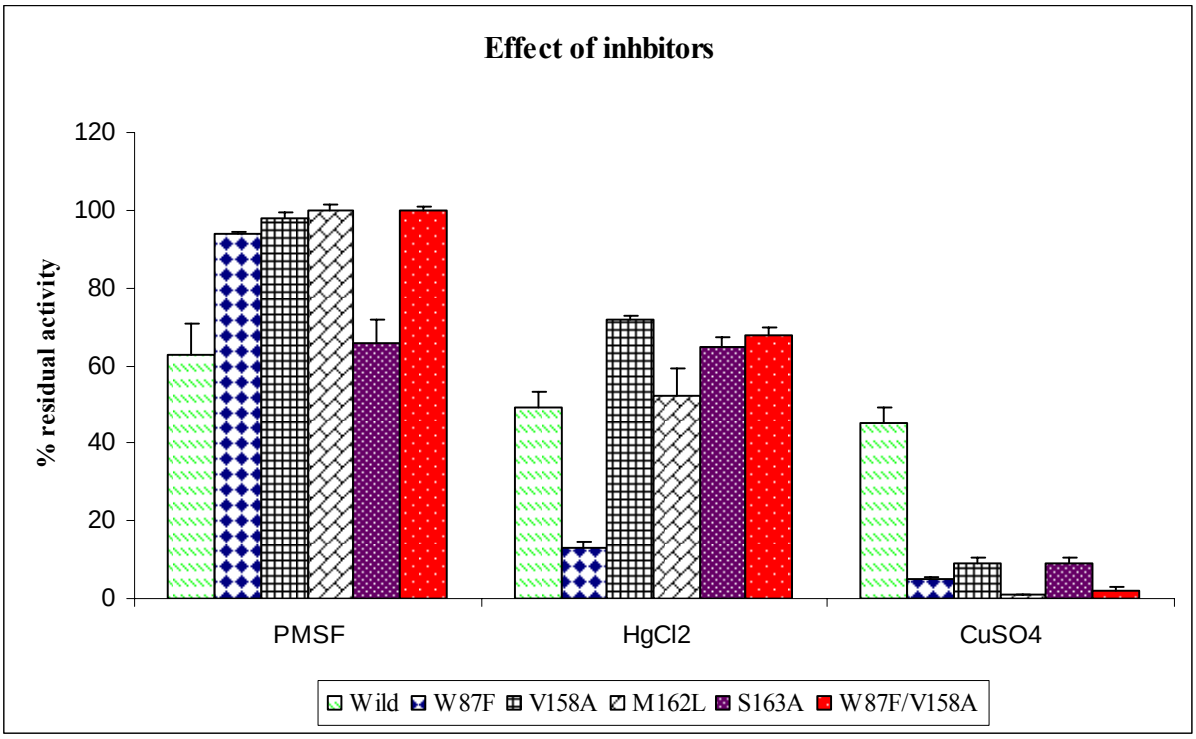


Fig. 3.37. Effect of PMSF, $HgCl_2$ and $CuSO_4$ on the mutants' stability. The residual activity was measured after incubating the enzyme for 1h with 10mM of the inhibitors at 20ºC. SD were derived from two experiments each with 4 replicates.

3.2.2.2.6. Directed evolution through random mutagenesis of Est A

The method of choice to produce random mutagenesis is to perform EP-PCR, which is a PCR reaction with high error rates. This can be achieved through using non proof reading Taq and changing the normal PCR conditions in various ways e.g. using unequal dNTP concentrations, adding Mn^{2+} ions 0.05-0.5mM and/or high Mg^{2+} ions concentration up to 10mM

The random mutagenesis was carried out using EP-PCR. I have cloned several genes within pET-16b, so standard primers were constructed that I can use it for the amplification of any of my genes cloned in pET-16b. EP-PCR was done as described in section 2.11.2. The PCR fragment (Fig 3.38) was digested with *Bgl*II/*Xho*I and the *Bgl*II/*Xho*I cassette was cloned again in pET-16b. A library of 6336 clones was constructed.

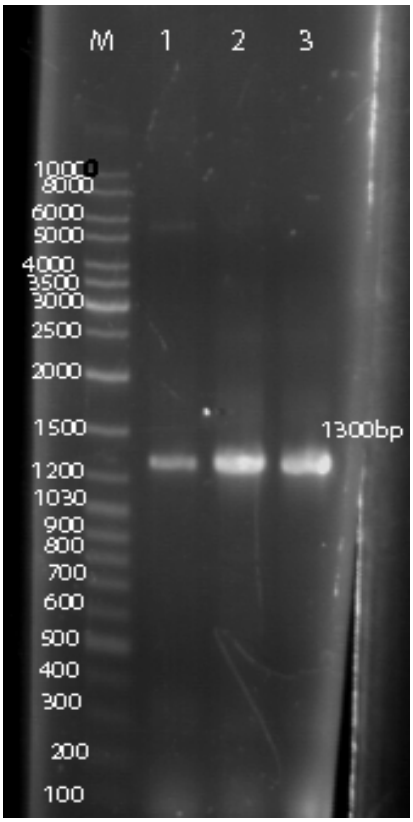


Fig. 3.38. EP-PCR. M; marker ladder mix, lane 1; DNA template 10ng, lane 2; DNA template 30ng and lane 3; DNA template 60ng.

1056 mutants were screened for activity against *p*-nitrophenyl esters, only 4% showed activity. All the active mutants had an activity pattern similar to the wild type except only one mutant (XXVF7) showed alteration of substrate specificity (Table 3.11). The mutant XXVF7 exerted activity against the tributyrin, when the activity against triglycerides was tested using plate and well method (Fig 3.39). The thermostability curve of XXVF7 was almost identical to that of the wild type (Fig 3.40). The mutant XXVF7 was sequenced to determine the mutation(s) occurred. The sequencing revealed that 5 point mutations were induced which leaded to 5 amino acid changes; L76R, L146P, S196G, W213R and L267R (Fig 3.41).

Table 3.10. Substrate specificity of the random mutant XXVF7. SD were derived from two different experiments each with four replicates.

Substrate	%Activity ±SD
PNP-Acetate C2	100±4
PNP-Butyrate C4	46±4
PNP-Caproate C6	28±6
PNP-Caprylate C8	14±2
PNP-Caprate C10	10±2

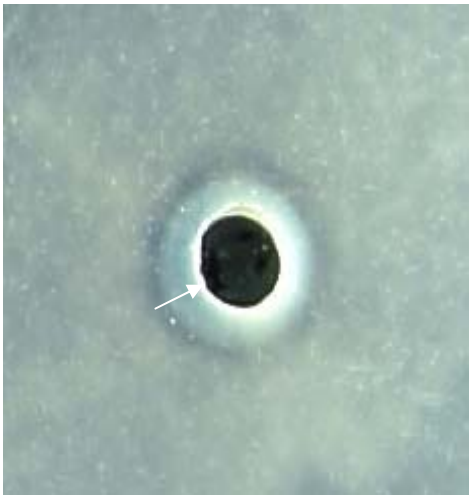


Fig. 3.39. Activity of XXVF7 against tributyrin. A zone of clearing was produced (indicated with the white arrow).

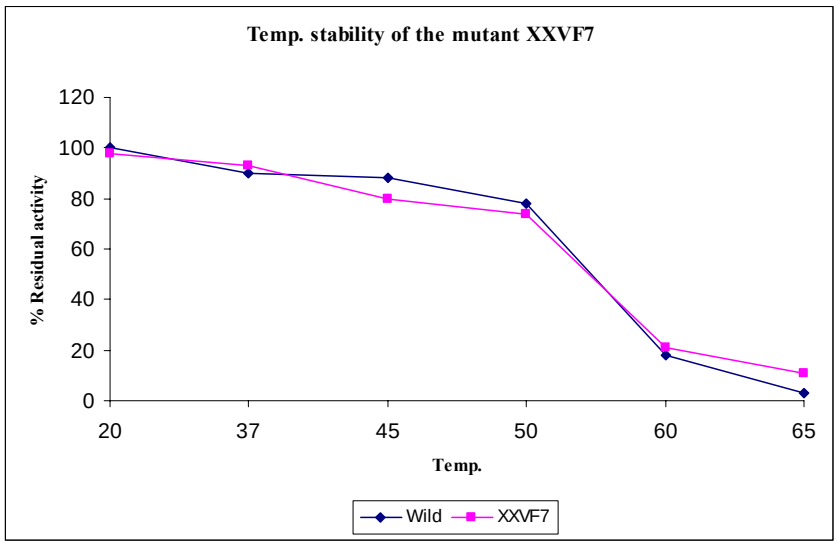


Fig. 3.40. The thermostability of the mutant XXVF7 compared to the wild type.

```
                *         20         *         40         *
Wild   : MSDIVLEPAAQDFADATAKPPLLYELGVEGARKLLDDVQSGPVEKPDVDE :   50
XXVF7  : MSDIVLEPAAQDFADATAKPPLLYELGVEGARKLLDDVQSGPVEKPDVDE :   50
         MSDIVLEPAAQDFADATAKPPLLYELGVEGARKLLDDVQSGPVEKPDVDE

               60         *         80         *        100
Wild   : KWITVPVEVGDVRVRIVKPAGTTGVLPVVLYVHGGGWILGNAGTHDRLVR : 100
XXVF7  : KWITVPVEVGDVRVRIVKPAGTTGVRPVVLYVHGGGWILGNAGTHDRLVR : 100
         KWITVPVEVGDVRVRIVKPAGTTGV●PVVLYVHGGGWILGNAGTHDRLVR

                *        120         *        140         *
Wild   : ELAVGAEAAVVFVEYDRSPEAKYPVAIEQAYATAQWVTTKGAEEGLDGSR : 150
XXVF7  : ELAVGAEAAVVFVEYDRSPEAKYPVAIEQAYATAQWVTTKGAEEGPDGSR : 150
         ELAVGAEAAVVFVEYDRSPEAKYPVAIEQAYATAQWVTTKGAEEG●DGSR

               160         *        180         *        200
Wild   : MVVAGDSVGGNMSAALTHMAKRRGDVTFLHQSLYYPVTDAGQDTESYRLF : 200
XXVF7  : MVVAGDSVGGNMSAALTHMAKRRGDVTFLHQSLYYPVTDAGQDTEGYRLF : 200
         MVVAGDSVGGNMSAALTHMAKRRGDVTFLHQSLYYPVTDAGQDTE●YRLF

                *        220         *        240         *
Wild   : AHGPHLTAKAMEWFWNAYAPDPAERDQITASPLRATPEDLQGLPPAFVVV : 250
XXVF7  : AHGPHLTAKAMERFWNAYAPDPAERDQITASPLRATPEDLQGLPPAFVVV : 250
         AHGPHLTAKAME●FWNAYAPDPAERDQITASPLRATPEDLQGLPPAFVVV

               260         *        280         *        300
Wild   : DENDVLRDEGEAYARKLIQAGVPTTSVRYNASLHDFMMLNPVRGTQASTA : 300
XXVF7  : DENDVLRDEGEAYARKRIQAGVPTTSVRYNASLHDFMMLNPVRGTQASTA : 300
         DENDVLRDEGEAYARK●IQAGVPTTSVRYNASLHDFMMLNPVRGTQASTA

                *        320         *
Wild   : AIEQAIHVLRSALGTD---------------- : 316
XXVF7  : AIEQAIHVLRSALGTD---------------- : 316
         AIEQAIHVLRSALGTD
```

Fig. 3.41. Five point mutations (•) have been detected within the XXVF7, L76R, L146P, S196G, W213R and L267R.

3.2.3. Overexpression and biochemical characterization of a cold active esterase from *Streptomyces coelicolor* A3(2) "Gene locus SCO 6966"

3.2.3.1. Est B expression and purification

The SCO 6966 gene was called *est B* and the protein called esterase B (Est B), The plasmid pSHS811 was transferred into BL21 (DE3) and the expression conditions were adjusted (the culture was induced 0.5mM IPTG for 4h at 30ºC using) and the protein was purified by affinity chromatography on Ni-agarose as in case of Est A. The enzyme preparation was analyzed by SDS-PAGE (Fig 3.42) and native PAGE with activity staining (Fig 3.42). The theoretical Mr of the recombinant protein including the His-tag was 31.443kD whereas the analysis of the esterase SDS-PAGE has shown this Mr was 31.9 kDa. Removing the His-tag using factor Xa digestion did not work (Fig 3.42).

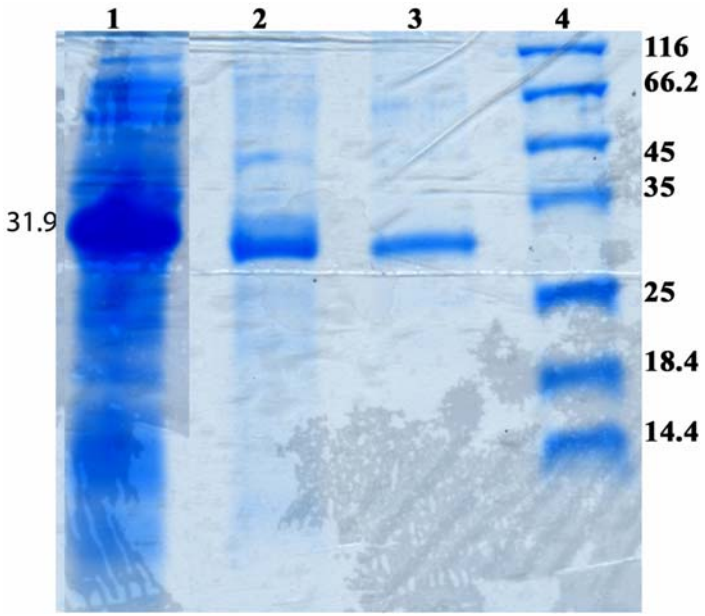


Fig. 3.42. SDS PAGE of Est B. Lane 1, Est B total cell protein; lane 2, Est B purified on Ni column; Lane 3, Est B purified on Ni column and treated with factor Xa; lane 4, protein molecular weight Marker.

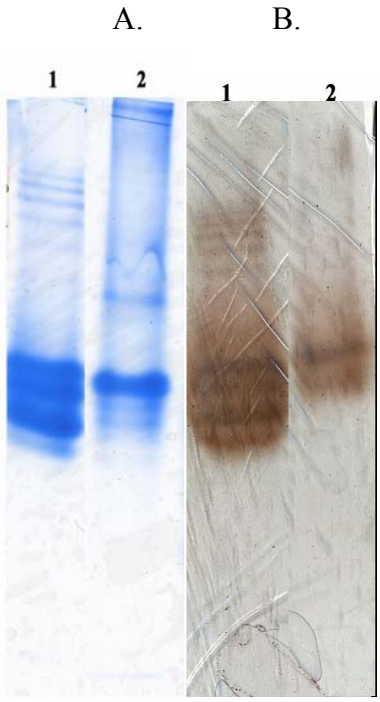


Fig. 3.43. Native PAGE of Est B. Lane 1, cell extract; lane 2, affinity column purified protein. (A) Gel stained with Coomassie brilliant blue. (B) Gel after activity staining.

3.2.3.2. Est B characterization

3.2.3.2.1. Substrate specificity

Substrate specificity of esterase B was investigated using *p*-nitrophenyl esters of different alkyl chain length. The enzyme was active against short and medium chain esters and showed a high activity towards short chain fatty acids (C_2-C_6), it exerted the maximum activity against *p*-nitrophenyl acetate, and showed also high activity towards *p*-nitrophenyl butyrate and caproate 90% and 75% respectively (Fig 3.44). Est B did not show any activity towards triglycerides or olive oil.

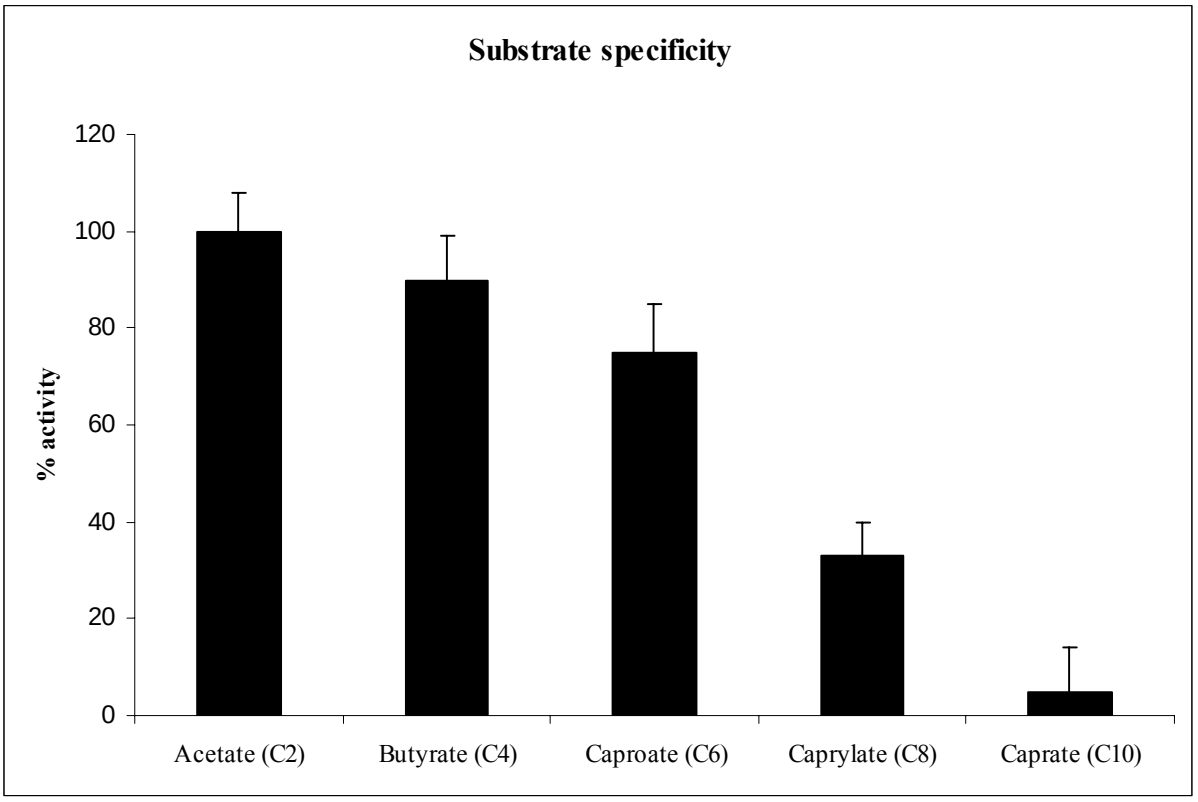


Fig. 3.44. Substrate specificity. SD was derived from two different experiments each with four replicates.

3.2.3.2.2. Effect of Temperature on the activity and stability of Est B

The enzymatic activity was measured at different temperatures (Fig 3.45) using a 5 minute assay and phosphate buffer pH 8. The activity increased with temperature reaching its maximum at 30ºC. The enzyme retained more than 25% of the activity at 4ºC and more than 70% at 15ºC, whereas it showed little activity at high temperature (at 50ºC it had 28%). In order to assess its thermostability, the enzyme was pre-incubated at different temperatures for 1h before assaying the residual activity. Fig 3.46 showed that there was little loss of activity with 58% retention of activity after one hour at 45°C, followed by abrupt decrease in activity by incubation for one hour at more than 50°C. The enzyme was quite stable at temperatures up to 30ºC where it retained more than 90% of its activity.

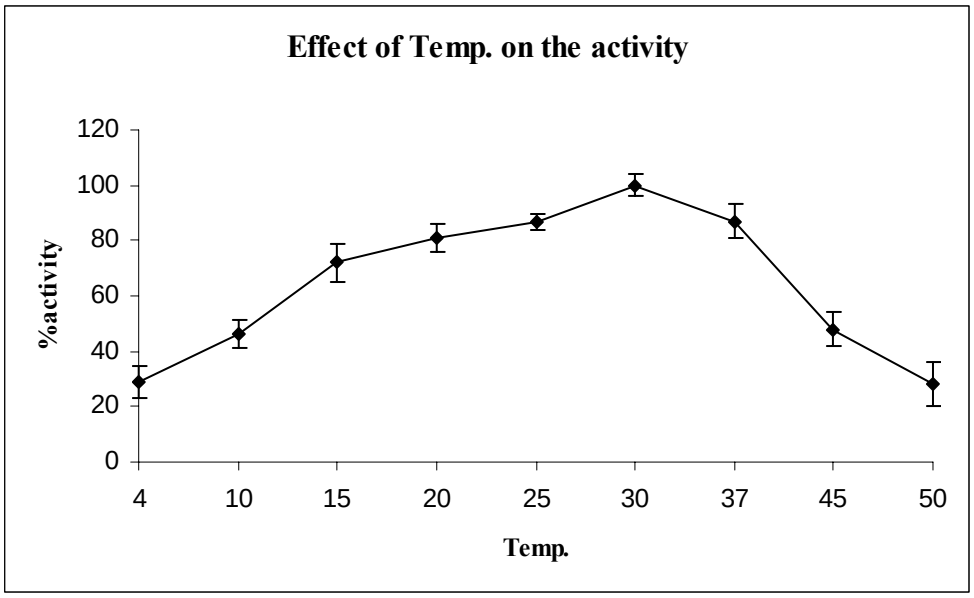


Fig. 3.45. Effect of temperature on activity. Activity is represented as percentage of the maximum activity. Standard deviations were derived from two different experiments each with four replicates.

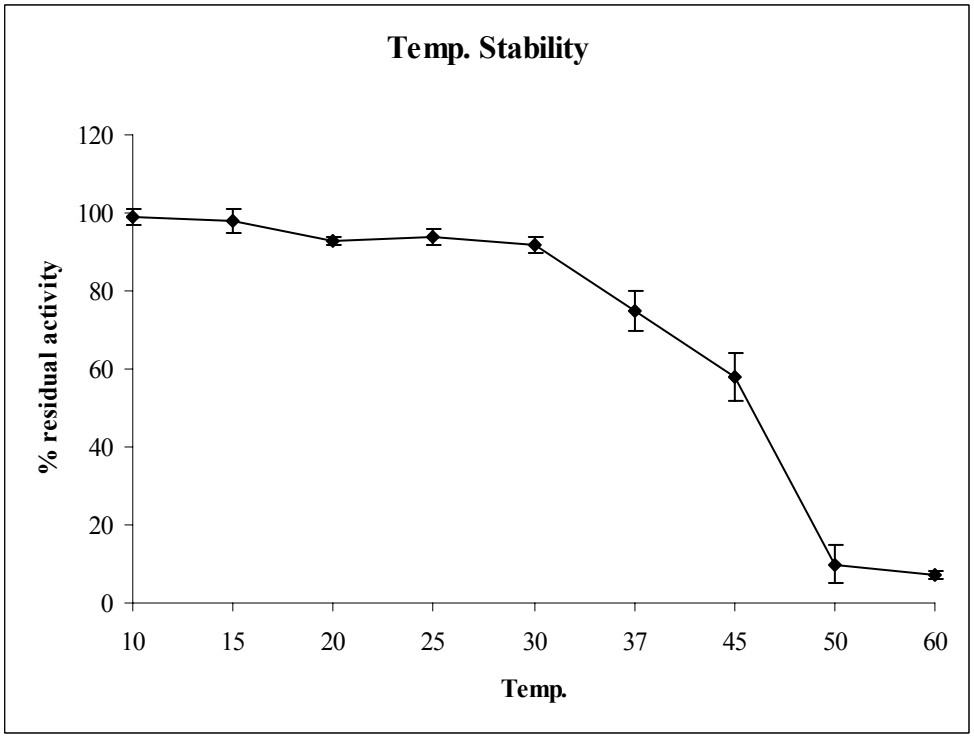


Fig. 3.46. Effect of temperature on stability. Stability was measured by incubation for 1 h at the stated temperature with determination of the residual activity at 37°C. Standard deviations were derived from two different experiments each with four replicates.

3.2.3.2.3. Effect of pH on the activity and stability of Est B

The esterase enzyme was assayed on the substrate *p*-nitrophenyl acetate for 2 minutes at 25°C at a range of pH values. The enzyme preferred alkaline conditions with an optimum activity at pH8-8.5 (Fig 3.47) and very little activity (< 20%) at a pH lower than 6.5. The enzyme retained substantial activity at pH values up to 9. The enzyme was stable on storage at pH values between 7.5 and 11 for 24h at 15°C, Est B showed low stability in acidic pH; less than 50% activity at pH range 3.5-5 was retained (Fig 3.48).

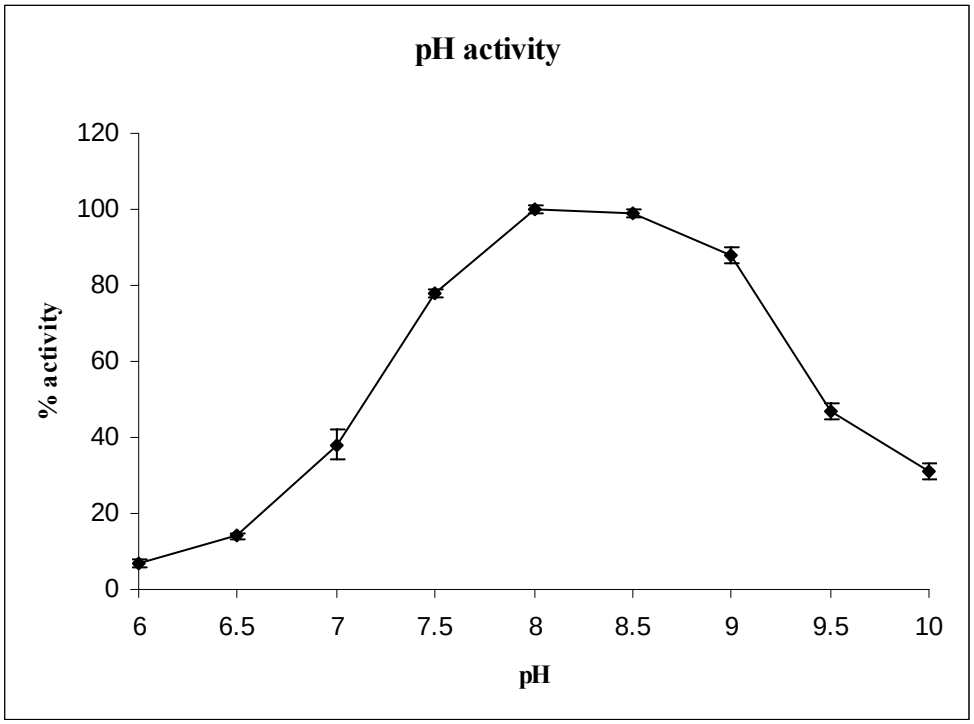


Fig. 3.47. Effect of pH on activity. Activities are shown as percentages of the maximum activity. Standard deviations were derived from two different experiments each with four replicates. SD were indicated as error bars. The absence of an error bar indicated a deviation less than the symbol size.

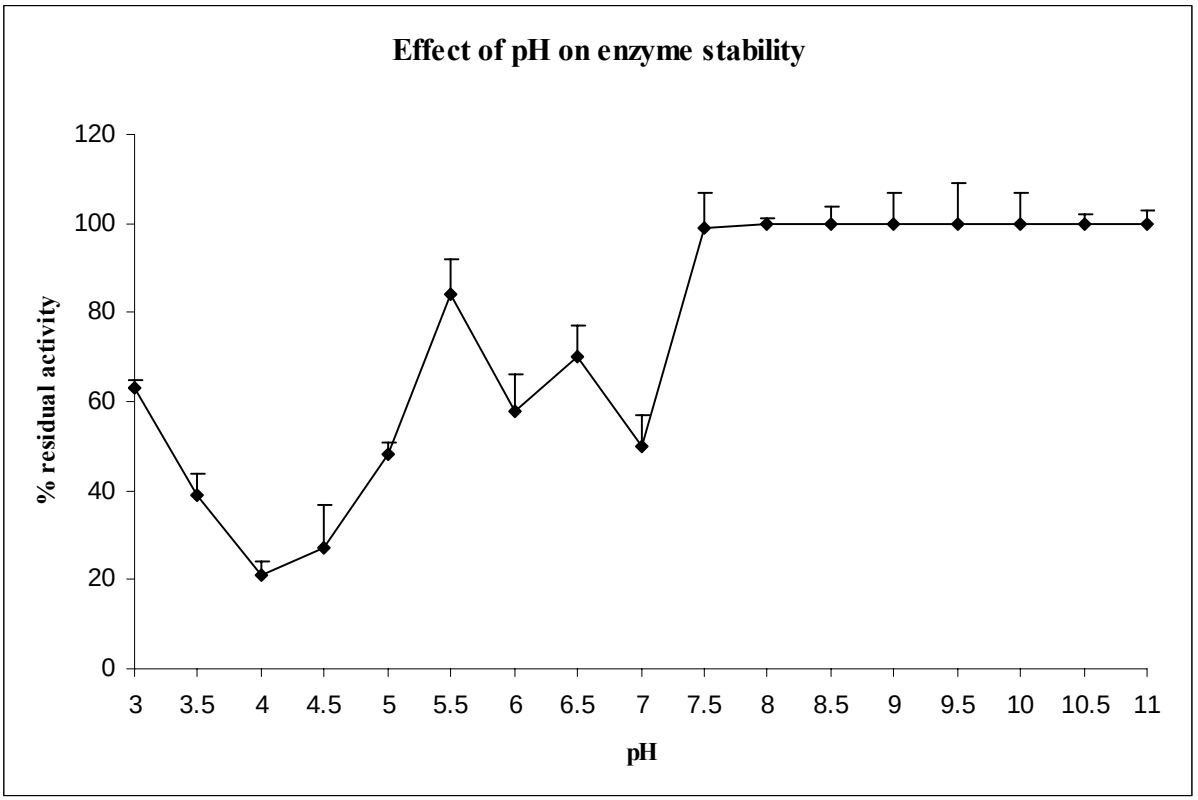


Fig. 3.48. Effect of pH on stability. Stability was measured after 24h incubation in different buffers at 15˚C. Standard deviations were derived from two different experiments each with four replicates. SD were indicated as error bars. The absence of an error bar indicated a deviation less than the symbol size.

3.2.3.2.4. Effect of metal ions and inhibitors of Est B

The effect of pre-incubation with different metal ions and inhibitors on the enzymatic activity was tested (Fig 3.49). There was no obvious effect of any of the used metals and inhibitors at concentration 1mM after 1h incubation except the Hg^{+2} and PMSF, they produced a marked decrease in the activity (39% and 46% respectively), however preincubation in 10mM PMSF, mono- and divalent ions decreased the activity greatly. $CuSO_4$ decreased the activity to 31% and the $HgCl_2$ to only 7%. PMSF 10mM decreased the activity to 29%. There was a small increase of activity in the presence of DTT.

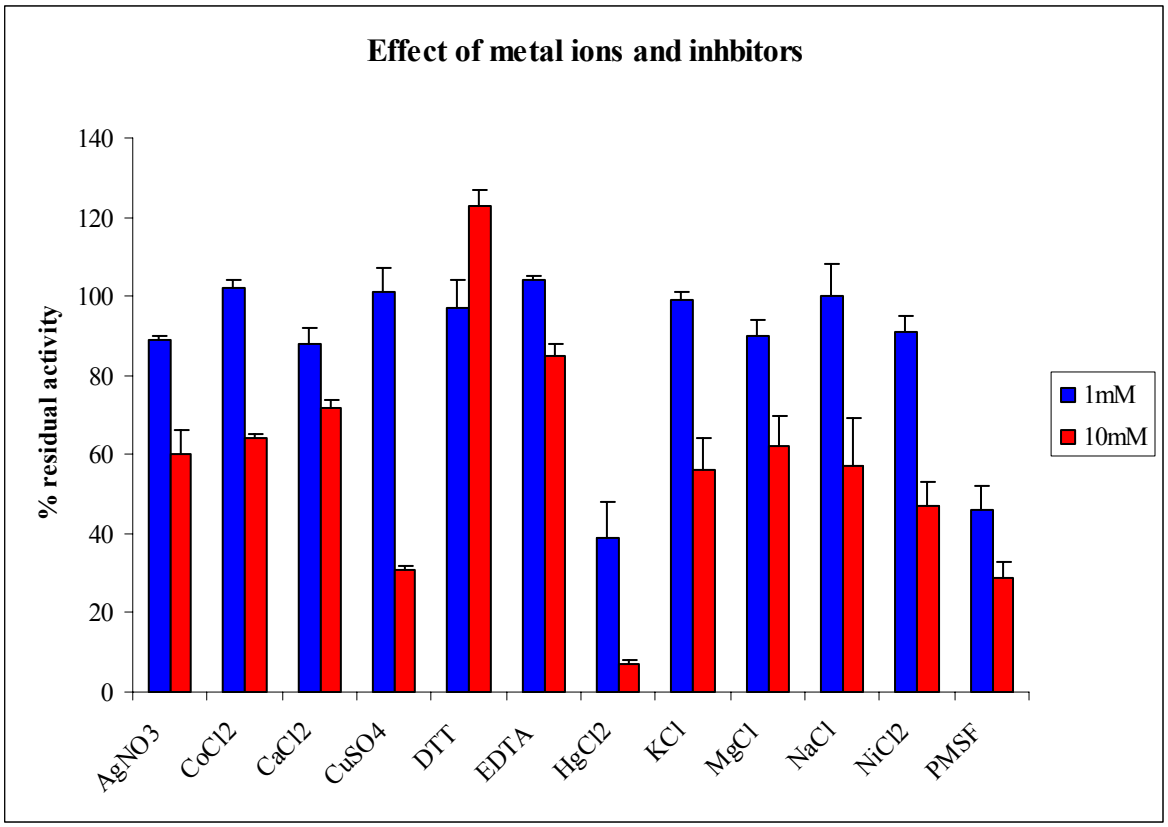


Fig. 3.49. Effects of metal ions and inhibitors on activity of esterase B. All reagents were tested using 1 mM (blue bars) and 10 mM (red bars). The final concentration in reactions was always ≤2%. Standard deviations were derived from two different experiments each with four replicates.

3.2.3.2.5. Effect of organic solvents on the activity and stability of Est B

Reaction mixtures contain 10% of water miscible organic solvents had reduced the enzyme activity significantly (Table 3.12). The effect of organic solvents on stability was as shown in (Table 3.13). The enzyme was quiet stable for 1h at 15°C in 20% organic solvent solutions except isopropanol, which reduced the activity around 50%, however the enzyme was unstable in 50% organic solutions, where it retained 7-18% only of its activity.

Table 3.11. Activity in different organic solvents at concentration 10%; SD were derived from two different experiments each with four replicates

Organic solvent	Activity±SD In 10% organic solvent
acetonitrile	14±4
acetone	16±3
DMF	15±4
DMSO	37±1
isopropanol	11±1
ethanol	19±3
methanol	12±1

Table 3.12. Stability in different organic solvents; SD were derived from two different experiments each with four replicates

Organic solvent	Stability±SD	
	20%[a]	50%[a]
acetonitrile	86±2	7±2
acetone	98±1	13±2
DMF	94±2	18±1
DMSO	96±6	17±5
isopropanol	54±4	7±2
ethanol	100±1	13±1
methanol	90±4	10±1

[a]the concentration of the organic solvents in the reaction mixture was <1%

3.2.3.2.6. Kinetic parameters

The K_m and V_{max} were estimated using *p*-nitrophenyl acetate, butyrate, caproate and caprylate as substrates. Table 3.14 confirmed that Est B has a higher affinity towards the short chain esters than towards the middle chain.

Table 3.13. K_m and Vmax. SD was derived from two different experiments each with four replicates.

Substrate	K_m±SD mM	V_{max}±SD mM/min
Acetate (C2)	0.90±0.06	0.008±0.00004
Butyrate (C4)	0.93±0.011	0.005±0.00028
Caproate (C6)	2.53±0.51	0.011±0.00033
Caprylate (C8)	3.00±0.28	0.003±0.00212

3.2.3.2.7. Enantioselectivity profile of Est B

Est B was tested for the enantioselectivity against the same chiral compounds as used for Est A (the enantioselectivity screening was done by our collaborators in CSIR regional research institute Jammu, India). Est B did not show a significant enantioselectivity as the enantiomeric excess (ee) value of the hydrolysed products/starting materials was low. For secondary alcohols enzyme Est B showed preference for acyl esters of 'R' enantiomer to give 'R' alcohol. In case of alkyl esters of acids the enzyme preferred 'S' enantiomer to give enriched'R' ester and 'S' acid (Appendix B).

3.2.3.2.8. Classification of Est B

Analyzing the sequence of Est.B revealed that it contains all the conserved motifs of group V according to the Arpigny and Jaeger classification (Fig 3.50) these enzymes originate from bacteria occupying various habitats. Some are mesophilic (e.g. *H. influenza*), cold adapted bacteria (e.g. *Psy. immobilis*) and heat adapted bacteria (*Sulfolobus acidocaldarius*).

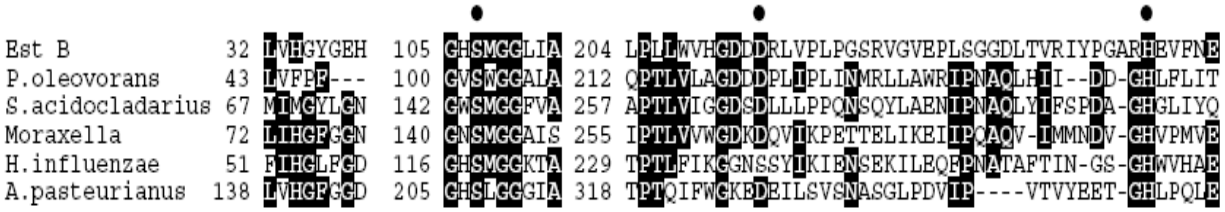


Fig. 3.50. Alignment of the predicted Est B amino acid sequence with members of the family V of esterases and lipases (Arpigny *et al*., 1999). The three critical amino acids in the active centre are shown (•). The conserved amino acids are highlighted in black. The enzymes are from *Streptomyces coelicolor* (Est B), *Pseudomonas oleovorans*, *Sulfobus acidocaldarius*, *Moraxella sp* L11, *Haemophilus influenzae*, *Acetobacter pasteurianus*.

3.2.4. Other Enzymes from *S. coelicolor* and *S. avermitilis*

3.2.4.1. Overexpression and biochemical characterization of an esterase from *Streptomyces coelicolor* A3(2) "Gene locus SCO 3644"

The plasmid pSHS971 was transferred into BL21 (DE3) and the expression conditions were adjusted and the protein was purified by affinity chromatography on Ni-agarose as in case of Est A and Est B.

The enzyme did not show any activity against the triglycerides and the olive oil, however activity was observed against the synthetic *p*-nitrophenyl esters. The maximum activity was measured against the acetate ester, whereas the butyrate and caproate showed respectively only 23% and 6% of the maximum activity. The K_m for *p*-nitrophenyl acetate was estimated to be 0.32mM and that for butyrate ester 0.76mM.

The enzyme behaved at 35ºC as optimum temperature, and it retained more than 30% of its activity at 5ºC. The enzyme proved to be highly unstable with temperature the activity decreased to 16% after 1h preincubation at 25ºC and to only 9% at 30ºC. However the preincubation 30min showed activity 65% and 56% and the preincubation 15 min showed activity 78% and 73% at 25ºC and 30ºC respectively.

The enzyme showed its optimum activity at pH 8. It was not possible to measure the pH stability for 24h because of the high instability of the enzyme, so stability was measured after only 1h preincubation at different pH. The enzyme was highly stable over pH range 6.5-10.

3.2.4.2. Overexpression of an enzyme from *Streptomyces coelicolor* A3(2) "Gene locus SCO 1265"

A trial for expression and characterization of the product of SCO 1265 was done by Rao Ren during his "Diplomarbeit". The gene SCO 1265 was overexpressed in pET-16b. The enzyme produced from this gene was soluble protein and showed activity against some short to middle chain *p*-nitrophenyl esters when the crude cell extract was tested. However he failed to purify the enzyme using the Ni affinity columns as the enzyme lost the activity after purification.

3.2.4.3. Some insoluble lipolytic enzymes from *Streptomyces coelicolor and Streptomyces avermitilis*

The genes SAV 469, SAV 1549 (by Rao Ren during his "Diplomarbeit"), SAV 7089, SAV 3461 (by Qi Zhang during his "Diplomarbeit"), SCO 4799 and SCO 3219 were expressed in pET-16b, and I could observe an extra band corresponding to the expected molecular weight in the induced total cell protein and not in the control, whereas there was no extra band in the clear lysate. Which means I could overexpress these genes but the produced proteins either form inclusion bodies or highly insoluble proteins. This correlates with the calculated theortical solubility (see Table 3.3). Several attempts were made to increase the solubility through changing the induction conditions and adding some solubility enhencers like Triton-x100, betaine and sorbitol but we could not achive marked increase in the solubility (data not shown).

3.2.4.4. Expression of some genes using the expression vector pET-23b

I have tried to express some genes; SAV 1549, SAV 3461 (done by Qi Zhang), SAV 7089 SCO 3644 and SCO 7131 in pET-23b to obtain the protein in native form. There was no sign for successful expression, as I could not find any extra band compared to the control in the induced total cell protein in any of the tested genes. It seems this vector was not suitable for expression of the target genes, so I stoped using this vector and continued to work with the pET-16b which produces His-tagged protein.

4. Discussion

Enzymes play an important role physiologically, as they direct all the biochemical reactions in living organisms. Enzymes work normally under mild conditions, and this property made them good candidates for replacing the classical catalysts in industry. As enzymes' employment in several applications is expanding, the demand for new enzymes is increasing. Esterases and lipases can be widely used in organic chemical processing, detergent formulations, synthesis of biosurfactants, oleochemical industry, dairy industry, cosmetics, pharmaceuticals production, fine chiral compounds synthesis, etc.

Streptomyces are rich in secondary metabolites production, therefore they represent untapped source of interesting esterases and lipases. The two *Streptomyces* strains; *S. coelicolor* and *S. avermitilis*, which genome projects were completed, possess 31 and 20 putative esterases and/or lipases genes respectively. Among these 51 genes only one, which codes for *S. coelicolor* lipA, had been studied (Valdez *et al*., 1999).

This study is the first step to uncover this hidden pool of lipolytic enzymes. We have managed to clone 14 different putative lipolytic genes from both strains in an expression vector, expression and product characterization of three of these genes was finished and furthermore the directed evolution of one of these enzymes, which we thought it might have interesting catalytic activity, was started.

4.1. Biochemical characterisation of Est A

Although the gene (locus SCO 7131) was annotated as a putative lipase, the biochemical characterization showed it is an esterase. The enzyme was assigned as esterase and not lipase, because it did not exhibit the "interfacial activation" phenomena and it was active exclusively against C_2 esters and not the longer ones.

4.1.1. Substrate specificity and Est A kinetics

A number of lipolytic enzymes belonging to HSL family were extensively studied during the last few years. Although the family members show homology with human HSL, all the so far characterized bacterial enzymes were esterases and not lipases (Chahinian *et al*., 2005). Moreover the bacterial HSL carboxylesterases are exerting their best activity against C_6 esters e.g lipP from *Pseudomonas* sp strain B11-1 showed best activity towards C_4 and C_6 (Choo *et*

al., 1998), EST2 from *Alicyclobacillus acidocaldarius* had the maximum activity against C_6 esters (Manco *et al*., 1998), AFEST from *Archaeoglobus fulgidus* used C_6 esters as best substrate (Manco *et al*., 2000), also the recently isolated thermostable HSL from metagenomic library showed best catalysis towards C_6 esters (Rhee *et al*., 2005). My Est A was different from the aforementioned HSL enzymes and has shown unique substrate specificity, it was active only against C_2 esters. Also the affinity of Est A towards *α*-naphthylacetate was 10-fold its activity towards *β*-naphthylacetate and 3-fold its activity towards *p*-nitrophenylacetate i.e. Est A possesses not only unique high substrate specificity but also regioselectivity. In 2005 Mandrich *et al*. have highlighted the possible role of the N terminus on enzyme specificity in the HSL EST2 from *Alicyclobacillus acidocaldarius* for the first time. The 3D structure of EST2 is known as well as the HSL lipase from *Burkholderia cepacia*. When the Mandrich group superimposed both structures they realized that the active site in the case of the *Burkholderia cepacia* lipase is freely accessible, whereas the EST2 active site is covered with the N terminal (α1 and α2 strands). They produced a truncated mutant lacking the N terminal, and it did act on longer chain esters and triglycerides. They also investigated an HSL esterase from *Sulfolobus solfataricus*, which is a homologue of EST2 with natural deletion of the N terminal, and it exhibited the same pattern of substrate specificity as the truncated EST2. Moreover EST2 wild type exhibited regiospecificity against the preacetylated monosacchrides, a property which is not shown by the truncated mutant. The previous results emphasize the role of the N terminal on the specificity of EST2 and may be applied for the other HSL family members. Taking in consideration that the N terminal of *S. coelicolor* is not homologue to *Alicyclobacillus acidocaldarius* EST2 or any other known HSL enzyme, we can speculate that the N terminal of *S. coelicolor* Est A plays a role of its unique high specificity towards the C_2 esters and the observed regioselectivity. The N terminal of Est A may produce steric hindrance around the enzyme pocket more than that produced by EST2, allowing only the small substrates to access the active site and favour one orientation on the others. This could be investigated by constructing deletion derivatives.

4.1.2. Effects of temperature on Est A activity and stability

Est A has shown interesting behaviour with temperature. The optimum temperature was 55ºC and the enzyme retains more than 70% of its activity after preincubation 1h at 55ºC. The half life of the enzyme at 50ºC was 4.5h. The previous observation seems to be interesting when we remember that Est A is obtained from a mesophilic bacteria which has optimum growth temperature 30ºC and maximum growth temperature 40ºC.

It is thought that amino acids composition has an influence on the protein thermal adaptation. Amino acids composition analysis of the HSL family pointed out some amino acids changes going from psychrophilic to hyperthermophilic enzymes; more specifically a decrease in the Cys, Asn and Thr, as well as an increase in Glu and Pro content (Mandrich *et al*., 2004). Table 4.1 shows a comparison of amino acid composition between Est A and EST2 of *Alicyclobacillus acidocaldarius* as a thermophilic member of HSL family and the esterase Aes of *E. coli* as a mesophilic member. We can easily observe that the content of the amino acids thought to play a role on thermal stability in Est A is not identical with any of the thermophilic or the mesophilic counterpart, however it is a mixture between both of them; Asn and Cys resemble the thermophilic enzyme and the rest are similar to the mesophilic enzyme.

Table 4.1. A comparison of amino acids composition between Est A and EST2 of *Alicyclobacillus acidocaldarius* as thermophilic member of HSL family and esterase Aes of *E. coli* as a mesophilic member.

Residue	Est A 316aa		EST2 310aa		Aes 319aa	
	No	Mol%	No	Mol%	No	Mol%
Ala	41	13.0%	37	11.9%	32	10.30%
Arg	17	5.4%	16	5.2%	18	5.64%
Asn	**6**	**1.9%**	**6**	**1.9%**	**8**	**2.51%**
Asp	23	7.3%	21	6.8%	21	6.58%
Cys	**0**	**0.0%**	**1**	**0.3%**	**9**	**2.82%**
Gln	11	3.5%	12	3.9%	18	5.64%
Glu	**21**	**6.6%**	**20**	**6.5%**	**17**	**5.33%**
Gly	26	8.2%	22	7.1%	21	6.58%
His	8	2.5%	8	2.6%	6	1.88%
Ile	9	2.8%	10	3.2%	12	3.76%
Leu	26	8.2%	35	11.3%	37	11.60%
Lys	10	3.2%	12	3.9%	8	2.51%
Met	7	2.2%	2	0.6%	10	3.13%
Phe	7	2.2%	12	3.9%	15	4.70%
Pro	**20**	**6.3%**	**28**	**9.0%**	**20**	**6.27%**
Ser	13	4.1%	13	4.2%	13	4.08%
Thr	**20**	**6.3%**	**8**	**2.6%**	**19**	**5.96%**
Trp	5	1.6%	4	1.3%	5	1.57%
Tyr	11	3.5%	16	5.2%	17	5.33%
Val	35	11.1%	23	7.4%	13	4.08%

In addition to the amino acid composition (Mandrich *et al*., 2004) have identified six possible amino acids replacements playing a role in protein thermostabilization Gln-Arg, Gly-Ala, Gly-Arg, Ser-Ala, Thr-Arg and Asp-Glu. They found 9 of these replacements which favour the thermostability within the alignment of EST2 and brefeldin A. Placing EstA in this alignment revealed that 5 replacements out of 9 were conserved in Est A (Fig 4.1). From the previous observation concerning the amino acids composition and the amino acids replacements, which improve thermostability, we can conclude that Est A is a transition stage between mesophilic and thermophilic enzymes within HSL family, and this can account for the recognized pattern of Est A stability and activity with temperature.

```
EST2            ------------------------------------------MPLDPVIQQVLDQLNR 16
brefeldin       MTVTPERSTYTPPGRLGDESSGPRTDPRFSPAMVEALATFGLDEVAAAPPVSASDDLPTV 60
SCESTA          ---------------------------------------MSDIVLEPAAQDFADATAK 19
                                                        :   *  .   *

EST2            MPAPDYKHLSAQQFRSQQSLFPPVKKEPVAEVREFDXDLPGRTLKVRXYRPEGVEPPYPA 76
brefeldin       LAAVGASHDGFQAVYDSIALDLPTDRDDVETSTETILGVDGNEITLHVFRPAGVEGVLPG 120
SCESTA          PPLLYELGVEGARKLLDDVQSGPVEKPDVDEKWITVPVEVG-DVRVRIVKPAGTTGVLPV 78
                 .               .     *..:  *           *  : ::  :* *.    *

EST2            LVYYHGGGWVVG-DLETHDPVCRVLAKDGRAVVFSVDYR----LAPEHKFPAAVEDAYD 130
brefeldin       LVYTHGGGMTILTTDNRVHRRWCTDLAAAGSVVVMVDFRNAWTAEGHHPFPSGVEDCLA 179
SCESTA          VLYVHGGGWILG-NAGTHDRLVRELAVGAEAAVVFVEYD----RSPEAKYPVAIEQAYA 132
                ::* ****  :   :  .*      **  . :.*. *::        .  :* .:*:.

EST2            ALQWIAERAADFHLDPARIAVGGDSAGGNLAAVTSILAKERG---GPALAFQLLIYPSTG 187
brefeldin       AVLWVDEHRESLGLS--GVVVQGESGGGNLAIATTLLAKRRGRLDAIDGVYASIPYISGG 237
SCESTA          TAQWVTTKGAEEGLDGSRMVVAGDSVGGNMSAALTHMAKRRG---DVTFLHQSLYYPVTD 189
                :  *:  :  .  *.   :.* *:* ***:: . : :**.**        .  : *   .

EST2            YDPAHPPASIEEN----AEGYLLTGGXXLWFRDQYLNSLEELTHPWFSPVLYP--DLSGL 241
brefeldin       YAWDHERRLTELPSLVENDGYFIENGGMALLVRAYDPTGEHAEDPIAWPYFASEDELRGL 297
SCESTA          AGQDTESYRLFAH------GPHLTAKAMEWFWNAYAPDPAERDQITASPLRATPEDLQGL 243
                           *  :       :   *     .  .    *   .  :* **

EST2            PPAYIATAQYDPLRDVGKLYAEALNKAGVKVEIENFEDLIHGFAQFY-SLSPGATKALVR 300
brefeldin       PPFVVAVNELDPLRDEGIAFARRLARAGVDVAARVNIGLVHGADVIFRHWLPAALESTVR 357
SCESTA          PPAFVVVDENDVLRDEGEAYARKLIQAGVPTTSVRYNASLHDFMMLNPVRGTQASTAAIE 303
                **  :.. : * *** *  :*. * :*** .        :*.   :     . *  : :.

EST2            IAEKLRDALA----- 310
brefeldin       DVAGFAADRARLRRP 372
SCESTA          QAIHVLRSALGTD-- 316
                 .  .
```

Fig. 4.1. Structural alignment of Est A, EST2 and brefeldin A. the replacement positions which improve thermostability are marked red

4.1.3. Effects of pH on Est A

The optimum pH of Est A was pH 7.5, and it showed high stability in the pH range 5.5-10 when stored for 24h at 20ºC. In these aspects Est A did not differ greatly from other esterases and lipases either these belonging to HSL family or those which do not. e.g. the esterase lipP from *Pseudomonas* sp showed optimum pH 8 and stability over pH range 6-9 (Choo *et al*., 1998). The (GDSL) family lipase from *S. rimosus* had optimum pH 9.5 and was stable over pH range 4-10 (Abramic *et al*., 1999).

4.1.4. Effect of metal ions and inhibitors

The serine protease inhibitor PMSF 10mM decreased the activity to 63% after 1h preincubation. Although all the esterases and lipases have a serine in their active site, they behave differently to the potent serine active site inhibitor PMSF. Some are resistant to PMSF e.g. the extracellular lipase of *Pseudomonas* sp strain ATCC 21808 was unaffected by PMSF (Kordel *et al*., 1991), others are very sensitive; the extracellular lipase from *B. subtilis* 168 was strongly inhibited with 0.1mM PMSF preincubation for only 10min (Lesuisse *et al*., 1993).

Preincubation in 10mM K^+ lead to a significant increase in activity (138%), whereas 10mM Na^+ lead to only a small increase (118%). The optimum concentration of K^+ in the reaction mixture was 10mM, which leaded to increase the activity to 129%. The effect of K^+ can be argued as inorganic ions may bind to some of the ionic side chains of a protein. This kind of interaction, although not affecting the three dimensional shape of the enzyme in a substantial manner could make it easier for a substrate molecule to locate or bind to the active site of the enzyme. Thus the presence of the ion in optimum concentrations could alter the rate of the reaction.

4.1.5. Enantioselectivity profile of Est A

Est A did not show marked enantioselectivity against the so far tested compounds. Est A was tested against 43 chiral compounds, and was active against only 16 compounds, but the levels of stereo-selectivity were low for all the 16 substrates and even their conversion rates were low. We have proved before that Est A has high substrate specificity and it acts only against acetate esters as synthetic substrates. This means Est A activity scope is limited to the small sized esters, and this may account for Est A being active against only a small number of the

tested substrates with low conversion rates. None of the screened substrates was an ideal substrate for Est A, and we need to focus our enantioselectivity screening on the small sized chiral ester compounds to find the proper substrates.

Despite the observed enantioselectivity being low, it is also possible to improve such low enantioselectivity through directed evolution. Reetz and Jaeger (2000) have reported increasing the enantioselectivity of a lipase from 2% ee (E = 1) to >90% ee (E = 25) through several rounds of random mutagenesis and screening.

4.1.6. HSL family conserved motifs

According to the known sequences and the available data in 1999 Arpigny and Jaeger have determined 4 conserved motifs in the HSL family. 3 of them are containing the catalytic triad (S, D and H), and their role in catalysis is well known. The 4th motif which is HGGG where HG is a part of the oxyanion hole involved in the stabilization of the transition state, mutagenesis of the G residue resulted in drastic decrease in enzymatic activity (Manco *et al*., 1999). Our analysis of the protein data base revealed the presence of another 2 conserved motifs within HSL family (YRLAPE---P) and (Q-L-YP), whose residues are highly conserved 75-100% and 85-100% respectively (Table 3.9). As we have mentioned previously the role of the catalytic triad and the oxyanion hole is known, here a question arises what is the role of these conserved motifs and in particular the P residues P1 in the 1st motif as well as the P residue in 2nd motif, which are 100% conserved in the 119 enzymes from the HSL family.

Proline is the most rigid amino acid, usually proline acts as a structural disruptor in the middle of regular secondary structure elements such as alpha helices and beta sheets.The P1 residue of the conserved motif (YRLAPE---P) lies away from the enzyme pocket between strand β4 and the helix α4 either in the homology model of Est A or in the EST2 3D structure. The P residue in the conserved motif (Q-L-YP) in both Est A and EST2 exists inside the enzyme pocket between the strand β6 and the helix α6 (Fig 4.2). Probably these P residues are essential for the correct folding of this group of enzymes. It is interesting question to answer if these residues also share directly or indirectly in the catalysis or in the transition state stabilization.

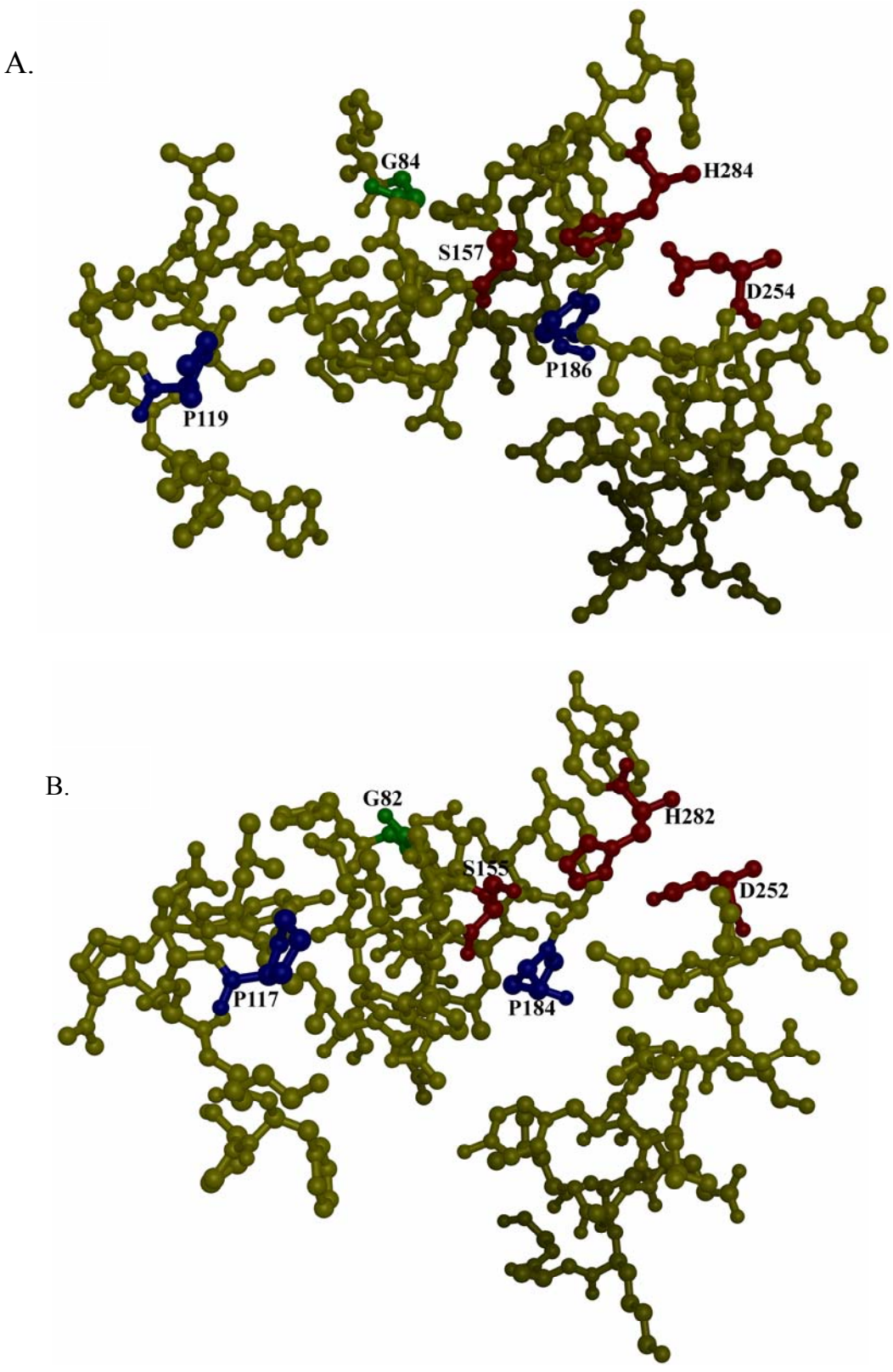


Fig. 4.2. The spatial arrangement of enzyme pocket and the two conserved motifs (YRLAP) and (Q-L-YP). A. Est A *S. coelicolor* and B. EST2 *Alicyclobacillus acidocaldarius*. The catalytic triad is red, the proline residues are blue and the oxyanion hole G is green. The proline in the motif (YRLAP) is P117/119 and in the motif (Q-L-YP) is P184

4.2. Site directed mutagenesis

I have produced 5 different site directed mutants; W87F, V158A, W87F/V158A, M162L and S163A. We can classify these mutants into 2 groups; the first group leaded to minor or insignificant changes in the substrate spectrum and enzyme kinetics and the second group which showed marked alterations in the substrate specificity and kinetics. I have compared the mutagenesis sites with their homologues in the known 3D structure of EST2 *Alicyclobacillus acidocaldarius* and AFEST *Archaeoglobus fulgidus* (Fig 4.3).The 2 mutants M162L and S163A are belonging to the first group. However M or S is a larger than L or A respectively and lying near S active site, the mutagenesis L162M or A163S did not lead to a significant change in substrate spectrum or enzyme kinetics. I think that the reason was the orientation of LA/MS; these residues are oriented away from the enzyme pocket, and hence are not involved directly in the catalysis, or interfering with the substrate movement or the transition state stabilization.

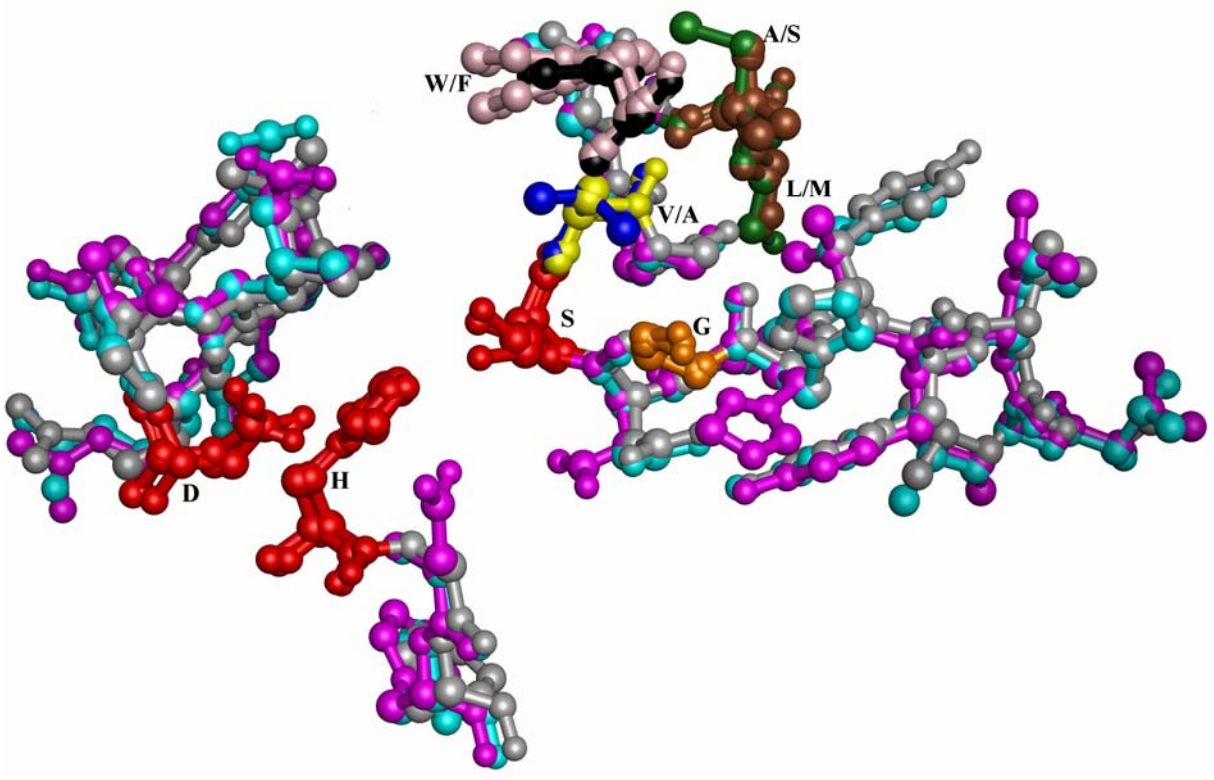


Fig. 4.3. Superimposition of enzymes' pocket; EST2 *Alicyclobacillus acidocaldarius* (grey), ESTA *Streptomyces coelicolor* (Magenta) and AFEST *Archaeoglobus fulgidus* (cyan), showing the mutated residues in Est A. The catalytic triad S/D/H are red, the oxyanion hole G is orange, the A active site is yellow/ V is blue, F is pink/W is black and the LA are sienna/ MS are green.

The mutants W87F, V158A and the double mutant W87F/V158A belong to the 2nd group. The mutant W87F reduced the affinity 4-fold with no change in substrate spectrum, in contrast to the double mutant W87F/V158A which widened the substrate spectrum with no change in kinetics, whereas the mutant V158A increased both the affinity and the substrate spectrum. By studying the position of V/A (Fig 4.4) we can realize that 2 side chains in valine are protruding inside the enzyme pocket; one towards the oxyanion hole glycerine and both just above the serine active site. Substitution of valine with alanine which did not possess these side chains may produce more space around the active site and hence allow the mutant V158A to act against larger substrates like *p*-nitrophenylbutyrate and *p*-nitrophenylcaproate. Another possible explanation concerns the role of the oxyanion hole and especially the G residue. Maybe the presence of the valine side chain in the space between the S active site and the G of the oxyanion hole interferes with this stabilization process allowing only the stabilization of the acetate esters and not the larger substrates, whereas in the V158A mutant such interference was absent and hence the enzyme acted on larger substrates.

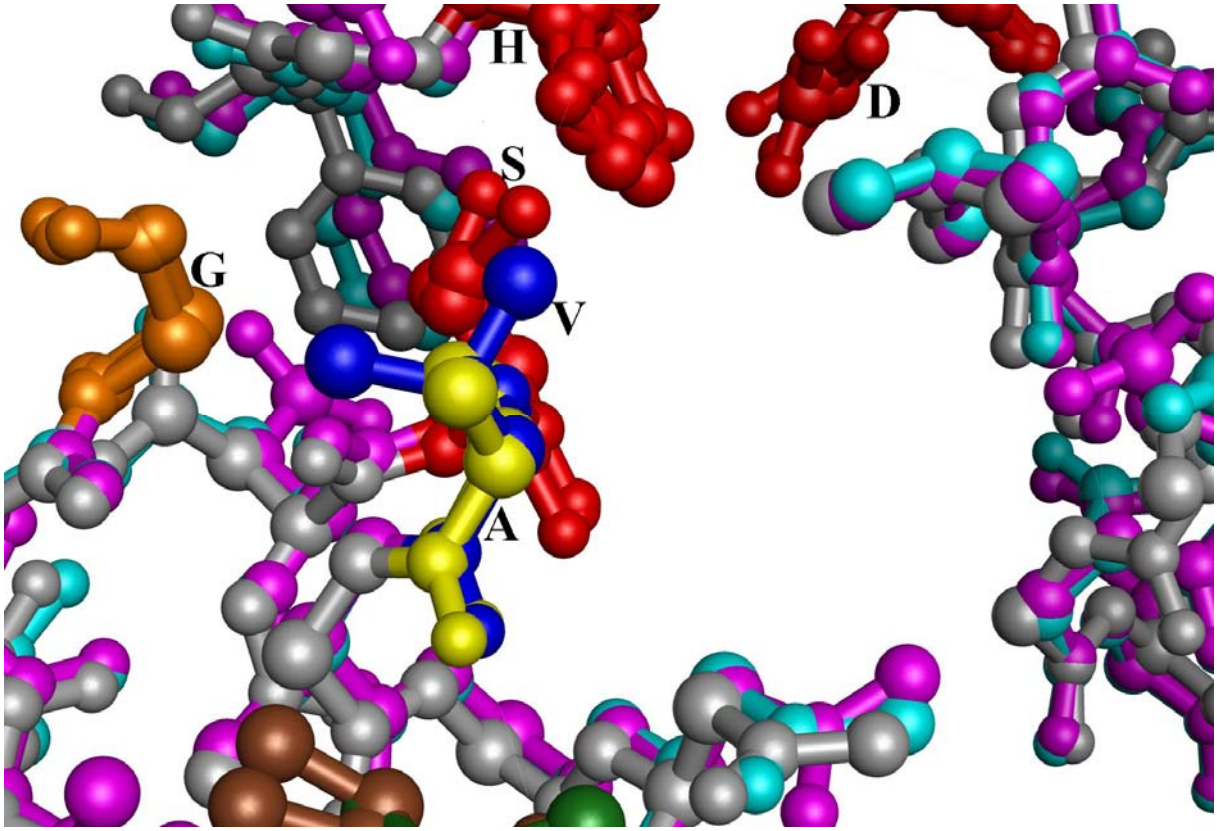


Fig. 4.4. Close up on the enzymes' pocket EST2 *Alicyclobacillus acidocaldarius* (grey), Est A *Streptomyces coelicolor* (Magenta) and AFEST *Archaeoglobus fulgidus* (cyan), showing the position and orientation of the V/A residues (blue/yellow) from the S active site (red) and the G oxyanion hole (orange) within the 3 enzymes.

The residues W/F are lying to some extent away from the enzyme pocket (Fig 4.3); maybe that is why the mutant W87F did not show any change in substrate spectrum. Combining both mutations (W87F/V158A) resulted also in increase in substrate spectrum, due to the effect of the A instead of the V.

The mutant V158A shows a 6-fold increase in the affinity whereas the mutant W87F shows 4-fold decrease in the affinity. It is possible that the hydrophobicity of the amino acids accounts for these effects. In both cases, the more hydrophobic amino acid (F or V respectively) showed a lower affinity.

All the mutants showed a lower thermostability than the wild type except the mutant V158A, which exhibited better thermostability. V158A retained almost all its activity after 1h incubation at 50ºC and had a half life 24h at the same temperature compared to 4.5h for the wild type enzyme. Interestingly, the presence of A at this position instead of L was associated with thermostability as shown earlier fig 4.1 (Mandrich *et al*., 2004).

4.3. Random mutagenesis

We have managed to construct library mutants of Est A. After screening 1056 mutants of the library, less than 4% of the screened mutants had activity. Only one mutant (XXVF7) showed a marked change in the substrate spectrum without affecting its thermostability. Sequencing the mutant XXVF7 showed five mutated residues (L76R, L146P, S196G, W213R and L267R). The protocol used resulted in a high mutation rate around 5 mutations per 1000 base pairs, this accounts for the high knockout rate we have observed within the library.

To optimize yield of mutants, the random mutagenesis should be done using a less drastic protocol aiming to decrease the knock out rate from 96% to around 37%, in other words to produce a mutation rate of 1-2 substitutions per 1000bp.

It is most probably one or more of the five mutations caused conformational changes in the enzyme active site, leading the enzyme to act on larger substrates.

The mutated residue L76R exists near the oxyanion hole (residues 79-84). The oxyanion hole is responsible for the stabilization of the transition state, so it is possible that a nearby mutation in the residue 76 from the less hydrophobic amino acid L to the most hydrophobic

amino acid R helped in stabilization of the transation state of the longer esters which possess a more hydrophobic side chain.

The mutated residue L146P is located 11 residues away from the serine active site (S157), in the turn connecting the strand β5 (which contains the S157) and the helix α5. It is known that the P is a rigid amino acid and its presence causes significant conformational changes. Maybe the P in position 146 leaded to a conformational change affected the β5 strand and hence the S157 was accessed easier by longer substrates.

The mutated residue L267R is a part of the helix α10 which is a conserved motif and contains the active site aspartate (D254). So again is the most hydrophilic amino acid R in this position responsible or at least share in increasing the spectrum of Est A.

In 2001 Manco *et al*. have reported the production of a mutant from EST2 *Alicyclobacillus acidocaldarius* which is able to use substrates with acyl side chain longer than the wild type. This mutant was produced by site directed mutagenesis based on the known 3D structure of EST2 and a snapshot of the enzyme sulphonate complex which mimics the second stage of the enzyme catalytic reaction. The EST2 double mutation was M211S/R215L. When we compared the residues in the reported EST2 mutation and the mutated residues in our Est A, interestingly we found that one of the five mutated residues in ESTA (W213R) is lying in the same region where the double mutation (M211S/R215L) of EST2 occurred. Moreover the position of Est A mutation is central to the position of the EST2 double mutation (Fig 4.5) (notice that the largest amino acid W was replaced with a smaller one R, which means the steric hindrance in the mutant was reduced). If the mutation W213R alone was responsible for the alteration of the substrate specificity of Est A, then we can speculate that this helix, where the residues 211,213 and 215 are existing, is very important for the determination of substrate specificity of HSL family and not only in EST2. Consequently we can predict that the catalytic reaction of Est A will be somehow similar to that of EST2. However all these conclusions still require experimental evidence, which can be achieved through production of W213R as single mutant. It would be worthwhile also to carry out saturation mutagenesis at the residues 211,213 and 215 to confirm their role in substrate specificity determination.

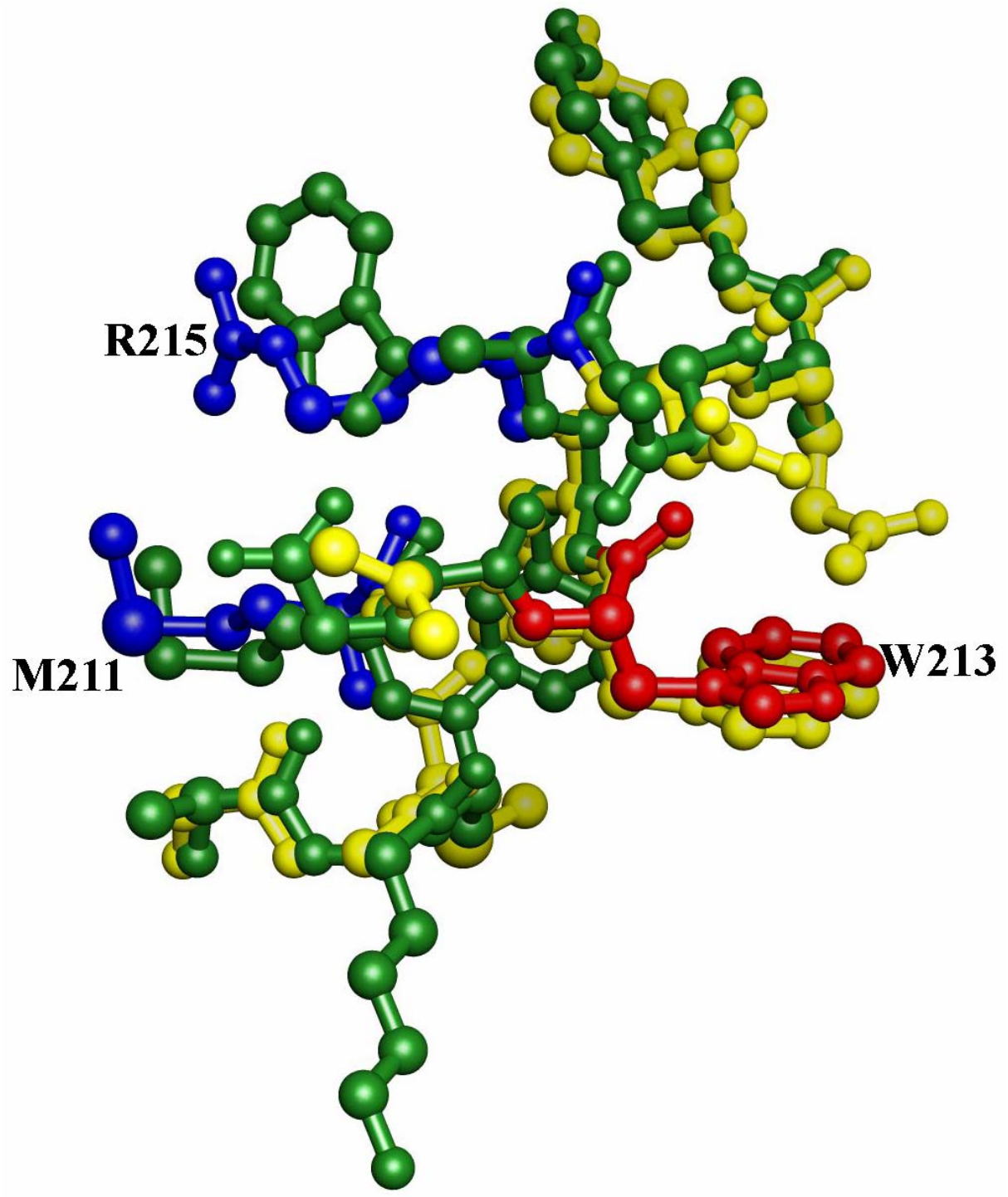


Fig. 4.5. Superimposition of Est A *S. coelicolor* (green) and EST2 *Alicyclobacillus acidocaldrius* (yellow). Showing the relative positions of EST2 double mutation residues (211 and 215) and ESTA W213.

4.4. Biochemical characterisation of Est B

Substrate specificity of Est B was investigated using *p*-nitrophenyl esters of different alkyl chain length. The enzyme showed a high activity towards short chain fatty acids (C2-C6), it exerted the maximum activity against the acetate ester. A similar specificity was found for the cold adapted lipase of *Pseudomonas sp* strain B11-1 (Choo *et al*., 1998). The K_m and V_{max} were also estimated on different substrates and showed a pattern concedes to that observed with activity measurements.

Est B retained more than 25% of the activity at 4ºC and more than 70% at 15ºC it is not surperising that the optimum temperature for Est B was 30°C, which is the optimum growth temperature of *Streptomyces coelicolor A3(2)*. There was little loss of activity with 58% retention of activity after one hour at 45°C. The enzyme was quite stable at temperatures up to 30ºC where it retained more than 90% of its activity; again it is the optimum growth temperature of *Streptomyces*. The enzyme not only showed a good activity at low temperatures, but also had good thermostability compared with other cold-adapted enzymes e.g. the low-temperature lipase from psychotropic *Pseudomonas sp*. strain KB700A, which loses 70% of its activity if incubated only 5 min at 60ºC (Rashid *et al*., 2001). Although high catalytic activity at low temperature is usually associated with thermosenstivity (Narinx *et al*., 1997). However, directed evolution studies to improve the thermostability of cold adapted enzymes revealed that there did not exist a strict correlation between the two (Wintrode *et al*., 2000; Mavromatis *et al*., 2002).

The effect of pre-incubation with different metal ions and other chemicals on the enzymatic activity was tested. None of the metal ions tested stimulated enzyme activity and the insensitivity to EDTA suggested that no divalent cations are needed for enzyme activity. The enzyme was sensitive to Hg^{2+} and there was a slight stimulation of activity with DTT, which suggests that one or more thiol groups is important for the activity. The enzyme was inactivated by PMSF, which is explained by the fact that Est B, like other lipolytic enzymes, has a serine residue in the active site in a conserved pentapeptide G-X-S-X-G (Brady *et al*., 1990; jaeger *et al*., 1999). A 10% concentration of water-miscible organic solvents reduced the enzyme activity significantly. However, the enzyme was fairly stable in 20% solutions of the most of the solvents tested compared to a large loss of activity observed with 50% solutions. This is in contrast to some lipolytic enzymes, which were activated by pre-incubation in organic solvents (Shimada *et al*., 1993; Choo *et al*., 1998).

An important application of lipolytic enzymes is to produce chiral precursors for the synthesis of pharmaceutical products. Est B was tested for its ability to hydrolyse some interesting substrates for this purpose. The levels of stereo-selectivity are low for all the substrates. An enantiomeric excess (ee) of more than 90% would be necessary for a useful enzyme for chiral synthesis. However it is possible to improve the (ee) through the directed mutagenesis (Reetz and Jaeger 2000).

4.5. Other enzymes

4.5.1. Activity of the esterase from the gene locus SCO 3644

This esterase had also high activity at low temperatures, it retained more than 30% of its activity at 5ºC, and this was even more than Est B. However it differed from Est B in thermostability. SCO 3644 esterase was thermosenstive, its activity decreased to 9% after 1h incubation at 30ºC, its thermosenstivity was comparable to that of typical pscyorphilic enzymes e.g. the low-temperature lipase from psychotropic *Pseudomonas sp*. strain KB700A (Rashid *et al*., 2001). Whereas Est B showed activity similar to psychrophilic enzymes and stability similar to mesophilic enzymes, SCO 3644 esterase was typical psychrophilic enzyme in activity and stability.

4.5.2. The enzyme produced from the gene locus SCO 1265

The over-expressed protein from this gene did not suffer a solubility problem and it showed esterase activity in its crude extract, but it was not possible to continue the characterization as the protein lost the activity completely after the purification. There is no degradation happened for the protein during the purification procedure as the PAGE and Western blot showed a unique band after purification on Ni column of ≈35kDa (theortical Mwt of the His-tagged protein is 34kDa). There may be several reasons for loss of activity: maybe the protein needs a co-factor (either metal ions or co-enzyme) which exists in the crude extract but is lost during the purification on the Ni column and the ultrafiltration processes; it could also be a problem caused by one of the components of the buffers used for purification or the enzyme is unstable in the storage buffer used.

4.5.3. The insoluble enzymes

Low solubility is one of the most frequently encountered problems when using *E. coli* as a host for production of recombinant proteins. According to recent data more than 50% of the recombinant proteins aggregate in insoluble inclusion bodies, when they are over-expressed in *E. coli* (Waugh 2005). We encountred this problem with the genes SAV 469, SAV 1549, SAV 7089, SAV 3461, SCO 4799 and SCO 3219, they produced highly insoluble proteins, which were resistant to the traditional manipulations used to increase the solubility (e.g. changing the expression conditions and adding solubility enhancers). It was also reported that these manipulations are not sufficient to enhance the solubility of many proteins (Schein 1989).

It is valuable to try to increase the solubility through the unconvenatial methods like co-expression of chaperone molecules in the host e.g. DnaK, ClpB, GroEL ,lbpA or lbpB (Sorensen and Mortensen 2005). Another approach may it worth to go through is the subcloning of these genes in a solubility fusion expression vector e.g. NusA, MBP, etc (Waugh 2005). It is worth noting that not every protein can be rendered soluble by fusion to a solubility enhancing tag. Also some proteins still form insoluble aggregates after splitting off the solubility enhancing partner.

4.6. Future prespectives

Streptomyces coelicolor and *Streptomyces avermitilis* have 51 genes (31 and 20 genes respectively) annotated as esterases and/or lipases. In this study after excluding the genes from *S. avermitilis* which have a homologoue in *Streptomyces coelicolor*, the number was reduced to 35 genes. Then the number of the genes was reduced again to 15 genes due to removing the genes which did not fit the planned work strategy (the start codon could be incorporated in an *Nde*I recognition site with a mismatch primer, and the gene sequence does not have a *Bam*HI or *Xho*I recognition site). One of these 15 genes did not produce a correct PCR fragment so only 14 out of the 51 genes were cloned from both strains. Four out of these 14 produced soluble protein (ca. 29%), and one of them had stability problem so it ends up that only three genes (ca. 21%) of the cloned genes were characterized taking in consideration that four genes were not checked yet (ca. 29%). Around 40% of the cloned genes produced insoluble proteins and, as mentioned in 4.5.3, using a suitable fusion vector may solve that problem for some but not all of the genes. In such cloning and heterologous protein

expression experiments protein solubility is a critical factor. It is possible to predict the solubility of the overexpressed protein in *E. coli* using the service provided by http://www.biotech.ou.edu/ University of Oklahoma. If we go back to Tab 3.3 we find that all the genes, which were predicted to be soluble (SCO 7133, SCO 6966, SCO3644 and SCO1265), have indeed produced soluble proteins. Whereas other genes e.g. SAV1549, SAV3461 and SCO4799 were highly insoluble and all attempts to solublize their overexpressed protein failed. So in future work it would be sensible to use solubility prediction as the primary criterion when selecting genes for cloning. Then we should develop further criteria to select genes, which are likely to possess interesting enzyme activities. We can use bioinformatic tools to find genes which are different from the known ones (e.g. Est A on the sequence level was different from other characterised members of its family HSL and it showed a unique substrate specificity which is not exhibited by other members of the family). It would also be interesting to clone genes which are likely to be involved in the production of secondary metabolites; these can be identified by software designed to identify secondary metabolite clusters, which contain characteristic combinations of genes. When microarray data are available, it will be possible to look for enzymes with interesting expression profiles. The availability of a wide range of cloning vectors means that selection on the basis of cloning strategy will play at most a minor role. This approach should produce a good yield of interesting enzymes and the huge expansion in sequencing data together with improved bioinformatics may well make this a strategy of choice in the future.

5. Summary

Esterases and lipases are widely used as industrial enzymes and for the synthesis of chiral drugs. Because of their rich secondary metabolism, *Streptomyces* species offer a relatively untapped source of interesting esterases and lipases. *S. coelicolor and S. avermitilis* contain 51 genes annotated as esterases and/or lipases. In this study I have cloned 14 different genes encoding for lipolytic enzymes from *S. coelicolor* (11 genes) and *S. avermitilis* (four genes). Some of these genes were over-expressed in *E. coli.* Three of the produced enzymes, which were produced by the genes SCO 7131, SCO6966 and SCO3644, were characterized biochemically and one of them was subjected for directed evolution.

The gene *estA* (locus SCO 7131) was annotated as a putative lipase/esterase in the genome sequence of *S. coelicolor* A3(2), but does not have a homologue in the genome sequence of *S. avermitilis* or in other known *Streptomyces* sequences. *estA* was cloned and expressed in *E. coli* as a His-tagged protein. The protein was purified and could be recovered in its non-tagged form after digestion with factor Xa. The relative molecular weight was estimated to be 35.5kDa. The enzyme was only active towards acetate esters and not on larger substrates. It had a stereospecificity towards α-naphathylacetate. It was thermostable, with a half-life at 50°C of 4.5 hours. Est A showed stability over pH range 5.5-10, and had optimum pH of 7.5. Its activity was drastically decreased when it was pre-incubated in 10mM PMSF, Cu^{+2} and Hg^{+2}. It was not very stable in most organic solvents and had only slight enantioselectivity. Est A belongs to the HSL family whose founder member is the human hormone-sensitive lipase. I have developed a protein profile for the HSL family modifying the conserved motifs found by Arpigny and Jaeger (1999). Due to the presence of several HSL members with known 3D structure and good homology to Est A, I was able to make a homology model of Est A.

Five different mutants of Est A were produced through site directed mutagenesis: W87F, V158A, W87F/V158A, M162L and S163A. The mutants M162L and S163A did not produce a significant change either in substrate specificity or enzyme kinetics. The mutants V158A and W87F/V158A could act on the larger substrates *p*-nitrophenylbutyrate and caproate and tributyrin. The mutant V158A had improved thermostability and its $t_{1/2}$ at 50ºC increased to 24h. The affinity of V158A towards *p*-nitrophenyacetate increased 6-fold when compared with the wild type, whereas the affinity of W87F decreased 4-fold.

Directed evolution of Est A was done through random mutagenesis and ER-PCR. A library of 6336 mutants was constructed and screened for mutants with a broader spectrum of substrate specificity. The mutant XXVF7 did show alteration in the substrate specificity of Est A. The mutant XXVF7 had 5 amino acids changes L76R, L146P, S196G, W213R and L267R.

The gene locus SCO 6966 (*estB* gene) was cloned and expressed in *E. coli* as a His-tagged protein. It was not possible to remove the His-tag using factor Xa. The tagged protein had a molecular weight 31.9kDa. Est B was active against short chain fatty acid esters (C_2-C_6). Its optimum temperature was 30ºC and was stable for 1h at temperatures up to 37ºC. The enzyme had maximum activity at pH 8-8.5 and was stable over pH range 7.5-11 for 24h. It was highly sensitive for PMSF, Cu^{+2} and Hg^{+2}. The enzymatic activity deceased in presence of organic solvents, however it was fairly stable for 1h in 20% organic solvents solutions.

A third esterase was produced from the gene locus SCO 3644. This esterase was a thermosensitive one with optimum temperature of 35ºC.

The three characterized enzymes included a thermophilic, mesophilic and psychrophilic ones. This indicates the high variation in the characters of *Streptomyces* lipolytic enzymes and highlighting *Streptomyces* as a source for esterases and lipases of interesting catalytic activity.

This study was an initial trial to provide a strategy for a comprehensive use of genome data.

6. Literature

Abramic, M., Lešcic I, Korica T, Vitale L, Saenger W and Pigac J (1999). "Purification and properties of extracellular lipase from *Streptomyces rimosus*." Enzyme Microb Technol **25**: 522-529.

Allardet-Servant, A., Michaux-Charchon, S., Jumas-Bilak, E., Karayan L. and Ramuz M. (1993). "Presence of one linear and one circular chromosome in the *Agrobacterium tumefaciens* C58 genome." Journal Bacteriology **175**: 7869-7874.

Arpigny, J.L., and Jaeger K-E (1999). "Bacterial lipolytic enzymes: classification and properties." Biochem J **343**: 177-183.

Banerjee, S., Katz, J., Levitz, M., and Finlay, T.H. (1991). "Purification and properties of an esterase from human breast cyst fluid." Cancer Res **51**: 1092-1098.

Bao, K., and Cohen S (2003). "Recruitment of terminal protein to the ends of Streptomyces linear plasmids and chromosomes by a novel telomere binding protein essentail for linear DNA replication." Genes and development **17**: 774-785.

Barbier, M., Prevot P, and Soyer-Gobillard M-O (2000). "Esterase in marine dinoflagellates and resistance to the organophosphate insecticide parathion." Internatal Microbiol **3**: 117-123.

Bentley, S., Chater KF, Cerdeno-Tarraga AM, Challis GL, homson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, urphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J and Hopwood DA (2002). "Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2)." Nature **417**: 141-147.

Bentley, S.D., Brown, S., Murrphy, L.D., Harris, D.E., Quail, M.A., Parkhill, J., Barrel, B.G., McCormick, J.R., Santamaria, R.I., Losick, R., Yamasaki, M., kinashi, H., Chen, C.W., Chandra, G., Jakimowicz, D., kieser, H.M., kieser, T. and Chater, K.F. (2004). "SCP1, a 356 023 bp linear plasmid adapted to the ecology and developmental biology of its host, *Streptomyces coelicolor* A3(2)." Mol Microbiol **51**: 1615-1628.

Berdy, J. (2005). "Bioactive microbial metabolites." J Antibiotics **58**: 1-26.

Bibb, M.J., Findlay, P.R. and Johanson, M.W. (1984). "The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequence." Gene **30**: 157-166.

Bornscheuer, U. (2002). "Microbial carboxyl esterases: calssification, properities and application in biocatalysis." FEMS Microbiol Rev **26**: 73-81.

Borodina, I., Krabben, P. and Nielsen, J. (2005). "Genome-scale analysis of *Streptomyces coelicolor* A3(2) metabolism." Genome Res. **15**: 820-829.

Bradford, M.M. (1976). "A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-254.

Brady, L., Brozozowski A.M., Derewenda, Z.S., Dodson E., Dodson G., Tolley S., Turkenburg J.P., Christiansen L., Huge-Jensen B., Norskov L., thim L. and Menge U. (1990). "A serine protease triad forms the catalytic center of a triacylglycerol lipase." Nature **343**: 767-770.

Bullock, W.O., Fernandez J M and Short J M (1987). "XL1-blue: A high efficiency plasmid transforming recA *Escherichia coli*." Biotechniques **5**: 376-379.

Burg, R., Miller, B., Baker, E., Birnbaum, J., Currie,S., Hartman, R., Kong, Y-L., Monaghan, R., Olson, G., Putter, I., Tunac, G., Wallick, H., Stapley, E., Oiwa, R., and Omura, S. (1979). "Avermectins, New Family of Potent Anthelmintic Agents: Producing Organism and Fermentation." Antimicrob Agents Chemother **15**: 361-367.

Cane, D., and Watt, R. (2002). "Expression and mechanistic analysis of a germacradienol synthase from *Streptomyces coelicolor* implicated in geosmin biosynthesis." PNAS **100**: 1547-1551.

Chahinian, H., Ali, Y., Abousalham, A., Petry, S., Mandrich, L., Manco, G., Canaan S., and Sarda, L. (2005). "Substrate specificity and kinetic properities of enzyme belonging to the hormone sensitive lipase family: Comparison with non lipolytic and lipolytic carboxylesterases." Biochim Biophys Acta **1738**: 29-36.

Chater, K.F. (2001). "Regulation of sporulation in *streptomyces coelicolor* A3(2): A checkpoint multiplex?" Curr Opin Microbiol **4**: 667-673.

Chen, C.W. (1996). "Complications and implications of linear bacterial chromosomes." Trends Gent. **12**: 192-196.

Chen, C.W., Haung, C., Lee, H., Tasi, H. and Kirby, R. (2002). "Once the circle has been broken: dynamics and evolution of *Streptomyces* chromosomes." Trends Gent. **18**: 522-529.

Cherry, J., and Fidantsef, A. (2003). "Directed evolution of industrial enzymes: an update." Curr Opin Biotech **14**: 438-443.

Choo, D.-W., Kurihara T., Suzuki T., Soda K. & Esaki N. (1998). "A cold-adapted lipase of an Alaskan psychrotroph, *Pseudomonas sp*. strain B11-1: Gene cloning and enzyme purification and characterization." Appl. Env. Microbiol. **64**: 486-491.

Christova, K., Sholeva, Z. and Chipeva, V. (1995). "Application of molecular biological methods in taxonomy of genus *Streptomyces*." Journal of culture collections **1**: 3-10.

Chung, S.-T. (1982). "Isolation and characterization of *Streptomyces fradiae* plasmids which are prophage of the actinophage SF1." Gene **17**: 239-246.

Claessen, D., Jong, W., Dijkhuizen, L. and Wösten H. (2006). "Regulation of *Streptomyces* development: reach for the sky!" Trends in Microbiology **14**: 313-319.

Copeland (2000). Enzymes: a practical introduction to structure, mechanism and data analysis, John Wiely & Sons, Inc.

Crespi, M., Messens, E., Caplan, A.B., Montagu, M.V. and Desomer, J. (1992). "Fasciation induction by the phytopathogen *Rhodococcus fascians* depends upon a linear plasmid encoding a cytokinin synthase gene." EMBO J. **11**: 795-804.

Cruz, H., Pérez C, Wellington E, Castro C and Servín-González L (1994). "Sequence of the *Streptomyces albus* G lipase-encoding gene reveals the presence of a prokaryotic lipase family." Gene **144**: 141-142.

Davidson, B., Macdougall, J. and Girons, I.S. (1992). "Physical map of the linear chromosome of the bacterium Borrelia burgdorferi 212, a causative agent of lyme disease, and localization of rRNA genes." J. Bacteriol. **174**: 3766-3774.

Dower, W.J., Miller, J.F., and Ragsdale, C.W. (1988). "High efficiency transformation of E.coli by high voltage electropration." Nucleic Acids Res **16**: 6127-6145.

Freemann, R.F., Bibb, M.J. and Hopwood, D.A. (1977). "Chloramphenicol acetyltransferase-independent chloramephenicol resistance in *Streptomyces coelicolor* A(3)2." J Gen Microbiol **98 (2) : 453-465**.

Friedmann, H. (1997). From Friedrich Wöhler's urine to Eduard Buchner's alcohol. New beer in an old bottle: Eduard Buchner and the growth of biochemical knowledge. *Valencia*, universitat de Valencia**:** 67-122.

Gravius, B., Glocker, D., Pigac,J., Pandža,K., Harnueli, D. and Cullum, J. (1994). "The 387kb linear plasmid pPZG101 of *Streptomyces rimosus* and ist interaction with the chromosome." Microbiol **140**: 2271-2277.

Guex, N., and Peitsch, M. C. (1997). "SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modelling." Electrophoresis **18**: 2714-2723.

Hayakawa, T., Otake, N., Yonehara, H., Tanaka, T. and Sakaguchi, K. (1979). "Isolation and characterization of plasmids from *Streptomyces*." J. Antibio. (Tokyo). **32**: 1348-1350.

Hopwood, D.A. (1999). "Forty years of genetics with *Streptomyces*: from in vivo through in vitro to in silico." Micribiology **145**: 2183-2202.

Hopwood, D.A. (2006). "Soil to genomics: *Streptomyces* chromosome." Annu Rev genet **40**: 1-23.

Hotta, Y., Ezaki S, Atomi H and Imanaka T (2002). "Extremely stable and versatile carboxylesterase from a hyperthermophilic *Archaeon*." Appl Env Microbiol **68**: 3925-3931.

Hu, L., Deeney, J.T., Nolan, C.J., Peyot, M.L., Ao, A, Richard, A.M., Luc, E., Faergeman, N.J., Knudsen, J., Guo, W., Sorhede-Winzell, M., Prentki, M., and Corkey, B.E. (2005). "Regulation of lipolytic activity by long chain acyl-coenzyme A in islets and adipocytes." Am J Physiol Endocrinol Metab **289**: 1085-1092.

Hutchinson, C.R., and Fujii, I. (1995). "Polyketides synthase gene manipulation: a structure- function approach in engineering novel antibiotics." Annu. Rev. Microbiol. **49**: 201-38.

Ikeda, H., Ishikawa J, Hanamoto A, Shinose M, Kikuchi H, Shiba T, Sakaki Y, Hattori M, and Omura S (2003). "Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*." Nature Biotechnology **21**: 526 - 531.

Jaeger, K.-E., and Eggert, T. (2002). "Lipases for biotechnology." Curr Opin Biotech **13**: 390-397.

jaeger, K.-E., Dijkstra BW and Reetz M T (1999). "Bacterial biocatalyst: molecular biology, three-dimensional structures, and biotechnological applications of lipases." Annu Rev Microbiol **53**: 315-351.

Jäger, S., Demleitner, G. and Götz, F. (1992). "Lipase of Staphylococcus hyicus: Analysis of the catalytic triad by site-directed mutagenesis." FEMS microbiol Lett **100**: 249-254.

Janowsky, D.S., Davis, J.M., and Overstreet, D.H. (2005). "Anticholinesterase (DFP) toxicity antagonism by chronic donepezil: a potential nerve agent treatment." Pharmacol Biochem Behav **81**: 917-922.

Jensen, R., DeJong, F., and Clark, R., Determination of lipase specificity. lipids, 1983. **18**(3): p. 239-252.

Kendall, K., and Cohen, SN (1988). "Complete nucleoitide sequence of the *Streptomyces Lividans* plasmid pIJ101 and correlation of the sequence with genetic properties." J Bacteriol **170**: 4634-4651.

Kendall, K., and Cullum, J (1986). "Identification of a DNA sequence associated with plasmid integration in *Streptomyces coelicolor* A3(2)." Mol Genet **202**: 240-245.

Kieser, H.M., Kieser, T. and Hopwood, D.A. (1992). "A combined genetic and physical map of the *Streptomyces coelicolor* A3(2) chromosome." J. Bacteriol **174**: 5496-5507.

Kieser, T., Bibb MJ, Buttner MJ, Chater KF and Hopwood DA (2000). Practical Streptomyces genetics: a laboratory manual. *Norwich.*, The John Innes Foundation.

Kieser, T., Hopwood, D.A., Wright, H.M. and Thompson, C.J. (1982). "pIJ101, a multi-copy broad host range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors." Mol Gen Genet **185**: 223-238.

Kordel, M., Hofmann B, Schomburg D, and Schmid RD. (1991). "Extracellular lipase of *Pseudomonas sp*. Strain ATCC21808: Purification, characterization, crystallization and preliminary X-ray diffraction data." J. Bact. **143**: 4836-4841.

Koshland, D. (1994). "Key and lock theory and the induced fit theory." Angw chem inl Ed Ingl **33**: 2375-2378.

Laemmli, U. (1970). " Cleavage of structural proteins during the assembly of the head of bacteriophage *T4*." Nature **227**: 680-685.

Lawrence, R.C., Fryer, T.F., and Reiter, B. (1967). "rapid method for the quantitative estimation of microbial lipases." Nature **213**: 1264-1265.

Leblond, P., Fischer, G., Francou, F.-X., Berger, F., Guerineau, M. and Decaris, B. (1996). "The unstable region of *Streptomyecs ambofaciens* includes 210kb terminal inverted repeats flanking the extremities of the linear chromosomal DNA." Mol Microbiol **19**: 261-271.

Lesuisse, E., Schank, K., and Colson, C., (1993). "Purification and perliminary characterization of the extracellualr lipase of *Bacillus subtils* 168, an extremely basic pH tolerant enzyme." Eur J Biochem **216**: 155-160.

Lezhava, A., Mizukami, T., Kajitani, T., Kameoka, D., Redenbach, M., Shinkawa, H., Nim, O. i and Kinashi, H. (1995). "Physical map of the linear chromosome of *Streptomyces griseus*." J Bacteriol **177**: 6492-6498.

Lin, Y.-S., Kieser, H.M., Hopwood, D.A. and Chen, C.W. (1993). "The chromosomal DNA of *Streptomyces lividans* 66 is linear." Mol Microbiol **10**: 923-933.

Lomovaskaya, N.D., Mkrtumain, N.M., Gostimskaya, N.L. and Danilenko, V.N. (1972). "Characterization of temperate actinophage C31 isolated from *Streptomyces Coelicolor* A3(2)." J Virol **9**: 258-262.

Lorenz, P., and Eck, J. (2005). "Metagenomics and industrial applications." Nat Rev Micro **3**: 510-516.

Manco, G., Adinolfi E, Pisani FM, Ottolina G, Carrea G, Rossi M. (1998). "Overexpression and properties of a new thermophilic and thermostable esterase from *Bacillus acidocaldarius* with sequence similarity to hormone-sensitive lipase subfamily." Biochem J **332**: 203-212.

Manco, G., Fabbraio, F., Adinolfi, E., and Rossi, M. (1999). "Homology modeling and active site residues probing of the thermophilic *Alicaclobacillus acidocaldrius* esterase2." protein Science **8**: 1789-1796.

Manco, G., Giosue, E., D'Auria, S., Herman, P., Carrea, G., and Rossi M. (2000). "Cloning, overexpression and properties of a new thermophilic esterase with sequence similarity to hormone sensitive lipase subfamily from the archaeon *Archaeoglobus fulgidus*." Arch Bioch Biophy **373**: 182-192.

Manco, G., Mandrich, L., and Rossi, M. (2001). "Residues at the active site of esterase 2 from *Alicyclobacillus acidocaldarius* involved in substrate specificity and catalytic activity at high temperature." J Biol Chem **276**: 37482-37490.

Mandrich, L., Merone, L., Pezzullo, M., Cipolla, L., Nicotra, F., Rossi, M., and Manco, G. (2005). "Role of the N terminus in enzyme activity, stability and specificity in thermophilic esterase belonging to the HSL family." J Mol Biol **345**: 501-512.

Mandrich, L., Pezzullo, M., Vecchio, P., Barone, G., Rossi, M., and Manco, G. (2004). "Analysis of thermal adaptation in the HSL enzyme family." J Mol Biol **335**: 357-369.

Marangoni, A. (2003). Enzyme kinetics a modern approach. *New Jersey*, A John Wiely & Sons, Inc., publication.

Mavromatis, K., Tsigos L., Tzanodaskalaki M., Kokkinidis M. and Bouriotis V. (2002). "Exploring the role of a glycine cluster in cold adaptation of an alkaline phosphatase." Eur. J. Biochem. **269**: 2330-2335.

Nardini, M., and Dijkstra, B. (1999). "α/ß Hydrolase fold enzymes: the family keeps growing." Curr Opin Struc Biol **9**: 732-737.

Narinx, E., Baise E and Gerday C. (1997). "Subtilisin fron psychrophilic antarctic bacteria: characterizationa and site-directed mutagenesis of residues possibly involved in the adaptation to cold." Protein Engineering **10**: 1271-1279.

Nelson, D., and Cox, M. (2005). Lehninger Principles of biochemistry. *New York*, W.H. Freeman and company.

Panda, T., and Gowrishankar, B.S. (2005). "Production and applications of esterases." Appl Microbiol Biotechnol **67**: 160-169.

Pandaza, S., Biukovic, G. , Paravic, A. , Dadbin, A., Cullum, J. and Hranueli, D. (1998). "Recombination between the linear plasmid pPZG101 and the linear chromosome of *Streptomyces rimosus* can lead to exchange of ends." Mol. Microbiol. 28 (6): 1165-1176.

Paradkar, A., Trefzer, A., Chakraburtty, R., and Stassi, D. (2003). "Streptomyces genetics: a genomic perspective." Critcal review in biotechnology **23**: 1-27.

Pasqualini, E., Caillol, N., Mas, E., Bruneau, N., Lexa, D., and Lombardo, D. (1997). "Association of bile-salt-dependent lipase with membranes of human pancreatic microsomes is under the control of ATP and phosphorylation." Biochem. J. **327**: 527-535.

Patel, J., Wallace, R., Brown-Elliott, B., Taylor, T., Imperatrice, C., Leonard, D., Wilson, R., Mann, L., Jost, K. and Nachamkin, I. (2004). "Sequence-based identification of aerobic actinomycetes." J Clin Micro **42**: 2530-2540.

Pathom-aree, W., Stach, J., Ward, A., Horikoshi, K., Bull, A. and Goodfellow M. (2006). "Diversity of actinomycetes isolated from challenger deep sediment (10,898m) from the Mariana Trench." Extremophiles **10**: 181-189.

Peitsch, M.C. (1995). "Protein modeling by E-mail." Bio/Technology **13**: 658-660.

Pérez, C., Juarez K, Garcia-Castells E, Sóberon G and Servín-González L (1993). "Cloning, characterization and expression in *Streptomyces lividans* 66 of an extracellular lipase encoding gene from Streptomyces sp. M11." Gene **123**: 109-114.

Philip, B., Sunna, A., Gibbs, M.D., Curach, N.C., Nevalainen, H. and Bergquist, P.L. (2002). "Prospecting for novel lipase genes using PCR." Microbiol **148**: 2283-2291.

Posner, I., and Desanctis, J. (1987). "The kinetics of heparin inhibition of the esterase and basal lipase activities of lipoprotein lipase." Arch Biochem Biophys **253**: 475-485.

Rashid, N., Shimada Y., Ezaki S., Atomi H. and Imanaka T. (2001). "Low-temperature lipase from *psychrotrophic Pesudomonas* sp. strain KB700A." Appl. Env. Micro. **67**: 4064-4069.

Raymer, G., Willard JMA and Schottel JL (1990). "Cloning, Sequencing and regulation of expression of an esterase gene from the plant pathogen *Streptomyces scabies*." J Bact **172**: 7020-7026.

Reetz, M., and Jaeger, K-E. (2000). "Enantioselective enzymes for organic synthesis created by directed evolution." Chem Eur J **6**: 407-412.

Revie, D., Smith DW and Yee TW (1988). " Kinetic analysis for optimization of DNA ligation reactions." Nucleic Acids Res **16**: 10301-10321.

Rhee, J.-K., Ahn D.-G., Kim Y.-G. and Oh J.-w. (2005). "New thermophilic and thermostable esterase with sequence similarity to the hormone senstive lipase family, cloned from a Metagenomic library." Appl. Env. Micro. **71**: 817-825.

Rosenstein, R., and Gotz, F. (2000). "Staphylococcal lipases: biochemical and molecular characterizattion." Biochimie **82**: 1005-10014.

Rubin-Pitel, S., and Zhao, H. (2006). "Recent advances in biocatalysis by directed evolution." Combinatorial chemistryand high throughput screening **9**: 247-257.

Ryding, N.J., Kelemen, G.H., Whatling C.A., Flärdh, K., Buttner, M.J. and Chater, K.F. (1998). "A developmentally regulated gene encoding a repressor-like protein is essential for sporulation in *Streptomyces coelicolor* A3(2)." Mol. Microbiol. **29**: 343-357.

Sakaguchi, K. (1990). "Invertons, a class of structurally and functionally related genetic elements that includes linear DNA plasmid, transposable elements and genomes of adeno-type viruses." Microbiol. Rev. **54**: 66-74.

Sakaguchi, K., Hirochika, H. and Gunge, N. (1985). "Linear plasmid with terminal inverted repeats obtained from *Streptomyces rochei* and Kluvermoyces lactis." Basic life Sci **30**: 433-451.

Sambrook, J., Fritsch, F., and Maniatis, T. (1989). Molecular cloning: A Laboratory Manual. *New York*, Cold Spring Harbor.

Sanger, F., Nicklen S and Coulson AR (1977). "DNA sequencing with chain terminating inhibitors." Proc Natl Acad Sci USA **74**: 5463-5467.

Scharf, S.J., Horn, G.T., and Erlich, H.A. (1986). "Direct cloning and sequence analysis of enzymatically amplified genomic sequences." Science **233**: 1076-1078.

Schein, C.H. (1989). "Production of soluble recombinant proteins in bacteria." Bio/Technology **7**: 1141-1148.

Schmid, A., Dordick, J., Hauer, B., Kiener, A., Wubbolts, M. and Witholt, B. (2001). "Industrial biocatalysis today and tomorrow." Nature **409**: 258-268.

Schrempf, H., Bujard, H., Hopwood, D.A.and Goebel, W. (1975). "Isolation of covalently closed deoxyribonucleic acid from *Streptommyces coelicolor* A3(2)." J Bacteriol **121**: 416-421.

Schwarz, D.C., and Cantor, C.R. (1984). "Separation of yeast chromosome-sized DNAs by pulsed field gel electrophoresis." Cell **37**: 67-75.

Schwede, T., Kopp, J., Guex, N., and Peitsch, MC. (2003). "SWISS-MODEL: an automated protein homology-modeling server." Nucleic Acids Research **31**: 3381-3385.

Sharma, R., Chisti, Y. and Banerjee, U.C. (2001). "Production, purification, characterization and application of lipases." Bitechnol Adv **19**: 627-662.

Shimada, Y., Koga C., Sugihara A., Nagao T., Takada N., Tsunasawa S., and Tominaga Y. (1993). "Purification and characterization of a novel solvent tolerant lipase from *Fusarium heteroporum*." J. Ferment. Bioeng. **75**: 349-352.

Shuman, S. (1994). "Novel Approach to Molecular Cloning and Polynucleotide Synthesis Using *Vaccinia* DNA Topoisomerase." J Biol Chem **269**: 1994.

Sommer, P., Bormann C and Götz F (1997). "Genetic and biochemical characterization of a new extracellualr lipase from *Streptomyces cinnamomeus*." Appl Env Microbiol **63**: 3553-3560.

Sorensen, P.H., and Mortensen , K. (2005). "soluble expression of recombinant proteins in the cytoplasm of *E. coli*." Microbial cell factories **4**: 1.

Stinson, S. (2001). Chiral pharmacutocals. chemical and engineering news. **79:** 79-97.

Stoddard Hatch, M., Brown, W.M., Deck, J.A., Hunsaker, L.A., Deck, L.M., and Vander Jagt, D.L. (2002). "Inhibition of yeast lipase (CRL1) and cholesterol esterase (CRL3) by 6-chloro-2-pyrones: comparison with porcine cholesterol esterase." Biochim Biophys Acta **29**: 381-391.

Sumner, J. (1946). The chemical nature of enzymes, Nobel lecture.

Tesch, C., Nikoleit K, Gnau V, Götz F and Bormann C (1996). "Biochemical and molecular characterization of the extracellular esterase from *Streptomyces diastatochromogenes.*" J Bact **178**: 1858-1865.

Towbin, H., Staehelin, T., and Gordon, J. (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications." BioTechnology **24**: 145-149.

Umezawa, H. (1982). "Low molecular weight enzyme inhibitors of microbial origin." Ann. Rev. Microbiol. **36**: 75-99.

Valdez, F., González G, Kieser HM and González LS (1999). "The *Streptomyces coelicolor* A3(2) lipAR operon encodes an extracellular lipase and a new type of transcriptional regulator." Microbiol **145**: 2365-2374.

Voet, D., and Voet, J. (1995). Bioechmistry. *Toronto*, John Wiley and Sons, Inc.

Vujaklija, D., Schröder W, Abramic M, Zou P, Lešcic I, Franke P and Pigac J (2002). "A novel streptomycete lipase: cloning, sequencing and high level expression of the Streptomyces rimosus GDS(L)-lipase gene." Arch Microbiol **178**: 124-130.

Walsh, G. (2004). Proteins "Biochemistry and biotechnology". *West Sussex*, John Wiely and Sons, Ltd.

Wang, G.-Y., Michailides TJ and Hammock BD (2000). "Affinity purification and characterization of a cutinase from the fungal plant pathogen Monilinia fructicola (Wint) honey." Arch Bioch Biophy **382**: 31-38.

Waugh, D.S. (2005). "Making the most of affinity tags." Trends Biotechnol **23**: 316-320.

Weiner, M.P., Anderson C, Jerpseth B, Wells S, Johnson-Browne B and Vaillancourt P (1994). "Studier pET system vectors and hosts." Strategies Mo Bio **7**: 41-43.

Whitford, D. (2005). Proteins "Structure and Function". *West Sussex*, John Wiley & Sons, Ltd.

Willey, J.M., Willems, A., Kodani, S. and Nodwell, J.R. (2006). "Morphogeneticsurfactants and their role in the formation of aerial hyphae in Streptomyces coelicolor." Molecular Microbiology **59**: 731-742.

Williams, S.T., Goodfellow, M., Alderson, G., Wellington, E.M.H., Sneath, P.H.A. and Sackin, M.J. (1983). "Numerical classification of Streptomyces and related genera." J. Gen. Microbiol. **129**: 1743-1813.

Wintrode, P.L., Miyazaki K. and Arnold F.H. (2000). "Cold adaptation of a mesophilic subtilisin-like protease by laboratory evolution." J. Biol chem. **275**: 31635-31640.

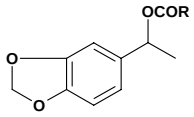
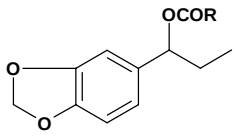
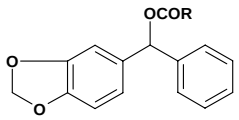
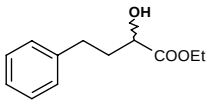
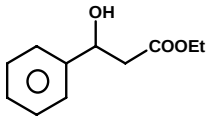
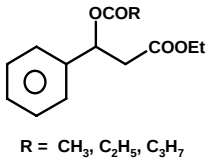
Yamasaki, M., and Kinashi, H. (2004). "Two chimeric chromosome of *streptomyces coelicolor* A3(2) generated by single crossover of the wild type chromosome and linear plasmid SCP1." J Bact **186**: 6553-6559.

Zhang, H., Lee, Y., Zhang, W. and Lee, H. (2006). "Culturable actinobacteria from the marine sponge *Hymeniacidon perleve*: isolation and phylogenetic diversity by 16S rRNA gene-RFLP analysis." Antonie van Leeuwenhoek **online first**: 1-11.

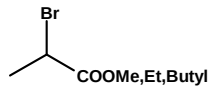
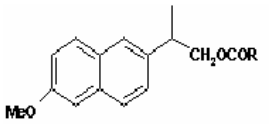
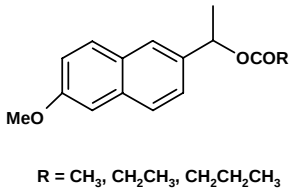
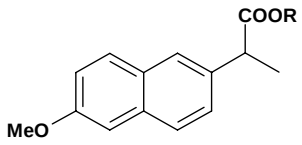
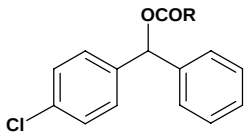
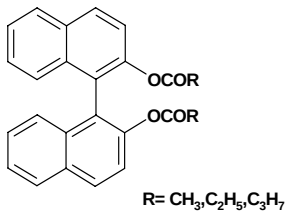
Zorn, H., Bouws, H., Takenberg, M., Nimtz, M., Getzlaff, R., Breithaupt, D.E., and Berger, R.G. (2005). "An extracellular carboxylesterase from the basidiomycete Pleurotus sapidus hydrolyses xanthophyll esters." Biol chem **386**: 435-440.

7. Appendix

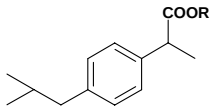
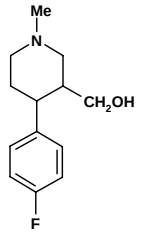
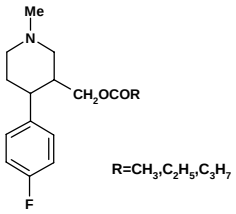
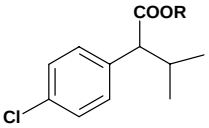
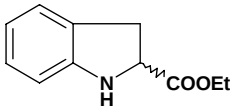
A: The hydrolysis of some chiral compounds using Est A

Substrate	R	Conv. (%)	ee (%) of the product	Cofiguration
 I- Acyl ester of 1-(3,4- methylenedioxyphenyl)-ethanol.	CH_3 C_2H_5 C_3H_7	18 13 9	21 12 6	R R R
 II- 1-(3,4- methylenedioxyphenyl)-propan-1-ol	CH_3 C_2H_5 C_3H_7	18 13 9	21 12 6	R R R
 III- Acyl esters of 1-(3,4-methylenedioxy-5-yl) benzylalcohol	CH_3 C_2H_5 C_3H_7	No hydrolysis		
 IV- Ethyl- 2-hydroxy-4-phenyl butanoate		64	16	R ester
 V- Ethyl 3-hydroxy-3-phenyl-propanoate		No hydrolysis		
 VI- Ethyl 3-(acyl)hydroxy-3-phenyl-propanoate	CH_3 C_2H_5 C_3H_7	No hydrolysis		

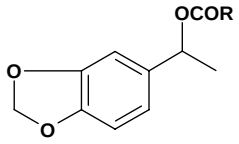
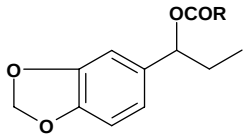
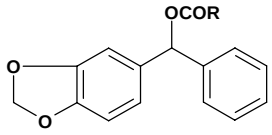
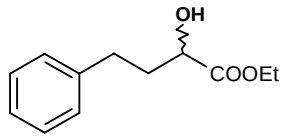
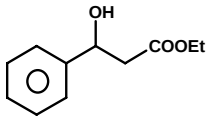
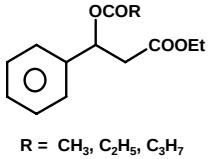
Continue Appendix A: The hydrolysis of some chiral compounds using Est A

Substrate	R	Conv. (%)	ee (%) of the product	Cofiguration
 VII- Acyl esters of 2-bromo propanoic acid	CH_3 C_2H_5 C_4H_9	15 25 32	11.3 15.7 21.8	S(acid) S (acid) S(acid)
 VIII- Acyl esters of 2-(6-methoxy-2-naphthyl)-propan-1-ol	CH_3 C_2H_5 C_3H_7	9 8 3	9 7 ND	R R ND
 IX- Acyl esters of 1-(6-methoxy-2-naphthyl) -ethanol.	CH_3 C_2H_5 C_3H_7	8 6 6	2 5 7	R R S
 X- alkyl esters of 2-(6-methoxy-2-naphthyl)-propanoic acid	CH_3 C_2H_5 C_3H_7	No hydrolysis		
 XI- Acyl ester of 1 – (p-chlorophenyl)-benzylalcohol.	CH_3 C_2H_5 C_3H_7	No hydrolysis		
 XII- Acyl esters of bisnapthol	CH_3 C_2H_5 C_3H_7	No hydrolysis		

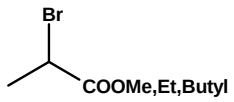
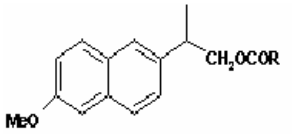
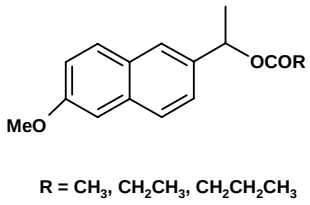
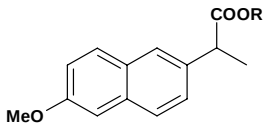
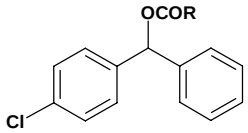
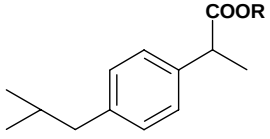
Continue Appendix A: The hydrolysis of some chiral compounds using Est A

Substrate	R	Conv. (%)	ee (%) of the product	Cofiguration
 XIII- 2-(p-(2-methyl propyl)-2-phenyl) – propanoic acid	CH_3 C_2H_5 C_3H_7	No hydrolysis		
  XIV- n-methyl 4-(p-flurophenyl)-3-acyloxymethylcyclohexamide	CH_3 C_2H_5 C_3H_7	No esterification No hydrolysis		
 XV- 1-(p-chloro phenyl)–2-methyl propanoic acid.	CH_3 C_2H_5 C_3H_7	No hydrolysis		
 XVI- Ethyl (indol-2-yl) formate		No hydrolysis		

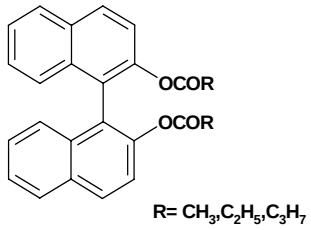
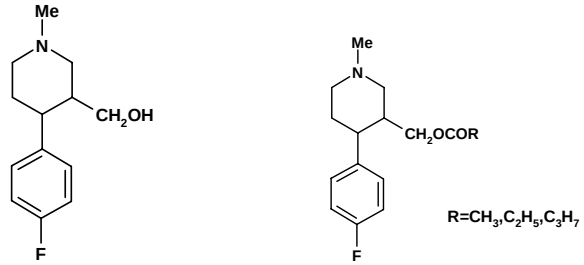
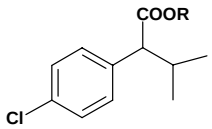
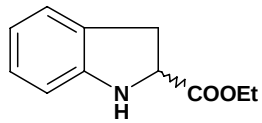
B: The hydrolysis of some chiral compounds using Est B.

Substrate	R	Conv. (%)	ee (%) of the product	Configuration
 I- Acyl ester of 1-(3,4-methylenedioxyphenyl)-ethanol.	CH_3 C_2H_5 C_3H_7	21 25 27	16 22 27	R R R
 II- 1-(3,4- methylenedioxyphenyl)-propan-1-ol	CH_3 C_2H_5 C_3H_7	28 24 23	17 13 19	R R R
 III- Acyl esters of 1-(3,4-methylenedioxy-5-yl) benzylalcohol	CH_3 C_2H_5 C_3H_7	No hydrolysis		
 IV- Ethyl- 2-hydroxy-4-phenyl butanoate		60	11	R ester
 V- Ethyl 3-hydroxy-3-phenyl-propanoate		No hydrolysis		
 R = CH_3, C_2H_5, C_3H_7 VI- Ethyl 3-(acyl)hydroxy-3-phenyl-propanoate	CH_3 C_2H_5 C_3H_7	No hydrolysis		

Continue Appendix B: The hydrolysis of some chiral compounds using Est B

Substrate	R	Conv. (%)	ee (%) of the product	Configuration
 VII- Acyl esters of 2-bromo propanoic acid	CH_3 C_2H_5 C_4H_9	45 42 37	1.8 3.8 3.8	S(acid) S (acid) S(acid)
 VIII- Acyl esters of 2-(6-methoxy-2-naphthyl)-propan-1-ol	CH_3 C_2H_5 C_3H_7	7 5 5	12 8 6	R R R
 IX- Acyl esters of 1-(6-methoxy-2-naphthyl) -ethanol.	CH_3 C_2H_5 C_3H_7	6 5 3	8 5 6	R R R
 X- alkyl esters of 2-(6-methoxy-2-naphthyl)-propanoic acid	CH_3 C_2H_5 C_3H_7	No hydrolysis		
 XI- Acyl ester of 1 – (p-chlorophenyl)-benzylalcohol.	CH_3 C_2H_5 C_3H_7	No hydrolysis		
 XII- 2- (p-(2-methyl propyl-2-phenyl) – propanoic acid	CH_3 C_2H_5 C_3H_7	No hydrolysis		

Continue Appendix B: The hydrolysis of some chiral compounds using Est B

Substrate	R	Conv. (%)	ee (%) of the product	Configuration
 XIII- Acyl esters of bisnapthol	CH_3 C_2H_5 C_3H_7	No hydrolysis		
 XIV- n-methyl 4-(p-flurophenyl)-3-acylmethylcyclohexamide	CH_3 C_2H_5 C_3H_7	No esterification No hydrolysis		
 XV- 1- (p-chloro phenyl) –2- methyl propanoic acid.	CH_3 C_2H_5 C_3H_7	No hydrolysis		
 XVI- Ethyl (indol-2-yl) formate		No hydrolysis		

Curriculum Vitae

Name Sameh Hamdy Soror

Day of birth 17.09.1974

Place of birth Cairo

Nationality Egyptian

Education:

2002-2003 "Diplombiologie" Dept. of Genetics, Faculty of Biology, University of Kaiserslautern, Germany, "Very good"

1999-2000 Pre-master courses Dept. of Biochemistry and molecular biology, Faculty of Pharmacy, University of Helwan, Cairo, Egypt, "Very good"

1992-1997 "Bachelor of pharmaceutical sciences" Faculty of Pharmacy, University of Cairo, Cairo, Egypt "excellent honor".

1989-1992 High school Elibrahimiah high school, Cairo, Egypt

1987-1989 Elhelmiah prep-school, Cairo, Egypt

Research experience:

06.2003-today	Research assistant and PhD student at Dept. of Genetics, Faculty of Biology, University of Kaiserslautern, Germany.
08.2002-05-2003	Master student at the Dept. of Genetics, Faculty of Biology, University of Kaiserslautern, Germany,
07.1998-10-2001	Demonstrator at the Dept. of Biochemistry and molecular biology, Faculty of Pharmacy, University of Helwan, Cairo, Egypt,.
10.1997-06.1998	Research assistant at "The national organization of drug control and research" Cairo, Egypt.

Publications

-A cold-active esterase of *Streptomyces coelicolor* A3 (2): from genome sequence to enzyme activity.

Sameh Soror, V. Verma, Ren Rao, Shafaq Rasool, S. Koul, G.N. Qazi and John Cullum Submitted.

-Cloning, overexpression and characterisation of an (HSL) acetylesterase of *Streptomyces coelicolor* A3(2)
Sameh Soror and John Cullum Submitted.

Presentations

-Cloning and characterization of an (HSL) acetylesterase from *Streptomyces coelicolor* A3(2)

Sameh Soror and John Cullum

158th meeting of SGM Warwick United kingdom. April 2006.

-A novel acetylesterase from *Streptomyces coelicolor* A3(2)

Sameh Soror and John Cullum

10th international congress on genetics of industrial microorganisms "GIM 2006". June 2006

Acknowledgment

I would like to thank Prof. Dr. J. Cullum for the excellent guidance and supervision, the careful reading of the thesis, precious comments and advises. I would also like to thank him for the trust and support and making it possible for me to carry out my “PhD” in Kaiserslautern.

Many thanks for Prof. Dr. Regina Hackenbeck for accepting to be the second corrector for this work.

I would like to thank Prof. Dr. J. Herrmann for being the chairman of my examination committee.

I would like also to thank HD. Mathias Redenbach for his help and support throughout the few years I have spent in the genetic department and for the proof reading of my thesis.

My thanks for Dr. V. Verma and his team at CSIR institute Jammu, India, for carrying out the enantioselectivity screening.

I am grateful for Dipl. Biol. Kerstin Wolf for her friendship and for being there in the difficult times.

Many thanks for all the PhD students, the Diplom students, and all the members of genetics Dept., whom I have worked with through my PhD work.

Great thanks for my parents, my brother and my sister for their love, support and encouragement.

At the end, there is no enough words to thank my small family “Chahira and Shorouk” for all their love, support, patience and for everything..... love you.

Hiermit versichere ich, die vorliegende Dissertation in der Abteilung Genetik der Universität Kaiserslautern selbständig durchgeführt und keine anderen als die angegebene Quellen und Hilfsmittel verwendet zu haben.

Kaiserslautern, im

Sameh Soror