การทำให้บริสุทธิ์และลักษณะสมบัติของไลเพสจากราเอนโดไฟต์

Fusarium oxysporum ไอโซเลต PTM 7

นางสาวตวงพร ปานอุทัย

PURIFICATION AND CHARACTERIZATION OF LIPASE FROM ENDOPHYTIC FUNGUS *Fusarium oxysporum* ISOLATE PTM 7

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ตวงพร ปานอุทัย : การทำให้บริสุทธิ์และลักษณะสมบัติของไลเพสจากราเอนโดไฟต์ *Fusarium oxysporum* ไอโซเลต PTM 7 (PURIFICATION AND CHARACTERIZATION OF LIPASE FROM ENDOPHYTIC FUNGUS *Fusarium oxysporum* ISOLATE PTM 7) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : อ.ดร.อภิชาติ กาญจนทัต, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร.ประกิตติ์สิน สีหนนทน์, ผศ.ดร. จิตรตรา เพียภูเขียว, 86 หน้า.

ทดสอบการสร้างไลเพสจากราเอนโดไฟต์จำนวน 65 ไอโซเลต พบว่ามีเพียง 10 ไอโซเลต ที่สร้างไลเพสโดยมี *Fusarium oxysporum* ไอโซเลต PTM 7 ซึ่งแยกได้จากใบ *Croton oblongifolius* Roxb (เปล้าใหญ่) สามารถสร้างไลเพสได้สูงสุด ค่ากิจกรรมไลเพสของราไอโซเลต PTM 7 สูงสุด เมื่อเลี้ยงในอาหารเลี้ยงเชื้อ basal medium โดยเติม 1 เปอร์เซ็นต์ น้ำมันมะกอก (ปริมาตร/ปริมาตร) 1 เปอร์เซ็นต์ เปปโทน (น้ำหนัก/ปริมาตร) และ 0.5 เปอร์เซ็นต์ โซเดียมไนเตรต (น้ำหนัก/ปริมาตร) เป็นแหล่งคาร์บอน   แหล่งอินทรีย์และอนินทรีย์ไนโตรเจนตามลำดับ เมื่อทำให้บริสุทธิ์โดยการ ตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟต 80 เปอร์เซ็นต์ โครมาโทกราฟีแบบแลกเปลี่ยนประจุชนิด DEAE และ โครมาโทกราฟีแบบแยกตามขนาดโมเลกุลชนิด Superdex-75 ได้ความบริสุทธิ์เพิ่ม 41.4 เท่า และมีน้ำหนักมวลโมเลกุลเท่ากับ 37.4 กิโลดาลตัน แต่ผลผลิตสุดท้ายลดลงเหลือเพียง 2.21 เปอร์เซ็นต์ เสถียรภาพของไลเพสมีค่ากิจกรรมสูงสุดที่พีเอช 8.0 และอุณหภูมิ 30 องศาเซลเซียส โดยไลเพสรักษาเสถียรภาพที่อุณหภูมิสูงถึง 40 องศาเซลเซียส และกิจกรรมของไลเพสถูกกระตุ้น ด้วยไอออนของ Ca^{2+}, Mg^{2+} โดยเฉพาะ Mn^{2+} (133 เปอร์เซ็นต์) ที่ความเข้มข้นต่ำ (1 มิลลิโมลาร์) แต่ ถูกยับยั้งด้วย Cu^{2+}, Fe^{2+}, Hg^{2+} และ Zn^{2+} ที่ความเข้มข้น 1 – 10 มิลลิโมลาร์ สำหรับ EDTA ที่ความ เข้มข้น 5   และ 10   มิลลิโมลาร์ ยับยั้งกิจกรรมของไลเพสได้ ขณะที่ความเข้มข้น 1 มิลลิโมลาร์ กระตุ้นกิจกรรมของเอนไซม์ เมื่อใช้พารา-ไนโตรฟีนิล ปาล์มมิเตทเป็นสับสเตรทมีค่าคงที่ของไม เคิลลิส-เมนเทนและอัตราเร่งปฏิกิริยาสูงสุดค่าค่อนข้างต่ำคือ 2.78 มิลลิโมลาร์ และ 9.09 โมล/นาที/ มิลลิกรัมโปรตีน ตามลำดับ ขณะที่การเร่งปฏิกิริยาทรานส์เอสเทอริฟิเคชันเพื่อผลิตไบโอดีเซลที่ อัตราส่วนโมลน้ำมันต่อเมทานอล 1 : 6 และเอนไซม์ 6 ยูนิต ให้ผลผลิตค่าค่อนข้างต่ำเพียง 28.4 เปอร์เซ็นต์ของผลผลิตไบโอดีเซล

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TUANGPORN PANUTHAI : PURIFICATION AND CHARACTERIZATION OF LIPASE FROM ENDOPHYTIC FUNGUS *Fusarium oxysporum* ISOLATE PTM 7. ADVISOR : APHICHART KARNCHANATAT, Ph.D., CO-ADVISOR : ASSOC. PROF. PRAKITSIN SIHANONTH, Ph.D., ASST. PROF. JITTRA PIAPUKIEW, Ph.D., 86 pp.

Sixty five endophytic fungal isolates, were tested for extracellular lipase production. Only ten isolates were found to produce lipase activity. *Fusarium oxysporum* isolated PTM 7, which was isolated from *Croton oblongifolius* Roxb. (Plao yai leaves), gave the highest activity. Highest lipase activity PTM 7 was shown in the basal culture medium was supplemented with 1% (v/v) olive oil, 1% (w/v) peptone and 0.5% (w/v) sodium nitrate as the carbon, organic and inorganic nitrogen sources, respectively. Purification PTM 7 extracellular lipase by precipitated in 80% saturation ammonium sulfate and molecule separation by DEAE-cellulose anion exchange and Superdex-75 gel filtration chromatography increased 41.4 in time apparent homogeneity but final yield decreased to 2.21 percent. The enriched lipase showed maximum stability at pH 8.0 and 30°C, was reasonably stable up to 40°C and at pH between 8.0 to 12.0. One mM Ca^{2+} and Mg^{2+} stimulated enzyme production and especially Mn^{2+} stimulated 133%, but inhibited by Cu^{2+}, Fe^{2+}, Hg^{2+} and Zn^{2+} 1 - 10 mM. 5 and 10 mM EDTA inhibited lipase activity but stimulatory at 1 mM. The K_m and V_{max} values were rather low at 2.78 mM and 9.09 mol/min/mg protein, respectively, when using *p*-nitrophenyl palmitate as the substrate. Enzymatic transesterification, which ratio of oil : methanol mole (1 : 6) and 6 U enzyme for biodiesel production was rather low only 28.4% FAMEs yield.

Field of Study: Biotechnology   Student's Signature _____

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                                Co-advisor's Signature _____

                                Co-advisor's Signature _____

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%	percentage
°C	degree celsius
µg	microgram
µl	microlitre
BSA	bovine serum albumin
CTAB	cetyltrimethylammonium bromide
kDa	Kilodalton
EDTA	ethylenediamine tetraacetic acid
FAMEs	fatty acid methyl esters
g	gram
GC	gas chromatography
h	hour
ITS	internal transcribed spacer
kDa	kilodaton
K_m	Michaelis-Menten constant
l	litre
M	molar
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MW	molecular weight
N	normal
nm	nanometer
NaCl	sodium chloride
pNP	para-nitrophenol
pNPP	para-nitrophenyl palmitate
PAGE	polyacrylamide gel electrophoresis

rpm	revolution per minute
SDS	sodium dodecyl sulfate
TEMED	*N,N,N',N'*-tetramethyl ethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
U	Unit activity
V	volt

CHAPTER l

INTRODUCTION

The biological relevance and variability of lipids is matched by the diversity of lipid-degrading enzymes throughout all kingdoms of life. Lipases (EC.3.1.1.3, triacylglycerol acyl-hydrolases) are a group of water-soluble acyl-hydrolases, which have the ability to hydrolyze triacylglycerols at an oil-water interface to release free fatty acids (polar lipids) and glycerol. Because of an opposite polarity between the enzyme (hydrophilic) and their substrates (lipophilic), lipase reactions occur at the interface between the aqueous and the oil phases (Reis *et al*., 2009). Lipases are considered to be some of the most important biocatalysts due to both their widespread biological functions and their biotechnological potential (Bornscheuer, 2002), and are present in microorganisms, plants and animals.

Lipases catalyze a wide range of reversible reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis (Babu and George, 2008). In the presence of organic solvents, the enzymes are effective catalysts for various inter-esterification and trans-esterification reactions. Furthermore, microbial lipases show regiospecificity and chiral selectivity (Gupta *et al*., 2003). In particular, microbial lipases have different enzymological properties and substrate specificities. Their biotechnological potential relies on their ability to catalyze not only the hydrolysis of a given triglyceride, but also on its synthesis from glycerol and fatty acids. Therefore, microbial lipases have many potential industrial applications (Jager *et al*., 1999), but their temperature stability is the most important characteristic for industrial use (Choo *et al*., 1997).

Interest in microbial lipases has markedly increased over the past few years. Microbial lipases are widely diversified in their enzymatic properties and substrate specificities, which make them very attractive for industrial applications (Gandhi, 1997). Indeed, lipases and especially fungal lipases are used in the oil and fat industry for the synthesis of structural triacylglycerols (Macrae, 1983), and in the pharmaceutical and agrochemical industries for the production of optically pure products (Godtfiedsen, 1990), and other diverse industries, such as detergents, beverages, dairy products and so on (Jager *et al*., 1998; Hiol *et al*., 2000). Lastly,

inexpensive waste materials can be used as fermentation media, they can be produced in a large quantity, the harvest process is not complicated and for many applications absolute purity is not required (Sharma *et al*. 2001). Filamentous fungi are the preferred source of lipases since they produce extracellular lipases. As a consequence a large number of lipase producing filamentous fungi have been characterized, including members from the genera *Aspergillus* (Mhetras *et al*., 2009; Saxena *et al*., 2003; Mayordomo *et al*., 2000; Gulati *et al*., 1999), *Fusarium* (Long *et al*., 2010; Hala *et al*., 2010; Maia *et al*., 2001), *Metarhizium anisopliae* (Silva *et al*., 2005), *Mucor* (Abbas *et al*., 2002; Hiol *et al*., 1999), *Penicillium* (Lima *et al*., 2004; Tan *et al*., 2004) and *Rhizopus* (Shukla *et al*., 2007; Hiol *et al*., 2000; Essamri *et al*., 1998).

Endophytic fungi are fungal microorganisms, which live inside plant tissues for at least part of their life cycle, typically without causing any disease symptoms in the host. Within hosts, fungal endophytes may inhabit all available tissues, including leaves, petioles, stems, twigs, bark, xylem, root, fruit, flower and seeds. The relationship between the endophyte and its host plant may range from latent phytopathogenesis to mutualistic symbiosis (Petrini, 1992). In the literature, the main studies of endophytic fungi have centered on screening for secondary metabolites, antimicrobial and enzyme activity. Enzyme expression tests may help us to understand the functional roles of endophytes and test whether fungi can change their mode of life from an endophyte, to a saprobe or pathogen. Endophytic fungi are considered potent microbes, producing hydrolytic enzymes (e.g. Torres *et al*., 2003; Maria *et al*., 2005).

Although the enzymes vary from isolate to isolate, all the endophytic fungi tested synthesize *in vitro* the enzymes necessary for penetrating and colonizing their plant hosts (Schulz *et al*., 2002). Such enzymes include pectinases, xylanase, cellulases and lipases, whilst proteases and phenol oxidase have also been documented with some endophytes (Tan and Zou, 2001). Lipases were produced by *Acremonium sp*., *Alternaria sp*., *Aspergillus sp*., *Fusarium sp*. and *Pestalotiopsis sp*., while amylase and protease were produced by a few of them. Endophytic fungi obtained from the leaves, stems and roots of *Annona sp*. that produced lipase included fungal genus *Acremonium*, *Aspergillus*, *Chaetomium*, *Colletotrichum*, *Fusarium*, *Cylindrocladium*, *Glomerella*, *Nigrospora* and *Phomopsis* (Silva *et al*., 2006). However, very little has been reported about lipase production by endophytic fungi up to date. The objective of this work was to isolate endophytic fungi capable of

producing lipase and describe the purification and characterization of a lipase from endophytic fungi.

CHAPTER ll

LITERATURE REVIEWS

2.1 Lipids

2.1.1 Sources

Lipids were a broad group of naturally occurring molecules. Most lipids, substances ranging from the simple fatty acids to the complex sphingo lipids, from the sterols and steroids to the pigments and vitamins, are naturally occurring in the forms of animal fat and vegetable oils. Moreover, lipids as a large and diverse group of naturally occurring organic compounds in living organisms, that were related to play an important roles in biological membrane of animals, plants and microorganisms. For example, glycerides serve as excellent sources of potentially chemical energy source and thermal insulator in mammals. Furthermore, lipids as important signaling molecules.

2.1.2 Physical properties of lipids

Definition of lipids depends on physical properties than on chemical structure or function. Generally, the lipids were a large and diverse group of naturally occurring organic compounds that are related by their solubility in nonpolar organic solvents (e.g. ether, chloroform, acetone & benzene) and general insolubility in water. This distinctive properties of lipid depends on long hydrocarbon chains that were a main parts of their structure. Melting points of each lipid were also determined by ratio of unsaturated fatty acids to saturated fatty acid (Hardley, 1985).

2.1.3 General aspects of triglycerides

The neutral fats and phospholipides were two biologically important groups of lipids, which had a glycerol backbone bonded to three fatty acids. Glyceride or acyglycerol was composed mainly of mono-, di- and tri-substituted glycerol, the most well-known being the fatty acid esters of glycerol (triacylglycerols), also known as triglycerides. In these compounds, the three hydroxyl groups of glycerol were each esterified, usually by different fatty acids. Because they function as a food store, these lipids comprise the bulk of storage fat in animal tissues. The common structure of mono-, di-, and triglyceride is shown in Figure 2.1.
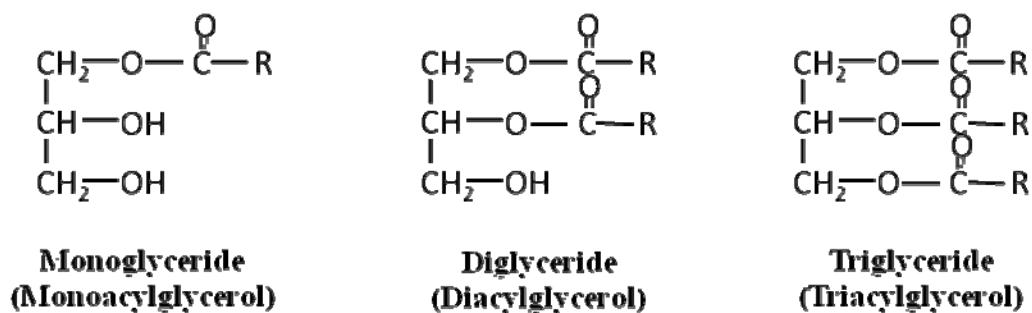
Figure 2.1 Chemical struture of mono-, di-, and triglyceride.

These triglycerides (or triacylglycerols) were found in both plants and animals, and compose one of the major food groups of our diet. Triglycerides occured in the solid, semisolid, liquid form at the room temperature that depends on the nature of constituent of fatty acid. For example, a large proportion of unsaturated fatty acids, i. e.. oleic, linoleic or linolenic acids, triglyceride in the most plants are found in the form of lipids and e called oils. However, triglycerides in animals, which contain a higher proportion of saturated fatty acid such as palmistic and steric acids were semisolid or solid. Furturemore, the reactivity of ester bond and degree of unsaturation in hydrocarbon chain were determined by the chemical reactivities of triglycerides. Acid and base hydrolysis of triglycerides was differently occurred in ester linkage, i.e., difference of this is acid hydrolysis reaction is reversible while base hydrolysis is irreversible (Conn and Stumpf, 1989)

2.2 Enzymatic hydrolysis of triglycerides

2.2.1 Lipases

Lipids constitute a large part o the earth's biomass, and lipolytic enzymes play an important role in the degradable of these. In the recent years, enzymes that degrade triglyceride lipids by hydrolysis of esters, especially esters of carboxylic acids, had been receiving much attention due to their potential use in industrial processes. During the past decades, many investigators have extensively studied the activity of lipolytic enzyme called lipases or triacylglycerol acylhydrolase (E.C. 3.1.1.3) in both academic and industrial viewpoints.

Lipase are part of the family of hydrolases that act on carboxylic ester bonds. Their physiological role is to hydrolyze triglycerides to diglycerides, mono glycerides, fatty acids and glycerol (Figure 2.2).

$$\text{Triglyceride}$$
$$\Updownarrow$$
$$\text{Diglyceride + Free fatty acid}$$
$$\Updownarrow$$
$$\text{Monoglyceride + Free fatty acid}$$
$$\Updownarrow$$
$$\text{Glycerol + Free fatty acid}$$
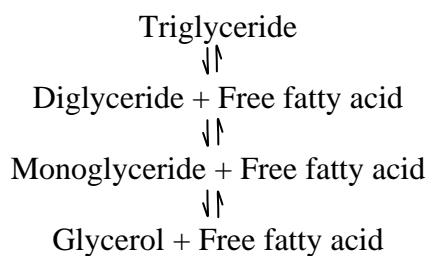
Figure 2.2 The lipase reaction.

Lipolytic enzymes composed of two major groups, the first group called lipases and the other group called phospholipases (phosphoglyceride acylhydrolases). Phospholipases are a class of four enzymes: phospholipases A_1 (E.C.3.1.1.32), phospholipases A_2 (E.C.3.1.1.4), phospholipases C (E.C.3.1.4.3), and phospho lipases D (E.C.3.1.4.4). Lipase was responsible for hydrolysis of the fatty acid ester bonds of triglycerides whereas the phospholipases hydrolyze the phosphoglycerol ester bonds. Each lipase, like the other enzyme, has its own optimum pH ranging from acid to alkaline. Besides, thermostable lipases were also different considerably due to their sources (Yamane, 1987).

2.2.2 Sources of lipases

Lipases were widely distributed in animals, plants and microorganisms (Sztajer and Maliszewska, 1989), which could produce lipase, either alone or in combination with esterases. Pancreatic lipase one of the exocrine enzymes of pancreatic juices, is obtained from human and pig pancreas. Plant lipases could find from seed and grain, which is collected in lipid-bodies and glyoxysome form. Lipases from plant are high specific substrate than lipase from mammalian and microorganisms (Huang *et al.*, 1988). However, microbial lipase could often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yield possible ease of genetic manipulation, regular supply due to absence of seasonal flucluations and rapid growth of microorganisms on inexpensive media. Microbial enzymes were also more stable than their corresponding plant and animal enzymes and their production was more convenient and safer (Fariha *et al.*, 2005). These lipase producing microorganisms have been isolated from various sources, like palm fruit, raw milk, raw meat, fermented sausages, oil-contaminated area, hot springs, soil samples, contaminated water samples, crude oil contaminated, spoiled coconut, etc. Nowadays, several lipases are currently commercialized but the majority are produced by fungi, yeast

and bacteria due to the facility to cultivate these microorganisms on a large scale (Table 2.1).

Table 2.1 Microorganisms produced lipases for commercial

Bacteria	Fungi	Yeasts
Achromobacter sp.	*Absida corymbifera*	*Candida sp.*
Achromobacter lipolyticus	*Absida hyalospora*	*Candida antarcea*
Acinetobacter sp.	*Amylomyces rouxii*	*Candida auricularia*
Acinetobacter seudoaligenes	*Aspergillus sp.*	*Candida curvata*
Alcaligenes sp.	*Aspergillus flavas*	*Candida lipolitica*
Alcaligenes denitrificans	*Aspergillus fumigates*	*Candida deformans*
Arthrobacter sp.	*Aspergillus nidulans*	*Candida folicrum*
Bacillus cereus	*Aspergillus niger*	*Candida humicula*
Bacillus megaterium	*Aspergillus oryzae*	*Candida rugosa*
Bacillus laterosporus	*Chaetomium sp.*	*Candida tsukubaensis*
Bacillus sphereicus	*Coelomyceles*	*Pichia miso*
Bacillus stearothermophilus	*Fusarium oxysporum*	*Saccharomyces fragilis*
Bacillus thaiminolyticus	*Fusarium solani*	*Saccharomyce*
Bacillus thermonocatenulatus	*Geotrichum*	*fibuligera*
Bacillus thermoleovocans	*Glomus versiforme*	*Saccharomyce*
Burkhoderia cepacia	*Hansenula anomala*	*lipolytica*
Burkhoderia chocolatum	*Humicola grisea*	*Saccharomyce*
Burkhoderia viscosum	*Humicola lanuginose*	*cerevisae*
Corynebacterium acnes	*Microthrix parvicella*	*Schizosaccharomyces*
Crytococcus laurentii	*Mucor javanicus*	*Talaromyces*
Enterococcus faecalis	*Mucor lipolyticus*	*thermophilus*
Flavobacterium arborescen	*Mucor miehei*	*Thielavia minor*
Flavobacterium ferruginem	*Mucor pusillus*	*Torula thermophila*
Lactobacillus sp.	*Neurospora sitophila*	*Ustilago maydis*
Leishmania donovani	*Nocardia amarae*	
Malbrancheae pulcella	*Penicillium crustosum*	
Micrococus frendenreichii	*Penicillium camembert*	
Mycobacterium chaelonae	*Penicillium cyclopium*	

Table 2.1 (Cont.) Microorganisms produced lipases for commercial

Bacteria	Fungi	Yeasts
Myxococus xantus	*Penicillium roquefortii*	
Propionibacterium acnes	*Penicillium candidum*	
Propionibacterium granulosum	*Penicillium citrinum*	
Protaminobacter alboflavus	*Penicillium simplicissimum*	
Pseudumonas sp.	*Penicillium solitum*	
Pseudumonas aeroginisa	*Penicillium uriticae*	
Pseudumonas cepacia	*Phycomyces nitens*	
Pseudumonas flurescen	*Rhizomucor meihei*	
Pseudumonas fragi	*Rhizopus sp.*	
Pseudumonas pseudoalcaligens	*Rhizopus chinensis*	
Pseudumonas stutzeri	*Rhizopus delemar*	

Reference: Godtfredsen (1990), Pandey *et el.* (2001), and Mayordomo *et el.* (2000).

2.2.3 Classification and specificity of extracellular microbial lipases

Owing to the versatility and high potential of microbial lipases used in biomedical and industrial applications, and also their inexpensive production, microbial lipases had been developed continuously. Most of the microorganisms, i.e., bacteria, fungi and yeasts that use neutral oils and fats as a carbon sources for their growth by breaking down the oils and fats prior to their digestion could excrete extracellular lipases through the external membrane into the circumstance. Because microbial lipases are inducible and extracellular enzymes, the properties of the enzyme producer as well as ratio of extracellular to intracellular lipases depend on culture conditions, individually inducible substrates such as olive oil. In addition, Chen *et al.* (1993) found that lipase production of fungi was also enhanced by hydrocarbon such as linear and n-alkanes.

According to the international nomenclature, lipase (E.C. 3.1.1.3 – triacyl glycerol acylhydrolases) are hydrolases cleaving the ester bonds in a triglyceride molecule. In the classification of lipases have a number of unique characteristics such as fatty acid specificity, stereospecificity, regiospecificity and positional specificity so the microbial lipases based on their specificity could be divided into three groups,

depending on the positions and structures of the fatty acids in the triglyceride molecule, which include the following (Macrae, 1983):

2.2.3.1 Nonspecific lipases

This group of lipases showed no marked specificity both as regards the position on the glycerol molecule and releases fatty acids from all three position of glycerol molecule (for example, lipases from *Candica rugosa and Oospora lactis*). These lipases catalyzed complete breakdown of triglycerides to free fatty acid and glycerol, although 1,2(2,3)-diglycerides, 1,3-diglycerides and mono- glycerides appear as intermediates in the reaction (Figure 2.3).

2.2.3.2 1,3-Specific lipases

This lipolytic enzyme catalyzed the release of fatty acid specifically from the outer 1- and 3-positions of glyceride (lipase from *Rhizopus mirosporus, Mucor miehei,* etc.). Therefore, triglycerides were hydrolysed to give free fatty acids, 1,2(2,3)-diglycerides and 2-monoglycerides as reaction products (Figure 2.3). Because 1,2(2,3)-diglycerides and especially 2-monoglycerides were chemically unstable and undergo acyl migration to give 1,3-diglycerides and 1-monoglycerides, respectively, prolonged incubation of a fat with a 1,3-specific lipase would give complete breakdown of some the triglycerides with formation of glycerol.

2.2.3.3 Free fatty acid specific lipases

The last group catalyses the specific release of a particular type of fatty acid from glyceride molecules. The one extracellular microbial lipase which had been shown to posses a very marked specificity for the hydrolysis of esters of a particular type of long-chain fatty acid is that derived from *Geotrichum candidum*. This lipases preferentially released from triglycerides long chain fatty acids containing a *cis* double bond in the 9 position. Saturated fatty acids and unsaturated fatty acids without a double bond in the 9 position are only slowly released. Among these groups, 1,3-specific lipase was commonly found in microbial lipases, while fatty acid specific lipase is little on extracellular microbial lipases.

1. Reaction catalyzed by non- specific lipases:

Triglyceride   **Fatty acid**   **Glycerol**
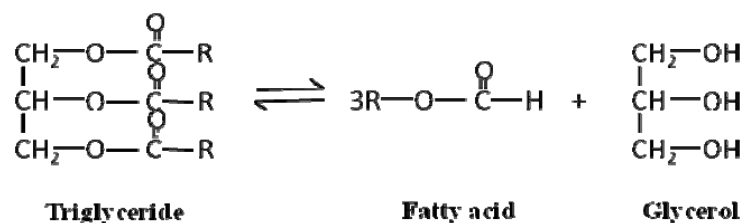
2. Reaction catalyzed by 1,3-specific lipases:

Triglyceride   **Fatty acid**   **1.2(2.3)-diglyceride**   **2-monoglyceride**   **Fatty acid**
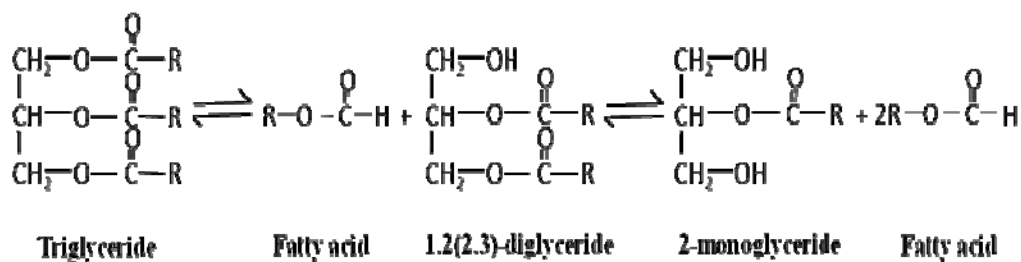
Figure 2.3 Positional specificity of microbial lipases (Macrae, 1983)

2.3 Catalytic reaction of lipases

2.3.1 Mechanism of action at oil-water interface

Lipases belong to the class of serine hydrolases and contain the consensus amino acids sequence $G-X_1-S-X_2-G$ at active site, where G = glycine, S = serine, X_1 = histidine and X_2 = glutamic or aspartic acid (Svendsen, 1994). The natural substrates of lipases were triglycerides of long-chain fatty acids. These triglycerides were insoluble in water and lipases are characterized by the ability to rapidly catalyze the hydrolysis of ester bonds at the interface between the insoluble substrate phase and the aqueous phase in which the enzyme in soluble (Figure 2.4). Under certain experimental conditions, such as in the absence of water, they were capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol.

Interestingly, lipases function at the oil-water interface involves two equilibrium processes in which the overall reaction is illustrated in Figure 2.5, i.e.,

1) Change in enzyme conformation

The lipases must pass through the interface to act so the adsorption of lipase to that site involves a conformation change in the enzyme resulting in an increase in activity.
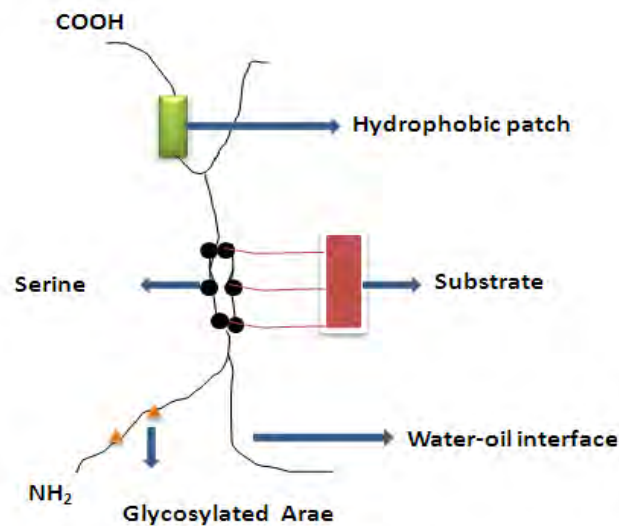
Figure 2.4 Diagrammatic representation of a lipase molecule showing its main features. Substrate can be any triglyceride (Saxena *et al.,* 1999).

2) Production of enzymatic products

When the enzyme-substrate complex is formed, the catalytic reaction occurs, the products are formed and the enzyme is generated because of desorption of the enzyme from the micelle and repenetration to the interface for substrate binding in each catalytic turnover.

Furthermore, the amount of oil available at the interface determines the activity of the lipases (Verger, 1997). This interface area could be increased substantially to its saturation limit by the use of emulsifier as well as by agitation. The saturation limit depends on the ingredients used as well as the physical conditions deployed. Thus, the activities of lipases could be pronouncedly increased by use of emulsifying agent as well as by methods that increase the size of the emulsion micelles. Therefore, the activity of lipases depended on the concentration of micellar substrates at the interface, while the activity of esterases, which catalyze only on the water-soluble substrate is dependent of substrate concentration.
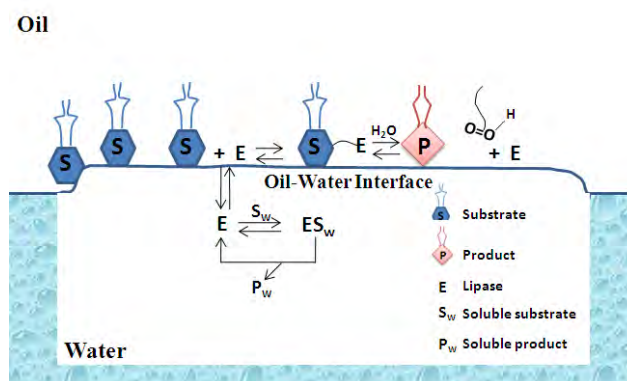
Figure 2.5 Lipolytic reaction at the oil-water interface (Saxena *et al.,* 1999)

2.3.2 Activation and inhibition of lipases

By binding of calcium to the lipase enzyme resulting in conformation change, promoting adsorption of the lipase to the substrate-water interface and/or dissociating from the interface fatty acid products of hydrolysis that might reduce end-product inhibition of the reaction, stimulation of lipase-catalyzed hydrolytic activity might be occurred. However, microbial lipase activity influenced by calcium might be different depending on the enzyme source and assay conditions. Nevertheless, inhibition of lipase activity is appeared by various substances, i.e., anionic surfactants, certain protein, metal ions and phosphorus-containing compounds such as phenylmethyl sulfonylfluoride. In addition, inhibition of lipase activity in the presence of anionic surfactant, was also conducted (Andree *et al.*, 1980).

2.3.3 Biochemically catalytic reaction of triglyceride lipases

Although triglyceride lipases normally catalyze the reaction of lipolysis, they also catalyzed the reverse hydrolysis in reaction media with low water content or in the presence of organic media. Therefore, biochemical reactions catalyzed by lipases involve with three types of reactions (as below). In water soluble media, lipases catalyzed hydrolysis of ester whereas in media with low water content, the lipases catalyzed a wide variety of transesterification and interesterification reactions such as alcoholysis and aminolysis.

1) Hydrolysis

$R_1COOR_2 + H_2O \longrightarrow R_1COOH + R_2OH$

2) Ester synthesis

$R_1COOH + R_2OH \longrightarrow R_1COOR_2 + H_2O$
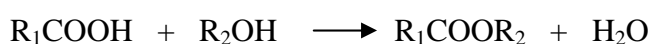
3) Transestrification

3.1) Acidolysis

$$R_1COOR_2 \ + \ R_3COOH \longrightarrow R_3COOR_2 \ + \ R_1COOH$$

3.2) Alcoholysis

$$R_1COOR_2 \ + \ R_3COH \longrightarrow R_1COOR_3 \ + \ R_2COH$$

3.3) Ester exchange

$$R_1COOR_2 \ + \ R_3COOR_4 \longrightarrow R_1COOR_4 \ + \ R_3COOR_2$$

3.4) Aminolysis

$$R_1COOR_2 \ + \ R_3NH_2 \longrightarrow R_1CONHR_3 \ + \ R_2OH$$
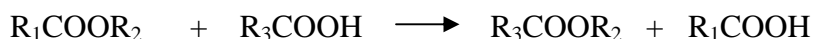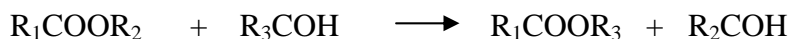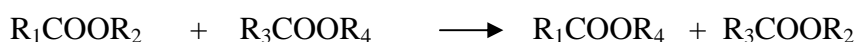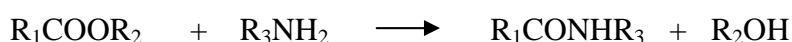
2.4 Endophytic fungi

Endophytic fungi were fungi, which live inside plant tissues for at least part of their life cycle without causing any disease symptoms in the host (Wilson, 1995). Within hosts, fungal endophytes might inhabit in all plant parts including leaves, petioles, stems, twigs, bark, xylem, root, fruit, flower and seeds (Figure 2.6). The relationship between the endophyte and its host plant may range from latent phytopathogenesis to mutualistic symbiosis (Petrini, 1992). Endophytic fungi could be found in all plant species, including algae (Hawksworth, 1988), ferns (Fihser, 1996), and conifers (e.g. Legaul *et al.*, 1989), as well as angiosperms, including grasses (e.g. Clay, 1988), palms (Rodrigues, 1996), and a variety of dicotyledonous shrubs (e.g. Petrini *et al.*, 1986). The ubiquity of fungal endophytes evidence that they might act as mutualists with their host plants ender certain conditions. Endophytes colonizing inside plant tissues usually get nutrition and protection from the host plant. In return, they confer profoundly enhanced fitness to the host plants by producing certain functional metabolites. Endophyte-infected plants often grow faster than non-infected ones (e.g. Carroll, 1988). This effect was at least in part due to the endophytes production of phytohormones such as indole-3-acetic acid (IAA), cytokines, and other plant growth promoting substances, and/or partly owing to the fact that endophytes could have enhanced the hosts uptake of nutritional elements such as nitrogen and phosphorus. In addition, metabolites exploited in pharmaceutical and agricultural industries were widespread among the endophytic fungi (Petrini *et al.*, 1992). For example, the anticancer drug, taxol, which was previously thought to be produced only by the plant genus Taxus (yew) had been found in many genera of endophytic fungi such as *Alternaria*, *Fusarium*, *Monochaetia*, *Pestalotia*,

Pestalotiopsis, *Pithomyces* and *Taxomyces* (Strobel *et al*., 1996). Endophytic fungi have proven to be an important source of new bioactive compounds (Lin *et al*., 2001) and enzymes. Such enzymes including pectinases, xylanase, cellulases, lipases, protinases, phenol oxidase, amylases, proteases, laccase, and asparaginases (Tan *et al.*, 2001, Theantana *et al.*, 2007, and El-Zayat, 2008). Endophytes might involve in decomposition when the tissue becomes senescent or die. The majority of the endophytic species which have been successfully identified are Ascomycetes and Deuteromycetes with a few Basidiomycetes and a very small number of Oomycetes (Issac, 1992).

In the literature, the main studies of endophytic fungi are screened for secondary metabolite, antimicrobial and enzyme activity. Endophytic fungi were considered potent microbes producing hydrolytic enzymes such as a screening of endophytic fungi isolated from Mediterranean plant, which were produced by *Rhizopus oryzae* (Torres *et al*., 2003). Maria *et al.* (2005) studied antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. They tested enzyme activity on agar plates by a clear zone around the colony. Lipases were produced by *Acremonium* sp., *Alternaria chlamydosporus*, *Alternaria* sp., *Aspergillus* sp., *Fusarium* sp. and *Pestalotiopsis* sp., while amylase and protease were produced by a few of them. Silva *et al.* (2006) studied endophytic fungi of *Annona* spp. Endophytic fungi were isolated from leaves, stems and roots of 110 sweetsop and 90 soursop plants from Pernambuco and analyzed for production of extracellular enzymes by qualitative assay in petri dishes containing specific solid media. Nineteen isolates showed lipolytic activity (*Acremonium, Aspergillus, Chaetomium, Colletotrichum, Cylindrocladium, Fusarium, Glomerella, Nigrospora* and *Phomopsis*) while five showed proteolytic activity, cellulolytic and amylolytic activity were not detected. In addition, eleven isolates of the genera *Acremonium*, *Colletotrichum*, *Phomopsis*, *Cylindrocladium, Chaetomium* and *Fusarium* efficiently improved plant growth. Maccheroni *et al.* (2004) studied the effect of ambient pH in the secretion of lipase by endophytic, phytopathogenic, and entomopathogenic isolates belonging to several species of *Colletotrichum* in a plate-clearing assays. However, lipase production by endophytic fungi had relatively received little study. Therefore, the aims of this study work to screen and produce lipase from endophytic fungi.
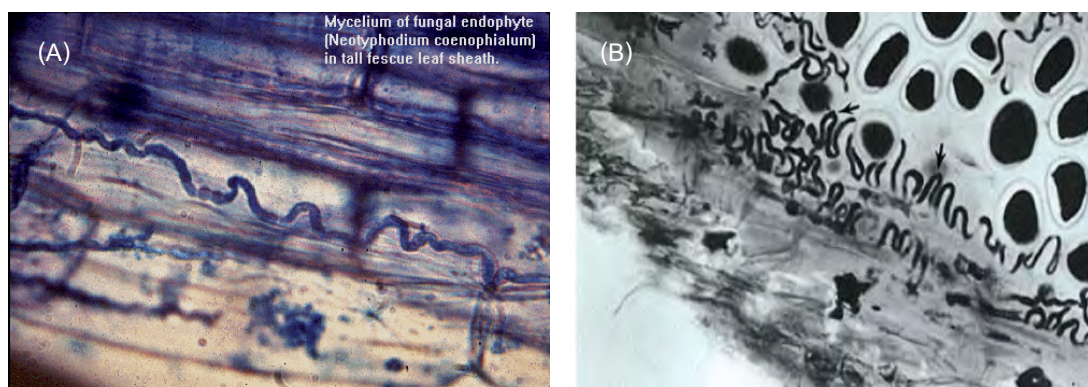
Figure 2.6 Vegetative growth in endophytic fungi. (A) Mycelium of fungal endophyte (*Neothyphodium coenophialum*) in tall feature leaf sheath. (B) Tangential section through seed of *Festuca arundinaceae* showing mycelium within seed (800×). (References: Bouton, 2002 and White, 2000)

2.5 Lipases producing microorganisms

As each industrial application needs lipases with specific properties, there was yet an interest in search for lipase that could be used in new applications of industries. Each industrial supplication requires unique lipase properties with respect to specificity, stability, temperature, pH dependence and ability to catalyze synthetic ester reactions in organic solvents. Therefore, isolation of new lipase-secreting microorganisms and studies on their enzyme production, purification, and characterization could provide new lipases with better quality and wider range of applications.

2.5.1 Screening of lipase producing microorganisms

Screening of lipase activity was tested into primary and secondary screening. In primary screening, several methods have been developed in solid media as qualitative detection. Solid media methods are mainly applied in the detection of microbial lipases and are classified into two categories. First, lipolysis was observed directly by changes in the appearance of the substrate such as tributyrin (Figure 2.7 A) and triolein (e.g. Cardenas *et al.*, 2001) but disadvantage of this method was substrate specific on esterases and hydrolases group. Second, methods where lipase activity was detected with indicator dyes: visualization of lipolytic activity on solid media could be determined by using dyes such as Victoria blue B, Spirit blue (Figure 2.7 B), and Nile blue sulfate (Shelley *et al*., 1987). The drop in pH due to the fatty acids released was observed by the change in color of indicators. This technique was very

convenient for rapidly screening of lipolytic microorganisms but acidification of the medium due to the generation of acidic metabolism other than free fatty acids (FFAs), which were released by microbial lipases, could give false results. In addition, an idea substrate dye rhodamine B in agar plate had been used either in identifying lipase activity (Kouker and Jaeger, 1987). Since the formation of fluorescent products was occurred from triacylglycerol hydrolysis by lipase but the molecular mechanism is still unknown. The suggested mechanism by Kouker and Jaeger (1987) are that an orange fluorescent complex formation with an excitation wavelength of 350 nm is the reaction between cationic rhodamine B and the fatty acid ions (Figure 2.7 C). In addition, this rhodamine B plate method provided the basis of specific and colonies visible upon UV irradiation at 350 nm is caused by hydrolyzed triacylglycerol, the rhodamine B plate method was insensitive to pH changes. Moreover, it also allowed reisolation of organisms, which show no growth inhibition or even change of physiological properties (Kouker and Jaeger, 1987).
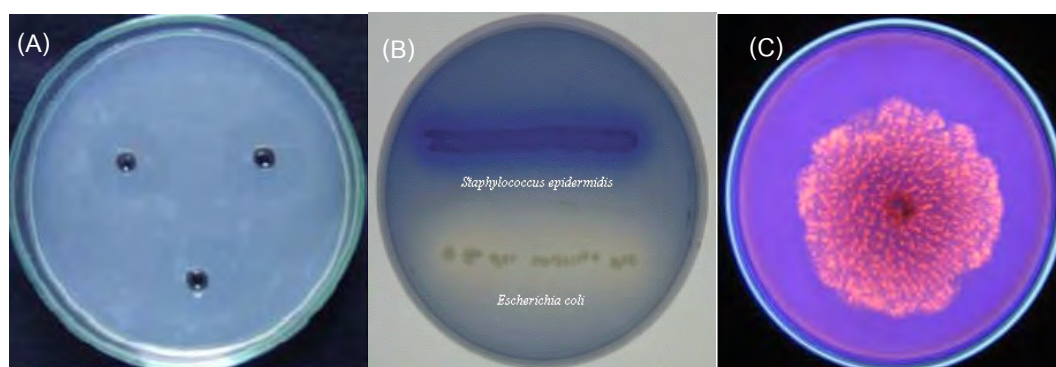
Figure 2.7 Several methods have been developed in solid media. (A) Lipase production is indicated by the formation of clear zone on tributyrin agar plate around the colonies. (B) Visualization of lipolytic activity of *Stahylococcus epidermidis* (positive reaction) and *Escherichia coli* (negative reaction). (C) Detection lipase activity on fluorescent dye rhodamine B agar plate (Reference: Malilas, 2006)

Secondary screening as quantitative methods, which have been developed to measure lipase activity and most of them are base on the principle of product appearance such as titrimetry, photometry, conductimetry, chromatography and IR spectroscopy methods. Turbidimetric method measures change oil/water interface properties. These methods are presented in Table 2.2.

Table 2.2 Assays for the quantification of lipase activity

Lipase assay	Principle
Titrimetry	
pH-stat	Titration by NaOH of Free fatty acids (FFAs) released with time during hydrolysis
Spectroscopy	
Photometry	Conversion of FFAs to copper soaps and reading of absorbance
	Measure of appearance of colored hydrolytic products such as *p*-nitrophenol (synthetic substrates)
Fluorimetry	Measure of the increase with time of the fluorescence intensity of the product at a given wavelength
Infra Red	Monitoring the lipolysis by recording the Fourier-transform IR spectrum of the reaction mixture
Chromatography (HPLC/GC/TLC)	Analysis and quantification of the hydrolytic products (FFAs, Mono-and diacylglycerols)
Radioactivity	Use of triacylglycerols containing radio-labelled acyl chains
Interfacial tensiometry	Monitoring the decrease in surface pressure due to reduction of surface density during hydrolysis of monomolecular substrate film
Turbidmetry	Monitoring the rate of clearing the substrate emulsion
Conductimetry	Measure of conductance change in the reaction mixture during lipolysis
Immuno-chemistry	Use of antibodies recognizing an epitope on a lipase
Microscopy	*In situ* Detection of FFAs by electron microscopy

Reference: Ali *et al.* (2006)

2.5.2 Culture media for lipases production

Lipases are secondary metabolism with the function to initiate the metabolism of lipids when they became available in the extracellular environment. Lipids, insoluble in water, must then be hydrolyzed to more polar compounds, which were furthermore absorbed as nutrients by microbial cells (Haas *et al.*, 1999). The production of lipases was mostly extracellular and microbial lipases are produced

mostly by submerged culture because submerged cultures were usually not difficulty in easy separation from the fermentation broth. However, solid state fermentation methods could be used also and immobilized cell culture had been used in a few cases.

Being excreted through the external membrane into the culture medium of extracellular microbial lipases, the quantities of lipase is influenced by the different factors such as medium composition (carbon and nitrogen sources), temperature, pH, size and age inoculum. Each factor could have various effects on lipase production depending on culture conditions and microorganism under examination (Haas and Bailey, 1993). However, the sources of carbon and nitrogen exerted a particular strong influence on the properties of the enzymes produced by the strains (Rogalska *et al.*, 1990).

Carbon and nitrogen were the fundamental substances for the growth of the microorganisms. Normally, they are supplied from natural sources. Production lipases were mostly induced in presence of fats and oil as the carbon sources. Substrate in the form of lipids must be present in the medium that enhances the production of lipase. It was shown that the physical state of substrates is influenced by temperature and in turn, influences the rates of substrate hydrolysis (Sugiura and Isobe, 1975). Substrate specificity may depend on the chemical structure of substrate molecules and the physical properties of the emulsion or other surface (Espostio *et al*., 1973). The effect of different carbon sources and trace elements added to basal medium was observed with the aim of improving enzyme production of lipase from a Brazilian strain of *Fusarium solani* FSI. When the production medium was supplemented with olive oil, corn oil and sesame oil, the lipase specific activity was optimum (Maia *et al*., 2001). *Mucor* sp. lipase was also produced constitutively in absence of lipids in batch culture, but the regulatory system of lipase synthesis seemed to be stimulated in presence of palm oil (Abbas *et al.*, 2002).

Olive oil was the most used lipid substrate to induce lipase production by bacteria, yeasts, and fungi. Gao and Breuil (1995) compared different plant oils for lipase production from the sapwood staining fungus *Ophiostoma piceae*. High lipase activity was obtained when vegetable oils such as olive, soybean, sunflower, sesame, cotton seed, corn, and peanut oil were supplemented as the carbon source. Maximum lipase production occurred when olive oil was used. Similarly, a thermophilic *Bacillus* strain A30-1 (ATCC 53841) produced maximal levels of thermostable alkaline lipase

when corn oil and olive oil (1%) were used as carbon sources (Wang *et al.*, 1995). The best results in the production of lipase from *Candida rugosa*, were obtained with the use of olive oil as the carbon source in the presence of nitrogen sources. Biomass concentration was also high in the presence of olive oil (Fadiloglu and Erkmen, 2002). One study explored 56 strains of molds for the ability to produce lipase (Costa and Peralta, 1999). A strain identified as *Penicillium wortmanii* was determined to be the best lipase producer (Costa and Peralta, 1999). Maximum lipase production (12.5 U/ml) was obtained in a 7-day culture using olive oil (5% w/v) as the carbon source. The optimal pH and temperature for the crude lipase activity were 7.0 and 45 °C, respectively (Costa and Peralta, 1999).

With additional olive oil supplementation, the volumetric productivity of lipase from *Acinetobacter radioresistens* could be improved; however, the lipase yield decreased with increasing concentrations of olive oil. The increase in the rate of lipase formation by olive oil in the presence of n-hexadecane was attributed to its enhancement of the uptake of n-hexadecane; the hydrolytic products of olive oil promoted the emulsification of n-hexadecane (Chen *et al.*, 1998). Sarkar *et al.* (1998) showed that olive oil at a concentration of 7% (v/v) resulted in an increase in lipase production by *Pseudomonas* sp., but at higher concentration the activity ceased drastically. The inhibition of lipase synthesis at higher olive oil concentrations could be due to poorer oxygen transfer into the medium which can alter fungal metabolism and consequently the production of lipases (Elibol and Ozer, 2000) In view of the reports reviewed, the production of lipase is mostly inducer-dependent, and in many cases, oils act as good inducers of the enzyme.

Various nitrogen sources were tested in the study of lipase production. A minimal medium could be used with an organic nitrogen sources such as urea (Deive *et al.*, 2003), corn steep liquor (Gao *et al.*, 2000), or an inorganic nitrogen sources such as sodium nitrate (Kanwar *et al.* 2002), ammonium sulfate and ammonium nitrate. Also, a rich medium containing peptone (Fadioglu and Erkmen, 2002, Benjamin and Pandey, 1997), tryptone or yeast extract can be used. For an extracellular lipase of *Penicillium citrinum*, Sztajer and Maliszewska (1989) obtained maximal production in a medium that contained 5% (w/v) peptone (pH 7.2). Nitrogen sources such as corn steep liquor and soybean meal stimulated lipase production but to a lesser extent than peptone. Urea and ammonium sulfate inhibited lipase synthesis (Sztajer and Maliszewska, 1989). Lipolytic activity (1120 U/l) was determined by

titration of the free fatty acids released from olive oil incubated with the cell-free broth. The superior nitrogen sources for production of lipase from *Bacillus cereus* were ammonium sulfate (nitrogen level 21.2 mg/100 ml), peptone (nitrogen level 297 mg/100 ml), and urea (nitrogen level 46.62 mg/100 ml) in combination, respectively (Dutta and Ray, 2009). Thermostable lipase of *Pseudomonas* sp. KW1-56 was produced in a medium that contained peptone (2% w/v) and yeast extract (0.1% w/v) as nitrogen sources (Izumi *et al*., 1990). *Acremonium structum* produced a large amount of lipase under stationary conditions in a medium containing 35% (w/v) soybean meal as nitrogen source (Okeke and Okolo, 1990). Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used. One exception reported is *Rhodotorula glutinis* (Papaparaskevas *et al*., 1992). Although good growth of *Rhodotorula glutinis* seems to require organic nitrogen sources (e.g., yeast extract and tryptone), an inorganic nitrogen source such as ammonium phosphate appears to favor lipase production (Papaparaskevas *et al*., 1992). In agreement with other authors, Salleh *et al.* (1993) obtained maximal production of extracellular lipase by the thermophilic fungi, *Rhizopus oryzae*, when the medium contained peptone as the nitrogen source. In studies of thermostable lipase production from thermophilic fungi *Emericella rugulosa, Humicola* sp., *Thermomyces lanuginosus*, *Penicillium purpurogenum*, and *Chrysosporium sulfureum*, use of yeast extract as the nitrogen source gave consistently high lipase production (Venkateshwarlu and Reddy, 1993). A Brazilian strain of *Peicillium citrinum* produced a maximal lipase activity of 409 U/ml in a medium that contained yeast extract (0.5%) as nitrogen source (Pimentel *et al*., 1994). A decrease in yeast extract concentration reduced the attainable lipase activity. Replacement of yeast extract with ammonium sulfate diminished lipase production (Pimentel *et al*., 1994). Lipase production from *Aspergillus niger* increased when the medium was supplemented with an inorganic nitrogen source (ammonium nitrate) (Pokorny *et al*., 1994). Similarly, the addition of ammonium sulfate and peptone into the medium enhanced lipase production by *Ophiostoma piceae* (Gao and Breuil, 1995). Wang *et al*. (1995) reported production of a highly thermostable alkaline lipase by *Bacillus* strain A 30-1 (ATCC 53841) in a medium that contained yeast extract (0.1%) and ammonium chloride (1%) as nitrogen sources.

2.6 Purification and characterization of lipases

    2.6.1 Purification

The importance of lipases has been wildly recognized in numerous fields of application. Although for most of these applications, a high degree of purity is not required, in some instances, when purified biocatalyst is indeed needed, industry looks for large quantities of highly stable and active enzyme preparations purified with a minimum number of steps that will keep the price of process down. The source and nature of enzyme, the eventual application and degree of purify needed contribute to determine the purification schemes. This subject has been recently and extensively reviewed (Saxena *et al.*, 2003, Taipa *et al.*, 1992). Enzyme purity is evaluated after each step with the measurement of overall activity (U) and specific activity (U/mg). The purification efficiency is estimated by the determination of the enzyme activity yield and purification factor. As a general rule, four steps of chromatography are needed to reach high purity for lipase (Palekar *et al., 2000*). In general, purification of lipases from animals or higher plants needs more steps than lipase from microorganisms to obtain a similar degree of purity. However, they seem to be more stable during the entire process.

Some conclusions from Saxena *et al.* (2003) and Taipa *et al.* (1992) can be drawn about the major methods used in the purification of these enzymes. Prepurification steps, most of the microbial lipases are extracellular and the fermentation process is usually followed by the removal of cells from the culture broth, either by centrifugation or by filtration. The cell-free culture broth is then concentrated by ultrafiltration, lyophilization and precipitation method. The precipitation is usually used as a fairly crude separation step often during the early stages of a purification procedure and it can be used as a method of concentration proteins prior to purification step. About 80% of the purification schemes attempted thus far have used a precipitation step, with 60% of these using ammonium sulphate and 35% using ethanol, acetone or an acid (usually hydrochloric) followed by the chromatographic steps. Increase in lipase activity depends on the concentration of ammonium sulfate solution used (Pabai *et al*., 1995). Frequently, it is necessary to remove salts or change the buffer after one step in purification for the next step to work efficiently. This is often achieved by dialysis.

Chromatographic methods have been the most used techniques for lipase purification. Most of the time, a single chromatographic step is not sufficient to get

the required level of purity. Hence, a combination of chromatographic steps is required. The separation of protein on the basis of their charge in ion-exchange chromatography that the separation is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium while gel filtration is a form of partition chromatography used for separating molecules of different sizes. Furthermore, a striking characteristic of many proteins is their ability to bind specific molecules tightly but not covalently by affinity chromatography.

Saxena *et al.* (2003) and Taipa *et al.* (1992) can be drawn about the major methods used in the chromatographic steps. Ion exchange chromatography is the most common chromatographic method; used in 67% of the purification schemes analyzed and in 29% of these procedures, it is used more than once. The most frequently employed ion-exchangers are the diethylaminoethyl (DEAE) group in anion exchange (58%) and the carboxymethyl (CM) in cation exchange (20%). Gel filtration is the second most employed purification method, being used in 60% of the purification schemes and occurring more than once in 22% of the purification processes. Affinity chromatography has been used as a purification step in 27% of the schemes for example; Concanavalin A (Con A) and heparin (Farooqui *et al*., 1994) are employed for purification of fungal and mammalian lipases on account of the glycoprotein nature of these lipases (Tombs and Blake, 1982, Aires-Barros and Cabral, 1991). Affinity methods can be applied at an early stage, but as the materials are expensive, the less costly ion exchange and gel filtration are usually preferred after the precipitation step. Although gel filtration has the lower capacity for loaded protein, it can be used at an early stage in the purification or as one of the last steps for fine polishing of the protocol. Various lipase purification procedures shows that no conclusions can be drawn regarding an optimal sequence of chromatographic methods that maximizes recovery yields and purification fold. Based on the nature of the lipase produced by the organism, one has to design the protocol for purification involving precipitation and chromatographic steps. However, the purity of the enzyme required for its usage.

2.6.2 Characterization

Many lipases have been extensively purified and characterized in terms of their activity and stability profiles relative to pH, temperature, effects of metal ions and chelating agents and molecular weight of enzyme. Characterization of lipase would determine the suitability of lipase in different environments and industries etc.

Ohnishi *et al*. (1994) reported an *Aspergillus oryzae* strain that produced at least two kinds of extracellular lipolytic enzymes, L1 and L2. The enzyme L1 was purified to homogeneity by ammonium sulfate and acetone fractionation, ion exchange chromatography, and gel filtration. Lipase L1 was a monomeric protein (24 kDa molecular weight) and preferentially cleaved all the ester bonds of triolein. A lipase from *Penicillium roqueforti* IAM 7268 was purified to homogeneity by a procedure involving ethanol precipitation, ammonium sulfate precipitation, and three chromatographic steps on different matrices (DEAE-Toyopearl 650 M, Phenyl Toyopearl 650 M, Toyopearl HW-60). The molecular mass of purified lipase was 25 kDa by electrophoresis (Mase *et al*., 1995). Hiol *et al*. (2000) purified an extracellular lipase produced by *Rhizopus oryzae* by ammonium sulfate precipitation, sulfopropyl Sepharose chromatography, Sephadex G-75 gel filtration, and a second sulfopropyl Sepharose chromatography step. The enzyme was purified 1200-fold and had a molecular mass of 32 kDa by SDS-PAGE and gel filtration.

The rate of a reaction approximately doubles for each 10°C increase in temperature. Assuming the enzyme is stable at elevated temperatures, the productivity of the reaction can be enhanced greatly by operating at a relatively high temperature. Consequently, thermal stability is a desirable characteristic of lipases (Janssen *et al*., 1994). The stability of this enzyme at elevated temperatures makes it very useful. Activity of lipase II from *Penicillium cyclopium* (Chahinian *et al*., 2000) and *Penicillium wortmani* (Costa and Peralta, 1999) is stable at pH 7.0 and 40–45°C. Activity is lost at temperatures above 50°C. The lipases from *Penicillium roqueforti* 141 were stable to heating (up to 55°C) and within a wide pH range. *Penicillium cyclopium* lipase was also found to be stable at 35°C for 60 min and has maximal activity in a pH range of 8–10 (Ibrik *et al*., 1998). The optimum temperature for enzyme activity of lipases from a Brazilian strain of *Penicillium citrinum* was also found in the range of 34–37 °C. However, after 30 min at 60°C, the enzyme was completely inactivated. The enzyme also showed optimum at pH 8.0 (Pimentel *et al*., 1994). The Brazilian strain of *Fusarium solani* FSI lipase activity was stable below 35°C but above this temperature activity losses were observed (Maia *et al*., 2001).

The optimum pH of the solvent-tolerant lipase from *Fusarium heterosporum* at 40°C and optimum temperature at pH 5.6 were 5.5–6.0 and 45–50°C, respectively, when olive oil was used as the substrate. The lipase was stable over a pH range of 4–10 at 30°C for 4 h, and up to 40°C at pH 5.6 for 30 min (Shimada *et al*., 1993).

Lipases are active and stable under alkaline conditions and over a broad temperature range, which makes them ideal for incorporation in various applications such as detergent industry. Lipases are also stable in detergents containing protease and activated bleach system (Gormsen *et al*., 1991). The purified *Aspergillus terreus* lipase showed excellent temperature tolerance (15–90°C) and was highly thermostable, retaining 100% activity at 60°C for 24 h. It showed good pH tolerance (3.0–12.0) and was stable over a pH range of 4.0–10.0 for 24 h (Yadav *et al*., 1998). Alkaline lipase produced by *Acinetobacter radioresistens* had an optimum pH of 10 and was stable over a pH range of 6–10; it could have great potential for application in the detergent industry. The optimal temperature and pH for lipase fermentation were 30°C and 7, which were related to cell growth, and stability and reactivity of lipase (Chen *et al*., 1998)

K_m and V_{max}, the latter was the maximum rate of reaction and K_m was a measure of the affinity of an enzyme for a particular substrate. A low K_m value represents a high affinity. The K_m values of the enzyme range widely, but for most industrially relevant enzymes, K_m ranges between 10^{-1} and 10^{-5} M (Fullbrook, 1996). K_m of lipase from Brazilian strain of *Fusarium solani* FSI using *p*NPP (*p* -nitrophenyl palmitate) as substrate was 1.8 mM with a V_{max} of 1.7 umol/min/mg protein (Maia *et al*., 2001). The values of K_m and V_{max} of a lipase from *Aspergillus niger* F044 calculated from the Lineweaver–Burk plot using *p*NPP as hydrolysis substrate were 7.37 mM and 25.91 umol/min/mg protein, respectively (Shu *et al*.,2007).

Metal ions generally form complexes with ionized fatty acids, changing their solubility and behavior at interfaces. Release of fatty acids to the medium is rate determining and could be affected by metal ions. However, the effects of metal ions depended on particular lipase. Mase *et al*. (1995) studied the effect of metal ions (1 mM concentration) on a purified lipase of *Penicillium roqueforti* IAM7268. The lipase activity was not affected by Ca^{2+}, Mg^{2+}, Mn^{2+}, Na^+, K^+, Cu^{2+}, and EDTA. In contrast, the enzyme was inhibited by Ag^+, Fe^{2+}, and Hg^{2+}. Hiol *et al*. (2000) studied the effect of various compounds and enzyme inhibitors on *Rhizopus oryzae* lipase. Among the metal ions, Fe^{2+}, Fe^{3+}, Hg^{2+}, and Cu^{2+} ions strongly inhibited the enzyme. Monovalent ions had little effect on lipolytic activities of both enzymes, Lipase I and II from *Geotrichum candidum*, while divalent ions at concentrations above 50 mM inhibited lipase activities in a concentration-dependent manner (Veeraragavan *et al*., 1990). Neither lipase from the fungus *Penicillium* sp. requires the presence of bivalent

metals in order to exhibit activity, while such cations as Zn^{2+}, Cu^{2+}, and Hg^{2+} are powerful inhibitors (Davranov and Khalameizer, 1997).

2.7 Applications

In the present day industry, lipases have made their potential realized owing to their involvement in various industrial reactions either in aqueous or organic systems, depending on their specificity. Lipases were widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals. The most important commercial use of lipases was in detergents. Approximately 1000 tons of lipases were added to 13 billion tons of detergents produced every year (Jeager and Reetz, 1998). Major applications of lipases are summarized in Table 2.3.

Table 2.3 Uses of lipases in industry

Industry	Used for	Products	Action
Bakery	Improvement of flavor/qualities and shelf life extension	Bakery products	Hydrolysis
Brewing	Improvement of aroma and acceleration of fermentation through lipids removal	Alcohol beverages	Hydrolysis
Cosmetic	Removal of lipids	General Cosmetic (emulsifiers, moisturizers)	Synthesis
Dairy	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Flavoring agents for dairy products milk, cheese and butter	Hydrolysis
Detergent	Removal of oil stains, spots and lipids from fabrics	Detergents for laundry and household applications	Hydrolysis
Fat and oils	Hydrolysis of oil and fats Transesterification of natural oils	Fatty acids, di-,mono-glycerides Reagents for lipid analysis; Oil and fats, such as formulated cocoa butter, margarine, fatty acids , and glycerol	Hydrolysis and Transesterification
Fine chemicals	Synthesis of esters	Ester	Synthesis

Table 2.3 (Cont.) Uses of lipases in industry

Industry	Used for	Products	Action
Food dressing	Quality improvement by lipid hydrolysis	Mayonnaise, dressing and whippings	Hydrolysis
Fuel	Conversion of vegetable oil to esters	Biodiesel	Transesterification
General cleaning	Fat removal	Cleaning agents	Hydrolysis
Leather	Removal of fats from animal skins	Leather products	Hydrolysis
Meat and fish processing	Development of flavor and removal of excess fats	Meat and fish products	Hydrolysis
Medical	Quantification of triglycerides	Diagnostic kits for blood triglyceride assay	Hydrolysis
Paper	Remove pitch from pulp	Paper with improved quality	Hydrolysis
Pharmaceutical	Digestion of oils and fats in foods	Digestive aids, specialty lipids	Hydrolysis and Transesterification
Polymer	Catalyst for polymer synthesis	Biopolymer	Synthesis

Reference: Gunassekaran and Das, 2005

An alternative source of energy for public transport was the so-called biodiesel, which has been produced chemically using oil from various plants (e.g. rapeseed oil). Biodiesel fuel originates from renewable natural resources and concomitantly reduced sulfur oxide production. The conversion of vegetable oil to methyl- or other short-chain alcohol esters could be catalyzed in a single transesterification reaction using lipases in organic solvents. Enzymatic transesterification using lipase had proved more attractive for biodiesel fuel production, since the glycerol produced as a byproduct could easily be recovered and the purification of fatty methyl ester is simple to accomplish. However, the industrial scale production failed so far because of the high cost of the appropriate biocatalyst. Two strategies were presented recently to solve this problem: immobilization of *Pseudomonas fluorescens* lipase increased its stability even upon repeated use (Iso *et al.*, 2001) and cytoplasmic overexpression of *Rhizopus oryzae* lipase in *Saccharomyces cerevisiae* with subsequent freeze-thawing and air drying resulted in a

whole-cell biocatalyst that catalyzed methanolysis in a solvent-free reaction system (Matsumoto *et al.*, 2001).

CHAPTER lll

EXPERIMENTAL

3. Material and methods

3.1. Materials and Chemicals

Acetic acid (Merck Ag Darmstadt, Germany)

Acrylamind (Plusone Pharmacia Biotech, Sweden)

Agar (Sigma, USA)

Ammonium chloride (Sigma, USA)

Ammonium hydrogen phosphate (Sigma, USA)

Ammonium persulfate (Sigma, USA)

Ammonium sulfate (Sigma, USA)

Bis-acrylamide (Promega, USA)

Bovine serum albumin (Sigma, USA)

Bromophenol Blue (USB, USA)

Calcium chloride (Sigma,U.S.A)

Cetyltrimethylammonium bromide (CTAB)

Coconut oil

Copper sulfate (Sigma,U.S.A)

Coomassie Brilliant Blue G-250 (Sigma, USA)

Corn steep liqour

Rhodamine B (Sigma, USA)

Corton oblongifolius Roxb. (Plao-yai) leaves

Dextrose (Sigma, USA)

Di- Ammonium Hydrogen Phosphate (Sigma, USA)

Di- Potassiumhydrogen phosphate (Merck Ag Darmstadt, Germany)

Ethylenediaminetetraacetic acid, EDTA (Sigma, USA)

Ethanol (Merck Ag Darmstadt, Germany)

Ethyl acetate (Ajax Finechem, New Zealand)

Gum Arabic (Sigma, USA)

Hydrochloric acid (J.T. Baker, USA)

Heptane (Sigma, USA)

Iron sulfate (Sigma,U.S.A)

ITS1F and ITS4 primers (Fermentas, Califonia, USA)

Magnesium sulfate (Sigma,U.S.A)

Magnesium chloride (Sigma,U.S.A)

Manganese chloride (Sigma, USA)

2-Mercaptoethanol (Sigma, USA)

Metanol (Sigma, USA)

Methyloctanoate (Sigma,U.S.A)

Methylumbelliferyl butyrate (Fluka, Swit-zerland)

Olive oil

Palm oil

PCR master Mix (Fermentas, Califonia, USA)

Peptone (Sigma, USA)

Phosphoric acid (J.T. Baker, USA)

p-nitrophenyl palmitate (Sigma, USA)

p-nitrophenol (Sigma, USA)

Potassium dihydrogen phosphate (Merck Ag Darmstadt, Germany)

Potato

Sodium azide (Merck Ag Darmstadt, Germany)

Sodium carbonate (Sigma, USA)

Sodium chloride (Merck Ag Darmstadt, Germany)

Sodium hydroxide (Merck Ag Darmstadt, Germany)

Sodium hypochlorite (Sigma, USA)

Sodium nitrate (Merck Ag Darmstadt, Germany)

Soybean powder (Sigma, USA)

Soybean oil

Standard Molecular Weight Marker (Sigma, U.S.A)

Sunflower oil

Tetramethylethylenediamine, TEMED (Plusone Pharmacia Biotech, Sweden)

Tissue paper

Tris (USB, U.S.A)

Triton X-100 (Sigma, USA)

Urea (Sigma, USA)

Yeast extract (Sigma, USA)

Zinc sulfate (Sigma, USA)

3.2 Equipment

Autoclave (Taladlab, Thailand)

Auto pipette (Pipetman, Gilson, France)

Centrifugation (Beckman Coulter, U.S.A)

Dialysis bag (Snake Skin Dialysis Tubing, Pierce, U.S.A)

Electrophoresis unit (Hoefer mini VE, Amersham Pharmacia Biotech, Sweden)

Filter paper (Whatmann NO.1)

Flask

Forcept

Gas chromatography (Aligent Technologies 6890 N)

Gel Electrophoresis unit (Hoefer model miniVE, Pharmacia Biotech, UK)

Gel filtration resin, superdex G-75 (AKTA prime, Sweden)

High Speed Refrigerated Centrifuge (Kubota 6500, Japan) formance Liquid

Hot ait oven (Memmert, Germany)

Hot plate stirrer (HL instrument, Thailand)

Ion exchange resin, DEAE-cellulose (AKTA prime, Sweden)

Laminar Flow (Safety Lab, Asian Chemical and Engineering Co., Ltd., Thailand)

Micro-centrifuge (Tomy MTX-150)

Orbital Shaker (OS-10 Biosan, Latvia)

PCR NucleoSpin® (Macherey-Nagel Inc., Easton, USA)

Petridish plate

pH meter (Mettler Toledo, U.S.A)

Pipette tips (Bioline, U.S.A)

Shaking incubator (Vision Scientific)

Spectrophotometer (Synergy HT Biotek, USA)

Speed vacuum centrifuge (Heto-Holten, Denmark)

Suction

Ultrasonic (leaner D200, D.S.C, U.S.A.)

UV chamber

Vortex mixer (Vortex-Genie2, Scientific Industries, U.S.A)

Water Bath (NTT-1200 Tokyo kikakikai, Japan)

96-well microtiter plate (greiner, USA)

3.3 Microorganisms

3.3.1 Endophytic fungi isolated from *Croton oblongifolius*

Isolation Endophytic fungi from *Croton oblongifoliu* was done by using a modified of Petrini's method (Petrini, 1986). The healthy *Croton oblongifolius* leaves were rinsed cleaned with running tap water and dried in a laminar air flow. The specimens were cut into small pieces (5 × 5 mm). Surface sterilization was prepared by immersing pieces sequentially into 95% (v/v) ethanol for 1 min, 12% (w/v) sodium hypochlorite for 5 min and then 95% (v/v) ethanol for 30 second. Finally, they were washed in sterilized water twice, dried with sterile tissue paper and placed on the surface of potato dextrose agar (PDA) plates. Plates were then incubated at room temperature and examined subsequently for fungal growth. Endophytic fungi were subcultured to fresh PDA medium plates by hyphal tip transfer, and incubated for 7 - 14 days at room temperature to obtain pure cultures. The fungal purity was examines under stereo microscope. Different morphology was collected for further study.

3.3.2 Endophytic fungi isolated from stock cultures

Twenty two fungal endophyte cultures isolated from mangrove leaves and six fungal endophyte cultures isolated from palm leaves were obtain from culture collection of Department Microbiology, Faculty of Science, Chulalongkorn University.

3.4 Screening of endophytic fungi for extracellular lipase production

All endophytic fungal isolates were grown on PDA plates for 7 days. The mycelium agar plugs diameter 5 mm were cut by cork borer from the edge of colony and were them transferred onto the test agar media PDA plates containing 1% (v/v) olive oil and supplement with 0.001% (w/v) rhodamine B. The cultures were incubated at room temperature (30°C) for 7 days. Lipase production was identified as by orange halo around the colonies under UV light at 350 nm (Samad *et al*., 1989). Selective high lipase activity strains were cultured in 100 ml of basal medium (appendix A). Each flask was inoculated with three 5 mm-diameter agar plugs and incubated for 30°C on a rotary shaker with speed 150 rpm. Mycelial were collected by filtered through filter paper (Whatmann No. 1). The filtrates were used for testing lipase activity.

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3.5 Assay for lipase activity

Lipase activity was performed by a modification to the method described by Winkler and Stuckmann (1979) by measuring the increase in the absorbance at 410 nm in a visible spectrophotometer caused by the release of *p*-nitrophenol after hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) as the substrate at 37°C for 30 min. Thirty mg of *p*NPP dissolved in 10 ml isopropanol was emulsified in 90 ml of 50 mM Tris-HCl pH 7.0 containing 1.8% (v/v) Triton X-100 and 100 mg of gum arabic. To initiate the reaction, 0.1 ml of the enzyme solution was mixed with 0.9 ml of the *p*NPP containing emulsion and left for 30 min at 37°C whereupon the reaction was stopped by the addition of 1 ml of 1 M Na_2CO_3. One unit (U) was defined as the amount of enzyme that liberated 1 µmol *p*-nitrophenol per min. Values are given as the mean ± standard error (SE) in triplicate for each point.

3.6 Identification of endophytic fungi

The endophytic fungal strain which showed the highest level of lipase production was then identified to species using morphological and molecular systematic approaches.

3.6.1 Morphological identification

Morphological identification were done by using macroscopic and microscopic characters.

1) Macroscopic features

Colony characteristics of endophytic fungal isolated PTM 7 such as shape, size, color, margin, pigment, and others were studied.

2) Microscopic features

- Preparation of specimen for light microscope

Material preparation for slide culture technique was done onto V-shaped glass rods on filter paper in Petri dishes. The material was sterilized in an autoclave Potato Dextrose Agar was poured in to sterile Petri dishes and when set the agar was cut into 1 x 1 cm size, then aseptically put transferred on to the prepared glass slide and inoculated with the culture of endophytic fungal isolated PTM 7 on the middle of the four edges of agar, that the piece of agar was then covered with a cover slip and sterile distilled water added to maintain moisture inside the Petri dishes. The slide cultures were incubated at room temperature (25-30°C) until the fungus grew onto the glass slide and coves slip.

Semipermanent slides (from slide cultures) for light microscopy were mounted in lactophenol-cotton blue for observation. The microscopical mycelia structure was examined using light microscope.

- Preparation of specimen for scanning electron microscope

Endophytic fungal isolated PTM 7 cultured on PDA was sent for observation and photography with scanning electron microscope at the Scientific and Technological Research Equipment Center, Chulalongkorn University.

The cultures of endophytic fungi isolated PTM 7 was fixed in a solution of 2% (v/v) osmium tetraoxide for 1 hour. The sample were coated with gold using a sputter coater model and observed and photographed with a JSM-5410 LV scanning electron microscope.

3.6.2 Molecular identification

Molecular identification was confirmed and done by based upon the DNA sequence similarity of the internal transcribed spacer (ITS) regions of the rDNA, comparing this isolate to those in the NCBI GenBank database. Genomic DNA was prepared from fresh mycelial cultures of the selected endophytic fungal isolate and extracted with cetyltrimethylammonium bromide (CTAB), as described in Zhou *et al.* (1999). PCR amplification of the internal transcribed spacer (ITS) was performed in a total volume of 35 µl which was comprised of approx. 100 ng genomic DNA, $1 \times$ PCR master Mix (Fermentas, Califonia, USA), and the ITS1F and ITS4 primers. The amplification was performed in a thermocycler with a PCR profile of 94°C for 5 min, followed by 38 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 1 min, plus a final extension of 72°C for 5 min. The PCR reactions were purified using the NucleoSpin® (Macherey-Nagel Inc., Easton, USA) and were direct sequenced on both the leading and lagging strands (using the ITSF1 and ITS4 primers, respectively) commercially by Macrogen (Seoul, Korea). The complete consensus sequence was then used to BLAST search the NCBI GenBank database using the default settings, with the top 100 highest sequence similarity hits being recorded and compared. Species annotation of the deposited ITS sequences in the GenBank database were taken on trust and used to convert the molecular operational taxonomic unit (MOTU) designation of the fungal isolate to a likely species designation where the % sequence similarity was high enough (>97%).

3.7 Lipase production

For lipase production, the selected endophytic fungal isolates were cultivated in a modified basal medium, as described in section 3.4., and the effect of the carbon and nitrogen sources on the extracellular lipase (activity) production level were observed. Previous investigations into the extracellular lipase production in a wide variety of microorganisms have been reported that oils but not sugars as carbon sources enhances the lipase production level (Annibale *et al*., 2006; Brozzole *et al*., 2009; Cihangir and Sarikya, 2004; Hiol *et al*., 2000; Lima *et al*., 2003; Rifaat *et al*., 2010) In this work, various types of oils were used to determine their effects on lipase production over a 20 day culture period, replacing the olive oil in the basal medium (section 3.4) with similar concentrations of one of coconut, sunflower, rice bran, palm and soybean oil. Next, the peptone was replaced in the basal media by one of soybean powder, yeast extract, corn steep liquor and urea as the organic nitrogen source, or sodium nitrate was replaced by ammonium sulfate, ammonium persulfate, ammonium hydrogen phosphate and ammonium chloride as the inorganic nitrogen source. Note that this was performed as a univariate analysis and not a multivariate, and so any potential interaction between these components is not ascertained. Various concentrations of each selected carbon (0.5, 1 and 2% (v/v) oil), organic (0.5, 1 and 2% (w/v)) and inorganic (0.1, 0.2 and 0.5% (w/v)) nitrogen sources were also tested. All experiments were done in three replicates, with the results were reported as the mean ± SE.

3.8 Protein content determination

Protein contents were determined by the Bradford assay (Bradford, 1976), using 0-0.3 mg/ml of bovine serum albumin (BSA) as the standard to construct the calibration curve. For each serial two-fold dilution of the sample in deionized water, 50 µl aliquots were transferred into each of three wells of a microtiter plate and 50 ul of Bradford's reagent (100 ml contains: 10 mg Coomassie Brilliant Blue G-250 and 10 ml of 85% (v/v) phosphoric acid, dissolved in 95% (v/v) ethanol) were added to each well. The plate was shaken (Biosan, OS-10, Latvia) for 5 min and then left for 10 min before reading the absorbance at 595 nm using an ELISA plate reader (Biotek Synergy HT, Biotek instrument, USA). The obtained OD was converted into the protein concentration using the linear equation computed from the standard curve.

During the column chromatographic separations, the elution peak profiles of proteins were determined by measuring the absorbance at 280 nm.

3.9 Purification of lipase

All the procedures were performed at 4^oC, unless otherwise stated.

3.9.1 Lipase precipitation with $(NH_4)_2SO_4$

To 5 liters of culture supernatant, $(NH_4)_2SO_4$ was slowly added with stirring to a final concentration 80% saturation and then left to stand overnight at 4^oC. The precipitate was collected by centrifugation at $10,000 \times g$ for 30 min (Beckman Coulter, USA), and dissolved in dialysis bag (3,500 MWCO) in 5 L distilled water, with against 3 changes at 4^oC and then concentrated by speed vacuum centrifuge (Heto-Holten, Denmark) to ~ 80 mg/ml, which is referred to hereafter as the "ammonium sulfate cut fraction".

3.9.2 DEAE-cellulose ion exchange chromatography

DEAE-cellulose ion exchange chromatography was performed with a 1.6 cm $\times$ 15 cm column using an automatic liquid chromatography system (AKTA prime, Amersham Pharmacia Biotech, Sweden). The column was equilibrated with 5 column-volumes of 50 mM Tris-HCl (pH 7.0). Thereafter, 5 ml samples (400 mg protein) of the ammonium sulfate cut fraction were injected into the column and eluted with the same buffer at a flow rate of 2.0 ml/min, collecting 10 ml fractions before a linear 0 - 1.0 M NaCl gradient in the same buffer was applied over the next 55 fractions. The eluted fractions were monitored for protein content with a UV detector at 280 nm and for lipase activity as described in section 3.5. The fractions containing lipase activity from the column were pooled, dialyzed against 3 changes of 5 L of distilled water and concentrated to ~ 25 mg / ml, and is referred to as the "post-DEAE-cellulose lipase fraction".

3.9.3 Superdex-75 gel filtration chromatography

The post-DEAE-cellulose lipase fraction was then further enriched by preparative Superdex-75 column (1.6 cm $\times$ 60 cm) chromatography. The column was equilibrated with two column-volumes of 100 mM NaCl / 50 mM Tris-HCl (pH 7.0), and then 2 ml of the post-DEAE-cellulose lipase fraction solution (50 mg protein) was injected and eluted in the same buffer at a flow rate of 0.5 ml/min and collecting 5 ml fractions. Fractions were monitored for protein with a UV detector at 280 nm and for

lipase activity as described in section 3.5. The lipase active fractions were pooled, dialyzed against 3 changes of 5 L of distilled water and concentrated to ~ 5 mg / ml, and is referred to as the "enriched lipase fraction".

3.10 Determination of enzyme purity by native-PAGE and lipase activity staining

The enzyme from each step of purification was analyzed by its native protein pattern and its purity according to the method of Bollag *et al*. (1996). Electrophoresis conditions, protein and activity staining are described below.

3.10.1 Non-denaturing gel electrophoresis

Native PAGE was performed with 10% and 5% (w/v) acrylamide separating and stacking gels, respectively, with 100 mM Tris-glycine (pH 8.3) as the electrode buffer. Electrophoresis was run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit (Hoefer model miniVE, Pharmacia Biotech, UK). After electrophoresis, proteins in the gel were visualized by Coomassie blue R-250 (Sigma) staining and activity staining.

3.10.2 Coomassie blue staining

Native (section 3.10.1) and reducing SDS-PAGE (section 3.11) gels were stained by immersion in 0.1% (w/v) Coomassie blue R-250 in 10% (v/v) acetic acid / 45% (v/v) methanol for 45 min. Destaining was performed by immersing the gel in 10% (v/v) acetic acid / 45% (v/v) methanol, with several changes of this destaining solution until the background was clear.

3.10.3 Staining for lipase activity

After native-PAGE resolution the gel was directly immersed in 2.5% (v/v) Triton X-100 / 50 mM Tris-HCl (pH 7.0) at room temperature for 30 min. The gel was then washed rapidly in 50 mM Tris-HCl (pH 7.0) before being immersed in a 100 µM solution of methylumbelliferyl butyrate (MUF-butyrate). Blue florescent bands, indicating lipase activity, were visualized using a UV transilluminator at 365 nm (Prim *et al*., 2003).

3.11 Molecular weight determination by SDS PAGE

Discontinuous reducing 0.1% (w/v) SDS-PAGE was performed according to the procedure of Laemmli (1970) using 15% and 5% (w/v) acrylamide resolving and stacking gels, respectively. Samples were treated with reducing (2-mercaptoethanol containing) sample buffer and boiled for 5 min prior to application to the gel.

Electrophoresis was run with a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. High and low molecular weight standards were coresolved on each gel and used to determine the subunit molecular weight of the enriched lipase enzyme. After electrophoresis, proteins in the gel were visualized by staining with Coomassie blue R-250 as described in section 3.10.2

3.12 Effect of temperature on the lipase activity and thermostability

The effect of temperature on the lipase activity of the enriched lipase fraction (post-superdex-75) was determined by incubating the enriched lipase fraction in 50 mM Tris-HCl (pH 7.0) at various temperatures (-20 - 100°C at 10°C intervals) for 30 min. The thermostability of the lipase was investigated by preincubating the enriched lipase fraction at various temperatures (-30 - 60°C in 10°C intervals) in the same buffer for the indicated fixed time intervals (10 - 120 min), cooling to 4°C and then assaying the residual lipase activity as described above.

3.13 pH-dependence of the lipase activity

Incubating the enriched lipase fraction in buffers of broadly similar salinity levels, but varying in pH from 2 - 14, was used to assess the pretreatment pH stability of the lipase. The buffers used were (all 20 mM) glycine-HCl (pH 2 - 4), sodium acetate (pH 4 - 6), potassium phosphate (pH 6 - 8), Tris-HCl (pH 8 - 10) and glycine-NaOH (pH 10 - 12). The enriched lipase fraction was mixed in each of the different pH-buffer compositions, plus the control (50 mM Tris-HCl (pH 7.0)). For pH stability, the above lipase-buffer mixtures were left for 30 min at room temperature and then adjusted back to 50 mM Tris-HCl (pH 7.0) and assayed for lipase activity as in section 3.5. The control incubation was set at 100% activity and the activity of the samples from the different pH buffers were expressed relative to that of the control (100% activity).

3.14 Effect of metal ions on the lipase activity

The effect of preculture with different divalent metal cation salts (mostly chloride anions but also two sulfate anions) and the chelating agent ethylenediamine tetraacetic acid (EDTA), on the lipase activity of the enriched lipase fraction was evaluated. The enriched lipase fraction was incubated for 30 min with one of chloride salt of cation Ca^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, sulfate salt of cation Cu^{2+} or Zn^{2+} or

EDTA, at one of three concentrations (1, 5 and 10 mM) with continuous shaking. The residual lipase activity was then evaluated (section 3.5), and from this the relative lipase activity (%) was calculated taking the residual lipase activity found in the control samples (without the addition of metal salts or EDTA) as 100%.

3.15 Determination of kinetic parameters

The Michaelis constant (K_m) and maximum velocity (V_{max}) values of the enriched lipase fraction were determined by measuring the rate of pNPP hydrolysis under standard assay conditions. The reaction mixture was 50 mM Tris-HCl (pH 7.0) with the pNPP substrate at concentrations ranging from 0.25 to 6 mM. The values for K_m and V_{max} were then determined from the Lineweaver-Burk plot.

3.16 Preliminary biodiesel production

The ability of this enriched lipase fraction to catalyze the transesterification of palm oil with methanol was evaluated in 10 ml screw-capped vessels containing 1 g of palm oil and 5, 5.5 or 6 Units of the enzyme, and incubated at 30°C for 24 h with shaking at 200 rpm. The transesterification reaction is reversible and so can be driven by increasing in the amount of one of the reactants to obtain a higher fatty acid methyl ester (FAMEs) yield, with theoretically at least 3 molar equivalents of methanol being required for the complete conversion of oil to FAMEs. Thus, the role of the oil: methanol mole ratio on the transesterification efficiency of palm oil, was evaluated at 1:3, 1:4, 1:5 and 1:6 oil: alcohol mole ratios. After incubation the sample was taken from the reaction mixture and centrifuged at 15,000 × g for 30 min to separate the phases. The upper FAMEs containing layer was harvested, its volume was measured and an aliquot mixed thoroughly for gas chromatography (GC) analysis to determine the FAMEs composition using methyl octanoate as the internal standard. For the time course studies, an aliquot of 40 - 50 mg of reaction medium was diluted in n-heptane for GC analysis. The methyl ester was determined on a Aligent Technologies 6890 N GC equipped with an innowax column and a flame ionization detector, using an increasing temperature gradient (30°C to 180°C at 10°C / min, then increasing at 5°C / min to 200°C, at 0.5°C / min to 205°C and hold at 205°C for 2 min, increasing at 5°C / min to 250°C and hold for 5 min). Helium was used as the carrier gas and all GC measurements were performed in triplicate.

CHAPTER IV

RESULT AND DISCUSSION

4.1 Isolation and screening of lipase producing from endophytic fungi

Totally 65 of endophytic fungi isolates were selected on the basis of having different colony morphologies on PDA plates (section 3.3) were screened for extracellular lipase production by growing on PDA plates supplemented with rhodamine B(section 3.4). Only 10 isolates showed a clear zone of fluorescence at 350 nm by signifying the release of fatty acids during hydrolysis of triacyglycerols that then form a complex with rhodamine B producing an orange-fluorescent color under UV light (Kouker *et al*., 1987). Among the 10 active isolates, isolated (PTM 7) produced highest zone of fluorescence than the others (Figure 4.1 and data not shown). Nevertheless, to confirm selection, ten isolates was best lipase producer was selected, all ten of these isolates were evaluated in a liquid culture in the basal medium (section 3.4) and subjected to quantitative analyses of the lipase activity level in the culture media. Endophytes, isolated PTM 7 from *C. oblongifolius* (Plao yai) was found to produce the highest extracellular lipase activity at about 4.3 U/ml after 6 days (data shown in appendix E). The qualitative lipolytic activity closely reflected the quantitative lipase evaluation, with endophytic fungal isolated PTM 7 being the best lipase producer. Therefore, this isolate was selected more detail in order to evaluate species identification. The factors influencing the lipase production, enzyme activity, and kinetics.
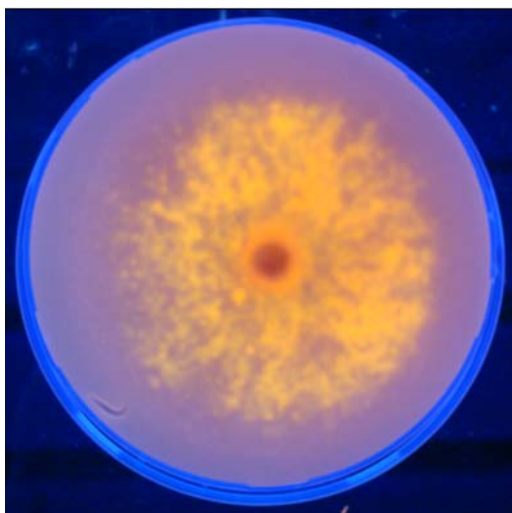
Figure 4.1 Showing the growth of lipase production endophytic fungal isolated PTM 7 on Rhodamine B agar medium with a halo orange fluorescent.

4.2 Identification of endophytic fungi

The isolated PTM 7, showed the highest lipase activity production, was identified to likely species level based on morphological and molecular systematics. With respect to morphological identification, the isolate showed a white to pale violet mycelia and produced a dark violet pigment on PDA medium (Figure 4.2A and 4.2B). Light microscopic examination revealed the presence of septate hypha (Figure 4.2C), whilst the macrocondia were relatively cylinderical, short to medium length with a straight to slightly curved shape (Figure 4.2D). In addition the apical cell is tapered and curved with septate. The microcondia were abundant on the aerial mycelia with an oval shape and without septate (Figure 4.2D). Scanning electron microscograph (SEM) of the macrocondia are shown in Figure 4.2E. Chlamydospores were not detected. From the above characteristics the isolate was identified as a member of the genus *Fusarium,* but these morphological characters are insufficient to identify isolated PTM 7 unequivocally beyond *Fusarium* sp. to the species level. Thus, the isolate was identified by help of molecular systematics using the DNA sequence of the rDNA ITS region. The BLASTn search revealed several highly similar (>97% identity) ITS sequences but these were all from *F. oxysporum* isolates, with the highest sequence identity being to *Fusarium oxysporum* Schlect (emend. Synder & Hansen) strain PY-HLG-2 (GU445378.1) at 99% sequence identity.
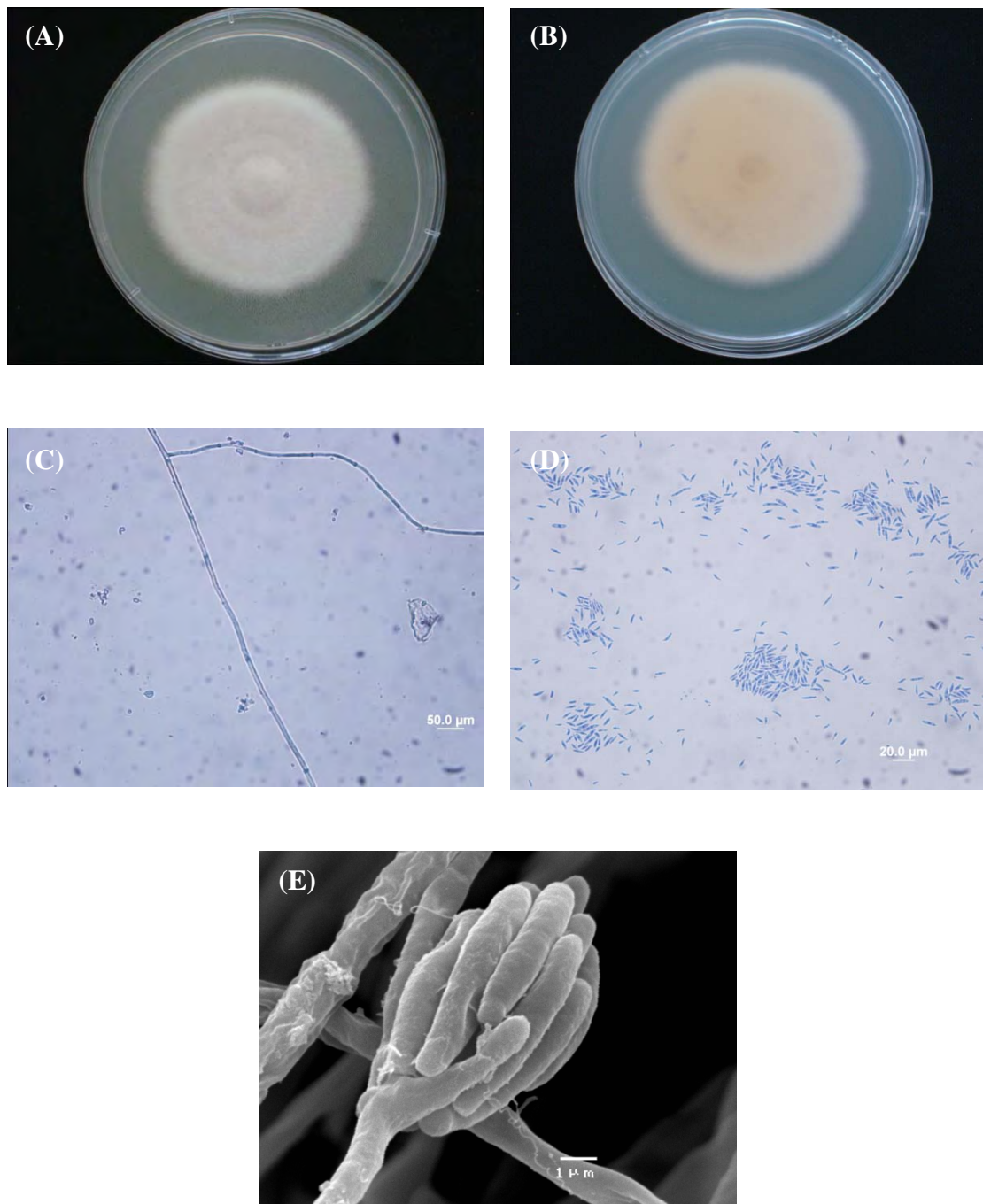
Figure 4.2 Morphology of the selected lipase-positive endophytic fungal isolated PTM 7 showing colony characteristic PDA plate (A) top view, (B) bottom view, (C) light micrograph of septate mycelium (400×) and (D) macrocondia and microcondia (400×) and (E) scanning electron micrograph of macrocondia of fungus isolated PTM 7 (10000×).

Based on the colony characteristics, microscopic structure and nucleotide sequencing analysis of the ITS region of the rDNA encoding DNA, isolated PTM 7 was identified as an isolate of the *Fusarium oxysporum* complex. *Fusarium* is a large genus of filamentous fungi that are commonly occurred in the soil and typically in association with plants. Most species are harmless saprobes and are relatively abundant members of the soil microbial community. Some strains produce mycotoxins in cereal crops that can affect human and animal health if they enter the food chain. The main toxins produced by these *Fusarium* species are fumonisins and trichothecenes (Gordon *et al*., 1997 and Stoner, 1981).

The *F. oxysporum* complex is genetically a heterogenous polytypic morphospecies, but as such is one of the most abundant and widespread microbes of the global soil microflora. However, *F. oxysporum* strains are ubiquitous soil inhabitants that have the ability to exist as saprophytes, and degrade lignin (Rodriguez *et al*., 1996) and complex carbohydrates (Christakopoulos *et al*., 1995) associated with soil debris. They are also pervasive plant endophytes that can colonize plant roots (Gordon *et al*., 1989), and may even protect plants or be the basis of disease suppression (Larkin *et al*., 1993). Although the predominant role of these fungi in native soils may be as harmless or even beneficial plant endophytes or soil saprophytes, many strains within the *F. oxysporum* complex are pathogenic to plants, for example, an outbreak of *F. oxysporum* that affected coca plantations in Peru, and other crops planted in the area, lead the United States to propose the use of the agent as a mycoherbicide in drug eradication.

4.3 Production of lipase

4.3.1 Effect of carbon source on lipase production

The activity of extracellular lipase of *F. oxysporum* was monitored at the initiation of culture and then after every 2 days of shaking incubation in basal medium (section 3.4) containing 1% (v/v) of a different vegetable oil as the carbon source (section 3.7) over a 20-day period (Figure 4.3A). The highest lipase production was obtained with olive oil (4.30 U/ml) after 6 days of incubation, and this then declined slowly over the next 4 - 6 days to the same lipase activity level as the next highest (sunflower oil as the carbon source), before rapidly falling. Indeed, a marked decrease in the lipase activity was observed with all the different oil based carbon sources during the later incubation periods, although the kinetics varied between oil types,

probably due to the increasing presence of proteases in the culture medium. The worst carbon source, in terms of lowest lipase activity production, was found with soybean oil, but whether this may reflect other components in the oil or the oil composition itself is not clear.
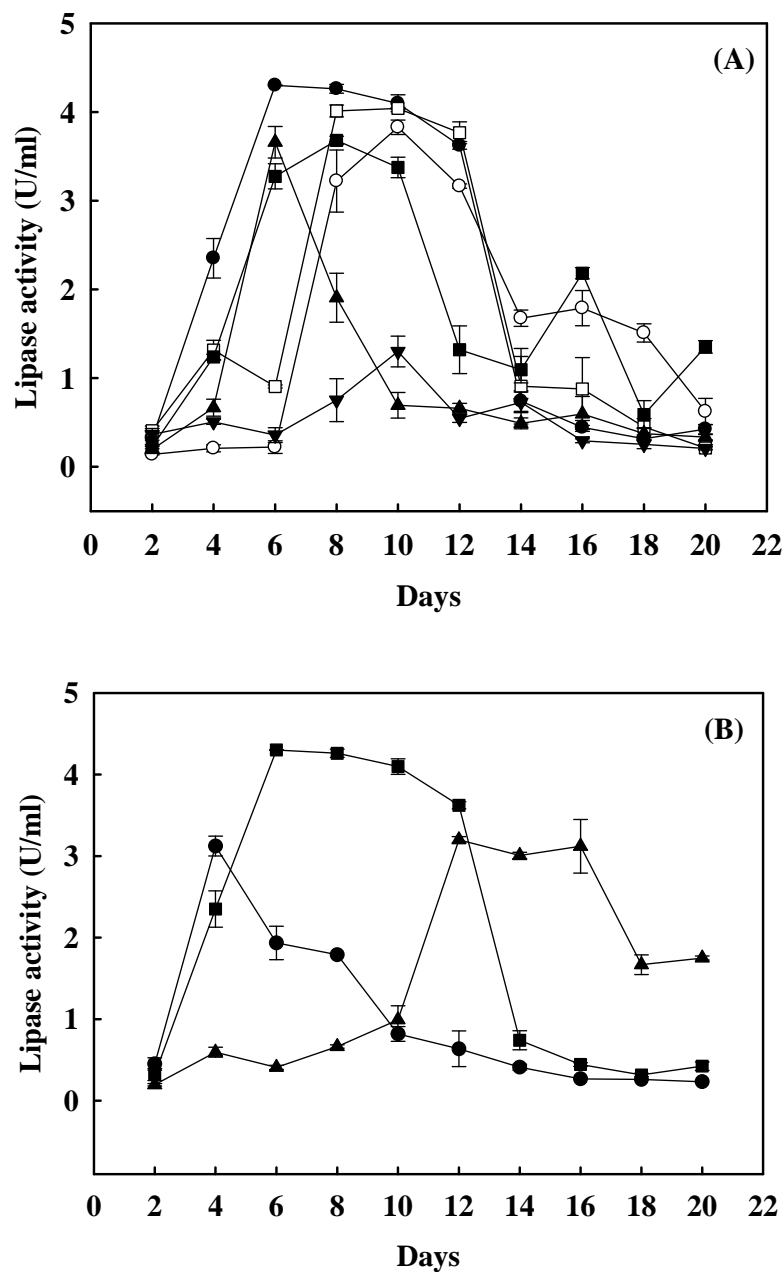
Figure 4.3 Effect of the oil-based carbon source on the lipase production by the endophytic fungi *F. oxysporum* isolated PTM 7. (A) Various carbon sources (1% (v/v) vegetable oils) as: olive (●), coconut (○), sunflower (□), rice bran (■), palm (▲) and soybean (▼). (B) Various concentrations (v/v) of olive oil: 0.5% (●), 1.0% (■) and 2.0% (▲). For both panels the data are shown as the mean of the three replicates $\pm$ SE.

That olive oil was found to be the best carbon source for the synthesis of lipase has been previously reported for *F. oxysporum*, although the activity yield obtained was significantly high at 17.0 U/ml (Hala *et al*., 2010), as well as by *Mucor racemosus* (Nadia *et al*., 2010), *Penicillium wortmanii* (Costa and Peralta, 1999) and *Trichoderma reesei* (Rajesh *et al*., 2010). In slight contrast, an even higher lipase activity was obtained from another *F. oxysporum* isolate on sunflower oil (35.8 U/ml), whereas corn and olive oils showed a relatively moderate yield (22.9 and 21.8 U/ml), respectively (Moataza *et al*., 2005). In terms of lipid yield or activity, it is of note that the evaluation method of Winkler and Stuckmann (1979) used here differs from that Licia *et al*. (2006) and Maia *et al*. (1999) used by some other authors and so differences between this and other studies are for comparative and not qualitative purposes.

In general, lipase production in microorganisms is enhanced by varying not only the lipid source but also its concentration. The effect of the concentration of the carbon source on lipase production by this isolated PTM 7 was studied with the addition of three different concentrations (0.5, 1.0 and 2.0% (v/v)) of olive oil to the basal medium (section 3.4), and then cultivated as before (section 3.7). The olive oil concentration was observed to have a strong influence on the amount of lipase produced (Figure 4.3B), where an increase in the olive oil concentration delayed the peak lipolytic activity attained, and whilst the peak activity obtained increased as the olive oil concentration increased from 0.5 to 1% (v/v), it was lower at 2% (v/v) oil, suggesting an inhibitory effect on the production of lipase from *F. oxysporum*. This could be due to a lower oxygen transfer into the medium, which can alter fungal metabolism and consequently the production of lipases (Elibol and Ozer, 2000), or the lower production of lipase by increasing olive oil concentrations might be due to the inhibition effect of the increasing concentration of released fatty acid in the culture medium as result of olive oil hydrolysis (Akhtar *et al*., 1980).

4.3.2 Effect of nitrogen source on lipase production

Both organic and inorganic nitrogen play an important role in enzyme synthesis. Inorganic nitrogen sources can be exhausted from the culture media quickly, while organic nitrogen sources can supply many cell growth factors and amino acids, which are needed for cell metabolism and enzyme synthesis. Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used. However, Pokorny *et al*. (1994) reported that the production of lipase by *Aspergillus*

niger increased when the medium was supplemented with an inorganic nitrogen source. Therefore, the effect of both organic and inorganic nitrogen sources on the lipase production level was evaluated using five different organic nitrogen sources each at one of three concentrations.

The highest production of lipase activity (2.20 U/ml), and so the best organic nitrogen source, was found to be supplemented with 1% (w/v) peptone (Figure 4.4A), although 0.5% (w/v) soybean powder was almost as effective. The effect upon lipase production levels of all five organic nitrogen sources were dose-dependent, typically decreasing with the higher doses, except for peptone, whilst urea was the worst. The superiority of peptone seen here is in accord with that reported previously for *F. oxysporum* (Moataza *et al*., 2005), *F. globulosum* (Ruchi *et al*., 2005) and *Aspergillus sp*. (Cihangir *et al*., 2004), as well as *Penicillium restrictum* (Freire *et al*., 1997) and *Rhizopus homothallus* (Rodriguez *et al*. 2006). To explain the superiority of peptone as an organic nitrogen source over other complex nitrogen sources such as yeast extract, Freire *et al*. (1997) suggested that peptone contains certain co-factors and amino acids that match the physiological requirement for lipase biosynthesis.

The role of five different inorganic nitrogen sources on lipase production was evaluated at three concentrations (0.1 - 0.5% (w/v)), and revealed that sodium nitrate at 0.5% (w/v) gave the highest lipase activity (2.58 U/ml) compared to the four other inorganic nitrogen sources and concentrations (Figure 4.4B). Indeed, sodium nitrate was superior at all three concentrations tested, whilst ammonium chloride was the least effective supplement. This correlates with the optimum growth of mycelium (data not shown).

However, the effect of the inorganic nitrogen sources was unstable over culture time since the lipase production level decreased over time when the same culture was used for serial propogation in basal culture media (data not shown), presumably since the fungi adapt as changes in both the morphological and physiological characteristics were observed (data not shown). Therefore, for increased lipase production the stock culture should be cultured on PDA plates containing with 1% (v/v) olive oil to induce enzyme activity before transferring to the liquid basal culture media for lipase production.
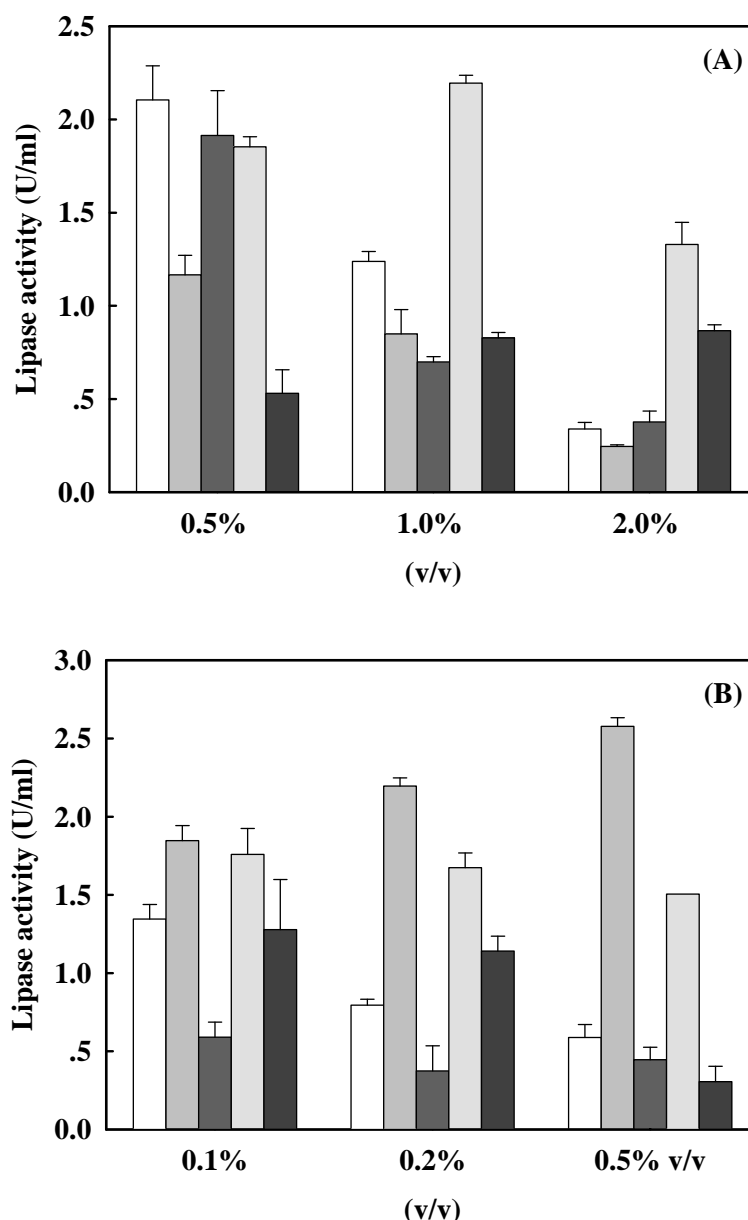
Figure 4.4 Effect of (A) organic and (B) inorganic nitrogen sources and their concentrations on the lipase produced level by *F. oxysporum* isolated PTM 7. The fungal isolate was cultured in basal media with the indicated organic or inorganic substitutions for 6 days. (A) Organic nitrogen: soybean powder (white), yeast extract (grey), corn steep liquor (dark grey), peptone (light grey) and urea (black). (B) Inorganic nitrogen: ammonium sulfate (white), sodium nitrate (grey), ammonium persulfate (dark grey), ammonium hydrogen phosphate (light grey) and ammonium chloride (black). For both panels A and B the data are shown as the mean of three replicates $\pm$ SE, and are derived from three repeats. Means with a different lower case letter above them are significantly different ($p<0.05$; Duncan's multiple means test).

4.4. Purification of Lipase

At the end of the cultivation period for six days, mycelia were removed by filtration through Whatmann No.1 chromatography paper. Lipases from other sources have previously been purified by conventional purification strategies employing ammonium sulphate precipitation and chromatography. Moataza *et al.* (2005) reported the partial purification of a *F. oxysporum* lipase was optimal with an initial 80% saturation ammonium sulfate precipitation (3.92-fold purification). Thus, an initial 80% ammonium sulfate cut was performed (section 3.9.1), resulting in a reduction in the total protein content of ~75%, but with a loss of ~58% of lipase activity and so only a 1.64-fold enrichment (Table 4.1).

Ammonium sulfate fraction was cut then subjected to DEAE-cellulose anion exchange chromatography (section 3.9.2.). The lipase active fraction was adsorbed onto the DEAE-cellulose column, allowing separation from the unbound proteins, and eluted from the column at 200 – 375 mM NaCl, whereas the non-lipase active bound protein eluted as a double peak at lower and equal salt levels (Figure 4.5A). Thus, the elution pattern showed a single lipase activity peak which was harvested and pooled. Compared to the ammonium sulfate cut fraction, the post-DEAE-cellulose lipase fraction showed a 63% reduction in the total protein content for only a loss of 16% lipase activity (Table 4.1), but the preparation was still not homogenous (Figure 4.6 A).

Thus, the post-DEAE-cellulose lipase fraction (section 3.9.2) was further fractionated using Superdex-75 gel column chromatography (section 3.9.3), where a sharp peak was eluted free of most of the other lipase activity negative proteins (Figure 4.5B). Compared to the post-DEAE-cellulose lipase fraction, although the post-Superdex-75 fraction (enriched lipase fraction) showed a 99.4% reduction in the total protein content this was achieved at the cost of a 93.4% loss of lipase activity, resulting in a 10.9-fold activity enrichment (Table 4.1). Overall, a 41.4-fold enrichment for a 2.21% yield was obtined after the three enrichmnt stages, compared to the crude culture filtrate (Table 4.1). The enriched lipase fraction (post-Superdex-75; section 3.9.3.), with a specific activity of 156.3 U/mg of protein (Table 4.1) and was enriched to or near to apparent homogeneity (Figure 4.6A), was used for all further enzyme characterization. The final specific activity obtained here was high compared to that reported for some other reported lipases from fungi, such as 6.1 U/mg for *F. oxysporum* f. sp. lini (Hoshino *et al.*, 1992), 11.1 U/mg for

R. rhizopodiformis (Razak *et al*., 1997) and 63 U/mg for *Pythium ultimum* (Mozffar and Weete, 1993) but was broadly similar to that (146 U/mg) reported for *Rhizopus chinensis* (Yasuda *et al*., 1999).
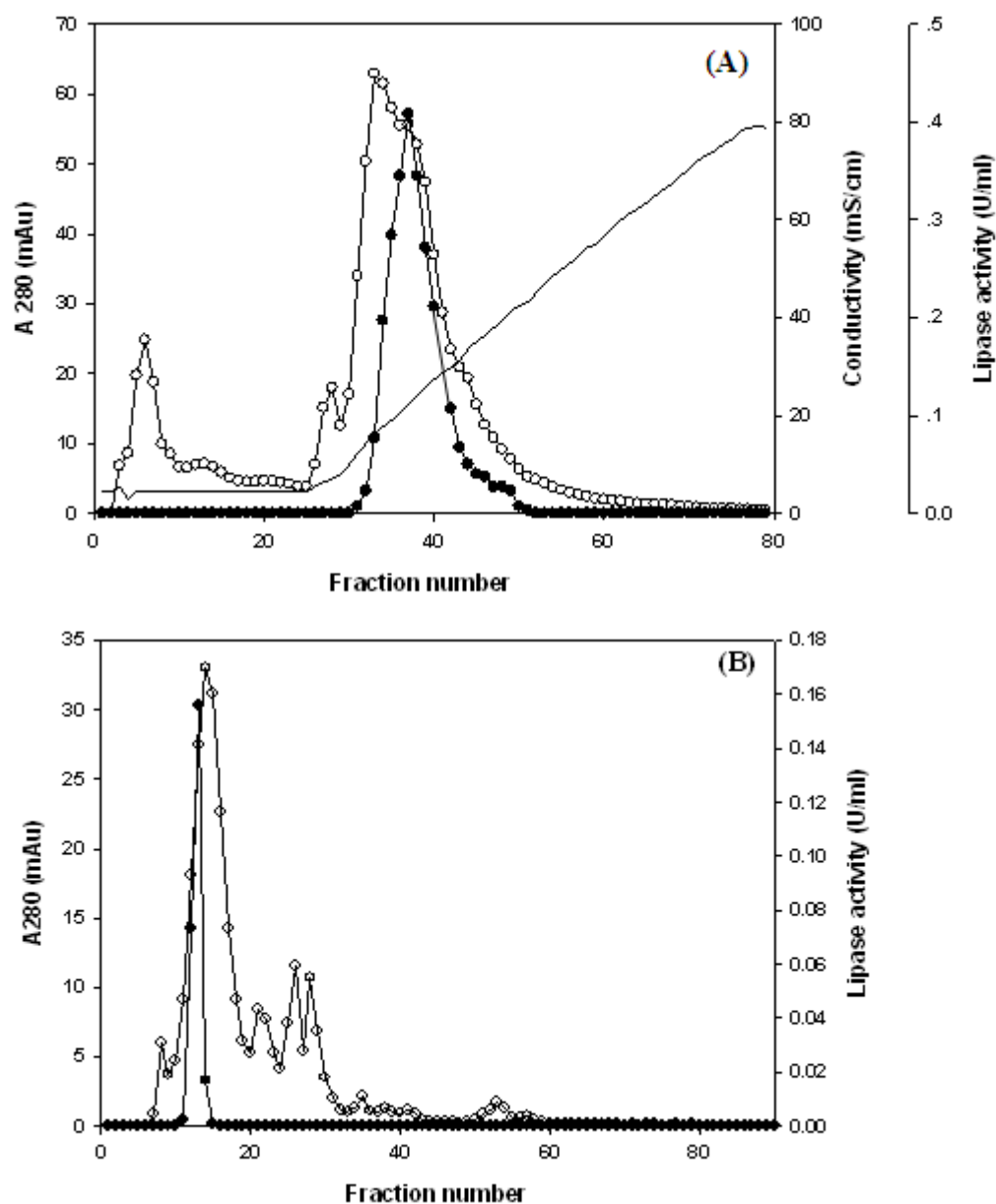
Figure 4.5 Profile of the enrichment of *F. oxysporum* isolated PTM 7 extracellular lipase extract by; (A) DEAE-cellulose ion-exchange chromatography of the ammonium sulfate cut fraction (400 mg protein) eluted in 20 mM Tris-HCl (pH 7.0) with a 0 - 1 M NaCl linear gradient; and (B) Superdex-75 gel chromatography of the post-DEAE-cellulose lipase fraction (50 mg) eluted in 100 mM NaCl / 20 mM Tris-HCl (pH 7.0). For both panels a and b; absorbance at 280 nm (○), lipase activity (●).

Table 4.1 Enrichment summary for the lipase from *F. oxysporum* isolated PTM 7

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	2996.25	11315.25	3.78	100.00	1.00
(NH$_4$)$_2$SO$_4$ precipitation	765.00	4740.00	6.20	41.89	1.64
DEAE- cellulose	280.00	4000.00	14.29	35.35	3.78
Sephadex-75	1.60	250.00	156.25	2.21	41.37

4.5 Determination of enzyme purity and protein pattern on native-PAGE

The lipase from each step of enrichment was analyzed for purity and protein pattern by native-PAGE, with protein and enzyme activity staining (Figure 4.6A). Whilst the post-DEAE-cellulose lipase fraction still showed multiple components, the enriched lipase fraction (post-Superdex-75 lipase fraction) showed a single protein band on native-PAGE, suggesting a high degree of purity, with only a enzyme (fluorescence) band seen when using methylumbelliferyl butyrate as the substrate, and at the same position (R$_f$), supporting that the enriched lipase fraction was a pure or near pure enzyme.

4.6 Determination molecular weight of lipase from *F. oxysporum* isolated PTM 7

Discontinuous reducing SDS-PAGE, a relatively sensitive technique for lipase separation, revealed a single strong band with an apparent molecular weight of 37.4 kDa after Coomassie blue R250 staining (Figure 4.6B). This supports enrichment to near homogeneity and suggests that the purified lipase could be a monomeric protein, or at least if a multimeric one that dissociates into subunits under these enrichment conditions, that this 37.4 kDa subunit has lipase activity alone.

(A)   **(B)**
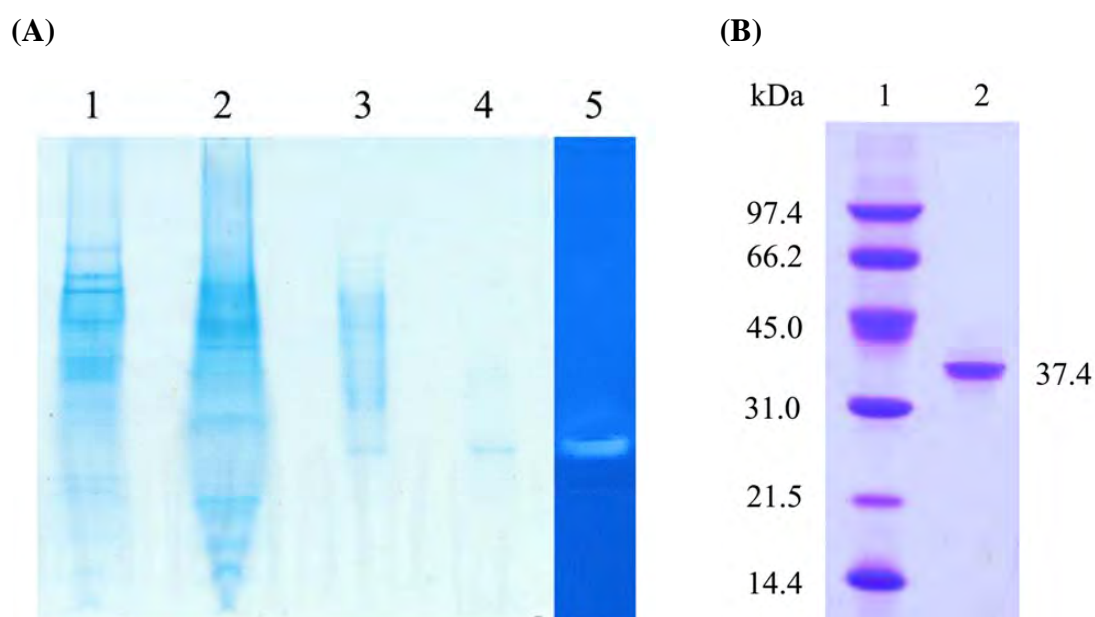
Figure 4.6 (A) Coomassie blue stained native-PAGE analysis of the *F. oxysporum* isolated PTM 7 lipase fractions from each step of the enrichment and stained for protein by coomassie blue (Lanes 1 - 4) or for lipase enzyme activiy (Lane 5). Lane 1, crude enzyme (20 µg of protein); Lane 2, ammonium sulfate cut fraction (20 µg of protein); Lane 3, post-DEAE-cellulose lipase fraction (15 µg of protein); Lanes 4 & 5, enriched lipase fraction (post-Superdex-75) (10 µg of protein). (B) Reducing SDS-PAGE analysis, after coomassie blue staining, of the enriched lipase fraction (post-Superdex-75) from *F. oxysporum* isolated PTM 7: Lane 1, Low molecular weight protein markers; Lane 2, enriched lipase fraction (5 µg of protein).

4.7 Effect of temperature on lipase activity and thermostability

To determine the effect of temperature on the enriched lipase fraction from *F. oxysporum* isolated PTM 7, the enzyme activity was estimated over the temperature range of -20 – 90°C at pH 7.0 for 30 min. The maximum activity was observed at 30°C (Figure 4.7A), declining to about 89% by 120 min (Figure 4.7B), with increasing temperatures above 30°C, a reduction in the lipase activity was noted, especially with increasing exposure time to the elevated temperature. Indeed, the lipase did not appear to be very thermoresistant, losing 25% and 29% activity during 10 or 20 min incubation at 50°C and 33% and 60% at 60°C, respectively.

According to the above data, the maximum thermostability of the lipase was observed at 30°C and whilst it showed a high stability at low temperatures, retaining

94, 75, 68, 85 and 94 % relative activities after 30 min at -20, 0, 4, 10 and 20°C, respectively (Figure 4.7A), at temperatures above 30°C a significant loss in enzyme activity was observed with increasing temperature and only 6% relative activity remained after 30 min at 90°C. This decrease in enzyme stability might be due to denaturation (Maria de Mascena *et al*., 1999). In general, earlier studies have shown that fungal lipases are not stable at temperatures above 30 - 40°C (Lima *et al*., 2004; Chahinian *et al*., 2000). However, some exceptional cases have been reported, such as the lipases produced by *Penicillium wortmanii* (Costa and Peralta, 1999) and *F. solani* (Maia *et al*., 2001).

4.8 Effect of pH on lipase activity and stability

The stability pH of the purified lipase from *F. oxysporum* was determined by measuring its activity at different pH values for 30 min. The enzyme was reasonably active over a broad pH range of 6 - 12, with a weaker activity level at pH 3 - 6, and the maximum activity was observed at pH 8.0 (Figure 4.8A). However, the results with respect to the effect of pH were compounded by a significant buffer-dependent effect, especially between the potassium phosphate and Tris-HCl buffers at pH 8.0, as well as between the glycine-HCl and sodium acetate buffers (cf. pH 4.0 in Figure 4.8A).

The pH stability of the enriched lipase fraction was determined by incubating the enzyme in the respective buffers at different pH values, and revealed that the enzyme was more stable at pH 8 and this activity declined with increasing incubation time at each pH, and with increasing pH values, but at pH 10.0 - 12.0 the relative lipase activity was only slightly decreased from that at pH 9, in contrast to the larger decreae from pH 8 to 9 (Figure 4.8B). The lipase retained around 90% and 67% activity after incubation 30 min at pH 9.0 and 12, respectively, compared to ~75% and 4% at 120 mins, respectively. A similar result was reported for the extracellular lipases from other isolates of *F. oxysporum* (Hoshino *et al*., 1992; Maria de Mascena *et al*., 1999) and *Mucor spp*. (Abbas *et al*., 2000). The lipase exhibited pH and temperature kinetics that are potentially suitable for the detergent industry, as the enzyme is active at neutral to alkaline pH and also within the 10 – 30°C temperature range. Further characterization of the lipase was therefore carried out to evaluate it as a potential additive.
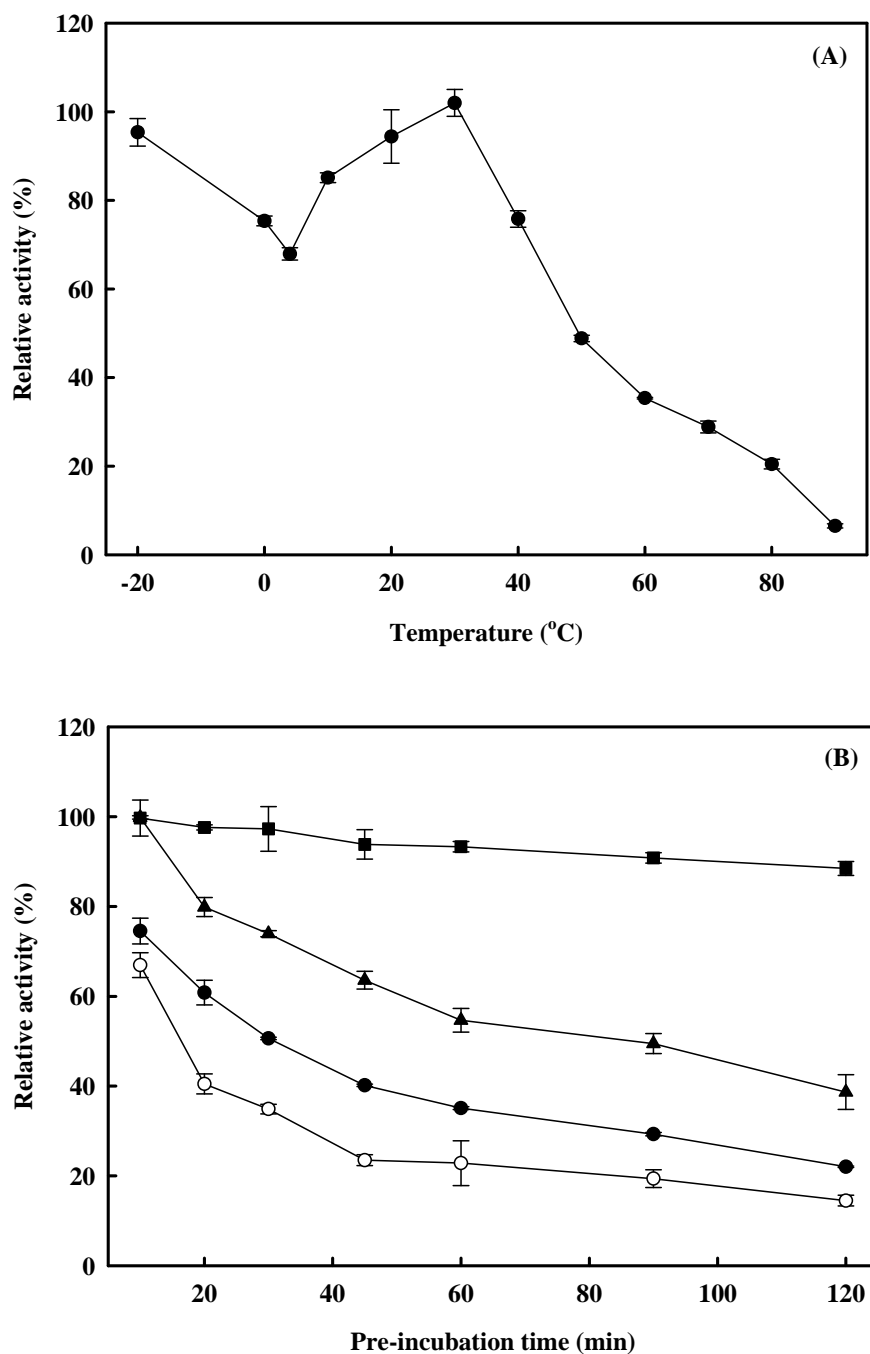
Figure 4.7 The (A) thermostability of the enriched lipase fraction from *F. oxysporum* isolated PTM 7 at various temperature for 30 min (B) thermostability of the enriched lipase fraction from *F. oxysporum* isolated PTM 7, assayed in 50 mM Tris-HCl (pH 7.0) at (■) 30°C, (▲) 40°C, (●) 50°C and (○) 60°C. For both panels A and B the data are shown as the mean $\pm$ SE, and are derived from three repeats. Means with a different lower case letter above them are significantly different (p<0.05).
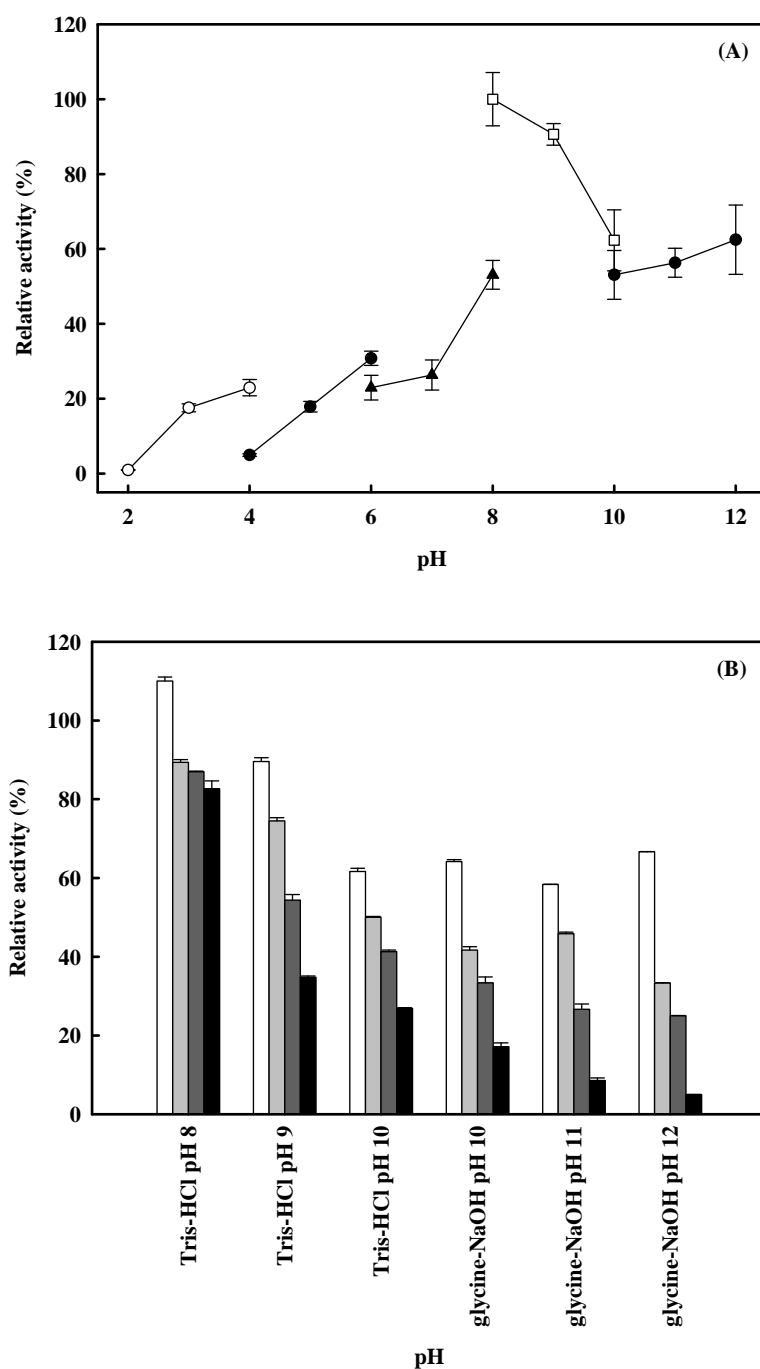
Figure 4.8 Effect of pH on the (A) the effect of pH on lipase activity was evaluated for 30 min in (all 20 mM) glycine-HCl (pH 2.0 - 4.0), sodium acetate (pH 4.0 - 6.0), potassium phosphate (pH 6.0 - 8.0), Tris-HCl (pH 8.0 - 10.0) and glycine-NaOH (pH 10.0 - 12.0) buffers. (B) The effect of pH on stability showing the relative lipase activity after incubation at various times in the presence of 20 mM Tris-HCl (pH 8.0 - 10.0) or 20 mM glycine-NaOH (pH 10.0 - 12.0). Data represent mean of three replicates $\pm$ SE.

4.9 Effect of metals ion and reagents on lipase activity

The effect of the addition of seven different divalent cation salts (five as chlorides but two as sulfates see Table 4.2 or the chelating agent EDTA, at one of three concentrations on the lipase activity is shown in Table 4.2 Ca^{2+}, Mg^{2+} and especially Mn^{2+} ions stimulated the lipase activity, while Cu^{2+}, Fe^{2+}, Hg^{2+} and Zn^{2+} all showed a dose-dependent inhibition of lipase activity, with a greater lipase inhibition being observed at higher salt concentrations. The approximately 140% increase in the lipase activity in the presence of Mn^{2+} ions is remarkable, since very little information is available about the promotion of lipolytic activity by Mn^{2+}. Indeed, in contrast, Mn^{2+} at 5 mM produced an expressive inhibitory effect (64%) of enzyme activity from *Metarhizium anisopliae* (Walter *et al*., 2009), compared to the 136% activity seen here with the same concentration of Mn^{2+}.

The lipase activity was inhibited by Fe^{2+} and Hg^{2+} is consistent with that reported for the *Mucor sp*. lipase (Abbas *et al*., 1999). Perhaps the Fe^{2+} and Hg^{2+} ions form a complex with the ionized fatty acids and change their solubility and behaviors at the oil-water interface. In addition, that Hg^{2+} inhibited the lipase activity could suggest the presence of at least one sulfhydryl group, most likely a cysteine amino acid residue, at the active site. Oxidation of this group by cations destabilizes the conformation folding of the enzyme, or leads to formation of disulfide bonds at irregular positions within the protein (Bera-Maillet *et al*., 2000). The metal chelating agent EDTA at 5 and 10 mM inhibited the lipase activity, which is consistent with the lipase being a metalloprotein and requiring Mn^{2+}. However, somewhat oddly, at 1 mM EDTA was not inhibitory but in contrast actually stimulated the lipase activity (127%).

Table 4.2 The effect of divalent cation salts and the chelating agent EDTA on the lipase activity of the enriched lipase fraction from *F. oxysporum* isolated PTM 7.

Reagent	% Relative lipase activity[a]		
	1 mM	5 mM	10 mM
Control[b]	100.0 $\pm$ 0.00	100.0 $\pm$ 0.00	100.0 $\pm$ 0.00
CuSO$_4$	74.0 $\pm$ 0.39	60.0 $\pm$ 0.45	14.0 $\pm$ 1.41
ZnSO$_4$	82.0 $\pm$ 0.28	68.0 $\pm$ 0.73	48.0 $\pm$ 0.81
CaCl$_2$	123.3 $\pm$ 0.18	100.0 $\pm$ 0.28	93.3 $\pm$ 1.09
MnCl$_2$	138.9 $\pm$ 0.69	136.1 $\pm$ 1.34	133.3 $\pm$ 0.94
HgCl$_2$	82.0 $\pm$ 1.53	68.0 $\pm$ 0.50	42.0 $\pm$ 0.74
MgCl$_2$	106.7 $\pm$ 0.27	113.3 $\pm$ 1.20	46.7 $\pm$ 0.33
FeCl$_2$	97.6 $\pm$ 0.42	60.2 $\pm$ 0.38	40.7 $\pm$ 1.98
EDTA	126.7 $\pm$ 0.25	80.0 $\pm$ 0.25	77.8 $\pm$ 1.65

[a]The relative activity was determined by measuring the lipase activity at 37°C in 50 mM Tris-HCl (pH 7.0) after pre-incubation at 30°C for 30 min with the indicated reagents and concentrations, [b]using the activity seen in the absence of such reagents in 50 mM Tris-HCl (pH 7.0) alone as 100%. Mean of three replicates $\pm$ SE. Means within a column or across a row that are followed by a different lower case letter are significantly different.

4.10 Determination of kinetic parameters

Lipases show different kinetic behaviors, depending on the substrate concentration. From the Lineweaver-Burk plot, when using *p*NPP as the substrate, the K_m and V_{max} values for the enriched lipase fraction from the endophytic fungi *F. oxysporum* isolated PTM 7 were rather low at 2.78 mM and 9.09 µmol/min/mg protein, respectively (Figure 4.9). Thus, the K_m value of the lipase from *F. oxysporum* was appreciably lower than those reported from other sources such *as Aspergillus niger* F044 (7.37 mM), *Burkholderia cepacia* ATCC 25609 (11 mM) and *Pseudomonas aeruginosa* (589 mM) when also using *p*NPP as the hydrolysis substrate (Shu *et al*., 2007; Dalal *et al*., 2008; Gaur *et al*., 2008, respectively).
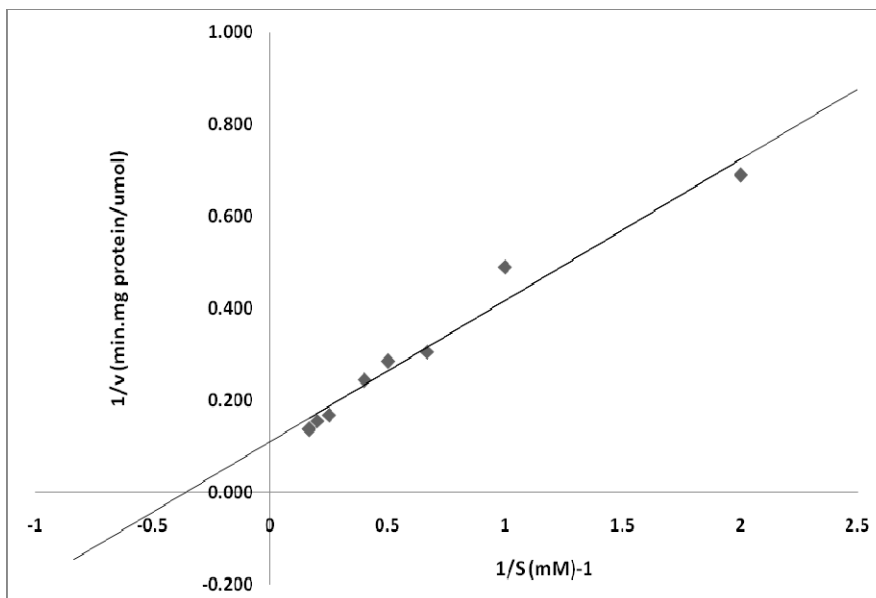
Figure 4.9 Lineweaver–Burk plot of the enriched lipase fraction from *F. oxysporum* isolated PTM 7. The lipase fraction was incubated with different concentrations of pNPP (0.25 - 6.0 mM) as substrate. Data are shown as the mean $\pm$ SD, and are derived from three repeats.

4.11 Preliminary biodiesel production

The enzymatic transesterification of palm oil with methanol by this enriched lipase fraction was evaluated with four different oil: methanol mole ratios and three different enzyme loadings, as outlined in section 3.16. The yield of biodiesel as FAMEs content increased with increasing lipase loadings from 5 to 6 Units for all four different oil: methanol ratios tested (Table 4.3). The theoretical oil: methanol mole ratio for the complete transesterification reaction is 1:3, but excessive concentrations of short-chain alcohols, such as methanol, could strongly and irreversible denature the lipase (Qin and Yunjan, 2010). Thus, oil: methanol molar ratios of 1:3 to 1:6 were evaluated in order to confirm the optimal ratio. The FAMEs yield was observed to increase with both increasing enzyme concentrations, as mentioned above, and with increasing oil: methanol mole ratios, with the highest FAMEs yield (28.4%) could be obtained at the oil: methanol ratio of 1:6 and with 6 U of enzyme. However, the yield obtained was still low.

Table 4.3 Transesterification of palm oil with methanol catalyzed by the enriched lipase fraction of the *F. oxysporum* isolated PTM 7.

Oil : methanol	Fatty acid methyl ester (%)[a]		
	5 Units	**5.5 Units**	**6 Units**
1:3	0.7 ± 0.0	2.1 ± 0.7	3.6 ± 0.5
1:4	1.1 ± 0.4	5.3 ± 1.1	11.7 ± 0.4
1:5	4.1 ± 1.2	5.4 ± 1.2	12.1 ± 1.4
1:6	4.5 ± 1.5	6.7 ± 1.3	28.4 ± 2.3

[a]Transesterification reactions were carried out at 30°C for 24 h. The data are shown as the mean ± SE and are derived from three repeats. Means within a column or across a row that are followed by a different lower case letter are significantly different ($p < 0.05$; Duncan's multiple means test of log transformed data).

CHAPTER V

CONCLUSION

Fungal endophytes were important microorganism source and could produce lipases, which demanded for industrial applications and organic synthesis. Enrichment of the lipase from the PTM 7 fungal isolated, a member of the *F. oxysporum* complex and selected on the basis of a high extracellular lipase activity, was attained by a simple three stage process, 80% saturation ammonium sulfate precipitation, DEAE-cellulose anion exchange and Sephadex-75 gel chromatography. If the low yield (~2.2%) could be improved this may prove a useful preparative technique. The 37.4 kDa lipase, or at least the active subunit if it is multimeric, revealed a relatively low K_m and V_{max} for pNPP compared to some previously reported lipases, but interestingly was strongly stimulated by Mn^{2+}, and has the potential to be an alternative lipase for enzymic biodiesel production by transesterification.

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APPENDICES

APPENDIX A

MEDIA

The media were prepared by sterilization in the autoclave at 121°C for 15 minutes.

1. Potato dextrose agar (PDA)

Potato, peeled and diced	200	g
Glucose	20.0	g
Agar	15.0	g
Distilled water	1,000	ml

Boil 200 g of peels, dried potato for 1 hr in 1000 ml. of distilled water. Filter, and make up the filtrate to one liter. Add the glucose and agar and dissolve by streaming.

2. Potato dextrose agar (PDA) containing olive oil and rhodamine B

Potato, peeled and diced	200	g
Glucose	20.0	g
Agar	15.0	g
Rhodamine B	0.01	g
Olive oil	10	ml
Distilled water	1,000	ml

Boil 200 g of peels, dried potato for 1 hr in 1000 ml. of distilled water. Filter, and make up the filtrate to one liter. Add the glucose, rhodamine B, olive oil and agar and dissolve by streaming.

3. Basal medium

Peptone	10	g
KH_2PO_4	1.5	g
$NaNO_3$	2.0	g
NaCl	0.5	g
$MgSO_4 \cdot 7H_2O$	0.5	g
$FeSO_4 \cdot 7H_2O$	0.001	g
$CuSO_4 \cdot 5H_2O$	0.001	g
$ZnSO_4 \cdot 5H_2O$	0.001	g
Olive oil	10	ml
Distilled water	1,000	ml
Final pH 7.0		

The basal medium was prepared by suspending all ingredients in distilled water and then warm slightly to dissolve completely. Later, the medium was autoclaved at $121^{\circ}C$ for 15 minutes.

APPENDIX B

Preparation for denaturing polyacrylamide gel electrophoresis

1. Stock solutions

2 M Tris-HCl (pH 8.8)

    Tris (hydroxymethyl)-aminomethane   24.2 g

    Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1 M Tris-HCl (pH 6.8)

    Tris (hydroxymethyl)-aminomethane   12.1 g

    Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

10% SDS (w/v)

    Sodium dodecyl sulfate (SDS)   10 g

50% Glycerol (w/v)

    100% Glycerol   50 ml

    Added 50 ml of distilled water

1% Bromophenol blue (w/v)

    Bromophenol blue   100 mg

    Brought to 10 ml with distilled water and stirred until dissolved.

    Filtration will remove aggregated dye.

2. Working solution

Solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide)

Acrylamide	29.2	g
N,N,-methylene-bis-acrylamide	0.8	g
Adjust volume to 100 ml with distilled water		

Solution B (1.5 M Tris-HCl pH 8.8, 0.4% SDS)

2 M Tris-HCl (pH 8.8)	75	ml
10% SDS	4	ml
Distilled water	21	ml

Solution C (0.5 M Tris-HCl pH 6.8, 0.4% SDS)

1 M Tris-HCl (pH 6.8)	50	ml
10% SDS	4	ml
Distilled water	46	ml

10% Ammonium persulfate

Ammonium persulfate	0.5	g
Distilled water	5	ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS)

Tris (hydroxymethyl)-aminomethane	3	g
Glycine	14.4	g
SDS	1	g

Dissolved in distilled water to 1 litre without pH adjustment (final pH should be 8.3)

5x sample buffer

(60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 14.4 mM 2-mercaptoethanol)

1 M Tris-HCl (pH 6.8)	0.6	ml
Glycerol	5	ml
10% SDS	2	ml
1% Bromophenol blue	1	ml
2-mercaptoethanol	0.5	ml
Distilled water	0.9	ml

3. SDS-PAGE

15% Separating gel

Solution A	10.0	ml
Solution B	5.0	ml
Distilled water	5.0	ml
10% Ammonium persulfate	100	μl
TEMED	10	μl

5.0% Stacking gel

Solution A	0.67	ml
Solution B	1.0	ml
Distilled water	2.3	ml
10% Ammonium persulfate	30	μl
TEMED	5.0	μl

APPENDIX C

Bradford Method

Preparation for protein concentration determination by Bradford Method

1. Stock solutions

Ethanol 95%	100	ml
Phosphoric acid 88%	200	ml
Brilliant Blue G	350	mg

2. Working solutions

Ethanol 95%	15	ml
Phosphoric acid 88%	30	ml
Stock solution	30	ml
Distilled water	425	ml

Calibration curve for protein determination by Bradford method
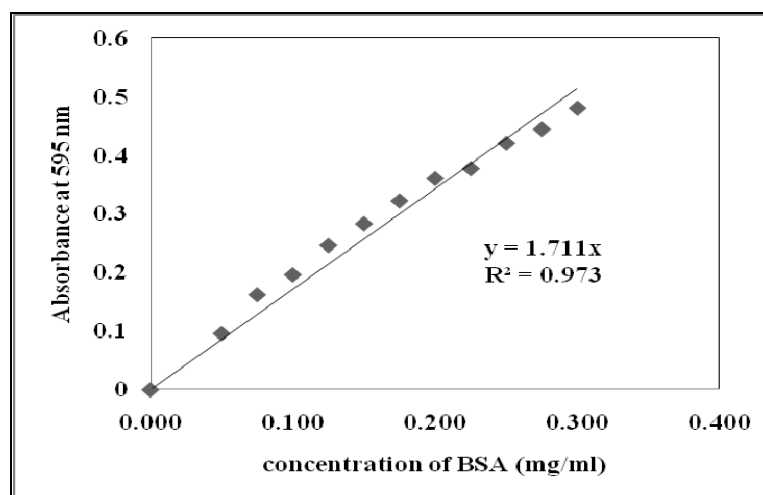
Calculation method

$$\text{Protein (mg/ml)} = \frac{\text{absorbance read}}{\text{slope constant}}$$

APPENDIX D

p -Nitrophenol Method

Preparation for lipase activity by *p* -Nitrophenol Method

1. **1 M Tris-HCl (pH 7.0)**

   Tris (hydroxymethyl)-aminomethane   121   g

       Adjusted pH to 7.0 with 1 M HCl and adjusted volume to 1000 ml with distilled water

2. **50 mM Tris-HCl (pH 7.0)**

   Diluted from 1 M Tris-HC1 and adjusted volume to 1000 ml with distilled water

3. **Solution A**

   p -nitrophenyl palmitate   30   mg

   isopropanol   10   ml

4. **Solution B**

   Triton x-100   1.8   ml

   Gum Arabic   100   mg

   50 mM Tris-HCl (pH 7.0)   90   ml

5. **Substrate for assay**

   Solution A   10   ml

   Solution B   90   ml

Calibration curve of *p* -nitrophenol detected by *p* -Nitrophenol Method
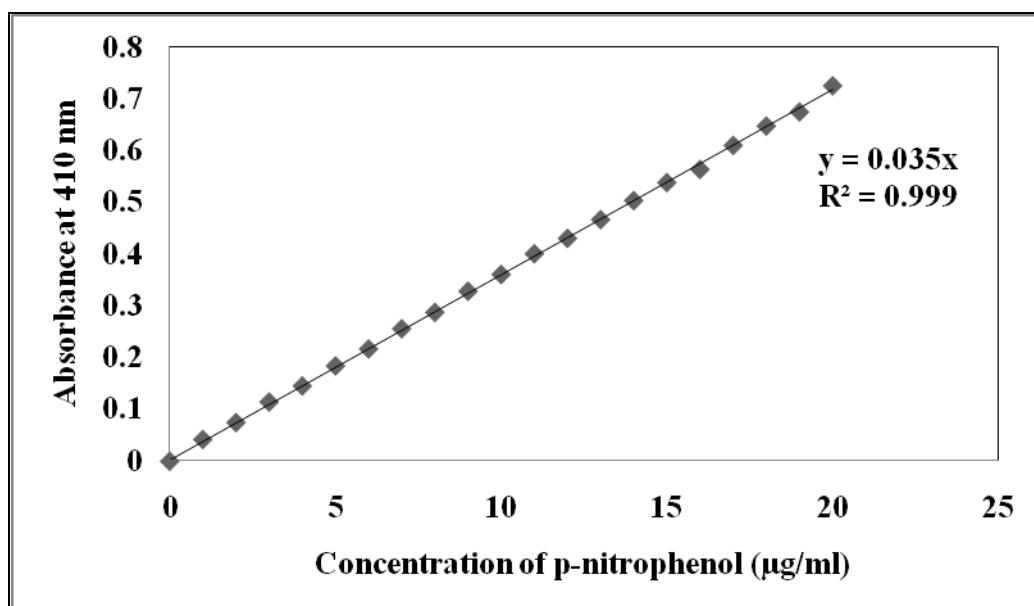
Calculation method

Lipase activity   =   (absorbance read/ slope constant) x total volume

(Unit/ml)   ml of enzyme used x incubation time x molecular weight of *p* -nitrophenol

Specific activity =   Lipase activity/ mg of protein

(Unit/mg)

One unit (U) was defined as the amount of enzyme that liberated 1µmol *p*-nitrophenol per min

APPENDIX E

Secondary screening of endophytic fungi for extracellular lipase production

After primary screening for extracellular lipase production using rhodamine B - PDA plates, all ten of these isolates were evaluated in a liquid culture in the basal medium.

APPENDIX F

Fatty acid methyl esters

Determination fatty acid methyl esters (FAMEs) by Gas chromatography using internal standard (methyl octanoate, C8:0)
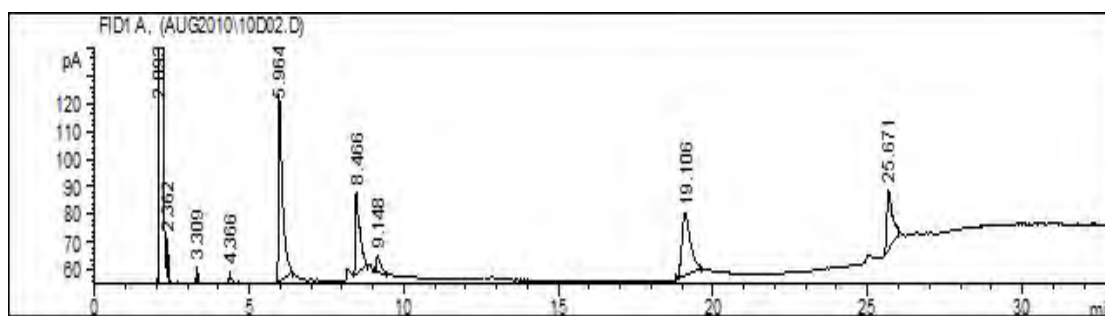
Methyl ester	Retention time	Area (%)
C8:0	2.362	1.4250
C12:0	3.309	1.1760
C14:0	4.366	0.9790
C16:0	5.964	37.454
C18:1	8.466	16.2991
C18:2	9.148	3.2740
C20:0	19.106	24.4152
C22:0	25.671	14.9796

Calculation method

$$\% \text{ Fatty acid methyl ester} = \frac{(\sum A) - A_{EI}}{A_{EI}} \times \frac{C_{EI} \times V_{EI}}{W} \times 100$$

$\sum A$ = total area C8:0 to C22:0 A_{EI} = area of C8:0 (Internal standard)

C_{EI} = concentration of C8:0 (mg/ml) V_{EI} = volume of C8:0 (ml)

W = weight of sample (mg)

$$\% \text{ Fatty acid methyl ester} = \frac{100 - 1.4250}{1.4250} \times \frac{10 \times 0.02 \times 100}{48.7} = 28.41$$

APPENDIX G

Fatty acid composition of each oils

	Coconut Oil	Rice brane Oil	Palm Oil	Olive Oil	Soybean Oil	Sunflower Oil
A. Saturated						
C10:0 Capric	6	-	-	-	-	-
C12:0 Lauric	47	-	-	-	-	-
C14:0 Myristic	18	-	1	-	-	-
C16:0 Palmitic	9	15	45	13	11	11
C18:0 Stearic	3	2	4	3	4	5
B. Unsaturated						
C16:1 Palmitoleic	-	-	-	-	-	-
C18:1 Oleic	6	43	40	71	24	28
C18:2 Linoleic	2	39	10	10	54	51
C18:3 Linoleic	-	1	-	1	7	5
% Unsaturated	**8**	**83**	**50**	**82**	**85**	**84**

Reference: Zamora (2005)

APPENDIX H

Molecular identification

Fusarium oxysporum was sent for identification by molecular methods. The rDNA ITS region of *Fusarium oxysporum* was amplified with the conserved fungal primer ITS_{1F} and ITS_4. *Fusarium oxysporum* produced a single band. The length of corresponding fragment was 558 bp, containing a part of the 18S, ITS1, 5.8S and 28S rDNA is shown.

1

5´ TCCGTTGGTG AACCAGCGGA GGGATCATTA CCGAGTTTAC
AACTCCCAAA CCCCTGTGAA CATACCAATT GTTGCCTCGG
CGGATCAGCC CGCTCCCGGT AAAACGGGAC GGCCCGCCAG
AGGACCCCTA AACTCTGTTT CTATATGTAA CTTCTGAGTA
AAACCATAAA TAAATCAAAA CTTTCAACAA CGGATCTCTT
GGTTCTGGCA TCGATGAAGA ACGCAGCAAA ATGCGATAAG
TAATGTGAAT TGCAGAATTC AGTGAATCAT CGAATCTTTG
AACGCACATT GCGCCCGCCA GTATTCTGGC GGGCATGCCT
GTTCGAGCGT CATTTCAACC CTCAAGCCCC CGGGTTTGGT
GTTGGGGATC GGCGAGCCCT TGCGGCAAGC CGGCCCCGAA
ATCTAGTGGC GGTCTCGCTG CAGCTTCCAT TGCGTAGTAG
TAAAACCCTC GCAACTGGTA CGCGGCGCGG CCAAGCCGTT
AAACCCCCAA CTTCTGAATG TTGACCTCGG ATCAGGTAGG
AATACCCGCT GAACTTAAGC ATATCATAAC CCGCACGA 3´
584

A blast search was performed to find a similar sequence to ITS region of fungal isolate PTM7 in the Genblank DNA database, available from http://blast.ncbi.nlm.nih.gov. The results revealed that ITS region of isolate PTM7 was similar to 99% identity of *Fusarium oxysporum* strain PY-HLG-2 (GU445378.1) 18S rRNA gene. Alignment data of part of the 18S, ITS1, 5.8S and 28S rDNA of *Fusarium oxysporum* is shown.

gb|GU445378.1|*Fusarium oxysporum* strain PY-HLG-2 18S ribosomal RNA gene,

partial sequence; internal transcribed spacer

1, 5.8S ribosomal RNA gene, and internal transcribed spacer

2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Length=582 Score = 1009 bits (546),

Expect = 0.0, Identities = 549/550 (99%),

Gaps = 1/550 (0%)

 Strand=Plus/Plus

```
Query  1    TCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25   TCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAA  84

Query  61   CATACCAATTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  85   CATACCAATTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAG  144

Query  121  AGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  145  AGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAA  204

Query  181  CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  205  CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAG  264

Query  241  TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265  TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCA  324

Query  301  GTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCCCGGGTTTGGT  360
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Sbjct  325  GTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCCCGGGTTTGGT  384

Query  361  GTTGGGGATCGGCGAGCCCTTGCGGCAAGCCGGCCCCGAAATCTAGTGGCGGTCTCGCTG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  385  GTTGGGGATCGGCGAGCCCTTGCGGCAAGCCGGCCCCGAAATCTAGTGGCGGTCTCGCTG  444

Query  421  CAGCTTCCATTGCGTAGTAGTAAAACCCTCGCAACTGGTACGCGGCGCGGCCAAGCCGTT  480
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Sbjct  445  CAGCTTCCATTGCGTAGTAGTAAAACCCTCGCAACTGGTACGCGGCGCGGCCAAGCCGTT  504

Query  481  AAACCCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGC  540
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Sbjct  505  AAACCCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGC  564

Query  541  ATATCA-TAA  549
            |||||| |||
Sbjct  565  ATATCAATAA  574
```

BIOGRAPHY

Miss Tuangporn Panuthai was born on January 7, 1985 in Bangkok, Thailand. She graduated with a Bachelor Degree of Science in field of General Science from Faculty of Science, Kasetsart University in 2006. She had been studies for a Master Degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2007.

Academic presentation;

1) Panuthai, T., Sihanonth, P., Piapukiew, J., Sangvanich, P., and Karnchanatat, A. Screening and production of lipase from endophytic fungi, The 22nd Annual Meeting of the Thai Society for Biotechnology International Conference on Biotechnology for Healthy Living, 20-22 October 2010, Prince of Songkla University, Trang Campus, Thailand,

2) Panuthai, T., Sihanonth, P., Piapukiew, J., Sangvanich, P., and Karnchanatat, A. Screening and production of lipase from endophytic fungi, The 12th Graduate Research Conference Khon Kaen University 2011, 28 January 2011, Khon Kaen, Thailand.