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โดยกระบวนการชักนำให้เกิดการกลายพันธุ์แบบสุ่ม

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ENHANCEMENT OF LIPASE ACTIVITY FROM *Stenotrophomonas maltophilia* BY RANDOM MUTAGENESIS

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จากการคัดเลือกและแยกแบคทีเรียจากตัวอย่างดิน 12 แหล่งและตัวอย่างปุ๋ย 10 แหล่ง โดยนำมาเพิ่มจำนวนเชื้อแบคทีเรียที่สามารถผลิตไลเพสได้ ในอาหารที่มีน้ำมันมะกอกเป็นองค์ประกอบ จากนั้นจึงนำมาคัดเลือกแบคทีเรียที่สามารถผลิตไลเพสขั้นต้นบนอาหารไตรบูไทริน สามารถคัดแยกเชื้อแบคทีเรียได้ทั้งหมด 95 ไอโซเลต จึงนำแบคทีเรียที่ได้มาทดสอบอีกครั้งบนอาหารเลี้ยงเชื้อแข็งที่มีน้ำมันมะกอก และโรดามีน บีเป็นส่วนผสมอยู่ พบว่ามีแบคทีเรียจำนวน 55 ไอโซเลตสามารถสร้างไลเพสได้ จากนั้นนำแบคเรียที่ได้มาเลี้ยงในอาหารเหลวสำหรับการสร้างไลเพสโดยใช้น้ำมันมะกอกเป็นตัวชักนำในการสร้างไลเพส จากนั้นเมื่อทดสอบความสามารถในการเร่งปฏิกิริยาของไลเพสจากเชื้อที่คัดเลือกได้พบว่า *Stenotrophomonas maltophilia* CU22 สามารถให้ค่าไฮโดรไลติกแอคติวิตีสูงสุดและเปลี่ยน น้ำมันปาล์มให้เป็นเมทิลเอสเทอร์ได้ดีเมื่อเปรียบเทียบกับสายพันธุ์อื่น จึงนำมาชักนำให้เกิดการกลายพันธุ์ โดยใช้รังสี UV พบว่าเมื่อฉายรังสี UV เป็นระยะเวลา 10 วินาทีมีอัตราการตาย 96.67 % ได้พันธุ์กลาย 47 ไอโซเลต และเมื่อทดสอบความสามารถในการเร่งปฏิกิริยาของไลเพสพบว่า 11 ไอโซเลตสามารถให้แอกทิวิตี จำเพาะสูงขึ้น จึงนำแบคทีเรียทั้ง 11 ไอโซเลตมาตรวจสอบความเสถียร พบว่ามี 10 ไอโซเลตที่ยังคงให้ค่าแอกทิวิตีจำเพาะสูงกว่าสายพันธุ์ตั้งต้นเมื่อทำการเลี้ยงไปผ่านไป 20 ชั่วรุ่น จึงนำมาทดสอบความสามารถในการผลิตเมทิลเอสเทอร์ด้วยปฏิกิริยาทรานส์เอสเทอริฟิเคชัน พบว่าสายพันธุ์กลาย 3 สายพันธุ์ที่ได้จากการฉายรังสี UV คือ UV107, UV1016 และ UV1048 สามารถเปลี่ยน เป็นผลิตภัณฑ์ได้มากกว่าสายพันธุ์ตั้งต้นโดยให้ร้อยละการเปลี่ยนแปลงเป็นผลิตภัณฑ์เท่ากับ 7.56, 9.30 และ 7.37% ตามลำดับ ในขณะที่สายพันธุ์ตั้งต้นให้ร้อยละการเปลี่ยนแปลงเป็นผลิตภัณฑ์เท่ากับ 6.91% ดังนั้นสายพันธ์กลาย UV 1016 จึงมีความสามารถให้ร้อยละการเปลี่ยนแปลงเป็นผลิตภัณฑ์มากกว่าสายพันธุ์ตั้นต้นถึง 35% ซึ่งเมื่อตรวจสอบลำดับนิวคลีโอไทด์ของยีนไลเพสพบว่ามีการเปลี่ยนแปลง ของลำดับของนิวคลีโอไทด์ตัวที่ 1702, 1765, 1766 และ 1768 ซึ่งทำให้ลำดับของกรดอะมิโนเปลี่ยนแปลงไปด้วยคือตำแหน่งที่ 568 จากแอสปาราจีนเป็น แอสปาติกแอซิด, ตำแหน่งที่ 589 จากกลูตามีนเป็น ทริปโตฟาน และตำแหน่งที่ 590 จากอาร์จินีน เป็นไกลซีน

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NIRAMOL JUNTAWIENG: ENHANCEMENT OF LIPASE ACTIVITY FROM *Stenotrophomonas maltophilia* BY RANDOM MUTAGENESIS. THESIS ADVISOR: ASSOC. PROF. WARAWUT CHULALUKSANANUKUL, Ph. D., THESIS COADVISOR : ASST. PROF. ALISA S. VANGNAI, Ph. D., 120 pp.

The isolation of lipase-producing bacteria were isolated from sources of 12 soil samples and 10 composts in Thailand, using enrichment media containing olive oil. The primary screening was carried out employing tributyrin agar plate and Rhodamine B supplemented with olive oil was used for secondary lipase screening. Fifty-five lipase-producing isolates were determined lipase activity by using *p*-nitrophenyl palmitate as substrate. The results showed that *Stenotrophomonas maltophilia* CU22 exhibited the highest specific activity. Also, lipase from *S. maltophilia* was catalyzed transesterification of the palm oil and methanol in non-solvent media and showed 7% methyl ester conversion. To improve lipase activity, random mutagensis by UV irradiation was used. At 10 sec expose time, the death rate with 96.67%, 47 mutants was as subject to test hydrolytic assay. Ten mutants with higher lipase specific activity were subcultured twenty times and then investigated transesterification. UV107, UV1016 and UV 1048 showed %conversion higher than wild type, were 7.56, 9.30 and 7.3 %. DNA and amino acid sequencing of partial lipase gene of wild type and UV1016 , were compared. The result showed nucleotide substitution at 1765, 1766 and 1768 with different amino acid substitution, were obtained. There are Asn568->Asp, Gln589->Trp and Arg590->Gly.

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LIST OF ABBREVIATIONS

°C	Degree of Celsius
et al.	Et. Alii (latin), and others
g	gram
h	hour
l	liter
M	Molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mol	mole
nm	nanometer
OD	Optical density
rpm	round per minute
w/v	weight by volume
v/v	volume by volume

CHAPTER I

INTRODUCTION

1.1 Statement of problem

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyze the hydrolysis of triglycerides, and under certain conditions the reverse reaction, esterification, forming glycerides from glycerol and fatty acids. Some lipases are able to catalyze transesterification and enantioselective hydrolysis reaction. Plants, animals, bacteria and fungi produce lipases. However, Bacterial and fungi enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Wiseman, 1995).

Lipases are enzymes with general interest within many industrial applications. Lipases are used within the industry, e.g., detergent, oil and fats, baking, organic synthesis, hard surface cleaning, leather industry and paper industry. As each industrial application may require specific properties of the biocatalysts, there is still an interest in finding new lipases that could create novel applications.

The utilization of gene technology and of new production technologies have made industrial enzymes with improved properties or better cost performance available. Gene technology offers several benefits to the enzyme industry. This technology enables the use of safe, well-documented host organisms easy to cultivate, the microbial production of enzymes of animal and plant origin, the realization of

enhanced efficiency and high product purity, and also the production of enzymes with improved stability and activity.

Generation of mutations by directly damaging DNA with chemical and phsical agents has been used to dissect biological systems for many years. It dose provide a valuable point of comparison to other methodologies. The basis of mutagenesis by UV irradiation or alkylating agents is that the damaged DNA is incorrectly replicated or repaired leading to mutation. Genetic material that passes through these cells accumulates mutations at a vastly higher rate than usual. This is an effective and straightforward way of introducing mutations throughout a DNA construct. However, in common with physical and chemical mutagens the mutagenesis is indiscriminate (Neylon, 2004).

Within the screening of bacterial lipase, *Stenotrophomonas maltophilia*, previously known as *Pseudomonas maltophilia* or *Xanthomonas maltophilia*, displayed highest lipase activity. *S. maltophilia* is an emerging pathogen that has been reported to be involved in cystic fibrosis, bacteremia, endocarditis, central nervous system infection, skin and tissue infection, ocular infection, and urinary infections (Denton and Kerr, 1998). From the clinical point of view, *S.maltophilia* is also of noteworthy interest as an important nosocomial pathogen. From the ecological point of view, the habitat of *S. maltophilia* in the environment has been reported to be wide: water and soil samples, crops, vegetation, animals and other ecological niches inside and outside hospitals (Denton and Kerr, 1998). Even though it is such an ubiquitous microorganism, little information is currently available concerning the incidence of this pathogen in animal foods. To enhance lipase activity, hydolysis activity and transesterification, the random mutagenesis method by UV irradiation and NTG treatment were used.

1.2 Objectives

In this study, a lipase-producing bacterium was screened and isolated. The characterization of the lipase from *S. maltophilia* was investigated in term of hydrolytic and transesterification activities. Then, the lipase activity of the bacterial isolated was enhanced by random mutagenesis.

1.3 Scope of the study

1.3.1 To screen, isolate, select and identify a lipase-producing bacterium.

1.3.2 To enhance lipase activity by random mutagenesis method

1.3.3 To select lipase-producing mutants with higher lipase activity than wild-type

1.3.4 To characterize lipase produced by wild type and mutants

1.4 Expected results

This research may provide available mutants with higher activity than wild type, which can be further applied.

1.5 Thesis organization

This thesis comprises five chapters including this introduction. Chapter 2 gives the theoretical background and literature review are described. In Chapter 3, material and methods is explained. The results could be found in Chapter 4 and the Chapter 5 is the discussion. Finally, the conclusion and future works are orderly described.

CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 General aspects and definition

Lipases or triacylglycerol ester hydrolases (EC .3.1.1.3) are serine hydrolases, which catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases (Table 2.1), lipases are activated only when adsorbed to oil - water interface (Martinelle *et al.*, 1995) and do not hydrolyze dissolved substrates in the bulk fluid. A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. Lipases display little activity in aqueous solutions containing soluble substrates. In eukaryotes, lipases are involved in various stages of lipid metabolism including fat digestion, absorption, reconstitution, and lipoprotein metabolism. In plants, lipases are found in energy reserve tissues. How lipases and lipids interact at the interface is still not entirely clear and is a subject of intense investigation (Balashev *et al.*, 2001).

Table 2.1 Differences between lipases and carboxyl esterase (Bournschuer, 2002)

Property	Lipase	Esterase
Preferred substrates	Triglycerides (long-chain), secondary alcohols	Simple ester, Triglycerides (short-chain)
Interfacial activation/lid	Yes	No
Substrate hydrophobicity	High	High to low
Enantioselectivity	Usually high	High to low to zero
Solvent stability	High	High to low

The increasing interest in lipase research over the past decades has likely occurred for three reasons. The first is related to the molecular basis of the enzyme catalytic function or the lipase paradigm. Lipase catalyzes reactions involving insoluble lipid substrates at the oil – water interface. This capability is due to the unique structural characteristic of lipases. These latter indeed contain a helical oligopeptide unit that covers the entrance to the active site. This so-called lid only moves upon access to a hydrophobic interface such as a lipid droplet. The second reason is linked to the enzyme's medical relevance, and its importance in regulation and metabolism, since products of lipolysis such as free fatty acids and diacylglycerols play many critical roles, especially as mediators in cell activation and signal transduction. Lastly, it was discovered that lipases are powerful tools for catalyzing not only hydrolysis, but also various reverse reactions, such as esterification, transesterification, and aminolysis, in organic solvents. Such biocatalysts present some important advantages over classical catalysts. Indeed, their specificity, regioselectivity and enantioselectivity allow them to catalyze reactions with reduced side products, lowered waste treatment costs and under conditions of mild temperature and pressure. Accordingly, considerable attention has been given lately to the commercial use of lipases (Villeneuve *et al.*, 2000)

Because of their capability to preserve their catalytic activity in organic solvents, the activities of lipases as catalysts have been investigated to determine their potential for the conversion of surplus fats and oils into higher value products for food and industrial uses. However, the low stability, low activity or selectivity encountered occasionally with a number of these enzymes, and the relatively prohibitive cost of native enzyme have been the chief obstacle hindering more rapid expansion of industrial lipase technology on a large scale. Therefore, customization of lipases by

chemical and physical modifications has more recently been attempted to improve their catalytic properties in hydrolysis and synthesis involving aqueous and non-aqueous solvents. In addition, the cost of enzyme can be reduced by the application of molecular biological tools, such as recombinant DNA technology and protein engineering, which may allow the production of lipases in large quantities and with genetically enhanced properties. Each of the procedure involves a different degree of complexity and efficiency. Chemical methods feature the formation of covalent bonds between the lipase and the modifier, while physical methods are characterized by weaker interactions of the enzyme with the support material, or mechanical containment of the lipase within the support. Additionally, genetic engineering of lipase involves modification of the gene encoding the enzyme. Features of this technology include the ability to isolate and express genes of interest and the ability to change the amino acid occupying a single, or multiple, sites in a protein. This technology also allows the insertion or deletion of single or multiple amino acids, and the fusion of segments from different genes and different organisms.

2.2 Enzymatic reaction of lipase

The biological function of lipases is to catalyze the hydrolysis of esters, especially long chain triacylglycerols, to yield free fatty acids, di- and mono-acylglycerols, and glycerol. Lipases are also capable of catalyzing the reverse reaction, achieving esterification, transesterification (acidolysis, interesterification, alcoholysis, aminolysis, oximolysis and thiotransesterification in anhydrous organic solvents, biphasic systems and in micellar solution with chiral specificity. The equilibrium between the forward (hydrolysis) and the reverse (synthesis) reactions is controlled by the water activity of the reaction mixture.

2.2.1 Hydrolysis

Total hydrolysis of ester bonds in triacylglycerols may be accomplished at high temperatures and pressure in the presence of steam. Fatty acids can alternatively be produced by ambient pressure saponification or chemically catalyzed hydrolysis. However, the use of lipases for enzymatic splitting of fats in the presence of excess water (Figure 2.1) is more appealing since the reaction proceeds under mild conditions of pressure and temperature with specificity and reduced waste. This technology is currently employed in the production of fatty acids, diglycerides, monoglycerides, flavoring agents for dairy products and detergents for laundry and household uses

2.2.2 Esterification

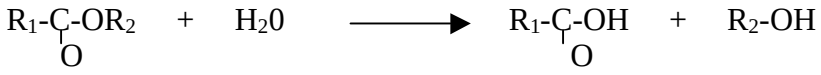
Esterification reactions between polyhydric alcohols and free fatty acids are catalyzed by lipases in water-poor organic solvents under conditions of low water activity or even solvent free systems Figure 2.1. Although ester synthesis can be done chemically with acid or base catalysis, the use of enzyme technology offers the advantages of mild conditions, reduced side reactions, and specificity.

2.2.3 Transesterification

The term transesterification refers to the process of exchanging acyl radicals between an ester and an acid acidolysis, an ester and another ester interesterification, or an ester and an alcohol alcoholysis Figure 2.1. Transesterification is accomplished industrially by heating a mixture of the anhydrous ester and another reactant species at relatively high temperatures. Alternatively, alkali metals or alkali alkylates may be used at lower temperatures. However, the

application of lipases for the modification of fats and oils by transesterification offers again the advantages of mild conditions, reduced side reactions, and specificity.

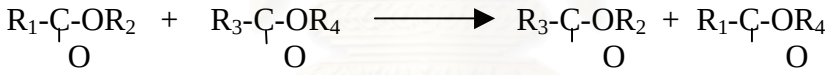
Hydrolysis



Ester synthesis

Acidolysis

Interesterification



Alcoholysis

Aminolysis

Figure 2.1 Enzymatic reactions of a lipase. Hydrolysis or synthesis of a triacylglycerol substrate catalyzed by lipase in aqueous and non-aqueous solutions. (Villeneuve *et al.*, 2000)

2.2.4. Catalysis on unnatural substrates

Lipases are not limited to catalysis of the synthesis and hydrolysis of carboxylic acid esters. They can utilize compounds other than water and alcohols as nucleophiles. Lipases are thus capable of catalyzing different reactions such as aminolysis, thiotransesterification, and oximolysis in organic solvents with selectivity. The selectivity of lipase in the aminolysis of esters in anhydrous media has been successfully used for peptide and fatty amide syntheses. These results hold promise for using lipase technology in the synthesis of optically active peptides, polymers, surfactants and new detergents at low cost.

2.3 Sources of lipases

Lipases are ubiquitous in nature and are produced by various plants, animals and microorganisms. Lipases of microbial origin, mainly bacterial and fungal, represent the most widely used class of enzymes in biotechnological applications and organic chemistry. A list of the common bacterial lipase producers is presented in Table 2.2. The extracellular bacterial lipases are of considerable commercial importance, as their bulk production is much easier. Although a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains (Jaeger *et al.*, 1994; Palekar *et al.*, 2000). Of these, the important ones are: *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium* and *Pseudomonas*. Of these, the lipases from Pseudomonas bacteria are widely used for a variety of biotechnological applications (Jaeger *et al.*, 1994; Pandey *et al.*, 1999; Beisson *et al.*, 2000). Several products based on bacterial lipases have been launched successfully in the market in the past few years (Gupta, Gupta and Rathi, 2004)

Lipase production from a variety of bacteria, fungi and actinomycetes has been reported (Sztajer *et al.,* 1988, Rapp and Backhaus 1992). The presence of lipases in bacteria had been observed as early as 1901 A.D. for *Bacillus prodigiosus*, *Bacillus pyocyneus* and *Bacillus fluorescens* (Jaeger *et al.,* 1994) which represent some of today's best studied lipase producers, now named, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens,* respectively. Lipase producers have been isolated mainly from soil, or spoiled food material that contain vegetable oils. Lipases also represent important virulence factor of many plant and animal pathogens. Lipases with novel properties have been discovered from microorganisms isolated from antarctic ocean (Feller *et al.,* 1990), hot springs (Gowland *et al.,* 1987, Lee *et al.,* 1999), compost heaps (Gowland *et al.,* 1987, Rathi *et al.,* 2000) and highly salty or sugary environments (Elwan *et al.,* 1985, Ghanem *et al.,* 2000). Lipase producers have been reported to grow at varied pH and temperatures. The fungi are usually reported to require acidic pH for growth and lipase production (Arima *et al.,* 1772, Pokorny *et al.,* 1994). Many bacteria are found to prefer neutral pH but there are reports of alkalophic (Gao *et al.,* 2000, Ghanem *et al.,* 2000) bacteria also. Psychrophilic and thermophilic organisms, as well as organisms having different oxygen demand (aerobic, microaerophilic and anaerobic) are reported to produce lipases. The occurrence of fungal and bacterial lipases is listed in Table 2.1

Table 2.1 Lipase producing bacteria (Gupta, 2004).

Bacteria	References
Achromobacter sp.	Mitsuda *et al.,* 1988
A. lipolyticum	Brune and Gotz 1992; Davranov, 1994
Acinetobacter sp.	Wakelin and Forster 1997; Barbaro *et al.,* 2001
A. calcoaceticus	Dharmsthiti *et al.,* 1998; Jaeger *et al.,* 1999; Pandey *et al.,* 1999; Pratuangdejkul and Dharmsthiti, 2000
A. radioresistens	Liu and Tsai, 2003
Alcaligenes sp.	Mitsuda *et al.,* 1988

Table 2.1 Lipase producing bacteria (continued).

Bacteria	References
A.denitrificans	Odera *et al.*, 1986
Arthrobacter sp.	Pandey *et al.*, 1999
Archaeglobus fulgidus	Jaeger *et al.*, 1999
Bacillussp.	Sidhu *et al.*, 1998a, 1998b; Pandey *et al.*, 1999; Sharma *et al.*, 2002a; Nawani and Kaur,2000
B. alcalophilus	Ghanem *et al.*, 2000
B. atrophaeus	Bradoo *et al.*, 1999
B.megaterium	Hirohara *et al.*, 1985
B.laterosporus	Toyo-Jozo, 1988
B. pumilus	Jaeger *et al.*, 1999
B.sphaericus	Toyo-Jozo ,1988
B. stearothermophilus	Bradoo *et al.*, 1999; Jaeger *et al.*, 1999
B. subtilis	Jaeger *et al.*, 1999
B. thaiminolyticus	Toyo-Jozo, 1988
B. thermocatenulatus	Jaeger *et al.*, 1999; Pandey *et al.*, 1999
Burkholderia glumae	Jaeger and Reetz, 1998; Reetz and Jaeger, 1998
Chromobacterium violaceum	Koritala *et al.*, 1987
C. viscosum	Jaeger and Reetz 1998; Jaeger *et al.*, 1999
Corynebacterium acnes	Brune and Gotz, 1992
Cryptocoocus laurentii	Toyo-Jozo, 1988
Enterococcus faecalis	Kar *et al.*, 1996
Lactobacillus curvatus	Brune and Gotz, 1992
L. plantarum	Lopes Mde *et al.*, 2002
Microthrix parvicella	Wakelin and Forster, 1997
Moraxella sp.	Jaeger *et al.*, 1999
Mycobacterium chelonae	Pandey *et al.*, 1999
Pasteurella multocida	Pratt *et al.*, 2000
Propionibacterium acnes	Jaeger *et al.*, 1999
P. avidium	Brune and Gotz, 1992
P. granulosum	Brune and Gotz, 1992
Proteus vulgaris	Jaeger *et al.*, 1999
Pseudomonas aureofaciens	Koritala *et al.*, 1987
P. fluorescens	Arpigny and Jaeger, 1999; Pandey *et al.*, 1999
P. fragi	Jaeger *et al.*, 1994; Schuepp *et al.*, 1997; Ghanem *et al.*, 2000
P. luteola	Arpigny and Jaeger, 1999; Litthauer *et al.*, 2002
P. mendocina	Jaeger *et al.*, 1999; Surinenaite *et al.*, 2002
P. nitroreducens var. thermotolerans	Ghanem *et al.*, 2000
P. pseudomallei	Kanwar and Goswami, 2002
P. wisconsinensis	Arpigny and Jaeger, 1999
Psychrobacter immobilis	Jaeger *et al.*, 1999
Staphylococcus aureus	Simons *et al.*, 1996; Jaeger *et al.*, 1999
S. epidermidis	Simons *et al.*, 1996; Jaeger *et al.*, 1999
S. haemolyticus	Oh *et al.*, 1999
S. hyicus	Jaeger *et al.*, 1999; Van Kampen *et al.*, 2001
S. warneri	Pandey *et al.*, 1999; Van Kampen *et al.*, 2001
S. xylosus	Pandey *et al.*, 1999; Van Kampen *et al.*, 2001
Serratia marcescens	Matsumae *et al.*, 1993,1994; Pandey *et al.*, 1999; Abdou, 2003
Streptomyces exfoliatus	Arpigny and Jaeger, 1999
Sulfolobus acidocaldarius	Jaeger *et al.*, 1999
Vibrio chloreae	Jaeger *et al.*, 1999

2.4 Screening and isolation of bacterial lipase

Screening is a very important issue for developing better lipase biocatalysts. Lipase units are generally based on their hydrolytic activity on tributyrin or triolein. A clear zone of tributyrin hydrolysis is indicative of either esterase or lipase activity. More recently, agar plates supplemented with olive oil have also been employed for screening lipase-positive colonies. Alternatively, an indicator is added to the agar that in turn forms complexes with the acids, and a colored zone is produced. The indicators used are Nile Blue Sulphate (Christen, 1984) and Victoria Blue (Gao,Cao and Zhanga , 2000). These tests are very convenient for fast screening of lipolytic microbes growing on agar plates. However, some false-positive results can result from acidification of the medium due to the generation of acidic metabolites other than free fatty acids that are released by microbial lipases. To overcome these limitations, Kouker and Jaeger (1987) employed the fluorescence dye Rhodamine B to observe the zone of lipolysis as an orange fluorescence under UV light at 350 nm. Rhodamine forms a fluorescent complex with free fatty acids. Thus the lipase-producing colonies give a flourescent halo that is visible under UV light. In place of triolein (trioleoylglycerol), olive oil has also been used in the agar medium. This fluorescent dye method allows quantification of very minute levels of lipase activity. However, their broad substrate specificity and the diversity of reactions catalyzed by these enzymes, make it difficult to define a universal test for lipase activity. Besides, there is a growing interest in synthetic biocatalytic transformations catalyzed by lipases.

2.5 Lipase production

Bacterial lipases are mostly inducible enzymes, requiring some form of oil, fatty acid, fatty acid alcohol or fatty acid ester for induction, which usually secreted out in the culture medium. Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production by submerged culture. The type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature, and the dissolved oxygen concentration influence lipase production. Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield; however, a few authors have produced good yields in the absence of fats and oils (Sharma, Chisti, and Banerjee, 2001)

2.5.1 Effect of carbon sources

Several other studies confirm enhanced lipase production when oils are used as enzyme inducers. Sugihara *et al.* (1991) reported lipase production from *Bacillus* sp. in the presence of 1% olive oil in the culture medium. Little enzyme activity was observed in the absence of olive oil even after prolonged cultivation. 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.

2.5.2 Effect of nitrogen sources

Nitrogen sources such as corn steep liquor and soybean meal stimulated lipase production but to a lesser extent than peptone. Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used. Lipase yield and stability could be improved by supplementing the preferred organic nitrogen source with ammonium (Cordenons *et al*., 1996). The extracellular lipase was measured using pNPP as the substrate (Vorderwiilbecke *et al*., 1992). Lin *et al.* (1996) reported an extracellular alkaline lipase produced by *P. alcaligenes* F-111 in a medium that contained soybean meal (1%), peptone (1.5%), and yeast extract (0.5%).

Table 2.2 Some of lipase production form bacteria.

Bacteria	C- source	N-source	Lipase (U/ml)	Assay method	Reference
Acinetobacter radioresistens	Olive oil and n-hexadecane	Tryptone, Yeast extract, NH_4Cl	2	Titrimetry using olive oil	Chen *et al*., 1998
Bacillus circulans	Soluble starch	Soybean meal, peptone	2.4	Titrimetry using tributyrin	Sztajer and Maliszewska, 1988
Bacillus thermoleovorans ID-1	Olive oil	Tryptone, yeast extract	0.7	Spectrophoto metry using *p*NP- butyrate	Lee *et al*., 1999

2.6 Lipase assay methods

Lipases can be viewed as lipolytic enzymes with catalysts for a large number of esters. The assay of lipases activity of can monitored the release of either fatty acids or glycerol from triacylglycerolsor fatty acid esters. The fatty acids released by lipase-mediated hydrolysis can be determined quantitatively using titrimetry, colorimetric assays, fluorescence, chromatographic procedures and immunological method. The Table 2.3 gives an overview of different lipase assay methods.

2.6.1 Titrimetry

Titrimetry is one of the oldest and most widely used quantitative assays, on account of its simplicity, accuracy and reproducibility. This is a reliable technique for characterizing lipase action and specificity, as well as the interfacial activation phenomenon (Ferrato *et al.*, 1997). The pH-stat method is a highly sensitive as well as a quantitative method that can measure the release of even 1 μmol of released fatty acid. However, at a pH value of less than 7, where free fatty acids are not fully ionized, pH-stat titration is either inaccurate or impossible to perform, even after introducing a correction factor (Gupta *et al.*, 2003 cited in Gargouri *et al.*, 1986). One unit of lipase activity is defined as the amount of enzyme that catalyses the release of 1 μmol of fatty acid from olive oil. However, titrimetry is extremely laborious and time- consuming. Colorimetric and fluorimetric assays provide the option of simpler and more rapid assays, although the substrates are expensive and therefore not used as widely as the standard assay protocols (Gupta *et al.*, 2003).

2.6.2 Spectrophotometric assay

The *p*-nitrophenyl esters of various fatty acids are generally used as substrates and measured of *p*-nitrophenol by spectrophotometrically at 410 nm. Short-chain esters are water-soluble, their hydrolysis are catalyzed by esterase rather than lipase. The lipase activity is measured using *p*-nitrophenyl palmitate. A major limitation of this assay is that the enzyme reactions cannot be performed at acidic pH owing to the lack of absorbance of *p*-nitrophenol at acidic pH (Kademi et al, 2000). Thus enzyme activity at only neutral and alkaline pH values can be ascertained by this procedure.

2.6.3 Fluorescence assay

Fluorescent compounds have also been used for lipase assay. The method involves measurement of the fluorescent fatty acid released because of lipase activity [45,46]. Duque *et al.* (1996) developed an assay using fluorogenic and isomerically pure 1-(3)-o-alkyl-2,3-(3,2)-diacylglycerols as substrates. This lipase assay is continuous and does not require separation of substrate and reaction products. This method could detect low lipolytic activity and was 6000-fold more sensitive than conventional titration, which measures activity in the range of μmol/min per ml. Jette and Ziomek (1994) described a quantitative fluorescence lipase assay based on the interaction of Rhodamine B with fatty acids released during the hydrolysis of triacylglycerols. This method is rapid and can be automated further, but expesive substrate limits its usage.

2.6.4 Chromatographic procedures

Chromatography is a direct determination method for measure of the release of fatty acids following lipase-catalyzed hydrolysis of a substrate. The TLC is a quantitative analysis of the released free fatty acids from triacylglycerols using densitometric or autoradiographic methods with radiolabelled triacylglycerols. These methods are very sensitive and can detect fatty acid to a few picomol (Ruiz and Rodriguez-Fernandz, 1982). The main disadvantage is that these procedures are very time-consuming and discontinuous. The GC quantified methyl esters, which are converted from the fatty acids in the official American Oil Chemists Society. The HPLC can also be easily identify the product of lipolysis and has been used for lipases from *Penicillium* sp. (Gulomova, 1996)

2.6.5 Immunological methods

Gupta *et al.* (2003) review immunological methods or ELISA-based procedures are highly specific and sensitive and can be adopted easily. However, they could not be adopted as routine laboratory assays, since purified enzyme samples as well as the raising of poly- or mono-clonal antibodies are pre- requisites. Aoubala *et al.* (1995) developed two sandwich ELISA techniques for evaluating the interfacial binding of human gastric lipase to lipid monolayers. This assay made it possible for to determine the corresponding interfacial access of the enzyme. The HPLC often recommended for substrate-specificity determination, however, the use HPLC depends upon availability of the instrument and time-consuming for routine analysis.

2.7 Applications of lipases

Microbial lipases are widely diversified in their enzymatic properties and substrate specificity. Bacterial lipases are generally more stable than animal or plant lipases. Lipases are active under ambient conditions and the energy expenditure required to conduct reactions at elevated temperatures and pressures is eliminated that reduces the destruction of labile reactants and products. Lipases are part of the family of hydrolases that act on carboxylic ester bonds, which catalyze esterification, interesterification, and transesterification reactions in non-aqueous media. This versatility makes lipases the enzymes of choice for potential applications. For example to produce cocoa butter from palm mid-fraction. Lipases can be used as biocatalyst in the production of useful biodegradable compounds, such as activated sludge and other aerobic waste processes. In textile industry, Lipases are used to assist in the removal of size lubricants, in order to provide a fabric with greater absorbency for improved levelness in dyeing. Also, lipase can reduce the environmental load of detergent products. Lipases have also been used for addition to food to modify flavour by synthesis of esters of short chain fatty acids and alcohols, which are known flavour and fragrance compounds. Lipase can be used as diagnostic tools and their presence or increasing levels can indicate certain infection or disease. Lipases may be used as digestive aids. Lipases are extensively used in the dairy industry for the hydrolysis of milk fat and have been used in the improvement of flavour in coffee whiteners to produce the creamy flavour, and buttery texture of toffees and caramel. In cosmetics, lipases have been used in hair waving. However, many new potential applications of lipases have been proposed and, since the methods for lipase production have been improved, some of these new uses will be economically viable.

2.7.1 Use of lipases in production of biodiesel

The limited resources of fossil fuels, increasing prices of crude oil, and environmental concerns have been the diverse reasons for exploring the use of vegetable oils as alternative fuels. The biodiesel fuel or monoalkyl ester from vegetable oil does not produce sulphur oxide and minimize the soot particulate. Because of these environmental advantages, biodiesel fuel can be expected as a substitute for conventional diesel fuel.

Conventionally the synthesis of biodiesel is accomplished by chemical transesterification or alcoholysis, which is the displacement of alcohol by another an ester. The transesterification reaction is represented by the general equation as Figure. 2.2.

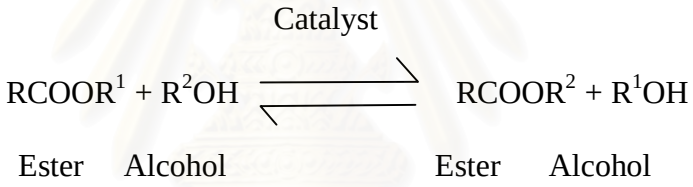


Figure 2.2 General equation of transesterification (Meher, Sagar and Naik, 2006)

Chemical methods give high conversion ratio of triacylglycerols to biodiesel in short times. However, chemical transesterification is connected with some drawbacks, for example, high-energy consumption, difficulty in glycerol recovery, and a high amount of, alkaline wastewater from the catalyst (Table 2.3). Lipase has been used as a biocatalyst for the synthesis of biodiesel from natural oils and can overcome the problems of conventional chemical processes (Fukuda, Kondo and Noda, 2001, Soumanou and Bornscheuer, 2003).

Since the cost of lipase production is the main hurdle to commercialization of the lipase-catalyzed process, several attempts have been made

to develop cost effective systems. Further enhancement of lipase production may be achieved by genetic engineering (Fukuda, Kondo and Noda, 2001).

Table 2.3 Comparison between alkali-catalysis and lipase-catalysis methods for biodiesel fuel production (Fukuda, Kondo and Noda, 2001).

	Alkali-catalysis process	Lipase-catalysis process
Reaction temperature	60-70°C	30-40 °C
Free fatty acids in raw materials	Saponified products	Methyl esters
Water in raw materials	Interference with the reaction	No influence
Yield of methyl esters	Normal	Higher
Recovery of glycerol	Difftcult	Easy
Purification of methyl esters	Repeated washing	None
Production cost of catalyst	Cheap	Relatively expensive

2.8 Mutation

A mutation is a heritable change in the sequence of organism's genome; the full complement of an organism's genetic material is referred to as genome. An organism that carries one or more mutations in its genome is referred to as a mutant.

2.8.1 Type of mutation

There are many ways in which the structure of the genetic material may change. A mutation will henceforth be considered to be any alteration in the base sequence of the nucleic acid comprising the genome of an organism, regardless of whether there is any phenotypic effect from the alteration. This definition is deliberately broad in order to encompass the tremendous variety of mutational types.

2.8.1.1 Base substitutions

The easiest type of mutation to visualize is that of base substitution, in which a single nucleotide base is replaced by another. If a purine is replaced with a purine (e.g., adnine for guanine) or a pyrimidine with a pyrimidine (e.g. thymine for cytosine), the change is referred to as a transition. If a purine is replaced by a pyrimidine or vice versas, the change is referred to as a transversion. When a single base change is the simplest kind of mutation to visualize, it may also be one the more difficult to detect. This is due to the fact that the genetic code is highly redundant, so that in many cases the same amino acid can be coded by many different codons.

2.8.1.2 Insertion and deletion mutations

Literally, a deletion mutation is the removal of one or more base pairs from the DNA, whereas an insertion mutation is the addition of one or more base pairs. In practice, deletion and insertion mutations usually involve considerably more than one base pair. Insertion or deletion of base pairs in multiples of three results in the addition or elimination of amino acids in the polypeptide chain.

2.8.1.3 Frameshift mutations

When a ribosome translates an mRNA molecule, it must accurately determine the reading frame, since all possible triplet codons are meaningful. It does this by recognizing a series of base on the RNA molecule adjacent to the initial AUG codon. Then it moves along the molecule in three base jumps. If an insertion or deletion og base pairs in other than multiples of three has occurred, then the reading frame shifts, and gibberish is produced instead of the normal amino acid.

2.8.1.4 Suppressor Mutations

A suppressor mutation is one which eliminates the phenotypic effect of another mutation but not its genotypic; i.e., a cell which carries one mutation has a mutant phenotype, but a cell which carries the original mutation plus a suppressor mutation has a normal phenotype but a doubly mutant genotype.

2.8.2 Mutagen (Birge, 1981)

A mutagen is anything, which increase the mutation rate of an organism. Mutagens are frequently used to increase the probability of finding a mutation by some selective process. A variety of mutagens will be discussed and some indication of their modes of action given which summarized in Table 2.4.

2.8.2.1 Radiation

Two type of radiation are commonly used: UV light and X rays. They differ greatly in terms of the energy involved and, therefore, in their effects. X rays are extremely energetic, and when they interact with the DNA, the result is usually a break in the phosphodimer backbone of the DNA. Ultraviolet light, on the other hand, catalyzes a reaction in which adjacent pyrimidine base (on the same stand) form dimers. The presence of a dimmer prevents the various polymerases from functioning until it is removed. Mutations may occur during the repair process.

2.8.2.2 Chemical Modifiers

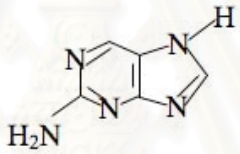
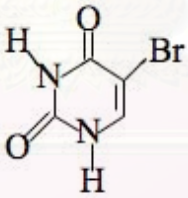
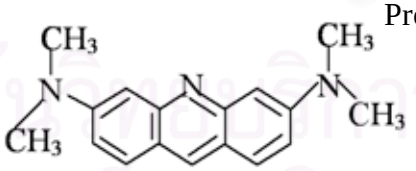
One of the earliest mutagens used on bacteria was nitrous acid, whose primary effect has been assumed to be a deamination of cytosine and quanine. The deamination would result in a change in the hydrogen bonding relationships so

that, at the next replication, adenine or thymine, instead of guanine or cytosine, would be inserted. Although it has sometimes been stated that nitrous acid specifically induced transitions of the GC to AT type, this is probably an oversimplification, as even transverstion are occasionally observed. Recently it has been suggested that nitrous acid may introduce intrastrand cross-links, which must be excised in the same manner as alkylated based.

Hydroxylamined is a moderately specific mutagen, which reacts primarily with cytosine but may also attack uracil or adenine. A typical effect of the chemical is to replace a cytosine with a thymine residue.

Numerous alkylating agents have mutagenic activity. They attach or methyl group at the 7-position on the purine ring which results in the excision of the base by removal from the deoxyibose moiety without distrupting the phosphate backbone. The resulting gap must then be filled. Examples of alkylating agents are ethyl methane sulfonate (EMS), methyl methane sulfonate (MMS), and the N-methyl-N'-nitro-N-nitrosoguanidine (NTG). The last, an extreamly potent mutagen which tends to act at the site of the replication fork on the DNA by production of 7-methylguanine. In a culture treated with this mutagen, as many as 15% of the cells may be mutated for a specific trait such as maltose utilization. In fact, the greatest problem with NTG mutagenesis is its tendency to produce mutation.

Table 2.4 Some common mutagens and their properties (Birge, 1981)

Mutagen	Structure	Mode of action
X ray	5 nm wavelength	Single- and double-strand breaks
UV light	254 nm wavelength	Pyrimidine dimers
Nitrous acid	HNO_2	Deamination; intrastrand cross-links
Hydroxylamine	NH_2OH	Hydroxylation od cytosine
Methyl metane sulfonate	$CH_3SO_3CH_3$	Alkylation of purines
2-aminopurine		May replace adenine; may hydrogen bond to cytosine
5-bromouracil		May replace thymine; may hydrogen bond to guanine
Acridine orange		Production of frameshifts
ICR 191 (a nitrogen mustard)		Production of frameshifts

2.8.2.3 Base Analogs

A base analog is a chemical that has a ring structure similar to one of the normal nucleic acid bases but which dose not have the same chemical properties. Some base analogs, such as 5-bromouracil (5-BU) or 2- aminopurine (2-AP) are also structure analogs and are incorporated directly into DNA in place of the normal bases (thymine and adenine, respectively). They tend to be more variable in their hydrogen-bonding properties and, therefore, may induce errors during replication, either by inserting themselves in the wrong position or else by causing an increase pairing when acting as a template. In addition, base analogs may increase the sensitivity of the molecule to other mutagenic treatments (e.g., 5-BU makes DNA more sensitive to UV light)

2.8.2.4 Cross-Linking Agents

Certain chemicals result in the production of interstand cross-links in the DNA, which obviously prevent DNA replication until they are repaired. Examples of cross-likers are mitomycin C and trimethyl psopalen. The latter compound has been widely used because it must be activated by expose to 360 nm light. This gives the experimenter good control of the timing of the crossing-linking events.

2.8.2.5 Transposons

Transposons are units of DNA which move from one DNA molecule to another, inserting themselves nearly at random. They are also capable of catalyzing DNA rearrangements such as deletions or inversion. An excellent exmple is bacteriophage Mu, which acts as a mutagen due to its propensity for inserting itself

randomly into the middle of a structural region of DNA during lysogenization, causing loss of the genetic function encoded rarely, leaves the DNA again. This mode of insertion is in marked contrast to that of a phage like lamda, which has a vary specific site of integration for its DNA.

2.8.2.6 Mutator Mutations

Certain types of mutations, which affect the DNA replication machinery, have mutagenic effects. These mutations affect the fidelity of the replication process but do not appear to significantly impair the polymerization reactions. Mutations have been isolated in *E. coli*, which tend to produces transition, transversion, deletions or frameshifts.

CHAPTER III

MATERIALS AND METHODS

Equipments

Autoclave (Ta Chang Medical Instrument, Taiwan)

Centrifuge, refrigerated centrifuge (Kubota 3700, Japan)

Centrifugal Filter Devices, 10KDa (Millipore, U.S.A)

Gel documentation (Vilber Lourmat,France)

Digitale Balance (Satorious, Germany)

Electrophoresis unit (Bio-rad Laboratories, USA)

Electroporation (Bio-rad Laboratories, USA)

Fast Plasmid Mini, 0032 007 653 (Eppendorf, USA)

Filter Paper, No 1, diam. 110 mm (Whatman, England)

High Performance Liquid Chromatrography Model: LC-20A (Shimadzu PLC, Japan)

High Performance Liquid Chromatrography's Column

Model: Apollo Silica 5U, length 250 mm, i.d. 4.6 mm

(Alltech Associates. Inc., U.S.A)

Incubator, Model 700 (Memmert GmbH, Germany)

Incubator shaker: Model G-600 (New Brunswick Scientific, U.S.A)

Microtitreplate 96 well, U bottom (Bibby Sterili Ltd, UK)

Shaking incubator (Vision Scientific., LTD, Korean)

Silica gel plate, F254 (Merck, Germany)

Spectrophotometer, Zenyth 200rt (Anthos Labtec Instruments, Austria)

UV lamp (Sylvania, Japan)

Laboratory chemicals

Acetic acid (BDH Chemical, England)

Acetonitile (Labscan, HPLC grad, Thailand)

Ammonium sulfate (Merck, Germany)

Bovine serum Albumin; BSA (Merck, Germany)

Coomassie blue G-250 (Sigma, USA)

Copper sulfate (Merck, Germany)

Di-potassium hydrogen phosphate (Riedel, Germany)

Eciosane (Aldrich, Germany)

Ethyl acetate (BDH Chemical, England)

Fast Plasmid TM Mini Kit (Eppendorf, Germany)

Folin-Ciocalteu's reagent (Carlo Erba Reagenti, France)

Glucose (Fluka, Switzerland)

Glycine (Scharlau Microbiology, Spain)

Hydrochloric acid (Merck, Germany)

Magnesium sulfate (Merck, Germany)

N,N,N',N'-Tetramethylene ethylene Diamine;TEMED (BDH, England)

Nutrient Broth (Difco, U.S.A.)

Oil

Corn oil (Chim Brand, Thailand)

Olive oil (Chumchon Community Pharmacy, Thailand)

Palm oil (Morakot Brand, Thailand)

Physic nut oil (Extracted)

Rice bran oil (Alfaone Brand, Thailand)

Rubber seed oil (Extracted)

Sesame oil (Namjai Brand, Thailand)

Sunflower oil (Cook Brand, Thailand)

p-nitrophenol (Fluka, Switzerland)

p-nitrophenyl palmitate (Sigma-Aldrich, U.S.A.)

Potassium dihydrogen phosphate (Merck, Germany)

Potassium sodium tartrate (Merck, Germany)

Protein molecular weight marker (Fermentas, Canada)

Sodium chloride (BDH, England)

Sodium hydroxide (Merck, Germany)

Tris (Hydroxymethyl) aminomethane (USB, U.S.A)

Triton X-100 (Scharlau, Spain)

Yeast extract (Scharlau, Spain)

Rhodamine B (Fluka, Switzerland)

β- mercaptoethanol (Sigma, U.S.A)

Sulfuric acid (RIEDEL-DE-HAEN, Germany)

Taq polymerase (Fermenatus, U.S.A)

Tag buffer (Fermenatus, U.S.A)

Research methodology

The research methodology can be summarized as followed:

3.1 Screening and isolation of lipase producing bacteria

3.1.1 Sample collection

Bacterial lipases were isolated from twelve soil samples in Thailand, which obtained from various sources such as natural resources and oil contaminated soil and ten compost samples. The samples were collected sequentially during April 2005 to September 2006. The detail of samples was shown in Appendix A. All samples were stored in icebox to laboratory and then kept at 4°C.

3.1.2 Enrichment of lipase producing microorganisms

One gram of soil sample or compost sample was suspended in 10 ml of sterile distilled water. After shaking, 5 ml suspension was added in a 250 ml of Erlenmeyer flask containing 25 ml of enrichment medium (Appendix A). The mixture was incubated at 30°C on a rotary shaker at 200 rpm for 5 days.

3.1.3 Screening and isolation of producing lipase bacteria

One ml aliquot of soil suspension was transferred to 9 ml of distilled water and performed serial dilutions; then spread on tributyrin agar plate (Appendix A). After that the plates were incubated for 3 days at 30 °C, colony with clear zone were selected and picked. The selected colony was reisolated by streaking on tributyrin agar plate. A single colony was cultivated at least 2 twice to assure the purity of culture. Secondary screening of bacterial isolates was tested on Rhodamine

B agar plates (Appendix A). Each isolated colony surrounded with orange fluorescent halo upon ultraviolet radiation at 350 nm was regarded as lipase producers.

3.1.4 Cell growth conditions and stock cell maintenance

Cell growth conditions and stock cell maintenance of isolated producing lipase bacteria in the laboratory are described as followed.

3.1.4.1 Preparation of growth media

The isolated producing bacteria were grown in nutrient broth at 30 °C. The nutrient broths was prepared by dissolving 8 g of nutrient broth powder (Difco Bacto, USA) in 1 L of distilled water and adjust pH to 7.2, then autoclaved at 121 °C for 15 min.

3.1.4.2 Preparation of stock cell culture

The lipases-producing bacterial were grown by picking a single colony into 5 ml of nutrient broth (section 3.1.4.1) at 30 °C, 200 rpm for 12 hours. The cell culture (0.7 ml) was taken into sterile 0.7 ml of 60% glycerol in 1.5 ml eppendorf as the cell stock. The cell culture stocks were stored at -80 °C.

3.2 Preparation of the cell-free extract from bacterial isolates

The selected single colony was grown in 5-ml nutrient broth and incubated at 30 °C, 200 rpm for 12 h. Then, the inoculum's size of 1.0% (v/v) was added to 100 ml of the olive oil medium (Appendix A) in a 250-ml Erlenmeyer flask and incubated at 30 °C, 200 rpm for 48 h as . The culture broth was centrifuged at 10,000 rpm for 15

min at 4 °C, and the supernatant were filtered through a filter paper (Whatman no.1). The filtrated supernatant were used as the cell free extract.

The cell free extract was concentrated using Ultracel -15K (Amicon Ultra-15, Milipore, Ireland) for transesterification.

3.3 Enzymatic assays for lipase

3.3.1 Hydrolysis activity

The hydrolysis activity of lipase was determined by monitoring the *p*-nitrophenol released from *p*-nitrophenyl palmitated (*p*NPP) as the substrate according to a method described by Bournchereuer *et al.* (1994) with slight modifications for microplate reader and can described as followed.

Solution A contained 0.71 mM *p*-nitrophenyl palmitated (*p*NPP) was dissolved in 2-propanol. Solution B contained 0.4% Triton X-100 and 0.1% gum arabic. The reaction mixture consisted of 1 part of solution A and 9 parts of solution B was freshly prepared before assay. A 20 μl volume of an appropriated dilution of enzyme solution was added to 180 μl of the reaction mixture. Finally, the lipase activity was analyzed at 410 nm after incubation at 37 °C for 30 min using a spectrophotometer (Zenyth 200rt, Anthos Labtec Instruments, Austria)

One unit of enzyme activity was defined as the amount of enzyme required to release 1 nmol of *p*-nitrophenol per min under the described conditions. Preparation of stock solutions and the standard curve of *p*-nitrophenol as well as calculation the unit of enzyme activity was described and shown in Appendix B.

3.3.2 Transesterification reactions in solvent-free conditions

The transesterification reactions were carried out in 35 mL glass-vials containing 3g of palm oil and the enzyme (10 mg of protein) and distilled water to 6 ml of solution (64.5%). The reaction mixture was preincubated at 40 °C for 30 min and agitated by mixing with a magnetic stir at 900 rpm in a water bath. The reaction was initiated by adding 1/3 mole of methanol (0.121 ml) to the reaction at 8^{th} hour. Then, at 12^{th} and 16^{th} , another 1/3 mole of methanol was separately added. The final concentration of methanol in the reaction was 3 moles. The reactions were at 40 °C and 900 rpm for 48 h. The sample were withdrawn and centrifuged at 13,000 rpm for 30 min., the methyl ester in the upper phase were further proceeded.

3.4 Identification of methyl ester

3.4.1 Thin-Layer Chromatography (Samukawa, 2000)

The methyl ester was analyzed by Thin-Layer Chromatography **(**TLC) with 20 x 20 cm silica gel 60 F254 (Merck, Dermstadt, Germany). The 1.0 µl of oil sample was spotted onto TLC plate. The developing solvent with slight modifications was composed of hexane: ethyl acetate: acetic acid = 90: 10: 2(v/v). A solvent consisting of sulfuric acid: methanol = 1:1 (by wt) was sprayed onto the plate and the spots were detect after plate baking at 110 °C for 30 min.

3.4.2 High Performance Liquid Chromatography (HPLC)

The HPLC device was equipped with Apollo Silica 5U: (250 mm × 4.6 mm) connected to a evaporative light scattering (ELSD) detector. The operating conditions of ELSD was as follows: drift tube temperature: 40 °C, gas

pressure 8 psig. A flow rate of 1.5 mL/min was used. The HPLC mobile phase was composed of a mixture of solvent A (hexane/isopropanol: ethyl acetate: formic acid; 85: 10: 10:10: 0.1 v/v) and solvent B (hexane: formic acid (100: 0.2 v/v) Data collection and analysis were performed using LC solution software (Shimadzu, Japan). An Eicosane was used as an internal standard. Sample was dissolved in hexane.

3.5 Determination of protein concentration

Protein concentration was determined by a modified Lowry method (Held and Hurley, 2001) in microplate reader where bovine serum albumin (BSA) was used as a standard protein. The reagent preparation and the protein standard curve were shown in Appendix C.

The reaction consists of 100 μl of the sample and 200 μl of mixed solution A and B (A: B = 50:1) were added and rapidly mixed. The reaction was incubated at room temperature for 10 min. Then, 20 μl of the solution C was added, rapidly mixed and incubated at room temperature for 30 min. Finally, the absorbance of clear blue solution was measured at 650 nm.

3.6 Identification of producing lipase bacteria

The lipase-producing bacteria were tested using biochemical test.

3.6.1. Biochemical test

Biochemical characterizations of bacteria were analyzed by the laboratory of Institution for Scientific Research, Department of Medical Science, Ministry of public Thailand. Biochemical test methods are show in Appendix D

3.6.2. Polymerase chain reaction (PCR)

Stenotrophomonas maltophilia showed highest lipase activity. Therefore, its identification was confirmed by PCR with species-specific PCR (SS-PCR) primer (Whiteby, 2000), SM1 (5'-CAGCCTGCGAAAAGTA-3') and SM4 (5'-TTAAGCTTGCCACGAACAG-3'), that targeting on specific region in 23S rRNA gene of *S. maltophilia.* The expecting PCR product size only was approximately 540 bp, which is a specific band of *S. maltophilia.*

Genomic DNA from the bacterial strain was extracted by a standard method (Sambrook *et al.*, 1989*).* A reaction mixture (total volume of 25v µl) contained a 1 µM concentration of each primer, 10 ng of genomic DNA, a 200 µM concentration of each deoxynucleotide triphosphate, and 1.25 U of *Taq* DNA polymerase in a 3 mM $MgCl_2$ PCR buffer (Idaho Technologies), in a total volume of 50 µl. All PCRs had an initial denaturation of 95°C for 5 min with a subsequent 30 cycle amplification. The cycle parameters consisted of annealing at 58°C for 10 s, extension at 72°C for 60 s, and denaturation at 95°C for 10 s. For the last cycle the extension step was 2 min. After amplification, 20 µl of each reaction mixture was subjected to electrophoresis in a 0.8% agarose gel in 0.5× Tris-borate-EDTA (TBE) buffer (pH 8.0) alongside a 100-bp ladder. The PCR products were visualized and photographed after ethidium bromide staining. Positive result was assessed by the amplification of a 531-bp product. *E.coli* was used as negative control.

3.7 Volume reduction of extracellular lipase produced by *Stenotrophomonas maltophilia* CU22

Lipase from *S. maltophilia* was concentrated using ammonium sulfate precipitation. After 72 h of culture, the supernatant of the culture were filtered

through filter paper. Ammonium sulphate was added to the supernatant to 80% saturation, with mild agitation at 4 °C. The extract was then maintained under gentle stirring at 4 °C for 8 h, after which it was centrifuged at 14,000 rpm for 30 min. The supernatant was removed and the precipitate was re-suspended in a minimal volume (approximately 5 ml) of 50 mM potassium phosphate buffer, pH 7.0. This suspension was dialyzed against 100x volume of the same buffer, at 4 ◦ C, at least 3 hours, with two exchanges. The molecular weight cut-off of the dialysis membrane was 14000 Da. The dialyzed solution retained within the bag was used as the crude lipase.

3.8 Lipase production for transesterification

Stenotrophomonas maltophilia was cultivated in lipase production media for enhance lipase production and lipase activity for transesterification.

3.8.1 Effect of oil in olive oil media

1.0% (v/v) of olive oil in olive oil media (Appendix A) was substituted by various oils: corn oil (Chim Brand), rubber seed oil, palm oil (Morakot Brand), physic nut oil, rice bran oil (Alfaone Brand), sesame oil (Namjai Brand) and sunflower oil (Cook Brand) to determine if it could enhance lipase production and lipase activity for transesterification with 1.0% (v/v). The inoculum's size of 1.0% was added to 100 ml of the each of oil medium in 250 ml Erlenmeyer flask, incubated at 30 °C and 200 rpm for 48 h. The culture broth was centrifuged at 10,000 rpm for 15 min at 4 °C, then filtered the supernatant through a filter paper (Whatman no.1). The filtrated supernatant was used as the cell free extract.

For transesterification, the cell free extract was concentrated using Aqacide and then dialyzed against 50 mM Potassium Phosphate buffer, pH 7.0. All operations were performed at 4 °C.

3.8.2 Effect of media in lipase activity and production

The single colony of *S. maltophilia* was inoculated in 100 ml of TGY media (Appendix A) at 30 °C, 200 rpm for 24 h. Then, 0.4% oil was added to the TGY medium in 250 ml Erlenmeyer flask and incubated at 30 °C, 200 rpm for 72 h. Olive oil and palm oil were investigated.

The culture broth was collected by centrifuged at 10,000 rpm for 15 min at 4 °C, and the supernatant were filtered through a filter paper (Whatman no.1). The filtrated supernatant was used as the cell free extract. For transesterification, the cell free extract was concentrated using Aqacide and then dialyzed against 50 mM Potassium Phosphate buffer, pH 7.0. All operations were performed at 4 °C.

3.9 Mutagenesis

3.9.1 UV radiation

A single colony of *S. maltophilia* from on a nutrient agar was grown in 50-ml nutrient broth at 30°C and 200 rpm. Four-milliliter cell suspension (~ 10^8 cells/ml) was placed under an ultraviolet lamp with distance of 32 centimeters and was radiated for different exposure time intervals between 4s and 99s. The suspension was mixed during the UV radiation. A 100-µl of irradiated suspension was transferred to 9 ml of nutrient broth in the tube covered with aluminum foil, to exclude light and incubate at 30°C, 200 rpm for 60 min. The culture suspension was then transferred to

sterile water for serial dilution and spreaded on nutrient agar and incubated at 30°C for 48 hours. After viable colony counting, the samples with a death rate of 95% or more was subject to subsequent isolation (Geo, Cao and Zhang, 2000)

3.9.2 NTG treatment

The starting inoculum (~ 10^8 cells/ml) was washed twice and resuspended with 0.2 M pH 6.0 potassium phosphate buffer (Geo, Cao and Zhang, 2000). NTG solution was added by varying dose of NTG to a final concentration of 10-200 μg/ml and the suspension was incubated at 30°C, 200 rpm, for 30 min. The cell was spun down and washed in phosphate buffer, pH 6.0. The samples was serially diluted with sterile water and plated on NA plate. Samples with a death rate of 90% will be subject to subsequent isolation.

3.9.3 Stability of mutant

S. maltophilia CU22 was transferred from a nutrient agar plate to a second nutrient agar plate and inoculated at 30 ºC for 24 h, cells from the second plate were transferred into the third plate and the third plate was cultured under same conditions as before. Each transfer represents one generation. The above culture was transferred many times and the lipase activity of each generation in lipase production media was assayed and the stability of the mutant for lipase production was determined.

3.10 Amplification of lipase gene from *S. maltophilia* CU22

3.10.1 DNA extraction

The *S. maltophilia* CU22 was cultured in nutrient broth at 30 °C for 12-18 hours. The cell were collected by centrifugation at 5,00 rpm for 10 min prior to lysis in lysis buffer (10 mM Tris-HCl, 20mM glucose and 2% SDS). Proteinase K of 5 μl was added to have the final concentration of 1 mg/ml and prior to incubated at 45 °C for 2 hours. Phenol/Chloroform was added to the solution and centrifuged to separate phases. The upper aqueous phase was transferred to a new tube, precipitated with 2 volumes of absolute ethanol and kept at -20°C for 1 hour. The solution was then centrifuged at 10,000 rpm for 10 min. DNA pellet was dissolved in TE buffer and stored at -20°C.

3.10.2 Amplification of partial lipase gene

The partial lipase gene from *S. maltophilia* CU22 was amplified by using PCR with a pair of modified primers (Jiang *et al*, 2006), JBPF1 (5'- CAG GAA ACA GCT ATG ACC CCS TGG AAY CCS GAY TCG GAA -3') and JBPR1 (5'- TGT AAA ACG ACG GCC AGT GCG GTT SAG GTC YTG SAC CCA -3') both of which containing the M13 forward and reverse primer sequences at 5' of JBPF1 and JBPR1, respectively. The PCR reaction was consist of 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of the four deoxyribonucleotide triphosphates, 1 uM each of primer and 0.03 U/ul *Taq* DNA polymerase. The reaction were allowed to proceed in a thermocycler (TP600; Takara, Japan) programmed to 95 °C for 4 min and then proceed with 35 cycles of 95 °C for 1 min, 58 °C for 30 sec and 72 °C for 1 min, final extension at 72 °C for 5 min. The PCR was holding at 4 °C. The PCR product was exised on 2% agarose gel in Tris-borate buffer at 100 volts for 30 min, followed by staining with ethidium bromide and

visualization on a UV transilluminator at 302 nm, then purification by using Qiaquick gel extraction kit.

The purified fragment was ligated into pTZ57R/T (Fermentus, Canada) and incubated at 16 °C for overnight, then transformed into *E. coli* JM109 using electroporation. The transformed cells were grown in LB-IPTG-X gal agar containing 100 μg/ml of ampicillin at 37 °C for 24 hours. The recombinant plasmid was checked for the insert fragment by performed a PCR with M13 primers and JBPF1+JBPR1 primer then double digestion with *Eco*RI and *Hin*dIII before subject to DNA sequencing by using primer M13-forward and reverse, respectively.

3.10.3 Amplification of lipase gene

The complete nucleotide sequence of lipase from *S. maltophilia* CU22 was determined by inverse PCR. HindIII or EcoRI digested genomic DNA of the bacteria. The purified-digested genomic DNA was performed a self-ligation by incubation at 16 °C for overnight. Of 7 ul of purified ligated product was use as the template for inverse PCR, which consist of 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of the four deoxyribonucleotide triphosphates, 1 uM each of primer pair [SMlip-iF (5'-ACCGCCTGCAAGATCGAT-3') and SM-iR1 (5'-GCTTCAGCGCACCCAC CAGA -3') and SMlip-iF1 (5'-GGCGGTCAGGTTGCTGCCGTCCGGCG-3') and SM-iR2 (5'-GAACTACGCGA CCGTCGTGCTGGG-3')] and 0.03 U/ul *Taq* DNA polymerase. The reaction were allowed to proceed in a thermocycler (TP600; Takara, Japan) programmed to 95 °C for 4 min and then proceed with 35 cycles of 95 °C for 1 min, 58 °C for 30 sec and 68 °C for 5 min, final extension at 72 °C for 10 min. The PCR was holding at 4 °C. The PCR product was checked by electrophoresed on 2% agarose gel in Tris-borate buffer

at 100 volts for 30 min, followed by staining with ethidium bromide and visualization on a UV transilluminator at 302 nm. The PCR product band was excised from agarose gel and purified by Qiaquick gel extraction. Purified fragment was performed DNA sequencing by using primer SMlip-iF and SM-iR1, respectively.

3.11 Minimum inhibitory concentration (MIC) values

A single colony of *S. maltophilia* CU22 wild type and mutants from on a nutrient agar was grown in 5-ml nutrient broth at 30°C and 200 rpm. 75 μl of inoculum (~ 10^5 cells/ml) was diluted with 75 μl of two-fold dilutions of antibiotics in each well of microtiter plate. Chloramphinical, kanamycin, ampicillin and penicillin were investigated, stock solution were sterilized using 0.2 μm pore size cellulose acetate filters (Satorious, Germany). The control of the adequacy of the broth to support the growth of the organism was inoculum and antibiotic-free broth. The control of the sterility was uninoculum and antibiotic-free broth. After overnight incubation at 30°C, 200 rpm, the microtiter plates were examined for visible evidence of bacterial growth in the form of turbidity at 540 nm using spectrophotometer. The lowest concentration of antibiotic that prevents visible growth represents the endpoint of the test, the MIC that defined as the lowest concentration of an antibiotic that will inhibit the visible growth of a microorganism.

3.12 Characterization of crude lipase

3.12.1 Molecular weight determination of lipase by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The denaturing gel was performed according to Bollag and Edelsetin (1993). The gel caster was assembled. The clean and transparent glass plate was cleaned with soft detergent, alcohol and allowed to air dry. The separating gel solution was prepared with o.1% (w/v) SDS in 10% (w/v) and 5.0% stacking gel. While Tris-glycine (pH 8.3) containing 0.1% SDS was use as electrode buffer (Appendix E). Samples to be analyzed were treated with sample buffer (Appendix E) and boiled for 3 min prior to gel application. The electrophoresis was performed at constant of 100 V, at room temperature on a Mini-Gel Electrophoresis unit from cathode towards anode.

After electrophoresis, proteins in denaturing gel were visualized by Comassie blue staining. Gel was stained with 0.1% (w/v) of Comassie brilliant R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid for at least 30 min. The slab gels were destained with a destining solution (10% methanol and 10% acetic acid) for 1-2 hours several times until the gel background was clear.

3.12.2 Effect of pH on lipase activity

The crude lipase was assayed as described in section 3.2.1 using *p*-nitrophenyl palmitate as a substrate and protein concentration (section 3.3) was carried out, but in various pH conditions. The 50 mM of acetate or phosphate or Tris-HCl or glycine-NaOH was used as reaction buffers for pH 4.0, 5.0-8.0, 8.0-9.0 and 9.0-11.0, respectively.

3.12.3 Effect of temperature on lipase activity

The crude lipase was assayed by the method as described in section 3.2.1 (*p*-nitrophenyl palmitate as a substrate) at 30, 40, 50, 60, 70, 80 and 90°C for 30 minutes. The protein concentration (section 3.3) was carried out.

3. 12.4 pH stability of lipase activity

The crude lipase was incubated at 4°C for 1, 12 and 24 hours, in 50 mM buffers at various pH values (the total volume of 1 ml). An aliquot of the enzyme solution was withdrawn at interval and the remaining activity was assayed as described in section 3.2.1 (*p*-nitrophenyl palmitate as a substrate). The 50 mM of acetate or phosphate or Tris-HCl or glycine-NaOH was used as reaction buffers for pH 4.0, 5.0-8.0, 8.0-9.0 and 9.0-11.0, respectively. The result was expressed as the percentage of the residue activity as the activity incubated in potassium phosphate buffer, pH 7.0, was used as 100% activity.

3.12.5 Temperatures stability of lipase activity

The thermostaility of the enzyme was investigated in the range of 30-90 °C. The partially purified lipase in 50 mM phosphates buffer, pH 7.0 was preincubuted at temperature 30, 40, 50, 60, 70, 80 and 90°C for 1, 12 and 24 hours, then the residual activity was assayed as described in section 3.2.1(*p*-nitrophenyl palmitate as a substrate). The result was expressed as the percentage of the residues activity as the activity preincubated at 37 °C was used as 100% activity.

3. 12.6 Effect of metal ions on lipase activity

Various concentrations (1 mM, 5 mM and 10 mM) of various types of monovalent ions ($Na^+$and K^+) and divalent ion (Mg^{2+}) were added to reaction mixture. The enzyme activity was assayed by the method as described in section 3.2.1, using *p*-nitrophenyl palmitate as a substrate.

CHAPTER IV

RESULTS

4.1 Screening, isolation and selection of lipase-producing bacteria

To screen bacterial strains capable of lipase production, twelve soil and ten compost samples were primarily enriched in olive oil media for 5 days at room temperature, and then investigated on tributyrin-NB agar plate. Ninety-five isolates forming clear zone on tributyrin plate were selected. Then, all isolates were subjected to the secondary screening on Rhodamine B agar plate. Fifty-five selected isolates showed an orange fluorescent halo when observed under 350 UV light, indicating the production of an extracellular lipase (Table 4.1 and Figure 4.1).

The isolated bacteria were cultured in olive oil media (Tanigaki *et al*., 1995) for lipase production. After 2 days, the supernatant was collected. The lipase activity was determined by monitoring the *p*-nitrophenol released from *p*-nitrophenyl palmitate (*p*NPP) as a substrate according to a method described by Bourncher *et al.* (1994) with slight modifications for assay in microplate reader described in section 3.3.1. The protein concentration was determined by the method as described in section 3.5 using BSA as a protein standard. The lipase specific activity and lipase production of isolated bacteria were calculated and showed in Figure 4.2 and 4.3, respectively.

Table 4.1 Isolation of lipase producing bacteria from soil and compost samples

Type	Source	Tributyrin agar*	Rhodamine B agar**
1.Soil	1.1 Ban Na, Nakhon Nayok (NA)	3	0
	1.2 Chulalongkorn University (CU)	3	1
	1.3 San Kamphaeng, Chiang Mai (SKP)	2	0
	1.4 Amphoe Pai, Mae Hong Son (PAI)	3	2
	1.5 Sediment from Thai Vegetable Oil PCL (SED)	6	4
	1.6 Nan (NAN)	6	4
	1.7 Fossils Shell Beach , Krabi (KB)	3	0
	1.8 Thung Salaeng Luang National Park, Phitsanulok (SL)	3	0
	1.9 Rain forest in Taksin Maharat National Park ,Tak (FR)	5	3
	1.10 Pine forest, Taksin Maharat National Park,Tak (TAK)	4	2
	1.11 Bang Pakong River, Chachoengsao (BP)	5	1
	1.12 Viang sa, Nan (WS)	3	1
2. Compost	2.1 Oil palm empty fruit bunches (CA)	8	6
	2.2 Compost from chicken dung (CB)	3	2
	2.3 Anthill (CC)	6	5
	2.4 Bamboo leave litter (CD)	5	3
	2.5 LongKong leave litter (CE)	6	5
	2.6 Waste from Straw mushroom cultivation (CF)	6	4
	2.7 Waste from Oyster mushroom cultivation (CG)	4	2
	2.8 Rice (CH)	4	3
	2.9 Rubber leave litter (CI)	5	4
	2.10 Mangosteen leave litter (CJ)	2	1
	Total	95	55

*****The number of isolates which showed a positive clear zone on tributyrin agar plate.

** The number of isolates that showed a positive orange fluorescent halo under 350 UV light on Rhodamine B agar plate.

Source

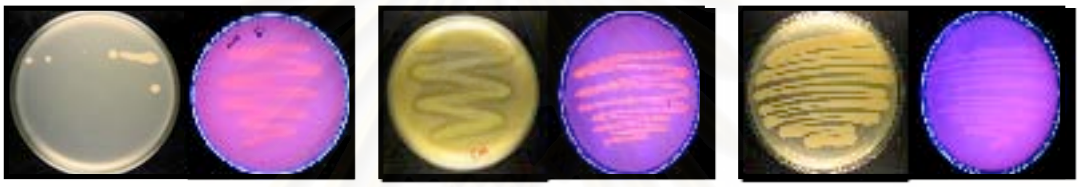
1. Soil

1.1 Ban Na, Nakhon Nayok (NA)

NA11 NA12 NA21

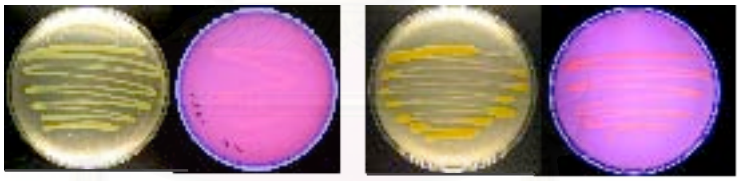
1.2 Chulalongkorn University (CU)

CU11* CU22* CU24



1.3 Amphoe San Kamphaeng, Chiang Mai (SKP)

SKP11 SKP13*



1.4 Amphoe Pai, Mae Hong Son (PAI)

PAI11 PAI12* PAI22*

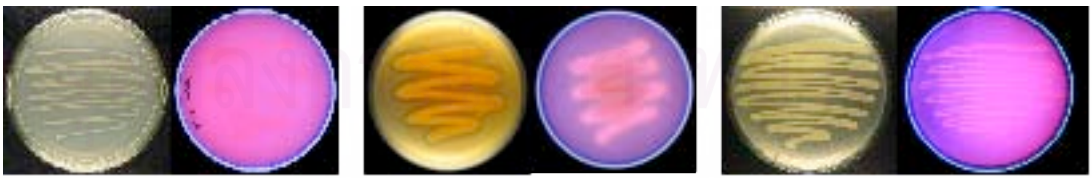
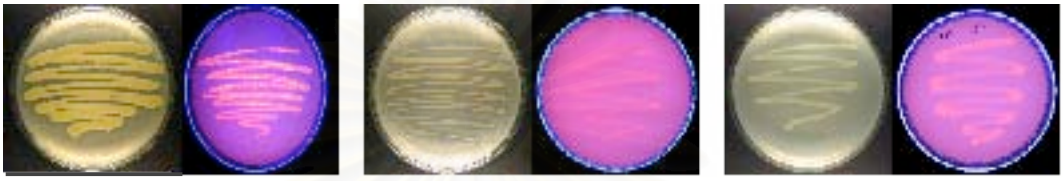


Figure 4.1 Lipase-producing bacteria isolated from soil and compost samples. Bacteria isolated showed an orange fluorescent halo when observed under UV light, which are represented by an asterisk (*).

1.5 Sediment from Thai Vegetable Oil PCL (SED)

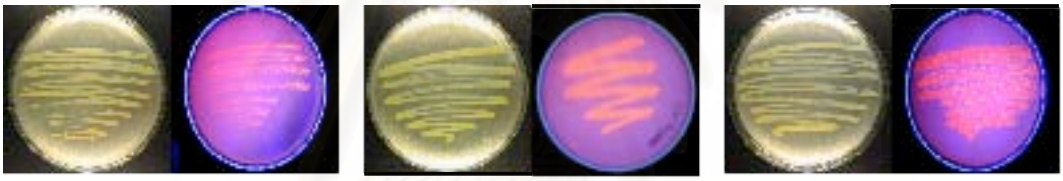
SED13* SED14* SED21*

SED22* SED23 SED24

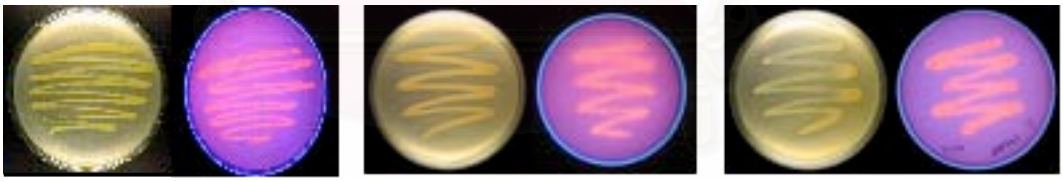


1.6 Nan (NAN)

NAN21* NAN24* NAN31*



NAN44* NAN244* NAN241*



1.7 Fossils Shell Beach , Krabi (KB)

KB11 KB12 KB13

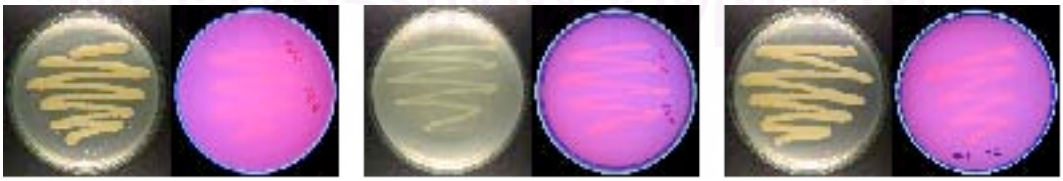


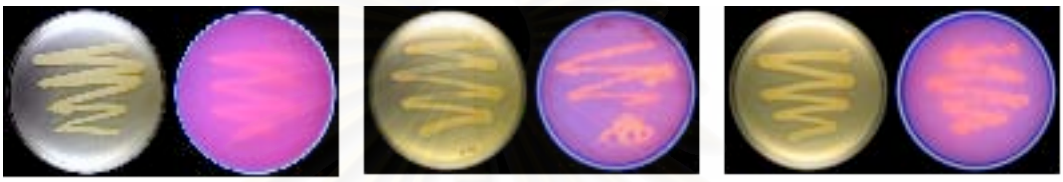
Figure 4.1 Lipase-producing bacteria isolated from soil and compost samples. Bacteria isolated showed an orange fluorescent halo when observed under UV light, which are represented by an asterisk (*) (continued).

1.8 Thung Salaeng Luang National Park, Phitsanulok (SL)

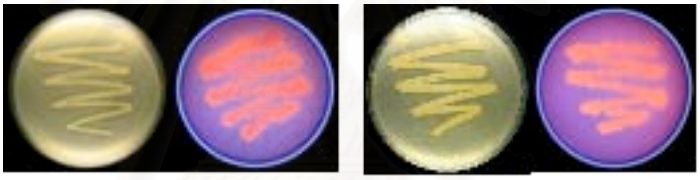
SL11 SL22 SL23

1.9 Rain forest in Taksin Maharat National Park ,Tak (FR)

FR11* FR42* FR421*

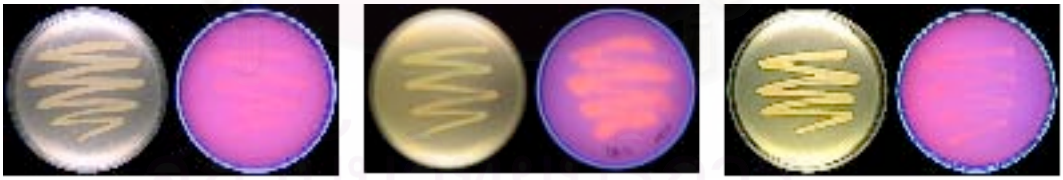


FR21* FR212*



1.10 Pine forest, Taksin Maharat National Park ,Tak (TAK)

TAK11* TAK32* TAK31

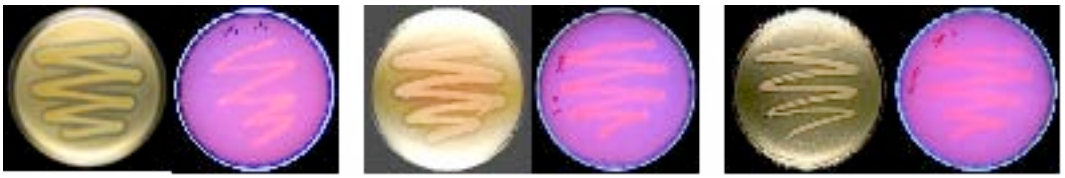


TAK33

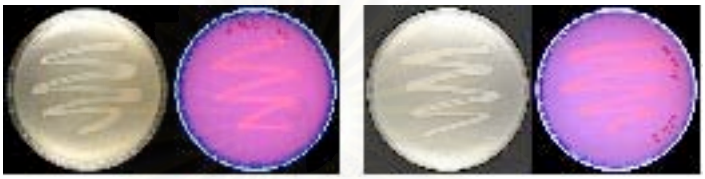
Figure 4.1 Lipase-producing bacteria isolated from soil and compost samples. Bacteria isolated showed an orange fluorescent halo when observed under UV light, which are represented by an asterisk (*) (continued).

1.11 Bang Pakong River, Chachoengsao (BP)

BP11* BP12 BP13



BP14 BP21



1.12 Viang sa, Nan (VS)

VS31* VS11 VS21

2. Compost

2.1 Oil palm empty fruit bunches (CA)

CA1022* CA1011* CA104*

Figure 4.1 Lipase-producing bacteria isolated from soil and compost samples. Bacteria isolated showed an orange fluorescent halo when observed under UV light, which are represented by an asterisk (*) (continued).

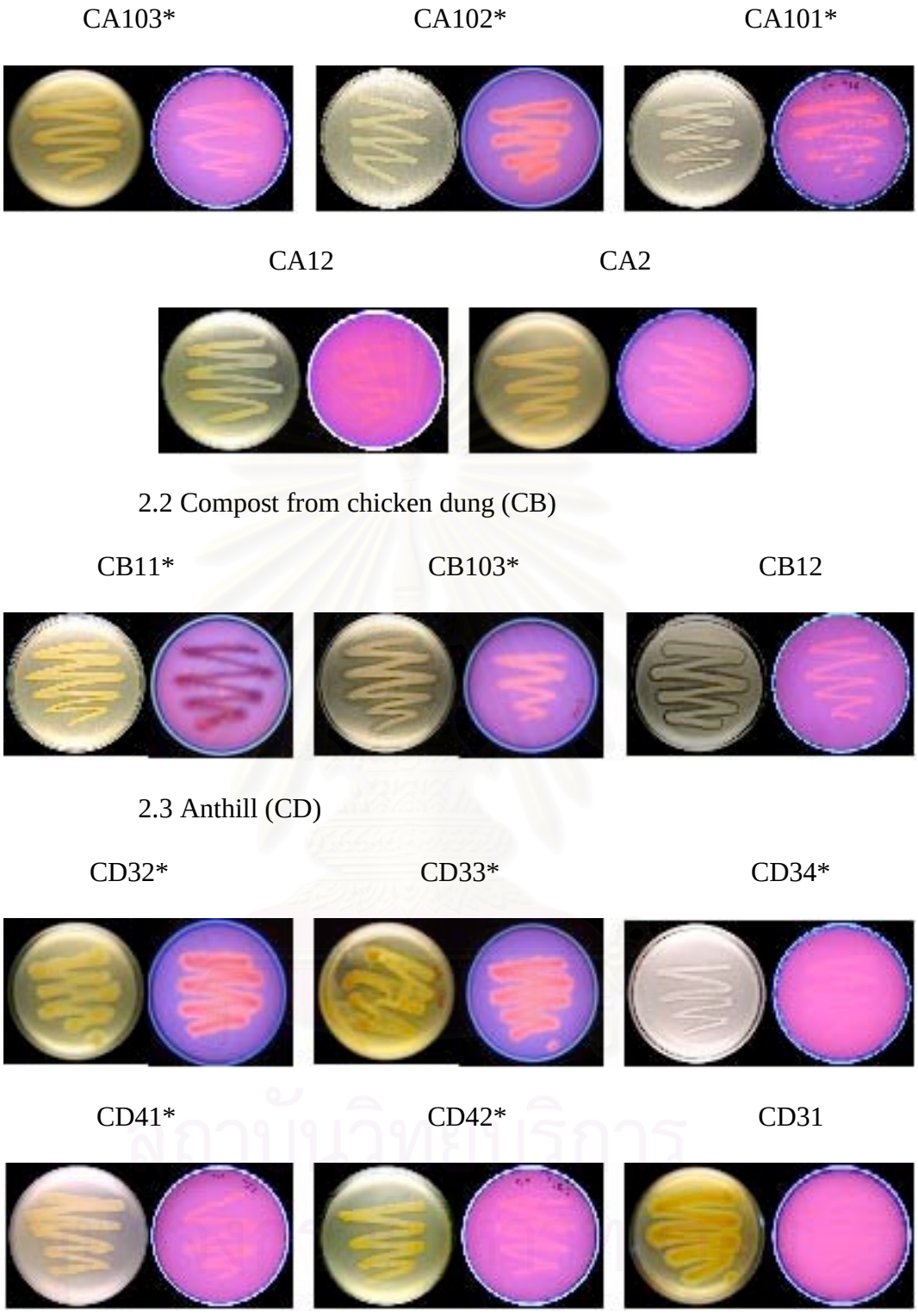
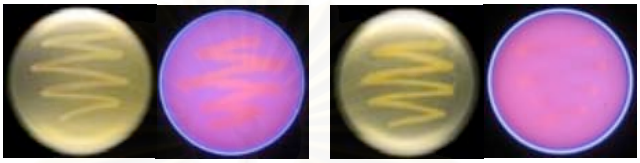


Figure 4.1 Lipase-producing bacteria isolated from soil and compost samples. Bacteria isolated showed an orange fluorescent halo when observed under UV light, which are represented by an asterisk (*) (continued).

2.4 Bamboo leave litter (CE)

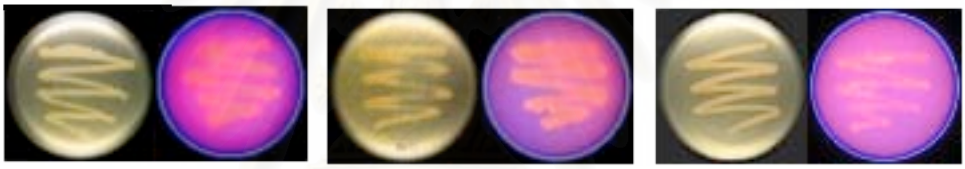
CE71* CE72* CE75*

CE74 CE73



2.5 Long Kong leave litter (CF)

CF1* CF2* CF3*



CF5* CF73* CF81

2.6 Waste from Straw mushroom cultivation (CG)

CG1* CG2* CG3*

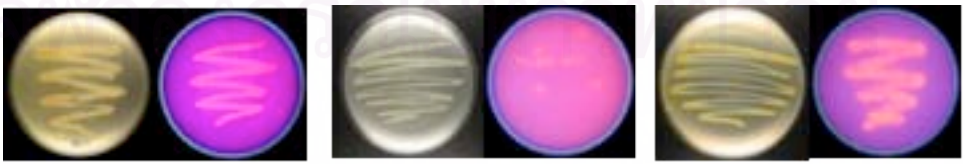
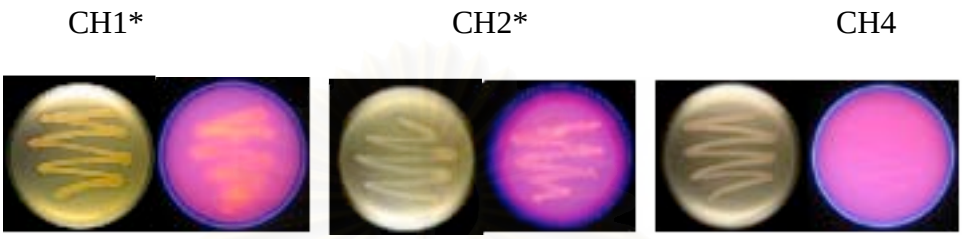
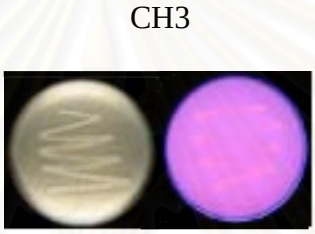


Figure 4.1 Lipase-producing bacteria isolated from soil and compost samples. Bacteria isolated showed an orange fluorescent halo when observed under UV light, which are represented by an asterisk (*) (continued).

2.7 Waste fro m Oyster mushroom cultivation (CH)





2.8 Rice (CI)

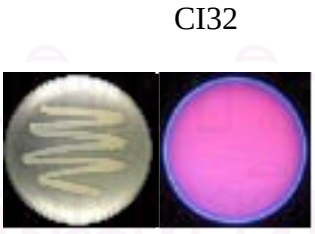
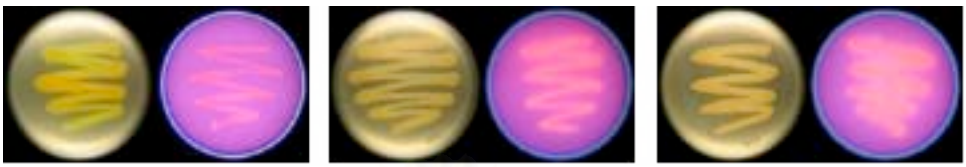


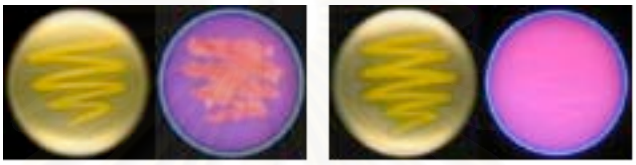
Figure 4.1 Lipase-producing bacteria isolated from soil and compost samples. Bacteria isolated showed an orange fluorescent halo when observed under UV light, which are represented by an asterisk (*) (continued).

2.9 Rubber leave litter (CJ)

CJ61* CJ62* CJ63*



CJ64* CJ65



2.10 Mangosteen leave litter (CK)

CK91 CK92*

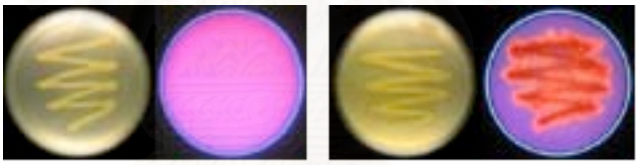


Figure 4.1 Lipase-producing bacteria isolated from soil and compost samples. Bacteria isolated showed an orange fluorescent halo when observed under UV light, which are represented by an asterisk (*) (continued).

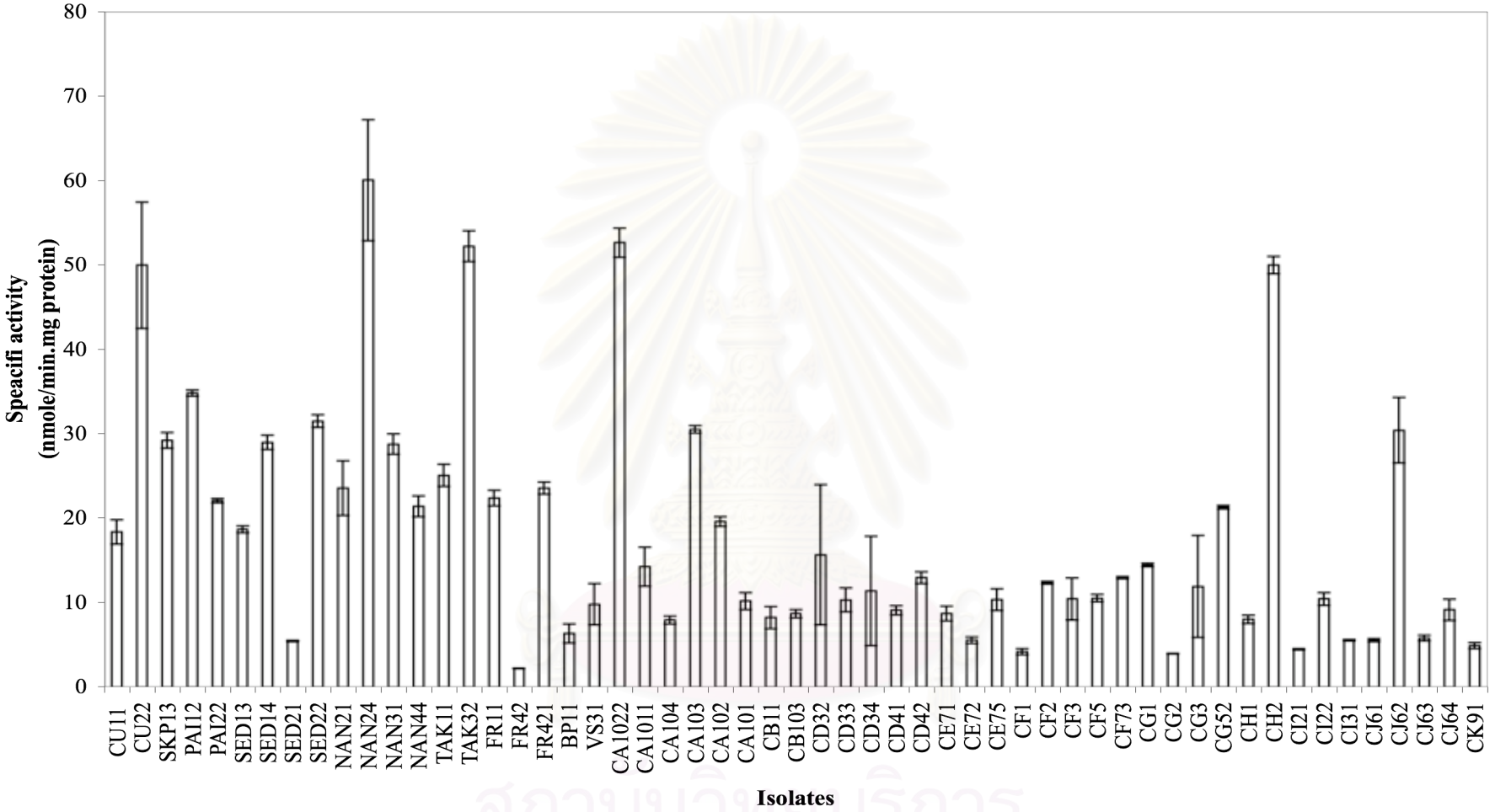


Figure 4.2 Lipase specific activities of positive lipase-producing bacterial isolated from 12 soils and 10 composts in Thailand.

Capital letters represent location of soil and compost samples shown in Table 4.1. Number represent numbers designated for each bacterial isolated.

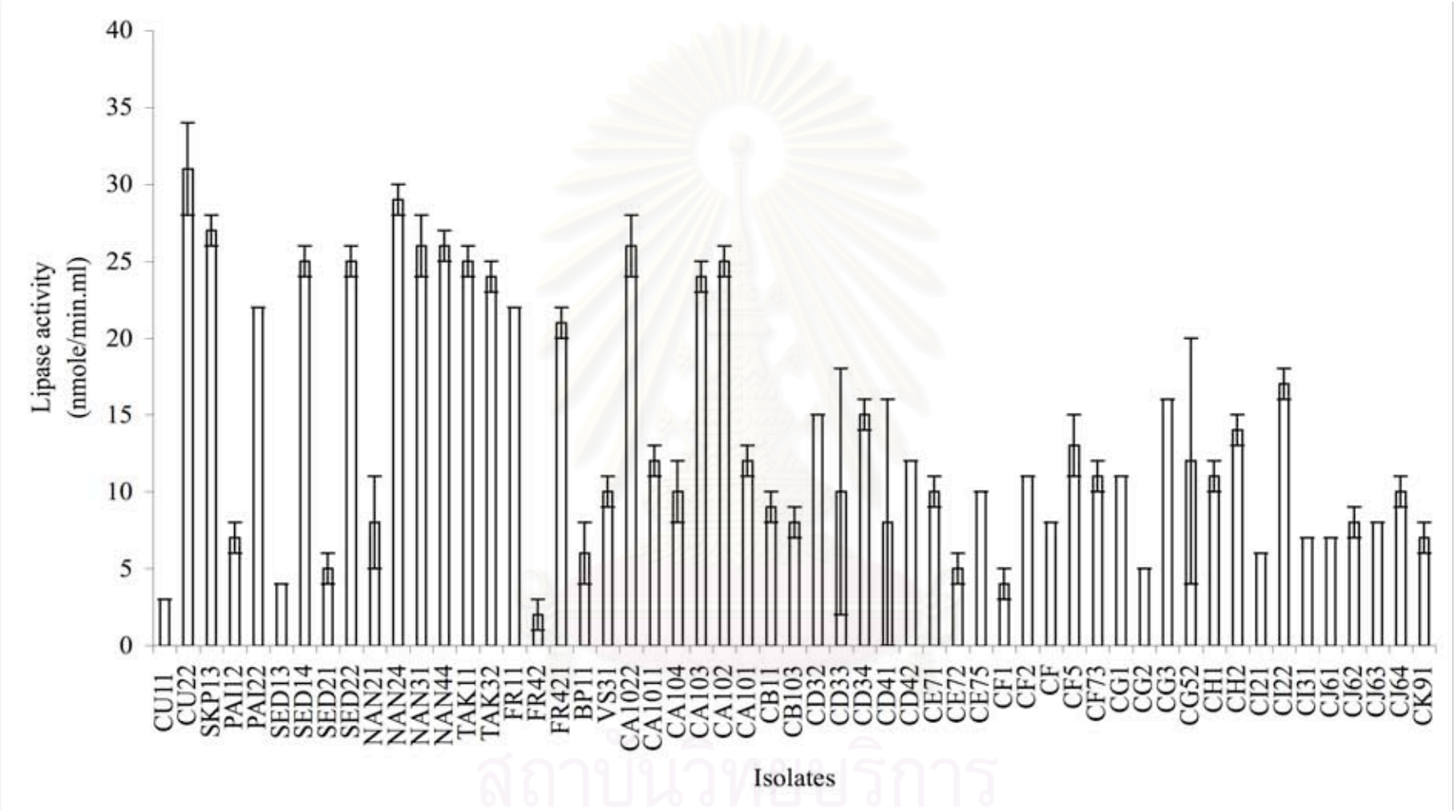


Figure 4.3 Lipase production of positive lipase-producing bacterial isolated from 12 soils and 10 composts in Thailand.

Capital letters represent location of soil and compost samples shown in Table 4.1. Number represent numbers designated for each bacterial isolated.

Since it was previously noted that lipase hydrolytic activity is not definitely related to transesterification, from the 55 isolates, randomly 23 isolates, with 5 mg of protein, having both high and low lipase specific activity were chosen for their transesterification abilities to produce methyl ester. Crude enzyme was prepared described in section 3.2 for transesterification analysis. Methyl ester formation was analyzed using TLC with hexane/ethyl acetate/acetic acid (90: 10:2) as mobile phase. Methanol: sulfuric acid (1:1, by volume) was used as a developing agent followed by heating the plate at 110°C for 30 minutes in order to detect compounds (Figure 4.4). According to the results on Figure 4.3, and retention factor (R_f) on Table 4.2, among the tested lipases, lipase from bacterial isolate CU22 showed a spot of methyl ester from the transesterification of palm oil with methanol, whereas other lipases showed very little or no activity of the transesterification reaction.

4.2 Identification of producing lipase bacterial isolated CU22

The CU22 was screened from cafeteria at faculty of Sciences in Chulalongkorn University where waste of oil-contaminated soil. This area is suitable lipase producing bacterial source because extracellular lipase is stimulated to produce inducible lipase.

The results of biochemical test from the laboratory of Institution for Scientific Research, Department of Medical Science, Ministry of public Thailand indicate that this test organism was *Stenotrophomonas maltophilia.* (Table 4.3) and SS-PCR, used as the confirmatory method, gave positive results for the CU22 as well as the control strain as shown in Figure 4.5. Therefore, it was designated as *S. maltophilia* CU22 that was finally selected for lipase characterization and mutagenesis.

The selected bacterial isolate CU22 having the ability to catalyze transesterification reaction was identified by biochemical tests (by the laboratory of Institution for Scientific Research, Department of Medical Science, Ministry of public Thailand) (Table 4.3). Then, the isolate was determined genetically.

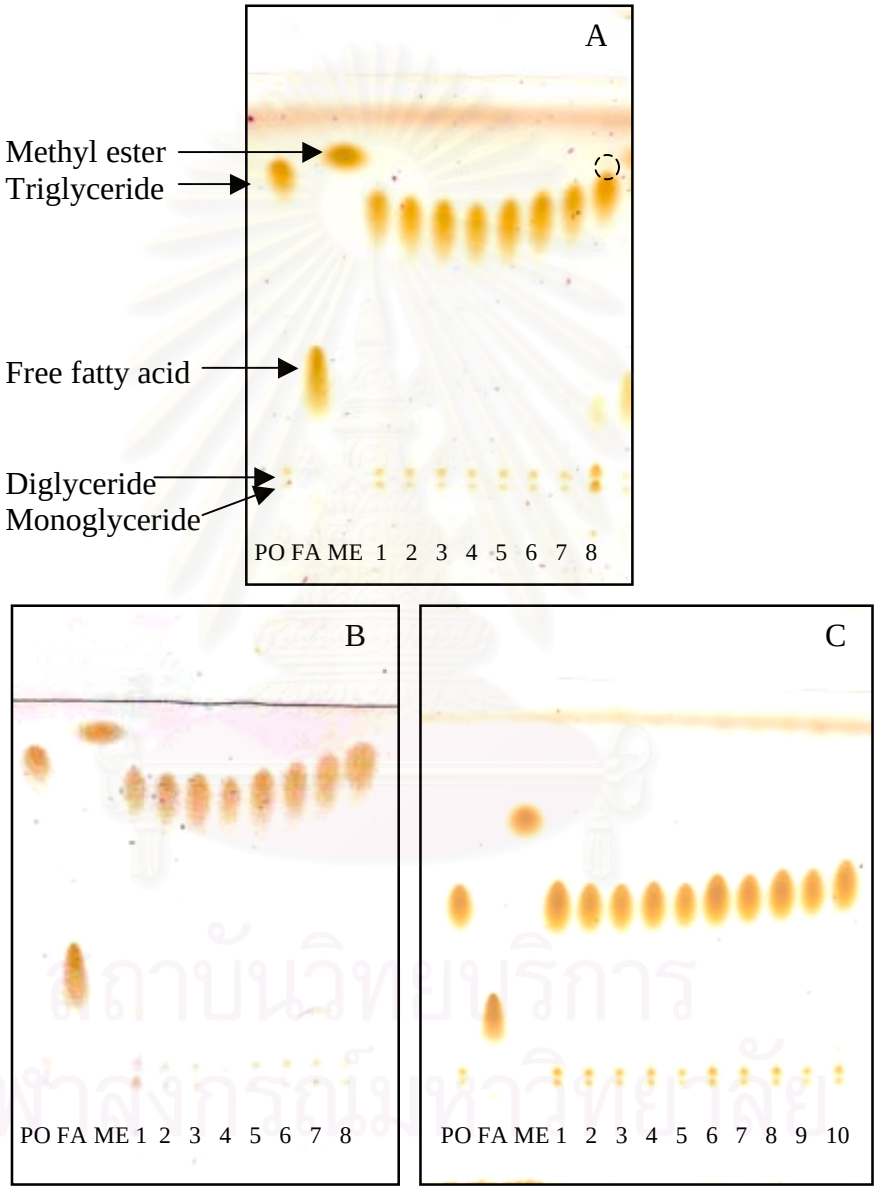


Figure 4.4 Thin-layer chromatography analysis of the reaction mixture during the transesterification catalyzed by using crude lipase.

(A) Lane 1-8: SKP13, PAI22, SED13, SED21, NAN21, NAN24, TAK32 and CU22, respectively

(B) Lane 1-8: FR42, BP11, VS31, CA103, CA102, CB103, CD32 and CD33, respectively

(C) Lane 1-10: CE72, CE75, CF5, CF73, CG3, CG52, CH1, CI21, CJ62 and CK91, respectively

Table 4.2 Retention factor (R_f) of compounds from transesterification detected by thin-layer chromatography*.

	Standard/ Samples	Distance of solvent (cm.)	Distance of sample (cm.)	Retention factor (R_f)
(A)	Palm oil	15.5	13	0.84
	Free fatty acid	15.5	6.5	0.42
	Methyl ester	15.5	13.4	0.86
	SKP13	15.5	12.7	0.82
	PAI22	15.5	12.5	0.81
	SED13	15.5	12.5	0.81
	SED21	15.5	12.5	0.81
	NAN21	15.5	12.4	0.80
	NAN24	15.5	12.5	0.81
	TAK32	15.5	12.7	0.82
	CU22	15.5	12.4	0.80
		15.5	13.2	0.85
(B)	Palm oil	15.0	12.7	0.85
	Free fatty acid	15.0	10.1	0.67
	Methyl ester	15.0	14	0.93
	FR42	15.0	12.3	0.82
	BP11	15.0	12.4	0.83
	VS31	15.0	12	0.80
	CA103	15.0	12.1	0.81
	CA102	15.0	12.3	0.82
	CB103	15.0	12.5	0.83
	CD32	15.0	12.4	0.83
	CD32	15.0	12.3	0.82
	CD33	15.0	12.2	0.81
(C)	Palm oil	14.5	8.4	0.58
	Free fatty acid	14.5	4.8	0.33
	Methyl ester	14.5	10.8	0.74
	CE72	14.5	8.4	0.58
	CE75	14.5	8.2	0.57
	CF5	14.5	8.4	0.58
	CF73	14.5	8.3	0.57
	CG3	14.5	8.5	0.59
	CG52	14.5	8.4	0.58
	CH1	14.5	8.6	0.59
	CI21	14.5	8.5	0.59
	CJ32	14.5	8.5	0.59
	CK91	14.5	8.5	0.59

* In order to TLC plates which were showed in Figure 4.3.

Table 4.3 Biochemical tests and selective media for identification of characteristics of CU22*

Biochemical Tests	Bacterial isolate: CU22
Catalase/Oxidase test	/-
TSI/H_2S production	K/K / -
SIM (H_2S/indole/motile)	-/-/+
Simmon's citrate	+
Urease test	-
Nitrate reduction /N_2 gas production	+/-
Esculin hydrolysis	+
Acetate utilization	+
Voges-Proskauer reaction	
Molonate	
Gelatinase	+
DNase	+
Fermentation test	
Glucose/gas	+/
Maltose	+
Lactose	+
Mannitol	-
D-xylose	-
Rhamnose	
Sucrose OF(Staph)	+
Mannose OF(NF)	
Adonitol SB	-
L-Arabinose ASS	
Inositol CTA	

OF(NF)

Biochemical Tests	Bacterial isolate: CU22
Sorbital	
Raffinose	
Fructose	+
Trehalose	
Salicin	
Starch	
Lysine Decarboxylase	+
Arginine Dihydrolase	-
Ornithine Decarboxylase	-
Bacterial indentification	*Stenotrophomonas maltophilia*

TSI: Triple sugar iron agar reacton, OF: Oxidation-fermentation basal medium, NF: Non-fermentation gram-neagative bacilli, SB: Sugar base, ASS: Ammonium salts sugar base, CTA: Cystine tryptic, + = positive result - = negative results, K/K: alkaline butt and alkaline slant, blank: not tested,

*Data from the laboratory of Institution for Scientific Research, Department of Medical Sciences, Ministry of Public Health in Thailand.

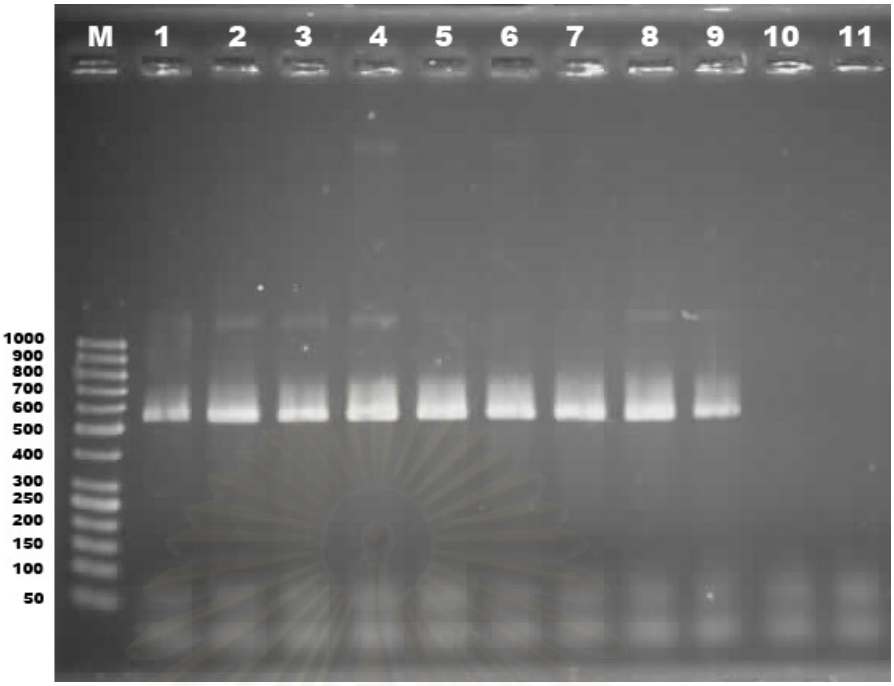


Figure 4.5. PCR product of 23S rRNA gene of *S. maltophilia* CU22. Showed a 540 bp; lane M = 50 bp DNA ladder, lane 1-4 = *S. maltophilia* CU22, lane 5 = *S. maltophilia* ATCC 13637t, lane 6 = *S. maltophilia* LMG00958t, lane 7 = *S. maltophilia* PT8/49 (WHO41), lane 8 = *S. maltophilia* DMST 4332, lane 9 = *S. maltophili*a DMST 3535, lane 10 = *E. coli* ATCC 25922, lane 11 = negative control (no template).

4.3 Effect of oil on lipase production and transesterification of *S. maltophilia* CU22

4.3.1 Effect of oil in olive oil media

Although olive oil supported cell growth and lipase production in *S. maltophilia* CU22, attempt to improve lipase production and transesterification reaction was carried out by growing cell in various type of oil, corn oil, rubber seed oil, palm oil, physic nut oil, rice bran oil, sesame oil and sunflower oil (1%, v/v) was substituted olive oil in the growth medium (section 3.2). Cell free crude enzyme was

prepared as described in section 3.3.1 and determined for its hydrolytic activity as well as transesterification reaction using palm oil as a reaction substrate (3.4.2). The lipase activity was determined by monitoring the *p*-nitrophenol released from *p*-nitrophenyl palmitated (*p*NPP) as the substrate according to a method described by Bornscheuer *et al.* (1994) with slight modifications for microplate reader (3.3.1). The specific activity of *S. maltophilia* CU22 grown in each media were calculated and showed in Figure 4.6.

For transesterification, the concentrated lipase solution (5 mg of protein was used) and distilled water to final volume of 1.335 ml of solution were pre-incubated in palm oil at 40 ◦C for 30 min and agitated by mixing with a magnetic stir at 900 rpm in a water bath. Methanol was added to the reaction 3 times during the reaction (three-step) to final concentration of 3 mole in the reaction. The reactions were kept at 40 °C, mixing at 900 rpm for 48 h. The sample were withdrawn at 24 and 48 hours and centrifuged at 13,000 rpm for 30 min. The methyl ester in the upper phase was collected and determined by TLC (Figure 4.6).

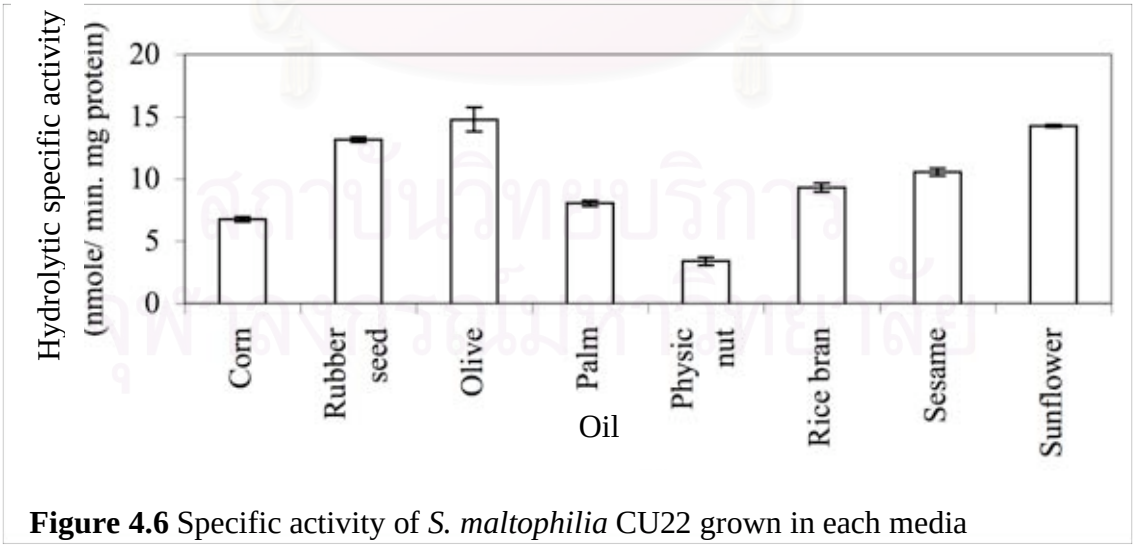


Figure 4.6 Specific activity of *S. maltophilia* CU22 grown in each media

After 24 hours of transesterification reaction time, methyl ester could not be detected using TLC. At 48 hours, the methyl ester was detected in the reaction containing crude lipase prepared from rubber seed oil or olive oil.

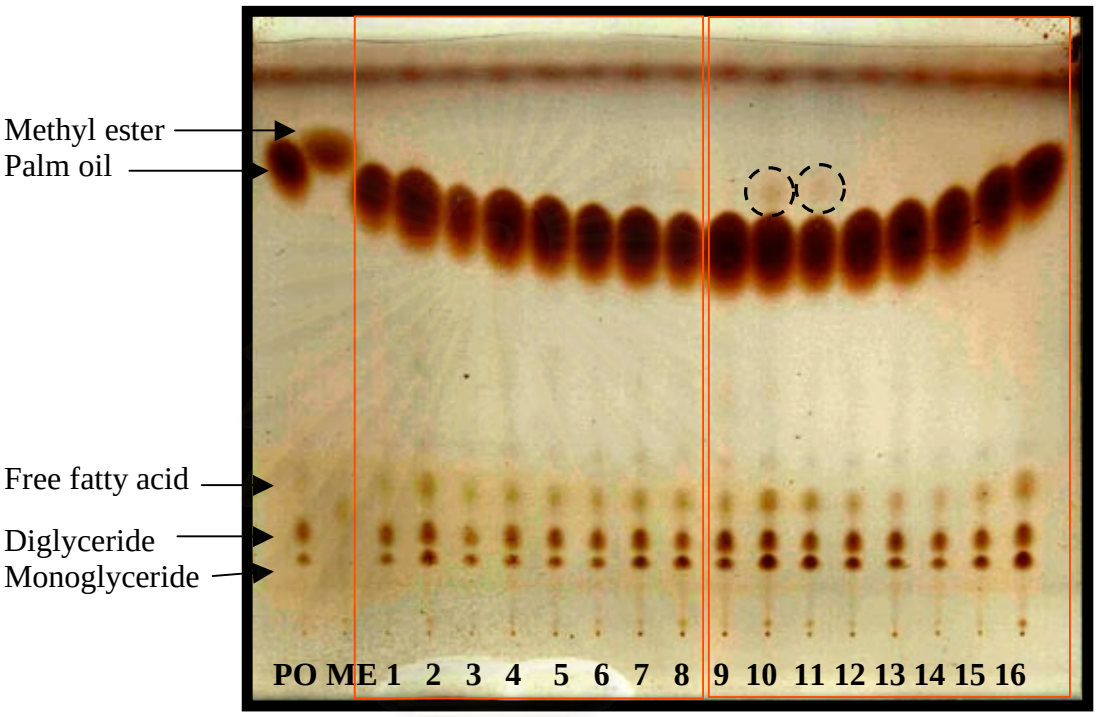


Figure 4.7 Thin-layer chromatography analysis of the transesterification reaction mixture catalyzed by lipase from cells grow on various type of oil. Dashed line represents methyl ester.

PO, Palm oil; ME, Methyl ester;

Lane 1-8 were transesterification samples, at 24 hours, catalyzed by lipase from cells grown on: corn oil, rubber seed oil, olive oil, palm oil, physic nut oil, rice bran oil, sesame oil and sunflower oil, respectively**;**

Lane 9-16 were transesterification samples, at 48 hours, catalyzed by lipase from cells grown on: corn oil, rubber seed oil, olive oil, palm oil, physic nut oil, rice bran oil, sesame oil and sunflower oil, respectively.

4.3.2 Effect of media in lipase activity and production

From the result of effect of oil in olive oil media in 4.3.1, *S. maltophilia* CU22 was induced with rubber seed oil and olive oil in oil media, which could detect methyl ester from transesterification reaction. However, olive oil in Thailand have expensive price and rubber seed oil is cultured in some areas in Thailand, so palm oil was investigated in TGY medium, compared with olive oil.

For hydrolytic specific activity, *S. maltophilia* CU22 was cultured in TGY medium containing palm oil was the hightest specific activity when assayed by *p*-nitrophenol palmitate as substrate (Figure 4.8). Also, for transesterification with palm oil could detect methyl ester from crude lipase of *S. maltophilia* CU22 (Figure 4.9).

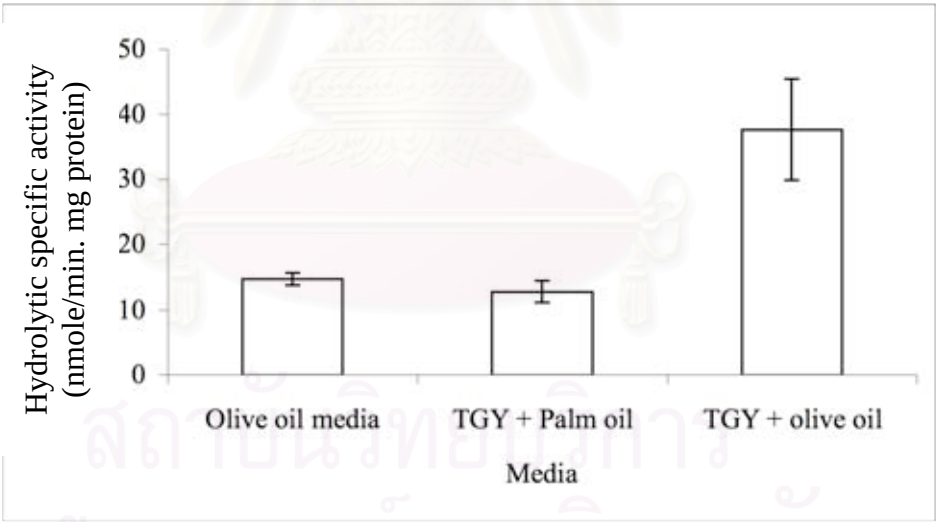


Figure 4.8 Specific activity of *S. maltophilia* CU22 in olive oil media and TGY media.

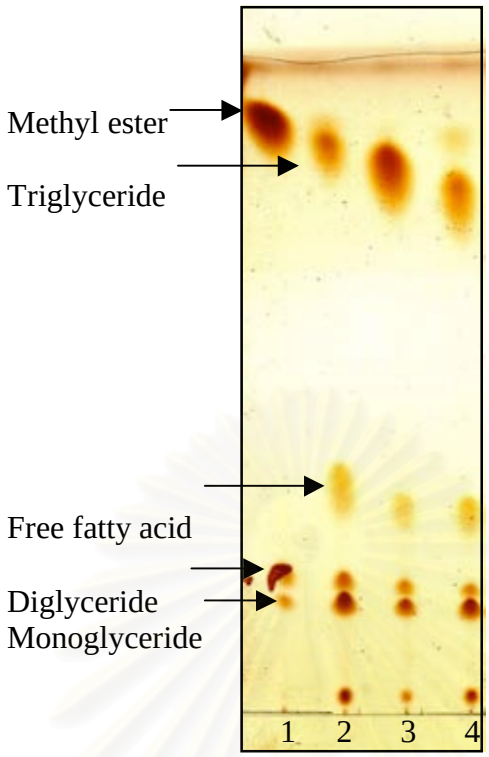


Figure 4.9 Thin-layer chromatography analysis of the transesterification reaction mixture using crude lipase in olive oil media and TGY media.

Lane 1: Methyl ester,

Lane 2-4: transesterification samples, at 24 hours, catalyzed by lipase from cells grown on olive oil media, TGY and olive oil, and TGY and palm oil, respectively.

4.4 MUTAGENESIS

4.4.1 Growth of *S. maltophilia* CU22

S. maltophilia CU22 was grown in 50 ml of nutrient broth and its growth was monitored via a spectrophotometer as culture turbidity (OD_{540}). Bacteria growth is shown in Figure 4.10. The mid-log phase of the bacteria when cultured at 200 rpm, 30 °C for 6 h was subjected to be a starter culture for mutagenesis (Ghribi, Zouari and Jaoua, 2004) and lipase production.

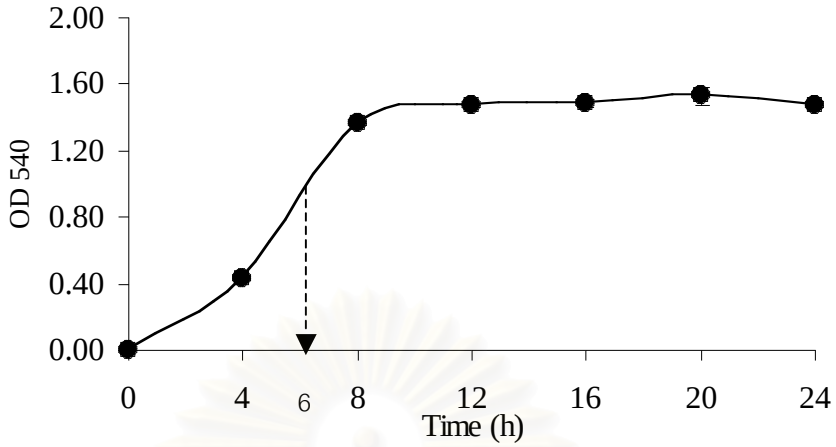


Figure 4.10 Growth curve of *S. maltophilia* in NB.

4.4.2 UV mutagenesis

4.4.2.1 Survival curve from UV radiation

S. maltophilia CU22 grown in 50 ml of nutrient broth at 200 rpm, 30 °C for 6 hour the mid-log phase was diluted to OD_{540} 0.1 cell suspension (~ 10^8 cells/ml). Four-milliliter cell suspension was placed under an ultraviolet lamp with distance of 32 centimeters and radiated for different time intervals between 4s and 99s. The viable colonies at the different exposure time were counted and survival graph was plotted. The percentage of death rate was shown in Table 4.4 and Figure 4.11. The survival colonies were shown in Figure 4.12. The survival colonies at 10s of irradiation time having a death rate of 96.67 % were selected.

Table 4.4 Survival colony and percent death rate after UV irradiation.

Exposure time (s)	Viable colony (× 10^3CFU/ml)	Death rate (%)
0	36000 ± 6557	0
4	18033 ± 15077	49.91
6	3100 ± 3378	91.39
8	2767 ± 2804	92.31
10	1200 ± 721	96.67
30	80 ± 17	99.78
60	5 ± 5	99.97
90	0	100
99	0	100

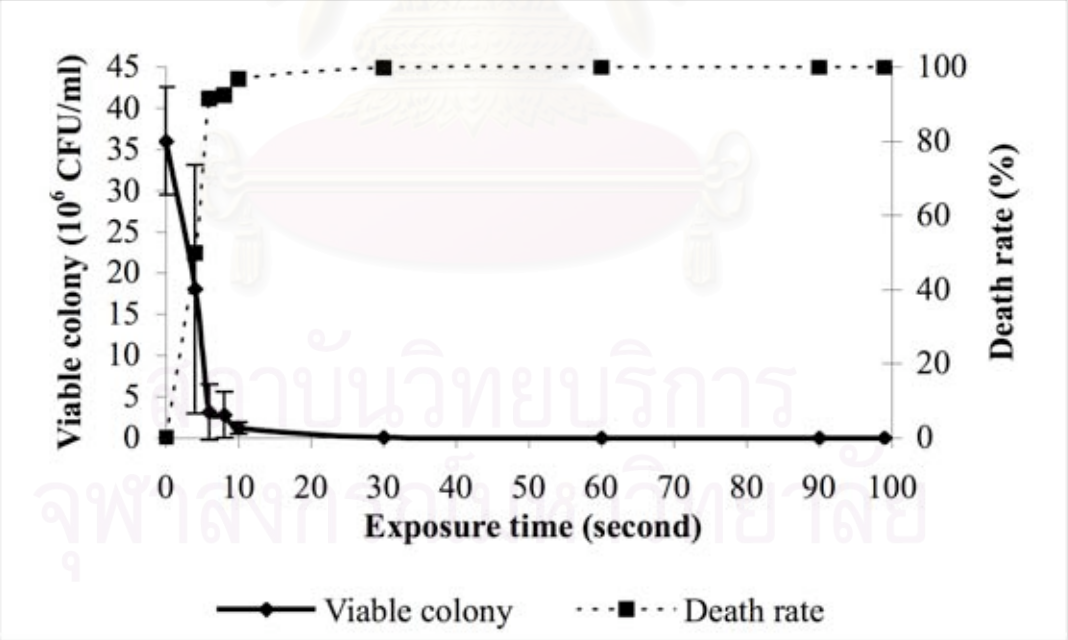


Figure 4.11 Survival curve of *S. maltophilia* CU22 after UV irradiated for 0-99s.

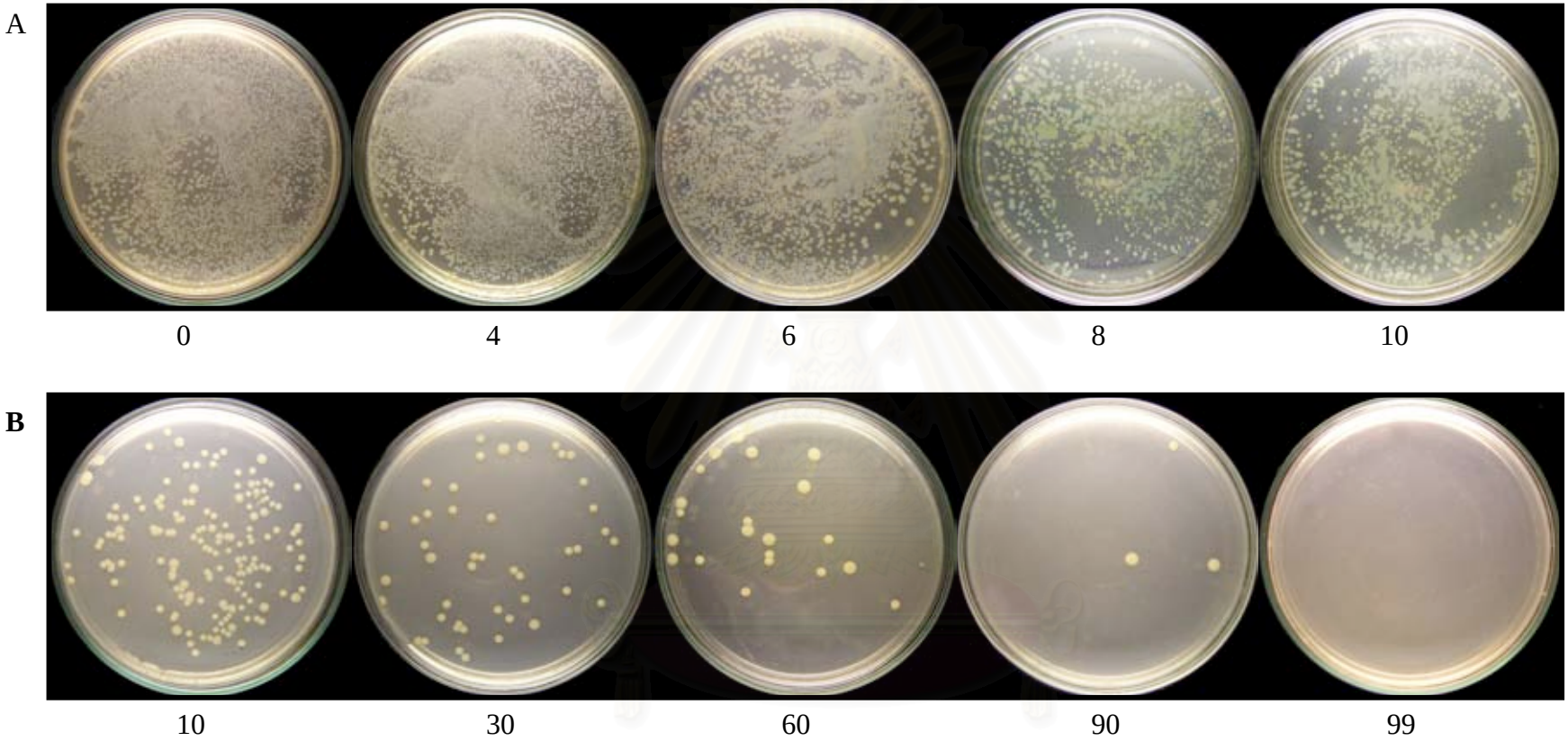


Figure 4.12 Survival colonies after UV radiation for at different exposure time between 4s and 99s

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4.4.2.2 Selection of mutants

S. maltophilia CU22 having the highest lipase specific activity among other isolates was chosen for further strain improvement using UV irradiation. The plates having less than 5% survival rate (10 sec) were selected for the isolation of mutants. A total of 47 mutants were selected and cultivated in 5 ml of TGY media. The lipase activity was determined by *p*-nitrophenyl palmitated (*p*NPP) as the substrate according to a method described in section 3.4.1 and 3.6. The specific activity of mutants were calculated and showed in Figure 4.12. Eleven UV out of 47 mutants (UV103, UV104, UV107, UV1016, UV23, UV28, UV29, UV35, UV47, UV 48 and UV 49) showed higher lipase activity and lipase production than the parent strain (Table 4.5).

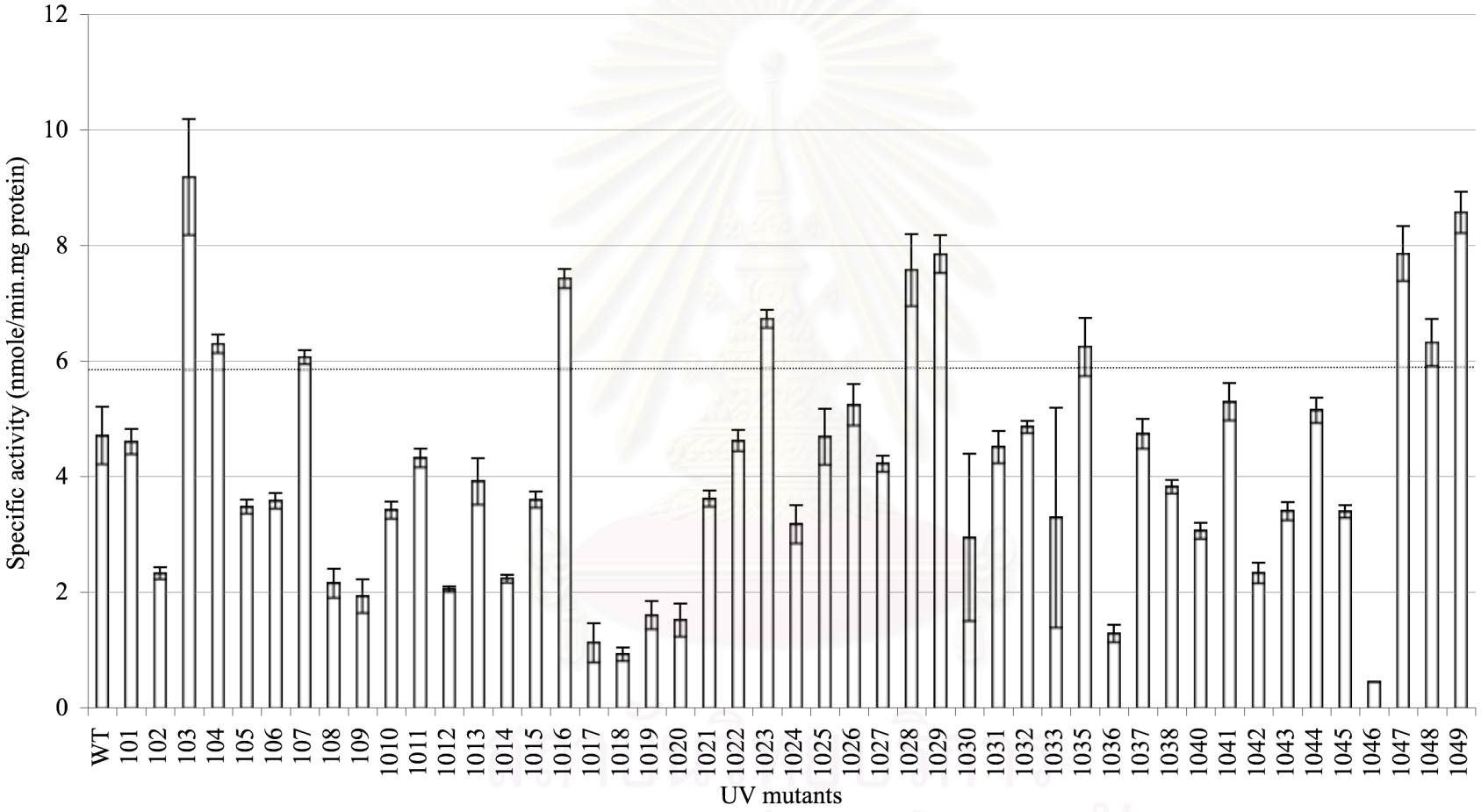


Figure 4.13 Specific activities of mutants from UV radiation for 10 sec.

Table 4.5 Lipase activity of mutants

Mutant	Activity (Unit/ml)	Protein (mg/ml)	Specific activity (Unit/mg)	% Relative	
				Activity (Unit/ml)	Specific activity (Unit/mg)
WT	7.15 ± 0.75	1.52 ± 0.13	4.71	100	100
UV103	13.64 ± 1.49	1.49 ± 0.29	9.18	191	195
UV104	8.85 ± 0.23	1.41 ± 0.17	6.29	124	134
UV107	9.35 ± 0.19	1.54 ± 0.07	6.06	131	129
UV1016	11.96 ± 0.26	1.61 ± 0.10	7.42	167	158
UV1023	11.39 ± 0.26	1.69 ± 0.03	6.73	159	143
UV1028	11.21 ± 0.93	1.48 ± 0.08	7.57	157	161
UV1029	11.99 ± 0.50	1.53 ± 0.18	7.85	168	167
UV1035	9.29 ± 0.74	1.49 ± 0.03	6.24	130	133
UV1047	11.37 ± 0.69	1.45 ± 0.05	7.85	159	167
UV1048	10.23 ± 0.67	1.62 ± 0.03	6.31	143	134
UV1049	13.48 ± 0.56	1.57 ± 0.15	8.57	189	182

4.4.2.3 Stability of mutants

The stability of the mutants was tested by determining lipase activity of each mutant generation (F) compare to that of the wild type strain (having specific activity 6-8 nmole/ min. mg protein).

One generation (F) is defined as one cycle of cell growth when the UV mutant was transferred from a nutrient agar to a new nutrient agar plate which was inoculated at 30°C for 24 h. From plate, a mutant at generation was grown in 5 ml of liquid medium and the lipase activity was assayed. Since the growth rates of the selected mutants were comparatively similar to that of the wild type, the lipase specific activity was determined as the stability of the mutant. With the exception of

UV104, all other mutants showed increased specific activity when compare with the wild type (Figure 4.14).

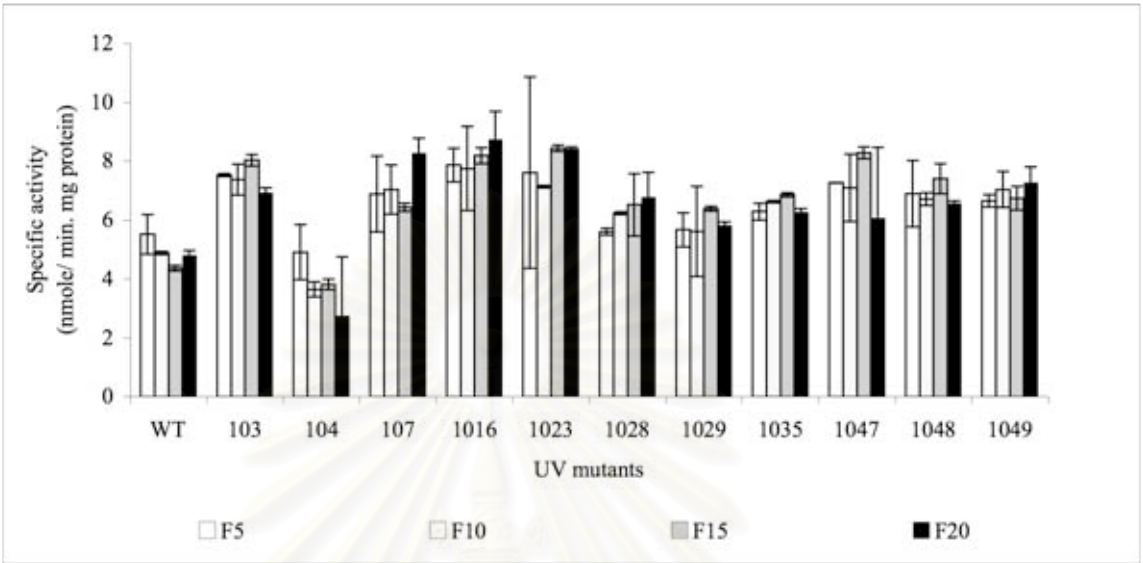
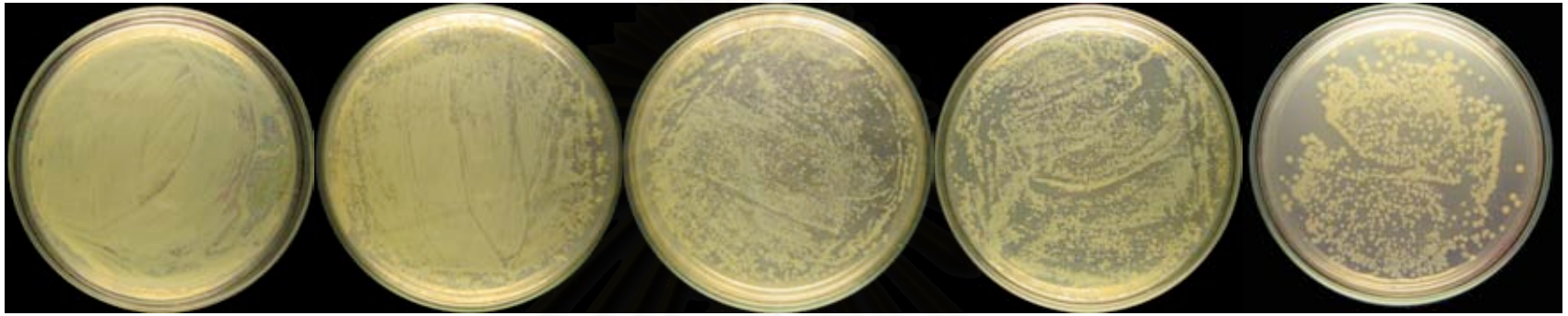


Figure 4.14 Stability of lipase production of the UV mutants. F represents number of generation.

4.4.3 NTG MUTAGENESIS

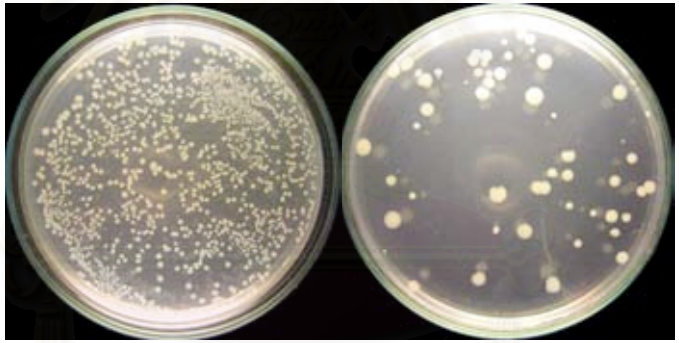
S. maltophilia CU22 was also subject to strain improvement by NTG treatment using various concentration of NTG (10, 20, 40, 60, 80, 100 and 200 μg/ml). Survival rate was determined at different dose of NTG. Results were expressed as percent of death rate (%death rate). However, even at high concentration of NTG at 200 μg/ml, *S. maltophilia* CU22 showed only 2.85% death rate (Figure 4.15). On the other hand, *E. coli* was as a control for potency of NTG showed a decrease of survival colony with 84.69% death rate at 100 μg/ml of NTG. Since, *S. maltophilia* CU22 showed high resistance to NTG. NTG was no longer used for the mutagenesis of *S. maltophilia* CU22.

(A)



0 μg/ml 10 μg/ml 80 μg/ml 100 μg/ml 200 μg/ml

(B)



0 μg/ml 100 μg/ml

Figure 4.15 Survival colony of (A) *S. maltophilia* after treated with NTG and (B) *E.coli* after treated by NTG

4.5 Transesterification of palm oil by *S. maltophilia* CU22 mutant

Ten mutants with high lipase specific activity were tested for transesterification of palm oil. Methyl ester production is shown in Figure 4.16. Although TLC result was not clearly observed, HPLC analysis result (Appendix F) showed increasing of percentage of conversion by mutant UV107, UV 1016 and UV1048 with 9.0, 9.5, and 9.4 %, respectively, while the wild type enzyme could catalyze only 7.3% conversion.

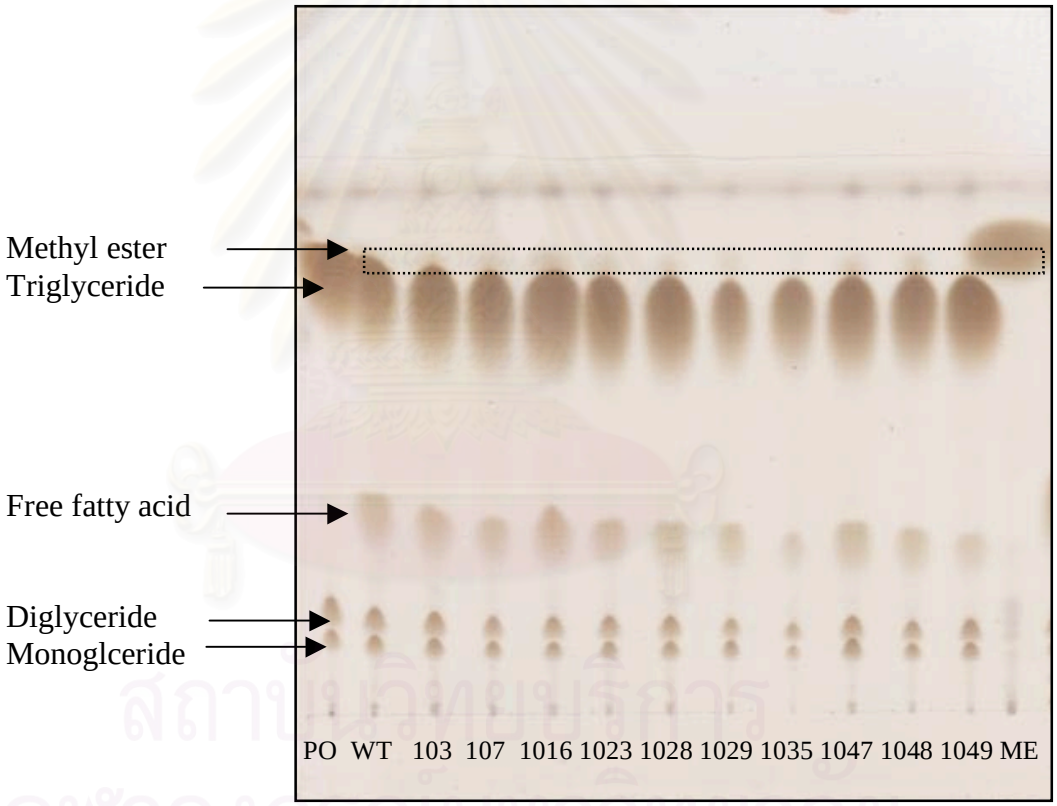


Figure 4.16 Thin-layer chromatography analysis of the transesterification reaction catalyzed by crude lipases of (10 mg) *S. maltophilia* wild type and mutants. PO, Palm oil; FA, Free fatty acid; ME, Methyl ester which indicated in box.

The mutants with higher %FAME conversion than wild type were repeated for transesterification three independent experiments. TLC and HPLC, shown in Figure 4.17 and 4.18, respectively were used to determine methyl ester formation.

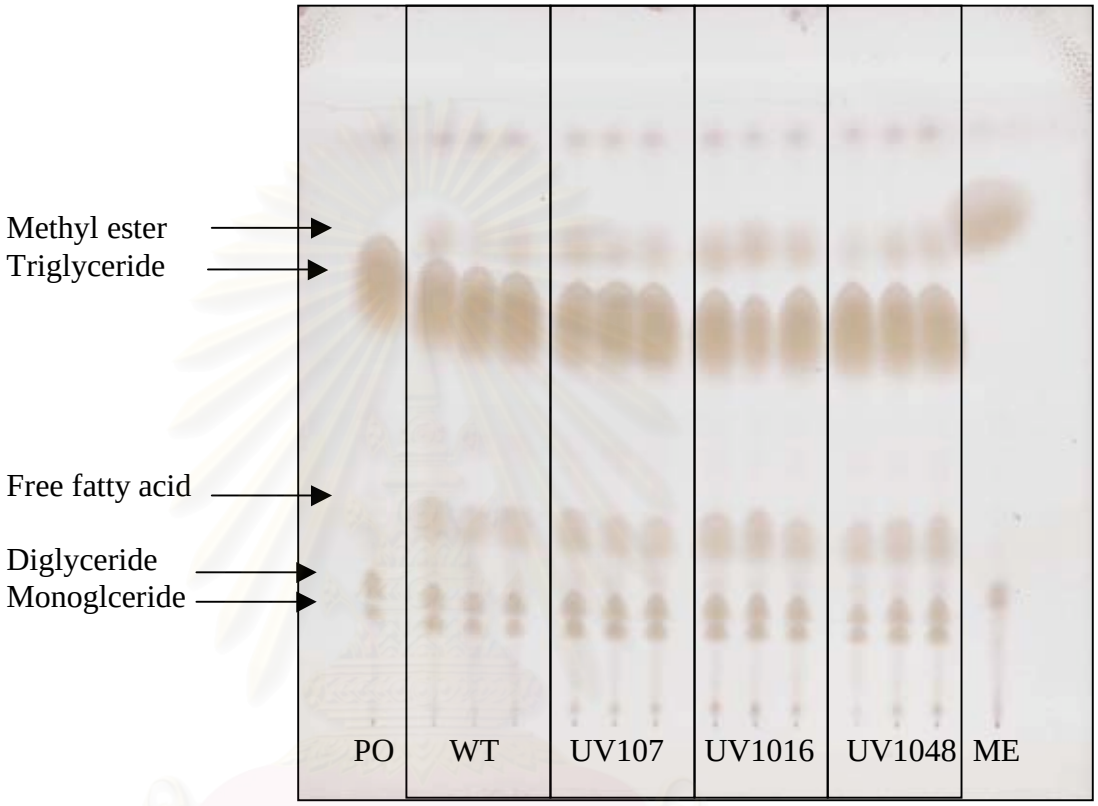


Figure 4.17 Thin-layer chromatography analysis of the concentrate lipase of 10 mg/ml *S. maltophilia* wild type and mutants. PO, Palm oil; ME, Methyl ester. The spot from three independent experimental were determined.

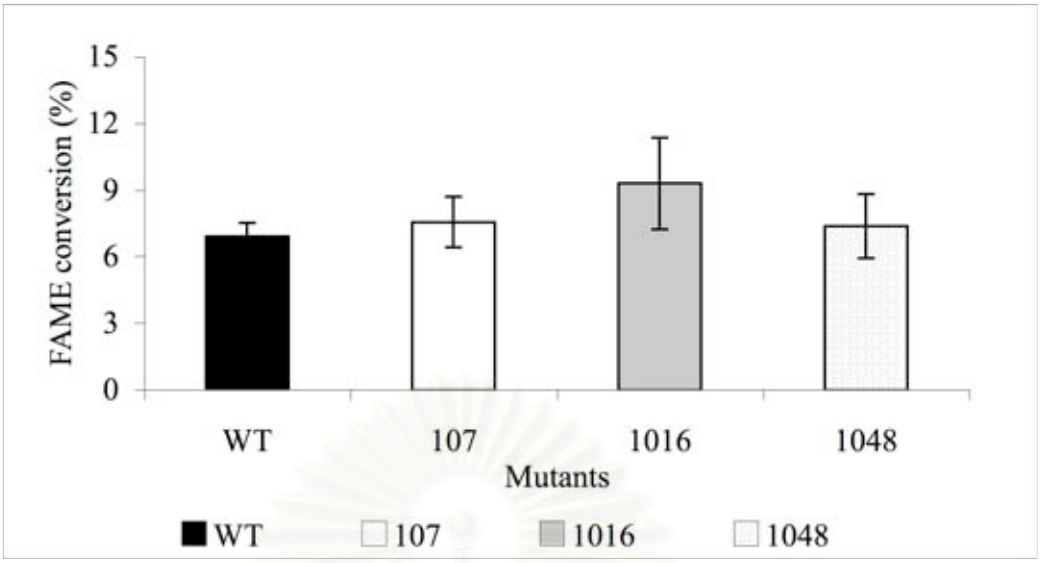


Figure 4.18 FAME formation (%) from transesterification of palm oil using 10 mg of crude lipase. Transesterification reaction was carried out at 40ºC for 48 hours. Methanol was three-time consecutively added into 3g-palm oil to the final molar ratio of 3:1 with 64.5% water (v/v). The data were means from three independent experimental with vertical bars representing standard error of the means (n=3)

4.6 Minimum inhibitory concentration (MIC) values

MIC defined as the lowest concentration of an antibiotic that will inhibit the visible growth of a microorganism to be used as genetic markers. The results of the response to antibiotics using broth culture showed in Figure 4.19. All of them, wild type and all mutants were resistant to amplicillin and penicillin. On the other hand, wild type and all mutants were sensitive to kanamycin and chloramphinicol. But UV107 was more sensitive to kanamycin than the other, and UV 1016 was more resistant to chloramphenicol than the other.

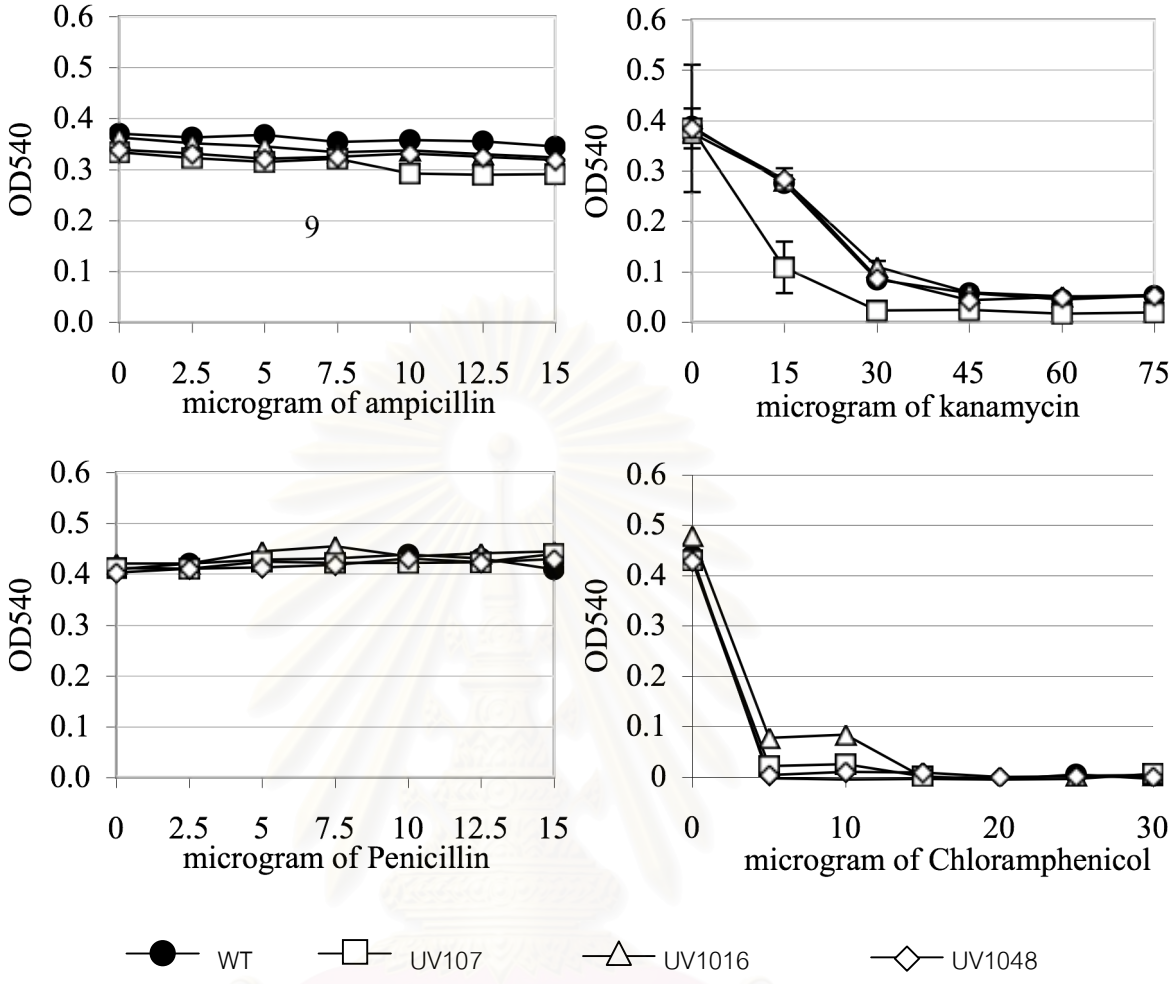


Figure 4.19 MIC of *S. maltophilia* CU22 wild type and the mutants.

4.7 Characterization of lipase from *S. maltophilia wild* type and the mutants

The properties of lipase produced by wild type and mutants (UV107, UV1016 and UV 107) of *S. maltophilia* stains were characterized. Extracellular lipase produced by *S. maltophilia* WT and the mutant UV107, UV1016 and UV1048 were prepared as followed. The culture broth (100 ml) was centrifuged and filtered through a Whatman No. 1 filter paper. The filtrate was then precipitated with 80% ammonium sulfate and dialyzed extensively against 0.05 M Phosphate buffer. The protein was determined in dialysate as described in section 3.8. The specific activity of the

concentrated enzyme was calculated and compared with that of the wild type crude enzyme.

The relative molecular mass of the concentrate lipase was estimated by SDS–PAGE (12.5%), according to the method of 3.91. Proteins were stained with Coomassie Brilliant Blue R-250. From the mobility on SDS-PAGE, the concentration of protein was increasing by ammonium salt precipitation. The result of SDS-PAGE analysis could not identify the band of lipase. However, the total protein of cell-free supernatant was concentrated by with ammonium sulfate precipitation.

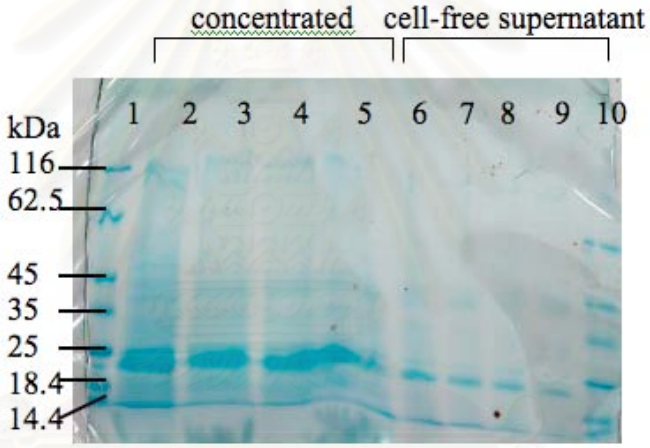


Figure 4.20 SDS–PAGE analysis of lipase from *S. maltophilia* WT, UV107, UV1016 and UV1048 on a 12.5 % acrylamide gel.

Lane 1 and 10: Protein molecular weight marker

Lane 2-5: 80% ammonium sulfate precipitate WT, UV107, UV1016 and UV1048 (50 μg protein loading)

Lane 6-9: cell-free supernatant WT, UV107, UV1016 and UV1048 (50 μg) protein loading)

4.7.1 Effect of pH on lipase activity

The pH optimum of lipase was determined as mentioned in section 3.10.2. Activities of the enzyme at different pHs were shown in Figure 4.21. In this study, the 0.05 M of acetate, phosphate, Tris H-Cl and glycine/NaoH were used as reaction buffers for pH 4.0-5.0, 6.0-7.0, 8.0 and 9.0-11.0, respectively. The specific activity of lipase was barely observed at pH below 7.0. Lipase activity was fairly similar between wild type and the mutants at pH ranging from 7-10. At pH11, wild type lipase showed significantly higher activity.

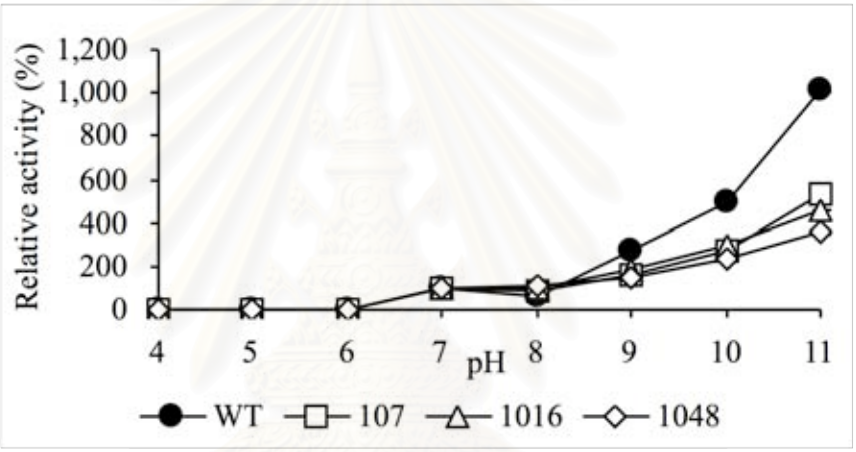


Figure 4.21 Effect of pH on lipase activity. 0.05 M of acetate, phosphate, Tris H-Cl and glycine/NaoH were used as reaction buffers for pH 4.0-5.0, 6.0-7.0, 8.0 and 9.0-11.0, respectively. Relative activity was expressed in comparison with the lipase specific activity at pH 7 that was taken as 100%.

4.7.2 Effect of temperature on lipase activity

The optimum temperature of the lipase was investigated by incubating the reaction mixture at various temperatures for 30 minutes (section 3.9.3). Wild type showed the highest activity at 80 °C, whereas UV 107 showed the highest activity at

60 °C. Both UV 1016 and UV 1048 showed the optimum temperature at 70 °C having higher specific activity than that of wild type. (Figure 4.22)

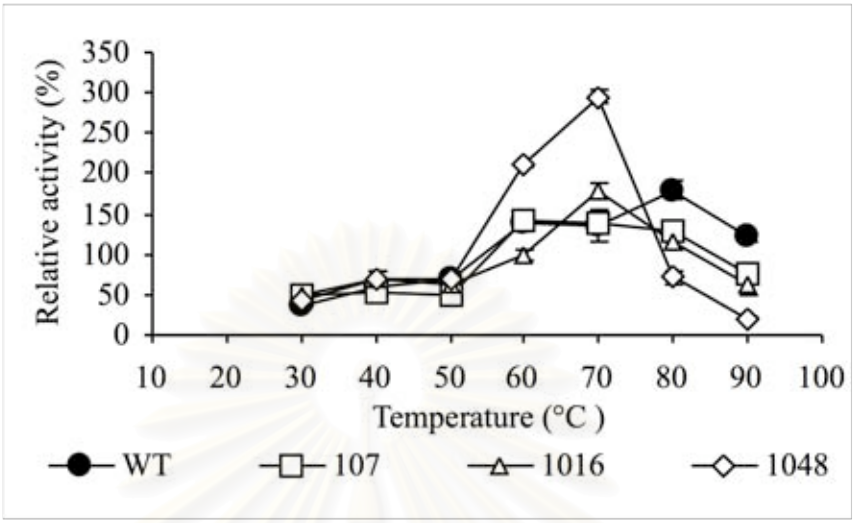


Figure 4.22 Effect of temperature on lipase activity. Relative activity was expressed in comparison with the lipase specific activity at 37 °C that was taken as 100%.

4.7.3 Effect of ion

The effect of ion on lipase activity was examined by adding different compounds to the standard reaction mixture. The result (Figure 4.23) showed that the lipase activities were decreased when 10mM of Mg^{2+}, K^{+} and Na^{+} was added. The wild type enzyme was strongly inhibited by these ions. On the other hand, lipase from UV107 was enhanced by 145% and 140%, respectively when 1 and 5mM Mg^{2+} and K^{+} was added. Similarly, UV1016 was enhanced by 139% and 170% in the presence of 1 and 5 mM Mg^{2+} and K^{+}, and by 144% in the presence of 1 mM Na^{+}. As for lipase of UV1048 enhancement of lipase activity was not observed.

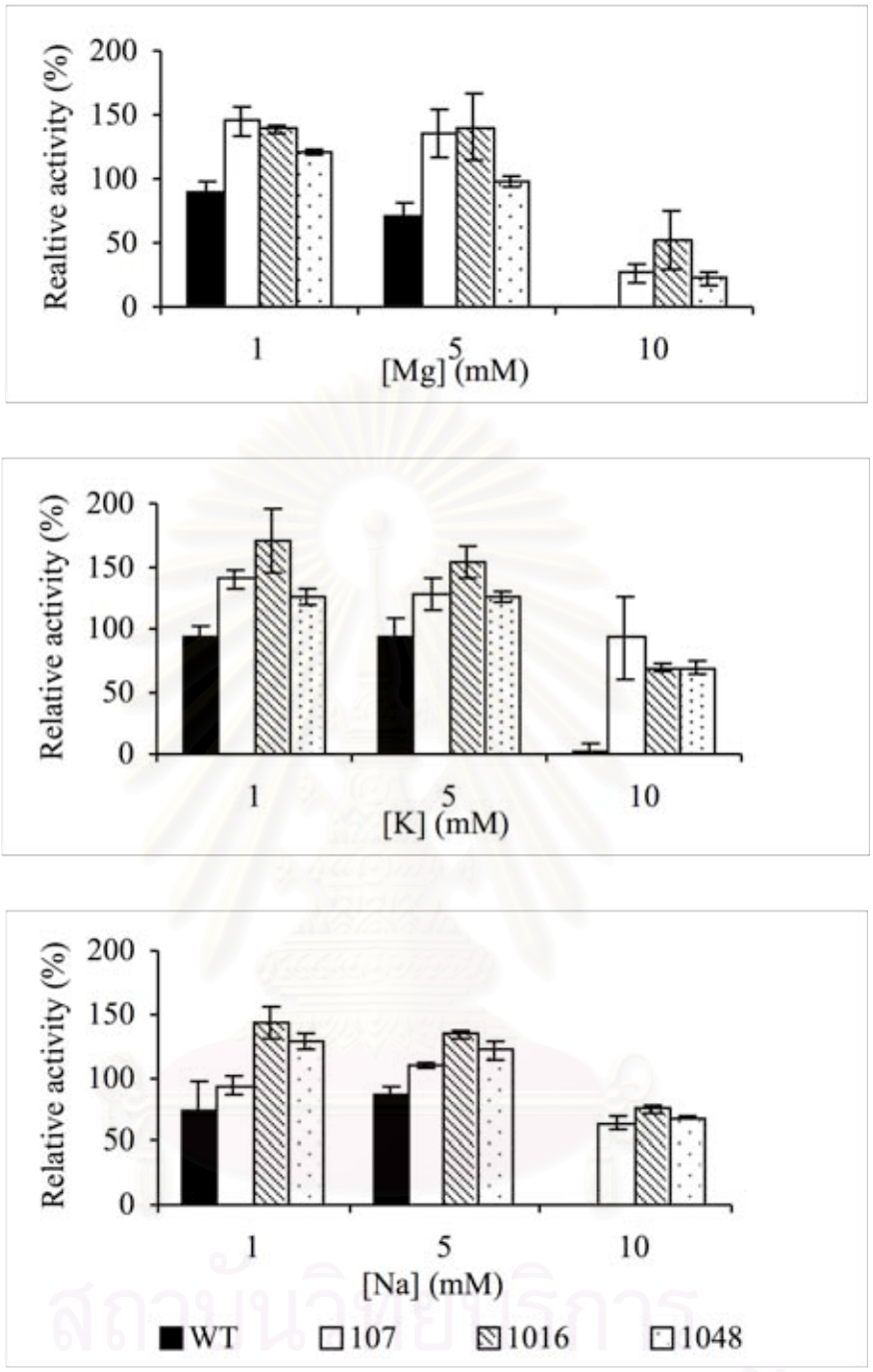


Figure 4.23 Effect of ion on lipase activity of wild type and mutant. Relative activity was expressed in comparison with the lipase specific activity of untreated ion that was taken as 100%.

4.7.4 pH stability of lipase activity

The pH stability of lipase was measured by incubation the enzyme at room temperature for 1, 6, 24 and 48 hours in 50 mM of potassium phosphate (pH 7.0) or Tris H-Cl (pH 8.0) or glycine/NaOH (pH 9.0-11.0), respectively (the total volume of 1 ml). Then, the residual enzyme activity was measured as described in section 3.10.4. All of them were stable in the pH range of 7 to 9 (Figure 4.24). The half-life of the lipase from wild type and mutant at pH 10, 30 °C is 6 hours.

4.7.5 Temperature stability of lipase activity

The temperature stability of lipase was investigated by preincubation at 30, 40, 50, 60, 70, 80 and 90 °C for 1, 6, 24 and 48 hours in 50 mM 0.05 M of phosphate followed by the measurement of residual lipase activity under the assay conditions as described in section 3.9.5. The temperature stability of enzyme was in the range 50 to 60 (Figure 4.25), while all enzymes completely lost their activity at higher temperature (>70 °C). The half-life of lipase from wild type and mutants are showed in Table 4.6.

Table 4.6 The half-life of lipase from wild type and mutants at 30-60 °C.

Temperature (°C)	WT	UV107	UV1016	UV1048
30	48h	24h	6h	24h
40	3h	3h	1h	1h
50	>48h	15h	6h	12h
60	15h	6h	6h	12h

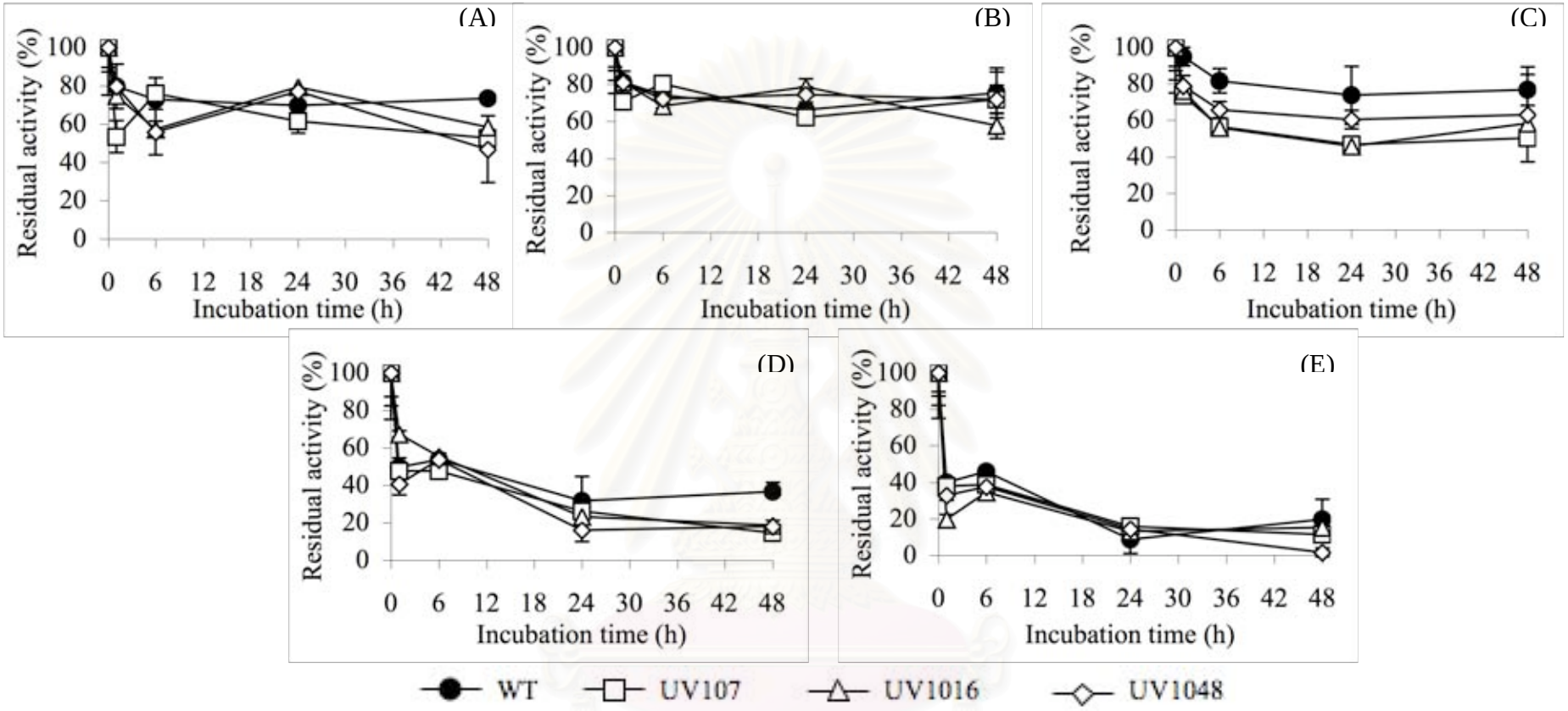


Figure 4.24 pH stability on lipase activity. The lipase activity was incubated in (A) 50 mM of potassium phosphate buffer, pH 7.0; (B) 50 mM of Tris-HCl buffer, pH 8.0; (C) 50 mM of glycine-NaOH buffer, pH 9.0, (D) 10.0 and (E) 11.0 at room temperature for 1, 6, 24 and 48 hours. The residual enzymes activity was measured. The enzyme activity incubated for 30 minutes in each buffer was used as 100% activity.

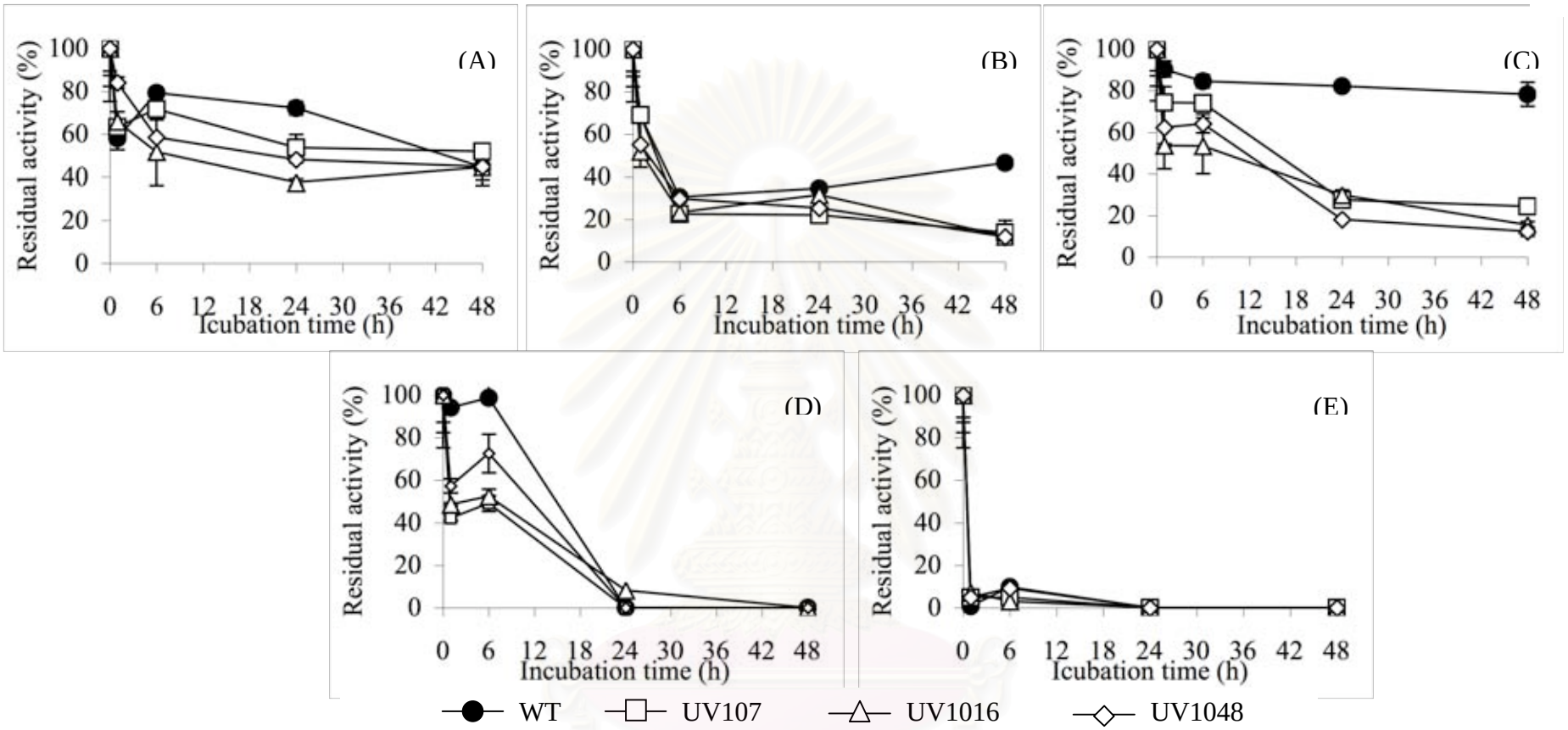


Figure 4.25 Temperature stability on lipase activity. The lipase activity was pre-incubated at (A) 30, (B) 40, (C) 50, (D) 60, and (E) 70 °C for 1, 6, 24 and 48 hours followed by the measurement of residual lipase activity under the standard assay conditions as described in section. The enzyme activity incubated for 30 minutes in each temperature was used as 100% activity.

4.7.6 Effect of EDTA

EDTA (1 mM) were added to reaction mixture to determine its effect to enzyme activity. The specific activity of each enzyme was calculated and showed in Figure 4.26. The results demonstrated EDTA inhibition on lipase activity of UV 1048, while EDTA was a less potent inhibitor on the lipase activity of wild type and UV 107. On the other hand, EDTA was shown to be an enzyme stimulator of UV 1016 by 123.9%.

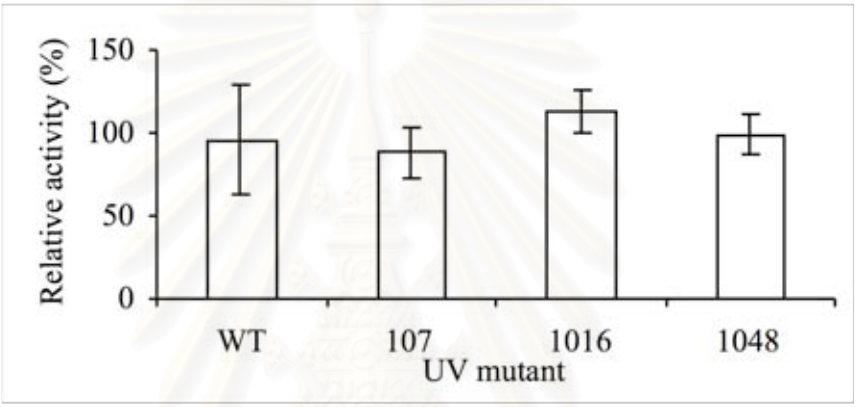


Figure 4.26 Effect of EDTA on lipase activity of wild type and mutants. Relative activity was expressed in comparison with the lipase specific activity of untreated EDTA that was taken as 100%.

4.8 Cloning of a partial lipase gene from *S. maltophilia* CU22.

4.8.1 PCR amplification of lipase gene

PCR amplification of lipase gene from S. maltophilia CU22 wild type, UV107, UV 1016 and UV1048 were performed as previously described in Material and Methods 3.11.2. The PCR product of 900 bp in size was observed as shown in Figure 4.27.

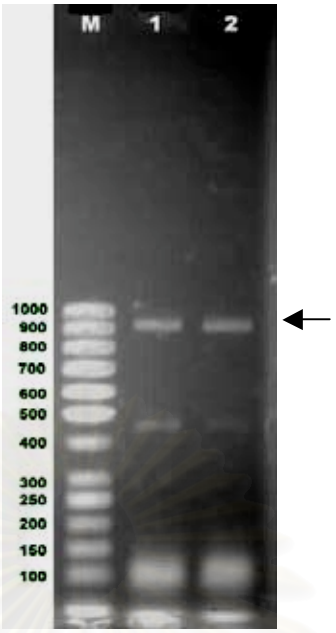


Figure 4.27 The PCR amplification of partial lipase gene from *S. maltophilia* CU22. PCR product of 900 bp (indicated by arrow, lane 1 and 2) was observed on 0.8% agarose gel eletrophoresis after ethidium bromide staining. Lame M: 100 bp marker.

4.8.2 Determination of lipase gene from *S. maltophilia* CU22

As shown in figure 4.26, the primer JBPF1 and JBPR1 could amplify a 900 bp size of lipase gene fragment. After cloning and sequencing, sequence analysis indicated that this 900 bp fragment has high similarity to lipase/esterase to from *Xanthomonas* spp. (64% similarlity), *Chromobacterium* sp. (31% similarlity), and also outer membrane autotransporter barrel of *S. maltophilia* strain 0450 by using blastx searching.

The completed nucleotide sequence was continued performed by inverse PCR as described in section 3.12.3. Figure 4.28 showed a completed nucleotide sequence and amino acid sequence of the open reading frame. The nucleotide sequence was 1,809 bp and protein sequence was 602 amino acids.

```
   1 atgcagctcagcaaacacccgatccgctccctgatggcggccgcg
     M  Q  L  S  K  H  P  I  R  S  L  M  A  A  A
  46 atcgcgctggccgcgcttccggccatggcaggcgaatccccgtat
     I  A  L  A  A  L  P  A  M  A  G  E  S  P  Y
  91 tccaaggccgtgttcttcggcgacagcctgaccgatgccggctat
     S  K  A  V  F  F  G  D  S  L  T  D  A  G  Y
 136 ttccgcccgctgctgccggccgatgtgcgtccggtgaccggccag
     F  R  P  L  L  P  A  D  V  R  P  V  T  G  Q
 181 ttcaccaccaaccccggatgggtgtggtcgcagcaggtcgccaac
     F  T  T  N  P  G  W  V  W  S  Q  Q  V  A  N
 226 tactatggcctcaatggcgccgccaatggcaacggccagagcggt
     Y  Y  G  L  N  G  A  A  N  G  N  G  Q  S  G
 271 gacaattacgcggtcggtggtgcccgcgttggcgtggacgtgccg
     D  N  Y  A  V  G  G  A  R  V  G  V  D  V  P
 316 agcgcaatggggactatcccctcgctgaagtcgcaggcggcccgt
     S  A  M  G  T  I  P  S  L  K  S  Q  A  A  R
 361 tatctggccgccaacggcggcaaggctgacggcaatgtcctgtac
     Y  L  A  A  N  G  G  K  A  D  G  N  V  L  Y
 406 acggtgtggggcggcgccaatgacctgttcgccgccgcagctgca
     T  V  W  G  G  A  N  D  L  F  A  A  A  A  A
 451 ccggcgcaggcacaggccatcatcggcgccgccgtcaccgaccag
     P  A  Q  A  Q  A  I  I  G  A  A  V  T  D  Q
 496 atcgctctggtgggtgcgctgaagcaggccggtgcgcagtacgtg
     I  A  L  V  G  A  L  K  Q  A  G  A  Q  Y  V
 541 ctggtgccgaacctgccgaacgtcggcctgactccggccttccgc
     L  V  P  N  L  P  N  V  G  L  T  P  A  F  R
 586 ggcccgaacgcggccaccgccaccgcgctgtcagccggctacaac
     G  P  N  A  A  T  A  T  A  L  S  A  G  Y  N
 631 aaggctctttatggtggcctgaagcaggccggcatcgagttcatc
     K  A  L  Y  G  G  L  K  Q  A  G  I  E  F  I
 676 ccgctcgataccttcaccgtgctgggcgaagtcgccgccaatccg
     P  L  D  T  F  T  V  L  G  E  V  A  A  N  P
 721 gccatgtacggcttcaccaacgtcaccagcaccgcctgcaagatc
     A  M  Y  G  F  T  N  V  T  S  T  A  C  K  I
 766 gatccggccaattcgactcagagcatcctgacctgcaatccgacc
     D  P  A  N  S  T  Q  S  I  L  T  C  N  P  T
 811 agctacgtcagcccggatgcggccaacacctatctgttcgccgat
     S  Y  V  S  P  D  A  A  N  T  Y  L  F  A  D
 856 ggcgtgcatccgaccacggcgggccaccagctgctgggccagtac
     G  V  H  P  T  T  A  G  H  Q  L  L  G  Q  Y
 901 gcggtctcggtgctggaagccccgcgcctgcagcaggtgctgagc
     A  V  S  V  L  E  A  P  R  L  Q  Q  V  L  S
 946 cactcggcacagaccatcggtcgttcgcgtgccgatcaggtcagc
     H  S  A  Q  T  I  G  R  S  R  A  D  Q  V  S
 991 atgcacctgggcggtcgtccggccgacggcctgtcctggtggggc
     M  H  L  G  G  R  P  A  D  G  L  S  W  W  G
1036 ggcgtccgcggtgacctgcagcgctacgaccacgcggatctgtac
     G  V  R  G  D  L  Q  R  Y  D  H  A  D  L  Y
1081 gacggcctggcgccggccggcctgttcggcatcgactgggcgcgc
     D  G  L  A  P  A  G  L  F  G  I  D  W  A  R
1126 gacggcatggtggtcggcggcttcgctggcttcggccgcctcaac
     D  G  M  V  V  G  G  F  A  G  F  G  R  L  N
1171 gccgacttcggcaacagccgtggcgatttcacccagaaggacacc
     A  D  F  G  N  S  R  G  D  F  T  Q  K  D  T
1216 accgccggcctgttcgccggctggtacggcgatcgcatctgggtg
     T  A  G  L  F  A  G  W  Y  G  D  R  I  W  V
1261 aacggccaggtcagctacacctggctgtcgtatgacgtgaaccgc
     N  G  Q  V  S  Y  T  W  L  S  Y  D  V  N  R
1306 aaggtccagctcggcccggccacccgcgagcacggtggttcgccg
     K  V  Q  L  G  P  A  T  R  E  H  G  G  S  P
```

```
1351 gacggcagcaacctgaccgccgccttgaacgccggttacgagttc
     D  G  S  N  L  T  A  A  L  N  A  G  Y  E  F
1396 ggcaccgaaggcggcttccgtcacggtccgatcgcttcggtgatc
     G  T  E  G  G  F  R  H  G  P  I  A  S  V  I
1441 tggcagaaggtgaagatcgatggttacaccgaaagcgctgcggcc
     W  Q  K  V  K  I  D  G  Y  T  E  S  A  A  A
1486 ggcaccctggccaccgcgctgggctacgaccgccagaacgttgat
     G  T  L  A  T  A  L  G  Y  D  R  Q  N  V  D
1531 tcgaccgttggtcgcatcggttggcaggcccgcttcgatggcggc
     S  T  V  G  R  I  G  W  Q  A  R  F  D  G  G
1576 accctcaagccgtacgcgcagctgacctacgaccatgagttcgaa
     T  L  K  P  Y  A  Q  L  T  Y  D  H  E  F  E
1621 gacaccaagcagggcagcgcgtggctgcagaccctgccggaactg
     D  T  K  Q  G  S  A  W  L  Q  T  L  P  E  L
1666 ggcagctaccgtgtgccgggcctgaagttcgacaagaactacgcg
     G  S  Y  R  V  P  G  L  K  F  D  K  N  Y  A
1711 accgtcgtgctgggcgcccgcaccgaactgttcgggctgcagagc
     T  V  V  L  G  A  R  T  E  L  F  G  L  Q  S
1756 aacttcggccagagaagccaccgtgttcgtgaacttcagcggcaa
     N  F  G  Q  R  S  H  R  V  R  E  L  Q  R  Q
1801 cttctgtga 1809
     L  L
```

Figure 4.28 A complete nucleotide sequence and amino acid of lipase gene from *S. maltophilia* CU22.

By using blastx and blastn searching for the protein sequence, the result of both blast searching indicated this protein was similar to outer membrane autotransporter/lipase of *S. maltophilia* strain 0450 and also lipase/esterase from *Xanthomonas* spp., *Pseudomonas* spp., *Chromobacterium* sp. The interproScan was indicated this protein containing the part that likely to lypolytic enzyme of G-D-S-L family, esterase (SGNH hydrolase type), autotransporter beta-domain and outer membrane autotransporter barrel domain (Figure 4.29).

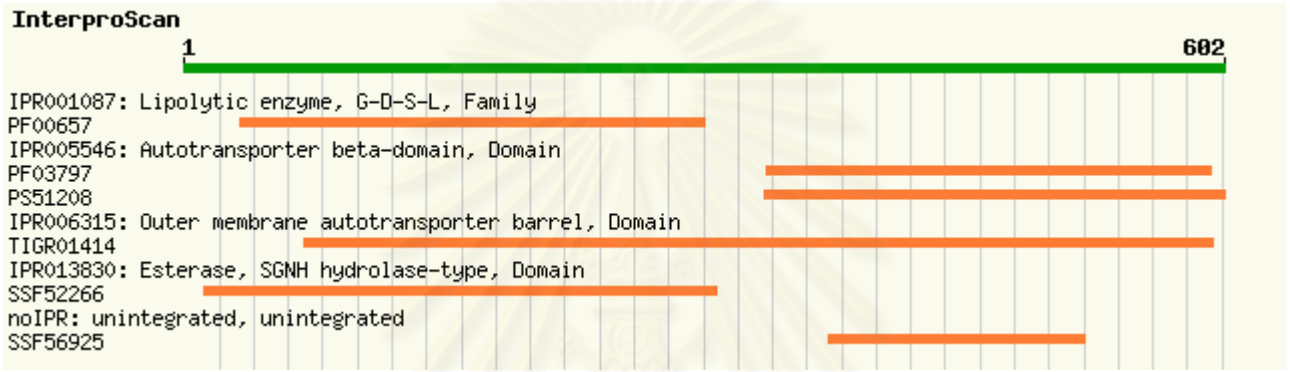


Figure 4.29 Similarity searching of amino acid sequence from lipase gene of *S. maltophilia* CU22

4.7.3 Determination of partial lipase gene from *S. maltophilia* UV1016

The partial lipase gene from *S. maltophilia* UV1016 was amplified by using primer Smal-lipF and lip-R4 and the PCR reaction was previous described as 3.12.4. The PCR product was purified and ligeated into pGEM-T vector, then transformed into *E.coli* JM109 using electroporation. The recombinant plasmid was extracted and sequenced by using with T7 primer and SMlip-iF, respectively. The partial lipase gene of *S. maltophilia* UV1016, 55% of whole lipase gene, was aligned using clustal W, compared wild type. DNA sequencing data have showed substitution at nucleotide 1702, 1765, 1766 and 1768 (Figure 4.30) and there are amino acid substitution from Arg to Asp at position 568, Gln to Trp at 589 and Arg to Gly at 590 of lipase gene (Figure 4.31).

```
WT              ACTCAGAGCATCCTGACCTGCAATCCGACCAGCTACGTCAGCCCGGATGCGGCCAACACC 840
UV1016          ----------------ACCTGCAATCCGACCAGCTACGTCAGCCCGGATGCGGCCAACACC 45
                                 ****************************************

WT              TATCTGTTCGCCGATGGCGTGCATCCGACCACGGCGGGCCACCAGCTGCTGGGCCAGTAC 900
UV1016          TATCTGTTCGCCGATGGCGTGCATCCGACCACGGCGGGCCACCAGCTGCTGGGCCAGTAC 105
                ************************************************************

WT              GCGGTCTCGGTGCTGGAAGCCCCGCGCCTGCAGCAGGTGCTGAGCCACTCGGCACAGACC 960
UV1016          GCGGTCTCGGTGCTGGAAGCCCCGCGCCTGCAGCAGGTGCTGAGCCACTCGGCACAGACC 165
                ************************************************************

WT              ATCGGTCGTTCGCGTGCCGATCAGGTCAGCATGCACCTGGGCGGTCGTCCGGCCGACGGC 1020
UV1016          ATCGGTCGTTCGCGTGCCGATCAGGTCAGCATGCACCTGGGCGGTCGTCCGGCCGACGGC 225
                ************************************************************

WT              CTGTCCTGGTGGGGCGGCGTCCGCGGTGACCTGCAGCGCTACGACCACGCGGATCTGTAC 1080
UV1016          CTGTCCTGGTGGGGCGGCGTCCGCGGTGACCTGCAGCGCTACGACCACGCGGATCTGTAC 285
                ************************************************************

WT              GACGGCCTGGCGCCGGCCGGCCTGTTCGGCATCGACTGGGCGCGCGACGGCATGGTGGTC 1140
UV1016          GACGGCCTGGCGCCGGCCGGCCTGTTCGGCATCGACTGGGCGCGCGACGGCATGGTGGTC 345
                ************************************************************

WT              GGCGGCTTCGCTGGCTTCGGCCGCCTCAACGCCGACTTCGGCAACAGCCGTGGCGATTTC 1200
UV1016          GGCGGCTTCGCTGGCTTCGGCCGCCTCAACGCCGACTTCGGCAACAGCCGTGGCGATTTC 405
                ************************************************************

WT              ACCCAGAAGGACACCACCGCCGGCCTGTTCGCCGGCTGGTACGGCGATCGCATCTGGGTG 1260
UV1016          ACCCAGAAGGACACCACCGCCGGCCTGTTCGCCGGCTGGTACGGCGATCGCATCTGGGTG 465
                ************************************************************

WT              AACGGCCAGGTCAGCTACACCTGGCTGTCGTATGACGTGAACCGCAAGGTCCAGCTCGGC 1320
UV1016          AACGGCCAGGTCAGCTACACCTGGCTGTCGTATGACGTGAACCGCAAGGTCCAGCTCGGC 525
                ************************************************************

WT              CCGGCCACCCGCGAGCACGGTGGTTCGCCGGACGGCAGCAACCTGACCGCCGCCTTGAAC 1380
UV1016          CCGGCCACCCGCGAGCACGGTGGTTCGCCGGACGGCAGCAACCTGACCGCCGCCTTGAAC 585
                ************************************************************

WT              GCCGGTTACGAGTTCGGCACCGAAGGCGGCTTCCGTCACGGTCCGATCGCTTCGGTGATC 1440
UV1016          GCCGGTTACGAGTTCGGCACCGAAGGCGGCTTCCGTCACGGTCCGATCGCTTCGGTGATC 645
                ************************************************************

WT              TGGCAGAAGGTGAAGATCGATGGTTACACCGAAAGCGCTGCGGCCGGCACCCTGGCCACC 1500
UV1016          TGGCAGAAGGTGAAGATCGATGGTTACACCGAAAGCGCTGCGGCCGGCACCCTGGCCACC 705
                ************************************************************

WT              GCGCTGGGCTACGACCGCCAGAACGTTGATTCGACCGTTGGTCGCATCGGTTGGCAGGCC 1560
UV1016          GCGCTGGGCTACGACCGCCAGAACGTTGATTCGACCGTTGGTCGCATCGGTTGGCAGGCC 765
                ************************************************************

WT              CGCTTCGATGGCGGCACCCTCAAGCCGTACGCGCAGCTGACCTACGACCATGAGTTCGAA 1620
UV1016          CGCTTCGATGGCGGCACCCTCAAGCCGTACGCGCAGCTGACCTACGACCATGAGTTCGAA 825
                ************************************************************

WT              GACACCAAGCAGGGCAGCGCGTGGCTGCAGACCCTGCCGGAACTGGGCAGCTACCGTGTG 1680
UV1016          GACACCAAGCAGGGCAGCGCGTGGCTGCAGACCCTGCCGGAACTGGGCAGCTACCGTGTG 885
                ************************************************************

WT              CCGGGCCTGAAGTTCGACAAGAACTACGCGACCGTCGTGCTGGGCGCCCGCACCGAACTG 1740
UV1016          CCGGGCCTGAAGTTCGACAAGGACTACGCGACCGTCGTGCTGGGCGCCCGCACCGAACTG 945
                ******************** ***************************************

WT              TTCGGGCTGCAGAGCAACTTCGGCCAGAGAAGCCACCGTGTTCGTGAACTTCAGCGGCAA 1800
UV1016          TTCGGGCTGCAGAGCAACTTCGGCTGGGGAAGCCACCGTGTTCGTGAA------------ 993
                ************************  * ********************
```

Figure 4.30 Nucleotide sequence comparison of the partial lipase gene of *S. maltophilia* CU22 and UV1016. Identical nucleotide are represented by an asterisk (*)

```
WT              AMYGFTNVTSTACKIDPANSTQSILTCNPTSYVSPDAANTYLFADGVHPTTAGHQLLGQY 300
UV1016          -------------------------TCNPTSYVSPDAANTYLFADGVHPTTAGHQLLGQY 35
                                         **********************************

WT              AVSVLEAPRLQQVLSHSAQTIGRSRADQVSMHLGGRPADGLSWWGGVRGDLQRYDHADLY 360
UV1016          AVSVLEAPRLQQVLSHSAQTIGRSRADQVSMHLGGRPADGLSWWGGVRGDLQRYDHADLY 95
                ************************************************************

WT              DGLAPAGLFGIDWARDGMVVGGFAGFGRLNADFGNSRGDFTQKDTTAGLFAGWYGDRIWV 420
UV1016          DGLAPAGLFGIDWARDGMVVGGFAGFGRLNADFGNSRGDFTQKDTTAGLFAGWYGDRIWV 155
                ************************************************************

WT              NGQVSYTWLSYDVNRKVQLGPATREHGGSPDGSNLTAALNAGYEFGTEGGFRHGPIASVI 480
UV1016          NGQVSYTWLSYDVNRKVQLGPATREHGGSPDGSNLTAALNAGYEFGTEGGFRHGPIASVI 215
                ************************************************************

WT              WQKVKIDGYTESAAAGTLATALGYDRQNVDSTVGRIGWQARFDGGTLKPYAQLTYDHEFE 540
UV1016          WQKVKIDGYTESAAAGTLATALGYDRQNVDSTVGRIGWQARFDGGTLKPYAQLTYDHEFE 275
                ************************************************************

WT              DTKQGSAWLQTLPELGSYRVPGLKFDKNYATVVLGARTELFGLQSNFGQRSHRVRELQRQ 600
UV1016          DTKQGSAWLQTLPELGSYRVPGLKFDKDYATVVLGARTELFGLQSNFGWGSHRVRE---- 331
                ***************************:******************  ******

WT              LL 602
UV1016          --
```

Figure 4.31 Amino acid comparison of of the partial lipase gene of *S. maltophilia* CU22 and UV1016. Identical nucleotide are represented by an asterisk (*) and similar ones by colon (:).

CHAPTER V

DISCUSSIONS

5.1 Screening and isolation of lipase producing bacteria

Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media (Hansan, Shah and Hameed, 2006). Morover, genetic manipulation to increase cells yield and/or enzyme activity (Demain, 1971), to increase the enzyme activity may be employed using microbial cells because of their short generation times, their relatively simple nutritional requirements.

In this study, in order to screen and isolate lipase-producing bacteria from a wide population, it conventionally screen on tributyrin agar plate. Tributyrin is a water-soluble. A clear zone of tributyrin hydrolysis is indicative of esterase or lipase activity. Since lipases are usually defined as enzymes that hydrolyse esters of long-chain aliphatic acids (triolein) from glycerol at oil/water interfaces (Macrae, 1985), thus olive oil containing 70% triolein is also generally used, as a substrate (Jensen, 1983). Further in this study, the secondary screening using an agar plate supplementing with olive oil and Rhodamine B have also been employed for screening lipase positive colony, by observing the orange fluorescence under UV light at 350 nm. Rhodamine forms a fluorescent complex with free fatty acids (Kouker and Jaeger, 1987). Thus, only the lipase-producing colonies gave a fluorescent halo that is visible under UV light.

These screening methods have been used successfully to screen both gram

positive (Sztajer and Maliszewska, 1988; Schmidt-Dannert, 1994) and gram negative bacteria (Labuschagne, Tonder and Litthauer, 1997) capable of lipase production.

According to the screening protocols used, ninety-five isolated bacteria were screened from twelve soils and ten composts. Based on the highest activity with *p*-nitrophenyl palmitate as substrate, gram-negative bacteria, namely CU22, was characterized by biochemical tests and identified to be *Stenotrophomonas maltophilia*.

5.2 Lipase production of *S. maltophilia* CU22

S. maltophilia is previously known as *Pseudomonas, Pseudomonas maltophilia* or *Xanthomonas maltophilia*, is an emerging pathogen that has been associated with a broad and increasing number of clinical syndromes (Robin and Janda, 1996). From the clinical point of view, *S. maltophilia* is also of noteworthy interest as an important nosocomial pathogen. From the ecological point of view, the habitat of *S. maltophilia* in the environment has been reported to be wide: water and soil samples, crops, vegetation, animals and other ecological niches inside and outside hospitals (Denton and Kerr, 1998). Even though it is such an ubiquitous microorganism, little information is currently available concerning the incidence of this pathogen in animal foods. Frank *et al.* (1985) reported the isolation of a strain of *P. maltophilia* from decomposed mahimahi (*Coryphaena hippurus*). More recently, other authors have described the presence of *S. maltophilia* in raw milk (Litopoulou-Tzanetaki and Vafopoulou-Mastro-jiannaki, 1995). Apart from this, the presence of *X. maltophilia* in non-carbonated mineral water has also been reported (Jayasekara *et al.*, 1998).

Hooker, Freedmen and Stacey (1997) describe the first reported study of lipase from the *S. maltophilia*, isolated from cow's manure contaminated with soil from a

private farm in Wafsal, Kuwait.

Ben-Gigirey *et al.* (2000) isolated two haundred and twenty-seven bacterial strains from fresh and fronzen albacore tuna, three strains of *S. maltophilia* were investigated for lipase production on nutrient agar tributyrin medium. Additionally, the production of extracellular lipase was investigated in tributyrin agar Victoria blue medium. All of three *S. maltophilia* strains showed to be the least extensive producer of extracellular lipase.

The major factor for the expression of lipase activity has always been carbon, since lipases are inducible enzymes (Lotti *et al.* 1998) and are thus generally produced in the presence of a lipid source such as oil. In this study, the result of the effect of oil on lipase activity to hydrolyze *p*-nitrophenyl palmitate and transesterification with palm oil was investigated. The highest hydrolytic specific activity of *S. maltophilia* CU22 was shown to be with olive oil, sunflower oil and rubber seed oil respectively (Figure 4.4)

Other studies have reported that soybean oil was as the best lipid carbon source for the enzyme production by a bacterial isolate (Gupta, Saroop and Jain, 2004). Furthermore, Kumar *et al.* (2005) studied the various carbon sources (olive oil, coconut oil, castor oil, ground nut oil, mustard oil, sunflower oil, Tween 20, Tween 80, cottonseed oil, and soybean oil for lipase production in a specific medium. Amongst the various carbon sources tested for lipase production of *B. coagulans* BTS-3, refined mustard oil (1%, v/v) was the best inducer followed by Tween 80, Tween 20, and olive oil.

Kumar *et al.* (2005) also tested different types of nitrogen sources to determine their effect on the production of lipase. The best lipase production was obtained with peptone (0.5% w/v) and yeast extract (0.5% w/v) in the production

broth. Peptone and yeast extract were the best nitrogen source for lipase production by *Candida rugosa* (Fadiloglu and Erkmen, 2001). While yeast extract alone increased lipase production in *Bacillus* sp. (Sidhu *et al.*, 1998) and *Cryptococcus* sp. (Kamini *et al.*, 2000).Therefore, lipase inducibility and production are dependent on type of carbon source and bacterial type.

5.3 Improvement of lipase activity of *S. maltophilia* CU22 by random mutagenesis

5.3.1 UV mutagenesis

UV irradiation is one the convenient mutagens, providing that the cells to be treated are appreciably transparent to it. Mutagenic wavelengths lie between about 200 and 300 nm, which is the peak of absorption by nucleic acids (Hopwood, 1970).

Two factors that can interfere with efficient UV mutagenesis are the presence of UV-absorbing nutrients (aromatic compounds such as tryptophan or nucleic acid bases) and the shielding of cells by high cell densities. Eisenstadt, Carlton and Brown (1994) suggested the protocol to avoid these factors by irradiating bacteria suspended in a UV-transparent buffer at low densities. An additional interfering factor is photoreactivation. Many bacteria are able to repair some kinds of UV-induced DNA damage (pyrimidine dimmers) in a visible-light-dependent reaction. To avoid photoreactivation, carry out UV irradiation and post-irradiation treatments are carried out in dim light (Hopwood, 1970). Higher UV doses will result in more killing than lower doses will. In this study, to prevent cell density effect, 10^8

cells more used to start UV irradiation. The results of UV radiation, the mutants at 10s of exposure time having a death rate of 96.67 % were selected.

5.3.2 NTG mutagenesis

NTG is in widespread use as a mutagen by bacterial geneticists, and appears to be the most potent chemical mutagen yet discovered.

Adelberg *et al.* (1965) showed the rate of mutagenesis of *E. coli* K12, was close to its maximum at an NTG concentration of 100 μg/ml; further increase in concentration decrease the survival without significantly increasing the mutation rate.

In this study, NTG was used up to concentration of 200 μg/ml. However, *S. maltophilia* CU22 could tolerate NTG, yielding only 2.85% death rate. This result is in agreement with previous reports with other NTG treatment with *S. maltophilia.*

S. maltophilia are difficult to treat with NTG due to the intrinsic antibiotic resistance of this bacterial species (Arpi *et al.*, 1996, Garrison *et al.*, 1996). A combination of reduced permeability (Yamazaki *et al.*, 1989) and expression of efflux pump(s) (Alonso and Martinez, 1997, Zhang, Li, and Poole, 2000) might account at least in part for *S. maltophilia* intrinsic resistance to drugs. Besides a reduced permeability to the drug, *S. maltophilia* can pump out the antibiotic through a multidrug efflux determinant (Alonso, Sanchez and Martinez, 2000)

5.3.3 Selection of mutants

The most important factor contributing to the successful isolation of a particular kind of mutants is an efficient screening procedure; that is a method for distinguishes the mutant strain from wild type. The ease with which different mutants can be recognized various enormously, but it is almost always possible to increase the

efficiency of screening, above the basal level of random testing of survivors, by considering the expected properties of the mutants.

Antibiotic-resistant mutants can be easily isolate from most bacteria (Kondo, Yamagishi and Oshima, 1991). The mutations are useful as genetic markers because they provide a readily selectable phenotype (Eisenstadt, Carlton and Brown, 1994). In the former, no single mutation confers a high level of resistance, but mutiple-muant strain can be built up in which the cumulative effects of several mutation results in a high level of resistance.

Since Minimum inhibitory concentration (MIC) is defined as the concentration of the drug that inhibited growth of the organism within a specified time period, it can be used to distinguish between wild type and mutant strain. However, mutant isolates with slow growth rates in the presence of the drug may not have grown sufficiently to be detected within the specified time period. Nevertheless, prolonged incubation will allow the resistant organism to grow, whereas, under the same conditions, the susceptible organism will fail to grow irrespective of the incubation time (Ruiz-Dez and Martı´nez Suarez, 2003). In this investigation, mutants strain UV107 and UV1016 exhibited slight different of MIC when kanamycin and chloramphenicol was used. Therefore the MIC could not be conclusive.

5.4 Characterization of lipase from *S. maltophilia* CU22 wild type and mutants

Catalytic activity of lipase depends on pH, temperature, and metal ion activator. Therefore, there factors affecting lipase from *S. maltophilia* CU22 were investigated. The pH optimum was barely observed at pH below 7.0. Lipase activity was fairly similar between wild type and the mutants at pH ranging from 7-10, while at pH11, wild type lipase showed significantly higher activity. Generally, bacterial

lipases are active at neutral pH with the exception of *P. fluorescens* SIK W1 lipase, which has an acidic optimum at pH 4.8 (Andersson *et al.* 1979). Lipases from Bacterial lipases possess stability over a wide range, from pH 4 to pH 11.

S. maltophilia CU22 wild type showed the highest hydrolytic activity at 80 °C, whereas the mutant UV107 showed the highest activity at 60 °C. Both UV 1016 and UV 1048 showed the optimum temperature at 70 °C with higher specific activity than that of wild type. Further determination at higher temperature than 70 °C was not carried out since *p*-nitrophenyl was degraded (autohydrolysis) at high temperature (Beisson *et al.*, 2000)

Bacterial lipases generally have temperature optima in the range 30–60°C. Thermal stability data are available only for species *of Bacillus* (Schmidt-Dannert *et al.*1996) and *Pseudomonas* (Dong *et al.*, 1999)

The effect of ion on lipase activity was examined by adding different ions to the standard reaction mixture. The result showed that the lipase activities from the wild type strongly inhibited when 10mM of Mg^{2+}, K^{+} and Na^{+} was added. On the other hand, lipase from the mutant UV107 was enhanced by 145% and 140% when 1 mM Mg^{2} and 5 mM K^{+} was added. Similarly, the mutant UV1016 was enhanced by 139% and 170% in the presence of 1 and 5 mM Mg^{2+} and K^{+}, and by 144% in the presence of 1 mM Na^{+}. As for lipase of the mutant UV1048, the enhancement of lipase activity was not observed. Cofactors are generally not required for lipase activity, but divalent cations such as calcium often stimulate enzyme activity. This has been suggested to be due to the formation of the calcium salts of long-chain fatty acids (Macrae and Hammond 1985; Godtfredsen 1990). Calcium-stimulated lipases have been reported in the case of *B. subtilis* 168 (Lesuisse *et al.* 1993), *B. thermoleovorans* ID- 1 (Lee *et al.* 1999), *P. aeruginosa* EF2 (Gilbert *et al.* 1991), *S.*

hyicus (Van Oort *et al.* 1989), *C. viscosum* (Sugiura *et al.* 1974) and *Acinetobacter* sp. RAG-1 (Snellman *et al.* 2002). In contrast, the lipase from *P. aeruginosa* 10145 (Finkelstein *et al.* 1970) is inhibited by the presence of calcium ions.

The results demonstrated EDTA inhibition on lipase activity of UV 1048, while EDTA was a less potent inhibitor on the lipase activity of wild type and UV 107. On the other hand, EDTA was shown to be an enzyme stimulator of UV 1016 by 123.9%. EDTA has been reported to inhibit activity of a few lipases; some of the lipases by EDTA was overcome by treatment with divalent metal ions like Ca^{2+} (Sharon *et al.* 1998), or less efficiently by Mg^{2+} and Ba^{2+} (Baral and Fox 1997) indicating that the enzymes might have a calcium or divalent cation-binding site. Bacterial lipases have been reported to have calcium-binding site that is important for maintaining their structure (Lang *et al.* 1996, Kim *et al.* 1997).

For transesterification, *S. maltophilia* CU22 wiltd type showed the conversion rate of palm oil to methyl ester, was 7.3%. The UV mutants with stable higher hydrolytic activity than wild type were tested for transesterification of palm oil. Only three of ten mutants showed increasing of %conversion by mutants UV107, UV1016 and UV 1048 with 23, 30 and 29% higher than of the wild type. The results revealed no correlation between the hydrolytic and synthetic activities of *S. maltophilia.* Also, in a previous report (Wu, Jääskeläinen, and Linko, 1996) showed nine commercially available powdered lipases were investigated for their catalytic ability to hydrolyze olive oil and synthesize I-butyl oleate. For all lipases the previous studied suggested little relationship between the hydrolytic and synthetic activities. The results, the hydrolytic lipase activity may be of little value in predicting the synthetic activity, and in extreme cases, a lipase may exhibit no synthetic activity while possessing a high hydrolytic activity.

5.5 Cloning of a partial lipase gene from *S. maltophilia* CU22

Cloning and sequencing of genes and mutant alleles contribute to a mechanistic understanding of their function and regulation. Sequence information also provides experiment tools for further analysis. For example, the nucleotide sequences of a gene can be altered via site-specific mutagenesis to test specific hypotheses about the structure and function of a gene product. Cloned genes and the sequences derived from them are also useful for developing molecular probes to characterize mutants, to study gene expression, and to identify related genes in other organisms.

Arpigny and Jaeger (1999) have classified 47 different bacterial lipases into six families on the basis of amino acid sequence homology.They have also compared the data available from three-dimensional structures of some enzymes and biochemical properties of different lipases reported by different researchers.

Lipase gene of *S. maltophilia* CU22 was classified in family II or GDSL family). The enzymes grouped in family II did not exhibit the conventional penta-peptide Gly- Xaa-Ser-Xaa-Gly but rather displayed a Gly-Asp-Ser-(Leu) [GDS(L)] motif containing the active-site serine residue. In these proteins, this important residue was found to lie much closer to the N-terminus than in other lipolytic enzymes. At this time, no report of lipase in this class was researched in transesterification reaction.

The partial lipase gene from *S. maltophilia* UV1016 was amplified and sequenced. DNA sequencing data have showed nucleotide substitution and there are amino acid from Arg to Asp at position 568, Gln to Trp at 589 and Arg to Gly at 590 of lipase gene. This result showed that UV1016 is a mutant with 35% conversion higher than wild type with amino acid substitutions, which may be effect to hydrolysis activity and transesterification of lipase.

CHAPTER VI

CONCLUSION

Ninety-five isolates of lipase-producing bacteria were isolated from 22 sources of soil samples in Thailand by using tributyrin-agar plate. Rhodamine B supplemented with olive oil was used for the secondary lipase screening. Fifty-five lipase-producing isolates were determined for their lipase activity by using *p*-nitrophenyl palmitate as a substrate. The results showed that the isolate CU22 yielded highest lipase hydrolytic specific activity and having activity of the transesterification reaction produce methyl ester, which showed a spot of methyl ester.

The bacterial isolate CU22 was biochemically identified as *Stenotrophomonas maltophilia*. To improve lipase production and transesterification reaction was carried out by growing cell in various type of oil, corn oil, rubber seed oil, palm oil, physic nut oil, rice bran oil, sesame oil and sunflower oil (1%, v/v) was substituted olive oil in the growth medium. The methyl ester was detected in the reaction containing crude lipase prepared from rubber seed oil or olive oil. However, the price of olive oil and rubber seed oil in Thailand were not cost production so palm oil was investigated in TGY medium. For hydrolytic specific activity, *S. maltophilia* CU22 was cultured in TGY medium containing palm oil was the highest specific activity and also, for transesterification with palm oil could detect methyl ester.

To improve either lipase hydrolytic activity or its synthetic activity, a classical random mutagenesis was employed. A total of 47 mutants, which obtained after ultraviolet radiation were selected and determined lipase activity. Among of them, ten stable UV mutant showed higher lipase activity and lipase production than the parent strain. *S. maltophilia* CU22 was also subject to strain improvement by NTG

treatment, even at high concentration of NTG at 200 μg/ml, *S. maltophilia* CU22 showed only 2.85% death rate. NTG was no longer used for the mutagenesis of *S. maltophilia* CU22.

Ten mutants with high lipase specific activity were tested for transesterification of palm oil. The mutants UV107, UV 1016 and UV1048 showed increase of % conversion with 23, 30, and 29 %, respectively, while the wild type enzyme could catalyze only 7.3% FAME conversion.

Three mutants were characterized compare wild type. The results of MIC showed UV107 was more sensitive to kanamycin than the other, and UV 1016 was more resistant to chloramphenicol than the other. The pH optimum of lipase was similar between wild type and the mutants at pH ranging from 7-10. At pH11, wild type lipase showed significantly higher activity. The optimum temperature of the lipase, wild type showed the highest activity at 80 °C, whereas UV 107 showed the highest activity at 60 °C. Both UV 1016 and UV 1048 showed the optimum temperature at 70 °C having higher specific activity than that of wild type. The effect of ion on lipase showed that wild type enzyme was strongly inhibited by Mg^{2+}, K^{+} and Na^{+} these ions. On the other hand, lipase from UV107 and UV1016 were enhanced when Mg^{2+} and K^{+} was added. The pH stability of lipase was showed all of them were stable in the pH range of 7 to 9. The temperature stability of enzyme was in the range 50 to 60 and the results of EDTA showed inhibition on lipase activity of UV 1048, while EDTA was a less potent inhibitor on the lipase activity of wild type and UV 107. On the other hand, EDTA was shown to be an enzyme stimulator of UV 1016 by 123.9%. The partial lipase gene from *S. maltophilia* UV1016 was compared with wild type and amino acid substitution from Arg to Asp at position 568, Gln to Trp at 589 and Arg to Gly at 590 of lipase gene.

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APPENDICES

APPENDIX A

Preparation for media

Enrichment medium (Gao *et al.*, 2000)

2.0% (w/v) olive oil

0.5% (w/v) $(NH_4)_2SO_4$

1.0% (w/v) peptone

0.5% (w/v) K_2HPO_4

0.1% (w/v) $MgSO_4 \bullet 7H_2O$

Tributyrin agar plate (Sztajer *et al.*, 1988)

0.5% (w/v) peptone

0.3% (w/v) yeast extract

1.0% (w/v) tributyrin

2.0% (w/v) agar

Rhodamine B agar plates (Schmidt-Dannert *et al.*, 1994)

Nutrient agar supplemented by 0.001% (w/v) Rhodamine B and 1% (w/v) olive oil in distilled water.

Olive oil media (Tanigaki *et al.*, 1995)

$(NH_4)_2SO_4$	0.5%
Peptone	0.5%
$MgSO_4 \bullet 7H_2O$	0.1%
KH2PO4	0.5%.
Yeast extract	0.1%
Olive oil	1%

TGY media (Lanser, Manthey and Hou, 2002)

Tryptone	0.5%
Yeast extract	0.1%
Dextrose	0.1%
K_2HPO_4	0.1%

The component was dissolved in 1 L of distilled water, adjusted pH to 7.0 and autoclaved at 121 °C for 15 min.

APPENDIX B

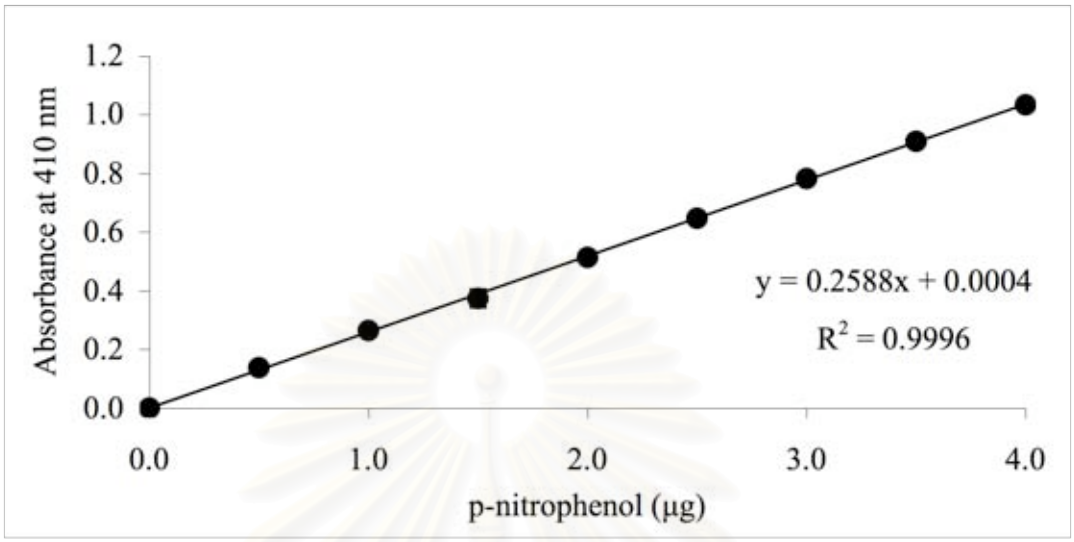
Preparation of stock solutions for hydrolysis assays

Procedure:

1. To prepare the stock solotion as described in following table.
2. Read the absorbance of each tube at 410 nm against the blank using the spectrophotometer.
3. Determinated the concentrantion of p-nitrophenol in the sample from a standard curve prepared by plotting the absorbances of the standards versus the concentration of p-nitrophenol.

Stock solution	[Final] (mM)	Volume added (μl)		
		Experiment	No substrate control	No enzyme control
A solution (2X) pNPP 180 mg isopropanol 30 ml	0.71	9	0	9
B solution (2X) Triton X-100 0.8% Gum arabic 0.2%		81	81	81
0.2 M Potassium phosphate buffer	50	50	50	81
Distilled water		40	49	60
Enzyme solution		20	20	0
Total volume		200	200	200

Standard curve of p-nitrophenol



Appendix C

Preparation and standard curve for protein determination

Reagent for determination of protein concentration by a modified Lowry method (Held and Hurley, 2001)

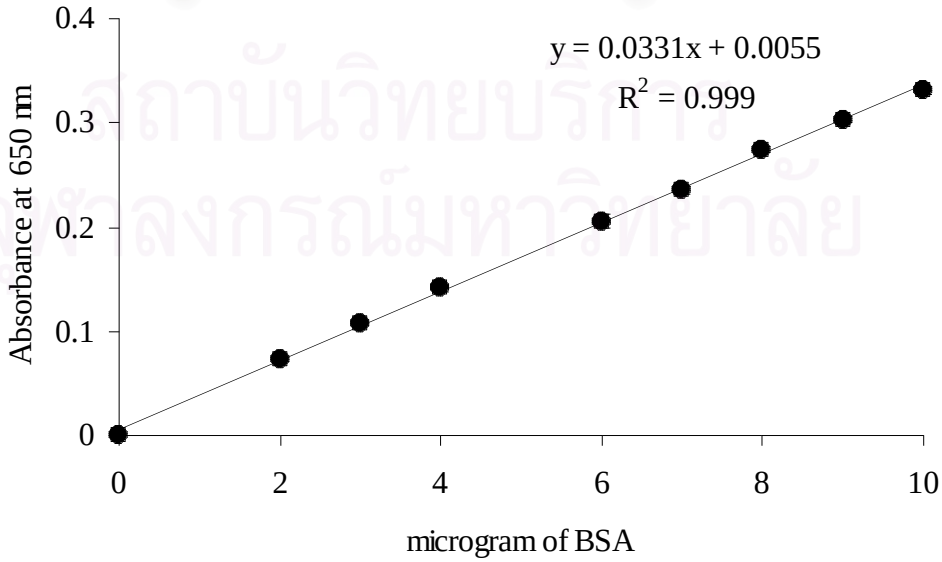
Biuret reagent:

1% copper sulfate	0.5 ml
2% Sodium potassium tartrate	0.5 ml
2% Sodium carbonate	
dissolved in 0.1 M sodium hydroxide	50 ml

Phenol reagent (Folin-Ciocalteu's reagent):

Folin-Ciocalteu's reagent used in this work was reagent grade from Carlo Erba Reagenti, France

Standard curve for protein determination



APPENDIX D

HPLC RESULTS

HPLC results from transesterification reaction

$$\%\text{conversion} = \frac{[\text{FAME}]}{\{[\text{FAME}]+([\text{TAG}]\text{x } 3)+[\text{FFA}]\ ([1,3\ \text{DAG}]\text{ x } 2)+([1,2\ \text{DAG}]\text{ x } 2)+[\text{MAG}]\}} \text{ x } 100$$

S. maltophilia wild type

Name	Ret. time	Area	Height	Area%	Conc.	Units
Ecosane	2.441	8441524	1058202	31.627	0.0000	mM
FAME	5.312	3927	648	0.015	1.251	mM
TAG	7.473	13940119	1124435	52.415	3.998	mM
FFA	8.996	1919081	188720	7.216	1.209	mM
1,3 DAG	10.933	1293758	89431	4.865	0.686	mM
1,2 DAG	11.601	1008206	92504	3.791	0.612	mM
MAG	19.867	19246	768	0.072	0.089	mM

S. maltophilia UV107

Name	Ret. time	Area	Height	Area%	Conc.	Units
Ecosane	2.430	8298886	1090996	35.632	0.0000	mM
FAME	4.891	5388	771	0.023	1.254	mM
TAG	7.317	13028922	1121311	55.941	3.790	mM
FFA	8.847	989827	108611	4.250	0	mM
1,3 DAG	10.938	477436	30484	2.050	0.254	mM
1,2 DAG	11.578	487041	44598	2.078	0.294	mM
MAG	20.004	6160	273	0.026	0.077	mM

S. maltophilia UV1016

Name	Ret. time	Area	Height	Area%	Conc.	Units
Ecosane	2.440	10153049	1123492	36.841	0.0000	mM
FAME	4.891	10158	1508	0.037	1.260	mM
TAG	7.328	14748176	1124570	53.515	3.511	mM
FFA	8.842	1715697	188102	6.226	0.543	mM
1,3 DAG	10.918	485092	32059	1.760	0.210	mM
1,2 DAG	11.571	442184	44792	1.605	0.217	mM
MAG	20.044	4397	324	0.016	0.075	mM

S. maltophilia UV1048

Name	Ret. time	Area	Height	Area%	Conc.	Units
Ecosane	2.445	8736236	1103005	36.490	0.000	mM
FAME	5.271	6751	851	0.028	1.256	mM
TAG	7.456	13090951	1123613	54.679	3.620	mM
FFA	9.028	107526	106313	4.491	0.026	mM
1,3 DAG	10.944	537009	34008	2.243	0.272	mM
1,2 DAG	11.640	492139	43344	2.056	0.283	mM
MAG	20.131	3119	190	0.013	0.074	mM

BIOGRAPHY

Miss Niramol Juntawieng was born on March 24, 1982 in Lumphun, Thiland. She graduated with the Bacherlor Degree og Science in Animal Science, King Mongkut's Institute of Technology Ladkrabang in 2004 and continued studying for Master's Degree of Science in Genetices at Chulalongkorn University in that year.