MOLECULAR STUDIES OF GENES ENCODING FOR ENZYMES CAPABLE OF HYDROLYZING CEPHALOSPORIN C AND LIPID

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
(MICROBIOLOGY)

IN
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
1996

THESIS
ENTITLED

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ACKNOWLEDGMENT

Without the help and encouragement of many people, this thesis would have never been accomplished. First and foremost, Dr. Vithaya Meevootisom, my major advisor for his guidance, encouragement, kindness and patience and giving me the opportunity to do this thesis work.. His kindness will always be remembered.

I owe a special debt of gratitude to Dr. Chuenchit Boonchird, my co-advisor, for her invaluable supervision, extensive guidance and comments with full of kindness during the time of studying.

I am most grateful to Dr. Watanalai Panbangred for her wise guidance, comments and suggestion throughout this project.

My sincere appreciation is expressed to Dr. Vichai Boonsaeng and Dr. Prasit Palittapongarnpim, M.D. for their worthy critiques of this thesis. I am also grateful to Dr. Soavanee Dharmsthiti for her kind suggestion and providing some chemicals used in enzyme assays and the purification process.

Many thanks to Ms. Patama Monkongdee, Ms. Duangrat Jul-ak-sorn, Mr. Thianchai Lakornrat and Mr. Suriya Ruethatip for their helps, sincerity and friendship.

Also to be acknowledged is Dr. Peerapan Tan- ariya and my colleagues at Srinakharinwirot university for their understanding, encouragement and friendship. My special thanks also to Ms. Anchana Meenoi and Ms. Daravadee Kongchum for their helps in typing this manuscript.

Finally and most important, I would like to thank my mother, sisters and husband for their loves, understanding, encouragement and patience. Without their physical and mental supports, this work would not have been possible.

Benjamas Thanomsub

ชื่อวิทยานิพนธ์ การศึกษาระดับโมเลกุลของยีนที่สร้างเอนไซม์สำหรับย่อยเซฟาโลสปอริน ซี และไขมัน

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บทคัดย่อ

เซฟาโลสปอริน ซี สามารถจะถูกย่อยได้โดยเอนไซม์ เอสเทอเรส ได้เป็นดีอะเซทิล เซฟาโลสปอริน ซี ซึ่งเป็นสารตัวกลางในการผลิตสาร เซฟาโลสปอรินกึ่งสังเคราะห์โดยการเปลี่ยนแปลงอนุพันธ์ที่คาร์บอนตำแหน่งที่ 3 เชื้อ *Bacillus subtilis* WRRL-B558 เป็นเชื้อแบคทีเรียที่สร้างเอนไซม์ที่มีความสามารถนี้ได้สูง (Abbot และ Fukuda, 1975) และได้ถูกนำมาใช้ในการศึกษาในครั้งนี้ ยีนที่กำหนดการสร้างเอนไซม์เอสเทอเรสจากเชื้อ *Bacillus subtilis* WRRL-B558 ได้ถูกโคลน และพบลำดับนิวคลีโอไทด์ จากการวิเคราะห์ลำดับนิวคลีโอไทด์จำนวน 1560 เบสของบริเวณ *Hin*dIII และ *Sal*I ซึ่งเป็นบริเวณที่กำหนดการสร้างเอนไซม์นี้ พบว่ามียีน 2 ยีนปรากฏอยู่ติดกันแต่ในทิศทางการแสดงออกของยีนตรงข้ามกันยีนทั้งสองจะมีลักษณะเฉพาะของ conserved sequences ของโปรโมเตอร์ที่ตำแหน่ง-35 และ -10 และ ribosome binding site รวมทั้ง transcription terminator ซึ่งมีลักษณะเป็น inverted repeats ของนิวคลีโอไทด์อยู่หลังรหัสหยุด TAA ในยีนที่ 1 สามารถกำหนดการสร้างเอนไซม์ที่ประกอบด้วย 120 กรดอะมิโน และมีน้ำหนักโมเลกุลของเอนไซม์เท่ากับ 13,101 ส่วนยีนที่ 2 สามารถกำหนดการสร้างเอนไซม์ที่ประกอบด้วย 212 กรด อะมิโน และมีน้ำหนัก

โมเลกุลของเอนไซม์เท่ากับ 22692 จากการศึกษาหาน้ำหนักโมเลกุลของโปรตีนที่สร้างโดยยีนที่ 1 และ 2 โดยวิธี SDS-PAGE พบว่ามี protein ขนาดเล็กประมาณ 14 kDa

จากการเปรียบเทียบ DNA homology ของยีนที่ศึกษาอยู่กับยีนอื่นที่บรรจุใน GenBank Database พบว่า ยีนที่ 1 มี 95% homology กับบางส่วนของ artificial lipase sequence ของ *Geotrichum candidum* ส่วนยีนที่ 2 แสดง 95% homology กับ *B. subtilis* 168 lipase gene และ 97% homology กับ *G. candidum* artificial lipase gene ยีนทั้งสองนี้ได้ถูก subclone เข้าใน pTTQ18 vector ซึ่งมี strong promotor คือ ptac และพบว่าในยีนที่ 1 สามารถเพิ่มการผลิตเอนไซม์ทั้งสองชนิดสูงขึ้นประมาณ 7.6 เท่า ถ้าไม่มีการใช้ IPTG inducer หรือเพิ่มขึ้น 38 เท่า ถ้าใช้ IPTG inducer ร่วมด้วย จากการวิเคราะห์คุณสมบัติด้านความจำเพาะกับ substrates ต่าง ๆ พบว่ายีนทั้งสองสร้างเอนไซม์เป็นชนิด lipase enzyme ซึ่งสามารถทำหน้าที่เป็นเอสเทอเรสได้ด้วย และสามารถย่อยเซฟาโลสปอริน ซี ได้ ดีอะเซทิล เซฟาโลสปอริน ซี ด้วย นอกจากนี้จากการศึกษา inhibitors ที่อาจมีผลต่อการทำงานของ enzyme ทั้งสองพบว่า PMSF ที่เป็น inhibitor ต่อ serine enzyme สามารถยับยั้งการทำงานของเอนไซม์ทั้งสองตัวนี้ได้ แต่ sulfhydryl agents เช่น β-mercaptoethanol ไม่สามารถยับยั้งการทำงานของเอนไซม์นี้

การทำให้เอนไซม์บริสุทธิ์โดยการผ่าน Sephadex G-200 column gel filtration พบว่าไม่ประสบผลสำเร็จเท่าที่ควร เพราะได้ความบริสุทธิ์เพิ่มขึ้นเพียง 2 เท่าใน peak ที่ดีที่สุด (pTL7-peak 3) แต่ได้เกิดความสูญเสียของเอนไซม์ไปสูงด้วย ทั้งนี้อาจเป็นเพราะเอนไซม์ทั้ง 2 ชนิดมีคุณสมบัติ hydrophobicity สูง ไม่เหมาะที่จะใช้ Sephadex G-200 gel filtration ในการแยก

Thesis Title Molecular Studies of Genes Encoding for Enzymes Capable of Hydrolyzing Cephalosporin C and Lipid. .

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ABSTRACT

Cephalosporin C can be hydrolyzed by an esterase resulting in formation of deacetylcephalosporin C, a key intermediate, by which substitution at C3' side chain produce potentially useful semisynthetic cephalosporins. *B. subtilis* WRRL-B558 was known to be a high producer of cephalosporin acetylesterase (Abbot and Fukuda, 1975). Genes from *B. subtilis* WRRL-B558 encoding for the enzymes was cloned and sequenced. DNA sequence of 1560bp from *Hin*dIII-*Sal*I region showed 2 ORFs (ORF1 and ORF2) located adjacent to each other in reverse orientation. Their nucleotide sequences were consisted of -35 and -10 promoter conserved sequence; putative ribosome binding site and 11-12 bp inverted repeats, and a Rho independent transcription terminator located, after the TAA stop codon. ORF1 encodes for a deduced protein of 120 amino acids and ORF2 for 212 amino acids with MW of 13, 23 kDa, respectively. This was in agreement with MW of the enzymes determined by SDS-PAGE to be around 14 kDa. DNA homology revealed 95% homology of ORF1 to part of *Geotrichum candidum* artificial lipase

sequence. ORF2 showed 95% with homology that of *B. subtilis* 168 lipase gene and 97% homology with *Geotrichum candidum* artificial lipase gene. The genes from both ORFs were subcloned into pTTQ18 vector which contains the ptac strong promotor. The subclone of ORF1 was found to have higher lipase activity around 7.6 times (uninduced with IPTG) and 38 times (induced with IPTG), than that of the original recombinant clones. Studies on substrate specificity of the two enzymes showed that they were of the lipase type that were able to act as esterase and able to hydrolyse cephalosporin C to form deacetylcephalosporin C. The enzymes were found to be sensitive to PMSF, a serine inhibitor but resistant to β-mercaptoethanol, a sulfhydryl agent. The enzymes produced from subclones were partially purified using Sephadex G-200 gel filtration. The purification process was found to be rather unsuccessful because the best pooled peak (pTL7-peak 3) showed only 2 times increase in purity of the enzyme while low percent recovery was achieved. The failure could be attributed to the strong hydrophobic nature of both enzymes.

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LIST OF ABBREVIATIONS

7-ACA	7-Aminocephalosporanic acid
7-ADCA	7-Aminodeacetoxycephalosporanic acid
ADC	Assumed deacetylcephalosporin C
Ap	Ampicillin
AMP	Adenosine- 5'- monophosphate
ATCC	American Type Culture Collection
ATP	Adenosine- 5'- triphosphate
bp	base pair
Cep C	Cephalosporin C
cm	centimeter
DC	Deacetylcephalosporin C
DTT	Dithiothreitol
et al	et. ali (latin), and other
Fig.	Figure
g	gram
h.	hour
HPLC	High pressure liquid chromatography
IPTG	Isopropyl β- D- thiogalactoside
Kb	Kilobases (1,000 bp)
L, l	liter
LB	Luria - Bertani medium
μ	micro
λ	wavelength
m	mili-
M	Molar
min	minute
MW	Molecular weight

α-NA	Alpha-naphthylacetate
NAD	Nicotinamide - adenine dinucleotide
NADH	Reduced NAD
nm	nanometer
OD	Optical density
ORF	Open reading frame
pNP	para-nitrophenol
pNPP	para-nitrophenylpalmitate
rpm	revolutions per minute
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TSB	Tryptic soy broth
U	Unit
V	Voltage
XL-1B	XL-1Blue

CHAPTER I
INTRODUCTION

Cephalosporin C is an important antibiotic which was originally produced by *Cephaloporium acremonium*. It can be used as a starting material to produce various cephalosporin derivatives. Cephalosporin derivatives are now becoming drugs of choices for use against various pathogenic bacteria which resist other common drugs. Therefore, searching for new cephalosporin derivatives with extended spectra of antibacterial activity is required to ensure us for future fights against emerging resistant pathogens. Cephalosporin derivatives are made by modification of the two side chains of cephalosporin C at C3 and C7 positions. Cephalosporin C is first removed of its side chains to form deacetylcephalosporin if the side chain at C3 was removed and 7-aminocephalosporanic acid if the side chain at C7 was removed. The removal could be achieved by using either chemical processes or enzymatic processes. Industrially, the removal of side chains is done by the chemical processes. However, more concerns of chemical pollutions are increasing, therefore, a competitive enzymatic process is needed. Research towards development of the competitive enzymatic process should be encouraged. It is the purpose of this work to study cephalosporin acetylesterase, an enzyme that removes the acetyl group at C3 position, at the molecular level. Result from this study may contribute to development of a competitive enzymatic process for production of deacetylcephalosporin and its derivatives and, that, consequently reduced chemical pollutions added to the environment.

The work selected to study the cephalosporin C acetylesterase gene of *B. subtilis* WRRL-B558 which was reported to be a high producer of the enzyme. The study would include characterization of the enzyme, gene

cloning and nucleotide sequencing and characterization of the cloned genes, the recombinant enzymes and the recombinant clones. Also, increase gene expression using a strong promoter would be attempted.

CHAPTER II
BACKGROUND

2.1 General properties of antibiotics

Antibiotics are products of secondary metabolism that are capable of inhibiting growth processes of some others even when being used at low concentrations. During World War II, the demand for chemotherapeutic agents to treat wound infections led to the development of a production process for penicillin and the beginning of the era of antibiotics research. This continues to be the most important area of industrial microbiology today. Intensive screening programs in industrial countries continue to increase a large numbers of new antibiotics which may be developed for future use.

Antibiotics vary enormously in their structures, but most are large, complex molecules, with hydrophobic regions that facilitate diffusion into cells. Their large sizes may offer broader area of interaction with macromolecular targets. Multiple ring structures are common and are developed to interact well with their targets (Davis *et al.*, 1990).

Most antibiotics are produced by bacteria, actinomycetes and fungi and their distribution within the taxonomic groups is shown in Table 1.

Table 1. Number of antibiotics produced by major groups of microorganisms (From Berdy, 1985).

Taxonomic group	Number of antibiotics
Bacteria, other than Actinomycetes	950
Actinomycetes	4600
Fungi	1600

For fungi, some antibiotics that are produced by the Aspergillaceae and Moniliales are of practical importance. Out of 10 of the known fungal antibiotics that are produced commercially only penicillins, cephalosporin C, griseofulvin and fusidic acid are clinically important (Crueger and Crueger, 1990). For bacteria other than actionomycetes, there are many taxonomic groups which produce various antibiotics of importance such as *Bacillus polymyxa* produces polymyxin B, *Bacillus licheniformis* produces bacitracin and *Latobacillus* spp. produces nisin. For actinomycetes, especially *Streptomyces* spp. produce variety of important antibiotics that are useful clinically, agriculturally and industrially. Examples of these are *Streptomyces clavuligerus* produces cephamycins and clavulanic acid which are important for clinical uses, and *Streptomyces fradiae* produces tylosin which are used as animal growth promotant in animal feed.

2.1.1 Classification of antibiotics

Antibiotics can be classified according to their antimicrobial spectra, mechanisms of action, producer strains, manners of biosyntheses or chemical structures. Table 2 shows a simplified classification according to their chemical structures.

Chemotherapeutic antibiotics can be either broad spectrum antibiotics that are active against many organisms or narrow spectrum antibiotics that are active aganist only a restricted range of organisms. Other than being used chemotherapeutically, antibiotics are used in agricultural, food, and other non-medical fields as summarized in Table 3.

Table 2. Classification of antibiotics according to their chemical structures. An example of each is given in parentheses. (from Berdy, 1985).

1. Carbohydrate-containing antibiotics	
Pure sugars	(Nojirimycin)
Aminoglycosides	(Streptomycin)
Orthosomycins	(Everninonicin)
N-Glycosides	(Streptothricin)
C-Glycosides	(Vancomycin)
Glycolipids	(Moenomycin)
2. Macrocyclic lactones	
Macrolide antibiotics	(Erythromycin)
Polyene antibiotics	(Candicidin)
Ansamycins	(Rifamycin)
Macrotetrolides	(Tetranactin)
3. Quinones and related antibiotics	
Tetracyclines	(Tetracycline)
Anthracyclines	(Adriamyacin)
Naphthoquinones	(Actinorhodin)
Benzoquinones	(Mitomycin)
4. Amino acid and peptide antibiotics	
Amino acid derivatives	(Cycloserine)
β-Lactam antibiotics	(Penicillin)
Peptide antibiotics	(Bacitracin)
Chromopeptides	(Actinomycins)
Depsipeptides	(Valinomycin)
Chelate-forming peptides	(Bleomycins)

5. Heterocyclic antibiotics containing nitrogen

Nucleoside antibiotics (Polyoxins)

6. Heterocyclic antibiotics containing oxygen

Polyether antibiotics (Monensin)

7. Alicyclic derivatives

Cycloalkane derivatives (Cycloheximide)

Steroid antibiotics (Fusidic acid)

8. Aromatic antibiotics

Benzene derivatives (Chloramphenicol)

Condensed aromatic antibiotics (Griseofulvin)

9. Aliphatic antibiotics

Compounds containing phosphorous (Fosfomycins)

Table 3. Applications of antibiotics (Zahner, 1978).

Application	Antibiotics available		New antibiotics needed
	Many	Some	
Medicine	Gram-positive	Gram-negative bacteria, including multiple resistance Dermatophyes	Systemic mycoses Viruses, Protozoa Parasites, Tumors
Non medical areas	-	Plant pathology (phytopathogenic fungi) Animal nutrition	Plant pathology - Phytopathogenic bacteria and viruses - Insects and mites - Nematodes - Food preservatives

In terms of volume of production and annual sales, antibiotics of the β-lactam group are the most importance. Their annual production and sales in 1980 were 17,000 tonnes (Table 4) accounted for more than 71% of the total amount of antibiotics produced and 380 million US$ accounted for around 43% of the total value of antibiotics sold respectively (Pirt, 1987). It should also be noted that, their annual projected growth rate of the market was one of the highest i.e., around 5% (Pirt, 1987). Therefore, due to their significance they are one of the most studied group of antibiotics.

Table 4. World market for antibiotics in 1980 (Data from Barber, 1980 and Kieslich, 1985).

	Annual Production (tonnes)	Annual value US$	Projected annual growth rate of market	% of market
Penicillin	17,000	380	+ 5.5	42
Tetracyclines	5,000	185	- 3	21
Cephalosporin C	12,000	100	+ 0.5	11
Erythromycins	800	80	+ 4	9
Rifamycin B		50	+ 7	6
Others	<1000	99	-	11

2.2 Beta-lactam

Beta-lactam antibiotics are specific inhibitors of bacterial cell wall synthesis that is unique to bacteria. Even when being used at high concentration, they do not have side effects on the host except in allergic patients (Davis , 1990). They combine specifically with the penicillin-binding proteins of the bacterial cell, by inhibiting the enzyme activity of

these proteins, causing the wall disorganization and follow by cell death. This group of antibiotics has been known for a long time by the first discovery of Penicillin by Alexander Fleming in 1929, when staphylococcal growth on a petri plate was inhibited by a contaminating *Penicillium notatum* culture (Crueger and Crueger,1990).

As shown in Figure 1, the β-lactam antibiotics can be divided into five distinct classes. Penicillins and the cephalosporins belong to the most effective of all therapeutic agents for the control of infectious diseases. In addition to the development of numerous semisynthetic β-lactams based on the known β-lactam rings, antibiotics with completely new β-lactam ring systems have been isolated in the past few years using new specific and sensitive screening methods.

2.3 Cephalosporin C

Cephalosporin C was discovered in culture filtrates of *Cephalosporin acremonium* in 1953. The strain isolated by Brotzu in 1945 was later classified as *Acremonium chrysogenum* and produces several antibiotics : cephalosporin C, penicillin N (a 6-APA derivative with D-α-aminoadipic acid as its side chain), and the steroid cephalosporins P1-P5. The cephalosporin antibiotics are also produced by other fungi, such as *Emericellopsis* and *Paecilomyces* (Crueger and Crueger, 1990). In 1971, cephalosporins were isolated from various *Streptomyces* species, such as *S. lipmanii*, *S. clavuligerus* or *Nocardia lactamdurans*.

Cephalosporins are valued not only because of their low toxicity but also because of their broad spectrum nature. With about 29% of the antibiotic market, the cephalosporins are the single most important group of antibiotics (Crueger and Crueger, 1990).

The basic structure of the cephalosporin C is 7-amino cephalosporanic acid (7-ACA) which consists of a dihydrothiazine ring

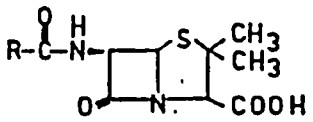
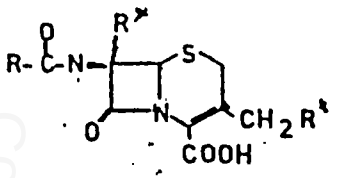
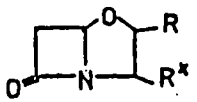
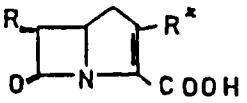
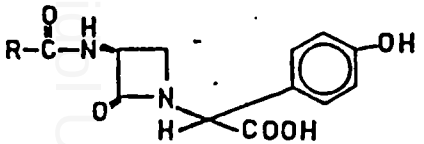
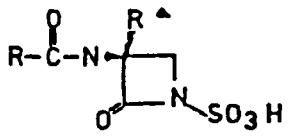
Basic structure	Antibiotic	Most important producing strains
Penam 	Penicillins	*Penicillium chrysogenum* *Aspergillus nidulans* *Cephalosporium acremonium* *Streptomyces clavuligerus*
Ceph-3-em 	Cephalosporins 7-Methoxycephalosporins	*Cephalosporium acremonium* *Nocardia lactamdurans* *Streptomyces clavuligerus*
Clavam 	Clavulanic acid	*S. clavuligerus*
Carbapenem 	Thienamycins Olivanic acids Epitheinamycins	*S. cattleya* *S. olivaceus* *S. flavogriseus*
Monolactam  	Nocardins	*Nocardia uniformis* subsp. *tsuyamenensis*
	Monobactams	*Gluconobacter* sp. *Chromobacter violaceum* *Agrobacterium radiobacter* *Pseudmonas acidophila* *Pseudomonas mesoacidophila* *Flexibacter* sp. *Acetobacter* sp.

Figure 1. The basic structure of the naturally occurring β-lactam antibiotics (from Crueger and Cruger, 1990).

with a condensed β-lactam ring (Figure 2). The 7-ACA carries a variable acyl moiety in position 7 causing changes in its stability and pharmacokinetic properties (Huber *et al.*, 1972). Modification of the side chain at C3 affects absorption of the drug (Mandell and Sande,1991). Modifications of cephalosporins were studied with the aim to improve the properties of cephalosporins.

Biosynthesis of cephalosporins (Figure 3) proceeds from δ- (α-aminoadipyl) - L- cysteinyl - D - valine to isopenicillin N. In the next stage, penicillin N is produced by transformation of the L-α-AAA side chain into the D- form, via the action of a very labile racemace. After ring expansion to form deacetoxycephalosporin C by the so-called "expandase" reaction, then hydroxylation via a dioxygenase to form deacetylcephalosproin C occurs. The acetylation of cephalosporin C by an acetyl CoA dependent transferase is the end point of the biosynthetic pathway in fungi (Crueger and Crueger, 1990). In streptomycetes further transformations occur : cephalosporin C or the carbamoyl derivative of deacetylcephalosporin C is converted in a two-step reaction with molecular oxygen and S-adenosylmethionine to 7-methoxy cephalosporin or cephamycin C.

2.4 Cephalosporin C derivatives

Since the beginning of the chemotherapic era, a number of resistant strains has increased by transmission of the resistant properties from one strain to the others by natural selection. Carelessly use of antibiotics accelerates rate of resistance. Currently, alternatives for overcoming the resistance problem are to use new antibiotics or to improve properties of the known compounds by modifying their molecules using either chemical or genetic means (Crueger and Crueger, 1990).

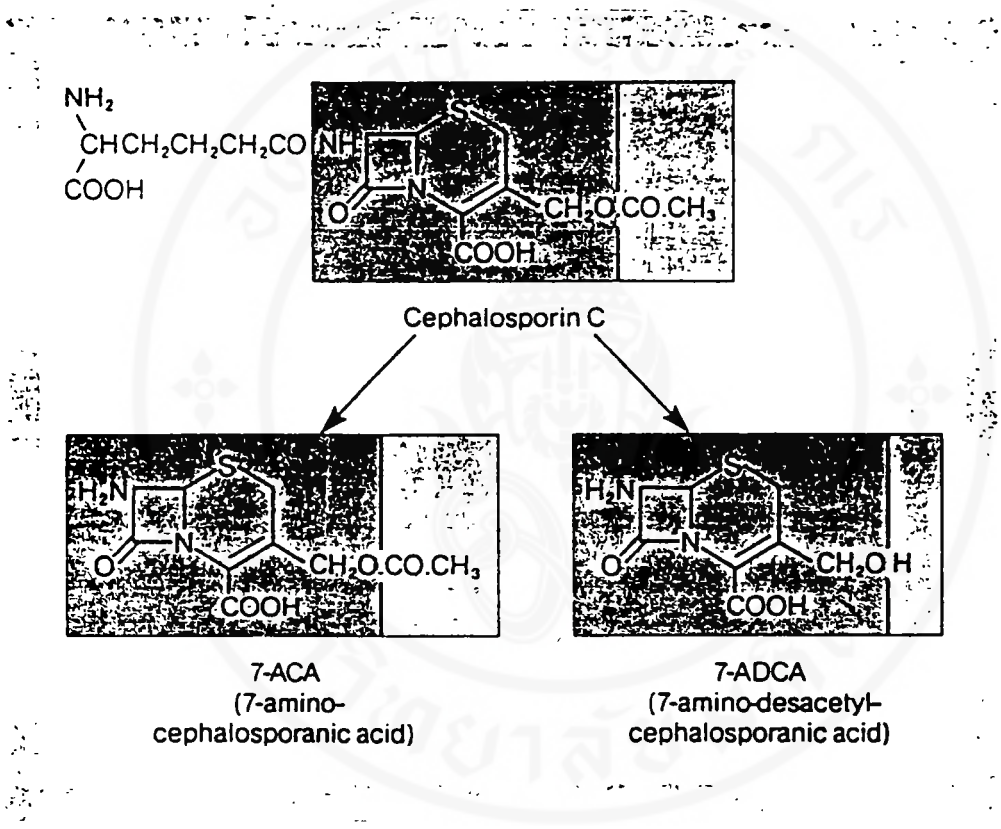


Figure 2. The generation of 7-amino-cephalosporanic acid (7-ACA) and of 7- amino-desacetyl-cephalosporanic acid (7-ADCA) from cephalosporin C (from Savidge, 1984).

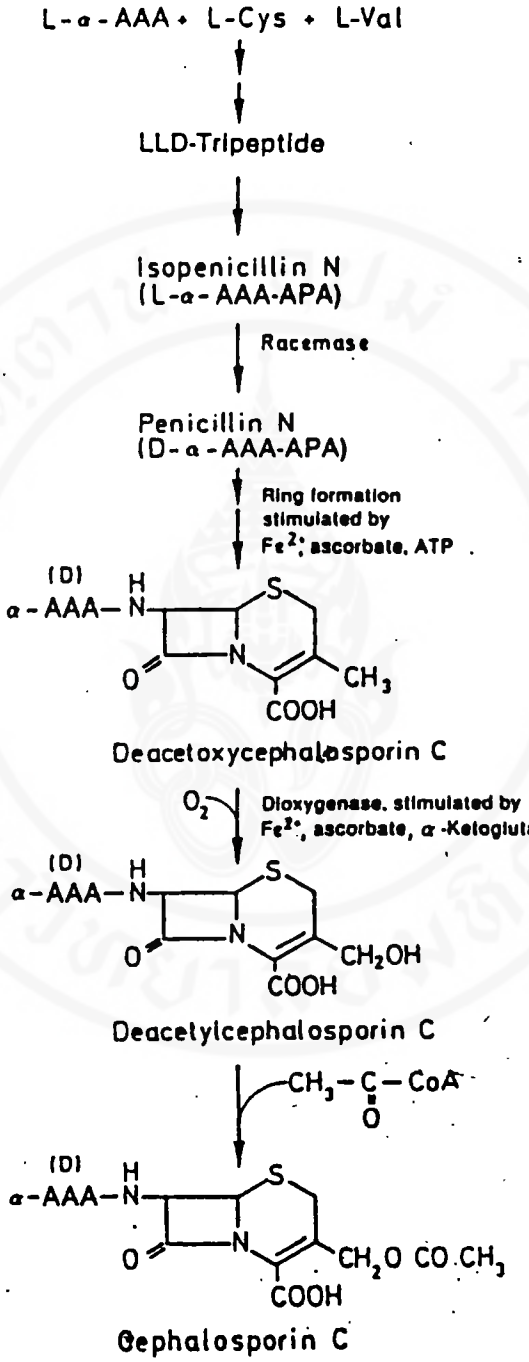


Figure 3. Biosynthesis of cephalosporin C by *Cephalosporium acremonium* (from Crueger and Crueger, 1990).

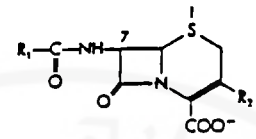
As mentioned earlier, suitable modification of cephalosporin C structure at C7 or C3 creates new potential antibiotics against sensitive organisms and some resistant strains, therefore, a number of therapeutically important semisynthetic cephalosporins are commercially produced. They are synthesized by either chemically or enzymatically splitting the natural cephalosporin C into 7-aminocephalosporanic acid (7-ACA), deacetyl-7-aminocephalosporanic acid or 7-aminodeacetoxycephalosporanic acid (7-ADCA) and its side chains. Then subsequent acylation at C7 or esterification at C3 position are done with appropriate new side chains to obtain the semisynthetic cephalosporins with new characteristics such as excellent β-lactamase stability and a broaden action spectrum.

2.4.1 Classification of cephalosporins

With the discovery of methods to modify cephalosporins, a new variety of cephalosporins are produced. They can be grouped into a simplified system by their chemical structure, clinical pharmacology, beta-lactamase resistance or anti-microbial activity spectrum. The well accepted system of classification by "generation" is very useful in clinical use, although committed somewhat arbitrarily (Mandell and Sande, 1991). The classification by generations is based on general features of antimicrobial activity (Donowitz and Mandell, 1990) as shown in Table 5.

The first-generation cephalosporins have good activity against gram-positive bacteria and relatively modest against gram-negative microorganisms. Most gram-positive cocci are susceptible except that of enterococci, methicillin-resistant *S. aureus* and *S. epidermidis*. Activity against *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* is good (Mandell and Sande, 1991). The second generation cephalosporins have somewhat increased activity against gram-negative bacteria but are

Table 5. Names, structural formulas, dosage and dosage forms of selected cephalosporins and related compounds (from Mandell and Sande, 1991).



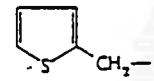
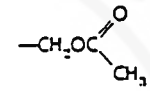
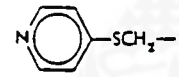
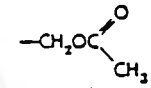
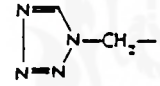
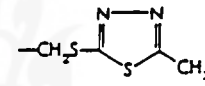
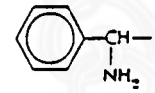
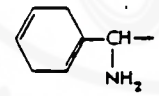
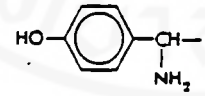
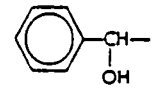
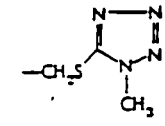
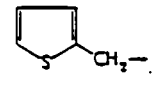
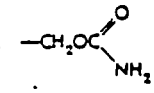
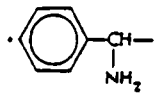
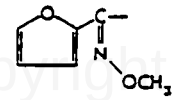
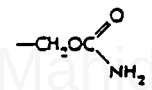
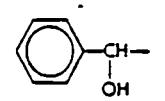
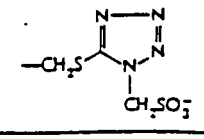
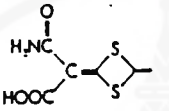
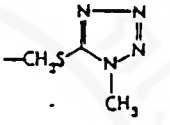
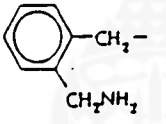
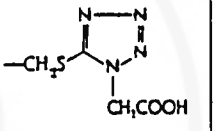
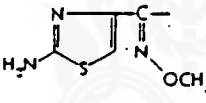
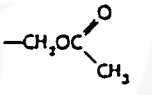
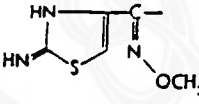
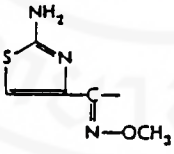
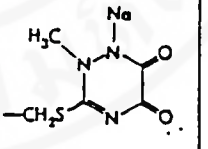
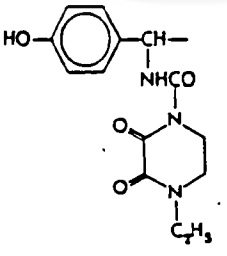
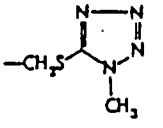
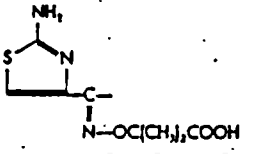
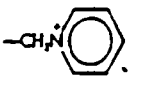
COMPOUND (TRADE NAMES)	R_1	R_2	DOSAGE FORMS, * ADULT DOSAGE FOR SEVERE INFECTION, AND HALF-LIVES
First Generation Cephalothin (KEFLIN)			I: 1 to 2 g every 4 hours $T_{1/2}$ = 0.6 hour
Cephapirin (CEFADYL)			I: 1 to 2 g every 4 hours $T_{1/2}$ = 0.7 hours
Cefazolin (ANCEF, KEFZOL, others)			I: 1 to 1.5 g every 6 hours $T_{1/2}$ = 1.8 hours
Cephalexin (KEFLET, KEFLEX)			C.T.O: 1 g every 6 hours $T_{1/2}$ = 0.9 hour
Cephradine (ANSPOR, VELOSEF)			C.O: 1 g every 6 hours I: 2 g every 6 hours $T_{1/2}$ = 0.8 hour
Cefadroxil (DURICEF, ULTRACEF)			C.T.O: 1 g every 12 hours $T_{1/2}$ = 1.1 hours
Second Generation Cefamandole (MANDOL)			I: 2 g every 4 to 6 hours $T_{1/2}$ = 0.8 hour
Cefoxitin† (MEFOXIN)			I: 2 g every 4 hours or 3 g every 6 hours $T_{1/2}$ = 0.7 hour
Cefaclor (CECLOR)			C.O: 1 g every 8 hours $T_{1/2}$ = 0.7 hour
Cefuroxime (KEFUROX, ZINACEF)			I: up to 3 g every 8 hours $T_{1/2}$ = 1.7 hours
Cefuroxime axetil ‡ (CEFTIN)			T: 500 mg every 12 hours
Cefonicid (MONOCID)			I: 2 g every 24 hours $T_{1/2}$ = 4.4 hours

Table 5. Names, structural formulas, dosage and dosage forms of selected cephalosporins and related compounds (continued).

COMPOUND (TRADE NAMES)	R_1	R_2	DOSAGE FORMS, * ADULT DOSAGE FOR SEVERE INFECTION, AND HALF-LIVES
Cefoletan (CEFOTAN)			I: 2 to 3 g every 12 hours $T_{1/2}$ = 3.3 hours
Ceforanide (PRECEF)			I: 1 g every 12 hours $T_{1/2}$ = 2.6 hours
Third Generation Cefotaxime (CLAFORAN)			I: 2 g every 4 to 8 hours $T_{1/2}$ = 1.1 hours
Ceftizoxime (CEFIZOX)		—H	I: 3 to 4 g every 8 hours $T_{1/2}$ = 1.8 hours
Ceftriaxone (ROCEPHIN)			I: 2 g every 12 to 24 hours $T_{1/2}$ = 8 hours
Cefoperazone (CEFOBID)			I: 1.5 to 4 g every 6 or 8 hours $T_{1/2}$ = 2.1 hours
Ceftazidime (FORTAZ, others)			I: 2 g every 8 hours $T_{1/2}$ = 1.8 hours

* T = tablet, C = capsule, O = oral suspension, I = injection.
† Cefoxitin, a cephamycin, has a —OCH$_3$ residue at position 7.
‡ Cefuroxime axetil is the acetyloxyethyl ester of cefuroxime.

much less active than the third-generation agents. Third-generation cephalosporins are generally less active than first-generation agents against gram-positive cocci, but they are much more active against the Enterobacteriaceae, including the beta-lactamase-producing strains. A subset of the third-generation agents is also active against *Pseudomonas aeruginosa* (Donowitz and Mandell, 1988).

Cephalosporins can be classified into 5 categories by another aspect on the basis of β-lactamase resistance, metabolic stability and acid stability (oral use) as shown in Figure 4.

Member of group 1 has general structure as shown in Figure 5. The 3-acetoxymethyl group is easily attacked by esterases and results in its conversion to 3-hydroxymethyl group with the diminution of antibacterial activity.

Group 2 cephalosporins all contain, in common with those of group 1 at position 7, a mono-substituted acetamido group at position 7. Consequently, they have similar antibacterial activity and are sensitive to β -lactamases of gram negative bactria but the substituents at position 3 render them stable to esterases resulting increase of the time the compounds remain in the body. The structures and characteristics of group 2 cephalosporins are shown in Figure 6.

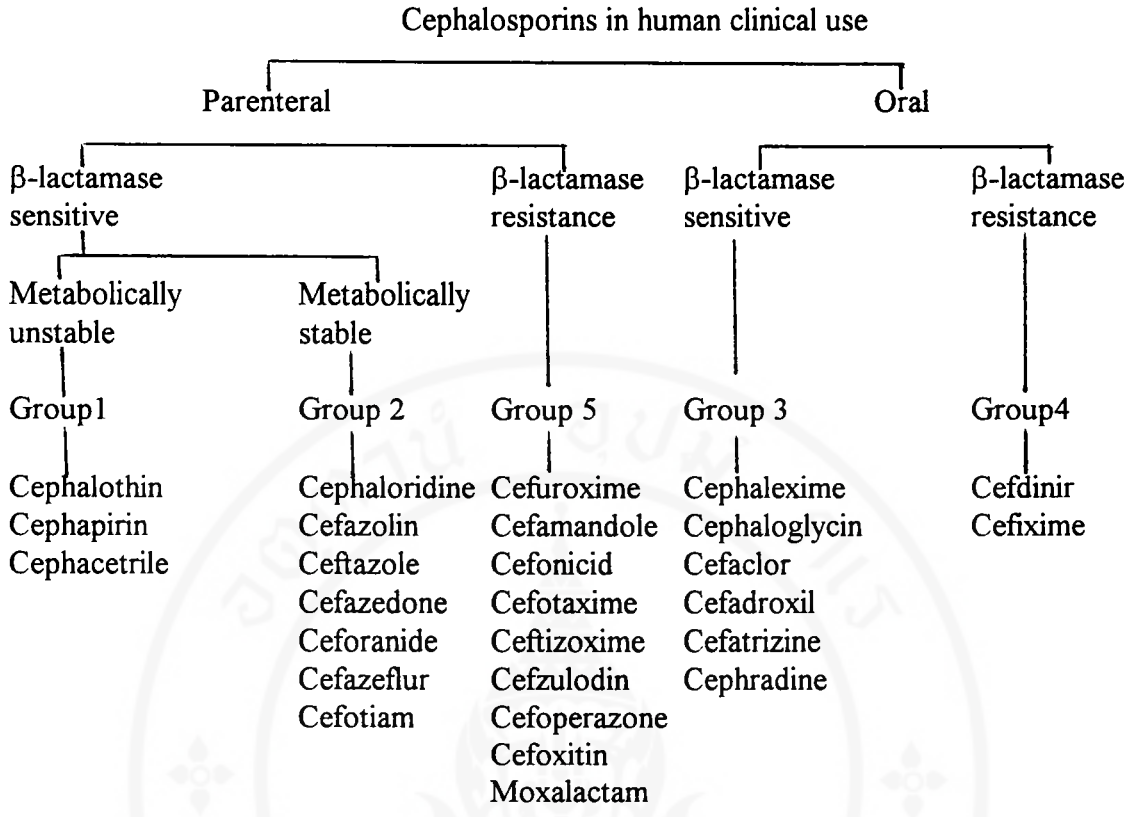
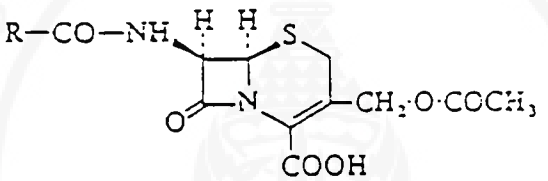


Figure 4. Classification of cephalosporins according to β-lactamase resistance and pharmacokinetic properties. (from Gale *et al.*,1981 which was adapted from O' Callaghan *et al.*,1979).

All orally absorbed cephalosporins of group 3 as shown in Figure 7 contain closely related 7-acyl groups which have amino substituent on the α-carbon and the six-membered carbocyclic ring. The free amino group is important for oral absorption properties. A small and polar group at position 3 help in oral absorption, with methyl group being optimum for oral absorption but worst for activity. Two orally absorbed cephalosporins which showed resistance to various β-lactamase of Group 4 were newly distributed in the market in Thailand in 1992 and 1994 namely cefdinir and cefixime, respectively. Cefixime, for example, contained a vinyl group at C3 position and a carboxymethoxyimino group at C7 position of 7-ACA which render them stable to various β-lactamase and has broad-spectrum



General structure

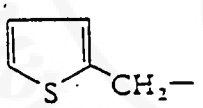
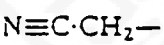
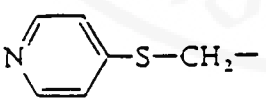
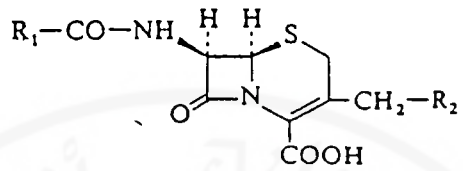
Name	R group	Characteristics
Cephalothin		All contain mono-substituted acetamido groups as the 7-acyl substituent.
Cephacetrile		Good activity against Gram-positive bacteria: active against non-β-lactamase-producing Gram-negative bacteria.
Cephapirin		Undergo deacetylation of group on position 3 *in vivo* resulting in lower activity against clinically-important organisms and more rapid elimination from the body.

Figure 5. Group 1 cephalosporins : their structures and characteristics (from Gale *et al.*, 1981).



General structure

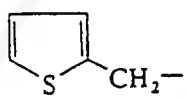
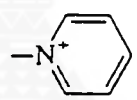
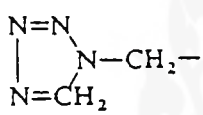
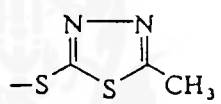
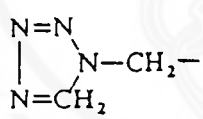
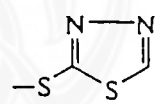
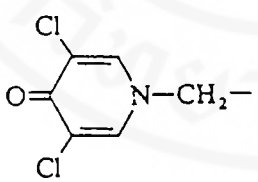
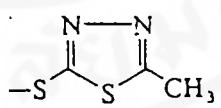
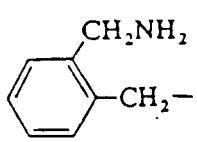
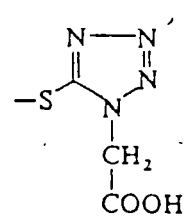
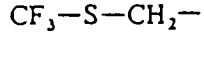
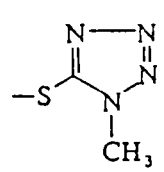
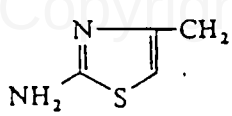
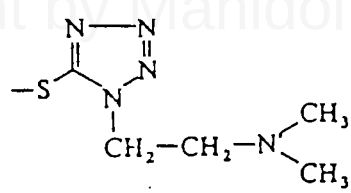
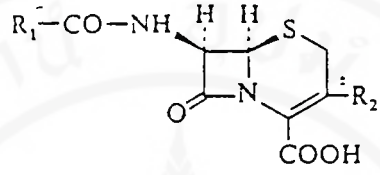
Name	R_1	R_2	Characteristics
Cephaloridine			All contain mono-substituted acetamido group as the 7-acyl substituent.
Cefazolin			All are metabolically stable (cephaloridine was the first to be synthesized).
Ceftezole			All possess good activity against Gram-positive bacteria. Activity against Gram-negative bacteria differs: cefotiam > cefazolin > cephaloridine
Cefazedone			
Ceforanide			Poor activity against Gram-negative β-lactamase producers. Ceforanide and cefotiam have some resistance to β-lactamases.
Cefazaflur			Cefotiam is the most active member of the group and has the broadest spectrum of activity, but is inactive against *P. aeruginosa*.
Cefotiam			Rapid bactericidal activity.

Figure 6. Structures and characteristics of group 2 cephalosporins (from Gale *et al.*, 1981).



General structure

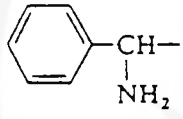
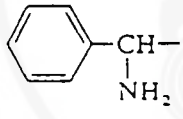
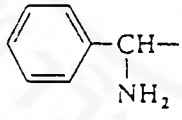
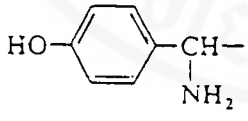
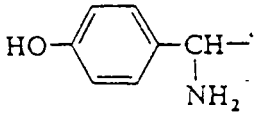
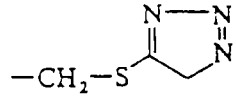
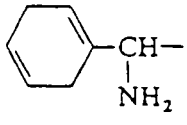
Name	R_1	R_2	Characteristics
Cephalexin		$-CH_3$	7-acyl groups are all closely-related structurally. All possess an amino group on the α-carbon atom of the sidechain and a 6-membered carbocyclic ring. The group at position 3 is small and polar to aid oral absorption. Bactericidal activity, but not expressed as rapidly as by the Group 2 cephalosporins. Morphological effect is to induce filamentation.
Cephaloglycin		$-CH_2-O-CO-CH_3$	
Cefaclor		$-Cl$	
Cefadroxil		$-CH_3$	
Cefatrizine			
Cephradine		$-CH_3$	

Figure 7. Structures and characteristics of group 3 cephalosporins (from Gale *et al.*, 1981).

activity against gram-positive and negative organisms (Fujisawa Pharmaceutical, 1995).

The member of group 5 cephalosporins, as shown in Figure 8, differ considerably in the structure but one very important factor they have in common is their resistance to β-lactamases and that contributes to excellent antibacterial activity. This property resides in the 7-β substituent, the methoxy group, at the 7-α-position which renders the drugs (eg. cefoxitin and cefmetazole) virtually totally resistant to β-lactamases.

2.4.2 Semisynthetic cephalosporins

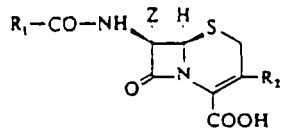
One factor that has stimulated a lot of work on the cephalosporins, is the penicillin-like low toxicity of these β-lactam antibiotics in man. Another is the opportunity to vary substituents in two positions in the cephem ring system to produce potentially valuable changes in biological properties and with high activity against a wide range of gram-negative bacteria, as well as the β-lactamase-producing staphylococcus.

In doing so, the starting key intermediates, 7-amino cephalosporanic acid (7-ACA), 7-aminodeacetoxy cephalosporanic acid (7-ADCA) and deacetyl-7ACA in which all contain the cepham nucleus are needed. Production of the key intermediates can be achieved in 2 ways, chemical and/or enzymatic methods.

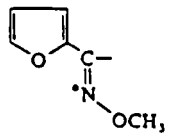
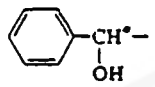
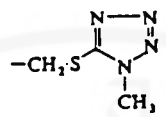
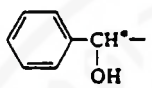
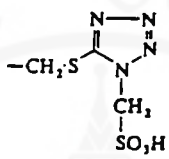
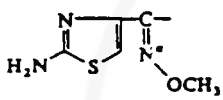
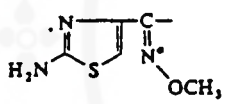
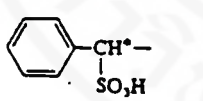
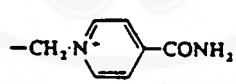
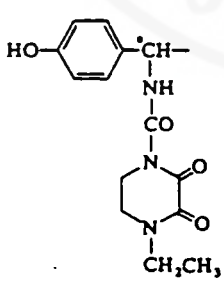
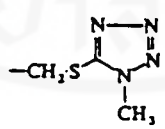
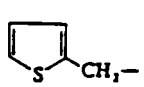
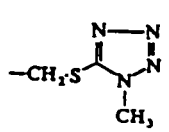
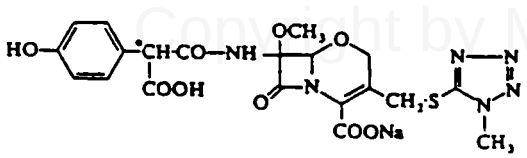
2.4.2.1 Production of 7-aminocephalosporanic acid (7-ACA)

7-ACA can be produced by chemical deacylation of cephalosporin C. The reaction is shown in Figure 9.

The direct enzymatic deacylation of cephalosporin C to 7-ACA is difficult possibly owing to the D-configuration of α-amino acyl side chain of cephalosporin C (Vandamme, 1980; 1981). However, D-



General structure

Name	R_1	R_2	Z	Characteristics
Cefuroxime		$-CH_2-OCONH_2$	H	General: Good metabolic stability. Several have *in vitro* activity better than that of carbenicillin. β-lactamase resistance varies; decreasing resistance from cefoxitin → cefotaxime → cefamandole
Cefamandole			H	Broad spectrum activity against Gram-negative bacteria including indole-positive *Proteus*. First cephalosporins with useful activity against *H. influenzae* and *N. gonorrhoeae*, including β-lactamase producers.
Cefonicid			H	
Cefotaxime		$-OCOCH_3$	H	α-Methoxyimino group gives increased β-lactamase resistance. Aminothiazolyl group results in good activity. Very good activity against enterobacteriaceae Cefotaxime is metabolized less rapidly on the 3-position than cephalothin.
Ceftizoxime		$-H$	H	
Cefsulodin			H	Cefsulodin has narrow spectrum of activity (see Table 3.10).
Cefoperazone			H	Cefoperazone has broader spectrum of activity: it is more resistant to cephalosporinases than penicillinases.
Cefoxitin		$-CH_2-O-CONH_2$	OCH_3	7-α-Methoxy group results in increased β-lactamase resistance but reduced antibacterial activity.
Cefmetazole	$N{\equiv}C-CH_2-S-CH_2-$		OCH_3	Cefoxitin is unique in being resistant to *Bacteroides fragilis* β-lactamase and also inhibits the enzyme.
Moxalactam				Moxalactam is an oxa-cepham compound—less chemically stable. Broad spectrum activity including *Ps aeruginosa*. Longer serum half-life than cefoxitin and less serum-bound.

* centres of asymmetry

Figure 8. Group 5 cephalosporins : their structures and characteristics (Gale *et al.*, 1981).

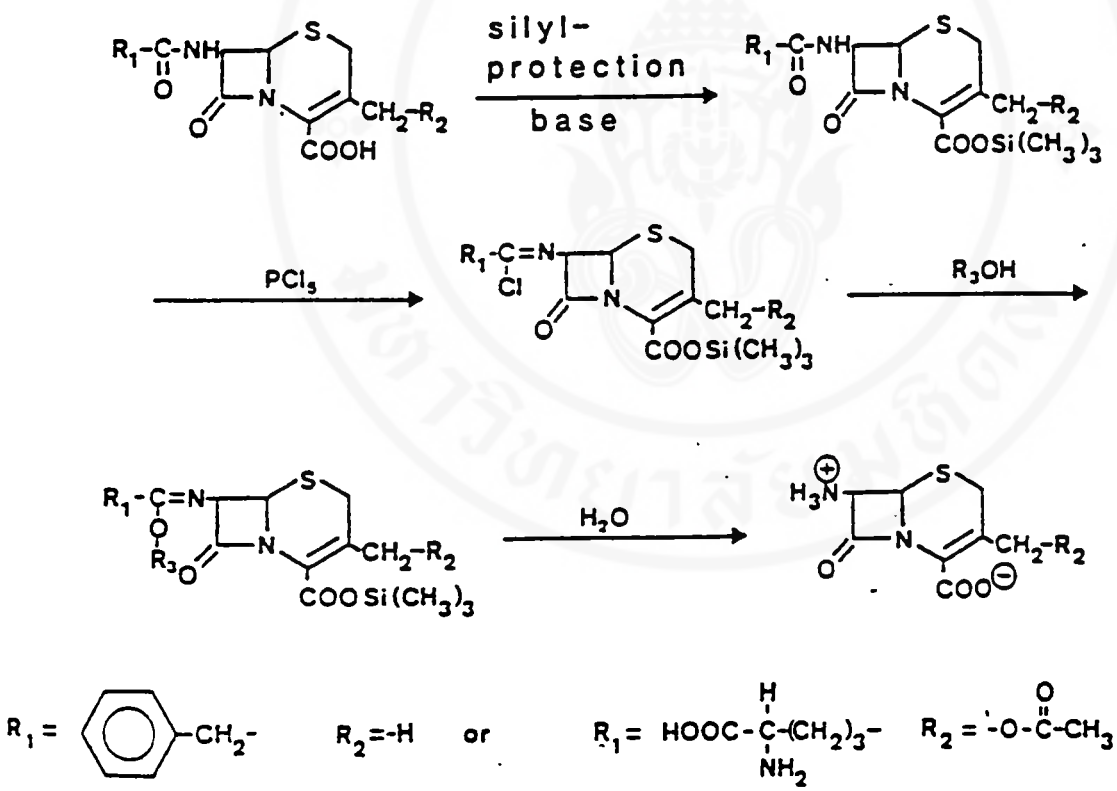


Figure 9. Chemical deacylation of cephalosporin C (from Hersbach *et al.*, 1984).

amino acid oxidases of bacterial origin have been isolated and the enzymes can convert cephalosporin C into glutaryl 7-ACA which is rapidly hydrolyzed by an acylase to 7-ACA (Toyo Jozo, Japenese Patent No. 117205, Shibuya *et al.*, 1981) (Figure 10). Mazzeo and Romeo 1972 have reported that 7-ACA might be obtained from cephalosporin C through oxidative deamination. It was suggested that 7-ACA might yielded from cephalosporin C by a sequential reaclion of the two enzymes. Recently, two acylase genes had been cloned from a soil isolate of *Pseudomonas*, one of which was claimed to have ability to do direct deacylation of cephalosporin C to 7-ACA (Matsuda *et al.*, 1987).

2.4.2.2 Production of 7-aminodeacetoxy cephalosporanic acid (7-ADCA)

7-ADCA can be prepared from cephalosporin C by hydrogenation and conversion of cephalosporin C into deacetoxy cephalosporin C and subsequent deacylation (Stedman *et al.*, 1964). In 1976, mutants that were deficient in the final stage of cephalosporin C biosynthesis had been isolated. They secreted deacetoxycephalosporin C in stead of cephalosporin C (Liersch *et al.*, 1976). Deacetoxycephalosporin C can, then , be converted into 7-ADCA, but the process is not economical . In 1960, it was demonstrated that the thiazolidine ring of penicillin could be expanded, although in low yield, into the dihydrothiazine ring of cephalosporin (Morin *et al.*, 1963). The production of 7-ADCA from penicillin G became preferable to other methods, when an efficient procedure with high yields (>70%) was found (deKoning *et al.*, 1975). The efficient method for production of 7ADCA from penicillin G involved three steps as shown in Figure 11; oxidation of penicillin G to its sulfoxide, ring expansion of penicillin G sulfoxide into 6-

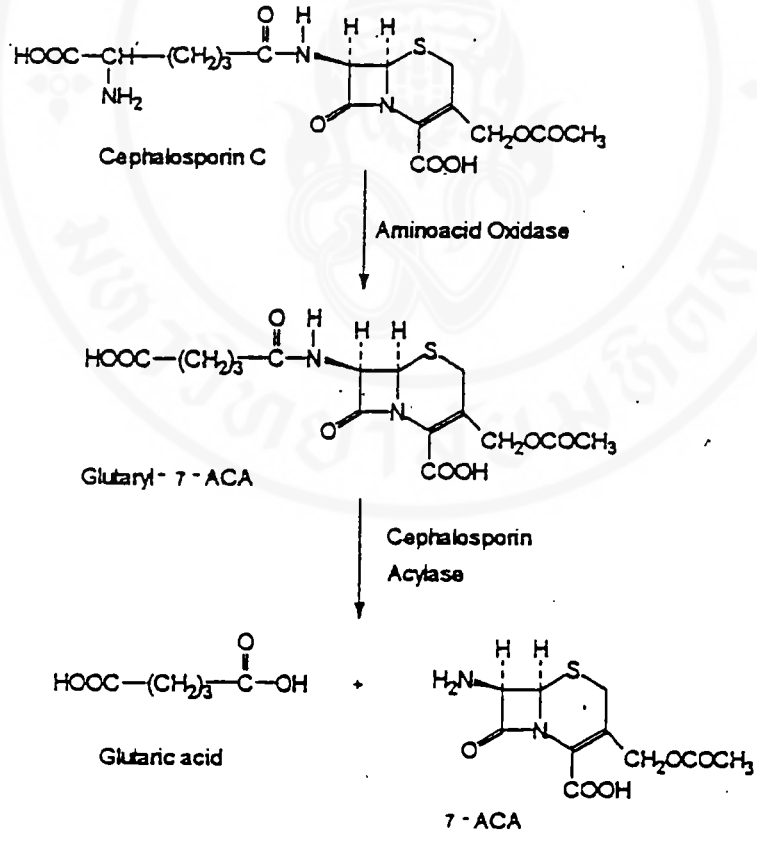


Figure 10. Enzymatic deacylation of cephalosporin to 7-ACA

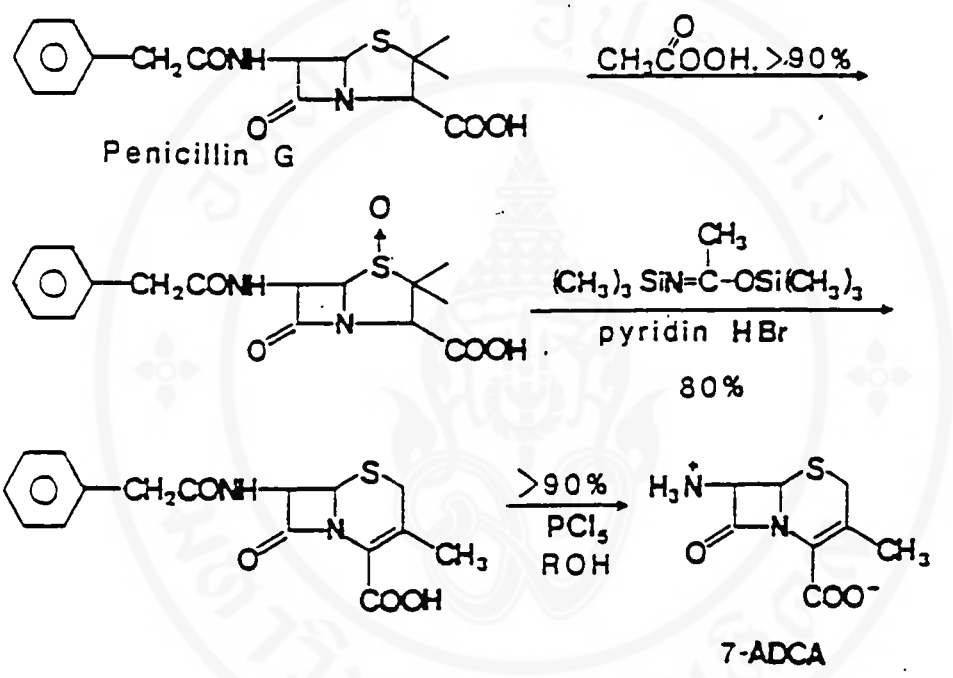


Figure 11. Conversion of penicillin G into 7-ADCA (from Hersbash *et al.*, 1984).

membered ring of dihydrothiazine ring with transient protection of the carboxyl group, and finally deacylation (Hersbach *et al.*, 1984)

The enzymatic production of 7-ADCA can be done by using either benzylpenicillin or phenoxypenicillin acylase as shown in Figure 12. Bayer (1975; 1977) used immobilized benzyl penicillin acylase which was covalently attached to cyanogen bromide-activated dextran to deacylate 7-phenylacetyl-ADCA (6%) at 37°C, pH 7.8. pH of the reaction was controlled by using ammonia. The reaction liquor was passed through a column containing the ion exchange resin, Lewatit MP500A in the chloride form, and the eluate was adjusted to pH 3.7. After leaving the eluate at room temperature for 24 h. 7-ADCA was precipitated out. The precipitated product was filtered off and washed successfully with water and acetone and then dried to give a 94% yield of 7-ACA. In the absence of the ion exchange treatment, the product was colored and difficult to filter.

In order to modify the substitution at the C-3 position of cephalosporin or 7-ACA it is first necessary to remove the acetate group resulting in deacetylcephalosporin C or a deacetyl-7ACA. This can be achieved chemically, but yields are low owing to β-lactam degradation and lactonization between the newly formed hydroxymethyl and adjacent carboxyl group. Thus, the carboxyl group of the nucleus is often required to be protected by blocking groups such as nitrobenzyl or trimethylsilyl, which may be difficult or expensive to be prepared and removed (Savidge,1984). Enzymatic hydrolysis may, therefore, be preferrable.

Acetylesterase, enzyme that catalyze removal of the acetate group at C-3 position has been isolated from several sources, for example, plants (Jeffery *et al.*, 1961), mammalian tissues (Demain *et al.*, 1963), fungi (Poutanen and Sundberg, 1988) and bacteria (O' Callaghan and

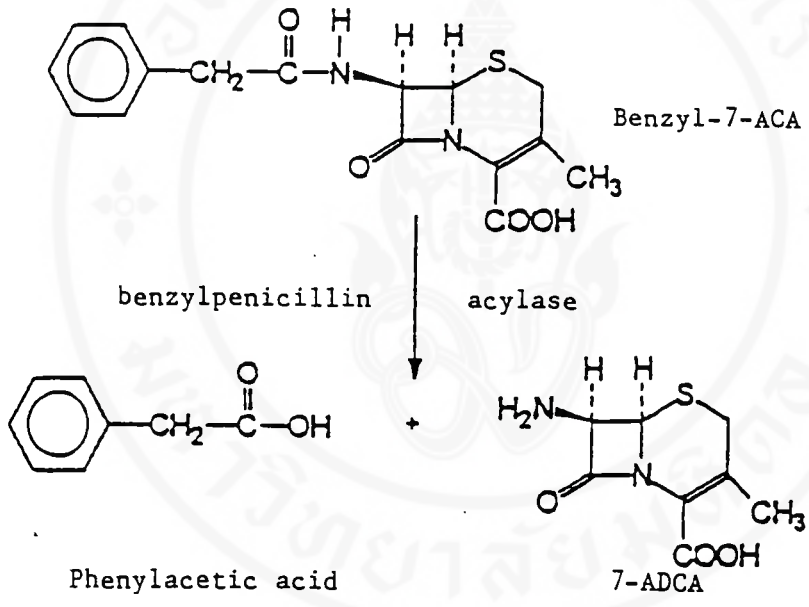


Figure 12. Enzymatic production of 7-ADCA

Muggleton, 1963; Castro *et al.*, 1992 and Higerd and Spizizen, 1973). Moreover, the cephalosporin acetylesterase has been reported from several microorganisms such as *Fusaria* sp., (Singh *et al.*, 1980), *Streptomyces clavuligerus* (Brannon *et al.*, 1972), *E. coli* (Nishida *et al.*, 1967, Nishiura *et al.*, 1978) and *B. subtilis* (Abbot and Fukuda, 1975). Particularly, the useful sources of the enzyme are the *Bacillus* species (Eli Lilly, 1976). The enzymatic deacetylation is shown in Figure 13.

The enzyme from *B. subtilis* WRRL-B558 has been extensively studied by Abbot and Fukuda (1975 a; b) and Abbot *et al.*, (1976). The organism was cultured in flasks containing tryptone soya medium for up to 22 h. at 30°C. The recovered extracellular enzyme which was purified by fractional precipitation with ammonium sulfate and ultrafiltration was assayed titrimetrically using cephalothin as a substrate and found to be very stable. It showed molecular weight of 190,000, a temperature optimum between 40 and 50°C, and a pH optimum of 7.0. Both reaction products were weakly inhibitory, but the reaction readily went to completion. Recently, the cephalosporin C acetylesterase was cloned from *B. subtilis* SHS0133 and expressed in *E. coli* (Mitsushima *et al.*, 1995) which showed MW. of 280,000. It was composed of eight identical subunits each with molecular weight of 35,000. This enzyme was stable up to 60°C for 30 min at pH 7.0. When 7-ACA was used as a substrate it was found that the products, 7-ACA and acetate were weak inhibitors. The pI value of the enzyme was 5.3 as determined by isoelectric focusing. This enzyme possessed different properties as compared to other cephalosporin C deacetylase enzymes as shown in Table 6.

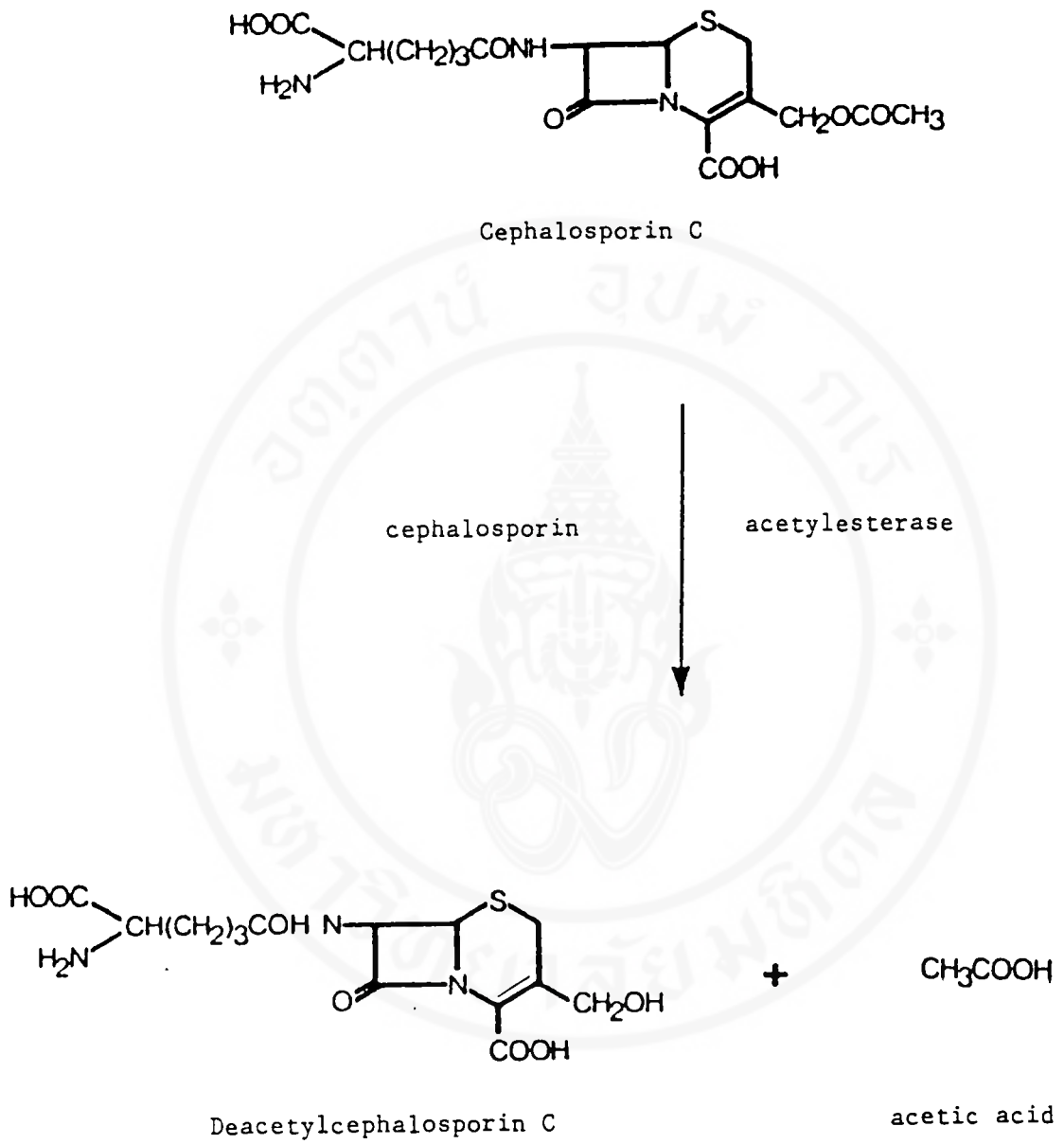


Figure 13. Enzymatic production of deacetylcephalosporin C.

Table 6. Comparison of some properties of the cephalosporin C acetylesterase from *B. subtilis* SHS0113 and those from other strains of *B. subtilis* (From Takimoto *et al.*, 1994).

Strain	SHS0133	WRRL-B558	ATCC6633
Molecular Mass	280kDa	190kDa	150kDa
Structure	octameric	ND	tetrameric
Optimum temperature	55°C	40-50°C	ND
Optimum pH	8.0-8.5	7.0	8.0
Isoelectric point	5.3	ND	4.7
Km (7-ACA)	7.9 mM	2.8 mM	6.5 mM
Ki (deacetyl-7-ACA)	171 mM	36 mM	ND

ND; Not determined

2.5 Esterases

2.5.1 General properties

Esterases are enzymes that catalyze the hydrolysis of a large number of uncharged carboxylic esters in the reaction

$$R_1\text{-}COOR_2 + H_2O \rightleftharpoons R_1\text{-}COOH + R_2\text{-}OH$$

Esterases have a broad specificity and thus create difficulties in classification and nomenclature. Many of the enzymes represented in subgroup 3.1.1. are groups of enzymes with closely related specificities (Myers, 1960). There is also an overlap in specificity with some enzymes listed in other groups. For example, p-nitrophenylacetate, which has been widely used for detecting esterase activity, is also hydrolyzed by more specific esterases such as acetylcholinesterase and by proteolytic enzymes such as chymotrypsin. However, in general, esterase is recognized as the enzyme catalyze the hydrolysis or synthesis of uncharged carboxylester or short chain fatty acid (Krisch, 1971). The reaction catalyses by esterases is similar to that by lipases. Both esterases and lipases are classified into the

group of enzyme of hydrolases which hydrolyse ester bonds. The classification of these enzymes are shown in Table 7.

2.5.2 Classification of esterases

The classification of esterases depends on the chemical nature of the alcohol and acid or of the radicals R_1 and R_2 found in the ester which confer special types of specificity among the esterases. In 1953, Aldridge proposed a classification of esterases based on the enzyme behavior toward organophosphorus compounds, such as dietyl-p-nitropheyl phosphate (=paraoxon; E600). The enzymes can be classified as follow :

Table 7. Classsification and reactions of enzymes hydrolyzing ester bond (From Damm, *et al.*, 1966).

Systematic name	Recommended trivial name	Reaction
3. HYDROLASES		
3.1 Acting on ester bonds		
3.1.1 Carboxylic Ester Hydrolases		
3.1.1.1		
Carboxylic-ester hydrolases	Carboxylesterase NRN-Ali-esterase, B-esterase	A carboxylic ester + H_2O ⇌ an alcohol + a carboxylate
[*Note: Wide specificity*]		
3.1.1.2		
Aryl-ester hydrolase	Arylesterase NRN-A-esterase, paraoxonase	A phenyl acetate + H_2O ⇌ a phenol + acetate
[*Note: Acts on many phenolic esters; the enzyme from sheep serum also hydrolyses paraoxon*]		
3.1.1.3		
Glycerol-ester hydrolase	Lipase	A triglyceride + H_2O ⇌ a diglyceride + a fatty acid ion
[*Note: The pancreatic enzyme acts only at an ester-water interface; the outer ester links are preferentially hydrolysed*]		

Systematic name	Recommended trivial name	Reaction
3.1.1.4		
Phosphatide acyl-hydrolase	Phospholipase A NRN- Lecithinase A	A lecithin + H_2O ⇌ a lysolecithin + an unsaturated fatty acid ion
[*Note: Also acts on phosphatidylethanolamine, choline plasmalogen and phosphatidates removing the fatty acid attached to the 2-position*]		
3.1.1.5		
Lysolecithin acyl-hydrolase	Lysophospholipase, phospholipase B NRN- Lecithinase B, lysolecithinase	A lysolecithin + H_2O ⇌ glycerolphosphocholine + a fatty acid ion
3.1.1.6		
Acetic-ester hydrolase	Acetylesterase NRN- C-esterase (in animal tissues)	An acetic ester + H_2O ⇌ an alcohol + acetate
3.1.1.7		
Acetylcholine hydrolase	Acetylcholin-esterase NRN-True cholinesterase	Acetylcholine + H_2O ⇌ choline + acetate
[*Note: Acts on a variety of acetic esters; also catalyses transacetylations*]		
3.1.1.8		
Acylcholine acyl-hydrolase	Cholinesterase NRN-Pseudo cholinesterase	An acylcholine + H_2O ⇌ choline + an anion
[*Note: Acts on a variety of choline esters and a few other compounds*]		
3.1.1.9		
Benzoylcholine hydrolase	Benzoylcholin-esterase	Benzoylcholine + H_2O ⇌ choline + benzoate
3.1.1.11		
Pectin pectyl-hydrolase	Pectinesterase	Pectin + n H_2O ⇌ n methanol + pectate
3.1.1.12		
Vitamin A-acetate hydrolase	Vitamin A esterase	Vitamin A acetate + H_2O ⇌ vitamin A + acetate
3.1.1.13		
Sterol-ester hydrolase	Cholesterol esterase	A cholesterol ester + H_2O ⇌ cholesterol + an anion
[*Note: Also acts on esters of cholesterol and some other sterols*]		

Systematic name	Recommended trivial name	Reaction
3.1.1.14		
Chlorophyll chlorophyllido-hydrolase	Chlorophyllase	Chlorophyll + H_2O = phytol + chlorophyllide
[*Note: Also catalyses chlorphyllide tranfer, e.g. converts chlorophyll in methanol into methylchlorophyllide*]		
3.1.1.15		
L-Arabinono-γ-lactone Hydrolase	Arabinonolactonase	L-Arabinono-γ-+ H_2O = L-arabinoate
3.1.1.16		
4-Carboxymethyl-4-hydroxyisocrotonolactone hydrolase	4-Carboxymethyl-4-hydroxyiso-crotonolactonase	4-Carboxymethyl-4-hydroxy-isocrotonolactone + H_2O = 3-oxoadipate
3.1.1.17		
D-Glucono-δ-lactone hydrolase	Gluconolactonase NRN-Lactonase	D-Glucono-δ-lactone+ H_2O = D-gluconate
[*Note: Also acts in D-glucono-δ-lactone-6-phosphate*]		
3.1.1.18		
D(or L)-Gulono-γ-lactone hydrolase	Aldonolactonase	D(or L)-Gulono-γ-lactone + H_2O = gluconate
3.1.1.19		
D-Glucurono-δ-lactone hydrolase	Uronolactonase	D-glucurono-δ-lactone + H_2O = D-glucuronate
3.1.1.20		
Tannin acyl-hydrolase	Tannase	Digallate + H_2O = 2 gallate
[*Note: Also hydrolyses ester links in other tannins*]		

Note NRN-non recommended name

A-esterases (EC3.1.1.2) known as arylesterases are the esterases that are not inhibited by organophosphorus compounds but instead hydrolyzed these compounds as substrates. Although A-esterases also catalyze hydrolysis of carboxylester as similar to that by B-esterases but the mechanism of their catalytic action may differ. This is supported by the finding that A-esterases are metalloenzymes, which are inhibited by chelating agents and activated by calcium ion, properties which are not found in B-esterases.

B-esterases (EC3.1.1.1) formerly known as ali-esterases are the esterases that are inhibited stoichiometrically by organo- phosphate compounds (without hydrolyzing them). Because of their capability of catalying the hydrolysis of a large number of carboxyl esters such as p-nitrophenyl acetate and phenyl acetate, this group of enzymes is also termed carboxylesterases.

C-esterases do not hydrolyze organophosphate compounds, nor they are inhibited by them. In contrast to A-and B-esterases, C-esterases are activated rather than inhibited by low concentrations of p-mercuribenzoate. However, the mechansim of the catalytic reaction is uncertain (Aldrige, 1953; Myer, 1960).

In addition to the criteria based on the basis of their reactions with characteristic inhibitors, these enzymes can also be classified by their abilities to hydrolyze undissolved substrates. However, it should be noted that none of the present classification of esterases is completely satisfactory and still unambiguous.

2.5.3 Substrate specificity

A. Hydrolysis of carboxyl ester

Esterases hydrolyze a wide variety of carboxyl esters. It is therefore difficult to definite their substrate specificity. However, Lery and Ocken (1969) categorized three groups of carboxyl ester substrate, namely, 1) unsubstituted monocarboxylate esters which have the highest relative velocity; 2) substituted monocarboxylate esters; and 3) dicarboxylate diesters and substituted diesters, of which only one ester group is hydrolyzed. This and many other indications is suggested that positive and also negative charged compounds are poor substrates for carboxylesterases and it can be assumed, therefore, that hydrophobic

bonds play a major role in enzyme substrate binding of carboxylesterases (Krisch, 1971).

Considering the hydrolysis of uncharged substrates, a wide range of aliphatic and neutral aromatic esters were exammined as substrate. The length and structure of the acyl and alkyl parts was shown to influence the specificity of enzymes to the substrates (Dixon, 1969). In case of the group, the affinity and reactivity of the enzyme increased together with increase in chain lengths up to a chain length of 4-5 carbon atoms but further increased in chain length caused a fall in reactivity but increased in affinity. For the alkyl group, maximum reactivity and affinity were found with 4-6 carbon atoms, a further increase in number of carbon atoms produced a fall in both reactivity and affinity.

B. Hydrolysis of thioesters

Esterases are also capable of splitting thioester linkages.

C. Hydrolysis of aromatic amides

The first indicaton that esterases may not only hydrolyze ester bonds but also amide bonds came from Myer *et al.*, (1957) and also Krisch (1963). In 1963, Krisch showed that highly purified liver esterases catalyzed the hydrolysis of certain aromatic amides such as acetanilide, phenacetine and several other anilide derivatives. The molecular activities towards anilide substrates, however, were generally very low.

D. Acyl group tranfer

Unspecific esterases are able to transfer acyl groups not only to water but also to several other nucleophilic acceptors. This finding came from many observations by a number of investigators (Bergmann and Wurzel, 1953; Krenitsky and Fruton, 1966 and Benorhr and Krisch, 1967).

2.5.4 Gene cloning and conserved amino acid sequence of the esterase genes

Esterase genes from various microorganisms have been cloned and sequenced. These includes an esterase gene of *Acinetobacter calcoaceticus* RAG-1, a phototrophic and oil-degrading microorganism, was shown to be 870bp long and its deduced protein was of MW 32,700 (Reddy *et al.*, 1989). An *est* gene coding for a cell bound esterase of *A. calcoaceticus* BD413 was cloned and sequenced (Kok *et al.*, 1993). Its nucleotide sequence revealed an open reading frame of 1130bp encoding a protein of 40 kDa. Mckay *et al.* (1992) cloned an *est* gene of a *Pseudomonas* sp. into *E. coli* and determined its nucleotide sequence. They found an ORF encoding for a polypeptide of 389 amino acid residues, with a molecular mass of 42272 Da. Another work of cloning the *est* gene from *Psuedomanas* sp. KWI-56 was done. The gene was sequenced revealing one ORF encoding for a deduced amino acid sequence of MW 29,011 which fit well with the estimated size obtained from SDS-PAGE (Shimada *et al.*, 1993).

Raymer *et al.* (1990) studied the gene that encoded for the extracellular esterase of *Streptomyces scabies*. From nucleotide sequence analysis, they predicted the enzyme to be consisted of 345 amino acids which was encoded by 1440 nucleotides. Recently, an esterase gene of a cyanobacterium, *Spirulina platensis* was cloned and sequenced. The gene was shown to have a 618bp ORF corresponding to a protein comprising of 206 amino acids with a MW of 23 kDa (Salvi *et al.*, 1994). So far, all the esterase genes that had been cloned were shown to have their deduced amino acid at the catalytic site analogous to that of serine proteases and lipases (Table 8). The conserved pentapeptide sequence (Gly-X_1-Ser-X_2-Gly) was usually found in the catalytic site of these serine active enzymes.

Table 8. Amino acid sequences at active sites of serine active enzymes.

(I) Penicillin-reactive proteins		
D-alanine carboxypeptidase	•	Ser
PB5 *E. coli*	DPASLTKMMT	AGC
PB6 *E. coli*	DPASLTKIMT	AGC
Streptomyces R61	RVGSVTKSFS	AGC
Beta-lactamase		
TEM tn3	PMMSTFKVLL	AGC
K. pneumoniae	PMVSTFKVLL	AGC
R. capsulata	LMNSTVKVPV	AGC
P. aeruginosa	PLNSTVKAFS	AGC
Streptomyces albus	PMCSVFKTLS	TCG
B. licheniformis	AFASTIKALT	TCG
I *B. cereus*	AFASTYKALA	TCA
III *B. cereus*	AFASTSKSLA	TCT
Staph. aureus	AYASTS*AIN	TCA
Oxacillinase		
OXA-2	SPASTFKIPH	TCG
OXA-1	APDSTFKIAL	TCA
PSE-2	LPASTFKIPN	TCA
ampC		
E. coli	ELGSVSKTFT	TCG
C. freundii	ELGSVSKTFT	TCG
blaR1 regulatory protein		
B. licheniformis	APASTYKVFS	TCC
Transglycolase-transpeptidase		
PBP1A *E. coli*	QVGSNIKPFL	TCC
PBP1B *E. coli*	SIGSLAKPAT	TCG
PBP3 *E. coli*	EPGSLVKPMV	TCA
(II) Alkaline phosphatase	•	
Human placenta	VPDSGATATA	AGT
Human intestine	VPDSAATATA	AGC
PHO8 yeast	VTDSAAGATA	TCA
E. coli	VTDSAASATA	TCG
(III) Subtilisin proteases	•	
B. licheniformis	NGTSMASPHV	TCA
B. amyloliquefaciens	NGTSMASPHV	TCA
Serratia	SGTSMAAPHV	TCA
Strep. cremoris	SGTSMASPFI	TCA
Thermus aquaticus	NGTSMATPHV	TCC
(IV) Esterases and lipases		
Esterases	•	Ser
ACE, *Drosophila*	MTLFGESAGSS	TCG
ACE, *Torpedo*	VTIFGESAGGA	AGT
Esterase D, human	MSIFGHSMGGH	TCC
CU, *C. capsici*	AVVAGYSQGTA	AGC
CU, *C. gloeosporides*	AIVSGYSQGTA	AGC
TE, duck uropygial gland	FALFGHSFGSF	AGT
TE, rat mammary gland	FAFFGHSFGSV	AGT
TE, rat FAS	YRVAGYSFGAC	TCT
Lipases		
Staphylococcus hyicus	VHFIGHSMGGQ	AGT
Lingual, rat	IHYVGHSQGTT	TCT
Hepatic, rat	VHLIGYSLGAH	AGC
Lipoprotein, mouse	VHLLGYSLGAH	AGC
Lipoprotein, guinea pig	VHLLGYSLGAH	AGC

(IV) Esterases and lipases (cont.)		
Transacylase		
LC, human	VFLIGHSLGCL	AGC
FAS, yeast		
Acetyl transacylase	KGATGHSQGLV	TCT
Malonyl transacylase	ATFAGHSLGEY	TCT
Related proteins		
Vitellogenin	•	
YP1 *Drosophila*	IHLIGQNVGAH	AAT
YP2 *Drosophila*	IHLIGSGPAAH	GGA
YP3 *Drosophila*	IHLIGQGISAH	GGA
Thyroglobulin	VTLAADRGGAD	CGT
(V) Serine proteases	In •	Ser
Trypsin, rat	SCQ^GDSGGPVVC	TCT
Chymotrypsin, rat	SCM^GDSGGPLVC	TCC
Elastase I, rat	GCQ^GDSGGPLHC	TCT
Elastase II, rat	SCN^GDSGGPLNC	TCC
Elastase III, human	GCN^GDSGGPLNC	TCT
CF B, human	TCR^GDSGGPLIV	TCT
Factor XII, human	ACQ^GDSGGPLVC	TCC
TPA, human	ACQ^GDSGGPLVC	TCG
UPA, human	SCQ^GDSGGPLVC	TCA
Factor XI, human	ACK^GDSGGPLSC	TCG
NGF alpha, mouse	TCK^GDSGGPLIC	TCA
NGF gamma, mouse	TCE^HDSGGPLIC	TCA
Kallikrein, mouse, mGK-1	TCV^GDSGGPLIC	TCA
Kallikrein, mouse, mGK-2	TCK^GDSGGPLIC	TCA
Kallikrein, mouse, mGK-5	TCA^GDSGGPLIC	TCA
Adipsin, mouse	TCR^GDSGGPLVC	TCC
MCP II, rat	AFM^GDSGGPLLC	TCT
CCPI, mouse	SFR^GDSGGPLIS	TCT
CCPII, mouse	SFE^GDSGGPLIS	TCT
CCP, mouse	SCN^GDSGSPLLC	TCT
PP, human	GCN^GDSGGPLNC	TCT
PAE, dog	TCK^GDSGGPLIC	TCA
TLP, *Drosophila*	ACQ^GDSGGPLVS	TCC
Snake, Drosophila	HCQ^GDSGGPIHA	TCC
V8, *Staph. aureus*	TTG^GNSGSPVFN	TCA
Prothrombin, human	ACE†GDSGGPFVM	AGT
Factor IX, human	SCG†GDSGGPHVT	AGT
Factor X, human	ACQ†GDSGGPHVT	AGC
Protein C, human	ACE†GDSGGPMVA	AGT
Plasminogen, bovine	SCQ^GDSGGPLVC	AGC
Plasminogen, human	SCQ^GDSGGPLVC	AGT
CF C1r, human	ACQ^GDSGGVFAV	AGT
CF C1s, human	SCK^GDSGGAFAV	AGT
TLP (hepsin), human	ACQ^GDSGGPFVC	AGC
apoLP, human	SCQ^GDSGGPLVC	AGT
Related sequences	•	
Haptoglobin, human	TCYGDAGSAFAV	GCG
Protein Z, bovine	ARVWVAGGAVVR	GCG

The active serine is starred and, in most cases, is that shown by experiment; otherwise it has been assigned by similarity of the sequence to the known active sequences. All the sequences are taken from the literature; a fully referenced and updated list may be obtained from the author. ACE, acetylcholinesterase; CU, cutinase; TE, thioesterase; LC, lecithin-cholesterol; FAS, fatty acid synthase; CF, complement factor; TPA, tissue plasminogen activator; UPA, urokinase plasminogen activator; CCP, cytotoxic T cell proteases; MCP, mast cell protease; PP, pancreatic protease; PAE, prostate arginine esterase; TLP, trypsin-like protease; apoLP, apolipoprotein. In, intron; ^, present; †, absent; *, cDNA sequence; ^, gene with no introns.

* From Brenner, 1988.

Interestingly, all serine proteases contain an aspartate at the X_1 position and the X_2 amino acid is almost invariably a glycine (Brenner, 1988). An inspection of deduced amino acid sequence of an esterase from *Spirulina platensis* showed a high degree of homology to that of *P. fluorescens* esterases (Salvi *et al.*, 1994). These sequences contained 11 highly conserved amino acid residues including the G F S Q G sequence which corresponded the G X S X G consensus motif (Salvi *et al.*, 1994). The conserved residues, G D S A G, were also found in *A. calcoaceticus* BD413 esterase (Kok *et al.*, 1993).

At the active site of serine proteinases a structure called " charge-relay system" was discovered (Blow, 1990). Later, this structure was also found in lipase enzymes (Schrag *et al.*, 1991, Brzozowski *et al.*, 1991; Winkler *et al.*, 1990). The charge-relay system or catalytic triad refers to the constellation of aspartic acid-histidine-serine which is formed by hydrogen bonding between ring nitrogen (N_1) of histidine with serine. The other ring nitrogen (N_2) of His is hydrogen-bonded to the carboxylate group of an aspartate (Blow, 1990). This charge relay system make serine nucleophilic and function as active residue to interact with substrates or inhibitors (Brzozowski *et al.*, 1991). Recently, a study in *Pseudomonas* sp. KWI-56 esterase gene revealed that the gene also contained the consensus sequence of active serine residue and their catalytic triads were believed to be composed of Ser, His and Asp/Gly (Shimada *et al.*, 1993).

2.5.5 Determination of activity of esterase

Today there are many reliable methods available for measuring carboxylesterase activities. Perhaps the most widely used assay is the titration of liberated acid equivalents by the pH-stat technique employing mostly aliphatic esters as substrates (Benohr *et al.*, 1966; Krisch, 1963; Benohr and Krisch, 1967; Horgan *et al.*, 1969; Bursch,

1954; Stoops *et al.*, 1969). Acid formation by esterases may also be followed by manometric determination of CO_2 liberated from a bicarbonate buffer (Aldridge, 1953; Connors *et al.*, 1950; Aldridge and Davison, 1952; Aldridge, 1954). Other convenient substrates are the nitrophenyl esters of several acids. The yellow (p- or o-) nitrophenolate ion liberated may be measured in a continuous photometric test (Benohr and Krisch, 1967; Franz and Krich, 1968; Krisch, 1966; Stoops *et al.*, 1969; and Huggins and Lapides, 1947). In addition, spectrophotometric assays in the UV range have been reported for ester (Krisch, 1963; Benohr and Krisch 1967; Barker and Jenecks, 1969; Franz and Krisch, 1968 and Hofstee, 1954) and amide substrates (Franz and Krisch, 1968; Reimann, 1969 and Eckert *et al.*, 1970). After enzymic hydrolysis of naphthyl esters the (α- or β-) naphthol formed may be coupled with diazonium salts to azodyes (Holmes and Masters, 1967; Hunter and Market, 1957; Uriel, 1961; and Ravin and Seligman, 1953). This method is suitable in particular for substrate staining of esterase bands after electrophoretic preparation. Analogously, the formation of aniline from acetanilide is measured by subsequent diazotization (Krisch, 1963). Whereas all methods mentioned so far measure product formation. It should be mentioned that the hydroxamic acid method of Hestrin (1949) measured rather the disappearance of (unhydrolyzed) substrate. However, it was not being used nowadays due to the assay method is less convenient as compared to those previously mentioned.

2.5.6 Possible role and application of esterases

Esterases are widely distributed in animals, plants and microorganisms. In animals, esterases are found in a wide range of tissues and organisms. The enzymes play a variety roles depending on their locations. A role of microsomal human esterase appears to be in the

detoxification of foreign compounds (Riddle *et al.*, 1991). In insects, it is found that esterases involve in detoxification of organophosphorus compounds in mosquito (Mouches *et al.*, 1986). In other insects such as the *Lepidoptera* (moths, butterflies) esterases play an essential role in hydrolyzing the methylester of juvenile hormone involves in metamorphosis and oogenesis (Hanzlik *et al.*, 1989; Wroblewski *et al.*, 1990). In plants, esterases are found to play a role in pectin degradation, Pectinase activity has been found in many plant tissues, indicating a general role in cell wall metabolism such as in pollen tube germination (Mu *et al.*, 1994), fruit ripening in tomato and peach fruits (Hall *et al* 1994., Glover and Brady, 1994).

In microorganisms, esterases are found to play essential roles in physiological significances, unspecific esterases obviously play a role in the detoxification of organophosphous compounds such as parathion, paraoxon, and diisopropylfluorophosphate (an acetylcholinesterse inhibitor, (Omburo *et al.*, 1992; Benning *et al.*, 1994; Dumas *et al.*, 1989). Some esterases are involved in pathogenic invasion of tissues in both plants and animals (Donnelly and Crawford, 1988; Magnuson and Crawford, 1992; Babcock *et al.*, 1992). Morever, esterases have received much attention in industrial importance. Esterases in a lactic acid bacterium, *Enterococcus faecium* play important roles in cheese ripening contributing for typical tastes and flavours (Tsakalidou *et al.*, 1994). In *Acenitobacter calcoaceticus* RAG-1, it was involved in production and release of an extracellular anionic heteropolysaccharide bioemulsifier termed emulsan. A mutant of *A. calcoaceticus* RAG-1 defective in esterase was found to be defective in emulsan production and release, too (Shabtai and Gutnick, 1985).

2.6 Lipases

2.6.1 General properties

Lipases are, as similar to esterases, considered to be carboxylesterases which hydrolyze the reaction of acid and alcohol esters. Lipases constitute a distinct group in the large and sometimes poorly defined esterase family because of their specific properties. The differences between lipases and ordinary esterases are still a matter of controversy. These are the differences in response to various inhibitors (Aldridge, 1953, Desnuelle, 1972) or in activity towards substrates with various lengths of the fatty acyl radicals and the physical states of the substrates to be hydrolysed.

Lipases are traditionally defined as enzymes capable of hydrolyzing esters of oleic acid (Brockerhoff and Jensen, 1974). They are considered to be hydrolytic enzymes due to their abilities to hydrolyse triglycerides. However, definition of a lipase as a hydrolytic enzyme originated primarily from its physiological function of triglyceride hydrolysis. Lipases are effectively able to catalyze not only hydrolytic reactions of ester but also the reverse reaction of synthesis as shown in equation 1.

$$R_1COOR_2 + H_2O \rightleftharpoons R_1COOH + R_2OH \qquad (1)$$

Additionally, the enzymes can also catalyze the transesterification reaction as shown in equation 2.

$$R_1COOR_2 + R_3OH \rightleftharpoons R_1COOR_3 + R_2OH \quad (2)$$

and interesterification reactions (between two acyl substrates such as acid + ester, or ester 1 + ester 2) leading to ester modification which is of interests in lipid application.

The role of water in lipase catalysis is of much importance. As seen from the reversibility of equation (1) at a given temperature, the extent of conversion depends on the amount of water present in the

reaction medium. Thus, hydrolysis is favoured in aqueous solution, while synthesis is favoured in reaction systems with minimal water. Comparing equations (1) and (2), it is clear that selectivity to transesterification over hydrolysis also increases in reaction systems with minimal water (John and Abraham, 1991). This ability of lipases to function in limited water systems under appropriated conditions is, thus, crucial to synthesis efficiency and to the direction of reaction selectivity.

In general, microbial lipases are acidic proteins with pI ranging from 4.0-6.0 (Gilbert *et al.*, 1991; Sugihara *et al.*, 1991; Wohlfarth *et al.*, 1992; Kejima *et al.*, 1994). However, a few microbes have been reported to produce neutral and basic lipases (Palmeros *et al.*, 1994; Lesuisse *et al.*, 1993; Muraoka *et al.*, 1982; Kolting *et al.*, 1983).

2.6.2 Substrate specificity

Studies of various kinds of lipases have indicated that some of them show preferential in hydrolyzing one kind of substrate over others. Substrate specificity of lipase can be divided into three classes; positional specificity or regiospecificity, fatty acid specificity and stereospecificity.

2.6.2.1 Positional specificity

On the basis of their positional specificity, microbial lipases can be divided into two groups. The first group is nonspecific lipases which catalyse the hydrolysis reaction of ester bonds at all three positions in the triglyceride molecules and release free fatty acids and glycerol as final products (Figure 14A). For this reaction, 1,2(2,3)-diglycerides, 1,3-diglyceride and monoglyceride appear as intermediates. The second group is lipases which catalyse hydrolytic reaction of ester bonds at the 1 and 3 positions of the glycerol moiety. With lipases in this group, triglycerides are hydrolyzed to give free fatty acids, 1,2(2,3)-diglycerides and 2-monoglycerides as products (Figure 14B). 1,2(2,3)-

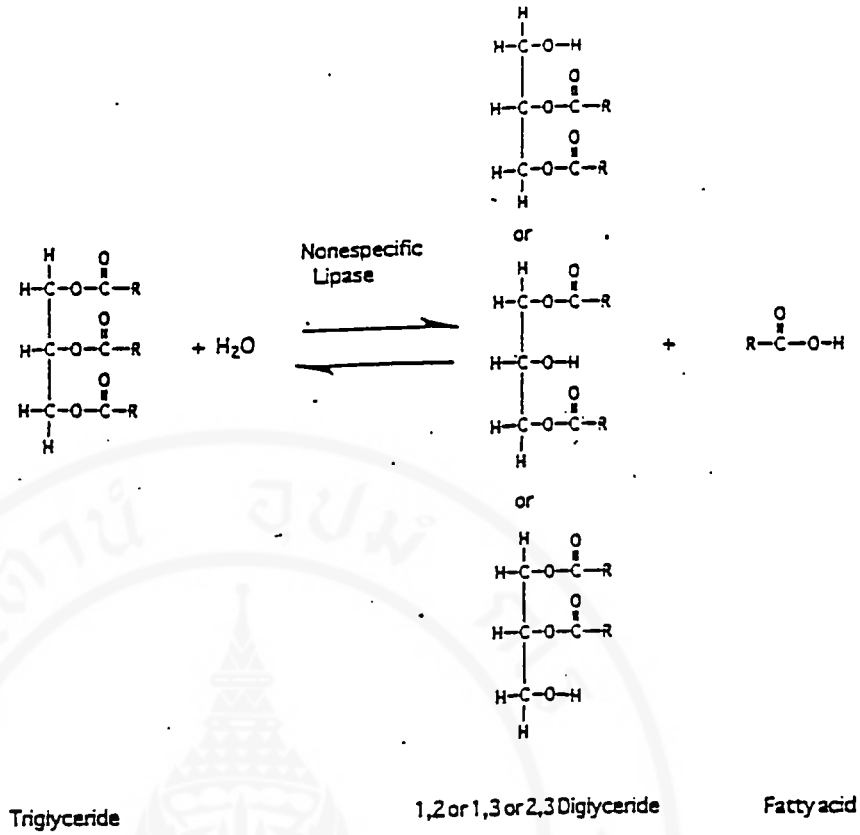


Figure 14 A. Hydrolytic reaction catalyzed by nonspecific lipase (from Sugihara *et al.*, 1991).

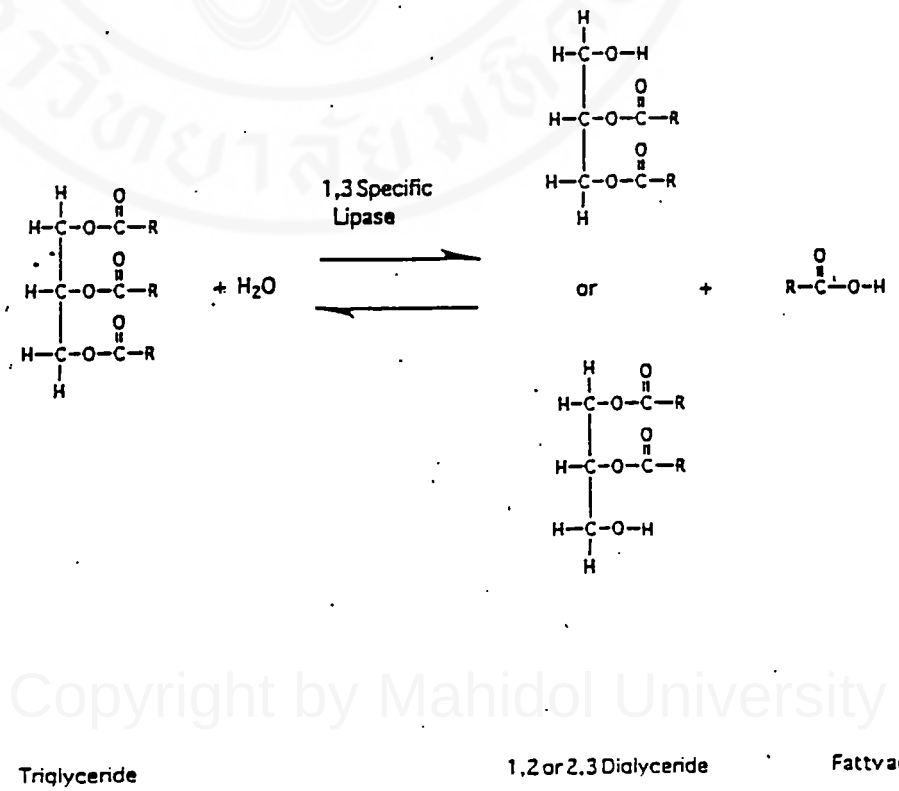


Figure 14 B. Hydrolytic reaction catalyzed by 1,3-specific lipase (from Sugihara *et al.*, 1991).

diglyceride and 2-monogyceride are unstable molecules which will undergo acyl-migration to from 1,3-diglycerides and 1-monoglyceride, respectively (Sugihara *et al.*, 1991; Lesuisse *et al.*, 1993; Ohnishi *et al*; 1994). However, it was reported that prolonged incubation of triglycerides with 1,3-specific lipases would give complete hydrolysis of the substrates (Sugihara *et al.*, 1991; Lesuisse *et al.*, 1993; Ohnishi *et al.*, 1994). As opposite to the previously described lipases, enzyme lipases that are specific to hydrolysis at the 2 position are very rare. One example of such a rare positional specificity of the enzyme is that from *G. candidum* (Sugihara *et al.*, 1991). The positional specificity of some microbial lipases are shown in Table 9. This positional specificity can be determined by analysis of the products formed with the use of triglyceride as a substrate. The products were analysed by using thin-layer chromatography on silica gel which could separate 1,2(2,3)-diglyceride from 1,3-diglyceride and 2-monoglyceride from 1-monoglyceride.

2.6.2.2 Stereospecificity or enantioselectivity

The presence of stereospecificity in several triacylglycerol lipases from animals and microorganisms toward enantiomeric alkyl-monoacylglycerol, an analogue of diacylglycerol, has been reported (Akesson *et al.*, 1983). Recently, many researchers demonstarted that microbial lipases can transesterify and esterify triglycerides, alcohols and chiral acids sterospecifically in nearly anhydrous organic solvent (Ihara *et al.*, 1991; Ilizumi and Murakami, 1994 and Katz *et al.*, 1993).

Table 9. Positional specificity of microbial lipases.

Microorganisms	Specificity	Ref.
Pseudomonas aeruginosa EF2	1,3 specific	Gilbert *et al* 1991
P.fragi	1,3 specific	Kugimiya *et al* 1986
P.fragi NRRL B-25	1,3 specific	Mencher *et al* 1967
P.cepacia	nonspecific	Sugihara *et al* 1992
P.fluorescens SIKW1	1,3 specific	Lee *et al* 1993
Bacillus subtilis 168	1,3 specific	Lesuisse *et al* 1993
B.thermocatnulatus	1,3 specific	Schmidt-Dannert *et al* 1994
Bacillus sp.	1,3 specific	Sugihara *et al* 1991
Serratia marcescens Sr41 8000	1,3 specific	Matsumae *et al* 1994
Propionibacterium acnes	nonspecific	Ingham *et al* 1981
*Staphylococcus aureus*26	nonspecific	Muraoka *et al* 1982
S.hyicus	nonspecific	van Oort *et al* 1989
Aspergillus oryzae L1	nonspecific	Ohnishi *et al* 1994
Penicillium simplicissimum	nonspecific	Sztajer *et al* 1992
P.expansum DSM 1994	nonspecific	Stocklein *et al* 1993
Mucor javanicus	1,3 specific	Ihara *et al* 1975
Trichosporon fermentans WUC-12 (lipase I&II)	nonspecific	Chen *et al* 1994
Fusarium heterosporum	1,3 specific	Shimada *et al* 1993
G. candidum	2-specific	Sugihara *et al* 1993

2.6.2.3 Fatty acid specificity

In the studies of fatty acid specificity, only monoacid triglycerides are used. This is to avoid the problem arises due to the physical state of the lipids. For example, the triglycerides of saturated long-chain fatty acids are solid at normal assay temperature while unsaturated fatty acids and short- to medium- chain saturated fatty acids are in liquid form. It has been shown that solid triglycerides are only slowly hydrolyzed by microbal lipases (Sugiura and Isobe, 1975b). It was also not practical to be used in the normal assay system. On the other hand, simple alkyl esters of fatty acid such as methyl esters are convenient substrates for the determination of fatty acid specificity. This was because they were liquid at normal assay temperature although the hydrolysis

reaction rate was usually slow as compared to that obtained with glycerides (Sugiura and Isobe, 1975b).

Studies on fatty acid specificity of several lipases have been reported. Almost all of them showed optimal specificity on medium-chain fatty acids (C6-12) (Table 8). Results made from several reports on a lipase from *G. candidum* that it preferentially released long-chain fatty acids containing a *cis* double bond in 9 position such as oleic acid (*cis*9-18:1), linoleic acid (*cis*9, 12-18:2), linolenic acid (all *cis*9,12,15-18:3) and palmitoleic acid (*cis*9-16:1) (Charton *et al.*, 1991). From the above finding and also with the results shown when incubated microbial lipases with common natural oils and fats indicating that most of them had low fatty acid specificity (Sugihara and Isobe, 1975b). However, there are exceptions for the long-chain (>C18) polyunsaturated fatty acids found in fish oils and the short-chain fatty acids found in milk fat (Sugihara and Isobe, 1975a). Fatty acid specificity of some microbial lipases are shown in Table 10.

Table 10. Fatty acid specificity of microbial lipases.

Microorganisms	Number of Carbon in Fatty acid Chain	Ref.
Pseudomonas fluorescens	10,16	Sugiura *et al* 1977
P.fluorescens SIKW1	6,8	Lee *et al* 1993
P.cepacia	<12	Sugihara *et al* 1992
P.cepacia	>8	Bornscheuer *et al* 1994
Pseudomonas sp KWI-56	10,14	Iizumi *et al* 1990
Aeromonas hydrophila	6,8	Anguita *et al* 1993
Bacillus subtilis 168	8	Lesuisse *et al* 1993
Bacillus sp.	8,10	Sugihara *et al* 1991
Serratia marcescens Sr41 8000	3-7	Matsumae *et al* 1994
Chromabacterium viscosum (lipase I)	4-12,18	Horiuti *et al* 1977
C.viscosum (lipase II)	6-12,18	Horiuti *et al* 1977
Staphylococcus aureus 226	4-12	Muraoka *et al* 1982
Rhizopus japonicus NR4000	8,10	Suzuki *et al* 1996
P.japonicus KY521 (lipase I&II)	10	Aisaka and Terada 1981
Geotrichum candidum (lipase I&II)	8	Sugihara *et al* 1990

2.6.3 Gene cloning and conserved amino acid sequences

Various lipase genes have been cloned from microorganisms by using either lipolytic activity or oligonucleotide probes corresponding to N-terminal amino acid sequence for screening the recombinant clones. Comparison of the cloned lipase genes revealed the variation of sizes of the ORF and translational products. Molecular weights of microbial lipases vary from 22777 for *B. subtilis*168 lipase (Dartois *et al.*, 1992) to 71832 for *Staphylococcus hyicus* (Got *et al.*, 1985). Results from comparison of numerous sequences showed that lipases shared very little homologies except within the presumed catalytic region which containing the conserved pentapeptide Gly-X-Ser-X-Gly (Table 11). This structure is reported to contain the nucleophilic serine residue essential for catalysis (Davis *et al.*, 1990; Moreau *et al.*, 1991) and is part of the catalytic triad Ser/Asp/His equivalent to the active residues seen in the serine proteases and also esterases (Brady *et al.*, 1990; Blow, 1990).

2.6.4 Methods for determining lipase activity

2.6.4.1 Qualitative detection

Qualitative detection for lipolytic activity can be done by use of agar plates containing substrates for lipases with or without an indicator. The most widely used methods are Tributyrin agar plate (Anguita *et al.*, 1993), Calcium-Triolein agar plate (Wohlfarth and Winkler, 1988) and Rhodamine B agar plate (Kouker and Jaeger, 1987). The first method uses tributyrin, a four-carbon chain triglyceride, homogenously emulsified in liquid agar media before pouring. Lipase-producing microorganisms can be identified by the formation of clear zones around their colonies due to hydrolysis of tributyrin by the enzyme. However, the disadvantage of this method is that esterases can also hydrolyze tributyrin and give the same result (Dowson *et al.*, 1986).

Table 11. The cloned lipase genes from various microorganims and their conserved catalytic sequence.

Microorganism	Protein sequence*										Reference
B. subtilis 168	V	D	I	V	**A**	H	**S**	M	**G**	G	Dartois *et.al.*, 1992
B. stearothermophilus	I	A	V	A	**G**	L	**S**	L	**G**	G	Kugimiya *et al.* 1987 Jap. Patent S62-233531
Staphylococcus hyicus	V	H	F	I	**G**	H	**S**	M	**G**	G	Gotz *et al.*, 1985
S. aureus	V	H	L	V	**G**	H	**S**	M	**G**	G	Lee and Iandolo, 1986
Pseudomonas fragi	V	N	L	I	**G**	H	**S**	Q	**G**	A	Kugimiya *et al.*, 1986
P.cepacia	V	N	L	V	**G**	H	**S**	Q	**G**	G	Jorgensen *et al.*, 1991
P.aeruginosa I and II	V	N	L	I	**G**	H	**S**	H	**G**	G	Nishioka *et al.*, 1990; Wohlfarth and Winkler, 1988
P. fluorescens	V	N	L	V	**G**	H	**S**	Q	**G**	G	Patent application of Amano : EPO0331376 (1989)
P. glumae	V	N	L	I	**G**	H	**S**	Q	**G**	G	Patent application Unilever WO 91/00910 (1991)
Moraxella I	L	G	A	I	**G**	W	**S**	M	**G**	G	Feller *et al.*, 1990
Moraxella II	T	H	V	G	**G**	N	**S**	M	**G**	G	Feller *et al.*, 1991 a
Moraxella III	I	V	L	S	**G**	D	**S**	A	**G**	G	Feller *et al.*, 1991 b
Mucor miehei	V	A	V	T	**G**	H	**S**	L	**G**	G	Boel *et al.*, 1988
Geotrichum candidum I	V	M	I	F	**G**	E	**S**	A	**G**	A	Nagao *et al.*, 1990
G. candidum II	V	M	I	F	**G**	E	**S**	A	**G**	A	Shimada *et al.*, 1990
G. candidum	V	D	I	V	**A**	R	**S**	M	**G**	G	Vandamme *et al.*, 1987 Eur. Patent 0243338
B. subtilis WRRL-B558 ORF2 lipase gene	V	D	I	V	**A**	H	**S**	M	**G**	G	This study
B. subtilis WRRL-B558 ORF1 lipase gene	F	T	F	G	**D**	G	**S**	A	**G**	V	This study

* Residues which are strictly conserved are in bold character.

Calcium-Triolein medium containing olive oil and calcium chloride. The medium can be used more specifically to detect for lipolytic activity (Wohlfarth and Winkler, 1988). Lipase-producing microbes can be isolated by the formation of white pellet of calcium oleate around the oil droplets in areas where colonies grow (Wolhfarth and Winker, 1988). Nonetheless, this method is sometime difficult to detect for the microorganisms which produce low amount of lipases. The more sensitive method would be that use a medium containing Rhodamine B as an indicator. This method use olive oil as a subtrate for the lipase reaction. Lipolytic activity can be visualized from orange fluoresence of the product, produced from an unknown reaction of free fatty acid and Rhodamine B, under a UV lamp (Kouker and Jaeger, 1987).

2.6.4.2 Quantitative detection

A. Titrimetric method

Different substrates have been used for the titration assay of lipase. However, the preferred methods use emulsion of insoluble triglycerides such as triolein. Olive oil can be substituted for triolein due to its low cost and its substrate nature is as nearly good as that of triolein. Stable emulsions of triolein or olive oil can be prepared by vigorously mechanic or ultrasonic mixing of the substrate. Calcium is usually added to the incubation mixture to precipitate the released fatty acids as their calcium soaps. This leads to a prevention of the inhibition on lipolysis by soluble soap (Macrae, 1983). At the end of the incubation period, the reaction is stopped by an addition of alcohol or acetone and the released fatty acids are titrated either using indicator such as thymol blue and phenophthalene or with a pH-stat at a set end point of pH 9.0.

B. Colorimetric method

Several colorimetric methods have been developed to improve the lipase assay sensitivity. The formation of copper soaps for detection of free fatty acids is one of the often used methods. The major disadvantage of this method is that it requires multiple steps of fatty acid extraction and copper soap formation h leads to the decrease in accuracy and sensitivity (Schmidt-Dannert *et al.*, 1994). Alternatively, substrates which yield chromogenic products produced directly from lipase catalyzed hydrolysis reaction such as naphthyl esters and p-nitrophenyl esters can be used. The most commonly used substrates are β-naphthyl acetate, β-naphthylcaprylate, p-nirophenyl palmitate. β-naphthyl ester is hydrolyzed into a carbon chain and β-naphthol which form an azo dye complex with Fast Blue BB salt. Then, the lipolytic activity can be measured by detection of absorbance at 540 nm (McKeller *et al.*, 1986). For p-nitrophenyl ester chromogenic p-nitrophenol is measured at absorbance of 410nm after hydrolysis reaction catalyzed by lipase. By comparing with their standard curves the optical density value of both β-naphthol-azo complex and p-nitrophenol can be converted to unit of products released per minute.

2.6.5 Possible role and application of lipases

Based on their three basic catalytic activities, such as hydrolysis, esterification and transesterification, many lipase applications have been developed in food industry, pharmaceutical industry, detergent industry, cosmetic and perfume industry and the treatment of sewage and biological wastes (Fernandezgarcia *et al.*, 1994; Akoh, 1994; Maase and Tilburg, 1983).

2.6.5.1 Lipase in food industry

Lipases are being used in oil and fat industry to modify triglycerides (Harwood, 1989; Li and Ward, 1993; Marangoni *et al.*, 1993). A good specific example of the use is the transesterification of cheap palm oils to produce cocoa butter equivalents. By use of the microbial lipase, transesterification of stearic acid and palmitic acid at 1,3-position of triglyceride molecules in palm oil resulted in the increase of the cocoa butter-like product (Harwood, 1989).

For the production of fatty acids, lipase-catalyzed hydrolysis of triglyceride has gained an increase in interest in industry because of its advantage over conventional high-pressure process. Furthermore, the enzymatic process gives cleaner products due to the more specific reaction (Linfield *et al.*, 1984 and Taylor *et al.*, 1986). Lipases can also be used to produce monoglyceride (MG), an important group of emulsifying agents widely used in food industry. Certain fatty acids released from milk are important in cheese ripening giving a characterisitc flavour. Specific flavours of numerous cheeses are due to various microbial lipases (Fernandezgarcia *et al.*, 1994; Stead, 1986; Peppler, 1976 and Arnold *et al.*, 1975). Lipases can also be used to modify fats in the bakery goods. The use of modified fats improves the flavour, color, softness and structure of bread (Sztajer and Zboinska, 1988). Partial hydrolysis of beef fat by lipases improves the properties of pet-food without influencing other components (Sztajer and Zboinska 1988).

2.6.5.2 Lipases in pharmaceutical industry

Recently, there has been a great deal of interest in biosurfactant such as a group of sugar alcohol esters produced from lipase-catalyzed reaction (Chopineau *et al.*, 1988; Akoh, 1994). These sugar alcohol esters possed a number of potential advantages over the

chemically manufacture counterparts, including lower toxicity, biodegradability, a wide variety of structures and ease of synthesis from inexpensive, renewable feedstocks. From the economic standpoint, biosurfactant can be produced from sugar and vegetable oil, both are surplus agricultural products. Many researchers tried to develop conditions for the synthesis of biosurfactants by using lipase-catalytic processes in various organic solvents (Chopineau *et al.*, 1988 and Schlotterbeck *et al.*, 1993) and under reduced pressure (Ducret *et al.*, 1995). For example, *C. viscosum* lipase was used for production of a number of biosurfactants from various plant and animal oils in dry pyridine. The products have been identified as primary monoester of sugar alcohols and fatty acids and found to exhibit excellent property to reduce interfacial and surface tension (Chopineau *et al.*, 1988). Consequently, biosurfactants may have applications in numerous areas, particularly in oil recovery, cosmetics and pharmaceutical preparations (Schlotterbeck *et al.*, 1993; Akoh, 1994). Another application of lipases in pharmaceutical industry is the use of an acid-stable microbial lipase, with therapeutic potential, in treating pancreatic lipase insufficiency in human (Newmark, 1988).

2.6.5.3 Lipases in laundry detergent industry

The importance of lipase in laundry detergent has been enhanced due to a decrease in usage of chemicals in detergents because chemical pollution is of environmental concerns (Andree *et al.*, 1980; Tatara *et al.*, 1985). At present, prospects for using lipases for laundry purposes appear to be good because of their high acitivity and stability under the conditions employed with laundering detergents (Newmark, 1988).

2.6.5.4 Lipases in paper manufacturing

The use of lipase enzymes in the paper manufacturing industry is expected to increase. In addition to the use of cellulases and ligninases, the use of lipases to improve the paper process has recently been explored (Skjold-Jorgenson and Lange, 1991). Deposition of resins on drying cylinders is a serious problem affecting paper quality and productivity. Treatment of the pulp with lipases leads to a considerable improvement in productivity and sustained high quality paper with less frequent cleaning of the drying cylinders being required. (Skjold-Jorgensen and Lange, 1991).

CHATER III
MATERIALS AND METHODS

3.1 Chemicals, reagents and media

All chemicals and solvents used in this study were of analytical or biotechnological grades and were obtained from Sigma (USA), Merck (Germany), Amersham (UK), Ameresco (USA) and BRL (USA). Restriction and modifying enzymes were acquired from BRL (USA), Boehringer Mannheim (Germany), and Pharmacia (Sweden). The acetic acid determination kit , the radiolabelling kit and the sequencing kit were purshased from Boehringer Mannheim (Germany), Promega (UK) and Pharmacia (Sweden), respectively. Partially purified acetylesterase (orange peel) and antibiotics were supplied by Sigma (USA). The radiolabelled compounds [^{32}P]-αdATP and [^{35}S] ATP were obtained from Amersham (UK). Microbiological media were obtained from Difco (USA) or Merck (Germany) and prepared according to the manufacturers' recommendation. Also enzymatic reactions used in this research work were performed as described in instructuion manuals provided by the suppliers.

3.2 Microorganisms and maintenance conditions.

Bacterial strains and plasmids used in this study are listed in Table 12. *Bacillus subtilis* WRRL-B-558 was obtained from National Center for Agricultural Utilization Research, USA. *Staphylococcus aureus* ATCC 25923 was kindly provided by Assoc. Prof. Malai Vorachit of Clinical Pathology Department, Ramathibodi Hospital, Thailand. pTTQ18 vector was a gift from by Dr. Watanalai Panbangred of the Department of Biotechnology Faculty of Science, Mahidol University, Bangkok, Thailand.

Table 12. Bacterial strains and plasmids used in this study.

Strains or Plasmids	Characteristic	Source or Reference
B. subtilis WRRL-B558	Wild type, DNA donor for cloning	NCAUR*
S. aureus ATCC 25923	Cephalosporin C sensitive organism, (a test organism)	ATCC
Escherichia coli XL-1Blue	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac* F' (*proAB*$^+$ *lacI*^{q} *lacZ* ΔM15) Tn (tet^r)	Stratagene
Escherichia coli JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ (*lac* - *proAB*) F' (*tra* D36 *proAB*$^+$ *lacI*^{q} *lacZ* ΔM15)	Stratagene
pBluescript (pBS)	2.9 Kb, *lacZ* promotor, Ap^r	Stratagene
pTTQ18	4.5 Kb, ptac promotor, rrnBt112terminator, Ap^r	Stark, 1987
pBT1	3.0 Kb *Bam*HI/*Sau*3AI fragment in pBS, Ap^r	this study
pBT4	2.5 Kb *Sma*I-*Sma*I fragment in pBS, Ap^r	this study
pBT5	2.5 Kb *Bst*XI-*Bst*XI fragment in pBS, Ap^r	this study
pBT6	2.5 Kb *Hin*dIII-*Hin*dIII fragment in pBS, Ap^r	this study

Table 12. Bacterial strains and plasmids used in this study (continued)

Strains or Plasmids	Characteristic	Source or Reference
pBT7	1.5 Kb *Bst*XI-*Hin*dIII fragment in pBS, Ap^r	this study
pBT8	1.4 Kb *Sal*I-*Sal*I fragment in pBS, Ap^r	this study
pBT9	1.5 Kb *Bst*XI-*Sal*I fragment in pBS, Ap^r	this study
pBT10	1.0 Kb *Bst*XI-*Sal*I fragment in pBS, Ap^r	this study
pBT11	0.6 Kb *Eco*RI-*Eco*RI fragment in pBS, Ap^r	this study
pBT12	0.6 Kb *Bst*XI-*Sal*I fragment in pBS, Ap^r	this study
pBL1	2.0 Kb *Hin*dIII-*Hin*dIII fragment in pTTQ18, Ap^r	this study
pBL2	2.0 Kb *Hin*dIII-*Hin*dIII fragment in pTTQ18 reverse orientation of pBL1, Ap^r	this study
pTL7	0.9 Kb *Hin*dIII-*Eco*RI fragment in pTTQ18 vector, Ap^r	this study
pTE12	0.6 Kb *Eco*RI-*Eco*RI fragment in pTTQ18vector, Ap^r	this study
pTLE28	1.5 Kb *Hin*dIII-*Eco*RI fragment in pTTQ18, Ap^r	this study
pTEL25	1.5 Kb *Eco*RI-*Hin*dIII fragment in pTTQ18, Ap^r	this study

* NCAUR = National Center for Agriculturel Utilization Research, USA

The microorganisms were usually cultivated in LB broth or on LB agar plates at 37°C for overnight to 24 hours. The test organism, *S. aureus* ATCC 25923, was grown on tryptic soy broth/agar at 37°C for overnight before use. LB agar supplemented with 50 μg/ml of ampicillin (Ap) was used for selection and maintenance of the recombinant *E. coli* clones.

If required, the freshly grown cultures on agar plates were kept at 4°C for later use. For a longer term of culture preservation, the freshly grow culture broth was mixed with glycerol to give a final concentration of 15% and the mixture was then aliquoted into small Eppendorf tubes and kept at -80°C.

3.3 Indicator media for detecting lipase producers

3.3.1 Tributyrin agar (Anguita et al 1993)

Tributyrin agar contained 1% tributyrin (1,2,3-tributyrylglycerol) in nutrient broth solidified with 2.0% (w/v) agar. After sterilization, the tributyrin (in the sterilized medium) was emulsified by vigorous stirring until homogenized appearance was seen and cooled to about 60°C, then, 20 ml of medium was poured into each petri dish. Lipase and/or esterase production on these plates was detected by clear zone formation around producer colonies.

3.3.2 Rhodamine B agar plate (Kouker and Karl-Erich, 1987)

After sterilization, nutrient agar containing 1% NaCl was allowed to cool down to about 60°C, then 0.04 mM Rhodamine B (sterilized by filtration) and 2% (v/v) olive oil were added and emulsified by vigorous stirring and mixing for 1 min. The medium was left for 10 min at 60°C to reduce foaming, after which 20 ml was poured into each petridish. Colonies producing lipase activity were detected by formation of fluorescent zone around the producer colonies under UV irradiation.

3.3.3 Calcium-Triolein agar (Wohlfarth and Winkler, 1988)

This media was also used for detection of lipase production. After sterilization, nutrient agar were allowed to cool down to about 60°C, then sterile 1% (v/v) olive oil and 0.01% (w/v) $CaCl_2$ were added with vigorously stirring and 20 ml the of mixture was poured into each petri dish. Colonies producing lipase were detected by formation of white precipitates of calcium salt around their colonies.

3.4 Enzymatic assay

3.4.1 Esterase activity assay

This method was developed according to Castro *et al.* (1992). 200 µl of the substrate (500 µg/ml α-naphthylacetate dissolved in ethanol : water in ratio of 1:2) was incubated with 100 µl the enzyme sample and 100 µl 50 mM phosphate buffer pH 7.0, at 37°C for exactly 30 min . The reaction was stopped by adding 50 µl of 4.0 M urea and made to a total volume of 2.8 ml with 50 mM borax in 40% ethanol (v/v). Subsequently, 200 µl of 1 mg/ml Fast Blue BB dye was added, mixed and incubated at room temperature for 10 min. The reaction mixture was measured with a spectrophotometer (Milton Roy Spectronic 301) at 515 nm wavelength against a blank which contained 50 mM phosphate buffer pH 7.0 or LB broth instead of the enzyme in the reaction mixture.

One unit of esterase activity was defined as the amount of enzyme required to released 1 µmole α-naphthol per ml per min. Standard curve was set up by using α-naphthol at concentrations between 0-0.2 µg/ml instead of the enzyme in the reaction mixture.

3.4.2 Lipase activity assay

The precedure of assay was that described by Kordel *et al.* (1991). The reaction reagent consisted of 1 part of solution A and 9 parts of solution B. Solution A was composed of (0.062 g of p-nitrophenylpalmitate (pNPP) in 10 ml isopropanol. The mixture was sonicated for 6 min before use. Solution B was composed of 0.4% (v/v) Triton X-100 and 0.1% (w/v) gum arabic in 50 mM Tris HCl. pH 8.0. The reaction reagent was prepared freshly before assay. A 200 µl of appropriately diluted enzyme solution was added to the 1800 µl reaction reagent . The reactions were measured at 410 nm wavelength after incubating at 37°C for 15 min. An OD_{410} of 1.0 is equivalent to 9.7 nmole of p-nitrophenol (pNP) released from pNPP per min. One unit of lipase activity was defined as nmole of pNP released per min per ml of the enzyme.

3.5 Cephalosporin C esterase production of *B. subtilis* WRRL-B558

The enzyme activity of the culture broth of *B. subtilis* WRRL-B558 or its cell suspension towards cephalosporin C was determined by assaying amount of product formed and the cephalosporin C remaining. Quantification was done by using both thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Culture of *Bacillus subtilis* WRRL-B558 was grown in LB broth at 30°C for 42 hours before being used for the assay. A commercial acetylesterase enzyme was used as a positive control especially for producing deacetylcephalosporin (DC) to be used as a reference in TLC and HPLC.

3.5.1 Thin layer chromatography (TLC)

The reaction mixtures were composed of 200 mg/ml cephalosporin C in 0.1M phosphate buffer pH 7.0 containing either 20 µl of commercial acetylesterase enzyme or 0.5 ml of *B. subtilis* WRRL-B558

broth or cell suspension. They were incubated at 37°C for 1 h. The mixtures were centrifuged and their supernates, 20 µl each, were applied onto silica gel 60-F_{254} plate (Merck). Also, standard cephalosporin C, and supernates of *B. subtilis* WRRL-B558 broth and cell suspension supernate were used to run in parallel to the tests and used as references. The TLC plate was developed with mixture of butanol : acetic : water (4:1:4). Spots of cephalosporin C and deacetylcephalosporin C were visualized under a UV light (254 nm).

3.5.2 High pressure liquid chromatography (HPLC)

A mixture of 0.5 ml phosphate buffer pH 7.0 containing 10 mg/ml cephalosporin C and either 20 µl of commercial acetylesterase enzyme or 0.5 ml of *B. subtilis* WRRL-B558 broth or cell suspension was incubated at 37°C for 1 h. At the end of incubation, the mixtures were centrifuged and the supernates were frozen at -20°C. Before loading to HPLC, the samples were thawed and applied, 20 µl each, to the reverse-phase HPLC column (Sepherisorb S5 OD52, BIORAD). Elution was performed with 0.002 M K_2HPO_4-KH_2PO_4 buffer pH 7.0. The absorbance was monitored at 265 nm.

3.6 Isolation of DNA

3.6.1 Isolation of bacterial genomic DNA (Miura, 1967)

The overnight culture of *B. subtilis* WRRL-B558 in LB broth was used as an inoculum (10% v/v) to inoculate into 200 ml LB broth and, then, the mixture was incubated at 37°C with shaking for another 4 h. The cell pellet was harvested and washed once with 50 ml TE buffer (25 mM Tris pH 8.0 and 5 mM EDTA). The cell pellet was resuspended with 20 ml TE buffer and lysed with 20 mg lysozyme at 37°C for 30 min. Then, 0.5 ml of 20% SDS and 1mg/ml Proteinase K were added and mixed slowly by inversion. After incubating at 37°C for 30 min, equal volume of phenol-

chloroform solution (1:1, v/v of phenol (pH 7.0) and chloroform) was added and mixed thoroughly. The mixture was centrifuged at 10,000 rpm for 5 min and the supernate was transferred to a new polypropylene tube. The step of phenol-chloroform extraction was repeated several times until the white interface disappeared. Then the aqueous phase was extracted with equal volume of chloroform. After centrifugation the aqueous supernate was then added with 2 volumes of cold ethanol and 10% (v/v) of 3 M sodium acetate. The mixture was kept at -20°C for overnight. The *B. subtilis* DNA was collected, after centrifugation at 10,000 rpm for 10 min, by using a sterile stirring rod. The DNA was washed once with cold 70% ethanol and centrifuged at 10,000 rpm for 10 min. The DNA was air dried and resuspended with 1 ml TE buffer. This DNA solution was treated with 1 mg/ml RNase at 37°C for 30 min and then extracted with phenol-chloroform repeatly until the white interface disappeared. The DNA in the aqueous solution was precipitated with cold absolute ethanol and washed again with cold 70% ethanol and air dried. This purified DNA was resuspended in 0.5 ml TE buffer and kept at 4°C.

3.6.2 Plasmid DNA preparation

3.6.2.1 Small scale preparation (Holmes and Quingley, 1981)

For *E. coli*, small scale plasmid preparation was done using the STET miniprep as a routine technique. Briefly, a single colony was inoculated into 5 ml of LB broth containing 50 μg/ml ampicillin, incubated overnight at 37°C with vigorous shaking. The cell pellet was collected from the overnight culture by centrifuging a few times at 10,000 g for 2 minutes each. The pellet was resuspended in 350 μl of STET solution (8% sucrose, 5% Triton X-100, 50 mM EDTA and 50 mM Tris HCl, pH 8.0) by vortexing.

A 25 µl of freshly prepared 10 mg/ml lysozyme solution was added to the cell suspension and immediately boiled for 40 seconds. The mixture was , then, centrifuged at 10,000 rpm for 10 min to pack the slimy pellet. The pellet was removed by picking with a toothpick. Then the aqeous mixture was extracted with phenol-chloroform repeatedly to denature the proteins. Then, the DNA in the aqueous supernate was precipitated with equal volume of cold 100% ethanol. The pellet was washed once of cold 70% ethanol, dried and resuspended with 20 µl of TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA). To remove RNA, 1 µl of 10 mg/ml boiled RNase A was added and incubated at 65°C for 10 min. The plasmid that was prepared by this method can be used for restriction enzyme analysis, cloning and sequencing.

3.6.2.2 Large scale preparation

This method was modified from the alkaline lysis method of Birnboim and Doly (1979) as follows. A 250 ml of overnight culture broth was centrifuged at 5,000 rpm for 5-10 min. The cell pellet was harvested and thoroughly suspended in 8 ml of Solution I (25 mM Tris HCl, pH 7.5, 10 mM EDTA, 15% sucrose, 2 mg/ml lysozyme), then incubated on ice for 20 min. A 16 ml of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added to the cell suspension, the mixture was mixed immediately and incubated on ice for another 10 min, to lyse the cells (SDS denatures bacterial proteins and NaOH denatures chromosomal and plasmid DNA). The mixture was subsequently neutralized by adding 12 ml of Solution III (3 M potassium acetate pH 5.2), mixed and maintained on ice for 5-10 min. The supernatant was collected by centrifugation at 10,000 rpm for 15-20 min, then filtered through layers of gauze cloth. DNA in the filtrate was then precipitated by adding 0.7% volume of isopropanol and centrifuged. The precipitate was dissolved in 10 ml TE and 0.5 volume of 7.5 M. ammonium acetate was

added, mixed and centrifuged at 10,000 rpm for 10 min. With all these steps, most of RNA would be precipitated while DNA would be in the solution. The supernatant was transferred to a new tube. The remaining RNA was digested by adding 50-100 μg/ml RNase and incubated at 65°C for 10 min. The protein and other impurities were removed by extraction with an equal volume of phenol : chloroform (1:1), vigorously shaked and centrifuged at 10,000 rpm 5 min. The process was repeated until the white interface disappeared. The final extraction was washed with equal volume of chloroform, vortexed and recentrifuged. The supernatant DNA was precipitated with 2 volumes of cold absolute ethanol, mixed and kept at -20°C for 1 h. to overnight. The DNA was collected by centrifugation at 10,000 rpm 5-10 min, then rinsed twice with cold 70% ethanol. The pellet was dried and dissolved with 0.5-1 ml TE and the DNA solution was stored at 4°C or -20°C.

3.7 DNA manipulation and analysis

3.7.1 Restriction enzyme digestion

Restriction enzyme digestion of plasmids and chromosomal DNA were performed according to the manufacturer's recommendations (BRL; Boehringer Mannheim). In principle, the reaction mixture was consisted of buffer (of particular pH) which was appropriated for particular restriction enzyme, the restriction enzyme (not more than 10% of total volume) and the DNA. The reactions was terminated either by heating at 65° C for 10 min or extracting with phenol-chloroform depended on the nature of the restriction enzyme. When a double digestion of DNA with 2 different buffer conditions was required, an ethanol precipitation was performed after the first enzyme digestion.

3.7.2 The agarose gel electrophoresis

To analyse and separate the DNA fragments, the electrophoresis of digested DNA using the horizontal type of mini-gel electrophoresis chamber (Mupid, minigel electrophoresis set, Toyo Co., Ltd. Japan) was used. DNA samples were mixed with 1/6 volumes of a DNA loading buffer (50% sucrose, 50 mM EDTA pH 8.0 and 0.05% Bromphenol blue) prior to loading into wells of 0.8% agrose gel in TBE buffer (Ameresco, USA). Electrophoresis was carried out at a constant voltage of 100 volts at room temperature for 20-30 min. The gel was soaked into 0.5 μg/ml ethidium bromide for 5-10 min before the DNA bands were visualized on a UV-transilluminator (Foto/PrepII, Fotodyne) and photographed using a polaroid camera (MP-4 Polaroid, Fotodyne).

3.7.3 Recovery of DNA fragments from an agarose gel

Plasmid DNA was digested with one or two restriction endonuclease(s) to yield interesting fragments. Isolation of the DNA fragments from the agarose gel was done by the Prep-A-Gene kit (BIORAD) according to manufacturer's recommendation. Briefly, the desired DNA band was excised with a razor blade in the smallest possible size and put into a 1.5 ml Eppendorf tube three volumes of the binding buffer were added onto the sliced gel and the tube was ,then, incubated at 37°C-55°C to dissolve the gel. 5 μl of matrix (1 μg DNA : 5 μl matrix was used) was added into the tube, mixed and standed at room temperature for 5-10 min with interval agitation for better purification. The tube was centrifuged at 10,000 rpm for 30 seconds and the supernatant was discarded. Then 250 μl (50 volumes of matrix) of the binding buffer was added, mixed and standed for 5-10 min. After centrifugation, the supernatant was discarded and the matrix was washed twice with 250 μl (50X volumes of matrix) of cold wash buffer (80%

ethanol in TE buffer). The matrix was completely dried and resuspended with elution buffer (distilled water or TE). The tube was then incubated at 37 °C-55°C for 5 min and centrifuged. The aqueous DNA was collected. The elution step was repeated twice to recover most DNA as much as possible.

3.7.4 Molecular cloning

B. subtilis DNA was partially digested with *Sau*3AI restriction endonuclease, 1:50 dilution of *Sau*3AI incubated with 30 μg of *B. subtilis* DNA with a suitable buffer at 37°C for 15 min. The reaction was, then, immediately extracted twice with phenol-chloroform solution. After the final extraction , the DNA was extracted with equal volume of chloroform. The purified DNA was loaded onto 0.8% agarose gel electrophoresis to examine the digestion pattern of DNA. A 10 μg of pBluescript vector was completely digested with *Bam*HI restriction enzyme and dephosphorylated with an alkaline phosphatase (Pharmacia, Sweden) in a suitable buffer for 1 h. at 37° C to prevent self ligation. The digested-dephosphorylated vector DNA was extracted with phenol-chloroform and checked for extent of digestion with agarose gel electrophoresis. A 1 : 3 ratio (μg/μg) of *B. subtilis* DNA and vector DNA was mixed and precipitated with cold absolute ethanol at -20°C overnight. The mixed DNA was washed with cold 70% ethanol and dried. The resuspended mixed DNA was ligated with 1 unit of T4 DNA ligase in a ligation reaction (30 mM Tris HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT and 0.5 mM ATP). The reaction was usually incubated at room temperature for 1-3 h. and then transferred to 4°C for overnight.

For subcloning experiment, the interesting fragments were recovered from the agarose gel as described previously and ligated to the completely digested-dephosphorylated vector in the traditional ratio of 3:1.

3.8 Transformation of recombinant plasmids into *E. coli*

3.8.1 Preparation of competent *E. coli*

The competent *E. coli* was prepared using the $CaCl_2$ method (Mandel and Higa, 1970). A single *E. coli* colony was inoculated into 5 ml LB broth and incubated at 37°C with shaking for overnight. A 1% of the overnight culture was subcultured into fresh 50 ml LB and incubated at 37°C for 3-4 h. until the OD_{600} was equal to 0.3-0.6. The culture was chilled on ice for 10 min and the cells were collected by centrifugation at 4,000 rpm for 10 min at 4°C. The supernate was discarded and the cell pellet was resuspended with 25 ml ice-cold 0.1M $CaCl_2$ and kept on ice for 30 min. The cells were centrifuged at 4,000 rpm for 10 min at 4°C. The supernate was discarded and the cell pellet was resuspended thoroughly with 3.3 ml ice-cold 0.1M $CaCl_2$ in 15% glycerol. The suspended cells were kept on ice for 15 min. Aliquots of 200µl were distributed in steriled ice-cold Eppendorf tubes and kept at -70°C until used.

3.8.2 Transformation technique

Transformation of *E. coli* was done using the standard method (Miniatis *et al.*, 1982). The competent *E. coli* cells were thawed on ice for 15-20 min, then a 20-100 ng ligated DNA was added and the tube was stored on ice for another 30 min. The tube was then transferred to a water bath set at 42°C for 90 seconds. The tube was cooled by putting it in an ice bath, for 1-2 min. A 800 µl of LB broth was added to each tube and the transformed cells was further incubated at 37°C for another 1 h.. A 100-200 µl of the transformed cells was spread on LA agar supplemented with 50 µg/ml ampicillin and incubated at 37°C, overnight.

3.9 Screening methods for esterase producing clones

3.9.1 Chemical method

The precedure used was that previously described by Higerd (1977). The white colonies grown on LB agar plate containing 50 μg/ml ampicillin were replica onto the same medium and incubated for 1-2 days, then the culture was overlaid with 10 ml of 0.8% LA soft agar in 0.5 M potassium phosphate buffer pH 7.5 containing 5 mg β-naphthylacetate and 5 mg Fast Blue BB dye. For *Bacillus subtilis* WRRL-B558, halo zones were observed around their colonies. In case of the esterase producing *E. coli,* their colonies turned into dark brown color compared to the light brown of the *E. coli* host.

3.9.2 Biological method

This method was developed for screening cephalosporin esterase producing bacteria. It was modified from the biological assay method as described by Meevootisom *et al.*, (1983). Recombinant bacteria was grown on LA agar containing 50 μg/ml ampicillin for 1-2 days, then, the culture was overlaid with 5 ml of LA soft agar (0.8%) containing 300 μg/ml cephalosporin C and 1% (v/v) *S. aureus* ATCC25923. The screening plates were incubated overnight at 37°C. Cephalosporin esterase producing clones were observed for the presence of larger satellite zone of *S. aureus* ATCC25923 around their colonies as compared to that of the *E. coli* host habouring the vector only.

3.10 Southern blot analysis

3.10.1 Southern blot transfer

After completion of electrophoresis, the gel was stained with 0.5 μg/ml ethidium bromide for 5-10 min. The gel was then washed in distilled water for 10-15 min and photographed under ultraviolet light (254 nm). A

ruler was placed alongside the gel in order to be able to match the DNA photograph of the autoradiogram. The gel was, then, soaked in a denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 45 min at room temperature with constant shaking. Subsequently, the gel was neutralized by soaking in the neutralizing solution (0.5 M Tris NaCl pH 7.4, 1.5 M NaCl) for 45 min at room temperature with shaking. Southern transfer was performed using Blot Transfer System (Gibco, BRL). A piece of Whatman 3 MM paper was cut to form a salt bridge. The wick was longer and wider than the gel and it was placed across the plastic bridge (standing on a tray containing 20XSSC) having both ends of the salt bridge immerging into 20XSSC. The gel was placed on the damp 3 MM paper and air bubbles were carefully removed. A wet nitrocellulose filter was cut exactly to the size of the gel and placed on top of the gel. All air bubbles trapped between the gel and the filter were removed with a glass rod.

A stack of paper towel (5-8 cm length) cut to the same size as the gel and the filter was placed on top of the nitrocellulose filter. An object was placed on top of the stack to weight the stack of towel down. The objective of this arrangement was to set up a flow of liquid from the reservior through the gel and the nitrocellulose paper, so that DNA fragment was transferred from the gel and deposited onto the nitrocellulose membrane by capillary force. DNA fragments were allowed to transfer for 12-24 h.. Next day, the position of wells were marked on the nitrocellulose membrane.

3.10.2 Radiolabeled DNA probe

The random primer DNA labelling method developed by Feinberg and Vogelstein (1983) was used. The method was based upon annealing of a mixture of all possible hexanucleotide primers to the target DNA. The complementary strand was synthesized from the 3'-OH termini of

the random hexanucleotide primer using the labelling grade Klenow DNA polymerase enzyme. Addition of modified deoxynucleotide triphosphate (^{32}P) dCTP (specific activity 3,000 Ci/m mole) was added to the denatured DNA in 50 µl total volume. The mixture was incubated at room temperature for at least 1 h.. At the end of incubation the reaction mixture was boiled for 2 min and then immediately transferred to ice. Then, 2 µl of stock 0.5 M EDTA solution (final concentration 20 mM) was added to the reaction mixture which was subsequently spun through Sephadex G-50 (equilibrated in TE buffer) column chromatography to separate non-incorporated radioactive nucleotides. This labelled DNA was ready for hybridization step or kept at -20°C until use.

3.10.3 Hybridization and autoradiography

The hybridization experiment was carried out by placing the dried filter in a hybridization bottle containing 20 ml of prehybridization buffer/100cm^2 of membrane and incubated at 65°C with constant shaking for at least 1 h. . Prehybridization buffer was then removed and fresh buffer was added. The radioactivity labelled probe was boiled for 5-10 min at 100°C to denature the DNA into single strands before being added to the hybridization bottle. Hybridization was performed overnight in an incubator at 65°C. After completion of the hybridization, the radioactive hybridization buffer was removed and the filter was washed for 5 min with 2 x SSC + 0.5% SDS at room temperature and followed by 2 x SSC + 0.1% SDS for 15 min at the same temperature. The filter was then washed with 0.1 x SSC + 0.5% SDS at 37°C with constant shaking for 30 min and repeated one more time of washing with the same solution at 65°C for 30 min. The filter was finally washed with 1 x SSC to remove SDS from the filter before being air dried on a sheet of 3 MM paper at room temperature. The filter was then exposed to

an X-ray film (Kodak) with an intensifying screen overnight or longer at -80° C. The film was developed and fixed according to the manufacturer's recommendations.

3.11 DNA sequencing

3.11.1 Construction of subclones for to be used in DNA sequencing

Some subclones in the deletion study were used for DNA sequencing. In order to sequence both strands of DNA, the subcloning experiments were carried out using restriction sites (s) in the restriction map of pBT1 to digest the pBT1 as sites for digesting of plasmid and pBluescript (pBS) vector. DNA fragments recovered from agarose gel electrophoresis were ligated to the partially digested-dephosphorylated pBS vector. The ligated DNA were transformed into the *E. coli* host. The plasmid DNA from the recombinant clones were extracted and analyzed by restriction mapping to check for the presence of the desired region of the DNA insert. Clones containing the desired DNA insert were grown and their for plasmid DNA was extracted as described previously. After removal of the RNA, the DNA was collected and used for sequencing.

In order to get complete sequence of 1560 bp *Hin*dIII-*Sal*I fragment, an additional sequencing of 250 bp from *Hin*dIII site was performed using the pBL1 plasmid and a universal primer.

3.11.2 Sequencing reaction by Sanger method (Sanger *et al* ., 1977)

Plasmid DNA of subcloned was isolated by STET or the modified rapid alkaline method, the DNA sequenced by using the T7 sequencing kit (Pharmacia, Sweden). The procedure for setting a sequencing reaction was carried out according to the manufacturer's recommendations. A

35 µl (5 µg) of template DNA was denatured by adding 8 µl of 2M NaOH and at incubating room temperature for 10 min and then the reaction mixture was precipitated with 2 volumes of ethanol and 3M sodium acetate (pH 4.8). The precipitated DNA was collected and washed with ice-cold 70% ethanol and dried. The pellet DNA was resuspended with 10 µl DW and annealed with 2 µl each of primer (5pg/µl) and annealing buffer (1M Tris HCl pH 7.6, 100 mM $MgCl_2$ and 160 mM DTT). The primers used in this study were either universal primer, T3 primer, reverse primer or oligonucleotide primers synthesized from the known sequence. The reaction mixture was then incubated at 65°C for 5 min and quickly transferred to 37°C and further incubated for 10 min. The tube was then left stand at room temperature for at least 5 min.

At labeling step, the annealed template-primer mixture was added with 3 µl of labeling mix-dATP (dCTP, dGTP, dTTP without ATP in solution) followed by addition of 1 µl of (^{35}S) dATP (10 µCi/µl) and 1 µl of diluted T7 polymerase (2 U). After mixing, the mixtures were incubated at room temperature for 5 min.

At termination step, a microtiter plate containing 2.5 µl of A' Mix-Short (ddATP in solution with dATP, dCTP, dGTP and dTTP), G' Mix-Short (ddGTP in solution with dATP, dCTP, dGTP and dTTP), C' Mix-Short (ddCTP in solution with dATP, dCTP, dGTP and dTTP), T' Mix-Short (ddTTP in solution with dATP, dCTP, dGTP and dTTP) in each well was prewarmed at 37°C. A 4.5 µl of labeling mixture (after 5 min incubation at room temperature) was added into each well and incubted at 37°C for 5 min. Then 5 µl of stop solution (deionized formamide solution containing EDTA, xylene cyanol and Bromophenol Blue) was added to terminate the reaction and all the mixtures were used immediately or kept at -20°C. Before loading

in polyacrylamide gel, the samples were either heated at 80°C-90°C for 3 min and cooled on ice then 2-5 µl of samples were loaded onto polyacrylamide gel.

3.11.3 Sequencing gel electrophoresis and autoradiography

Stock solution of acrylamide containing 7M urea was consist of 28.5 g acrylamide, 1.5 g bisacrylamide, 21.0 g urea and brought to 500 ml with H_2O and stirred. The completely dissolved mixture were added with 20 g of Amberlite MB-1 and stirred for 10 min then the solution was filtered through Whatman 3MM filter paper, degased and stored at 4°C.

The sequencing gel containing 6% acrylamide was prepared 1-20 h. prior to use. The gel solution (75 ml stock acrylamide, 0.075 g ammonium persulfate (Merck, Germany), and 35 µl TEMED (Sigma, USA) were mixed gently) was poured in between glass plates which were cleaned with a detergent, wash thoroughly in tap water, followed by wiping the glass surface twice with 90% ethanol and left dry. One shorter glass plate was further coated with dimethyldichlorsilane solution and the excess was removed by wiping the surface with 90% ethanol twice and left dry. After pouring the gel a shark tooth comb was inserted between glass plates immediately and allowed the gel to polymerize at room temperature for at least 1 h..

After pre-running vertically in 1xTBE buffer (Amersham, USA) at 1,500-2,000 V. for 15-30 min, the front wells were flushed out with TBE buffer to completely remove crystallized urea and unpolymerized acrylamide then 2-5 µl of each sequencing reactions (C, G, A, T, respectively) were loaded onto the gel and electrophoresed at 1,800 V with constant power for 1.5-5.5 h..

After electrophoresis for appropriate time, the gel was removed by twisting the plates with a metal spatula. When the upper glass plate was separated by allowing the gel attached to the lower glass plate. The gel on glass plate was placed to a shallow tray containing fixing solution (12% methanol and 10% acetic acid in water) and left for 30 min. The gel with glass plate was lifted up and the excess fixing solution was removed by tissue papers. The 3MM No. 1 paper was placed on the surface of the gel. After applying gentle pressure to firmly attach the gel to the paper, quickly flipped the paper and laid it on dry place by letting the gel side up and covered the gel with Saran Wrap plastic. The unwanted part was cut off. The gel was then dired in vacuum dryer (Hoefer, USA) at 80°C for 1 h.. The dired gel was then exposed to the X-ray film (CURIX XP) and kept at room tempature for 1-3 days before developing. The DNA sequence was analyzed by DNASIS^{TM} Software progam (Hitachi Software Engineering, Co., Ltd.).

3.12 Measurement of protein content in the samples

Protein content in enzyme samples was determined by the Lowry method (Lowry *et al.*, 1951). In brief, the protein in the 0.5 ml sample was incubated with a 2.5 ml mixture of alkaline copper sulfate solution (49 ml 0.2 M NaOH + 49 ml 4% Na_2CO_3 + 1 ml 1% $CuSO_4$. $5H_2O$ + 1 ml 2% sodium potassium tartrate) at room temperature for 10 min. A 0.25 ml of folin reagent (Folin-Ciocalteau reagent was diluted 1:1 with DW) was added and incubated further for 30 min at room temperature. The solution was read at OD_{750} against sample buffer run in the same condition as a blank. The concentration of protein in the sample was determined by a standard curve of bovine serum albumin concentration between 0.05-0.3 mg/ml.

3.13 Subcloning of the lipase genes in pTTQ18 vector.

The overexpression of the lipase genes was carried out by subcloning the fragment containing the ORF1 and ORF2 lipase genes into the pTTQ18 vector under ptac promoter. The subcloning procedure was as described in construction of subclones subjected to DNA sequencing section 3.11.1. The plasmid DNA were extracted from the recombinant clones and checked for the restriction map analysis and enzyme activity (qualitative and quantitative methods) compared to that of the vector control.

3.14 Polyacrylamide gel electrophoresis (PAGE)

Both native and SDS-PAGE used in this study were referred to the procedures described by Laemmli (1970).

3.14.1 Native PAGE

Native PAGE was carried out with a 7.5% separating gel which was prepared from a stock solution of 30% (w/v) acrylamide and 0.8% (w/v) N, N'-methylene bisacrylamide in 1.5 M Tris HCl pH 8.8. The gel also contained 0.05% w/v ammonium persulfate and 0.025% v/v of TEMED. After the separating gel were poured and saturated with DW, the gel was allowed to polymerize at room temperature for 30 min. A stacking gel was prepared 30 min before loading the protein. Constituents of the stacking gel were 4% acrylamide in 0.5M Tris HCl pH 6.8, 0.025% v/v TEMED and 0.05% w/v ammonium persulfate. The stacking gel was poured on top of the separating gel after DW was removed. The sample were prepared by diluting 4 folds in 4x sample solubilizing bufer (0.06M Tris HCl pH 6.8, 10% glycerol and 0.025% Bromphenol blue) and sometimes 0.05% Trition X-100 was added to reduce the protein aggregation. The wells were flushed with the electrophersis buffer (0.25 M Tris HCl containing 0.192 M glycine, pH 8.3), before 20 µl of samples with dye was loaded.

3.14.2 SDS-PAGE

SDS-PAGE was prepared similarly to that done for native PAGE except SDS were added into the gel, electrophoresis buffer and sample buffer. SDS-PAGE was carried out on 15% separating gel and 4% stacking gel, each containing 5 M urea, 0.5% (v/v) Triton X-100 and 1% SDS. SDS were added into the electrophoresis buffer and 4 x solubilizing buffer to the final concentration of 0.1% and 2%, respectively. Molecular weight markers (SDS7) used in this study was purchased from Sigma. The markers consisted of α-lactalbumin (Mr 14,200), trypsin inhibitor (Mr, 20,100), Trypsinogen (Mr 24,000) carbonic anhydrase (Mr 29,000), glyceraldehyde-3-phosphate dehydrogenase (Mr 36,000), egg albumin (Mr 45,000) and bovine albumin (Mr 66,000). The samples and molecular weight markers were diluted with 4x sample solubilizing buffer boiled for 3-4 min before being loaded into the flushed wells. The two gels were run in parallel at 150V for about 1 h.. One gel was stained for protein and the other was renatured immediately according to Rosenberg *et al.* (1975) by soaking in 0.04M Tris HCl, pH 6.0 containing 0.5% Triton X-100 and incubated at 4°C for overnight. Then, the solution was removed and the gel was washed with 0.1 M phosphate buffer pH 7.5 and shake at room temperature for 15 min before activity staining was performed.

3.14.3 Protein staining

The protein staining with Coomassie Brilliant Blue was done after removing the gel by soaking it in the staining solution (0.2% Coomasie Blue R250 in 40%(w/v) ethanol and 10% glacial acetic acid) for 30 min. Then, the gel was destained repeatedly in 40% methanol and 10%glacial acetic acid until the background was clear. For a long term storage, the gel was dried in

double layers of cellophane. This could be used for drying both SDS-and native gels.

3.14.4 Esterase/Lipase staining

For detection of esterase/lipase activity, the native and renatured gels were placed in freshly prepared the esterase staining solution composed of 20 mg β-naphthylactate dissolved in acetone and 50 mg Fast Blue BB dye in 0.1 M phosphate buffer pH 7.5. It should be noted that lipase could give a positive result with this esterase staining solution. The gel was soaked in the solution with constant shaking at room temperature for 1 h. (native) and 6 h. (renatured).

3.15 Partial purification of protein using gel filtration.

A 20 grams of Sephadex G-200 (Sigma) was swollen overnight in 0.1 M phosphate buffer pH 7.0 and degased by vacuum pump for 15 min. The gel was then packed in a 2.7 cm x 50 cm column (BIORAD). Following equilibration with 2 volumes (approximately 260 ml) of 0.1 M phosphate buffer, pH 7.0 in a cold room, the buffer above the gel in the column was drained to the gel surface. Five to fifteen ml of samples were loaded on the column and drained again to the gel surface. Then the column was filled with the same buffer. The buffer was applied with flow rate of 8 ml per hour. Two ml fractions each was collected for every 15 min by a fraction collector (Biorad). Protein content (OD_{280}) and lipase activity (using pNPP as a substrate) were measured for each fraction. Fractions with high lipase activity were pooled and stored at 4°C for further studies.

3.16 Substrate specificity of enzymes

The pool fractions of pTL7-peak3 and pTE12-peak1 were used to studied for substrate specificity using fatty acids of various chain length of

monoacyl triglycerides and cephalosporin C and for effects of inhibitors on enzyme activities.

3.16.1 Specificity of enzymes to various monoacyl triglycerides.

Substrate specificity of the lipases to various purified monoacid triglycerides was determined by modification of the method of Sugihara *et al.*, (1991). Sonicated cells in 1 ml suspension were prepared from 5 ml overnight culture of the *E. coli* host habouring recombinant plasmids or the vector only. They were used as enzyme sources in this study various chain-length monoacid triglyceride from C_4-C_{18} (tributyrin, tricaprylin, trilaurin, trimyristin, tripalmitin and tristearin) were used as substrates. Lipase activity was assayed by titration of fatty acids released from the substrates with alkali. The assay mixture contained 1 ml of substrate, 4 ml of 50 mM Tris-HCl (pH 7.8), 0.05 ml of 1 M $CaCl_2$ and 50 µl of enzyme solution. The mixture was incubated for 30 min at 37°C with shaking (200 rpm). The enzyme reaction was stopped by addition of 10 ml of 95% ethanol. Fatty acids released were determined by titration with potassium hydrogen-phthalate (KHP) standardized with 10 mM KOH using phenolphthaline as an indicator. One unit of lipase activity was defined as 1 nmole of fatty acid liberated per min by 1 ml of enzyme.

3.16.2 Specificity of enzymes to cephalosporin C

Partially purified enzymes from pTL7-peak 3 and pTE12-peak 1 were tested for ability to hydrolyze cephalosporin C by measuring the acetic acid liberated. Determination of acetic acid liberated was performed by using an enzymatic reagent kit (Boehringer Mannheim, Germany). Experiments were carried out by incubation of 0.5 ml of peak samples with the 0.5 ml phosphate buffer pH 7.0 containing 10 mg/ml cephalosporin C at 37°C for 3 h.. The reaction was then stopped by freezing the mixtures at -20°C and it

could be kept until the acetic acid assay was ready. When ready the frozen samples were thawed and assayed according to the manufacturer's recommendations. In principle, the acetic acid (acetate) was converted in the presence of enzyme acetyl-CoA synthetase (ACS), with ATP and coenzyme A (CoA) to form acetyl-CoA (reaction 1)

(1) Acetate + ATP + CoA $\xrightarrow{ACS}$ acetyl-CoA + AMP + pyrophosphate

Acetyl-CoA was then reacted with oxaloacetate to form citrate in the presence of citrate synthase (CS) (reaction 2)

(2) Acetyl-CoA + oxaloacetate + H_2O $\xrightarrow{CS}$ citrate + CoA

The oxaloacetate required for reaction (2) was formed malate and NAD in the presence of malate dehydrogenase (MDH) (reaction 3). It is in this reaction that NAD was reduced to NADH.

(3) Malate + NAD^+ $\underset{}{\overset{MDH}{\rightleftharpoons}}$ Oxaloacetate + NADH + H^+

Then, determination was based on the formation of NADH measured by the increase in absorbance at 340 nm. Since the amount of NADH formed was not linearly proportional to the acetic acid concentration. Thus, the concentration of acetic acid was derived from the calculation below.

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ (g/l)},$$

where ΔA = The absorbance difference calculated from absorbance measured at various steps of assay

V = final volume (ml)

v = sample volume (ml)

MW = molecular weight of the substance to be assayed (g/mol)

d = light path (cm)

ε = absorbance coefficient of NADH at 340 nm which is 6.3 l x mmol^{-1} x cm^{-1}

Thus, for acetic acid, the formulation would be

$$c = \frac{3.23 \times 60.05 \times \Delta A}{6.3 \times 1 \times 0.1 \times 1000} = 0.3079 \times \Delta A \text{ g/l}$$

3.17 Effect of inhibitors on enzyme activities

A serine inhibitor, PMSF, and a sulfhydryl inhibitor, β-mercaptoethanol, were used in this study. PMSF and β-mercaptoethanol were added to the pTL7-peak3 and pTE12-peak1 samples to a final concentration of 1mM each. After 2 hours of incubation at 30°C, the treated samples were assayed for lipase activity using pNPP as the substrate. The procedure of lipase activity assay was as described previously in section 3.4.2.

ε = absorbance coefficient of NADH at 340 nm which is 6.3 l x mmol^{-1} x cm^{-1}

Thus, for acetic acid, the formulation would be

$$c = \frac{3.23 \times 60.05 \times \Delta A}{6.3 \times 1 \times 0.1 \times 1000} = 0.3079 \times \Delta A \text{ g/l}$$

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ε = absorbance coefficient of NADH at 340 nm which is 6.3 l x mmol^{-1} x cm^{-1}

Thus, for acetic acid, the formulation would be

$$c = \frac{3.23 \times 60.05 \times \Delta A}{6.3 \times 1 \times 0.1 \times 1000} = 0.3079 \times \Delta A \text{ g/l}$$

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CHAPTER IV
RESULTS

4.1 Characteristics of *B. subtilis* WRRL-B558

The microorganism used as a donor DNA in this study was *B. subtilis* WRRL-B558 which was obtained from Dr. L.K. Nakamura, The National Center for Agricultural Utilization Research. This organism is a gram positive, aerobic endospore-forming bacterium of 0.7-1.2 x 1.2-2.6 μm in size. Although it is generally regarded as an aerobe, it can grow and sporulate slowly under a strictly anaerobic condition and can use nitrate as a terminal electron acceptor in an anaerobic respiration system (Gordon *et al.*, 1973). The organism appeared to grow well at both 30°C and 37°C. Its colony on an LA agar plate appeared as flat, opaque and irregular in shape. It could ferment D-glucose, D-mannitol and sucrose and with formation of gas but could not ferment galactose and lactose.

4.2 Production of esterase/lipase by *B. subtilis* WRRL-B558

B. subtilis WRRL-B558 was determined for its ability to produce esterase and lipase enzymes by using the colorimetric assays with α-naphthylacetate (α-NA) and p-nitrophenylpalmitate as substrates, repectively. This organism showed capability to produce enzymes with esterase activity (toward the α-NA substrate) of 20.6 U/mg protein and lipase activity (toward the pNPP substrate) of 2.76 U/mg protein. It should be noted that *B. subtilis* WRRL-B558 produced esterase detected in the culture broth and in the cells. In addition, the esterolytic activity towards cephalosporin C substrate was demonstrated with TLC and HPLC experiments. Since there was no standard deacetylcephalosporin C available in our laboratory, a comparable reaction of cephalosporin C with a commercial acetylesterase enzyme was run parallelly by assuming that the deacetylcephalosporin C would be formed from this reaction. Results from

TLC showed that Rf values of standard cephalosporin C and the assumed deacetylcephalosporin C (ADC) were 0.25 and 0.20, respectively (Table 13). The reaction mixture of cephalosporin C and *B. subtilis* WRRL-B558 using either culture broth or cell suspension when applied on TLC showed the same results with Rf values of 0.25 and 0.20 for cephalosporin C and its product, respectively. With the HPLC system, standard cephalosporin C, 7-ACA and 7-ADCA showed retention times at 7.45, 4.73 and 2.36 min, respectively and the ADC showed a retention time at 2.13 min (Figure 15, A-D). There were some background peaks (at retention times of 1.8-1.9 min) which were not clearly separated from that of the ADC in this solvent condition. The HPLC results of the reaction mixture of cephalosporin C and the *B. subtilis* cell suspension (Figure 16B) as compared to that of the control which contained only the *B. subtilis* cell suspension (Figure 16A) show an additional peak at 2.13 min.

From the above data it was suggested that *B. subtilis* WRRL-B558 produced an esterase enzyme which could hydrolyse cephalosporin C to form deacetylcephalosporin C(DC). Since *Escherichia coli* (XL-1B and JM109) would be used in the cloning experiments, its ability to produce an esterase enzyme active against cephalosporin C was determined by using the HPLC method. Results are shown in Figures 16 C and D that cephalosporin C was degraded as shown by a reduction in its peak height but no peaks of 7-ADCA, 7-ACA and DC were detected.

4.3 Cloning of cephalosporin C esterase gene of *B. subtilis* WRRL-B558

4.3.1 Properties of DNA used in cloning

Total DNA of *B. subtilis* WRRL-B558 was extracted as described in Materials and Methods. The purity of DNA was determined by its UV absorptions values of OD_{260} and OD_{280} nm (Shimadzu spectrophotometer, Japan) and by using an agarose gel electrophoresis. The

Table 13. Silica gel thin layer chromatography of cephalosporin C and the reaction products (ADC) of cephalosporin C with various enzyme sources.

Sample	Rf value**
Cephalosporin C	0.25
Cephalosporin C + acetylesterase commercial enzyme*	0.25, 0.20
Cephalosporin C + *B.subtilis* WRRL-B558 broth*	0.25, 0.20
Cephalosporin C + *B. subtilis* WRRL-B558 cell suspension*	0.25, 0.20

* The enzyme reactions were incubated at 37°C for 1h. before doing the test.

** The solvent system used was n-butanol : acetic acid : water at 4:1:4.

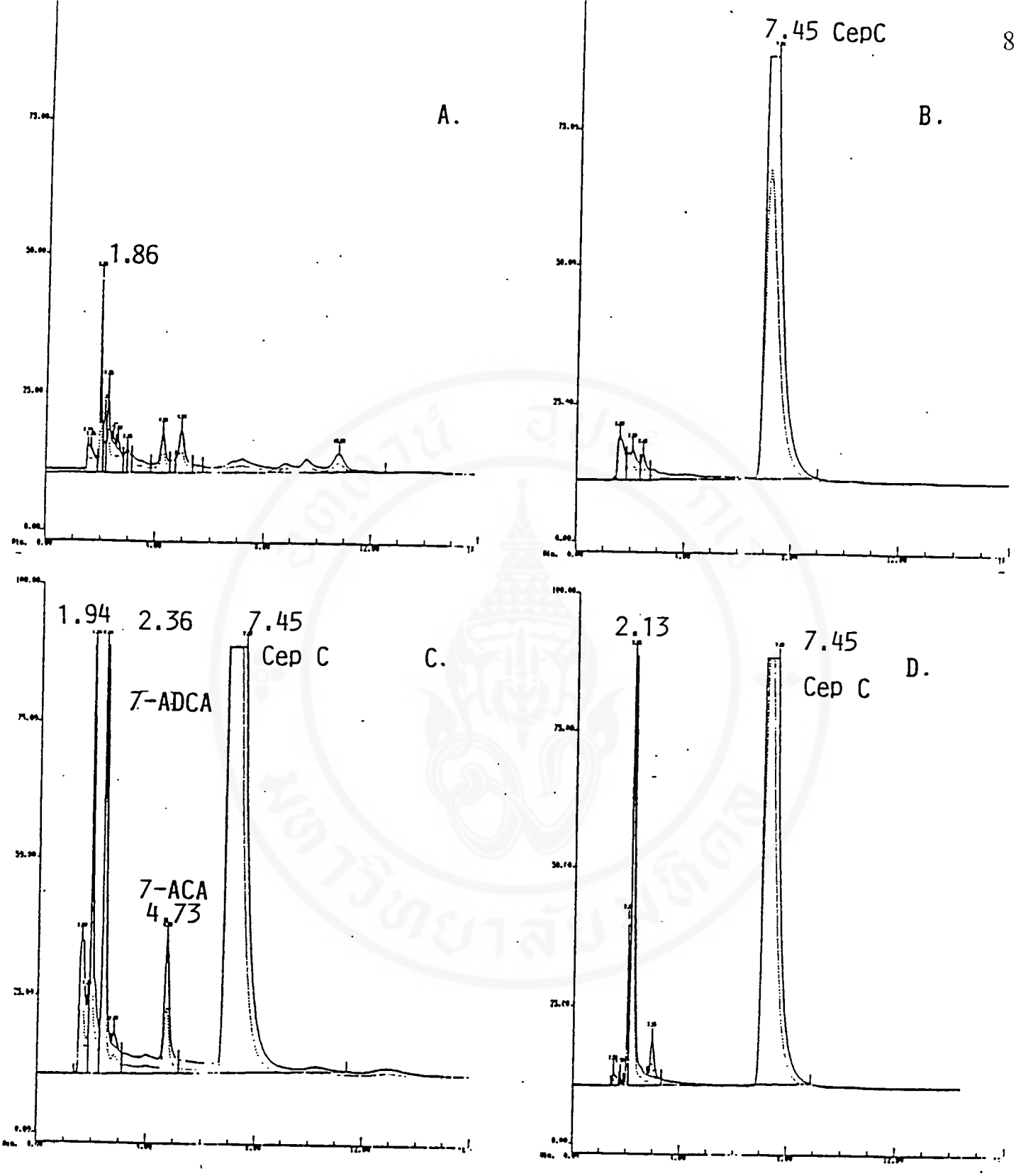


Figure 15. HPLC profile of A. LB broth; B. 1 mg/ml cephalosporin C in 0.1 M phosphate buffer pH 7.5; C.peak 1 standard 7-ADCA, peak2 7ACA and peak 3 cephalosporin C; D. 1 mg/ml cephalosporin C incubated with 10 µl of acetylesterase commercial enzyme at 37°C for 1 h.. The samples were filtered and injected 10 µl each into Spherisorb S5 OD52 column with 0.002M K_2HPO_4-KH_2PO_4 buffer pH 7.0 as the mobile phase.

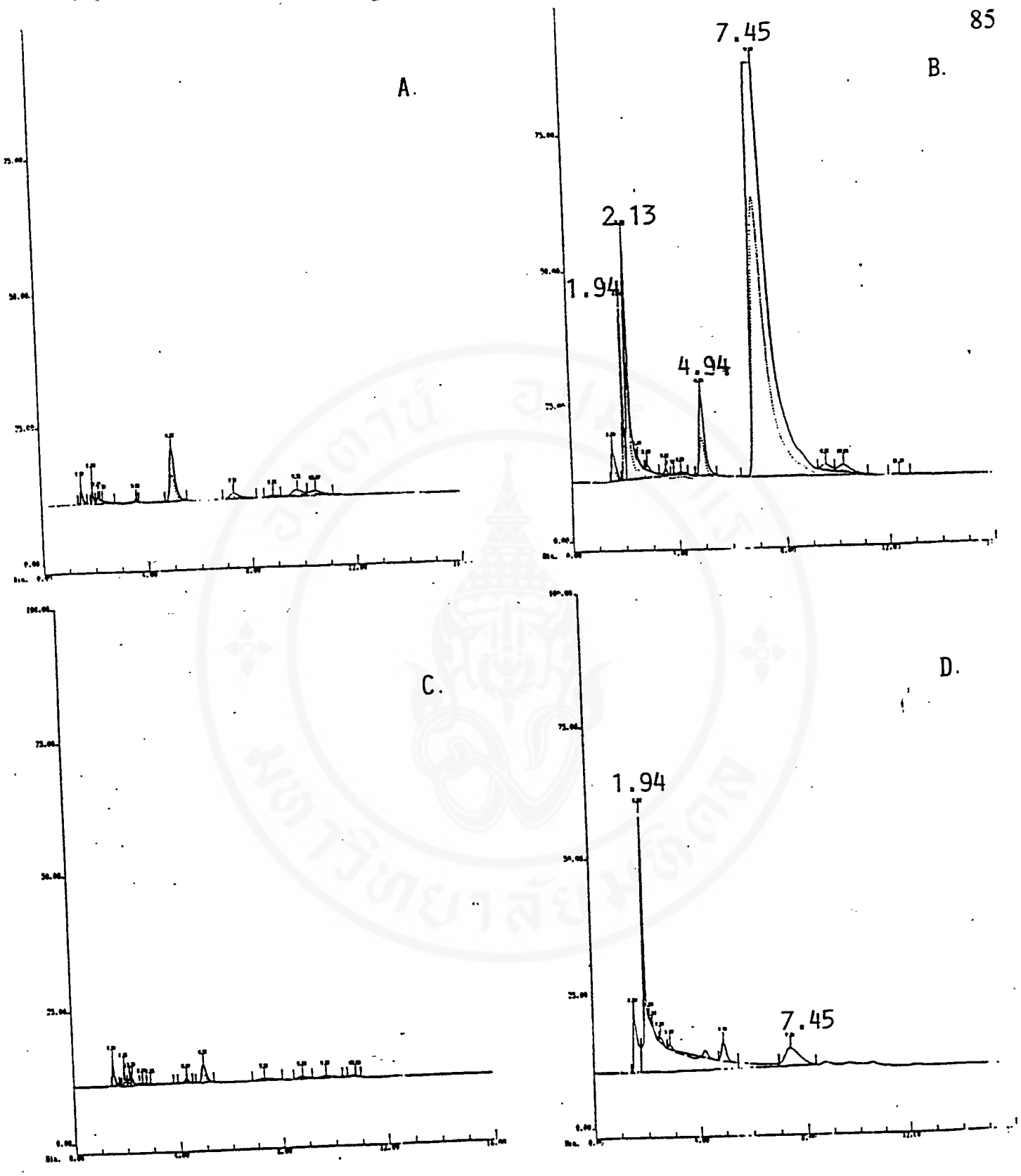


Figure 16. HPLC profile of supernates of A. *B. subtilis* cell suspension; B. *B. subtilis* cell suspension incubated with 1 mg/ml cephalosporin C ; C. *E. coli* XL-1B cell suspension; D. *E. coli* XL-1B cell suspension incubated with 1 mg/ml cephalosporin C. All samples were incubated at 37°C for 1 h. and the mixtures were filtered and 10 µl of each was injected into Spherisorb S5 OD52 column with 0.002 M K_2HPO_4-KH_2PO_4 buffer pH 7.0 as the mobile phase.

OD_{260}/OD_{280} ratio of the purified DNA was 0.73 and its concentration was estimated to be 400 ng/μl. No protein and RNA was observed in the agarose gel electrophoresis of the purified DNA sample.

The vector DNA, pBluescript was prepared in a large scale as described in Materials and Methods. The DNA preparation was found to have the OD_{260}/OD_{280} at 1.70 and its concentration was 5.8 μg/μl.

4.3.2 Molecular cloning

4.3.2.1 Cloning strategy

A short gun cloning was used to clone an esterase gene from chromosomal DNA of *B. subtilis* WRRL-B558.The purified genomic DNA was partially digested with *Sau*3AI restriction enzyme and ligated to *Bam*HI-dephosphorylated pBS vector as described previously. The ligated products were transformed into competent *E. coli* XL-1B. In transformants grown on ampicillin plates were tested for their esterase producing abilities by a 2 step overlaying techniques using two kinds of substrates. At first, the recombinant clones were screened with β-naphthylacetate and Fast Blue BB dye.Those which showed positive by having dark brown colonies (Figure 17A) were then selected and tested for ability to produce cephalosporin C esterase by overlaying with 300 μg/ml of cephalosporin C and 1% (v/v) of an overnight culture of *S. aureus* ATCC 25923. Those that produced cephalosporin C esterase would allow better growth of *S. aureus* ATCC 25923 on the top-layer medium. Of the 2,000 transformants selected, one recombinant clone which gave positive result to both substrates was selected. Figure 17B showed clearly that the recombinant clone(XL-1B+pBT1) could hydrolyse cephalosporin C and allow *S. aureus* ATCC 25923 to grow around their colonies much better than that done by the host or *B. subtilis* WRRL-B558. The plasmid which conferred the esterase activity was named pBT1 and analyzed for restriction mapping with various enzymes as shown in Figures 18A and 18B. Esterase activity of sonicated cells of the recombinant *E. coli*

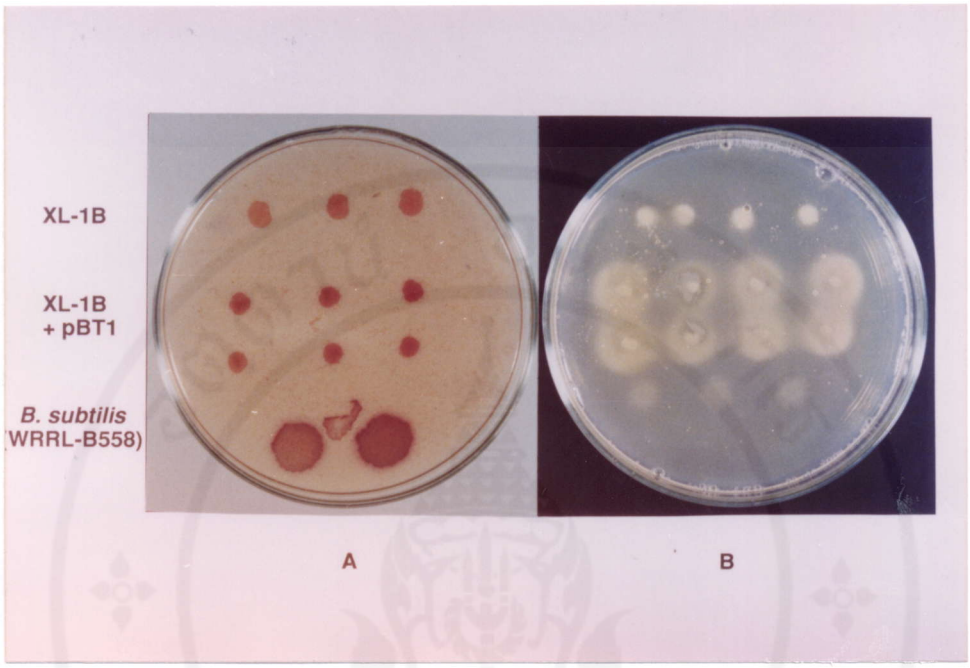
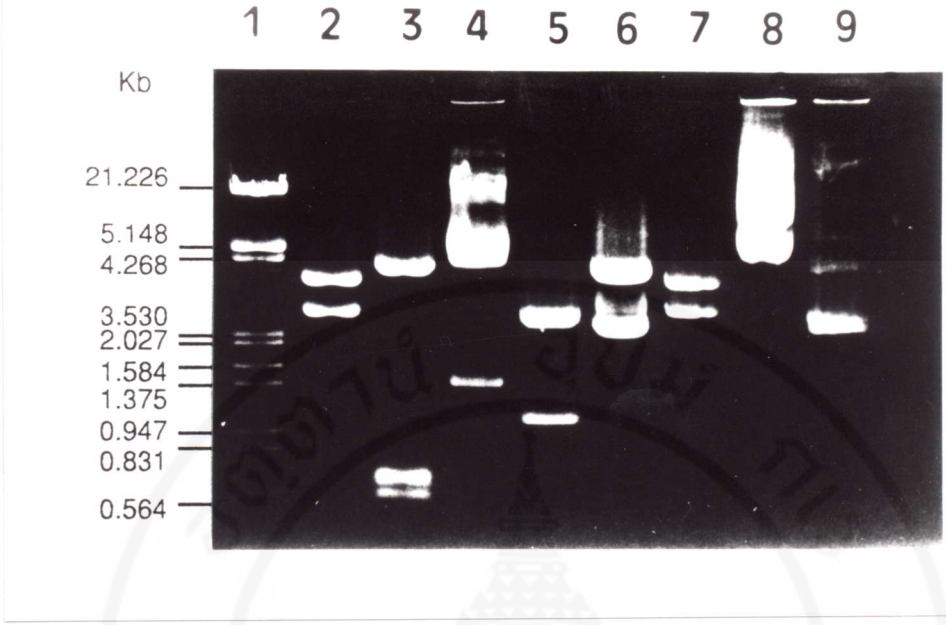


Figure 17. Screening methods for detecting cephalosporin acetylesterase producing clones. The esterase producing clones were overlayed with

A) β-naphthylacetate and Fast Blue BB dye, (the positive clones were detected as having dark brown colonies).

B) 300 μg/ml cephalosporin C and 1% (v/v) *S. aureus* ATCC 25923, (the positive clones were detected by having more sattellite growth of *S. aureus* ATCC25923 around their colonies.

The tests included a negative control (*E.coli* XL-1B host) and a positive control (*B. subtilis* WRRL-B558).



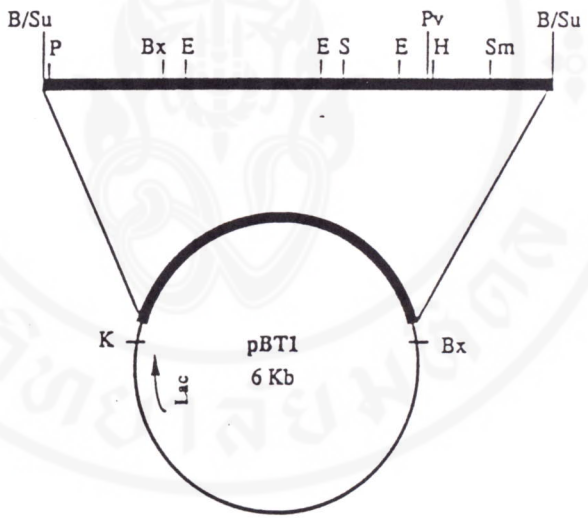


Figure 18. A) Agarose gel (0.8%) electrophoresis and of the recombinant plasmid pBT1 digested with various restriction enzymes.

Lane 1, λ DNA/*Hin*dIII - *Eco*RI; lane 2, pBT1 cut with *Bst*XI; lane 3, pBT 1 cut with *Eco*RI, lane 4, pBT1 cut with *Sal*I; lane 5, pBT 1 cut with *Pvu*II, lane 6, pBT1 cut with *Hin*dIII; lane 7, pBT1 cut with *Sma*I; lane 8, uncut pBT1; lane 9, pBS vector cut with *Eco*RI.

B) Physical map of pBT1 plasmid. Thick and thin lines represent *B. subtilis* DNA and vector pBluescript (pBS), respectively. The arrow indicates the direction of transcription of the lac promotor from the pBS (+ KS) Abbreviation of restriction enzymes: B, *Bam*HI; Bx, *Bst*XI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Su, *Sau*3AI.

was found to be about 1.2 times higher than that of the host habouring the vector only. However, this activity was still less than that of the culture broth and cell extracts of *B. subtilis* WRRL-B558 (Table 14).

4.4 Southern hybridization of *B. subtilis* WRRL-B558 chromosomal DNA

In order to be certain that the cephalosporin C esterase gene in pBT1 was derived from the genomic DNA of *B. subtilis* WRRL-B558, the chromosomal DNA of *B. subtilis* WRRL-B558 was prepared for Southern blot analysis, using pBT1 digested with *Bst*XI-*Hin*dIII restriction enzymes as a probe. The result of DNA hybridization shown in Figures 19 indicated that the inserted gene in pBT1 was derived from the *B. subtilis* WRRL-B558 chromosomal DNA. However, there were strong cross hybridizations between digested pBT1 and pBS vector which may be resulted from 11 nucleotides similarity of *Bst*XI restriction site that were found in the pBS vector and the DNA insert in pBT1 which was used as a probe.

4.5 Deletion study and esterase activity of subclones

To locate the esterase gene in the pBT1 plasmid, subclones of pBT1 were constructed according to the restriction sites in the pBT1 and tested for their abilities to express the esterase activity by overlaying with β-naphthylacetate and Fast Blue BB dye. Results of the deletion study were shown in Figure 20 which suggested that the esterase gene located on the *Bst*XI-*Sal*I fragment. These subclones were further tested with the biological method using the double layer technique with *S. aureus* ATCC 25923 as the test organism to determine the esterase activity towards cephalosporin C. All subclones except pBT11 showed good correlation of esterase activity as assayed by the two methods. The pBT11 habouring clone gave negative result with β-naphthylacetate but showed strong reaction against cephalosporin C (Figure 21). Thus, it seemed that the cephalosporin C

Table 14. Esterase activity of sonicated cells of pBT1 clone compared with *E.coli* XL-1B habouring pBS vector only and *B. subtilis* WRRL-B558 using α-naphylacetate as a substrate.

Organism/Plasmid	Esterase activity* (U/mg protein)
E. coli X L-1B/pBS	5.7
E.coli X L-1B/pBT1	6.7
B.subtilis WRRL-B558	20.6

* 1 unit of the esterase activity was expressed as 1nmol of α-naphthol released per min.

** Esterase activity of the culture broth of *B. subtilis* WRRL-B558 was 14.6 U/ml.

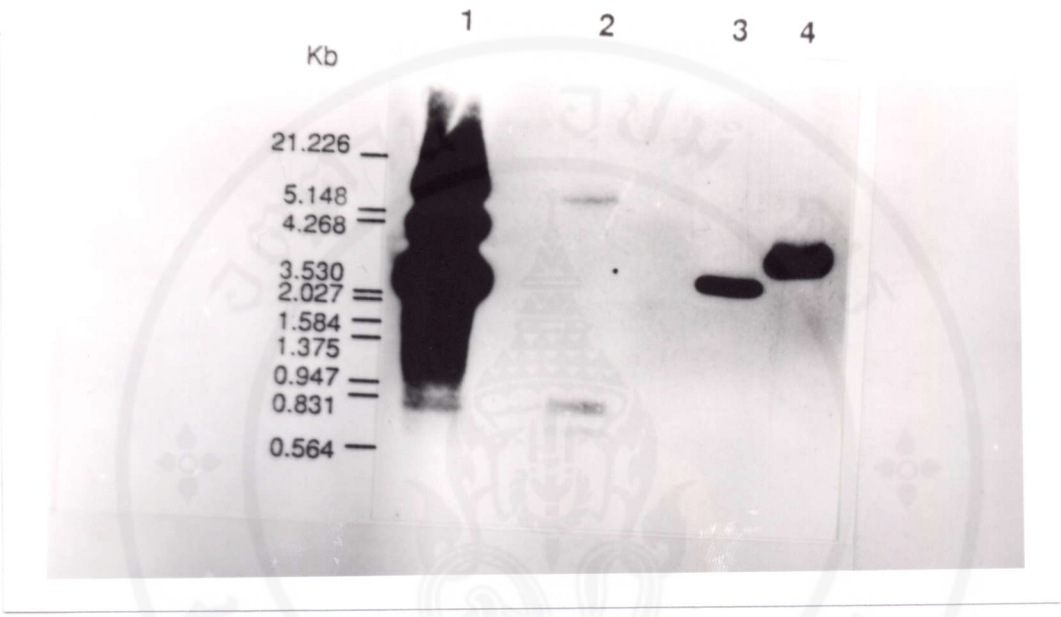


Figure 19. Southern hybridization of digested *B. subtilis* chromosome, pBluescript and pBT1 with a 1 Kb fragment of *Bst*XI-*Hin*dIII fragment Lane 1, pBT1-*Hin*dIII; lane 2, *B. subtilis* DNA-*Eco*RI; lane 3, *B. subtilis* DNA-*Hin*dIII; lane 4, pBluescript-*Hin*dIII.

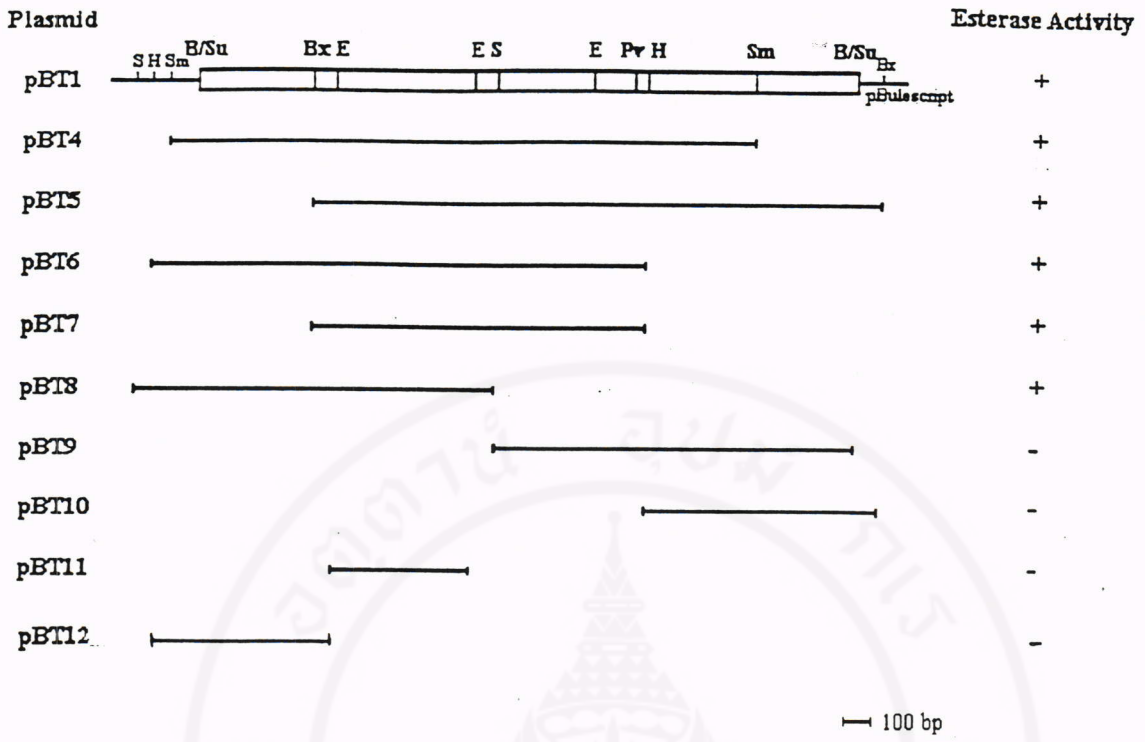


Figure 20. Deletion study of the 3Kb-*Sau*3AI fragment. The bars show subcloning fragments.
Symbol : +, colonies showed red halo zone (able to produce esterase); -, colonies did not showed red halo zone (unable to produce esterase) with β-napthylacetate and Fast Blue BB dye.
Abbreviation : B, *Bam*HI; Bx, *Bst*XI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S; *Sal*I; Su, *Sau*3AI; Sm, *Sma*I.

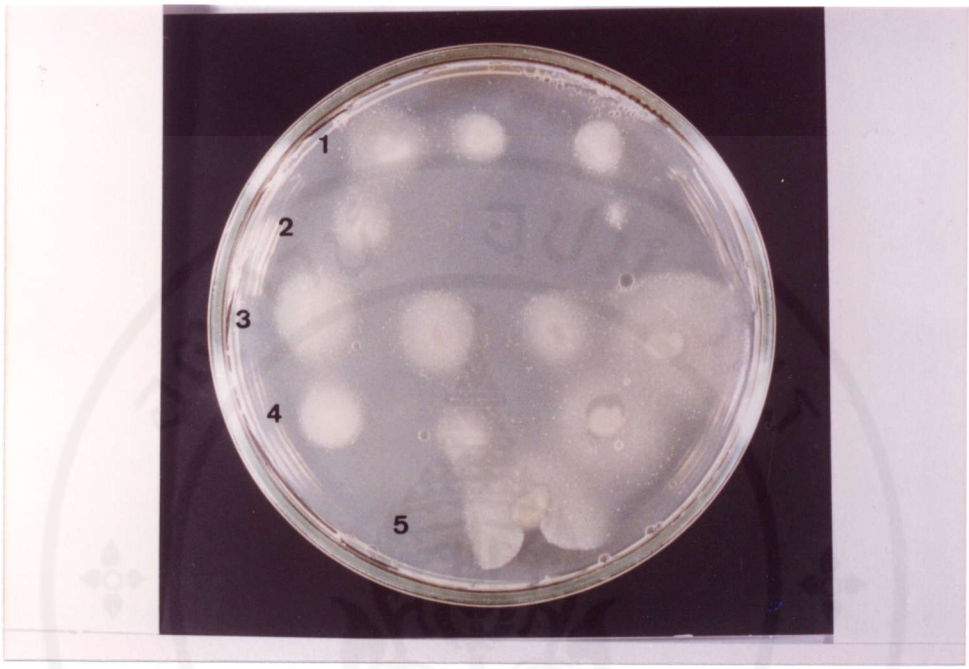


Figure 21. Detection of abilities to produce cephalosporin C esterase among subclones by overlaying the subclones with 300 μg/ml cephalosporin C and 1% (v/v) *S. aureus* ATCC 25923.
The rows of replica organisms were *E. coli* habouring plasmid of 1) pBluescript 2) pBT9 3) pBT11 4) pBT12 5) pBT1

esterase gene should be located in between the 600 bp of *Eco*RI-*Eco*RI fragment of pBT1.

4.6 DNA sequence of cephalosporin C esterase gene

The *Bam*HI/*Sau*3AI-*Bam*HI/*Sau*3AI fragment of pBT1 was used for sequencing. The strategy of DNA sequencing was done by using the subcloning method as shown in Figure 22. The DNA sequence of 1356bp between *Bam*HI/*Sau*3AI to *Sal*I fragment of pBT1 was analyzed by DNASIS^{TM} programme and shown to have one incomplete ORF and another complete ORF from the complementary strand of the DNA. A diagram showing the two ORFs and its direction of transcription is shown in Figure 23. The DNA translation with the deduced amino acid sequences of the complete ORF is shown in Figure 24. The gene consisted of -35 and -10 sequence of a promotor, putative ribosome binding site and inverted repeats downstream the TAA stop codon, presumably to be a transcription terminator structure. The complete ORF was named ORF1 and the incomplete ORF was ORF2. G/C content of ORF1 and ORF2 were found to be 43.1and 41.8%, respectively. DNA sequence homology of the two genes was found to be 51% homology and when compared to that reported in the GenBank Database, it was found that DNA sequence of ORF1 showed a 95% homology to the artificial lipase gene (AO2816) of *G.candidum* (Eur. Patent 0243338) and that of ORF2 showed 95% homology to a lipase gene of *B. subtilis* 168 (Dartois *et al.*, 1992) and 97% homology to the artificial lipase gene (A02815) of *G. candidum* (Eur Patent 0243338). Thus, the ORF1 in this study will be referred to as ORF1 lipase gene and the ORF2 will be referred to as ORF2 lipase gene.

4.7 Cloning of lipase gene from *B. subtilis* WRRL-B558 chromosome

In order to obtain the complete sequence of *B. subtilis* WRRL-B558 lipase gene, the *B. subtilis* chromosomal DNA was cloned again using 0.7 Kb *Eco*RI-*Eco*RI-pBT1 fragment as a probe in selecting recombinant

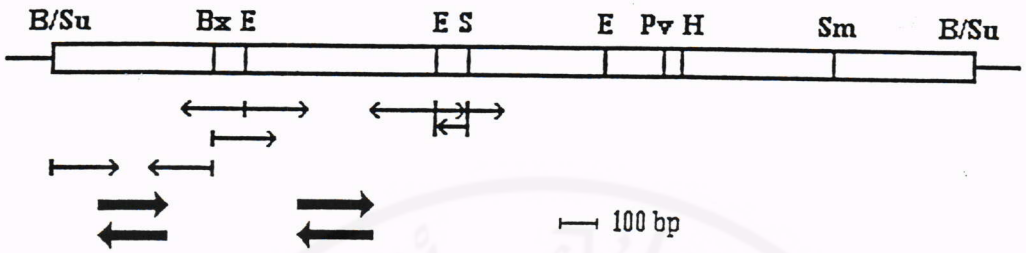


Figure 22. Sequencing strategy of the 1.4 Kb of *Bam*HI/*Sau*3AI -*Sal*IDNA fragment in pBT1.
Different subclones were generated using a restriction site in the polycloning site and another within the insert and cloned into pBS (SK) vector. The arrows indicate the nucleotide sequence obtained from each subclone using universal primer (leftward arrows) and reverse primer (rightward arrrows). Thick arrows represent sequences derived from the oligonucleotide primer synthesized from the known sequences.

Figure 23. Diagram shows two open reading frames in the 3 Kb-*Sau*3AI fragment of pBT1 plasmid. The arrows show the direction of the reading frames. The numbers indicate the open reading frames as described in the text.

DNASIS ***** DNA TRANSLATION LIST ***** DATE 03-15-9

*** INPUT INFORMATION ***

FILE NAME : 1.SEQ SEQUENCE : COMPLEMENTARY 1356 BP

CODON TABLE : UNIV.TCN

SEQUENCE REGION : 1 - 1356

TRANSLATION REGION : 337 - 699

*** DNA TRANSLATION ***

```
                SalI
   1  GTC GAC AAC TTT ACT TCC GCA GCA TTT TTT ATA TTT CTT GCC GCT      45
  46  GCC GCA TGG GCA AGG GGC ATT TCG TTT CAC TTT TCC CAC AAT AAA      90
                EcoRI
  91  CCC TCC GAA TTC TGT TGT AAC TTT TCT ATT TTA ACA AAA ATA ACT     135
 136  TCT GAG GGC TTG GTT TCG GGC TAA AAA AGA ATT TGG TAA TGT AAT     180
 181  AAG CTG GCT GAT TAT GCC AAT TTA CCC ATG ACA TTT TCA CCA ACT     225
 226  TTT GTG ATT TAA GGC TTA TCA CCT ATT CCC ACT TTA GGC CCT TCT     270
 271  TTT TTA CAA TCA TTA GGT ATT TAT TTA ACA TTT TAA GGA GTG TTT     315
   1                                  M   L   A   C   A   L   L   L       8
 316  CTA TTG GAA CTA GCT GGT TTC ATG CTG GCC TGC GCT CTT TTA CTT     360
   9   D   V   I   I   A   A   A   V   I   L   A   A   G   F   T      23
 361  GAT GTA ATC ATT GCC GCA GCA GTG ATT TTG GCA GCC GGA TTT ACG     405
  24   F   G   D   G   S   A   G   V   I   I   V   A   I   L   M      38
 406  TTT GGC GAT GGG TCC GCC GGT GTC ATT ATC GTC GCC ATA TTG ATG     450
  39   L   I   V   Y   P   L   L   M   P   L   T   N   W   K   G      53
 451  CTG ATT GTG TAC CCT TTG CTG ATG CCG CTG ACA AAT TGG AAA GGC     495
  54   T   L   G   K   K   I   I   G   L   Q   I   V   R   D   E      68
 496  ACA CTG GGC AAA AAA ATC ATC GGG CTG CAA ATC GTG AGG GAC GAA     540
  69   T   H   E   K   I   S   L   L   Q   A   I   V   R   Y   I      83
 541  ACA CAC GAG AAA ATT TCT CTC CTA CAG GCG ATT GTC CGC TAC ATC     585
  84   I   A   W   V   H   V   F   S   R   L   I   Y   L   T   A      98
 586  ATA GCG TGG GTG CAT GTG TTC TCC CGC CTG ATC TAT TTG ACT GCC     630
  99   A   F   T   K   K   K   Q   T   V   H   D   M   A   A   K     113
 631  GCA TTT ACG AAG AAA AAA CAA ACC GTT CAT GAT ATG GCT GCG AAA     675
 114   T   I   V   L   K   A   E   *                                  121
 676  ACC ATT GTG CTG AAA GCA GAA TAA CCT TGA AGA ATA GAA TTC TTC     720
                                                   EcoRI
 721  AAG GTT TTG TTT TTC ATT AAT TCG TAT TCT GGC CCC CGC CGT TCA     765
 766  GCC CTT CTT TAA TCA GGC TGT TGA CTT GGC TGC TGT ACA GAA GGC     810
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 811  CGA TGT GTC CAC CGC CAT GGA TTT GAA CGT TTC TAG CAC CGT CTA    855
 856  ATC TTG ATA AGT AAT TCA TGA CAA TCA TAT CGG CAC TGC TGT AAA    900
 901  TGG ATG TGT ATA AAA TCT TTT GAT TTG GAT CTG TTC CCG GAA GCG    945
 946  CCT TGC CTG TCG TCA AAC GGT TCG CGC CGC CAA CCG TCA CGA CGT    990
 991  TTG CAA CTT TAT TTC CGC CGT CCA GAT TTT TTA TGT AGT AAA GTG   1035
1036  TGT TCG CGC CCC CCA TGC TGT GAG CGA CAA TAT CCA CTT TTT TCG   1080
1081  CAC CCG TTT CAT CTA AAA CCT TTT GCA CAA ATC GTG ATA ATA CCG   1125
1126  GGC CAT TGT TAT AAT TTG TCC CTG TCT TGT CCC AAA AAT CAA CTG   1170
1171  CAT ACA GCT TGT CCG GCG ACC AGC CCT GAG ATA CGA GAT AGC TCT   1215
1216  TAA TTC CCG CAA AAT TGA ATG ATG CCC CTC CCA TAC CGT GAA CCA   1260
1261  TAA CGA CTG GAT TGT GTT CAG CGG CTT TTG CTG ACG GCT GCA GCG   1305
1306  CAA ACA GTG ATG TAA CAG ACA GCA TCA AAA TTG TTA CAA GCG CAA   1350
1351  TGA TCC                                                     1356
```

Figure 24. Complementary nucleotide sequence and deduced amino acid sequence of *B. subtilis* cephalosporin esterase gene (ORF1) from the 1356bp *Bam*HI-*Sau*3AI to *Sal*I fragment of pBT1 plasmid. Putative SD sequence and Pribnow box are indicated by solid lines. Arrows indicate the putative termination sequence. Sites specific for restriction enzymes are also indicated.

clones. Southern hybridization of various *Hin*dIII-digested recombinant plasmids are shown in Figure 25 which shows two recombinants positive clones (lanes 4 and 10). The two recombinant plasmids (of the two positive clones) were named pBL1 and pBL2. Restriction analysis of the two plasmids showed that both were of the same size but located in the plasmids in different orientation (Figure 26A). Thus, only restriction and physical maps of pBL1 are shown in Figure 26B). The pBL1 was, therefore, used for nucleotide sequencing to obtain a complete nucleotide sequence by using a universial primer to sequence the additional 5' region of the lipase gene. The nucleotide sequence of the lipase gene and its deduced amino acid sequence are demonstrated in Figure 27. Analysis of the lipase DNA sequence was shown to have 41.8% G/C content. The gene contained a putative promoter with -35 and -10 sequences, RBS and 12bp inverted repeats downstream the TAA stop codon. The total nucleotide sequence of 1,560bp between *Hin*dIII-*Sal*I fragment which contained both ORF1 and 2 is shown in Figure 28. The lipase activity of both pBL1 and pBL2 showed to have high activity (Table 15) suggesting that they expressed as its own promoter.

4.8 Comparison of DNA and amino acid sequences homology of the ORF1 and ORF2 lipase genes of *B. subtilis* WRRL-B558 with that of other lipases and esterases

As mentioned earlier, the DNA sequence of both ORFl and ORF2 lipase genes had high homology to nucleotide sequence of the artificial lipase genes (A02815 and A02816) of *G. candidum* (Eur. Patent 0243338) and also of *B. subtilis* 168 lipase gene (Dartois *et al.*,1992). The DNA sequence similarity between these three genes were demonstrated in Figures 29 and 30, respectively. The DNA sequence analysis of the artificial lipase genes (A02815 and A02816) demonstrated that they were complementary to each other. The ORF1 and ORF2 lipase of *B. subtilis* WRRL-B588 DNA and their deduced amino acid sequences were compared and found to have

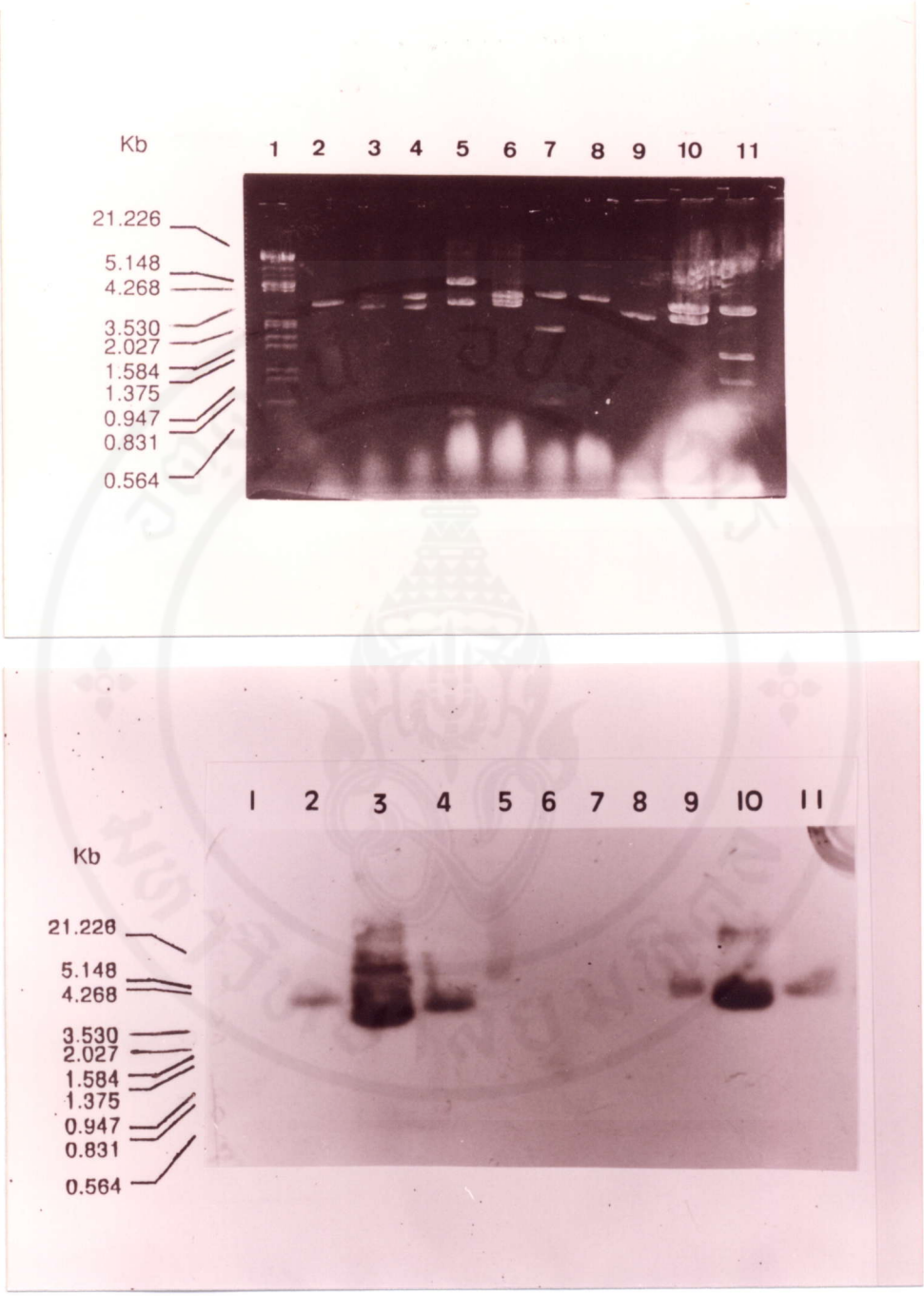
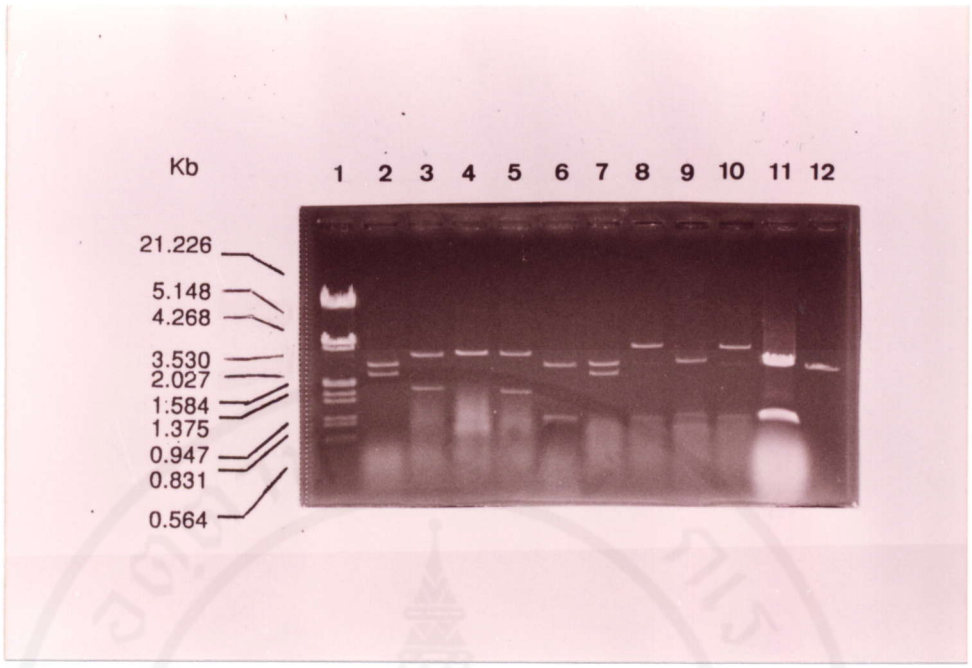


Figure 25. A. A 1.0% agarose gel electrophoresis of various *Hin*dIII digested recombinant plasmids derived from cloning *B. subtilis* DNA cut with *Hin*dIII and cloned into pBS-*Hin*dIII vector.
B. Southern blot DNA-DNA hybridization of various *Hin*dIII digested recombinant plasmids probed with 0.7 Kb *Eco*RI-*Eco*RI fragment which contained incomplete ORF2.
Lane 1, λ DNA/*Hin*dIII-EcoRI; lane 2, pBSK vector;
lane 3, pBT 1 cut with *Hin*dIII; lane 4, pBL1 cut with *Hin*dIII;
lane 5, pBL3 cut with *Hin*dIII; lane 6, pBL4 cut with *Hin*dIII;
lane 7, pBL5 cut with *Hin*dIII; lane 8, pBL6 cut with *Hin*dIII;
lane 9, pBL7 cut with *Hin*dIII; lane 10, pBL2 cut with *Hin*dIII;
lane 11, pBL8 cut with *Hin*dIII.



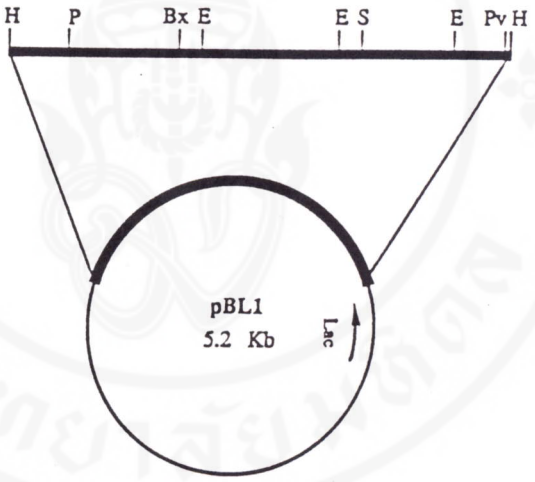


Figure 26. A) A 0.8% agarose gel elctrophoresis patterns of pBL1 and pBL2 plasmids.
Lane 1, λ DNA/*Hin*dIII-*Eco*RI; lanes 2 and 7, pBL1 and pBL2 cut with *Hin*dIII; lanes 3 and 8, pBL1 and pBL2 cut with *Bst*XI; lanes 4 and 9, pBL1 and pBL2 cut with *Eco*RI; lanes 5 and 10; pBL1 and pBL2 ; cut with *Sal*I lanes 6 and 11, pBL1 and pBL2 cut with *Bst*XI/*Sal*I; lane 12, pBSK vector cut with *Hin*dIII.
B) Physical map of plasmid pBL1. The thick and thin lines represent the *B. subtilis* and vector pBluescript (pBS) DNA, respectively. The arrow indicates the direction of transcription of the lac promotor from the pBS (+SK). Restriction enzymes : Bx; *Bst*XI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I.

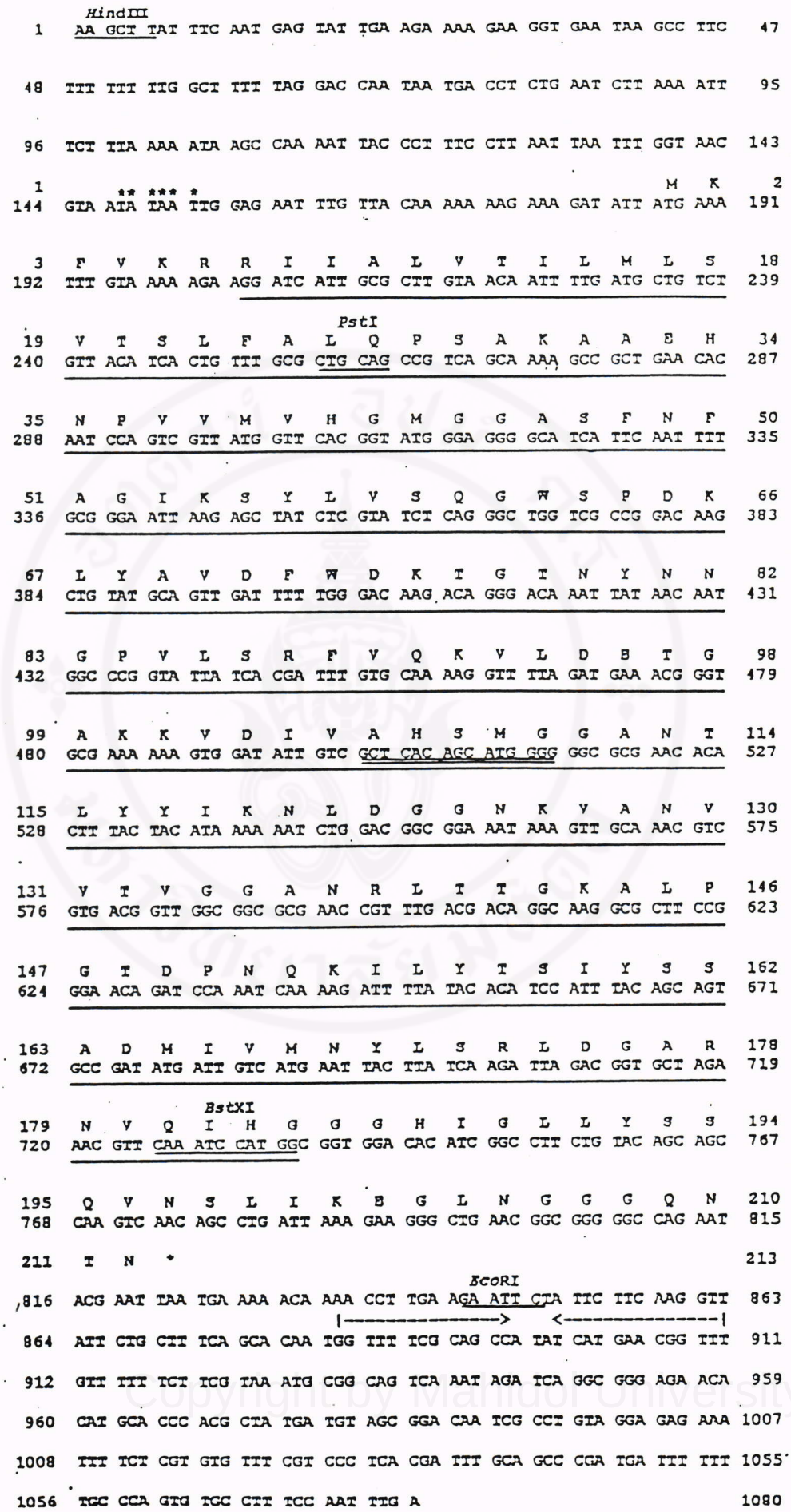


Figure 27. Nucleotide sequence and the deduced amino acids of lipase gene from *B. subtilis* WREL-B558. Symbols : _____, fragment use as probe; * putative promotor; _____ restriciton site; -----> <----- , inverted repeat; =, catalytic site

DNASIS ***** DNA SEQUENCE FILE LIST ***** DATE 03-15-96

*** INPUT INFORMATION ***

SEQUENCE : 1560BP; 504 A; 300 C; 349 G; 407 T.

*** COMMENT ***

LOCUS 0 1560 bp DNA 13-OCT-1995

BASE COUNT 504 A 300 C 349 G 407 T

```
          10          20          30          40          50          60
5' AAGCTTATTT CAATGAGTAT TGAAGAAAAG AAGGTGAATA AGCCTTCTTT TTTTTGGCTT
          70          80          90         100         110         120
   TTTAGGACCA ATAATGACCT CTGAATCTTA AAATTTCTTT AAAAATAAGC CAAAATTACC
         130         140         150         160         170         180
   CTTTCCTTAA TTAATTTGGT AACGTAATAT AATTGGAGAA TTTGTTACAA AAAAAGAAAG
         190         200         210         220         230         240
   ATATTATGAA ATTTGTAAAA AGAAGGATCA TTGCGCTTGT AACAATTTTG ATGCTGTCTG
         250         260         270         280         290         300
   TTACATCACT GTTTGCGCTG CAGCCGTCAG CAAAAGCCGC TGAACACAAT CCAGTCGTTA
         310         320         330         340         350         360
   TGGTTCACGG TATGGGAGGG GCATCATTCA ATTTTGCGGG AATTAAGAGC TATCTCGTAT
         370         380         390         400         410         420
   CTCAGGGCTG GTCGCCGGAC AAGCTGTATG CAGTTGATTT TTGGGACAAG ACAGGGACAA
         430         440         450         460         470         480
   ATTATAACAA TGGCCCGGTA TTATCACGAT TTGTGCAAAA GGTTTTAGAT GAAACGGGTG
         490         500         510         520         530         540
   CGAAAAAAGT GGATATTGTC GCTCACAGCA TGGGGGGCGC GAACACACTT TACTACATAA
         550         560         570         580         590         600
   AAAATCTGGA CGGCGGAAAT AAAGTTGCAA ACGTCGTGAC GGTTGGCGGC GCGAACCGTT
         610         620         630         640         650         660
   TGACGACAGG CAAGGCGCTT CCGGGAACAG ATCCAAATCA AAAGATTTTA TACACATCCA
         670         680         690         700         710         720
   TTTACAGCAG TGCCGATATG ATTGTCATGA ATTACTTATC AAGATTAGAC GGTGCTAGAA
         730         740         750         760         770         780
   ACGTTCAAAT CCATGGCGGT GGACACATCG GCCTTCTGTA CAGCAGCCAA GTCAACAGCC
         790         800         810         820         830         840
   TGATTAAAGA AGGGCTGAAC GGCGGGGGCC AGAATACGAA TTAATGAAAA ACAAAACCTT
         850         860         870         880         890         900
   GAAGAATTCT ATTCTTCAAG GTTATTCTGC TTTCAGCACA ATGGTTTTCG CAGCCATATC
         910         920         930         940         950         960
   ATGAACGGTT TGTTTTTTCT TCGTAAATGC GGCAGTCAAA TAGATCAGGC GGGAGAACAC
         970         980         990        1000        1010        1020
   ATGCACCCAC GCTATGATGT AGCGGACAAT CGCCTGTAGG AGAGAAATTT TCTCGTGTGT
        1030        1040        1050        1060        1070        1080
   TTCGTCCCTC ACGATTTGCA GCCCGATGAT TTTTTTGCCC AGTGTGCCTT TCCAATTTGT
        1090        1100        1110        1120        1130        1140
   CAGCGGCATC AGCAAAGGGT ACACAATCAG CATCAATATG GCGACGATAA TGACACCGGC
        1150        1160        1170        1180        1190        1200
   GGACCCATCG CCAAACGTAA ATCCGGCTGC CAAAATCACT GCTGCGGCAA TGATTACATC
        1210        1220        1230        1240        1250        1260
   AAGTAAAAGA GCGCAGGCCA GCATGAAACC AGCTAGTTCC AATAGAAACA CTCCTTAAAA
        1270        1280        1290        1300        1310        1320
   TGTTAAATAA ATACCTAATG ATTGTAAAAA AGAAGGGCCT AAAGTGGGAA TAGGTGATAA
        1330        1340        1350        1360        1370        1380
   GCCTTAAATC ACAAAAGTTG GTGAAAATGT CATGGGTAAA TTGGCATAAT CAGCCAGCTT
        1390        1400        1410        1420        1430        1440
   ATTACATTAC CAAATTCTTT TTTAGCCCGA AACCAAGCCC TCAGAAGTTA TTTTTGTTAA
        1450        1460        1470        1480        1490        1500
   AATAGAAAAG TTACAACAGA ATTCGGAGGG TTTATTGTGG GAAAAGTGAA ACGAAATGCC
        1510        1520        1530        1540        1550        1560
   CCTTGCCCAT GCGGCAGCGG CAAGAAATAT AAAAAATGCT GCGGAAGTAA AGTTGTCGAC 3'
```


Figure 28. Complete nucleotide sequence of 1560bp between *Hin*dIII-*Sal*I fragment from the 3Kb-*Sau*3AI fragment of which contains 2 open reading frames; ORF1 lipase gene; ORF2 lipase gene.

Table 15. Lipase activity of sonicated cells of *E. coli* habouring pBL1, pBL2 and pBT1 plasmids, *E.coli* host habouring the vector (pBS) only and *B. subtilis* WRRL-B558 using p-nitrophenylpalmitate as a substrate.

Sonicated cells of	Lipase activity * (U/mg protein)
JM 109 + pBS	0.7
JM 109 + pBT1	0.9
JM 109 + pBL1	41.8
JM 109 + pBL2	38.4
B. subtilis WRRL-B558	2.76

* 1 unit of the lipase activity was expressed as a nmol of p-nitrophenol released /ml./min.

```
LIP-ORF2.SEQ ATGAAATTTGTAAAAAGAAGGATCATTGCGCTTGTAACAATTTTGATGCTGTCTGTTACA
LIP-168.SEQ                                A
A02815.SEQ                                 A

LIP-ORF2.SEQ TCACTGTTTGCGCTGCAGCCGTCAGCAAAAGCCGCTGAACACAATCCAGTCGTTATGGTT
LIP-168.SEQ    G         T
A02815.SEQ     G         T

LIP-ORF2.SEQ CACGGTATGGGAGGGGCATCATTCAATTTTGCGGGAATTAAGAGCTATCTCGTATCTCAG
LIP-168.SEQ          T
A02815.SEQ                     C                   C           C

LIP-ORF2.SEQ GGCTGGTCGCCGGACAAGCTGTATGCAGTTGATTTTTGGGACAAGACAGGGACAAATTAT
LIP-168.SEQ            G                                       C
A02815.SEQ             G                                       C

LIP-ORF2.SEQ AACAATGGCCCGGTATTATCACGATTTGTGCAAAAGGTTTTAGATGAAACGGGTGCGAAA
LIP-168.SEQ          A
A02815.SEQ                      G

LIP-ORF2.SEQ AAAGTGGATATTGTCGCTCACAGCATGGGGGGCGCGAACACACTTTACTACATAAAAAAT
LIP-168.SEQ
A02815.SEQ                     G

LIP-ORF2.SEQ CTGGACGGCGGAAATAAAGTTGCAAACGTCGTGACGGTTGGCGGCGCGAACCGTTTGACG
LIP-168.SEQ
A02815.SEQ                                C

LIP-ORF2.SEQ ACAGGCAAGGCGCTTCCGGGAACAGATCCAAATCAAAAGATTTTATACACATCCATTTAC
LIP-168.SEQ
A02815.SEQ

LIP-ORF2.SEQ AGCAGTGCCGATATGATTGTCATGAATTACTTATCAAGATTAGACGGTGCTAGAAACGTT
LIP-168.SEQ                                           T
A02815.SEQ                                            T

LIP-ORF2.SEQ CAAATCCATGGCGGTGGACACATCGGCCTTCTGTACAGCAGCCAAGTCAACAGCCTGATT
LIP-168.SEQ              T
A02815.SEQ               T

LIP-ORF2.SEQ AAAGAAGGGCTGAACGGCGGGGGCCAGAATACGAATTAATGAAAAACAAAACCTTGAAGA
LIP-168.SEQ
A02815.SEQ

LIP-ORF2.SEQ ATTCTATTCTTCAAGGTTATTCTGCTTTCAGCACAATGGT
LIP-168.SEQ    G
A02815.SEQ     G         G         G         G
```

Figure 29. DNA sequence maximum homology list of the ORF2 lipase gene (LIP-ORF2.SEQ) of *B. subtilis* WRRL-B558, *B. subtilis* 168 lipase gene (LIP-168.SEQ) and artificial lipase gene of *G. candidum* (A02815.SEQ). The alphabets appeared at the two lower lines of each block indicate the different nucleotides.

AMINO ACID SEQUENCE MAXIMUM HOMOLOGY LIST

```
LIP-ORF2.AMI MKFVKRRIIALVTILMLSVTSLFALQPSAKAAEHNPVVMVHGMGGASFNFAGIKSYLVSQ
LIP-A.AMI                                                 I
A02815.AMI                                                I   P     T P


LIP-ORF2.AMI GWSPDKLYAVDFWDKTGTNYNNGPVLSRFVQKVLDETGAKKVDIVAHSMGGANTLYYIKN
LIP-A.AMI       R
A02815.AMI      R                                                 R


LIP-ORF2.AMI LDGGNKVANVVTVGGANRLTTGKALPGTDPNQKILYTSIYSSADMIVMNYLSRLDGARNV
LIP-A.AMI
A02815.AMI                L


LIP-ORF2.AMI QIHGGGHIGLLYSSQVNSLIKEGLNGGGQNTN
LIP-A.AMI        V
A02815.AMI       V
```

Figure 30. Amino acid sequence maximum homology list of the ORF2 lipase gene of *B. subtilis* WRRL-B558 (LIP-ORF2.SEQ), *B. subtilis* 168 lipase gene (LIP- 168.SEQ) and artificial lipase gene of *G. candidum* (A02815.SEQ). The alphabets appeared at the two lower lines of each block indicate the different amino acids.

51% and 24% homology, respectively. The deduced amino acid comparison of these two ORF lipase genes is shown in Figure 31.

Recently, Mitsushima *et al.* (1995) had cloned and reported a DNA sequence of cephalosporin C deacetylase from *B. subtilis* SHS0133. Comparison of its DNA/amino acid sequences to those of our lipases and those that mentioned above are summarized in Table 16. Compared with the microbial and mammalian lipases sequenced so far, *B. subtilis* WRRL-B558 ORF1/2 lipases appear to lack the conserved pentapeptide Gly-X-Ser-X-Gly purposed to play an essential role in catalysis. However, alignments of several microbial lipase sequences (Table 11) showed significant homology between this conserved peptide and the sequence Ala-His-Ser-Met-Gly (residue 106-110) present in the ORF2 lipase gene and Asp-Gly-Ser-Ala-Gly (residue 26-30) present in the ORF1 lipase gene of our *B. subtilis* WRRL-B558 which could conceivably carry out identical enzymatic function. There are a variation in the first Gly in this pentapeptide, from Gly in the conserved sequence to Ala and Asp in ORF2 lipase and ORF1 lipase, respectively (see Table 11). It is generally assumed that the central serine is the nucleophilic residue involved in the catalytic function. The DNA sequence/ deduced amino amino acid of ORF1 lipase gene from this study showed to have the smallest size compared to those of other lipases' sequence published so far.

4.9 Physical properties of the ORF1 and ORF2 lipase genes of *B. subtilis* WRRL-B558

The deduced amino acid sequences of the ORF1 and ORF2 lipase genes of *B. subtilis* WRRL-B558 were analysed by PROSIS^{TM} programme. The amino acid composition of the ORF1 lipase was high in glycine, alanine, valine, leucine, isoleucine, threonine and lysine with leucine the highest in mole % (14.17 Mole %) and cysteine and asparagine the lowest (0.83 Mole %). A somewhat similar amino acid profile is seen with

```
PROSIS      *****  AMINO ACID MAXIMUM HOMOLOGY LIST  *****        DATE 03-16-9

<<INFORMATION>>
   EQUIVALENT          NOTHING

   FILE1 NAME      START     END              FILE2 NAME      START     END

   ART-LIP1.AMI        1     120              LIP-E.AMI           1     212


   FILE NAME       1         10        20        30        40        50        6
   ART-LIP1.AMI    MLAC-----AL---L-LDVII--A----A-A-----VILAAGFTFGDG-S---AGVIIV
                   :     ::   : : :   :    : :       :     :   : : :   :: :
   LIP-E.AMI       MKFVKRRIIALVTILMLSVTSLFALQPSAKAAEHNPVVMVHGM--G-GASFNFAG-IKS
                   1         10        20        30        40        50        6

   FILE NAME      61         70        80        90       100       110       12
   ART-LIP1.AMI    ILMLIVYPLLMP--LTN---W-KGTLGKKII-G--L----QIVRDETHEK---I---SLI
                    :   :     : :     : : : :      : :     : : :::  :   :   :
   LIP-E.AMI       -L---VSQGWSPDKLYAVDFWDK-T-GTNYNNGPVLSRFVQKVLDETGAKKVDIVAHSMG
                  61         70        80        90       100       110       12

   FILE NAME     121        130       140       150       160       170       18
   ART-LIP1.AMI    QAIVRYII---------AWVH-VFS--RLIYLTA-AF--TKKKQ----TVH----DMA--
                    :   : :         : : :    ::   : :   :    :     :     ::
   LIP-E.AMI         GANTLYYIKNLDGGNKVANVVTVGGANRLT--TGKALPGTDPNQKILYTSIYSSADMIVM
                 121        130       140       150       160       170       18

   FILE NAME     181        190       200       210       220
   ART-LIP1.AMI    --------AKT--I-------------V--L-KAE.........
                           :  :             :  : : :
   LIP-E.AMI         NYLSRLDGARNVQIHGGGHIGLLYSSQVNSLIK-EGLNGGGQNTN
                 181        190       200       210       220

MATCHING PERCENTAGE
      TOTAL WINDOW          24%    (   56/  225)

      ALIGNMENT WINDOW      26%    (   56/  215)
```

Figure 31. Comparison of deduced animo acid sequence homology of the ORF1 lipase and that of the ORF2 lipase gene of *B. subtilis* WRRL-B558. The presumably catalytic sites were shown in boxes.

Table 16. Comparison of the DNA/amino acid sequence homology of the lipase/esterase genes and their products.

			G. candidum	
	B. subtilis 168 lipase gene	*B. subtilis* SHS0133 cephalosporinC deacetylase	artificial gene for lipases (A02815)	aritificial sequence for lipase (A02816)
B.subtilis WRRL-B558 ORF2 lipase gene	DNA (95%)* AA(98%)	DNA (57%) AA (26%)	DNA (97%) AA (96%)	DNA (51%) AA (25%)
B. subtilis WRRL - B558 ORF1 lipase gene	DNA (51%) AA (23%)	DNA (51%) AA (21%)	DNA (51%) AA (23%)	DNA (95%) AA (93%)
G. candidum artificial gene for lipase (A02815)	DNA (98%) AA (97%)	DNA (52%) AA (26%)	-	-
G. candidum artificial sequence for lipase (A02816)	DNA (50%) AA (24%)	DNA (52%) AA (21%)	-	-

* Numbers in parentheses showed the percentage of homology between DNA sequence (DNA) and amino acid sequence (AA).

Table 11. The cloned lipase genes from various microorganims and their conserved catalytic sequence.

Microorganism	Protein sequence*										Reference
B. subtilis 168	V	D	I	V	**A**	H	**S**	M	**G**	G	Dartois *et.al.*, 1992
B. stearothermophilus	I	A	V	A	**G**	L	**S**	L	**G**	G	Kugimiya *et al.* 1987 Jap. Patent S62-233531
Staphylococcus hyicus	V	H	F	I	**G**	H	**S**	M	**G**	G	Gotz *et al.*, 1985
S. aureus	V	H	L	V	**G**	H	**S**	M	**G**	G	Lee and Iandolo, 1986
Pseudomonas fragi	V	N	L	I	**G**	H	**S**	Q	**G**	A	Kugimiya *et al.*, 1986
P.cepacia	V	N	L	V	**G**	H	**S**	Q	**G**	G	Jorgensen *et al.*, 1991
P.aeruginosa I and II	V	N	L	I	**G**	H	**S**	H	**G**	G	Nishioka *et al.*, 1990; Wohlfarth and Winkler, 1988
P. fluorescens	V	N	L	V	**G**	H	**S**	Q	**G**	G	Patent application of Amano : EPO0331376 (1989)
P. glumae	V	N	L	I	**G**	H	**S**	Q	**G**	G	Patent application Unilever WO 91/00910 (1991)
Moraxella I	L	G	A	I	**G**	W	**S**	M	**G**	G	Feller *et al.*, 1990
Moraxella II	T	H	V	G	**G**	N	**S**	M	**G**	G	Feller *et al.*, 1991 a
Moraxella III	I	V	L	S	**G**	D	**S**	A	**G**	G	Feller *et al.*, 1991 b
Mucor miehei	V	A	V	T	**G**	H	**S**	L	**G**	G	Boel *et al.*, 1988
Geotrichum candidum I	V	M	I	F	**G**	E	**S**	A	**G**	A	Nagao *et al.*, 1990
G. candidum II	V	M	I	F	**G**	E	**S**	A	**G**	A	Shimada *et al.*, 1990
G. candidum	V	D	I	V	**A**	R	**S**	M	**G**	G	Vandamme *et al.*, 1987 Eur. Patent 0243338
B. subtilis WRRL-B558 ORF2 lipase gene	V	D	I	V	**A**	H	**S**	M	**G**	G	This study
B. subtilis WRRL-B558 ORF1 lipase gene	F	T	F	G	**D**	G	**S**	A	**G**	V	This study

* Residues which are strictly conserved are in bold character.

that of the ORF2 lipase gene, i.e., glycine, alanine, valine, leucine, isoleucine, threonine, lysine, serine and asparagine were present in high percentage with glycine the highest in mole % (11.79 Mole %) and cysteine the lowest (0.00 Mole %). The deduced protiens of the ORF1and ORF2 lipases showed molecular weights 13101 and 22692, respectively (Figures 32 and 33) and isoelectric points (pIs) of 10.48 and 9.98, respectively. The hydrophobicity analysis using the hydrophobicity index table file of Kyte-Thr in PROSIS^{TM} program showed that the ORF1 lipase was very hydrophobic with the mean index of 0.99 whereas the ORF2 lipase was less hydrophobic (mean index = -0.02) as shown in Figures 34 and 35, respectively. The hydrophobicity plot of amino acids of the ORF1 lipase shows similar patttern to that of A02816A . Also, a similar pattern of hydrophobicity plot was demonstrated for the amino acids of the lipase and that of *B. subtilis* 168 lipase and of A02815.

4.10 Subcloning of the ORF1 and ORF2 lipase genes of *B. subtilis* WRRL-B558 into pTTQ18 vector

4.10.1 Construction of overexpressing clones

Attempts to increase expression of the ORF1 and ORF2 lipase genes were done by subcloning each gene into pTTQ18 vector which contained the strong promotor of ptac. Construction strategy of subclones is shown in Figure 36. The resultant plasmids containing the ORF2 and ORF1 lipase genes in pTTQ18 vector were pTL7 and pTE12, respectively. Moreover, two more plasmids habouring both the lipase genes under the control of ptac promoter were constructed and named pTEL25 and pTEL28 as shown in Figure 36. An analysis of these plasmids was done by restriction mapping with various restriction enzymes used. Gel electrophoreses of the digested plasmids is shown in Figure 37. The restriction maps of subclones harbouring ORF1 and/or ORF2 in pBluescript or pTTQ18 vector are summarized in Figure 38.

```
PROSIS     ***** Amino Acid Composition / Molecular Weight *****    DATE 03-16-9

  *** INPUT INFORMATION ***

      FILE NAME : ART-LIP1.AMI

      SIZE      :   120 aa

  *** Amino Acid Composition Graph **

      Count  From     1  To    120 :  Total    120
```

Amino Acid		Count	Mol%	0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
Gly	G	7	5.83	***
Ala	A	16	13.33	*******
Val	V	11	9.17	*****
Leu	L	17	14.17	********
Ile	I	16	13.33	*******
Ser	S	3	2.50	**
Thr	T	8	6.67	****
Cys	C	1	0.83	*
Met	M	4	3.33	**
Asp	D	4	3.33	**
Asn	N	1	0.83	*
Glu	E	3	2.50	**
Gln	Q	3	2.50	**
Arg	R	3	2.50	**
Lys	K	9	7.50	****
His	H	3	2.50	**
Phe	F	4	3.33	**
Tyr	Y	3	2.50	**
Trp	W	2	1.67	*
Pro	P	2	1.67	*
Asx	B	0	0.00	
Glx	Z	0	0.00	
***	*	0	0.00	
Xxx	X	0	0.00	
(Total		120)		

```
*** Amino Acid Molecular Weight ***

    Molecular Weight(M.W) = 13101.22
```


Figure 32. Amino acid composition and molecular weight determination calculated from deduced amino acids of ORF1 lipase gene of *B. subtilis* WRRL-B558.

PROSIS ***** Amino Acid Composition / Molecular Weight ***** DATE 12-04-95

*** INPUT INFORMATION ***

FILE NAME : LIP-E.AMI

SIZE : 212 aa

*** Amino Acid Composition Graph **

Count From 1 To 212 : Total 212

Amino Acid		Count	Mol%	0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
Gly	G	25	11.79	******
Ala	A	16	7.55	****
Val	V	20	9.43	*****
Leu	L	20	9.43	*****
Ile	I	12	5.66	***
Ser	S	16	7.55	****
Thr	T	12	5.66	***
Cys	C	0	0.00	
Met	M	7	3.30	**
Asp	D	9	4.25	***
Asn	N	17	8.02	****
Glu	E	3	1.42	*
Gln	Q	7	3.30	**
Arg	R	6	2.83	**
Lys	K	14	6.60	****
His	H	5	2.36	**
Phe	F	6	2.83	**
Tyr	Y	9	4.25	***
Trp	W	2	0.94	*
Pro	P	6	2.83	**
Asx	B	0	0.00	
Glx	Z	0	0.00	
***	*	0	0.00	
Xxx	X	0	0.00	
(Total		212)		

*** Amino Acid Molecular Weight ***

Molecular Weight(M.W) = 22692.87

Figure 33. Amino acid composition and molecular weight determination calculated from deduced amino acids of ORF2 lipase gene of *B. subtilis* WRRL-B558.

```
PROSIS        ***** HYDROPHOBICITY ANALYSIS LIST *****

  *** INPUT INFORMATION ***
      FILE NAME : ARTI-LIP.AMI
      HYDROPHOBICITY INDEX TABLE FILE : KYTE.THR

  *** HYDROPHOBICITY INDEX ***
      WINDOW : 6          MEAN INDEX : 0.99    (FROM 1 TO 120)
      THRESHOLD LINE : 0.00
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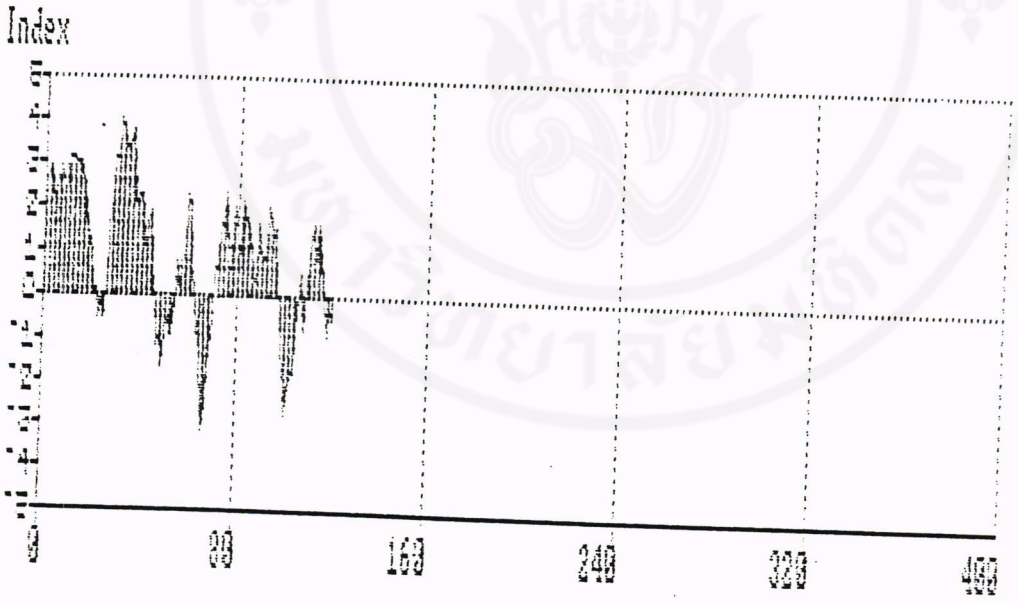

Figure 34. Hydrophobicity analysis of deduced amino acids derived from ORF1 lipase gene of *B. subtilis* WRRL-B558.

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PROSIS      ***** HYDROPHOBICITY ANALYSIS LIST *****

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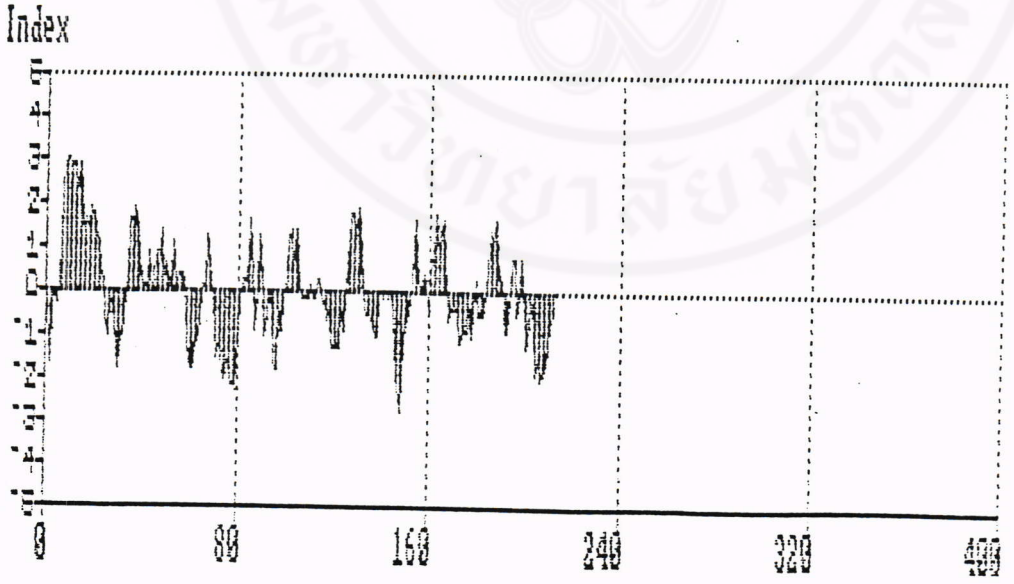


Figure 35. Hydrophobicity analysis of deduced amino acids derived from ORF2 lipase gene of *B. subtilis* WRRL-B558.

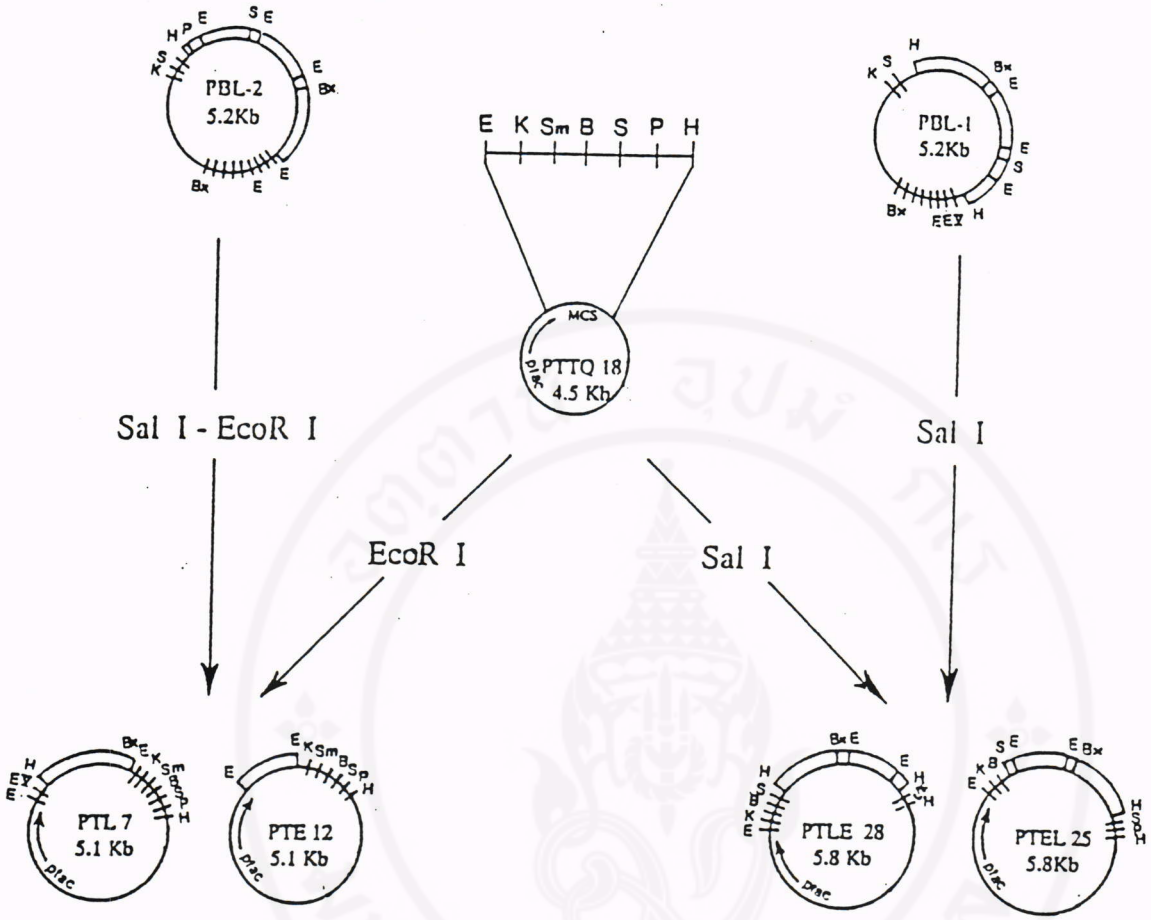


Figure 36. Construction of subclones pTL7, pTE12, pTLE28, and pTEL25 from pBL2 and pBL1 and the pTTQ18 vector. Restriction enzymes : B, *Bam*HI; Bx, *Bst*XI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I.

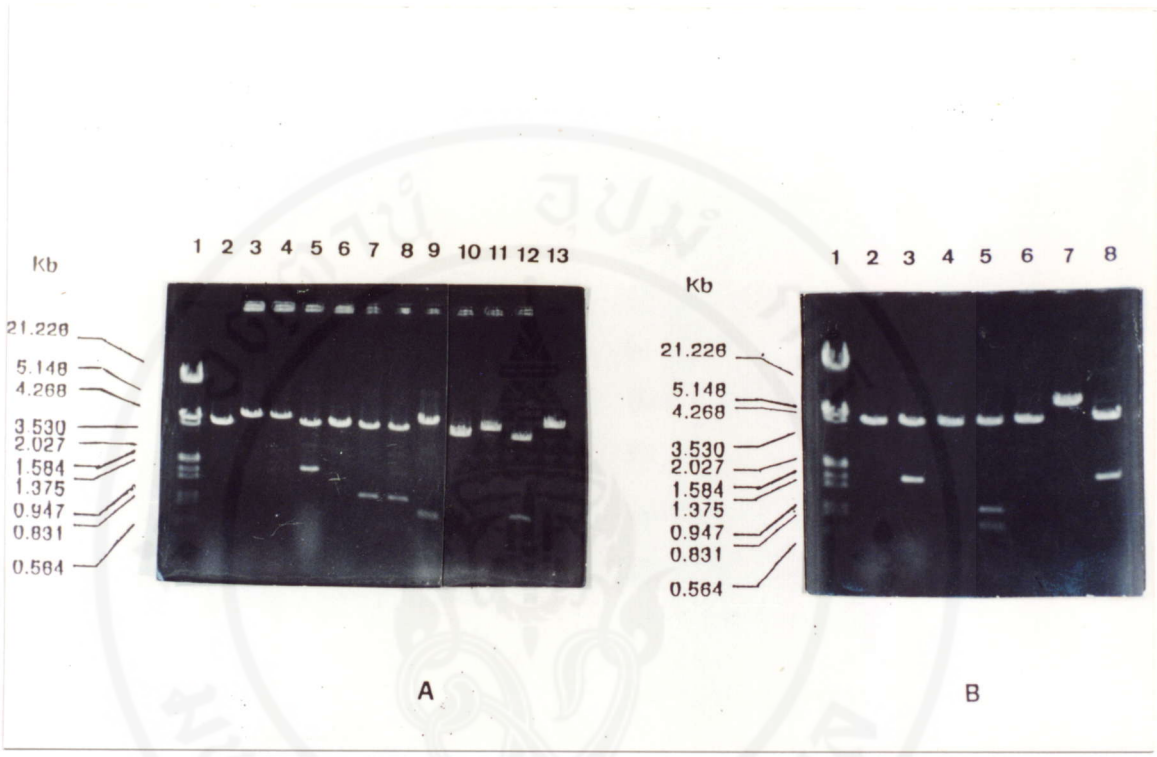
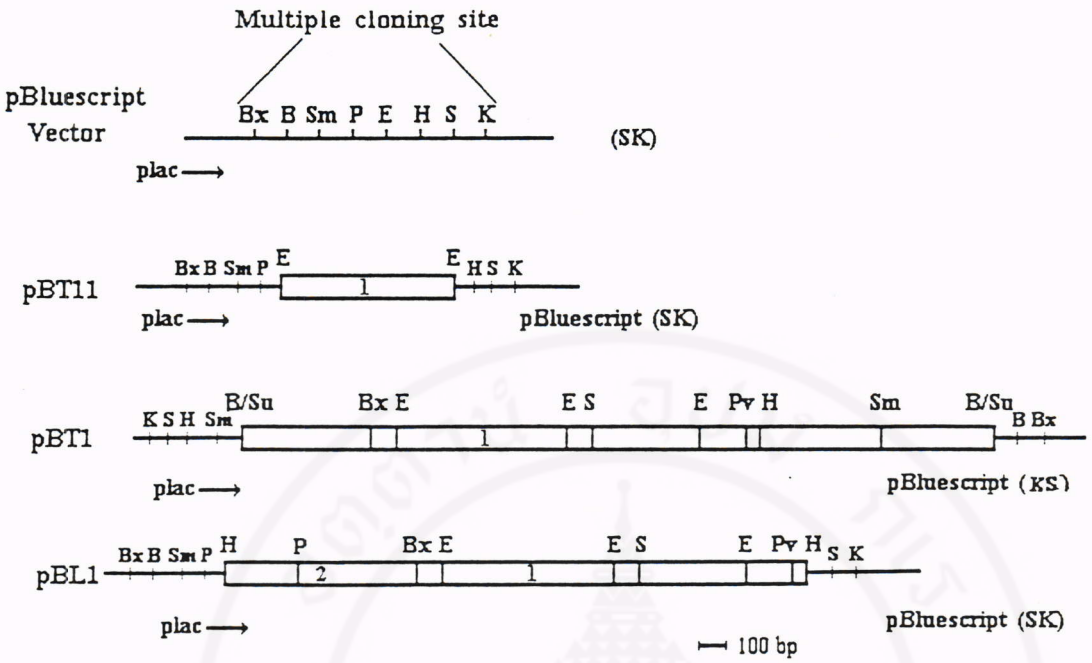


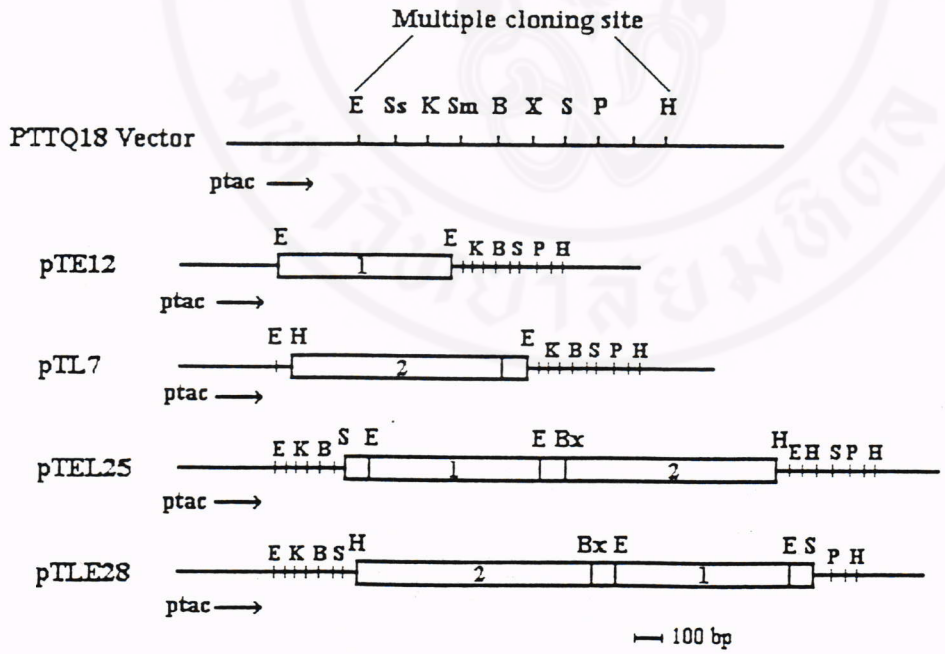
Figure 37. A 0.8% gel electrophoresis patterns of the recombinant plasmids pTE12,pTL7, pTEL25, and pTLE28.

A. Lane 1, λ DNA/*Hin*dIII-*Eco*RI; lane 2, pTTQ18 cut with *Sal*I; lane 3, pTE12 cut with *Sal*I; lane 4, pTL7 cut with *Sal*I; lane 5, pTEL25 cut with *Sal*I; lane 6, pTTQ18 cut with *Eco*RI; lane 7, pTE12 cut with *Eco*RI; lane 8, pTL7 cut with *Eco*RI ; lane 9, pTEL25 cut with *Eco*RI; lane 10, pTTQ18 cut with *Hin*dIII; lane 11, pTE12 cut with *Hin*dIII; lane 12, pTEL7 cut with *Hin*dIII; lane 13, pTEL25 cut with *Hin*dIII;

B. Lane 1, λ DNA/*Hin*dIII-*Eco*RI; lane 2, pTTQ18 cut with *Sal*I; lane 3, pTLE28 cut with *Sal*I; lane 4, pTTQ18 cut with *Eco*RI; lane 5, pTLE28 cut with *Eco*RI; lane 6, pTTQ18 cut with *Hin*dIII; lane 7, pTLE28 cut with *Bam*HI; lane 8, pTLE28 cut with *Hin*dIII.



A.



B.

Figure 38. A) Restriction maps of the ORF1 harbouring clones; pBT11, pBT1 under plac promotor in pBluescript vector.
B) Restriction maps of the overexpressing clones, pTE12, pTL7, pTEL25, pTLE28 under ptac promotor in pTTQ18 vector.
Restriction enzymes : Bx, *Bst*XI; E, site *Eco*RI; H, *Hind*III; S,*Sal*I.

4.10.2 Enzyme production of the overexpressing clones

To determine the enzyme activity of the overexpressed clones, various methods were employed. Indicator containing media, including Tributyrin agar plate, Rhodamine B plate and Calcium-Triolein agar plate were used to determine the lipolytic activity. Cephalosporin C and *S. aureus* ATCC 25923 were used to determined the cephalosporin C esterase activity. The overexpressed clones were compared to the *E. coli* harbouring vectors and the original clones in the pBluescript vector; pBT1 and pBT11. Results showed clearly that a clone harbouring the ORF1 lipase gene in pTTQ18 vector (pTE12) showed increased in lipase activity as seen with all the indicator containing media (Figure 39) and increased in cephalosporin C esterase as seen with the cephalosporin C- *S.aureus* overlaid medium (Figure 40) as compared to clones harbouring pBT1 and pBT11.

Quantitative measurement of the enzymes produced by the overexpressing clones were carefully performed. Lipase activity was assayed using p-nitrophenylpalmitate (pNPP) as a substrate and esterase activity was assayed using α-naphthylacetate (α-NA) as substrate. Results showed that marked increase in esterase (1.5 times) and lipases (7.6 times) activities was seen with the overexpressing clones (pTE12) when compared to their counterparts which contained the pBS vector (pBT11), except the clone containing pBL1 which showed high lipase activity (Table 17).

4.11 Overexpression of pTL7 and pTE12 under IPTG induction

To determine whether expression of the both lipase genes under the control of ptac would be increased with the induction of IPTG inducer, log phase cultures (4 h. of growth) of the recombinant overexpressing clones were added with IPTG to a final concentration of 10 mM and incubated further at 37°C for an additional 4 h. Sonicated cells of the overexpressing clones with and without IPTG induction were assayed for lipase activity using pNPP as a substrate compared to that of the *E. coli* harbouring only the

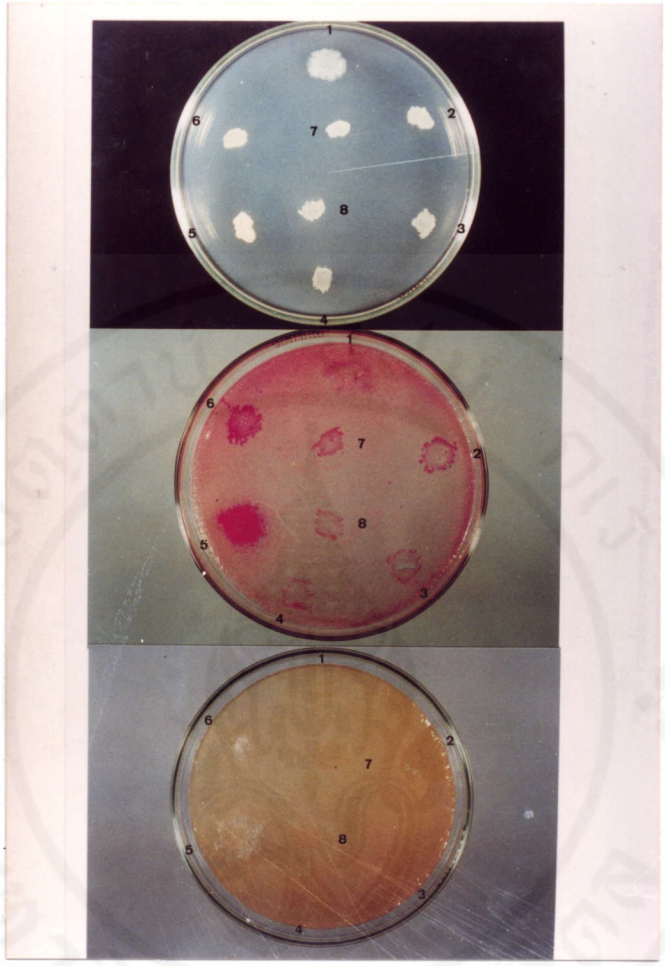


Figure 39. Phenotypic expression of various clones as determined by using A. Tributyrin agar plate B. Rhodamine B agar plate C. Calcium-Triolein agar plate. The culture were grown at 37°C for 48 h. and kept at 4°C for over 4 days.

1, *B. subtilis* WRRL-B558; 2, JM109/pTE12;
3, JM109/pBT11; 4, JM109/pBT1;
5, *S. aureus* ATCC25923; 6, JM109/pBL1;
7, JM109/pBS; and 8, JM109/pTTQ18.

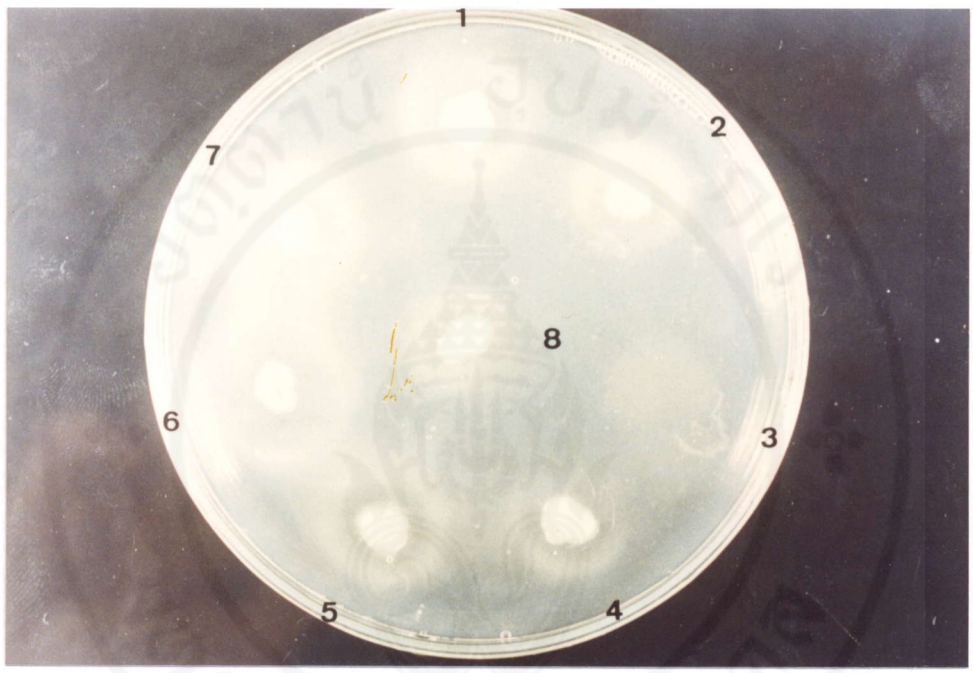


Figure 40. Determination of the cephalosporin C esterase activity of the producing clones. The replica colonies were grown at 37°C for 30 h.and overlayed with 1% (v/v) overnight culture of *S. aureus* ATCC 25923 and 300 μg/ml cephalosporin C. The plate was incubated at 37°C for 18 h.

1, JM109/pTE12; 2, JM109/pBL1;
3, *B. subtilis* WRRL-B558; 4, JM109/pBT11;
5, JM109/pTEL25; 6, JM109/pTL7;
7, JM109/pTLE28; and 8,JM109/pTTQ18 vector.

Table 17. Esterase and lipase activity of *E.coli* JM109 habouring ORF1, ORF2 or both with different orientation using α-naphthylacetate (α-NA) and p-nitrophenylpalmitate (pNPP) as substrates, respectively.

JM109 habouring	α NA* (nmol/min/mg protein)	pNPP* (nmol/min/mg protein)	pNPP/α-NA
pBS	5.7 ± 2.5	0.7 ± 0.2	0.12
pBS + ORF1 (pBT11)	5.8 ± 3.2	0.8 ± 0.4	0.14
pBS + ORF2-1 (pBL1)	15.8 ± 1.9	41.8 ± 2.7	2.65
pBS + ORF1 (pBT1)	6.7 ± 1.5	0.9 ± 0.3	0.13
pTTQ18	5.1 ± 2.1	0.6 ± 0.2	0.12
pTTQ18+ORF1 (pTE12)	8.5 ± 3.5	6.1 ± 0.8	0.72
pTTQ18 + ORF2(pTL7)	17.1± 0.3	47.5 ± 11.7	2.78
pTTQ18+ORF1-2 (pTEL25)	18.1± 8.9	32.6 ± 9.8	1.8
pTTQ18+ORF2-1 (pTLE28)	21.2 ± 17.9	40.4 ± 10.3	1.9
B. subtilis WRRL-B558	20.6 ± 13.2	2.76 ± 0.4	0.13

* The values shown above are the means of 6 samples from three experiments with 2 replicates each.

pTTQ18 vector. The IPTG induced pTL7 and pTE12 clones showed about 7 times and 5 times increased in lipase activity as compared to that under the non-induced condition (Table 18).

4.12 Polyacrylamide gel electrophoresis of the overexpressing clones

To characterize the nature of enzyme produced by clones harbouring pTL7 and pTE12, their sonicated (uninduced) cell extractes were used to run onto the 7.5% native gel electrophoresis. Controls included sonicated cells of the *E. coli* host with and without the pTTQ18 vector and the *B. subtilis* WRRL-B558. After the gel was stained for lipase activity, an extra positive smeared band appeared with samples of overexpressing clones compared to that of *E. coli* with the vector only (Figure 41). Since the positive bands of the overexpressing clones showed a smeared band pattern and stacked at the top of the gel suggesting that they might form aggregates. Therefore, to determine their molecular weights by using SDS-PAGE, a 15% SDS-gel was supplemented with 5M.urea and 0.5% Triton X-100. It should also be noted that there was a faint band at about the same position in the gel for each of the organisms studies.

The SDS-PAGE of the overexpressing clones (pTL7 and pTE12) was stained with Coomassie Brilliant Blue (Figure 42A) and its renatured counterpart gel was stained with the esterase activity stain (Figure 42B). The results showed that molecular weights of proteins that had lipase activity were about 14 kDa. for that of the recombinant clones. It was showed clearly that the activity-stained bands of pTL7 and pTE12 and 66 kDa for that of *B. subtilis* WRRL-B558.

4.13 Isolation and purification of the enzymes from recombinant clones pTL7 and pTE12

Sonicated cell extracts of *E. coli* pTL7 and *E. coli* pTE12 each was loaded onto a Sephadex G-200 column equilibrated with 0.1 M phosphate buffer pH 7.5. Chromatographic profiles of protein and lipase

Table 18. Lipase activity of sonicated cells of *E. coli* habouring pTTQ18, pTL7 and pTE12 plasmids.

	Lipase activity (Unit/mg protein)*	
	without IPTG	with IPTG**
JM109/PTTQ18	1.0	1.1
JM109/pTL7	1.6	11.6
JM109/pTE12	1.5	7.1

* Lipase activity was determined in duplicate using pNPP as a substrate.

** With IPTG induction, 4 h culture of each clone was grown in LB broth contain 50 μg/ml ampicillin and was supplemented with 10 mM IPTG, then, incubated further for an additional 4h..

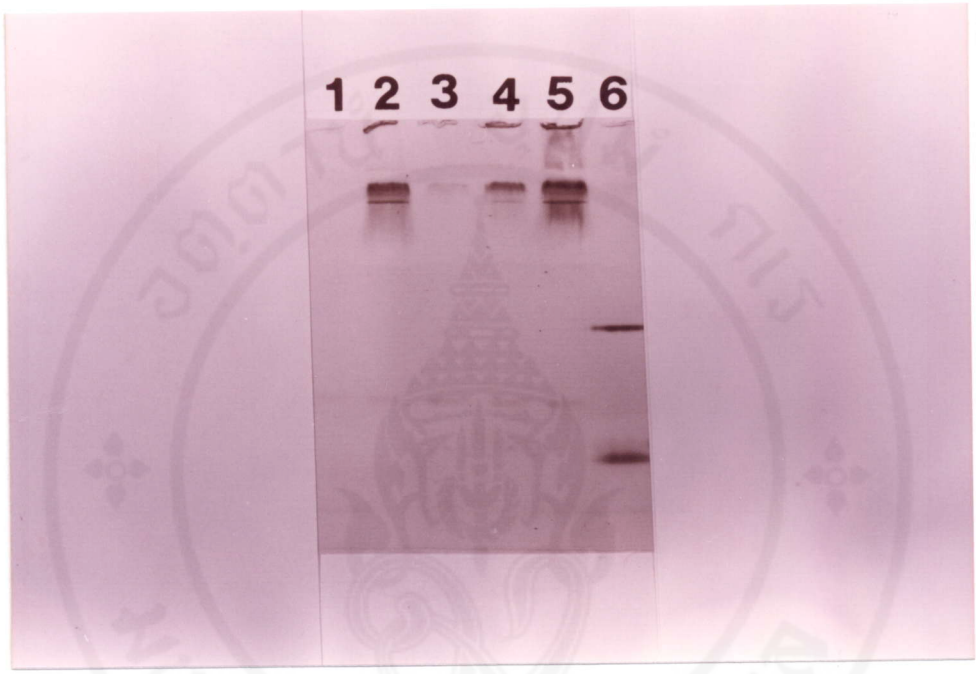


Figure 41. Native polyacrylamide gel electrophoresis of proteins from various clones. Twenty micrograms of protein from each sample was loaded onto 7.5% gel and stained for esterase activity. Lane 1, JM109/pTTQ18; lane 2, JM109/pTL7; lane 3, JM109/pTE12; lane 4, JM109/pTEL25; lane 5, JM109/pTLE28; lane 6, *B.subtilis* WRRL-B558.

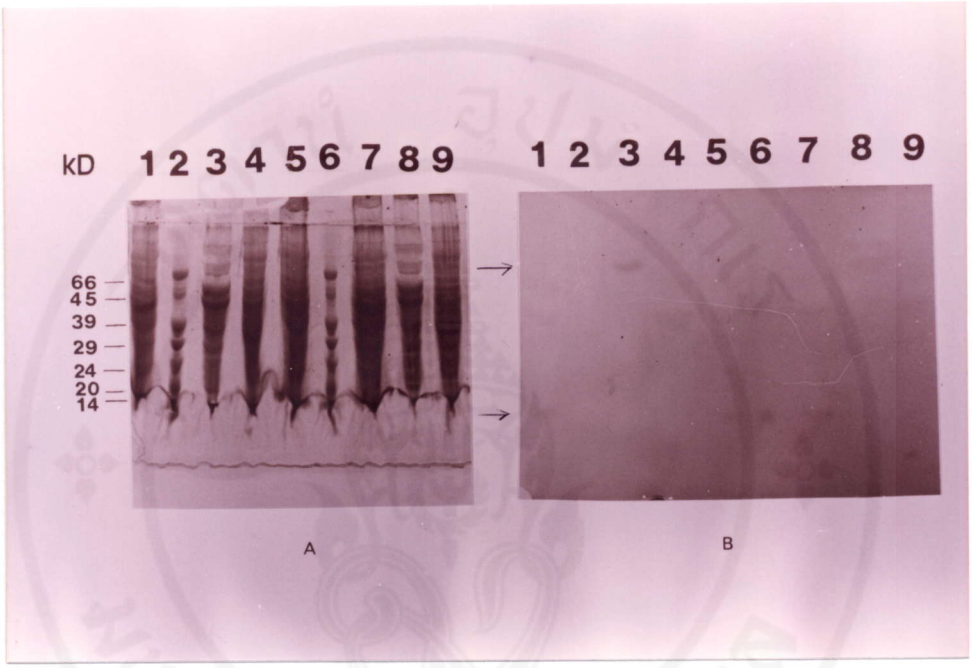


Figure 42. SDS-PAGE of sonicated cells of the overexpressed clones A. stained with Coomassie Brilliant Blue. B. Renaturation of the comparable SDS-PAGE in 0.04 M Tris HCL, pH 6.0 containing 0.1% Triton X-100 at 4°C overnight and stained with β-naphthyl acetate and Fast Blue BB dye in 0.1 M phosphate buffer pH 7.5 for esterase activity.
Lanes 1 and 5, JM109/pTL7; lane 2 Molecular weight standards; lanes 3 and 8, *B. subtilis* WRRL-B558; lanes 4 and 9, JM109/pTTQ18; lanes 6 and 7, JM109/pTE12.

activity of *E. coli* pTL7 and *E. coli* pTE12 are shown in Figures 43A and B, respectively. Two major of proteins were observed in both samples, one of which was eluted together with the void volume. On the other hand, there were many peaks of enzyme activity with *E. coli* pTL7 but only one peak with *E. coli* pTE12. Interestingly, the protein peaks that eluted at the void volume in both samples show high lipase activity. Fractions which had lipase activity were pooled then, the pooled samples were checked for homogeneity by nondenature PAGE. The results showed aggregated protein patterns on top of the gel and some bands with lipase activity (Figure 44). The pooled samples were also assayed for esterase and lipase activities using α-naphthylacetate and pNPP, respectively (Table 19). The results showed highest specific activity with the peak 3 sample from *E. coli* pTL7 but a disappointing result was obtained with the lower specific activity of the peak 1 sample of *E. coli* pTE12 as compare to that of the sonicated *E. coli* pTE12 sonicated cells. Details of enzyme purification for the lipase activity is shown in Table 20 and for the esterase activity is shown in Table 21. Purification was successful only with peak 3 of the pTL7 clones,.i.e., a two fold purification was achieved with a 36% recovery, the other peaks showed no better in purity of the enzyme than that of the sonicated cells.

4.14 Characterization of the partially purified ORF1 and ORF2 lipases from recombinant clones

The pTL7-peak 3 and pTE12-peak 1 samples were used to study for substrate specificity and inhibitor effects. Purified monoacid triglycerides, with various chain lengths were tested. Table 22 listed hydrolytic activities of pTL7-peak 3 and pTE12-peak2 towards the monoacid triglycerides when incubated at 37°C for 30 min. It was found that both enzymes showed broad specificity to various carbon chain lengths. They hydrolyzed esters of different fatty acid chain lengths at approximately the same rate with a slight preference for tristearin (C18).

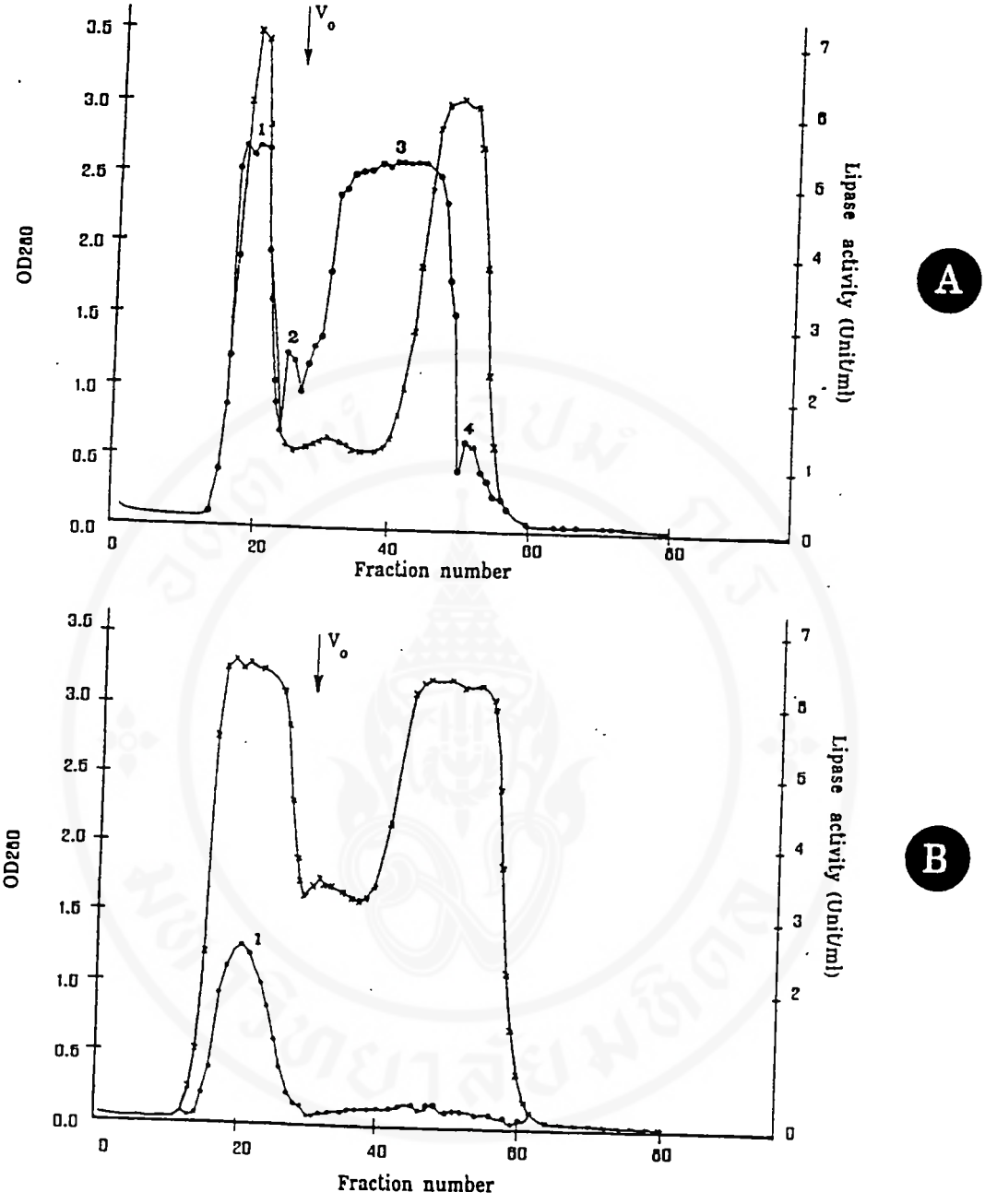


Figure 43. Profiles of Sephadex G-200 column chromatography of sonicated cell samples of A. JM109/pTL7 and B. JM109/pTE12. Two hundred ml of 18 h. old culture grown in LB broth containing 50 mg/ml of ampicillin was centrifuged and the pellet was washed once with 0.1M phosphate buffer pH 7.5 and resuspended with 20 ml of the same buffer. The suspension was treated with ultrasonic disintegration before being applied to the column. The column was equilibrated with 0.1M phosphate buffer pH 7.5. The elution was carried out with the same buffer at a flow rate of 8 ml/hr. Two ml fractions were collected at every 15 min.

x, Absorbance at 280 nm; •, lipase activity. Numbers are assigned for the interested peaks.

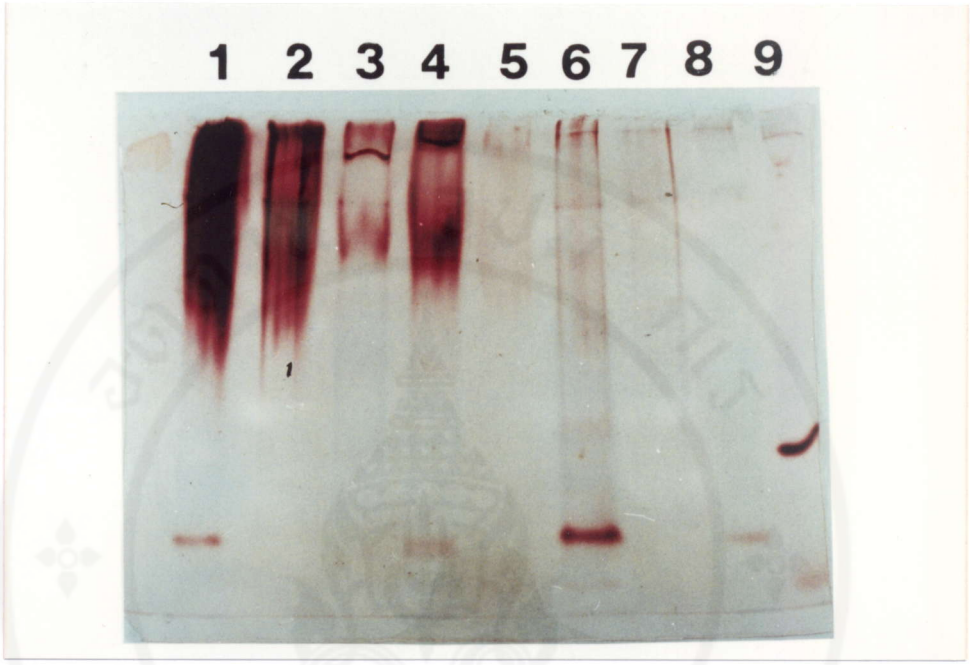


Figure 44. Esterase activity staining of protein samples obtained from pTL7, pTE12, pTTQ18 clones and *B. subtilis* WRRL-B558 on nondenature PAGE. The protein samples includes, the crude protein from sonicated cells and the partially purified protein's obtained after being purified with Sephadex G-200 column chromatography. The samples were mixed with sample buffer which contained 0.5% Triton X 100.
Lane 1, JM109/pTL7 sonicated cells; lane 2, peaks 1 of JM109/pTL7 sample;
lane 3, peak 2 of JM109/pTL7sample; lane 4, peak 3 of JM109/pTL7 sample;
lane 5, peak 4 of JM109/pTL7 sample; lane 6, JM109/pTE12 sonicated cells;
lane 7, peak 1 of JM109/pTE12 samples; lane 8, JM109/pTTQ18 sonicated cells;
lane 9, *B. subtilis* WRRL-B558 sonicated cells.

Table 19. Esterase and lipase activities of sonicated cells of JM109/pTL7 and JM109/pTE12 and pooled fractions from Sephadex G-200 chromatographic peaks.

Sample	Protein	α-naphthylacetate*		pNPP*	
	(mg/ml)	U/ml/min	U/mg protein	U/ml/min	U/mg protein
JM109/pTL7 sonicated cells	6.4	9.6	1.5	15.1	2.36
JM109/pTL7-peak 1	1.4	3.1	2.2	3.6	2.6
-peak 2	0.6	0.3	0.5	1.2	2.1
-peak 3	0.7	2.8	4	3.4	4.8
-peak 4	0.2	0.03	0.15	0.4	2.2
JM109/pTE12 sonicated cells	6.2	6.3	1	2.2	0.36
-peak 1	2.0	0.56	0.56	0.32	0.16

* 1 unit is expressed as 1 nmol α-naphthol or p- nitrophenol released/ml/min.

Table 20. Lipase activity of overexpressing clones, pTL7 and pTE12, before and after purification with Sephadex G-200 filtration. Lipase activity was measured with p-nitrophenylpalmitate as the substrate.

Type of protein	Volume (ml)	Activity* (U/ml)	Total Activity (U)	Protein (mg/ml)	Total Protein (mg)	Specific activity (U/mg protein)	Fold purification	% Recovery
pTL7-sonicated cells	20	15.1	302	6.4	128	2.36	1	100
Sephadex G-200								
pTL7- peak 1	12	3.63	43.6	1.4	16.8	2.6	1.10	14.4
pTL7- peak 2	4	1.27	5.1	0.6	2.4	2.1	0.89	1.7
pTL7- peak 3	32	3.4	109	0.7	22.4	4.8	2.03	36
pTL7-peak 4	4	0.43	1.72	0.2	0.8	2.16	0.9	0.5
pTE12-sonicated cells	20	2.25	45	6.2	124	0.36	1	100
Sephadex G-200								
pTE12-peak 1	22	0.317	7.0	2.0	44	0.16	0.44	15.5

* 1 unit was expressed as 1 nmole of p-nitrophenol released per/min at 37°C.

Table 21. Esterase activity of overexpressing clones, pTL7 and pTE12, before and after purification with Sephadex G-200 filtration. Esterase activity was measured with α-naphthylacetate as the substrate.

Type of protein	Volume (ml)	Activity* (U/ml)	Total Activity (U)	Protein (mg/ml)	Total Protein (mg)	Specific activity (U/mg protein)	Fold purification	% Recovery
pTL7-sonicated cells	20	9.6	192	6.4	128	1.5	1	100
Sephadex G-200								
pTL-7 peak 1	12	3.1	37.2	1.4	16.8	2.2	1.4	19.4
pTL7- peak 2	4	0.3	1.2	0.6	2.4	0.5	0.3	0.62
pTL7- peak 3	32	2.8	89.6	0.7	22.4	4	2.6	46.6
pTL7- peak 4	4	0.03	0.12	0.2	0.8	0.15	0.1	0.06
pTE12-sonicated cells	20	6.3	126	6.2	124	1.01	1	100
Sephadex G-200								
pTE12-peak 1	22	1.13	24.8	2.0	44	0.56	0.55	19.7

* 1 unit was expressed as 1 μmol α naphthol released per min at 37°C.

Table 22. Specificity of lipase from pTL7-peak 3 and pTE12-peak 1 to various purified monoacid triglycerides.

Substrate	Sample	Lipase acitivity* (Unit/ml)	Lipase specific acitivity (Unit/mg protien)
Tributyrin (C 4: 0)	pTL7-p3	24.4	34.9
	pTE12-p1	25.0	12.5
Tricaprylin (C 8 :0)	pTL7-p3	28.4	40.5
	pTE12-p1	22.7	11.4
Trilaurin (C 12 : 0)	pTL7-p3	30.1	43.0
	pTE12-p1	17.0	8.5
Trimyristin (C14 : 0)	pTL7-p3	17.1	24.4
	pTE12-p1	28.4	14.2
Tripalmitin (C16 : 0)	pTL7-p3	19.0	27.1
	pTE12-p1	26.7	13.3
Tristearin (C 18 : 0)	pTL7-p3	35.8	51.1
	pTE12-p1	34.1	17.0

* 1 unit of the enzyme activity was expressed as 1 μmole of fatty acid released / min / ml.

Table 23. Effect of phenylmethylsulfonylfluoride (pMSF) and β-mercaptoethanol on activity of lipase from pTL7-peak 3 and pTE12-peak 1.

Samples	Relative remaining activity (%)
pTL7-peak 3	100
pTL7-peak 3 + 1 mM PMSF	52
pTL7-peak 3 + 1 mM β-mercaptoethanol	96
pTE12-peak 1	100
pTE12-peak 1 + 1 mM PMSF	56
pTE12-peak 1 + 1 mM β-mercaptoethanol	100

Since lipase has been suggested to contain a serine catalytic site, the serine inhibitor, phenylmethyl-sulfonylfluoride (PMSF) was used to study the inhibitory effects. After 2 hour of incubation at 30°C, PMSF showed significant effect on the lipase activity of both enzymes (Table 23). Also, the effect of a sulfhydryl reducing agent, β-mercaptoethanol, was investigated by adding to the enzyme samples to a final concentration of 1mM. and the mixture was incubated for 2 h. at 30°C. The β-mercaptoethanol showed no effect on the lipase acitivity of both enzymes (Table 23).

In order to study the ability of the enzymes to hydrolyze cephalosporin C, pTL7-peak 3 and pTE12-peak 1 samples were incubated with cephalosporin C for 3 h. at 37°C and the reaction mixtures were determined for the acetic acid product liberated by using an enzymatic reagent kit (Boehringer Mannheim). The result (Table 24) showed that cephalosporin C acetylesterase activity of pTL7 peak 3 and of pTE12-peak1 was around twice higher than that of the sonicated cells of JM109 habouring the pTTQ18 vector. Nevertheless, the ability to hydrolyse cephalosporin C of the purified enzyme was found to be inferior to that of *B. subtilis* WRRL-B558 cells. The concentration of acetic acid liberated was measured as mg/l in the reaction samples and that it could be converted to mM of acetic acid which, in turn, could be converted to mM of cephalosporin C by the fact that 1M of cephalosporin C is hydrolysed by the enzyme resulted in 1M of acetic acid released.

Summary of lipase and esterase activities of pTL7-peak 3, pTE12-peak 1, sonicated cells of JM109/pTTQ18 and that of *B. subtilis* WRRL-B558 is shown in Table 25. The esterase activity of pTE12 peak 1 seemed to be more specific to cephalosporin C than that of pTL7 peak 3 and the others.

Table 24. Cephalosporin C acetylesterase activity of various clones The reaction mixture was consisted of 10 mg/ml cephalosporin C in 0.1 M. phosphate buffer pH 7.5 and sonicated cells were incubated at 37°C for 3 hr. Free acetic acid liberated was measured by an enzymatic reaction kit (Boehringer Mannheim).

Type of sonicated cells	Acetic acid liberated		
	mg/l	mM	μM/min
JM109/pTTQ18	110	1.83	10.16
JM109/pTL7-peak3	260	4.33	24.05
JM109/pTE12-peak1	210	3.5	19.44
B. subtilis WRRL-B558	369	6.16	34.22

The data shown was the result that was subtracted with blank (DW), Cep C in PBS and sonicated cells without cephalosporin C which were treated in parallel as that with the samples.

Table 25. Esterase and lipases of the pTL7 peak3, pTE12 peak1, sonicated cells of JM109/pTTQ18 and of *B. subtilis* WRRL-B558 to α - naphthylacetate, pNPP and cephalosporin C.

Sample	α-NA* (U/mg protein)	pNPP* (U/mg/protein)	cephalosporin C (mg/mg protein)	(μM/mg protein)
JM109/pTTQ18	1.0	1.9	110	10.1
pTL7-peak3	4	4.8	371.4	34.3
pTE12-peak1	0.56	0.16	105	9.7
B. subtilis WRRL-B558 Sonicated cells	18	3.0	369	34.2

* One unit = 1 nmole of the product released per min

Note Enzyme activity was expressed as the amounts of products; α-naphthol, p-nitrophenol and acetic acid released, respectively. For cephalosporin C substrate, the samples were incubated at 37°C with 1 mg/ml cephalosporin C for 1 hr. before doing the test.

CHAPTER V
DISCUSSION

Bacillus subtilis WRRL-B-558 used in this study was reported to be a high esterase producer organism (Abbot and Fukuda, 1975). The enzyme was reported to exhibit good kinetic properties and excellenat stability. This makes it attractive to be used for producing deacetylated cephalosporin, an intermediate in semi-synthetic cephalosporins synthesis. An approach to increase enzyme production for this purpose is the clone this gene and increase gene expression through genetic manipulation techniques.

At first, this work was to confirm the ability of *B. subtilis* WRRL-B558 to produce cephalosporin C esterase enzyme. Results obtained confirmed the previous report in that esterase activity of *B. subtilis* WRRL-B558 with α-naphtylacetate was high (20.6 U/mg protein) as compared to that of *E. coli* (5.7 U/mg protein). The enzyme could also hydrolyse cephalosporin C to form deacetylcephalosporin C as required. Although we do not have pure compound of deacetylcephalosporin C (DC), the use of a commercial esterase enzyme to hydrohyse cephalosporin C and use that as a standard DC was found to be reliable since the new peak formed had difficult retention time than that of Cep C, 7-ACA and 7-ADCA. Moreover, Reaction of cephalosporin C with *B. subtilis* WRRL-B558 yielded a similar peak with the same retention time. Therefore, we concluded that the product was real deacetylcephalosporin C and that the *B. subtilis* WRRL-B558 possessed high cephalosporin acetylesterase enzyme.

Since cephalosporin acetylesterase was the enzyme of interest in this project, the screening method for selecting recombinant clones using the double layered technique that contained cephalosporin C and or test organism on the second layer medium would be more specific than other general esterase plate tests. The test organism to be used must be cephalosporin resistant (cep^R) and deacetylcephalosporin sensitive (DC^S) or cep^S and DC^R. The selected *S. aureus* ATTCC25923 was found to be Cep ^{S} and DC^R thus it allowed *S. aureus* ATCC 25923 to grow as sattellite growth around the cephalosporin esterase producing clones.

The results from gene cloning showed that the selected recombinant clone, *E. coli* pBT1 was positive both with the β-naphthylacetate plate test and the biological double layer technique. Southern blot hydridization between the *B. subtilis* WRRL-B558 DNA and the probe made from *Bst*X-*Hin*dIII fragment of pBT1 confirmed that the inserted DNA was that dervied from the *Bacillus* DNA.

Result from deletion study (Figure 20) suggested that the gene was located on 0.9 Kb *Bst*XI-*Sal*I fragment. Nucleotide sequence of *Bam*HI/*Sau*3AI to *Sal*I site revealed that pBT1 plasmid contained a complete ORF1 gene and an incomplete ORF2 gene. Under *lacZ* promoter in pBS vector, the ORF2 was not in frame, thus, suggesting that the enzyme activity of pBT1 was derived from the ORF1 gene product only. The pBT11 clone containing 600bp *Eco*RI-*Eco*RI fragment gave negative result with β-naphthylacetate. This clone was found to have esterase acitivity with cephalosporin C substrate as shown in Figure 21. Since the reading of esterase activity using β-naphthylacetate and Fast Blue BB dye was rather difficult, therefore, it was believed that the negative result shown on Figure 20 on esterase activity of the pBT11 clone was a false negative. The factor that contributed to this could also be the substrate specificity of the enzyme, i.e., the esterase activity of the

pBT11 clone was more specific to cephalosporin C than β-naphthylacetate. Thus, the low activity together with the difficulty in reading results could easily cause a false result. Esterases with high substrate specificity were reported with the purified extracellular esterase enzyme from *Streptomyces viridosporus* T7A which showed that the enzyme could hydrolyse α-naphthylacetate, α-naphthylbutyrate and p-nitrophenylbutyrate but not p-nitrophenylacetate and p-nitrophenylpalmitate (Donnelly and Crawford, 1988). Another example was the spore esterase of *Bacillus coagulans* which had greater specificity (4 times more) for the α-naphthylacetate than β-isomer, although both naphthylacetate penetrate the spore equally well (Roberts and Rosenkrantz, 1966). Moreover, later study with the *Eco*RI-*Eco*RI fragment placed under the control of ptac (pTE12) showed that high esterase activity (against α-naphthylacetate) was obtained (Table 17).

When the complete ORF2 gene was cloned as in plasmid pBL1, significantly high lipase activity was detected (Table 15). This indicating that the ORF2 gene was a lipase encoding gene. Expression of the gene could be done by its own promoter as can be seen that pBL2 plasmid, the reverse orientation of pBL1, also gave high lipase activity (Table 15) eventhough it was not under the control of the lacZ promoter of pBS vector. This was also seen with pTLE28 and pTEL25 which were orientedly in opposite direction in the pTTQ18 plasmid vector. Clones of the two plasmids showed approximately the same lipase activity (Table 15). Thus the results could be interpreted also that self promoter of ORF-2 gene was a strong promoter since it could allow high expression for the ORF2 gene as similar to that of *lacZ* and ptac promoters (with no induction). However, the ORF1 was ORF2 genes were placed under the control of ptac and induced with IPTG. The lipase enzyme activity was increased to around 5 (ORF1-pTE12) to 7 (ORF2-pTL7) times higher

(Table 19). This indicated that higher yield of the lipase and esterase could be achieved through appropriate genetic manipulations. It should be pointed out that the clones studied for the lipase gene employed *E. coli* JM109 as the host because a recent report by Morikawa et al. (1994) showed that this strain of *E. coli* had low lipase activity and was a suitable host for lipase gene cloning studies. However, we found that both *E. coli* JM109 and *E. coli* XL-1B had similar (background of) lipase acitivity.

The very low pNPP/αNA ratio shown in Table 18 suggested that *B. subtilis* WRRL-B558 had high esterase and low lipase activities as opposite to that of the recombinant clones. And that instead of selecting the esterase producing clones we did select the lipase producing clones that, infortunately could be detected as the esterase producing clones, too.

Nucleotide sequence of both strands of the *Hin*dIII-*Sal*I fragment showed G/C content of the cloned gene to be around 40-50% similar to that of the *B. subtilis* genome (Gordon *et al.*, 1973). All Matching the 560 bp sequence of the *Hin*dIII-*Sal*I fragment to the artificial gene for lipase of *Geotrichum candidum*, a filamentous fungi (Eur. Patent 0243338) showed them to be very similar (98% homology). They both contained 2 ORFs in the same manner. Interestingly, the first 0.9Kb from *Hin*dIII site which contained an ORF (the ORF2) shared high homology to *B. subtilis* lipase gene reported by Dartois *et al.*, (1992). The significant homology of this gene among three the organisms suggested that this gene is conserved and, thus, the enzyme must play essential roles in these organisms. The 212 amino acids deduced from ORF2 contained the pentapeptide of A-H-S-M-G which was the same as that of *B. subtilis* 168 (Dartois *et al.*, 1992). One amino acid difference was found in *G.*

candidum artificial lipase, by having A-R-S-M-G. All these pentapeptides are presumably parts of the catalytic site of the enzymes.

The structure called "catalytic triad", which is a constellation of aspartic acid- histidine-serine by H-bonding activates the serine in this pentapeptide as catalytic residue, was also found in these deduced amino acids. The catalytic triad of the lipase gene of *B. subtilis* WRRL-B-558 was postulated to be Ser_{108}-$Asp_{164, 175}$ -$His_{183,187}$.

Previous reports showed X-ray analysis revealing atomic structures of two triacylglycerol lipases, unrelated in sequence : the human pancreatic lipase (hPL) (Winkler *et al.*, 1990) and a lipase enzyme isolated from the fungus *Rhizomucor meihei* (RmL) (Brady *et al.*, 1990). In both enzymes, the active centers contained structural analog of Asp-His-Ser triads (characteristic of serine proteinases). In addition, this structure was buried completely beneath a short helical segment or "lid". This helix structure exposed hydrophobic amino acid to the surrounding surface. The lid contained tryptophan sitting over the serine active site. It was proposed that interfacial activation generates movement of the lid and creates a catalytic competent enzyme able to attack the triglyceride molecules (Brzozowski *et al.*, 1991). The deduced amino acid of *B. subtilis* WRRL-B558 lipase gene showed the presence of the catalytic triad and Trp at positions 73 or 62 which might function as a lid. Another supportive evidence for the ORF2 gene product to be a lipase was that it was inhibited by a serine inhibitor agent, PMSF (Table 23).

Protein of the ORF1 lipase was rather small and quite hydrophobic (Figures 32, 34). It contained Ser_{28}-Asp_{67}-His_{108} which was supposed to form the catalytic triad. Although it contained no tryptophan but there were other hydrophobic amino acids surrounding the catalytic serine which was claimed to be important to lipase activity (Brzozowski *et al.*, 1991; Feller *et al.*, 1991). It was also inactivated by PMSF and able to

hydrolyze α-NA, pNPP and olive oil whose characteristics to were that activity of the lipase (Table 17 and Figure 39). Like the lipase of ORF2, it could also hydrolyze cephalosporin C (Table 24 and Figure 40).

The protein patterns of the overexpressing clones, pTL7 and pTE12 sonicated cells on native gel electrophoresis stained with the activity stain (Figure 41) showed smear patterns of protiens stacking at the uppermost part of the gel suggesting that the protein might aggregate. This was agreed with the hydrophobicity analysis shown in Figures 34 and 35 that these proteins were quite hydrophobic especially the ORF1 lipase. However, by adding 0.5% Triton X-100 into the sample buffer some protein bands could be demonstrated at the lower part of the gel suggesting that were non-aggregated enzymes or their subunits (Figure 44). The band location of active proteins from native gel electrophoresis of pTL7 and pTE12 sonicated cell samples (Figure 41) suggested that they were not the same protein of *B. subtilis* WRRL-B558. However, there was a faint (active) band at the same band position with each of the organism tested (Figure 44, *E. coli* pTE12 lane 6, *B. subtilis* WRRL-B558 lane 9 and *E. coli* host harbouring pTTQ18 lane 8). This enzyme was thought to be a lipase enzyme since it was found that *B. subtilis* WRRL-B558 produce high esterase and low lipase activity. With the esterase activity stain of the native gel of *B. subtilis* WRRL-B558 sample, the strong color band was thought to be the esterase band and the faint one was the lipase band. If the lipase was cloned into pTE12 or pTL7, then we would expected to see a much darker band at this position with E.coli pTE12 and/or *E.coli* pTL7. However, the results with the native gel did not support this idea. On the other hand, the same faint band was also detected with the sonicated of host harbouring the pTTQ18 only. This suggested that the faint bands in both recombinant clones was rather the expression of the *E. coli* (the host) gene. We could not be certain

whether or not the cloned lipases which were shown as protein aggregates in *E. coli* pTE12 and *E. coli* pTL7 were the same as *B. subtilis* lipase enzyme appeared as a faint band with the native gel. If it is the same enzyme(s), then, an answer is needed for the different behaviours appeared on the native gel. It could be that when the enzymes were produced in large quantity by the recombinant clones, they might form aggregates due to their hydrophobic nature. Moreover, different environment in the microbial cells(*B. subtilis* vs. *E. coli*) might prevent or assist the formation of aggregates. If once the aggregates were formed they might be more stable, therefore, very minute amount of free enzyme was detected.

Estimation of protein mass from activity staining of the renatured SDS-PAGE demonstrated that the active proteins had a molecular mass about 14 kDa, although the deduced MW of ORF1 and ORF2 were about 13,101kDa and 22,692 kDa, respectively. This discrepancy could just be due to the inaccuracy of the method and their difference in protein nature. However, the two proteins were of different sizes from that of the strongly positive band (around 66kDa) of *B. subtilis* WRRL-B558. This 66KDa band of *B. subtilis* WRRL-B558 was thought to be the esterase enzyme which was reported to be high in this organism (Abbot and Fukuda, 1975a) and because the activity stain was rather designed for detecting esterase activity (Table 14). The absence of an activity protein around 14 kDa for *B. subtilis* WRRL-B558 might be the result of low lipase activity of this organism (Table 15).

Partial purification of the enzymes was attempted by using Sephadex G-200 column chromatography. Since the enzymes were found mostly with microbial cells (no lipase activity could be detected in broths of the recombinant clones), only broth of *B. subtilis* WRRL-B558 show some enzyme activity, therefore, sonicated cells were used this study.

High lipase activity of both enzymes, the ORF2 lipase (pTL7 clone) and the ORF1 lipase (pTE12 clone) were detected with the protein peak appeared in the void volume indicating very large molecular weight proteins or the aggregation nature of the enzyme. Since the molecular weights of the deduced enzymes (ORF1 and ORF2 products) were known, they were considered to be aggregated proteins. The protein profile curve and the activity profile curve of pTL7 clones show high lipase activity at peaks 1 and 3 (Figure 43). Since the peak 3 activity showed low protein content, it was expected to have high specific activity which was confirmed by the results shown in Table 19. However, for pTE12 clones, the activity purfile showed only one peak at the void volume indicating that most enzyme was in the aggregated form. This result confirmed the data on hydrophobic nature of the ORF1 lipase (produced by pTE12 clones) which was strongly hydropholic in nature while the lipase (produced by pTL7 clones) was less hydrophobic. Purification process was considered unsuccessful since no better in purity obtained excepted with peak 3 of pTL7 clone and low percent recovery was obtained (Table 19).

The poor performance of Sephadex G-200 chromatography could be due to the fact that the Sephadex gel contained hydrophilic matrix which had low efficiency for gel filtration of hydrophobic substances. To reduce such polar effect, salt might be added to the running buffer to help blocking the polar groups (Pharmacia, USA). However, studies are needed to determine stability of the enzymes in the presence of salt. Other substances that may be used to reduce the polar effect are sugars such as sucrose or glycerol as was shown by Bowden and Georgiou (1990) and Sherrer *et al.* (1994). Other type of gels such as the hydrophobic gel, phenyl Sepharose, may be used to purify these hydrophobic enzymes as

that demostrated by Lesuisse *et al.* (1993). Again eluent used such as ethylene glycol may be toxic to the enzymes of interests.

For the inhibitor study, as expected the serine specific inhibitor, PMSF was an effective inhibitor of both enzymes (ORF1 and ORF2 lipase) because they had serine amino acid at their catalytic sites, on the other hand the sulfhydryl reagent, β-mercaptoethanol should have no effect on enzyme activities because only one cysteine was present in the ORF2 lipase and none was in the ORF1 lipase.

From the studies of substrate specificity (Tables 24 and 25) and the protein purification (Tables 20 and 21), it was concluded that if deacetylation of cephalosporin C was of interests we have two choices to do, that is, to do gene cloning again in order to fish out the *Bacillus* esterase gene (coding for the enzyme of 66 kDa) or to use either of the two lipases of our clones. If it is for the latter, a better method for isolation and purification of the enzymes is needed. However, there might be an advantage for using lipase to catalyse the ester bond at C3 position of cephalosporin C which can simultaneously incorporate a new side chain in the same reaction mixture, resulting in a new cephalosporin derivative. However, information about purity of the enzyme, stability, enzyme kinetics and even the difficulty relating to production and isolation of the enzymes an requried before final decision should be made.

CHAPTER VI
CONCLUSION

Cephalosporin C can be hydrolyzed by an esterase resulting in formation of deacetylcephalosporin C, a key intermediate, by which substitution at C3' side chain produce potentially useful semisynthetic cephalosporins. *B. subtilis* WRRL-B558 was known to be a high producer of cephalosporin acetylesterase (Abbot and Fukuda, 1975). Genes from *B. subtilis* WRRL-B558 encoding for the enzymes was cloned and sequenced. DNA sequence of 1560bp from *Hin*dIII-*Sal*I region showed 2 ORFs (ORF1 and ORF2) located adjacent to each other in reverse orientation. Their nucleotide sequences were consisted of -35 and -10 promoter conserved sequence; putative ribosome binding site and 11-12 bp inverted repeats, and a Rho independent transcription terminator located, after the TAA stop codon. ORF1 encodes for a deduced protein of 120 amino acids and ORF2 for 212 amino acids with MW of 13, 23 kDa, respectively. This was in agreement with MW of the enzymes determined by SDS-PAGE to be around 14 kDa. DNA homology revealed 95% homology of ORF1 to part of *Geotrichum candidum* artificial lipase sequence. ORF2 showed 95% with homology that of *B. subtilis* 168 lipase gene and 97% homology with *Geotrichum candidum* artificial lipase gene. The genes from both ORFs were subcloned into pTTQ18 vector which contains the ptac strong promotor. The subclone of ORF1 was found to have higher lipase activity around 7.6 times (uninduced with IPTG) and 38 times (induced with IPTG), than that of the original recombinant clones. Studies on substrate specificity of the two enzymes showed that they were of the lipase type that were able to act as esterase and able to hydrolyse cephalosporin C to form deacetylcephalosporin C. The enzymes were found to be sensitive to PMSF, a serine inhibitor but resistant to β-

mercaptoethanol, a sulfhydryl agent. The enzymes produced from subclones were partially purified using Sephadex G-200 gel filtration. The purification process was found to be rather unsuccessful because the best pooled peak (pTL7-peak 3) showed only 2 times increase in purity of the enzyme while low percent recovery was achieved. The failure could be attributed to the strong hydrophobic nature of both enzymes.

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