ARABIDOPSIS THALIANA LIPASES: CLONING AND EXPRESSION IN *PICHIA PASTORIS*

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ARABIDOPSIS THALIANA* LIPASES: CLONING AND EXPRESSION IN *PICHIA PASTORIS

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Abstract

Lipases are a group of enzymes that hydrolyze the carboxyl ester bonds in acylglycerols releasing organic acids and glycerol. There is growing interest in identifying plant lipases because literature reviews show that plant lipases can be highly substrate specific and their substrate specificities can be useful in industrial applications. Accordingly, the aim of this study is to screen *Arabidopsis thaliana* putative lipases. *Arabidopsis thaliana* is chosen since it is a model plant organism with its genome sequenced. The strategy followed begins by cloning open reading frames that have sequences similar to known lipases, obtained from Arabidopsis Biological Research Center into *Pichia pastoris* expression vectors. *Pichia pastoris* is the host of expression because it is a host that is suitable for high yield expression without the requirement of time-consuming purification steps. The sequence verified constructs being transformed into *Pichia pastoris* are expressed in small scale in order to screen their lipase activities through a robust fluorogenic activity assay using 4-methylumbelliferryl derived substrates. The screening

performed in this study investigated twenty seven putative *Arabidopsis* lipase activities and resulted in seven proteins of particular interest that can be further investigated through large scale expressions. The open reading frames screened in this study were successfully cloned into *Pichia pastoris* expression vectors; hence the constructs for large scale expressions are available. These constructs can be directly used for further investigation that may result in the annotation of new *Arabidopsis thaliana* lipases of commercial value in industrial applications.

ARABIDOPSIS THALIANA LIPAZLARI: KLONLAMA VE PICHIA PASTORIS'TE EKSPRESYONU

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Özet

Lipazlar asilgliserollarda bulunan karboksil ester bağlarını hidrolize eden enzimlerdir. Bitki lipazlarının bulunması ilgi duyulan bir alandır çünkü bu lipazların belli substratlara karşı spesifik olduğu düşünülmektedir ve bu özellikleri endüstride kullanılabilir. Bu çalışmanın amacı *Arabidopsis thaliana* lipazlarının taranmasıdır. *Arabidopsis thaliana* seçilmiştir çünkü bütün genomu sekanslanmış bir model organizmadır. Kullanılan strateji *Arabidopsis thaliana*'nın bilinen lipazlara benzer sekanstaki genlerini *Pichia pastoris* ekspresyon vektörlerine klonlamakla başlamaktadır. *Pichia pastoris* ekspresyon sistemi olarak seçilmiştir çünkü yüksek miktarda protein üretebilen bir sistemdir ve bu proteinler zahmetli pürifikasyon yöntemleri kullanılmadan çalışılabilir. Sekansı doğrulanmış klonlar *Pichia pastoris*'e aktarılıp küçük miktarlarda ekspres edilmiştir. Küçük miktarlarda, 4-metilumbelliferil substratları kullanılarak bu lipazlar floresan test ile taranmıştır. Bu çalışma sonucunda 27 *Arabidopsis* lipaz aktiviteleri çalışılmıştır ve 7

tanesinin daha ilerideki alışmalar da kullanılmasına karar verilmiştir. Bu klonlar *Pichia pastoris*'te yüksek miktarda üretilmeye hazır durumdalar; böylece hızlıca yüksek miktarlarda üretilip çalışılabilirler. Sonuç olarak endüstriyel uygulamalarda kullanılabilecek *Arabidopsis thaliana* lipazları bulunabilir.

"To my mother, father and brother"

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I would like to express my gratitude to my thesis supervisor Assoc. Prof. Dr. Ugur Sezerman for his invaluable guidance, motivation, support and patience throughout this study. I will always be sincerely thankful for his advices that were not merely related to my academic development but also related to personal development. I would definitely not want to lose his support throughout my life.

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TABLE OF ABBREVIATIONS

ABRC	Arabidopsis Biological Research Center
AOX	Alcohol Oxidase
EB	Elution Buffer
EC	Enzyme Commission
GAP	Glyceraldehydes-3-Phosphate Dehydrogenase
GC	Gas Chromatography
GLA	γ-linolenic acid
HPLC	High Performance Liquid Chromatography
LS-LB	Low Salt Luria Bertani Medium
MBP	Maltose Binding Protein
mg	Milligram
MU	Methylumbelliferyl
MUH	4-methylumbelliferyl heptanoate

PCR	Polymerase Chain Reaction
p-nitrophenyl	Para-nitrophenyl
RPM	Revolutions per minute
SOC	Super Optimal Broth with Glucose
SU	Sabanci University
TAG	Triacylglycerol
TLC	Thin layer chromatography
YPD	Yeast Peptone Dextrose
μg	Microgram

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1 INTRODUCTION

Lipases are a group of enzymes that hydrolyze the carboxyl ester bonds in acylglycerols releasing organic acids and glycerols. True lipases (EC 3.1.1.3) are classified as enzymes catalyzing the hydrolysis of long-chain acylglycerols at an oil/water interface. Our major concern in this study is triacylglycerol acylhydrolases that act on triacylglycerols to liberate fatty acids and glycerols.

Studies on the known structures of lipases led to the finding that they have a characteristic α/β hydrolase fold with an active site consisting of serine-aspartate or glutamate-histidine. Around the active site serine, a consensus sequence of Gly-Xxx-Ser-Xxx-Gly is found, where Xxx can be any amino acid except proline (Nardini et al., 1999; Ueda et al, 2002). This active site is covered by an amphipathic helix called the lid, involved in interfacial activation at an oil/water interface. Explicitly, for the hydrolysis reaction to occur, the lid is displaced so that the substrate reaches the active site.

Lipases are ubiquitous enzymes. Many lipases from microbial origins, animals and plants have been found and characterized (Prim et al., 2003) though little is known on plant lipases as compared to mammalian and microbial lipases. These enzymes are recognized as valuable biocatalysts because they can be used in various industrial applications. In food, detergent, cosmetics and pharmaceutical industries, lipases possess an important role. The great versatility of lipases in industrial applications is not only due to the hydrolysis reactions they catalyze but also due to the synthesis reactions they can catalyze. Their stability in organic solvents enables them to catalyze synthesis reaction in the absence of bulk water amounts. Moreover, they have a wide range of substrates and they do not require cofactors. Examples of lipase catalyzed reactions include esterification, transesterification, interesterification and aminolysis reactions (Krishna et al., 2007).

Unique properties of lipases that make them important biocatalysts are their substrate specificity that can be either through regiospecificity, fatty acid specificity and

enantioselectivity. That is, there are lipases that can distinguish between primary and secondary ester bonds. Additionally there are lipases that are specific toward certain fatty acids although these enzymes are few as compared to those that are fatty acid chain length specific. The most important specificity that is of utmost use in organic chemistry is the enantioselectivity of lipases. That is to say, lipases can be used in kinetic resolution of chiral enantiomers (Ghanem et al., 2005).

Although very little is known on plant lipases, their versatile substrate specificities make their identification and characterization an interesting area of research. Since in plants, triacylglycerol lipases are thought to play an important role during germination and post-germinative embryonic growth by hydrolyzing the triacylglycerol lipases into fatty acids, plant TAG lipases have been purified from seedling sources of oilseed plants such as rapeseed, castor bean, mustard and maize (El-Kouhen et al., 2005). In fact this physiological role of plant lipases is supported by the finding that patatin domain triacylglycerol lipase from *Arabidopsis* seeds is associated with the oil body of the seed extracts in vivo (Eastmond, 2006). The fact that plant lipases can be extracted from safe and cheap starting materials as germinating seeds by relatively simpler methods make them potential substitutes of microbial lipases in industrial applications. Rapeseed lipases and oat lipases are some major examples of lipases used as biocatalysts of commercial value. The drawback of studying plant lipases is their low activity and transient expression in germinating seeds until the plant can support itself photosynthetically.

Arabidopsis thaliana is also an oil seed plant; accordingly, as expected crude extracts of *Arabidopsis* seedlings have lipase activity. However, this activity is also low (Verger et al., 2000). When the lipase consensus sequence (PROSITE pattern PS00120) was searched for similarities in the *Arabidopsis* genome, approximately 50 proteins with sequence similarities towards TAG lipases were found. Experimentally though, three such genes were annotated as lipases through direct assays. These lipases were characterized by El-Kouhen et al., Padham et al., and Eastmond et al.

As mentioned, *Arabidopsis* lipases have a low activity; hence their activity should be determined through sensitive assays and specific assays. Accordingly, radiometric, chromogenic or fluorogenic methods should be preferred. Although traditional assays using radiolabelled triacylglycerols are very sensitive, these methods are not preferred because they cannot be measured continuously. Besides, these methods are expensive. On the other hand, chromogenic and fluorogenic methods are sensitive and continuous

but their drawback is that artificial esters can undergo non-enzymic hydrolysis. That is to say, these assays are non-specific but can be scaled to 96-well format. Due to these mentioned problems, Verger and others set up a lipase assay using a naturally fluorescent substrate, parinaric acid. This is a TAG purified from *Parinari glaberrimum* seed oil. Upon hydrolysis, the released parinaric acid shows increased fluorescence, in the presence of detergents, proportional to the amount of lipase added in the assay. This new method also can be scaled to microtiter-plate measurements and can detect low lipase levels (Verger et al., 2000).

The fact that *Arabidopsis thaliana* genome contains approximately 50 hits that share homologies with known lipases suggests that these genes can show unique substrate specificities with much abundance and versatility. Consequently, in this study the *Arabidopsis* genome was searched for cDNA's coding for proteins with amino acid sequences similar to the known lipase sequences. 40 such genes were purchased from The Arabidopsis Information Resource (TAIR) to be cloned, expressed and characterized.

The aim of this study is therefore to develop a high-throughput method to clone these 40 genes, to express them and to perform lipase activity assays on them as a means of starting to characterize them. Consequenly, to achieve this aim, *Pichia pastoris* is chosen as the expression host. This is so because *Pichia* provides an easy and fast processing of the expressed proteins if vectors that carry an extracellular signal sequence are used. In this study, pPICZ derived vectors carrying α-mating factor pre-pro leader sequence (α-MF) of *Saccharomyces cerevisiae* are used for extracellular expression. Moreover, the use of the *Pichia pastoris* expression system rather than *Escherichia coli* for the expression of eukaryotic proteins, as is the case in this study, is more advantageous because most post-translational modifications like glycosylation, disulfide bond formation and proteolytic processing can be performed (Daly and Hearn, 2005). After the expression of the putative *Arabidopsis* lipases, assays that can be performed in microtiter-plate format are done in order to enable the initial characterization of the putative lipases in a high-throughput manner. Fluorogenic assays using esters of 4-methylumbelliferone that are commercially available in various acyl chain lengths are more specific than chromogenic methods; consequently, fluorogenic assays are performed in this study as a means of rapid and sensitive activity determination against various substrates.

Development of such a technique and determination of new plant lipases with unique substrate specificities from cheap and biologically safe material can be commercially valuable since lipases with new catalytic properties may have tremendous potential in industrial applications.

2 OVERVIEW

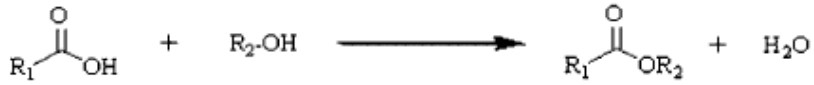
2.1 Historical Background on Lipases

2.1.1 Introduction to Lipases and Lipase Catalyzed Reactions

Lipases are a group of enzymes that belong to the class of alpha/beta hydrolases (Ollis et al., 1992). These enzymes express versatile activities; that is to say, they can show phospholipase, cutinase, amidase, cholesterol esterase and other esterase activities (Bornscheuer et al., 1999). Lipases of our concern are triacylglycerol acylhydrolases (belonging to the EC.3.1.1.3 group) catalyze the hydrolysis of triacylglycerols into fatty acids and glycerol at an oil-water interface (Schmid et al., 1998). Other than hydrolysis reactions, lipases can catalyze other reactions as well. These reactions are shown in the figure below.

Hydrolysis

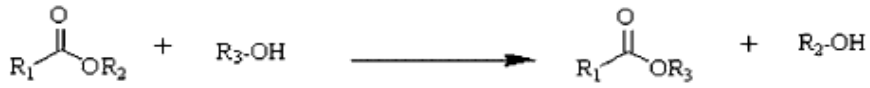
Esterification



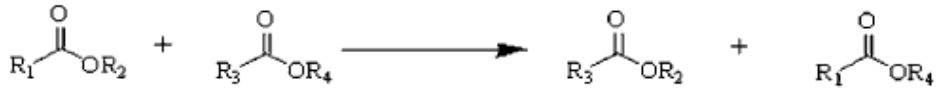
Transesterification

Acidolysis:

Alcoholysis:



Interesterification



Aminolysis

Figure 2-1 Lipase catalyzed reactions (Krishna et al., 2007).

The hydrolysis reaction of lipases is carried at an oil-water interface due to the fact that the natural substrates; of lipases, i.e. triacylglycerols, are insoluble in water (Gupta et al., 2004). This feature is called interfacial activation which was understood when the structural features of the lipases were elucidated. Lipase activity is increased at interface; nevertheless this does not mean that they do not show activity in bulk solutions (Verger, 1997).

2.1.2 Structural Features of Lipases

2.1.2.1 α/β Hydrolase Fold

The three dimensional structure of *Rhizomucor miehei* was the first structure to be solved in 1990 (Cygler et al., 1997). This result showed that this lipase had an α/β hydrolase fold. Later, it was elucidated that lipases with known 3-D structures showed the same fold (Ollis et al., 1992). That is, lipases contain a central β-sheet consisting of approximately eight paralel β-strands that are connected by around six α-helices (Schmidt-Dannert, 1999).

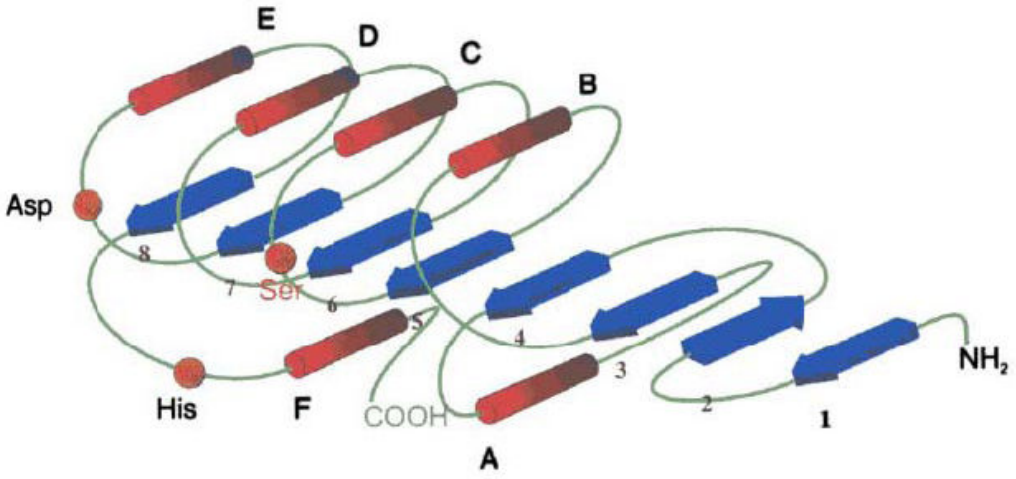


Figure 2-2 α/β hydrolase fold: Central β-sheet of eight β-strands (drawn as blue arrows) are connected by six α-helices (drawn as red cylinders) (Ollis et al., 1992).

2.1.2.2 The Catalytic Triad and the Lid

The configurations of the amino acid side chains were found to be stereochemically similar to that of serine proteases (Mukherjee et al., 2002). The active site of lipases consists of a catalytic triad of histidine, serine and aspartate or glutamate residues. These residues of the catalytic triad are shown in Figure 2-2

above. Similar to serine proteases, the catalytic residue in lipases is serine which is present on a loop as shown in figure 2-3.

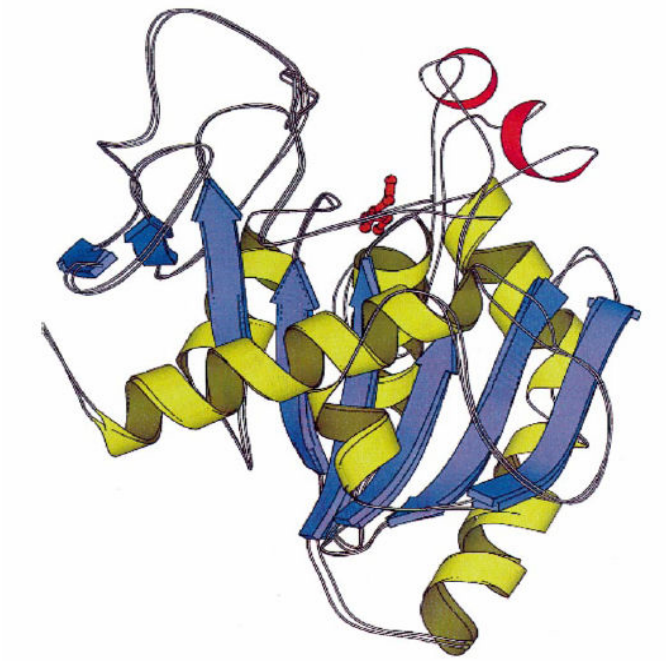


Figure 2-3 Overall Structure of lipase based on the crystal structure of *Humicola lanuginosa*; B-sheets are shown as blue arrows, α-helices are shown in yellow, catalytic Serine is shown in red sticks and the lid is shown in red (Svendsen, 2000).

Around the active site serine, a consensus sequence of Gly-Xxx-Ser-Xxx-Gly is found, where Xxx can be any amino acid except proline (Nardini et al., 1999; Ueda et al, 2002). However, in contrast to serine proteases, the active site of lipases is covered by a so-called "lid", which is an amphipathic helix (Mukeherjee et al., 2002). The solved 3-D structures of lipases showed that some, but not all, have a lid which has to be displaced from above the active site during interfacial activation. Although interfacial activation property of lipases had been used to define them and to distinguish them from esterases, it is no longer true due to the presence of lipases with no lids. Accordingly, lipases have been defined as carboxylesterases that hydrolyse long-chain acylglycerols (Verger, 1997).

Lipases can be of different origin. One of the most important features of diverse types of lipases from different sources is their substrate specificities. To find out the molecular mechanism behind substrate specificity, four main microbial lipase families have been analyzed with respect to the properties and shape of the fatty acid binding sites. These four main families are shown in Table 2-1. These families were classified according to their homology.

Table 2-1 Homologues microbial lipase families, sequences and X-ray structures (Schmidt-Dannert, 1999)

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

The members of the four lipase families are then divided into three subgroups according to their substrate binding sites. Namely, the subgroups are those lipases with crevice-like binding site near the surface, those with funnel-like binding site, and those with a tunnel-like binding site. These subgroups are shown in Table 2-2 below.

Table 2-2 Subgrouping of lipases according to their fatty acid binding sites (Schmidt-Dannert, 1999)

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

2.1.3 Reaction Mechanism of Lipases

The hydrolysis of esters is a five-step process. Firstly, a carboxylic acid ester binds via a hydrogen bond to the residues of the oxyanion hole. The Michaelis complex is formed.

In the resting state of the enzyme, the side chain hydroxyl proton of the active serine is part of a hydrogen bonding network comprising the catalytic triad. The oxygen of serine thus becomes more nucleophilic and can attack the carbon atom of the substrate. The negative charge of the serine is further moved to the ester oxygen and stabilised by H-bonds from different amino acid residues of the oxyanion hole. The resulting positive charge is located at the catalytic histidine and stabilized by the active site aspartate or glutamate, respectively. As a result, the first tetrahedral transition state is formed.

The active site histidine transfers the intermediately bound proton to the ester oxygen. The negative charge is then transferred from the oxyanion to the ester oxygen. The ester bond is then cleaved leading to an acylated enzyme and an alcohol molecule. Afterwards the alcohol in the binding site is replaced by a water molecule. Inverting the first steps, the water molecule binds to the acyl enzyme. A second tetrahedral intermediate is formed. Finally, the active site histidine transfers its proton to the serine and the ester has been cleaved (Figure 2-4).

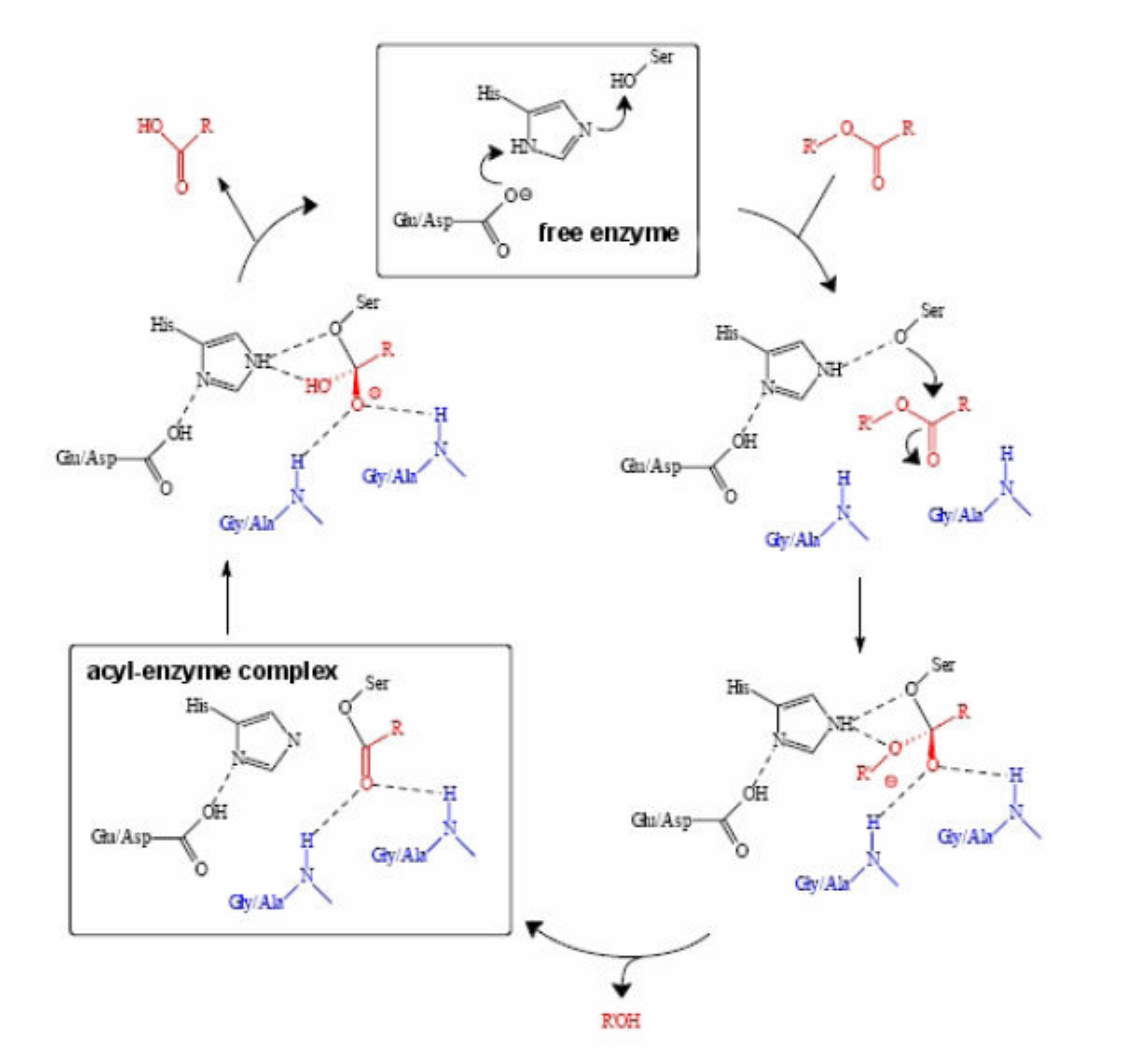


Figure 2-4 Reaction mechanism of lipases (Rusnak, 2004)

2.1.4 Substrate Specificity of Lipases

Substrate specificity can be achieved through regiospecificity, fatty acid specificity and enantioselectivity (Gupta et al., 2004).

2.1.4.1 Regiospecificity

Some lipases are regiospecific. That is, some lipases are 1,3 regiospecific; thus have the ability to distinguish between primary ester bonds (ester bonds at C1 and C3 positions) and secondary ester bonds (Stadler et al., 1995). Accordingly, they preferentially hydrolyze triacylglycerols at *sn*-1 and *sn*-3 positions rather than *sn*-2 positions and result in the production of 1,2(2,3)-diacylglyceride and 2-monoacylglyceride. The *sn* positions are seen in the figure below:

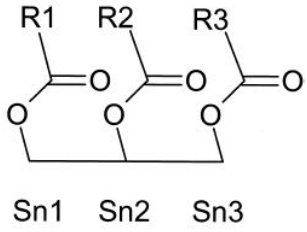


Figure 2-5 Positions of the glycerol moieties with the corresponding *sn* designations

For example, lipases from "*Bacillus* sp. (Sugihara et al. 1991; Lanser et al., 2002), *B. subtilis* 168 (Lesuisse et al., 1993), *Bacillus* sp. THL027 (Dharmsthiti and Luchai, 1999), *Pseudomonas* sp. f-B-24 (Yamamoto and Fujiwara, 1988, 1995), *P. aeruginosa* EF2 (Gilbert et al., 1991b) and *P. alcaligenes* 24 (Misset et al., 1994) were found to be regiospecific lipases.

2.1.4.2 Fatty Acid Selectivity

With respect to fatty acid selectivity, most lipases show fatty acid chain length specificity rather than fatty acid selectivity (Gupta et al., 2004). For example, lipases from *Bacillus* sp. (Wang et al., 1995), *P. alcaligenes* EF2 (Gilbert et al. 1991a, 1991b) and *P. alcaligenes 24* (Misset et al., 1994) were found to be specific for long-chain fatty acids; on the other hand, lipases from *B. subtilis* 168 (Lesuisse et al., 1993), *Bacillus* sp. THL027 (Dharmsthiti and Luchai, 1999), *P. Aeruginosa* 10145 (Finkelstein et al., 1970), *P. fluorescens* (Sugiura et al., 1977), *Pseudomonas* sp. ATCC 21808 (Kordel et

al., 1991), *C. viscosum* (Horiuti and Imamura, 1977) and *Aeromonas hydrophila* (Angultra et al. 1993) show preferential activity towards small or medium chain fatty acids. An example of a lipase that is known to show fatty acid selectivity is lipase from *Achromobacterium lipolyticum* (Davranov, 1994).

2.1.4.3 Enantioselectivity

Another important property of lipases based on substrate specificity is their enantioselectivity. That is, some lipases have the property of discriminating between the enantiomers of a racemic mixture (Reetz 2001). Most of the *Pseudomonas* family lipases have this property (Reetz and Jaeger 1998). This feature is quite important in the field of organic synthesis and pharmaceuticals.

2.1.5 Plant Lipases

Examples given above are microbial lipases. However, lipases are ubiquitous enzymes that can be obtained from microbial organisms, animals and plants. Although microbial lipases have been widely studied and have been found to be very important for industrial applications, there is growing interest in identifying plant lipases and using them as biocatalysts in industry. This is so because of the special properties of plant lipases, such as substrate specificity (Huang et al., 1988), and the cheap starting materials as dormant or germinating seeds (Kumarjee, 1994). Isolation of plant lipases by relatively simpler methods is another issue that makes the use of plant lipases in areas of commercial interest advantageous (Hassanien et al., 1986).

Major group of plant lipases consist of triacyglycerol acylhydrolases that hydrolyze the storage triacylglycerols in oil seeds. These lipases can be characterized as 'true' lipases. Plant lipases other than triacylglycerol acylhydrolases are characterized into two groups:

i) Non-specific acyl hydrolases that show the combinatorial effect of phospholipases A1 (EC 3.1.1.32), A2 (EC 3.1.1.4), B (EC 3.1.1.5), glycolipase, sulpholipase and monoacylglycerol lipase.

ii) Phospholipase C (EC 3.1.4.3) and phospholipase D (EC 3.1.4.4) (Mukherjee, 1994).

In plants, triacylglycerols have been purified and characterized from rapeseed (Hills et al., 1990, O'Sullivan et al., 1990), castor bean (Ory et al., 1962), peanut (Sanders et al., 1975), maize (Lin et al., 1983), oat (Martin et al., 1953) rice bran (Funatsu et al., 1971) and potato tubers (Hasson et al., 1976).

2.1.5.1 Applications of Plant Lipases

Applications of plant lipases in lipid biotechnology mainly involved the use of their "unique substrate specificities" (Kumarjee, 1994).

For example, in the case of oilseeds lipases were found to be abundant transiently in germinating oilseeds. Hence to obtain lipase preparations in a simple way, seedlings have been homogenized in Tricine buffer (pH 7.5) and then the sample has been centrifuged to obtain crude lipase preparations that can be directly used in biocatalysis reactions. (Hassanien, 1986)

Rapeseed lipase has been used in esterification and hydrolysis reactions to selectively discriminate against fatty acids with *cis*-6 or *cis*-4 double bond. That is, this lipase was used to increase the amount of γ-linolenic acid via kinetic resolution (Hills et al., 1989, Hills et al., 1990).

Explicitly,

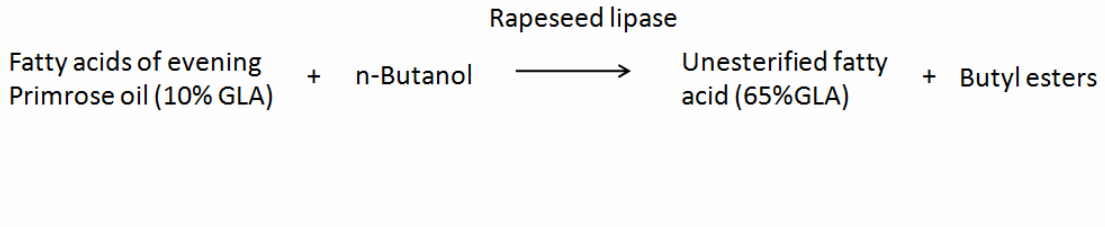


Figure 2-6 Esterification reactions of fatty acids of evening Primrose oil

Above is an esterification reaction that is carried out at 30°C in hexane. Since rapeseed lipase is selective against γ-linolenic acid, the amount of the fatty acid increased from 10% in the initial material to 65% after 72 hours of reaction (Hills et al., 1989). The same kind of increase in the relative proportion of γ-linolenic acid can be increased through a selective hydrolysis reaction as well (Hills et al., 1989).

The hydrolysis reaction is:

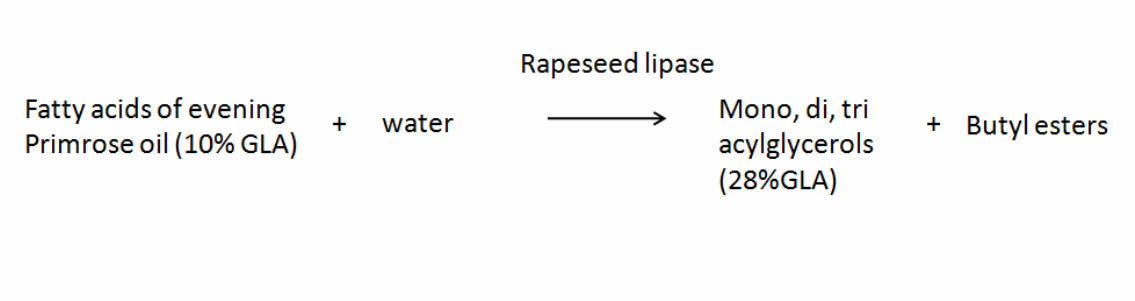


Figure 2-7 Hydrolysis reaction of triacylglycerols of evening Primrose oil

In the above selective hydrolysis reaction, the content of γ-linolenic acid in the unhydrolyzed acylglycerols increased from 10% to 28% after 60 minutes of reaction. Another important property of rapeseed lipases is that they can esterify acids into primary alcohols only; rather than secondary or tertiary alcohols (Hills et al., 1991). Consequently, "designed" esters can be synthesized through the use of rapeseed lipases.

Oat lipase is an example of another plant lipase that shows fatty acid chain length selectivity. It was found to rapidly hydrolyze oleoyl, elaidoyl, linoleoyl and linolenoyl moieties; on the other hand, palmitoyl, stearoyl and petroselenoyl moieties were selected against (Piazza et al., 1992).

Another plant lipase that shows regioselectivity is lipase from rice bran. This lipase preferentially cleaves fatty acids at *sn* 1, 3 positions (Funatsu et al., 1971). An additional example of a plant lipase used in biocatalysis is lipase from cotton plant that is used in transesterification reactions (Kadyrova et al., 1983).

Thanks to the above mentioned substrate specificities of different plant lipases, plants can be substitutes for microbial lipases in biocatalysis reactions (Palocci et al., 2003). However, the problem with plant lipases is that their activities are low. Usually, the hydrolysis rate was found to be less than 0.5μmol/min/mg. This problem was overcome through the characterization of lipases from *Euphorbia characias* latex or, in other words, laticifers (Palocci et al., 2003). Besides, as compared to microbial lipases, little is known about plant lipases. Their regulation, subcellular localisation and physiological roles are not known. However, it is known that they play an essential role during seed germination and embryonal growth following germination. Their role has been identified to be the mobilization of lipid storages in the seed (Bhardwaj et al.,

2001). Once plant lipases catalyze the hydrolysis triacylglycerols into free fatty acids and glycerol, the fatty acids are carried to glyoxysomes where they are converted to acyl-CoAs. Acyl-CoAs are then catabolyzed by β-oxidation into acetyl-CoA which is ultimately converted to sugar by gluconeogenesis (Eastmond, 2006). Since plant lipases are active during germination and post-germinative embryonal growth, their expression is transient (Huang et al., 1984). This poses another problem but once these problems are overcome, they can be important biocatalysts coming from a safe source that is available (Caro et al., 2000; Ncube et al. 1995).

2.1.5.2 *Arabidopsis thaliana* Lipases

Arabidopsis thaliana is a model dicot plant from mustard family. Since good lypolytic activity has been obtained from germinating mustard oilseeds (Mukherjee et al., 2002), then it has been suggested that *Arabidopsis thaliana* triacylglycerol (TAG) lipases can also show good activity.

The Arabidopsis Information Resource (TAIR) shows that there are 50 genes that are annotated as triacylglycerol lipases. However, only three of these were annotated based on direct enzymatic assays. The rest were annotated due to computational predictions.

The three triacylglycerol lipases were characterized by El-Kouhen et al., Padham et al., and Eastmond et al. El-Kouhen and others identified and characterized an *Arabidopsis* triacylglycerol lipase that is homologous to mammalian acid lipases. They used the lipase consensus sequence (PROSITE pattern PS00120) around the catalytic active site serine to search for *Arabidopsis* proteins. They identified more than 50 hits that shared homologies with known lipases and worked on At2g15230 gene that was shown to encode a protein that exhibits 30% identity towards human gastric and human lysosomal acid lipases.

The protein was extracted directly by the homogenization of the seedlings and lypolytic activity was isolated simply by centrifugation. This lipase was found to preferentially cleave long-chain fatty acids with a specific activity of 45 μmol/min/mg protein (El-Khouhen et al., 2005). This was found to be above the specific activities of

expressed plant cDNA's towards long chain triacylglycerols, which were found to be around 10^{-3} μmol/min/mg protein (Ishiguro et al., 2001; Hong et al., 2000).

Eastmond investigated the *sugar-dependent1* (*sdp1*) gene. This gene was identified by screening randomly mutagenised *Arabidopsis thaliana* seeds for stunted post-germinative growth that can be overcome by injection of sucrose. From these mutants, loci associated with the defective phenotype were found and one of them showing low activity in TAG hydrolysis was chosen for further investigation: *sdp1*. Positional cloning of *sdp1* led to the finding that the encoded protein contains a patatin-like domain (Pfam 01734). This domain has the "conserved Ser esterase motif (GXSXG)" (Eastmond, 2006). Although patatin is not active against TAG, patatin-like proteins from yeast and animals have been characterized as new TAG lipases (Anderson et al, 2002; Athenstaedt and Daum, 2003, 2005). Accordingly, the lypolytic activity of SDP1 was determined in vitro against TAG's using [^{14}C] triolein with a specific activity approximately 40 μmol/min/mg protein. This lipase was then characterized further by testing its activity against other substrates. It exhibited activity against long- and medium-chain saturated fatty acids and long-chain polyunsaturated fatty acids. The fact that the enzyme was more active against TAG than against diacylglycerol (DAG) and that it could not hydrolyze monoacylglycerol (MAG) suggested that this lipase is involved in the initial step of oil mobilization in germinating seeds. Consistent with this suggestion was the finding that SDP1 fused to GFP was localized with oil body membranes in vivo (Eastmond, 2006).

Padham and others used the full-length cDNA, At2g31690, from Arabidopsis that encodes a protein with GenBank accession number AAD24845. They obtained the cDNA by reverse-transcription PCR of the RNA from rosette leaves of 6 week old plants. This cDNA was chosen to be studied because the inferred amino acid sequence contained the active site sequence of lipases; i.e. [LIV]-X-[LIVAFY]-[LIAMVST]-G-[HYWV]-S-X-G-[GSTAC]. In addition, it was revealed by BLAST search that this protein showed 73% identity to another putative Arabidopsis TAG lipase. Based upon this computational prediction, to characterize this protein as a true lipase, the cDNA's were overexpressed in *Escherichia coli* as maltose binding protein (MBP) fusion proteins. MBP was used to purify the recombinant protein and to immobilize the fusion protein on amylose resin so that the activity tests can be applied in vitro. The rate of hydrolysis of TAG trilinolein (18:2) was measured in vitro. The mature protein AAD24845 fused to MBP was found to show increased activity when compared with

the activity of control MBP. By mature protein it was meant that the inferred protein AAD24845 originally had a transit peptide at its N-terminus that is cleaved; apparently for the protein to take on its active form. In this way, this protein was characterized as an Arabidopsis lipase. Besides, functional analysis had been made. Accordingly, it was revealed that this lipase was colocalized with neutral lipids in the chloroplast plastoglobuli and the suppression of the expression of this lipase curtailed growth (Padham et al., 2007). This is consistent with the predicted role of lipases in plants; that is, they mobilize fatty acids from plastoglobuli of chloroplasts.

Phospholipases have also been characterized in *Arabidopsis thaliana*. *DEFECTIVE IN ANTHER DEHISCENCE 1 (DAD1)* gene has been revealed to encode phopholipase A1, enzyme catalyzing the deesterification of *sn1* fatty acids of phospholipids (Ishiguro et al., 2001). Another Arabidopsis phospholipase that is UV-B inducible has also been characterized by Lo and others (Lo et al., 2004).

2.2 Methodological Background

In this section, a methodological background on lipase activity assay and *Pichia pastoris* expression system will be provided.

2.2.1 Methods to Measure Lipase Activities in Vitro

Lipases are enzymes that have a wide range of substrates; hence, there are a large number of assays developed to detect their activities. Since lipases are involved in a wide scope of physiological and industrial applications, development and optimization of sensitive and efficient in vitro assays becomes crucial for studying the activities of novel lipases. Here, an overview of common assays used for lipases will be presented with an emphasis on assays for lipases acting on triacylglycerols (Gilham and Lehner, 2004).

2.2.1.1 Titration

Titration is a traditional method to measure lipase activity. The amount of released fatty acids is measured via titration with sodium hydroxide (Stadler et al., 1995). The drawback of this method is that it is not very sensitive. Activities above 1 µmol/min are required for the assay to be considered reliable. Another problem is that it is not easy to measure acidic lipolysis since fatty acids are not totally ionized at low pH. Thus, this may lead to inacuurate measurements (Gilham and Lehner, 2004).

2.2.1.2 Chromogenic Assays

2.2.1.2.1 *Para*-Nitrophenyl Esters

p-Nitrophenol esters with various lengths of acyl side chains are commercially available. Laurate, palmitate and oleate are examples of long-chain esters whereas acetate and butyrate are short-chain esters. Acetate or butyrate esters are used to test esterase activity. To determine lipase activity, long-chain esters should be used. The problem with long-chain esters is that they are not readily solubilized in aqueous buffers; therefore emulsifying agents that would not inhibit lipase activity have to be utilized. For instance, the group of Gilham and Lehner used 0.01% Gum Arabic as the emulsifying agent (Kordel et al., 1991). Their reaction buffer contained 20mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.01% Triton X-100.

After the hydrolysis reaction, *p*-nitrophenol is released and the amount of liberated *p*-nitrophenol is measured spectrophotometrically at 410 nm. A blank without the enzyme should be used as a control. The advantages of this method are that it can be scaled into 96-well format and it requires commonly available equipments in the lab. Besides, kinetic measurements can be taken. The drawbacks of the method are that non-specific hydrolysis can occur; especially if short-chain esters are used. This kind of hydrolysis can be due to non-specific esterases or non-enzymatic proteins such as serum albumin or insulin. Another disadvantage is that the absorbance of p-nitrophenol varies

with pH but this problem can be overcome by using standards at different pH values (Gilham and Lehner, 2004).

2.2.1.2.2 Naphtyl Esters

Naphtyl esters of various chain lengths are also commercially available. Upon hydrolysis, naphtol is released. This molecule forms a colored product upon reacting with a diazonium salt. Absorbance of this colored product is measured at 560 nm in a ultraviolet-visible spectrophotometer (Gilham and Lehner, 2004).

For the reaction of lipases with napthyl esters, Gandolfi and others used a reaction medium of 100 mM Tris-HCl (pH 7.0) with 10 mg of Gum Arabic and 200 mg of sodium dioctyl sulfosuccinate (Galdolfi et al., 2000).

The advantages and the disadvantages of using these esters are the same as those for *p*-nitrophenyl esters (Gilham and Lehner, 2004).

2.2.1.2.3 Resorufin Esters

For another type of colorimetric assay, triacylglycerol analogues that have incorporated resorufin are used as chromogenic substrates for lipases. The figure below shows the structure of 1,2-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester and the products of hydrolysis occurring at the ester bonds.

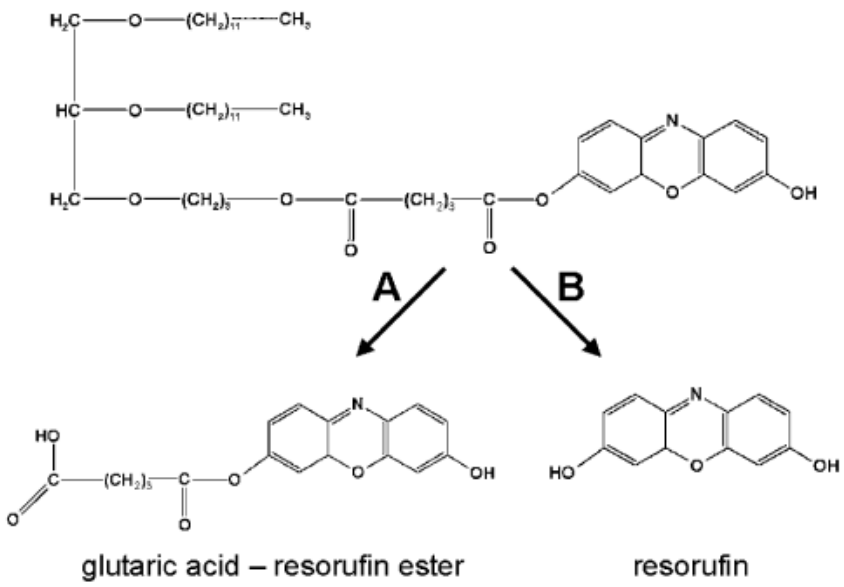


Figure 2-8 The chemical structure of 1,2-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester and the products of hydrolysis occurring at the ester bonds-glutaric acid – resorufin ester and resorufin (Gilham and Lehner, 2004).

Mass spectrometry results have shown that the abundant product resulting from this hydrolysis reaction by a lipase is free resorufin the absorbance of which can be measured spectrophotometrically at 572 nm (Bothner et al., 2000).

The reaction buffer used by Lehner and Verger consisted of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Triton X-100. Further dilution of the substrate was performed in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM sodium taurodeoxycholate (Lehner and Verger, 1997). This method can also be scaled down to 96 well format and kinetic measurements can be taken by taking absorbance measurements at 572 nm. A drawback of this assay is that since resorufin is polycyclic and not aliphatic as a fatty acid, it may not be hydrolyzed by some lipases (Gilham and Lehner, 2004).

2.2.1.3 Quantitation of Released Fatty Acids

2.2.1.3.1 Turbidometric Method

Turbidometric method is a spectroscopic assay that relies on increase in turbidity upon precipitation of the released fatty acids using calcium. Spectroscopic measurements are taken at 500 nm (Tigerstrom et al., 1989). This method was found to be more sensitive than titrimetric and chromogenic assays using *p*-nitrophenyl esters. Obviously this method cannot be used to measure lipase activity in turbid solutions, such as cell extracts. The reaction buffer for this assay consists of 20 mM Tris-HCl (pH 8.0), 1.8% Tween 20 (by volume) and 3 mM $CaCl_2$ (Gilham and Lehner, 2004).

2.2.1.3.2 Chromatography

Chromatographic methods are methods that directly determine the released fatty acids allowing the use of the naturally occurring lipid substrates. This is crucial for characterization of lipases. The problem with chromatographic methods is that only end point analyses can be made (Gilham and Lehner, 2004).

2.2.1.3.2.1 Thin Layer Chromatography

In TLC, radioactively labelled triacylglycerols are used and the quantity of released fatty acids are detected by densitometric, autoradiographic or scintillation counting. These methods carry the advantage of being very sensitive but include the disadvantages of radioactivity handling and time-consuming procedures (Gilham and Lehner, 2004).

2.2.1.3.2.2 Gas Chromatography

Mono-, di- and triacylglycerols and free glycerols released from lipase catalyzed hydrolysis reactions can be determined quantitatively using gas chromatography using an internal standard such as tridecanoylglycerol. This method is sensitive but requires

purified lipases to be used. The facts that the equipment is expensive and the procedure is laborious are other drawbacks of the method (Gilham and Lehner, 2004).

2.2.1.3.2.3 High Performance Liquid Chromatography

HPLC can be used to identify either the remaining acylglycerols or the released fatty acids in lipase catalyzed hydrolysis reactions. Identification and quantization of the fatty acids rely on the use of known standard. As in GC, HPLC is a useful method for determining the substrate specificities of lipases. However, this also is an expensive method and is time-consuming (Gilham and Lehner, 2004).

2.2.1.4 Fluorescent Assays

Fluorescent assay provide a quick, simple and sensitive method of activity determination through the use of fluorescently labeled substrates. The types of substrates used are listed below. Optimization of any one of the assays mentioned is very important for characterization of lipases and esterases. Since there are many non-specific esterases present in biological samples long acyl-chain acyl-glycerols should be used to determine true triacylglycerol lipase activity.

2.2.1.4.1 Pyrene

In fluorescent assays, once hydrolyzed, reaction products become fluorescent. The advantages of using fluorescent substrates are that kinetic measurements can be taken; moreover, fluorescent assays are very sensitive. Besides, they are less affected by the turbidity of reaction solutions (Gilham and Lehner, 2004).

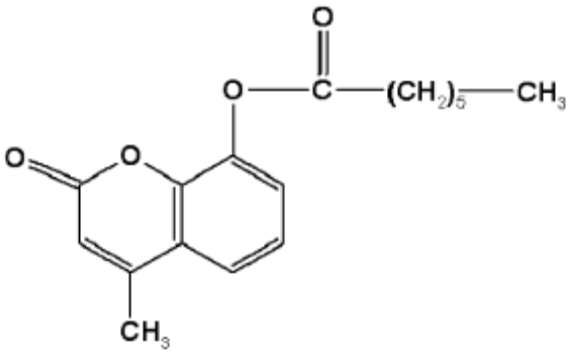
One type of fluorescent enzyme assay uses triacylglycerols labeled with a fluorescent moiety such as pyrene (Thuren, 1987). In this method a quencher should also be introduced into the substrate molecule to lower the basal level of fluorescence. Then as the substrate is cleaved by a lipase, the increase in fluorescence is measured

continuously at 375 nm (excitation 342 nm). Duque et al., proposed a protocol where the pyrene labeled substrate was dissolved in tetrahydrofuran (Duque et al., 1996).

Another method of measuring lipase activity with the use of pyrene involves 1,2-dioleoyl-3-(1-pyren-1-yl)decanoyl-*rac*-glycerol triacylglycerol analogue which contains a pyrene decanoic acid at a primary position of a triacylglycerol as one of the fatty acyl groups. This method gets use of the unique fluorescence property of pyrene that forms excimers when in close proximity. During the assay, the decrease in excimer fluorescence is measured as pyrene is liberated. Alternatively, an increase in pyrene fluorescence in the aqueous phase can be analyzed if the aqueous phase, containing liberated pyrene, is extracted with organic solvent. Since the ester linkage is at a primary position, total lipase activity will not be reflected by this method. Another drawback of the method is that triacylglycerols with modified pyrene moieties may be poorly hydrolyzed by some lipases due to steric hindrance. Despite these drawbacks, this assay is useful in the sense that it is sensitive and reproducible (Gilham and Lehner, 2004).

2.2.1.4.2 4-methylumbelliferone (4MU)

Esters of 4-methylumbelliferone are commercially available in various acyl chain lengths; hence can be used as substrate analogues of lipases in fluorogenic assays (Jacks et al., 1967). 4-methylumbelliferyl heptanoate (MUH) is commonly used in lipase assays rather than 4-MU-butyrate since MUH is insoluble in water and is not easily hydrolyzed by non-specific esterases. A protocol provided by Gilham and Lehner states that a stock solution of MUH is prepared in tetrahydrofuran and is diluted in a reaction buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA and 300 µM sodium taurodeoxycholate. As the ester bond is hydrolyzed, fluorescent compound is released and the increase in fluorescence can be measured kinetically at excitation/emission wavelengths 355/460 nm. The pros of this method are its sensitivity and the fact that it can be scaled down to 96-well format. The disadvantages include the substrate resembling monoacylglycerol rather than triacylglycerols (see Figure 7) and being spontaneously hydrolyzed (Gilham and Lehner, 2004).



4-methylumbelliferyl heptanoate

Figure 2-9 Chemical structure of MUH

2.2.1.4.3 Resorufin

Resorufin esters mentioned before as a chromogenic substrate can also be used as fluorescent substrates the hydrolysis of which can be measured kinetically at excitation/emission wavelengths 544/590 nm. The reaction buffer can be the same as that used for 4-MU esters (Gilham and Lehner, 2004).

2.2.2 Assaying *Arabidopsis thaliana* Lipases

Plant lipases are very low in abundance; thus, it is hard to purify amounts sufficient to study them in detail. That is why not too much is known on plant lipases. Moreover, *Arabidopsis thaliana* lipases show a very low activity even in the germinating oil seedlings extracts. Therefore development of sensitive and specific assays is crucial; furthermore a continuous assay is preferred. Accordingly, although assays using radiolabelled triacylglycerols are very sensitive, these methods cannot be measured continuously. Besides, these methods are expensive. On the other hand, chromogenic and fluorogenic methods are sensitive and continuous but their drawback

is that artificial esters can undergo non-enzymic hydrolysis. That is to say, these assays are non-specific (Verger et al., 2000).

Due to these mentioned problems, Verger and others set up a lipase assay using a naturally fluorescent substrate, parinaric acid. This is a TAG purified from *Parinari glaberrimum* seed oil. The excitation and emission wavelengths were found to be 324 and 420 nm, respectively. Almost half of the fatty acids obtained from *Parinari* oil contain the esterified form of parinic acid. Upon hydrolysis, the released parinaric acid shows increased fluorescence, in the presence of detergents, proportional to the amount of lipase added in the assay. This new method can be scaled to microtiter-plate measurements and can detect low lipase levels. The detection limit was found to be 0.1 ng of human pancreatic lipase in microtiter-plate format using *Parinari* TAG's as substrates (Beisson et al., 1999). The problem of this method is that the reaction should be carried under non-oxidative conditions because parinaric acid can be oxidized by atmospheric oxygen.

2.2.3 *Pichia pastoris* as an Expression System

For expression of heterologous proteins the methylotrophic yeast *Pichia pastoris* is a highly successful system. Pichia expression system has many important advantages:

The promoter derived from the alcohol oxidase I gene (AOX1) from *Pichia pastoris* is a very strong and very strictly regulated. The genetic manipulation of this system involves quite simple techniques. *Pichia pastoris* is extremely useful in the expression of eukaryotic proteins because most post-translational modifications like glycosylation, disulfide bond formation and proteolytic processing can be performed (Daly and Hearn, 2005). Furthermore, since *Pichia pastoris* is a haploid organism, any genetic modification is revealed in the phenotype of the next generations (Cregg et al., 2000). Another advantage of expressing protein in *Pichia pastoris* is that heterologously expressed proteins can be secreted into the medium through the use of secretion signal sequences, as the α-mating factor pre-pro leader sequence (α-MF) of *Saccharomyces cerevisiae* that is fused in frame to the gene of interest. Secretion of the expressed protein rules out the necessity of cell lysis and isolation of the expressed protein from cell extracts. This saves a lot of time. Moreover, high protein yields can be concentrated

in the supernatant because *Pichia pastoris* can grow to very high cell densities. Finally, another advantage of *Pichia* expression is the stability of the expression vectors due to the integration into the genome of *P. pastoris* (Daly and Hearn, 2005).

2.2.3.1 Strains and Phenotypes

The choice of a strain depends on the required application. Different strains that are available are shown in Table 2-3.

Table 2-3 Different strains of Pichia pastoris with their genotypes and phenotypes (Daly and Hearn, 2005)

Strain	Genotype	Phenotype
SMD1168	*His4, pep4*	Mut^+, His^-, $pep4^-$
GS115	*his4*	Mut^+, His^-
KM71	*his4, aox1: ARG4;arg4*	Mut^s, His^-
X-33	Wild type	—
MP-36	—	—
SMD1165	*His4, prb1*	Mut^+, His^-, $prb1^-$
SMD1163	*his4, prb1, pep4*	Mut^+, His^-, pep4, $prb1^-$
MC100-3	*arg4 his4 aox1Δ:: SARG4 aox2Δ:: Phis4*	Mut^-, His^-

The above table shows that strains GS115, SMD 118, SMD1165, SMD 1163 and KM71 do not have the ability to grow on a media that does not contain histidine because they are defective in the histidine dehydrogenase gene (*his4*). Those strains with Mut^+ phenotype are "methanol utilization plus" phenotypes and they grow on methanol at a normal, wild type rate. Strains with this phenotype have functional copies of the alcohol oxidase 1 and 2 genes (AOX1 and AOX2). On the other hand, strains with a Mut^s phenotype have a non-functional AOX1 but a functional AOX2 enzyme. AOX2 enzyme has a lower expression level; accordingly methanol is utilized slowly giving rise to a "methanol utilization slow" phenotype. Strains that are defective in both of the alcohol oxidase genes are said to have the Mut^-: "methanol utilization minus" phenotype (Daly and Hearn, 2005).

2.2.3.2 Expression Vectors

The choice of expression vector to be used also depends on the required application. There are many vectors available for both extracellular and intracellular expression. The first P. pastoris expression vectors are pHIL-D2 or pPIC9. They contain a functional copy of the histidine dehydrogenase gene that can be used for selection of positive transformants. From these vectors, pPIC9K was derived by adding GS418 (kanamycin) gene.

pPICZ derived vectors confer the antibiotic zeocin resistance though the *Sh ble* gene. These vectors have either AOX1 or GAP promoter. They may also contain α-mating factor pre-pro leader sequence (α-MF) for extracellular expression. Additionally, there are also vectors that do not contain signal sequence (Daly and Hearn 2005).

2.2.3.3 Choice of Promoter

The most widely used promoter is the AOX1 promoter because it is a strong promoter. The product of this gene is the alcohol oxidase enzyme and it regulates the methanol utilization of the cells (Cregg et al., 1993). A benefit of this promoter is that it can be switched off in the presence of glycerol or glucose. In this way toxic proteins may be expressed in *P. pastoris* because the protein expression will be induced once a biomass is established in repressive media (Waterham et al., 1997).

AOX2 is another promoter is *P. pastoris*; however it is a weak promoter. It is involved in only 10% of the production of the AOX enzyme. An alternative to the AOX promoters is the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter. This promoter is a constitutive promoter that leads to high levels of protein production. Nevertheless, this constitutive expression can lead to cytotoxic effects in the cells.

The promoter of the gene encoding formaldehyde dehydrogenase, FLD1, is another alternative promoter. This promoter is also induced by methanol and it requires a nitrogen source such as ammonium sulphate (Gellissen, 2000).

2.2.3.4 Culture Conditions

Culture conditions are important parameters that should be optimized for efficient *P. pastoris* expression.

In shake-flasks, limited aeration is one of the constraints of high level expression. For this reason baffled flasks are used to improve the level of expression (Vilatte et al., 2001).

A small-scale expression condition improving the level of expression in *Pichia* was developed by Barr et al. In this method the cells are grown to very high cell densities and then the cells are centrifuged and resuspended in small volumes of induction medium (Barr et al., 1992).

Another important parameter is the concentration of methanol since too much methanol may lead to methanol accumulation in the medium which may have adverse effects on the cells. Typically, 0.5-1.0% v/v methanol concentrations are used (Daly and Hearn, 2005).

Additionally, the components and the pH of the media affect the expression levels. For instance, when the medium was found to be buffered between pH 3.0 and 6.0, the amount of proteolysis was found to be reduced (Daly and Hearn, 2005).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All of the chemicals used are listed in Appendix.

3.1.2 Molecular Biology Kits

All of the molecular biology kits used for plasmid isolation, gel extraction and PCR purification are listed in Appendix.

3.1.3 Equipment

General laboratory equipments that are used are listed in Appendix.

3.1.4 Buffers and Solutions

Standard buffers and solutions are prepared according to *Molecular Cloning: A Laboratory Manual, Sambrook et al.,* 2001.

3.1.5 Strains

For subcloning purposes *Escherichia coli* XL1-Blue cells are used.

During expression studies, *Pichia pastoris* KM71H cells are used.

3.1.6 Media

E. coli strains were grown on LB (Luria-Bertani) plates or liquid media with the appropriate selection antibiotic. This mixture contains the tryptone, yeast extract, and sodium chloride, which are mixed in appropriate amounts. 20 g of LB Broth was used for preparation of 1 L liquid medium. The liquid medium was autoclaved at 121ºC for 20 min. before using.

Low Salt LB medium is used for strains grown under zeocin selection.

P. pastoris strains are grown on YPD (Yeast Peptone Dextrose) plates containing 1% yeast extract, 2% peptone, 2% dextrose. 2% agar is added if YPD plates are to be used. Plates older than a month were not used.

3.1.7 Plasmids and Vectors

Putative lipase ORF's purchased from ABRC (USA).

pPICZalphaA, pPICZalphaB and pPICZalphaC vectors are purchased from Invitrogen.

3.1.8 Primers

All primers are purchased from Microsynth (CH). The primers are diluted using PCR grade water according to the manufacturer's instructions.

F_(EcoRI)-pPICZaA

GATGAATTCCATCATCATCATCATCATAGGCCAGAAGGAGATATAACCATG

F_(PstI)-pPICZaB

GATCTGCAGGACATCATCATCATCATCATAGGCCAGAAGGAGATATAACCATG

F_(ClaI)-pPICZaC

GATATCGATCCATCATCATCATCATCATAGGCCAGAAGGAGATATAACCATG

R_(NotI)pPICZaABC

CTCGGCGGCCGCTAGAATTGTGAGCGCT

R_(XbaI)pPICZaABC

GATTCTAGATGTGAGCGCTCACAATTCTA

3.1.9 Enzymes

*Eco*RI, *Pst*I, *Cla*I, *Not*I, *Xba*I, *Bam*HI, *Hind*III, *Sac*I, *Pme*I restriction endonucleases (Fermentas)

T4 DNA ligase (Fermentas)

Klenow fragment (Fermentas)

Shrimp Alkaline Phosphatase (SAP) (Fermentas)

Pfu Polymerase (Fermentas)

Taq Polymerase (Fermentas)

3.1.10 Sequencing

Sequencing service was commercially provided by MacLab.

3.2 Methods

3.2.1 Cloning of the Putative Lipase Open Reading Frames into pPICZalphaA, pPICZalphaB and pPICZalphaC Vectors

3.2.1.1 Polymerase Chain Reaction by Pfu Polymerase

Those putative lipase genes that do not have an *Eco*RI site nor a *Not*I sit were amplified with **F_(EcoRI)-pPICZaA** and **R_(NotI)pPICZaABC.**

Those putative lipase genes that do not have an *Pst*I site nor a *Not*I site inside were amplified with **F_(PstI)-pPICZaB** and **R_(NotI)pPICZaABC.**

Those putative lipase genes that do not have an *Cla*I site nor a *Not*I site inside were amplified with **F_(ClaI)-pPICZaC** and **R_(NotI)pPICZaABC.**

The PCR conditions were as follows: initial denaturation at 94°C for 2 minutes followed by 30 cycles of denaturation step (at 94°C for 15 seconds), annealing step (at 53°C for 30 seconds) and an extension step (at 72°C for 120 seconds). These cycles were then followed by a final extesion step at 72°C for 7 minutes.

3.2.1.2 Gel Extraction of the PCR Products

The PCR products were run at 80V for 90 minutes using 1.2% agarose gels. Agarose gels were prepared using 1X TBE (Tris-Borate-EDTA) that had been prepared acording to the protocol in *Molecular Cloning: A Laboratory Manual, Sambrook et al.,* 2001. Qiagen gel extraction kit was then used.

3.2.1.3 Restriction of the PCR Products

Restriction of the PCR products were done according to the protocols provided by Fermentas.

3.2.1.4 Phenol/Chloroform Extraction of the Restriction Products

Restriction products were first diluted to 100 µl. Half volume of Phenol/Chloroform/Isoamylalcohol are added to the samples and mixed. The samples were then centrifuged for 10 minutes at 14,500 rpm and the upper phase was taken into fresh tubes. Then, to this phase 1/10 volume of 3M sodium acetate and 2.5 volumes of absolute ethanol was added. The mixture was centrifuged for 5 minutes at 14,500 rpm and the supernatant was discarded. The pellet was then rinsed with 70% ethanol twice and air-dired. Finally, the samples were eluted in appropriate volumes of elution buffer (EB).

3.2.1.5 Digestion of the Vectors

pPICZαA vectors were digested with *Eco*RI and *Not*I.
pPICZαB vectors were digested with *Pst*I and *Not*I.
pPICZαC vectors were digested with *Cla*I and *Not*I.
Digestions were done according to the protocols provided by Fermentas.

3.2.1.6 Gel Extraction of the Vectors

The cut vectors were run at 80V for 90 minutes using 1.2% agarose gels. Agarose gels were prepared using 1X TBE (Tris-Borate-EDTA) that had been prepared acording to the protocol in *Molecular Cloning: A Laboratory Manual,*

Sambrook et al., 2001. Qiagen gel extraction kit was then used to extract the vectors.

3.2.1.7 Ligation

Vectors extracted from gel were ligated with their corresponding phenol/chloroform extracted inserts in a 3:1 insert:vector ratio according to the protocol provided by Fermentas.

pPICZαA vectors were ligated with insert that are digested by *Eco*RI and *Not*I. Similarly, pPICZαB vectors were ligated with insert that are digested by *Pst*I and *Not*I and pPICZαC vectors were ligated with insert that are digested by *Cla*I and *Not*I.

3.2.1.8 Transformation into Competent XL1-Blue Cells

Competent cells were prepared according to the protocol in *Molecular Cloning: A Laboratory Manual, Sambrook et al.,* 2001. The competent cells were taken from the -80ºC refrigerator and kept on ice until they melt. Once the competent cells melt, 100µl of cells were added onto the ligation products in 1.5 ml eppendorf tubes cooled on ice before addition. The tubes were then kept on ice for 30 minutes. Heat shock was performed to the cells at 42ºC for 90 seconds. The tubes were then placed on ice again for 2 minutes and 750µl of SOC medium was added on the cells and the cells were incubated at 37ºC in the shaker for 45 minutes-1 hour, at 225 rpm. The cells were then centrifuged for 1 minute at 7000 rpm in a bench top centrifuge. Finally, in the laminar flow, the supernatant was removed until 100µl is left behind in each tube. The pellet is then gently resuspended in the 100µl of SOC medium and then 100 µl of the transformation mix was spread on LB-Zeocin plates containing 10-12 glass beads. Then the plates were incubated at 37ºC overnight for the cells to grow.

3.2.1.9 Colony PCR of Bacterial Cells

Colony PCR's on bacterial colonies were performed using Taq polymerase. The primers used were F_alpha and R_(NotI)-pPICZaABC.

To set up the PCR reaction a very small amount of the colonies were taken using a micropippette tip. Then the tip was the placed in the PCR tube and turned around firmly. Then to each tube 5 µl of ddH_2O was added and the tubes were kept at 95ºC for 5 minutes. Then the PCR master mix was added to each tube and the PCR reaction was started. The PCR conditions were as follows: initial denaturation at 94°C for 5 minutes followed by 32 cycles of denaturation step (at 94°C for 30 seconds), annealing step (at 53°C for 30 seconds) and an extension step (at 72°C for 90 seconds). These cycles were then followed by a final extesion step at 72°C for 7 minutes.

3.2.1.10 Culture Growth of Selected Colonies

Colonies that gave positive PCR result were chosen for growth in liquid media. Colonies were selected and grown overnight (for 12-16 hours) in LB medium containing 25 µg/ml zeocin at 37°C shaker. Glycerol stocks from these cultures were prepared according to *Molecular Cloning: A Laboratory Manual, Sambrook et al.,* 2001.

3.2.1.11 Plasmid Isolation

Qiagen miniprep kit was used for plasmid isolations.

3.2.1.12 Restriction Analysis and Sequencing

Restriction analysis of the isolated plasmids was done by digesting the plasmids with the appropriate enzymes and final confirmation of the clones was done by sequencing.

3.2.2 Transformation into *Pichia pastoris*

Restriction of positive clones with *Sac*I, *Pme*I or *Bgl*II

Restrictions were done according to the protocols provided by Fermentas. Those constructs containing only one recognition site of *Sac*I were restricted by *Sac*I preferably. If more than 1 restriction sites are present, then constructs are screened for restriction sites of *Pme*I and *BstX*I in the order of preference listed.

Transformation

The *P. Pastoris* KM71H strain was grown in 5 ml YPD overnight. The cells were then transferred into a larger volume of YPD such that the OD is 0.1-0.2. Once the OD reached 1.6 OD, 10 ml of cells were centrifuged at 2500 rpm, 10 min at 4°C for 1 transformation. The cells were resuspended in 8 ml of transformation buffer (100 mM LiAc, 100 mM DTT, 0.6 M Sorbitol, 10 mM Tris-HCl, pH 7.5) and incubated at room temperature for 30 minutes. Following a second centrifugation, the cells were resuspended in 1.5 mL of ice cold 1M Sorbitol and washed 3 times with 1.5 mL of 1M sorbitol. Finally, the cells are aliquoted in 80 µl of 1M sorbitol.

These competent cells were then mixed with 3-5 ng of restricted plasmids that will be transformed. The mixed samples were then electroporated at 1500 V, 175 Ω and 25 µF. Following electroporation, 1 mL of 1M sorbitol was added to the cells and the cells were shaken at 30ºC for one hour. Finally, they are spread on YPD plates containing 100 µg/ml zeocin.

Colony PCR

Colony PCR's on yeast colonies were performed using Taq polymerase. The primers used were F_alpha and R_(NotI)-pPICZaABC.

To set up the PCR reaction a very small amount of the colonies was taken using a micropippette tip. Then the tip was the placed in the PCR tube and the tubes were heated in a microwave at maximum heat for 3 minutes. Then the PCR master mix was added to each tube and the PCR reaction was started. The PCR conditions were as follows: initial denaturation at 94°C for 5 minutes followed by 32 cycles of denaturation step (at 94°C for 30 seconds), annealing step (at 53°C for 30 seconds) and an extension step (at 72°C for 90 seconds). These cycles were then followed by a final extesion step at 72°C for 7 minutes.

3.2.3 Expression

After the colony PCR positive *P. pastoris* clones were chosen for expression and grown in YPD overnight. The next day, 2 ml of the cells were transferred into 100 ml BMG (containing 100 mM potassium phosphate buffer, pH 6.0, 1.34% Yeast Nitrogen Base, 4 x 10^{-5} % biotin, and 1% glycerol in ddH_2O) and grown overnight. The next day, the cells were centrifuged at 2500 rpm for 5 minutes and the cells were then resuspended in 50 ml of BMM (containing 100 mM potassium phosphate buffer, pH 6.0, 1.34% Yeast Nitrogen Base,4 x 10^{-5} % biotin and 0.5% methanol in ddH_2O such that the starting OD for each culture is 30. First sample is taken. Then every 24 hour, a sample was taken and 5g/L of 100% methanol was given to the samples.

3.2.4 Lipase Activity Assays

The supernatants of the final samples of 50 ml expressions were used directly as the source of enzymes in the assays. SpectraMax Gemini XS from Molecular Devices (US) spectrofluorometer was used to measure fluorescence.

For each assay, standards giving the relative fluorescent units (RFU) corresponding to 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.13 µM, 1.56 µM, 0.78 µM, 0 µM of 4-MU in 100mM Tris-HCl buffer (pH 7.25) and ddH_2O.

For fluorescent assays, 96-well black microtiter plates (Costar) were used. To each well, 50 µl of 400 mM Tris-HCl (pH 7.25), 50 µl ddH_2O and 50 µl of the expressed samples were added. Then, just before the readings were initialized, 50 µl of 1mM 4-MU derived fluorescent substrates were added and the measurements were taken. The substrates used were 4-MU Butyrate, 4-MU Caprylate and 4-MU Caproate. During the assay, initial sample taken just after the methanol induction began was the blank of the assay for each expressed clone.

3.2.5 SDS-PAGE

SDS-PAGE gels were prepared and run according to the protocol in *Molecular Cloning: A Laboratory Manual, Sambrook et al.,* 2001.

3.2.6 Western Blotting

10% SDS-polyacrylamide gel was blotted on PVDF membrane at 200 mA constant current for 2 hours using the semi-dry blotting apparatus. Blotted membrane was blocked in blocking solution on orbital shaker overnight at 4ºC temperature. After washing the membrane in 1X TBS with 0.1% tween, the membrane was incubated with anti-his-HRP antibody (Roche) for 1 hour at room temperature. The membrane was then treated with ECL Advance Western Blotting Detection Kit (Amersham Biosciences) and resulting signals were analyzed by Hyperfilm ECL (Amersham Biosciences).

4 RESULTS

The 40 open reading frames (ORF's) were purchased from the Arabidopsis Biological Research Center were chosen such that they share similarities with known lipase sequences and are in pUNI51 vector (universal vector). The table below shows the ABRC codes of these ORF's and the Sabanci University (SU) codes given to them for this study along with the length of the sequences and the number of the restriction sites present within the sequences. This table also provides the expected molecular weight (MW) and the isoelectric point (pI) of the translation products of the ORF's.

Table 4-1 Open Reading Frames

		Restiction Sites						
SU Code	**ABRC Code**	**ClaI**	**PstI**	**EcoRI**	**NotI**	**MW (Da)**	**pI**	**length (bp)**
U001	U09178	0	1	1	0	39285	6,25	1087
U002	U10431	1	0	3	0	43024	4,97	1204
U004	U21413	0	1	0	0	41960	10,12	1149
U005	U21152	0	0	0	0	39575	6,26	1089
U006	U09850	0	0	0	0	35245	6,91	967
U007	U10780	0	1	0	0	39599	5,15	1117
U008	U10478	0	0	0	0	35705	7,83	985
U009	U09165	1	0	0	0	66123	6,51	1746
U010	U09272	0	2	0	0	69683	6,66	1870
U011	U09873	0	0	1	0	71691	5,85	1872
U012	U09966	0	0	1	0	50767	10,29	1423
U013	U10266	1	0	1	0	46057	4,88	1270
U014	U10446	1	0	0	0	60403	7,05	1621
U015	U12336	0	0	2	0	42427	6,30	753
U016	U12620	0	0	1	0	43611	8,62	1182
U017	U12709	1	0	1	0	36401	5,89	954
U018	U13183	1	2	0	0	40142	4,97	1095
U019	U13701	0	0	0	0	44061	7,82	1179
U020	U13852	0	0	0	0	41801	8,05	1119
U021	U14275	0	0	0	0	41595	7,72	1155
U022	U14432	1	0	1	0	96893	6,94	2547
U023	U14443	0	0	0	0	60428	6,18	1584
U024	U14794	0	0	1	0	39816	8,57	1056
U025	U14845	0	0	0	0	15161	5,42	420
U029	U18922	2	1	0	0	48124	9,22	1333
U030	U19788	0	0	1	0	41555	8,77	1162
U031	U21453	1	0	0	0	27156	6,42	729
U032	U21478	0	0	1	0	71737	5,76	1890
U033	U21703	0	1	1	0	58181	9,36	1572
U034	U22031	1	0	0	0	79533	7,01	2106
U101	U82581	0	0	1	0	44338	5,61	1182
U102	U60295	1	0	0	0	79533	7,01	1125
U103	U10742	1	0	1	0	80144	7,82	2146
U104	U22182	1	0	0	0	54882	9,05	1440
U105	U13565	1	1	0	0	50291	8,56	1335
U106	C104831	1	0	1	0	54205	5,17	1441
U107	U67611	0	0	0	0	40781	6,52	1077
U108	U13081	0	0	1	0	78494	5,17	2142
U109	U13874	1	1	0	0	73151	8,85	1950
U110	U20520	1	1	1	0	40004	4,90	1134

4.1 PCR Based Cloning of the Putative Lipase ORF's into pPICZalpha derived Vectors

Those genes not containing an *Eco*RI and *Not*I sites were cloned into pPICZalphaA vectors via PCR based cloning. Those clones and the primers are listed in the table below.

Table 4-2 SU Codes of the ORF's cloned into pPICZalphaA vector

SU Code	Primers
U002	F_(EcoRI)-pPICZaA, R_(NotI)-pPICZaABC
U006	
U007	
U008	
U010	
U014	
U017	
U018	
U020	
U021	
U029	
U109	

Table 4-3 SU Codes of the ORF's cloned into pPICZalphaB vector

SU Code	Primers
U005	F_(PstI)-pPICZaB, R_(NotI)-pPICZaABC
U009	
U011	
U012	
U013	
U015	
U016	
U017	
U019	
U022	
U023	
U024	
U025	
U030	
U031	
U032	
U034	
U101	
U102	
U103	
U104	
U106	
U107	
U108	

Table 4-4 SU Codes of the ORF's cloned into pPICZalphaC vector

SU Code	Primers
U001	F_(ClaI)-pPICZaC, R_(NotI)-pPICZaABC
U004	
U033	

The above clones were PCR amplified using *Pfu* DNA polymerase.

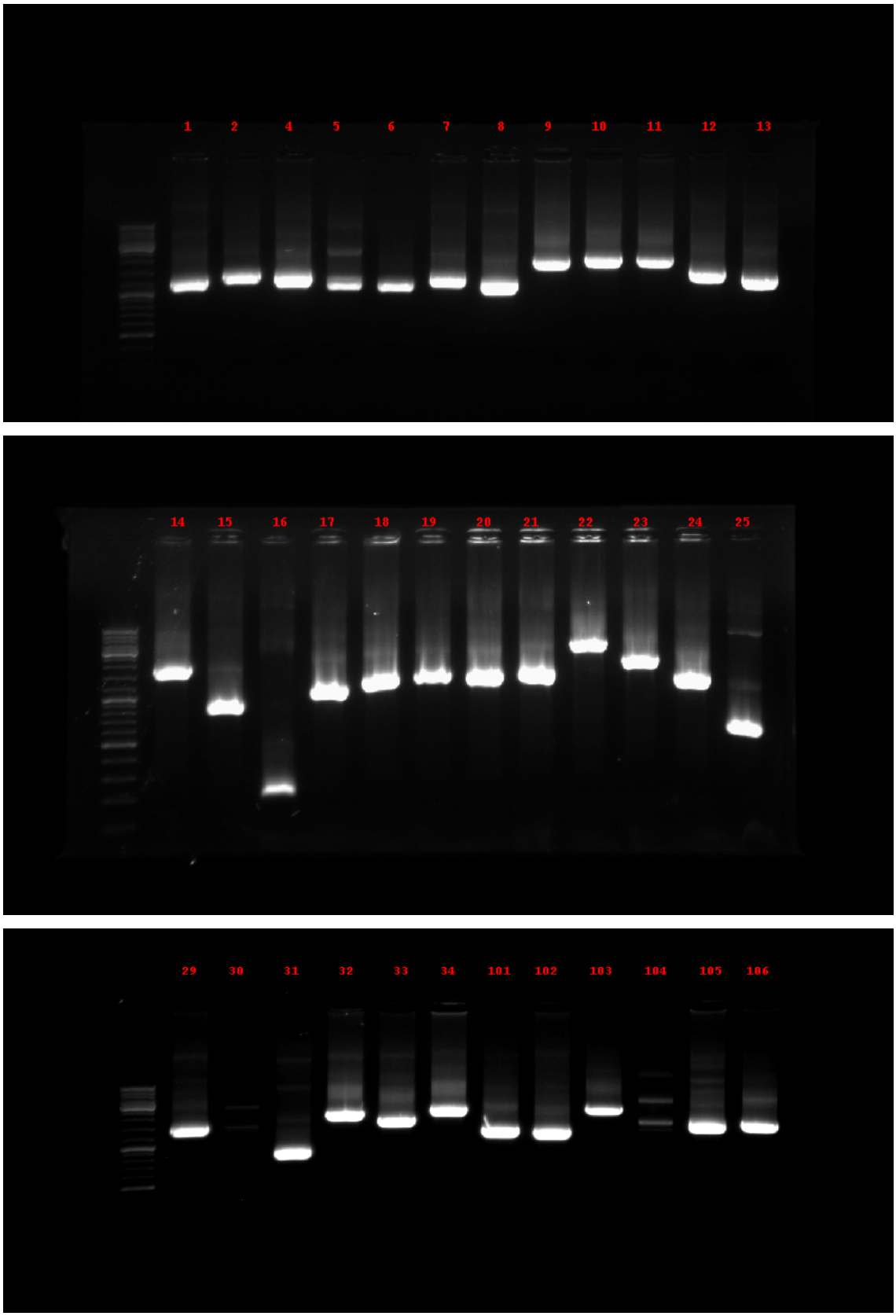


Figure 4-1 PCR results of the UXXX ORF's

All of the above were successfully amplified except U16 which gave a PCR result indicating a sequence length shorter than the length of the U16 ORF.

The PCR products were gel extracted, purified and digested. The ORF's in Table 4-1 were digested by *Eco*RI and *Not*I. Those listed in Table 4-2 were digested by *Pst*I and *Not*I. On the other hand, those in Table 4-3 were digested by *Cla*I and *Not*I. Restriction products were then phenol/chloroform extracted.

The vectors were digested at the same time. pPICZalphaA was digested by *Eco*RI and *Not*I. pPICZalphaB was digested by *Pst*I and *Not*I. On the other hand, pPICZalphaCwas digested by *Cla*I and *Not*I. Figure below shows the digestion results.

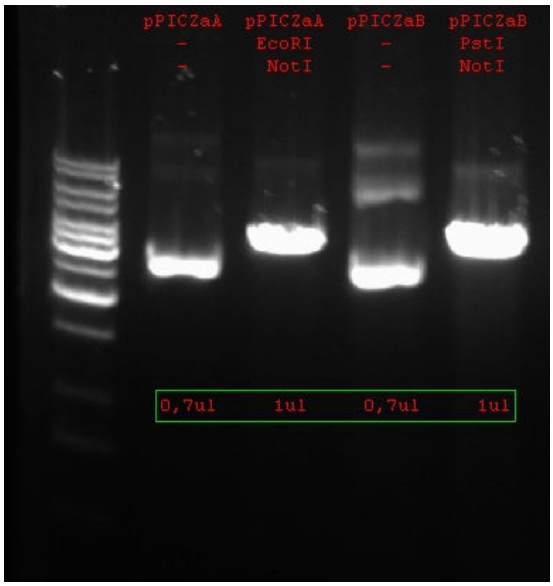


Figure 4-2 Digestion of Vectors

The PCR amplified and restricted inserts listed in Table 4-1 were ligated into pPICZalphaA vector. Similarly, those listed in Table 4-2 were ligated into pPICZalphaB vector and those listed in Table 4-3 were ligated into pPICZalphaC vector.

After the transformation of the ligation mixtures into *E. coli* XL1-blue strains, the colonies in LS-LB-Zeocin plates that give positive colony PCR results were chosen and confirmed further by restriction analysis. Since there are too many colony PCR results, these data are not shown. For further confirmation, the plasmids isolated from positive colonies were analyzed by restriction analysis. Insertion of the ORF's in table 4-1 was checked by restriction analysis with *Eco*RI and *Not*I. Likewise, Insertion of the ORF's in table 4-2 was checked by restriction analysis with *Pst*I and *Not*I and insertion of the

ORF's in table 4-3 was checked by restriction analysis with *Cla*I and *Not*I. Below are the results.

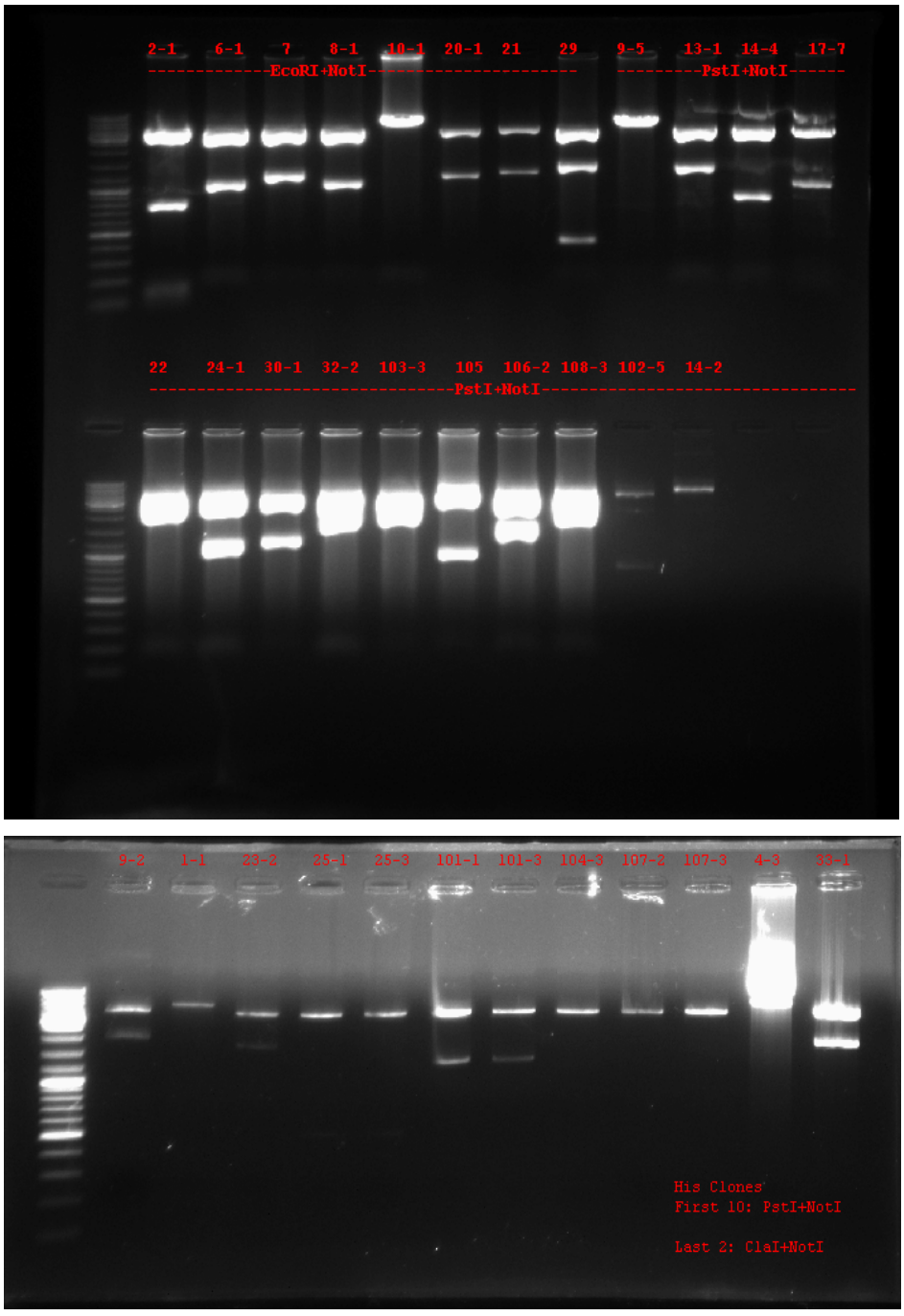


Figure 4-3 Restriction Analysis of Positive Clones

For further confirmation, the clones were also cut asymmetrically from the inside of the ORF's; however, the results of this analysis are not shown for simplicity.

Finally, sequencing results showed which ORF's were successfully inserted into pPICZalpha A, B and C vectors.

Table 4-5 Clones that are sequence verified

Clones	Sequence Verified	Confirmed by Restriction Analysis
U002	√	√
U006	√	√
U007	√	√
U008	√	√
U009	√	√
U010	√	√
U011	X	√
U013	√	√
U014	√	√
U015	√	√
U017	√	√
U018	√	√
U019	X	√
U020	√	√
U021	√	√
U022	√	√
U023	√	√
U024	√	√
U025	√	√
U029	√	√
U030	√	√
U031	√	√
U032	√	√
U033	√	√
U034	√	√
U101	√	√
U102	√	√
U103	√	√
U104	X	X
U105	√	√
U106	√	√
U107	X	X
U108	√	√
U109	X	X

Those clones that have been sequence verified were transformed into *Pichia pastoris*. Randomly chosen colonies on YPD-Zeocin plates were screened by colony PCR.

Colonies that have been shown to have the clones integrated in their genome were streaked 2-3 times on YPD-Zeocin plates prior to expression.

Expressions in *P. pastoris* were carried out for 6-7 days in 50 mL scale. In the end of the expression, lipase activity assays were performed directly using the supernatants.

4.2 Activity Assays

Fluorescent activity assays were performed using 4-MU derived substrates in SpectraMax Gemini-XS spectrofluorometer.

Prior to the assays, different types of substrates in our hands were tested for their stability in the reaction medium. 4-MU Caproate, 4-MU Laurate, 4-MU Caprylate, 4-MU Palmitate, 4-MU Elaidate, 4-MU Butyrate and 4-MU acetate were tested. In the end, 4-MU Butyrate was chosen as the substrate with medium chain length; whereas, 4-MU Caprylate was chosen as the substrate for long chain length since they were stable in reaction medium and resulted in low fluorescent readings.

Firstly, the assays were performed directly on 100 µl of the supernatants of *Pichia* expressions. As an example, one of the expressed clones will be taken into consideration and the calculations done will be shown on that clone. Since there are too many assays done, the results will be given directly for the rest. Taking the activity of U5-4 against 4MU-Butyrate into consideration, firstly a standard curve was drawn showing how much relative fluorescence unit corresponds to a certain concentration of 4-MU released. Different concentrations of 4-Mu's prepared were 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.625 µM, 7.8125 µM, 3.906 µM and 0 µM in Tris-HCl buffer (pH 7.25) and water. The points that distrupt the linearity were thrown, in this case the data for 250 µM, and a trendline was added. According to the equation of the trendline, the velocity values provided by the software of the fluorimeter are converted from milliunits per minute to µM of 4-MU released per minute. For U5-2, the standard curve is shown in Figure 4-4.

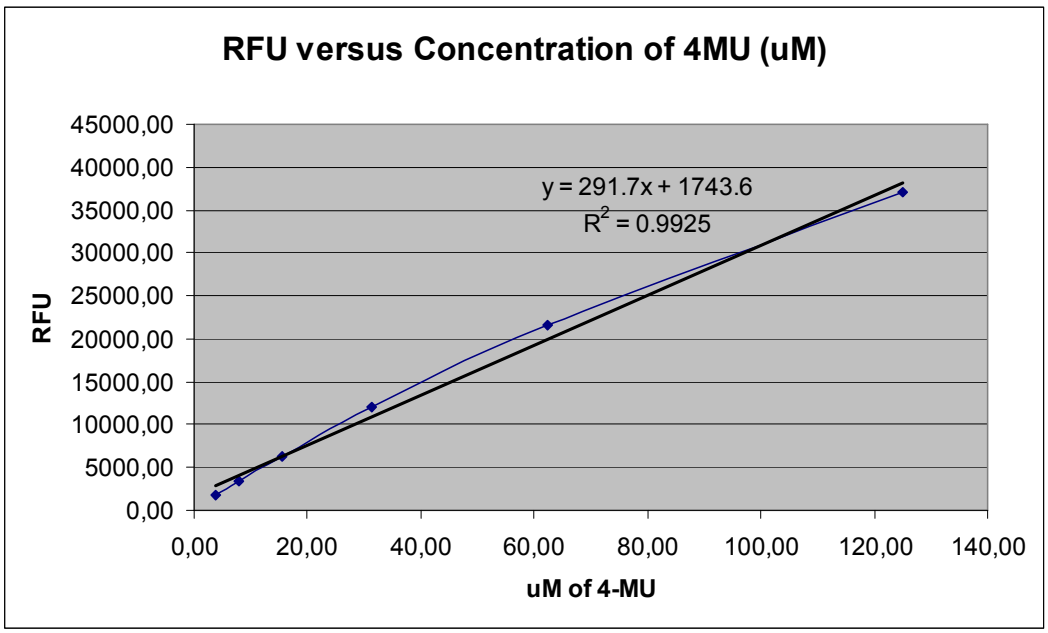


Figure 4-4 Standard Curve for U5-2 supernatant

The velocities of the final expression sample of U5-2 was given to be 474473.4 milliunits per minute. This results in (474473.4 -1743.6) / 291.7 = 1620.603 nM of 4-MU released per minute. The blank was the fluorescence provided by 4-MU-Butyrate in reaction buffer. The blank resulted in 56.48 µM of 4-MU released per minute. This value was subtracted from 1620.603 and the activity of U5-2 was found to be 1564.125 µM of 4-MU released per minute. For 200 microliter reaction 1nM 4MU equals to 0.2 nmol 4MU; so 1564.125 µM is equal to 312.825 nmol 4MU / min. Figure 4-5 also shows how the activity increases with increasing days of expression.

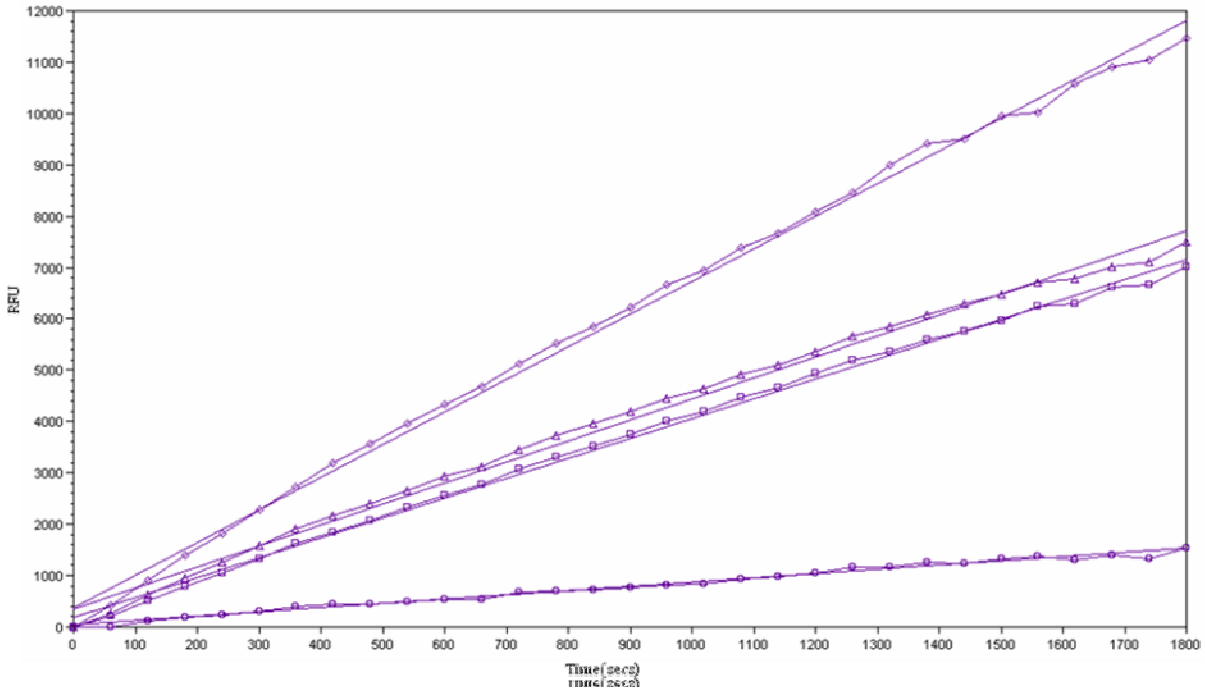


Figure 4-5 Kinetic Assay on U5-2 Expression Samples: ○ Blank Sample, □ Day 5 Sample, Δ Day 6 Sample, ◊ Day 7 Sample.

Similarly, the same calculation was applied to all samples. The table below shows only the significant activities obtained.

Table 4-6 Activities of the Given Samples against 4MU-Butyrate

Samples	nmol 4MU-Butyrate/min
U004	106.234
U005	312.824
U008	86.000
U010	55.830
U017	91.770
U020	94.084
U021	99.754
U022	36.568

The activities of some other samples were measured after freeze-thawing the samples. This resulted in very little activity.

Table 4-7 Activities of the given samples against 4MU-Butyrate after freeze-thawing

Samples	nmol 4MU-Butyrate/min
U002	3.360
U006	1.004
U007	2.484
U008	2.204
U009	0.000
U013	0.216
U015	0.502
U019	0.000
U024	2.644
U032	4.216
U103	2.818
U106	1.622
U108	2.576

To see whether the activity will increase if the samples are concentrated or not, all of the samples in our hands were lyophilized. 3 ml of the samples were lyophilized and dissolved in 300 µl of ddH_2O. The samples were then passed through desalting columns and their activities were measured against 4MU-Caprylate and 4MU-Butyrate. The figures below show that towards 4-MU Caprylate, the activity is increased. As blank, the substrates in reaction buffer are used.

Table 4-8 Activities of the given samples against 4MU-Caprylate and 4MU-Butyrate

Samples	Substrates	
	nmol 4MU- Caprylate/min	nmol 4MU-Butyrate/min
U002	51.657	40.756
U006	80.692	3.638
U007	70.028	2.592
U008	79.530	14.670
U009	7.376	7.660
U010	0.238	4.886
U013	3.178	7.300
U014	29.370	21.192
U017	2.428	7.164
U018	112.712	36.458
U020	0.000	4.444
U021	0.000	4.574
U024	0.306	4.020
U029	28.750	5.116
U030	94.842	24.392
U032	120.086	47.440
U103	55.572	3.870
U105	140.852	0.864

These assays were done in replicates and the values provided are the mean values.

The figures below clearly show that for the majority of the samples, activity is higher against 4MU-Caprylate than it is against 4MU-Butyrate.

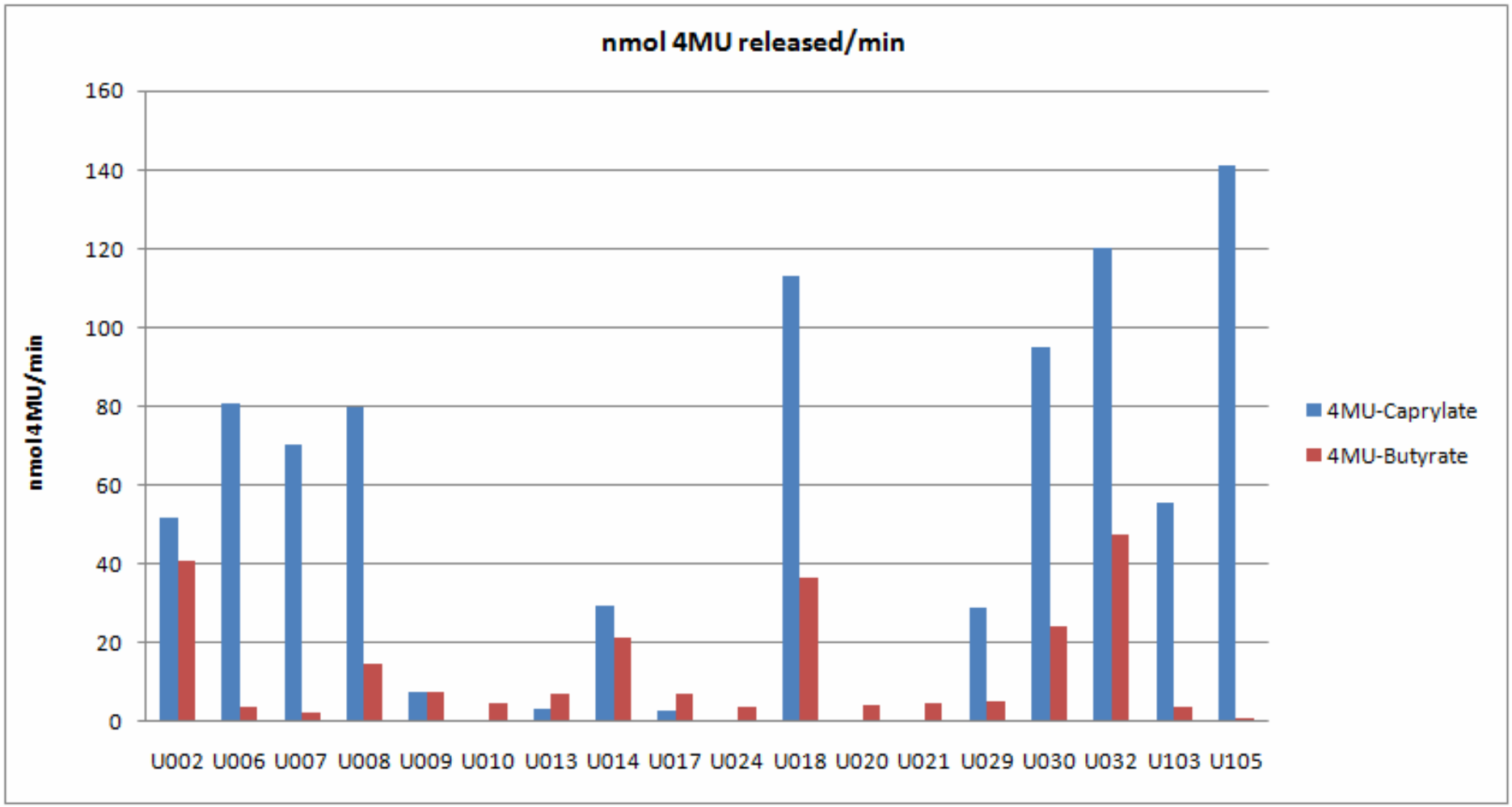


Figure 4-6 Comparison of the activities of the supernatants against 4MU-Caprylate and 4MU-Butyrate

4.3 Domain Search

The results show that U6, U7, U8, U18, U30, U32 and U105 show distinctive activity against caprylate rather than butyrate. On the other hand, U10, U20, U21, and U24 show little activity only towards butyrate. To check whether those proteins with significantly higher activity towards 4MU-caprylate have domains different from the ones not active against 4MU-caprylate, domains in the putative lipases were searched. U10 has Arabidopsis, phospholipase-like domain (InterPro: IPR007942). U20, on the other hand, is similar to putative *Arabidopsis thaliana* myrosinase-associated protein, and contains InterPro domain: GDSL-like lipase (InterPro: IPR001087). U21 is found to be PLA IIIB/PLP9 (Patatin-like protein 9) and contains InterPro domain Acyl transferase/acyl hydrolase/lysophospholipase (InterPro: IPR016035); additionally it contains the InterPro domain Patatin; (InterPro: IPR002641). U24 gives hit to esterase/lipase/thioesterase family protein; it is similar to esterase/lipase/thioesterase family protein of Arabidopsis thaliana (TAIR: AT3G62860.1) and contains the InterPro domain Alpha/beta hydrolase; (InterPro: IPR003089)

With respect to the clones showing high activity towards caprylate, U6 gives hit to alpha/beta fold family protein and is found similar to esterase/lipase/thioesterase family protein in *Arabidopsis thaliana* (TAIR:AT3G55180.1). It contains InterPro domain of alpha/beta hydrolase; (InterPro: IPR003089) as well as the alpha/beta hydrolase fold-1 (InterPro: IPR000073). U6 also gave good alignment results with known lipases. This result is shown in the appendix section. U7, on the other hand, has the GDSL-motif of lipase/hydrolase family proteins and contains the InterPro Lipase, GDSL domain: (InterPro: IPR001087). The alignment of U7 is also shown in the appendix section. These alignments show that U6 and U7 have the catalytic triad.

U8 is found similar to hydrolase, alpha/beta fold family protein of *Arabidopsis thaliana* (TAIR: AT2G39410.2) and contains the InterPro domain of alpha/beta hydrolase; (InterPro: IPR003089) as well as the alpha/beta hydrolase fold-1 (InterPro: IPR000073).

U18 is similar to GDSL-motif lipase/hydrolase family protein of *Arabidopsis thaliana* (TAIR: AT1G29670.1) and contains the InterPro GDSL-Lipase domain:

(InterPro: IPR001087). Likewise, U30 is similar to GDSL-motif lipase/hydrolase family protein of *Arabidopsis thaliana* (TAIR: AT5G37690.1) and contains InterPro domain Lipase, GDSL, active site; (InterPro: IPR008265 and InterPro: IPR001087).

U32 gives hit to lipase class 3 family protein / disease resistance protein-related and is similar to signal transducer/triacylglycerol lipase Arabidopsis thaliana (TAIR: AT3G48090.1) and contains InterPro domain Lipase, class 3; (InterPro: IPR002921). Similarly, U105 gives hit to lipase class 3 family protein and is similar to lipase class 3 family protein of *Arabidopsis thaliana* (TAIR:AT2G30550.2 and TAIR:AT2G30550.1). It contains InterPro domain of Lipase, active site; (InterPro: IPR008262) and InterPro domain of Lipase, class 3; (InterPro: IPR002921).

4.4 SDS-PAGE and Western Blotting

SDS-PAGE analysis of the expressed proteins did not reveal any distinct bands because the abundance of the proteins is too low. Even when the proteins were concentrated 10 times, they could not be detected by visualization of SDS-PAGE gels. The concentration levels of the expressed protein that reveal lipase activity against 4MU-butyrate and 4MU-caprylate are so low that even Western blotting could not detect all of the expressed proteins. However, for two of them, bands were observed but at a lower molecular weight position.

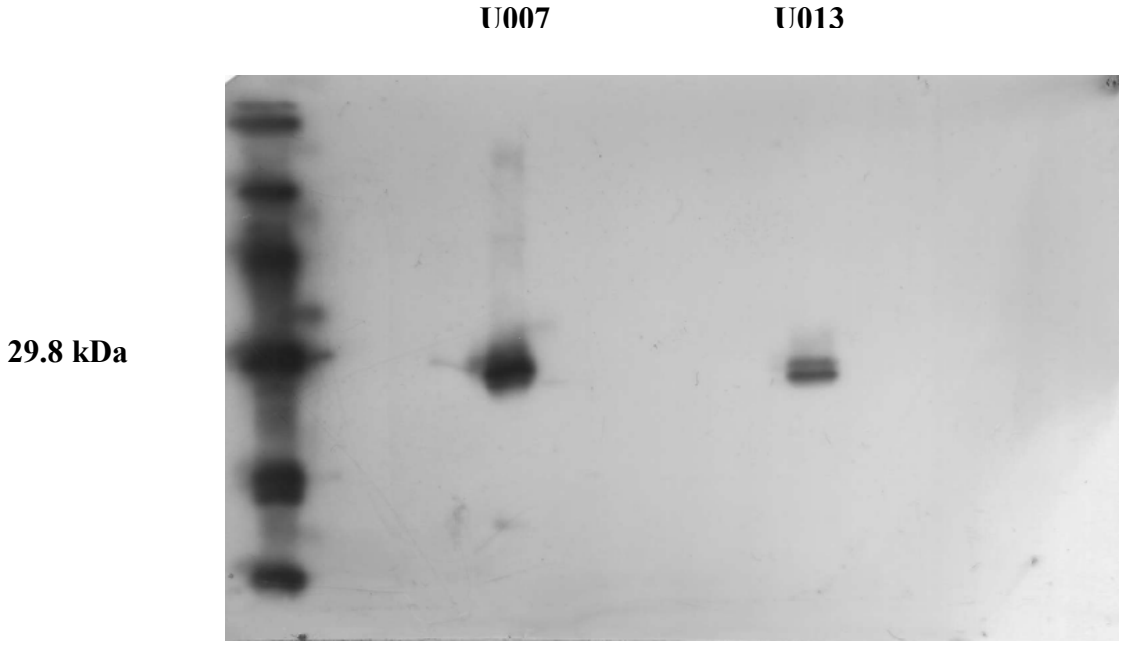


Figure 4-7 Western Blotting Results for U007 and U013

5 DISCUSSION

One of the aims of this study was to devise a high-throughput method for the cloning, transformation and expression of a wide range of open reading frames from Arabidopsis Biological Research Center (ABRC). It was to our advantage that the open reading frames from ABRC were cloned into pUNI51 (universal) vectors, cloning procedure could be carried out in a high-throughput manner through the use of three set of primers designed to suit the three different expression vectors: pPICZ alpha A, B and C. Since the aim was a rapid screening of all the putative lipase clones in our hands, optimization measures were not taken into account for the ORF's that could not be cloned through our setup. Similarly, no optimization was done for transformation into *Pichia pastoris* and expression in this host system. A method working for most of the

clones was set up and applied. In this manner, 29 out of 40 ORF's were successfully cloned into *Pichia* expression vectors and sequence verified. Two of the 40 clones were initially discarded because one of them, U016, was not the right open reading frame according to the size of the band resulting from PCR amplification and the other one, U110, contained all of the three restriction enzyme sites; that is, *Eco*RI, *Pst*I and *Cla*I, it should not contain in order to be in frame with the extracellular secretion signal of the *Pichia* expression vectors.

Pichia pastoris was chosen as a host organism for the expression of putative lipases. Transformation of expression vectors with the gene of interest into *Pichia pastoris* was performed quickly via electroporation and since the vectors are integrated into the genome of the host, a stabilized expression vector *Pichia* expression system suits our aim because it provides an easy and fast method of expressing sufficient amounts of proteins since they can grow to high cell densities. Some proteins were expressed in very high yields of up to 10 g / l (Cregg et al., 2000). Although *E. coli* expression is also a fast and easy expression system, it is not suitable for functional expression of eukaryotic proteins that need post-translational modifications like disulphide bond formation, glycosylation and proteolytic processing required for proper folding. Hence, *Pichia* expression system carries the advantages of both prokaryotic and eukaryotic expression systems. As prokaryotic systems, *P. pastoris* can be grown to large quantities in minimal media and as eukaryotic systems, posttranslational modifications are performed in *P. pastoris*. Furthermore, in *P. pastoris* expression, any toxic protein is not secreted into the extracellular medium, a great advantage for pharmaceutical use. *Pichia pastoris* expression is preferred over another eukaryotic expression system, *Saccharomyces cerevisiae*, because it results in higher yields of proteins. Moreover, processing of the expressed proteins is feasible if vectors that carry an extracellular signal sequence are used. In this study, pPICZ derived vectors carrying α-mating factor pre-pro leader sequence (α-MF) of *Saccharomyces cerevisiae* is used for extracellular expression (Daly and Hearn, 2003). Cloning the open reading frames in frame with the extracellular signal sequence theoretically should have led to extracellular expression of the encoded putative lipases in abundance. For this reason, without time-consuming procedures of cell lysing and protein purification, the supernatants of the expressions were used for SDS-PAGE gel analysis and activity assays.

In this manner, all of the ORF's successfully cloned into expression vectors were transformed into *Pichia pastoris*. Twenty five of these resulted in colonies in YPD-Zeo plates that were checked whether they contain the expression vectors integrated into the genome by colony PCR. Colonies giving positive colony PCR results were expressed (data not shown). As the expressions were finished at the end of the sixth or the seventh day, SDS-PAGE gels were run to see whether we could detect the expressed proteins. Bands corresponding to the putative lipases were not detected inferring that there was either no expression or the proteins were expressed at very low levels. Accordingly, it was suggested to perform activity assays on the supernatants.

Accordingly, activity assays on the supernatants of the 25 expression clones were performed. As the method of assay fluorogenic assays using 4-methylumbelliferyl derived substrates was chosen because this kind of assays are sensitive enough to detect low activities that *Arabidopsis* lipases exhibit. Besides, this method can be scaled down to microtiter-plate format and this is convenient for us because numerous samples can be screened for activity at once in a continuous fashion. Esters of 4-methylumbelliferone are commercially available in various acyl chain lengths; hence can be used as substrate analogues of lipases in fluorogenic assays. The disadvantages include the substrate resembling monoacylglycerol rather than triacylglycerols and being spontaneously hydrolyzed in reaction medium in the absence of lipase activity (Gilham and Lehner, 2004). Since literature survey shows that Arabidopsis lipases have low activity but high specificity, these may not be the best substrates to test their activity (Verger et al., 2000). Additionally, the fluorescent moiety in these artificial substrates may cause steric hindrance and this may be the cause of activities lower than expected.

Lipase activity assays are problematic in the sense that there are too many parameters that should be optimized for each enzyme in question. Firstly, the pH of the reaction medium and the temperature of the reaction should be optimized. However, these can be done once the best substrate for each enzyme is found. The preparation of the substrate is another important parameter that affects lipase activity. It is better if the substrates are emulsified in detergents forming micelles that provide the lipid/water interface where lipases can exhibit their activity. Of course this does not mimic the physiological conditions of lipases. The choice of the detergent used is crucial. Ma et al. reported that sodium taurocholate at a concentration of 7 mM increased the activity of the recombinant lipase they studied (Ma et al., 2000) but this should not be taken for

granted. For each lipase, the effects of different detergents should be tested since some can inhibit lipase activity. For example, Triton X resembles fatty acids and can inhibit lipase activity or Tween 20 may itself be hydrolyzed by lipases and compete with the intended substrate. Not to defeat the purpose of this study, we could not try the effects of different detergents for each of the 25 samples. Therefore, not to investigate the effects of different parameters in the activity assays we decided to use Tris-HCl buffer with pH 7.25 that is commonly used for most lipases.

Similarly, we also decided not to add any detergent to our reaction medium; so the substrates were prepared in water accordingly; however, they were prepared freshly to prevent their degradation. The substrates that remained stable in Tris-HCl buffer (pH 7.25) were 4MU-caprylate and 4MU-butyrate. Therefore these substrates were used in our assays.

In the first set of assays done on U4, U5, U8, U10, U17, U20, U21, and U22 the activities against 4MU-butyrate were investigated as shown in Table 4-6. These activities were better than the ones we did later as shown in Table 4-7. One of the reasons for this could be the detergent effect. For this assay, 0.02% Triton X-100 was added to the reaction medium and the substrate was also diluted in the same medium. The addition of this detergent might have had a positive effect but we could not have known this until the latter assays were done. Unfortunately, this first set of expressions were done in 10 ml expression scale and all the samples left after the assay was done were concentrated and used for SDS-PAGE analysis.

Seeing these promising activities towards 4MU-Butyrate in this first set of expressions, these expressions were repeated in a 50 ml expression scale. The rest of the clones were also expressed in a 50 ml scale. Unfortunately, U4, U5 and U22 could not be expressed again due to contamination problems. The rest of the clones were expressed for six days with 0.5% (v/v) of 100% methanol induction given each day. On the sixth day, the samples were collected and kept at -20°C. The second set of assays shown in Table 4-7 were done after thawing the samples. It was observed that the activities against 4MU-Butyrate were lower than the first set. As mentioned, this might be because of the absence of Triton X-100. Another reason for the decreased activity may be freeze-thawing the supernatants. It is also probable that the same conditions of expression for 10 ml scale may not be sufficient for good expression in 50 ml scale. Maybe this time lower amounts of proteins were expressed. Finally, in the second set of assays there was a problem with the spectrofluorometer. That is, fluorescence readings

corresponding to only 3.13 μM, 1.56 μM, 0.78 μM, 0 μM of 4-MU could be measured; the values were saturated for the rest. Accordingly, this did not result in an accurate standard curve. Instead of repeating this experiment, we decided to concentrate the expressed samples 10 times through lyophilization. Activity assay results against 4MU-caprylate and 4MU-butyrate on the concentrated samples are shown in Table 4-8. These results showed that activities against 4MU-butyrate were increased as compared to the results in Table 4-7. This increase is probably due to concentration of the samples. An interesting result is that most of the samples showed high activities towards 4MU-caprylate rather than 4MU-butyrate. Specifically, the data reveals that U6, U7, U8, U18, U30, U32 and U105 putative lipases exhibit substantially higher activity against 4MU-caprylate rather than 4MU-butyrate.

We believe that we were successful in expression because we see activities towards 4MU-caprylate in all samples except U20, U21 and U24. Moreover, all except U19 and U105 showed low but existing activities towards 4MU-butyrate. These samples showing no activities are important for us because they represent the controls in this assay. That is, it was mentioned that fluorogenic assays might be non-specific in the sense that the fluorescent substrates might undergo spontaneous hydrolysis in water, thus the activities in Table 4-8 might infer nonspecific hydrolysis. Nevertheless, U19, U20, U21, U24, and U105 show that the activities we see are not due to spontaneous hydrolysis or nonspecific esterases that may be present in the medium because the medium of all the supernatants in Table 4-8 is the same.

The finding that we observed activities when the supernatant were concentrated was expected because in general plant lipases have low activity. Plant lipases of commercial value having activities sufficient to be used in selective esterification, such as rapeseed lipases selectively discriminating against *cis*-6 and *cis*-4 double bonds, possessed such activities because they were directly extracted from plant seedlings during germination period when lipases are highly expressed. Hence in those extractions, lipases are very abundant. However, this expression is transient and occurs only during the germination period. On the other hand, we tried to achieve high yield of the expressed proteins in *Pichia pastoris* by placing the ORF's under the control of a strong promoter AOX1. However, apparently, putative lipases are not very abundant in the extracellular medium due to lack of optimization of the expression samples.

Arabidopsis lipases are even more problematic to detect than other oil plant lipases. Their activity is so low that it is difficult to detect them even via sensitive

assays such as fluorescent assays that we used. For this reason, Verger et al. suggested to use a naturally fluorescent substrate: parinaric acid. This is a TAG purified from *Parinari glaberrimum* seed oil. The excitation and emission wavelengths were found to be 324 and 420 nm, respectively. Upon hydrolysis, the released parinaric acid shows increased fluorescence, in the presence of detergents, proportional to the amount of lipase added in the assay. This new method can also be scaled to microtiter-plate measurements and can detect low lipase levels. If this substrate were used, maybe higher lipase activities could be detected.

The supernatants showing lipase activities against 4MU-caprylate and 4MU-butyrate could not be detected through SDS-PAGE. As mentioned, this is due to the low levels of the expressed proteins in the supernatant. The abundance of the expressed proteins was so low that the putative lipases could not be detected in concentrated supernatants by western blotting as well. U007 and U013 proteins detected by western blotting may have lower molecular weights due to degradation. Degradation from a site away from the active site might not have affected the activity.

One of the promising findings of this study was U6, U7, U8, U18, U30, U32 and U105 supernatants exhibiting distinctive activity against 4MU-caprylate rather than 4MU-butyrate. This provides us more insight with regards to substrate specificities of *Arabidopsis thaliana* lipases. This is consistent with literature surveys pointing out the potential use of plant lipases as biocatalysts due to heir “unique substrate specificities” (Kumarjee et al, 1994).

It was assumed that these lipases obey the Michaelis-Menten kinetics. Accordingly, the substrate concentration was in excess and the linear range of the reaction kinetics was used. It should be noted that the activities and specificities observed may be apparent. This is so because the substrates do not form a homogeneous phase; they form precipitates. This might be the reason why we could not see activities in some of the samples. The pH of the reaction may be another reason. These enzymes may show better activity at pH 6 for example. Different pH values can be tried but for 4MU substrates pH values between 6 and 8 are useful because at other pH values the substrates are unstable.

In future studies, optimization of larger scale expressions should be performed to detect the lipases, which have been shown to have activities, either in SDS-PAGE or Western blotting. Improvement of the activities of the lipases showing specific activity against 4MU-caprylate could be another area of growing interest that can be taken into

consideration in the future. Literature reviews show that even subtle differences in the active site of lipases may have preeminent effects on substrate utilization and selectivity (Gilham and Lehner, 2005). Since uncovering of new lipases with different catalytic properties, enantioselectivities or substrate specificities is crucial for biocatalysis reactions, screening of the open reading frames from ABRC was an important task. It pointed out the lipases that can be obtained from safe and cheap starting material and this finding can be taken under further investigation.

6 CONCLUSION

In this study, a high-throughput screening method was devised to clone putative lipase open reading frames into pPICZalpha A, B and C vectors for expression in *Pichia pastoris* system. Through fluorogenic assays in 96-well format, the activities of the expressed proteins were screened and those with considerable activities, especially towards 4MU-caprylate, which can be considered as a true lipase substrate, were found.

Seven of the open reading frames were found to distinctively prefer 4MU-caprylate cleavage over 4MU-butyrate cleavage. This pointed out one of the most

important properties of plant lipases that make them important substitutes of microbial lipases: their unique substrate specificities. This study provided us with the insight that ABRC open reading frames having sequences similar to known lipases can have important substrate specificities. Development of an efficient screening method for lipase activity is very important due to the growing interest in the potential use of lipases as biocatalysts. Properties of lipases such as their enantioselectivities, fatty acid selectivities and regiospecificities enhances growing interest on their identification and tailoring such that they suit the needs of pharmaceutical, cosmetic and detergent industries. Lipases coming from a plant source, as in the current study, are preferred since plants are a safe and cheap starting material.

The screening performed in this study investigated twenty seven putative *Arabidopsis* lipase activities and resulted in seven open reading frames of particular interest that could be further investigated in large scale expressions.

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8 APPENDIX

Chemicals

Chemical Name	Supplier	Catalog Number
Acrylamide/Bis-acrylamide	Amresco, USA	0254
Agar	Merck, Germany	101614
Agarose Low EO	Applichem, Germany	A2114
Ammonium persulphate	Sigma, Germany	
Ampicillin	Sigma, Germany	A9518
Biotin	Calbiochem, Germany	2031
Coomassie Brilliant Blue	Merck, Germany	115444
EDTA (Ethylene diamine tetraacetic acid)	Riedel-de Haen, Germany	27248
Ethanol	Riedel-de Haen, Germany	32221
Ethidium Bromide	Merck, Germany	OCO28942
D-(+)-glucose	Sigma, Germany	G-7021
Glycerol	Riedel-de Haen, Germany	15523
HCl	Merck, Germany	100314
Isopropanol	Riedel-de Haen, Germany	24137
Kanamycin	Sigma, Germany	K4000.102
KH_2PO_4	Riedel-de Haen, Germany	04243
K_2HPO_4	Merck, Germany	105099

KOH	Riedel-de Haen, Germany	06005
Liquid Nitrogen	Karbogaz, Turkey	
Lithium Chloride	Fluka, Switzerland	62478
Luria Agar	Sigma, Germany	L-3147
Luria Broth	Sigma, Germany	L-3022
2-Mercaptoethanol	Aldrich, Germany	M370-1
Methanol	Riedel-de Haen, Germany	24229
NaCl	Riedel-de Haen, Germany	13423
NaOH	Merck, Germany	106462
PageBlue Protein Stain	Fermentas, Lithuania	R0571
Peptone	Merck, Germany	107213
Phenol/Chloroform/ Isoamylalcohol	Applichem, Germany	A0889
Sodium Dodecyl Sulphate	Sigma, Germany	L-4390
Sodium Acetate Trihydrate	Riedel-de Haen, Germany	25022
D (-) Sorbitol	Applichem, Germany	A2222
TEMED	Sigma, Germany	T-7029
Triton X-100	Applichem, Germany	A1388
Tris	Fluka, Switzerland	93349
Tween 20	Merck, Germany	822184
Yeast Extract	Applichem, Germany	A1552
Yeast Nitrogen Base (with ammonium sulphate without amino acids)	Invitrogen, Germany	Q300-07
Zeocin	Invitrogen, Germany	R250

Molecular Biology Kits

ECL Advance Western Blotting Detection Kit	Amersham, Sweden	RPN2135
Qiaquick PCR Purification Kit	Qiagen, Germany	28106
Qiaquick Gel Extraction Kit	Qiagen, Germany	28706
Qiaquick Spin Miniprep Kit	Qiagen, Germany	27106
Qiagen Plasmid Maxi Kit	Qiagen, Germany	12165

Equipment

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
Balance:	Sartorius, BP211D, GERMANY Sartorius, BP221S, GERMANY Sartorius, BP610, GERMANY
Centrifuge:	Eppendorf, 5415C, GERMANY Eppendorf, 5415D, GERMANY Eppendorf, 5415R, GERMANY Kendro Lab. Prod., Heraeus Multifuge 3L, GERMANY Hitachi, Sorvall RC5C Plus, USA
Deepfreeze:	-80°C, ThermoForma, USA -20°C, Bosch, TURKEY
Distilled Water:	Millipore, Elix-S, FRANCE Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biorad Inc., USA
Electro Cell Manipulator	BTX, USA

Gel Documentation:	Biorad, UV-Transilluminator 2000, USA
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY Memmert, Modell 600, GERMANY Nuve, EN 120, TURKEY
Laminar Flow:	Kendro Lab. Prod., Heraeus, HeraSafe HS12, GERMANY
Lyophilizer	
Magnetic Stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY VELP Scientifica, Microstirrer, ITALY
Microliter Pipette:	Gilson, Pipetman, FRANCE Eppendorf, GERMANY
Microwave Oven:	Bosch, TURKEY
pH meter:	WTW, pH540 GLP MultiCal®, GERMANY
Power Supply:	Biorad, PowerPac 300, USA Wealtec, Elite 300, USA
Refrigerator:	4°C, Bosch, TURKEY
Shaker:	Forma Scientific, Orbital Shaker 4520, USA GFL, Shaker 3011, USA New Brunswick Sci., Innova 4330, USA
Spectrophotometer:	Schimadzu, UV-1208, JAPAN Schimadzu, UV-3150, JAPAN

Spectrofluorimeter	Molecular Devices, SpectraMax Gemini XS
Speed Vacuum:	Savant, Speed Vac® Plus Sc100A, USA Savant, Refrigerated Vapor Trap RVT 400, USA
Thermocycler:	Applied Biosystems, GeneAmp PCR System 9700, USA
Water bath:	Huber, Polystat cc1, GERMANY

Vectors

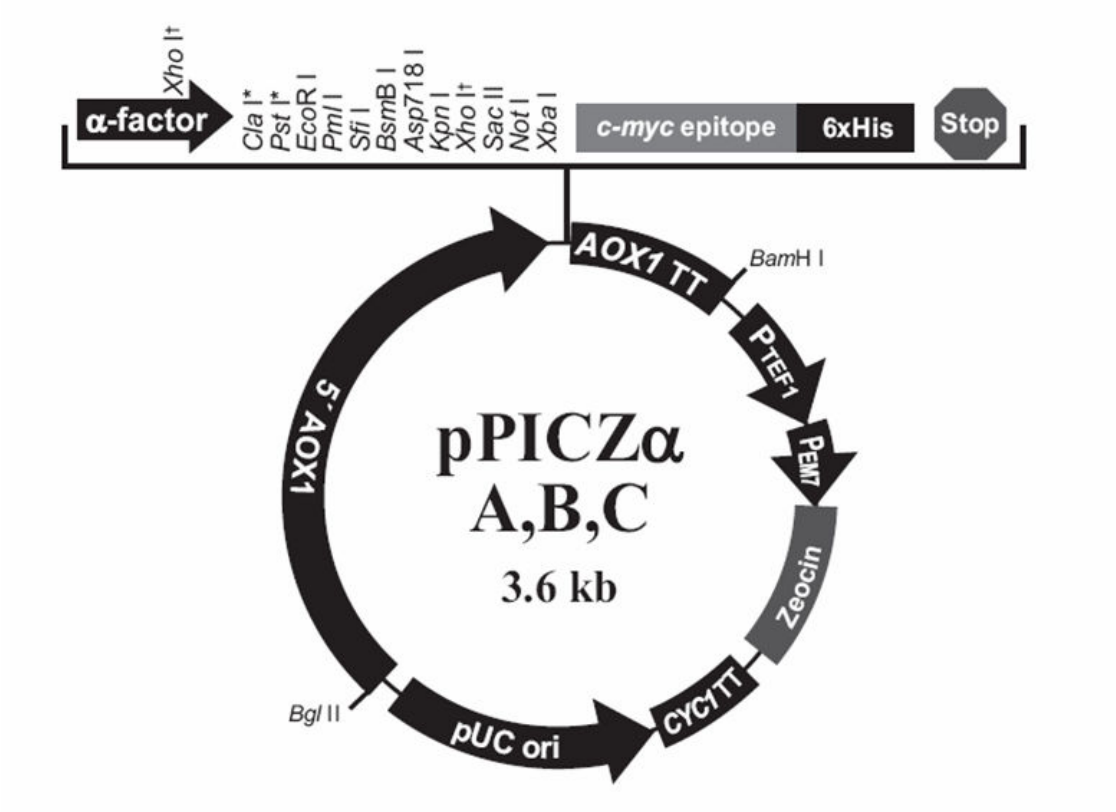

Figure 8-1 Ppıczalpha A,B and C vectors (Invitrogen Manual, 1997)

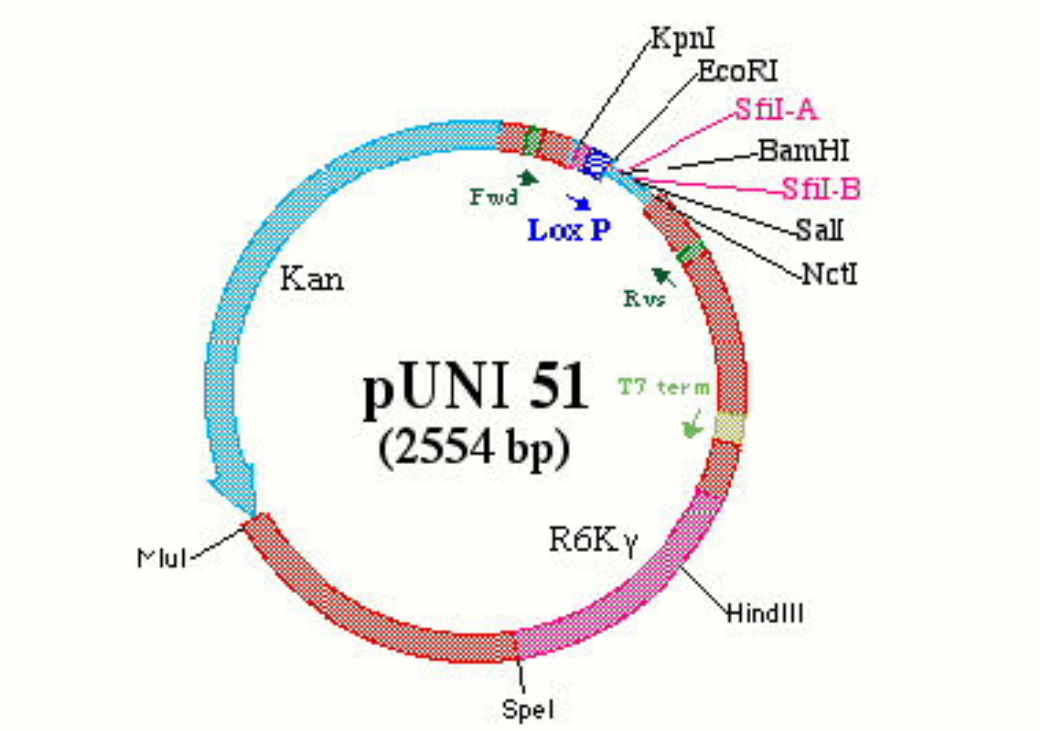

Figure 8-2 pUNI 51 vector (ABRC)

Alignments

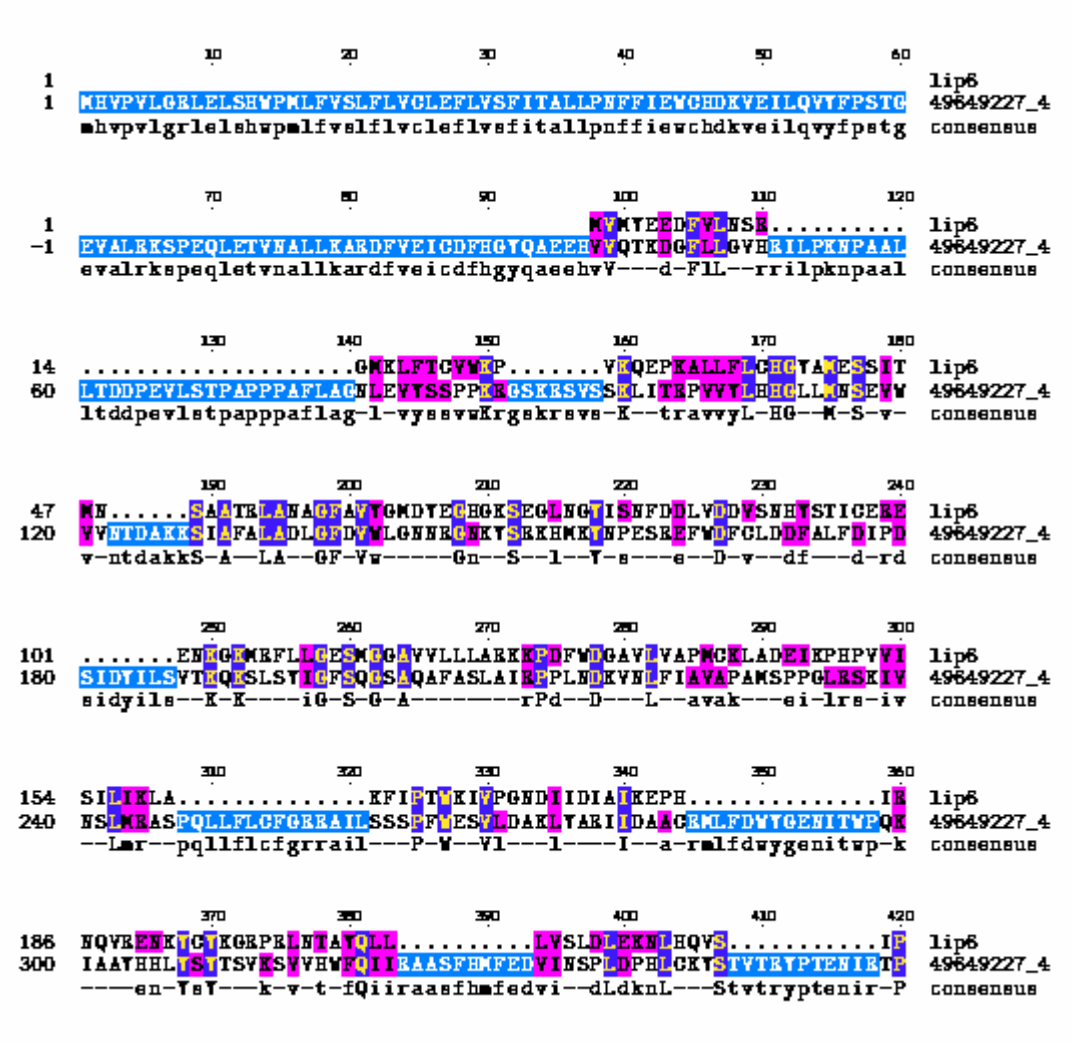

Figure 8-3 Alignment of U6 with a known lipase sequence

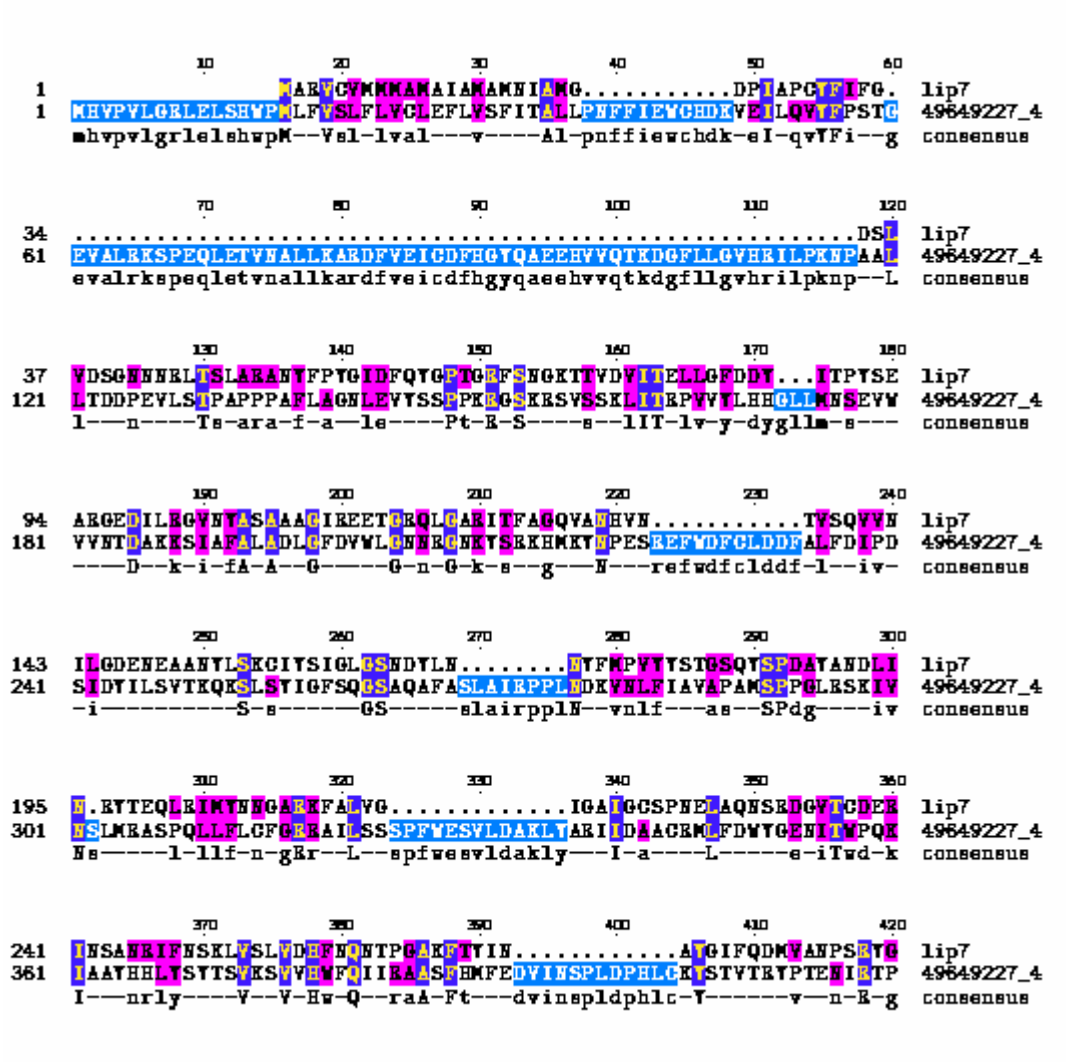

Figure 8-4 Alignment of U7 with a known lipase sequence