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ISOLATION, ANALYSIS, AND EXPRESSION OF CEPHALOSPORIN C DEACETYLASE GENE FROM *BACILLUS CEREUS* STRAIN BT-24

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KEY WORDS : *BACILLUS* / CEPHALOSPORIN C DEACETYLASE / ESTERASE / CLONING

SONGSAK ROEKRING : ISOLATION, ANALYSIS, AND EXPRESSION OF CEPHALOSPORIN C DEACETYLASE GENE FROM *BACILLUS CEREUS* STRAIN BT-24. THESIS ADVISORS : CHUENCHIT BOONCHIRD, Ph.D., VITHAYA MEEVOOTISOM, Ph.D., SUTHEP WIYAKRUTTA, Ph.D., p160. ISBN 974-663-807-6

Cephalosporin deacetylase (CAH) is an enzyme used for removal of the side chain at C-3 position of cephalosporin C. The product obtained is deacetylcephalosporin C that is useful for production of cephalosporin derivatives. In this study, high esterase producing strains isolated from soil samples were screened by the chemical and microbiological methods. Positive colonies were confirmed by HPLC for their ability to convert cephalosporin C to deacetylcephalosporin C. One strain showing highest amount of cephalosporin C deacetylase activity was chosen for the source of CAH gene cloning. The strain was identified as *Bacillus cereus*. The gene was cloned from *Sau*3AI digested genome using *Bam*HI dephosphorelated pBS II (SK+) as a cloning vector. A positive clone was detected by both the chemical and microbiological methods. Ability of the clone to remove the acetate group at C-3 position was also confirmed by HPLC. Study on the localization of the esterase gene with CAH activity in the cloned fragment showed that the gene was located on a 1.085 Kb *Xba*I-*Eco*RI fragment. DNA sequence of this gene fragment revealed that it contained one open reading frame (ORF) containing 903 nucleotides, which encoded for a polypeptide consisting of 301 amino acids. Analysis of the deduced amino acid sequence of the ORF showed that the cloned gene contained the common sequence Gly-X-Ser-X-Gly found in many esterases, lipases and serine proteases. The putative hexanucleotide sequence (-10 region, TATAAT) recognized by *Bacillus* RNA polymerase was found in the 5′ flaking region of the esterase gene. A Shine-Dalgarno sequence (GAGG) was also recognized. Study on expression of the cloned esterase using pKK223-3 vector showed that the level of expression increased about 3 folds with IPTG induction. Analysis by SDS-PAGE revealed that the cloned gene encoded for protein with MW 34.6 kDa. Activity staining of the renatured SDS-PAGE and Native-PAGE confirmed that the expressed protein possessed esterase activity. Comparison of the deduced amino acid sequence with those reported in the GenBank showed that the gene had low homology with many esterases. This gene was suggested to be a novel esterase which could convert cephalosporin C to deacetylcephalosporin C.

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ทรงศักดิ์ ฤกษ์หริ่ง : การแยก วิเคราะห์ และ การแสดงออกของยีน Cephalosporin C Deacetylase จากเชื้อ *Bacillus cereus* สายพันธุ์ BT-24 (ISOLATION, ANALYSIS, AND EXPRESSION OF CEPHALOSPORIN C DEACETYLASE GENE FROM *BACILLUS CEREUS* STRAIN BT-24). คณะกรรมการควบคุมวิทยานิพนธ์ : ชื่นจิตต์ บุญเฉิด, Ph.D., วิทยา มีวุฒิสม, Ph.D., สุเทพ ไวยครุฑธา, Ph.D. 160 หน้า. ISBN 974-663-807-6

เอนไซม์ Cephalosporin C deacetylase (CAH) สามารถย่อยสลายพันธะเอสเทอร์ที่ตำแหน่ง C-3 ของยาปฏิชีวนะ Cephalosporin C ให้ผลผลิตเป็น Deacetylcephalosporin C ซึ่งใช้เป็นตัวกลางสำหรับผลิตอนุพันธ์ของยา Cephalosporins หลายชนิด จากการทดลองทำการคัดเลือกสายพันธุ์แบคทีเรียซึ่งสามารถผลิตเอนไซม์ CAH ในปริมาณสูงจากตัวอย่างดินโดยวิธี Chemical และ Microbiological และทำการยืนยันโดยใช้ HPLC พบว่าสายพันธุ์ที่สามารถเปลี่ยน Cephalosporin C ไปเป็น Deacetylcephalosporin C ได้มากที่สุดคือ *Bacillus cereus* การโคลนยีน CAH ทำโดยการย่อยยีโนมด้วยเอนไซม์ตัดจำเพาะ *Sau*3AI ซึ่งต่อเข้ากับเวคเตอร์ pBS II (SK+) ที่ย่อยด้วย *Bam*HI และทำการตรวจหาโคลนโดยวิธี Chemical และ Microbiological ส่วนการทดสอบยืนยันความสามารถในการเคลื่อนย้ายหมู่ Acetate ที่ตำแหน่ง C-3 ของ Cephalosporin C ทำโดยใช้ HPLC เช่นเดียวกัน ผลจากการศึกษาหาตำแหน่งของยีน Esterase ที่มี CAH activity พบว่ายีนปรากฏอยู่บนชิ้นดีเอ็นเอช่วงเอนไซม์ตัดจำเพาะ *Xba*I และ *Eco*RV ซึ่งมีลำดับนิวคลีโอไทด์ จำนวน 1,085 คู่เบส เมื่อทำการหาลำดับนิวคลีโอไทด์ดังกล่าวพบว่า ยีนประกอบด้วย 903 คู่เบส ซึ่งกำหนดการสร้างโปรตีนที่ประกอบด้วยกรดอะมิโน 301 ตัว จากการวิเคราะห์ Deduced amino acid sequence แสดงให้เห็นว่าโปรตีนมี Common sequence คือ Gly-X-Ser-X-Gly ซึ่งพบได้ในเอนไซม์ Esterases, Lipases และ Serine proteases หลายชนิด นอกจากนี้ยังพบ Hexanulceotide sequence (-10 region, TATAAT) ที่มีความจำเพาะต่อเอนไซม์ RNA polymerase ของ *Bacillus* และยังพบ Shine-Dalgarno (SD) sequence (GAGG) บริเวณ 5′Flanking region ของยีนอีกด้วย ยีน Esterase ที่แสดง CAH activity นี้ได้ถูกโคลนเข้าเวคเตอร์ pKK223-3 เพื่อเพิ่มการผลิตเอนไซม์ พบว่าสามารถเพิ่มการผลิตประมาณ 3 เท่า เมื่อกระตุ้นด้วย IPTG ผลจากการทำ SDS-PAGE แสดงให้เห็นว่ายีนสร้างโปรตีนขนาดประมาณ 34.6 kDa และหลังจากทำการย้อม Activity ยืนยันว่าเป็นเอนไซม์ Esterase เมื่อทำการเปรียบเทียบกรดอะมิโนของยีนนี้กับของโปรตีนที่บันทึกอยู่ใน GenBank พบว่ายีนมี Homology กับเอนไซม์ Esterases หลายชนิดในระดับต่ำ ซึ่งแสดงให้เห็นว่ายีนที่ค้นพบนี้เป็น Esterase ชนิดใหม่ที่สามารถเปลี่ยน Cephalosporin C เป็น Deacetylcephalosporin C ได้

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LIST OF ABBRIVIATIONS

aa	amino acid
7-ACA	7-aminocephalosporanic acid
7-ADCA	7-aminodeacetoxycephalosporanic acid
Ap	ampicillin
Ap^r	ampicillin resistant
ATCC	American Type Culture Collection
bp	base pair
°C	degree Celsius
Cep C	cephalosporin C
CIP	calf intestinal alkaline phosphatase
cm	centimeter
DAC	deacetylcephalosporin C
CAH	cephalosporin C deacetylase
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
et al.	Et.alli (Latin), and others
etc.	ec cetera (Latin), and other things, and so on
fig.	figure
g	gram
h.	hour (s)
HPLC	High Performance Liquid Chromatography

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LIST OF ABBRIVIATIONS

(Continued)

IPTG	Isopropyl-β-D-thiogalactoside
i.e.	Ed est (Latin), that is
kb	kilobase (1,000 bp)
kDa	kilodalton
L, l	litre
LB	Luria-Bertani
λ	wavelength
m	mili-
mg	milligram
ml	milliltre
mM	millimolar
M	molar
min	minute
MW	molecular weight
Mr	relative molecular weight
α-NA	alpha-naphthylacetate
β-NA	beta-naphthylacetate
ND	not detectable
nm	nanometer
OD	optical density

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LIST OF ABBRIVIATIONS

(Continued)

ORF	open reading frame
pBS II (SK+)	Bluescript II SK+ plasmid
PCR	polymerase chain reaction
rpm	round per minute
SDS-PAGE	sodium dodecyl sulfate polyacrelamide gel electrophoresis
TB	terrific broth
TBE	Tris-Borate EDTA
TE	Tris-EDTA
TEMED	N-N-N'-N'-tetramethyl ethylenediamine
U	unit
UV	ultraviolet
V	voltage
v	volume
wt	weight
µg	microgram
µl	microlitre
%	percent

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CHAPTER I

INTRODUCTION

At the present time requirements for antibiotics as therapeutic agents are still high, especially, those in the β-lactam group. This is because β-lactam antibiotics are still one of the most effective antimicrobial agents against pathogenic bacteria with relatively low toxic to man. Examples of these are ampicillin, cloxacillin and amoxycillin, which are membered of the penicillin antibiotics and ceftiolene, cefodizine (Periti, 1996) which are membered of the cephem group.

Cephalosporin C is a natural cephem which is used as a starting material for production of various cephalosporin derivatives. These cephalosporin derivatives are often used as an alternative choice when treatment with penicillins failed due to problems of drug resistance or hypersensitivity. However, these antibiotics are more expensive than penicillins, therefore, more effective technologies for their production are needed. These include the technologies for production of 7-ACA and deacetyl 7-ACA, which are being used as the starting materials for production of all cephalosporins derivatives.

Production of 7-ACA from cephalosporin C may occur in one or two steps of enzymatic reactions, i.e., using only cephalosporin C acylase alone or that with the enzyme D-amino acid oxidase (Shibuga *et al.*, 1981; Matsuda and Komasu, 1985; Matsuda, 1987). Another enzyme, cephalosporin C deacetylase, is used to catalyse hydrolysis of the ester bond at 3′-position of cephalosporin C molecule to form

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deacetylcephalosporin C. This enzyme was produced by *Bacillus subtilis* (Abbot *et al.*, 1975; Konecny and Voser, 1977; Takimoto *et al.*, 1994). It was also found to deacetylate 7-ACA to form deacetyl-7-ACA. Deacetyl-7ACA is used as the starting material for production of a number of semisynthetic cephalosporins such as ceftiolene, cefuroxime (O'Callaghan *et al*, 1976) and S1108 (Totsuka *et al.*, 1992).

This study was designed to screen for cephalosporin C deacetylase producing microorganisms from soils collected from different areas in Thailand. The screening process was done by using both the chemical and microbiological methods for screening and the HPLC (High Performance Liquid Chromatography) analysis for confirmation. A positive isolate, which had the highest enzyme activity, was selected for further study at the molecular level. These included gene cloning, nucleotide sequencing and gene expression of the recombinant clone.

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CHAPTER II

LITERATURE REVIEW

1. General Aspects of β-lactam Compounds

Clinical uses of antibiotics were started with the discovery of the therapeutic efficacy of a β-lactam compound, which was perhaps considered to be the most important discovery in the history of therapeutic medicine. It was said that application of antibiotics in therapy of infectious diseases might have saved more lives than any other medical development (Heatley, 1990). Discovery of a β-lactam compound began in 1929, when Alexander Fleming published his observation about the inhibition of growth of *Staphylococcus aureus* on an agar plate contaminated with *Penicillium notatum* (Fleming, 1929). Three years later, it was shown that the growth inhibition was due to pencillin (Clutterbuck *et al.*, 1932) and the penicillin's structure was elucidated in 1945 by Hodgkin and Low using X-ray crystallography analysis to be composted of the β-lactam structure. During the late 1940s, the fungus *Cephalosporium acremonium* was isolated from the sea at Cagliari, Italy, by Guiseppi Brotzu (Brotzu, 1948). This fungus was found to produce new β-lactam compounds including penicillin N and cephalosporin C in the culture broth. The structure of cephalosporin C was later described by Abraham and Newton in 1961 (Abraham, 1961) and confirmed by X-ray crystallography analysis (Hodgkin and Maslen, 1961).

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The discovery of cephalosporin C generated a whole new group of clinically significant β-lactams. At present, β-lactams can be classified into five groups on the basis of their chemical structures (Figure 1). All of these compounds have in common the four-membered β-lactam ring. β-lactams consist of a bicyclic ring system except the monobactam group. The ability to synthesize β-lactams is widespread in nature. They have been found in fungi and in both Gram-positive and Gram-negative bacteria.

1.1 Biosyntheses of penicillins and cephalosporins

Pathways for biosyntheses of penicillins and cephalosporins have the first two steps in common (Figure 2). All naturally occurring penicillins and cephalosporins are formed from the same three amino acids: L-α-aminoadipic acid (L-α-AAA), L-cysteine, and L-valine. In the first reaction cycle, the amino acid precursors are condensed into the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). Reactions involved in the formation of this tripeptide include specific recognition of the amino acids, their activation via the formation of aminoacyl adenylates, and formation of the peptide bonds catalyzed by a single multifunctional enzyme designated ACV synthase according to the product formed (Figure 2). ACV synthase is encoded by a single structural gene designated *acvA* (*pcbAB*) (Figure 2). In the second step, oxidative ring closer of the linear tripeptide leads to the formation of a bicyclic ring structure, i.e., the four-membered β-lactam ring fuses to the five-membered thiazolidine ring, which is the characteristic of all penicillins. The resulting compound, isopenicillin N (IPN), possesses weak antibiotic activity and is the first bioactive intermediate of both penicillins and cephalosporins pathways. This reaction

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is catalyzed by isopenicillin N synthase (IPNS) which is encoded by the *ipnA* (*pcbC*) gene. IPN is the branch point of penicillins and cephalosporins biosyntheses.

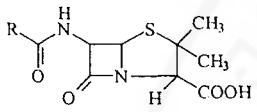
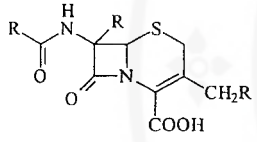
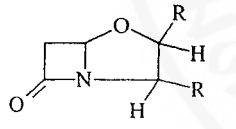
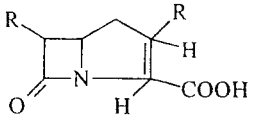
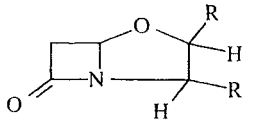
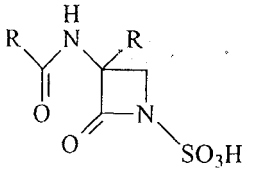
Classes of naturally occurring β-lactams	Antibiotics	Producing microorganisms (Examples)		
		Fungi	Bacteria	
Penam 	Penicillins	*Penicilium chrysogenum* *P. notatum* *Aspergillus nidulans*	Gram$^+$	Gram$^-$
Ceph-3-em 	Cephalosporins Cephamycins Cephabacins Chitinovorins	*Acremonium chrysogenum* *Paecilomyces persinicus*	*Streptomyces clavuligerus* *Nocardia lactamdurans*	*Flavobacterium sp.* *Lysobacter lactamgenus*
Clavam 	Clavulanic acid		*S. clavuligerus*	
Carbapenem 	Thienamycins Olivanic acid Epithienamycins		*S. clauligerus* *S. olivaceus*	*Erwinia carotovora*
Monolactam 	Nocardicines		*Nocardia uniformis* (subsp. *Tsuyamanensis*)	
	Monobactams			*Agrobacterium radiobacter* *Pseudomonas acidophila*

Figure 1. Naturally occurring classes of β-lactam antibiotics as compiled by O'Sullivan and Sykes (O'Sullivan and Sykes, 1986).

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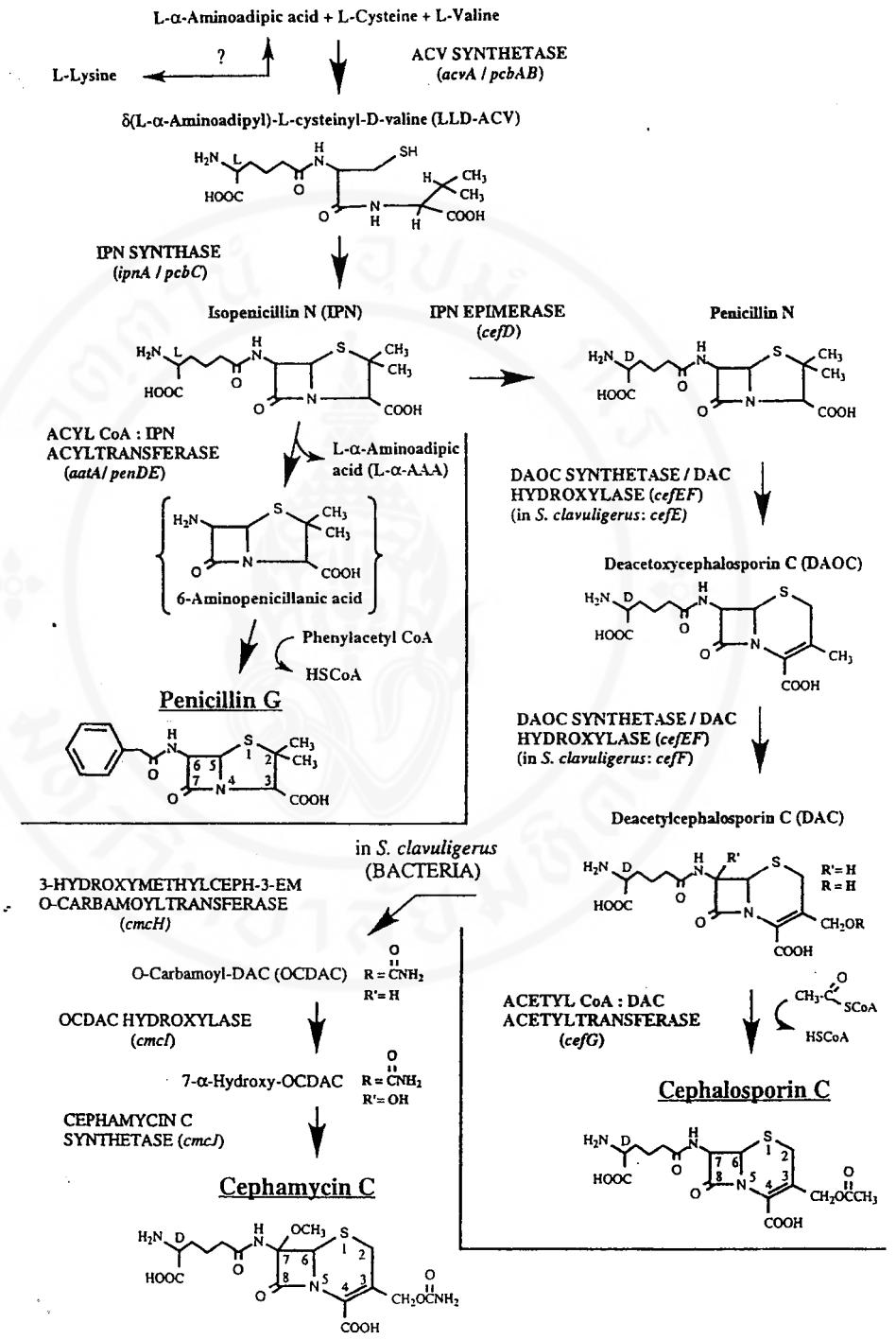

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Figure 2. Biosyntheses of penicillins, cephalosporin C, and cephamycin C (Brakhage, 1998). Genes and organisms names are italics, names of enzymes are in capital letters.

In the third and final step of penicillin biosynthesis, the hydrophilic L-α-AAA side chain of IPN is exchanged for a hydrophobic acyl group. The exchange is catalyzed by acyl coenzyme A (CoA): isopenicillin N acyltransferase (IAT). The corresponding gene has been designated *aat* (*penDE*).

The first step that commits to the production of cephalosporins is the isomerization of L-α-AAA side chain of IPN to its D enantiomer to give penicillin N. This reaction is catalyzed by IPN epimerase (Figure 2). Penicillin N is the precursor of antibiotics containing the ceph-3-em nucleus, i.e., cephalosporins and cephamycins (7-methoxycephalosporins) (Figures 1 and 2). Penicillin N undergoes a ring expansion to form deacetoxycephalosporin C (DAOC) by DAOC synthase (expandase) activity (Figure 2).

This ring expansion step involves the oxidative opening of the penam thiazolidine ring to give the six-membered dihydrothiazine ring, which is characteristic of all ceph-3-ems. In the next step, the methyl group at C-3 of DAOC if hydroxylated and oxidized until form deacetylcephalosporin C (DAC) (Figure 2) which are catalyzed by a single enzyme, DAOC synthetase (expandase)/DAC hydroxylase. This enzyme is encoded by *CefEF* gene, which was found in *A. chrysogenum*, and encoded by the two gene *cefE* and *cefF* in *Streptomyces clavuligeris*. In the last step of cephalosporin C biosynthesis an acetyl moiety from acetyl-CoA is transferred to the -OH group of DAC; this step is catalyzed by the product of *cefG*, acetyl CoA: DAC acetyltransferase (Figure 2). Several

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cephalosporins differ from cephalosporin C in the substituent attached to the 3′ oxygen have been isolated from a variety of microorganisms (Jensen and Demain, 1995). For the formation of cephamycin C, which have been studied in *S. clavuligerus*, start from the intermediate DAC (Figure 2). A carbamoyl group is attached to DAC to give *O*-carbamoyl-DAC (OCDAC). This reaction is catalyzed by 3-hydroxymethylceph-3-em *O*-carbamoyltransferase, which is encoded by the *cmcH* gene. Then, C-7 is hydroxylated by the action of OCDAC hydroxylase, encoded by *cmcI*. In the final step of cephamycin biosynthesis, the hydroxy group at C-7 is methylated to form cephamycin C, which the reaction is catalyzed by cephamycin C synthetase (*cmcJ*).

1.2 Basis of β-lactam activity

The activity of β-lactams against Gram-positive species depends on their affinity for the enzymes referred to as penicillin-binding proteins. Resistance of Gram-positive species to β-lactams is either due to altered penicillin-binding proteins or due to the presence of beta-lactamases, which are usually plasmid-mediated. The activity of β-lactams against gram-negative aerobic and anaerobic bacteria is the result of the way in which the compounds pass through the porin channels in the outer wall, resist inactivation by beta-lactamases, and bind to the penicillin-binding proteins. The newer cephalosporin agents have a remarkable spectrum of activity compared with the first cephalosporins. The factors involved in the antibacterial activity of β-lactam antibiotics are enumerated in Table 1. Basically, β-lactam antibiotics inhibit bacterial cell wall synthesis. The activity of β-lactam compounds against most Gram-positive

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species depends on their affinity for enzymes at the target site. These enzymes are currently referred to as penicillin-binding proteins (PBPs). Penicillin-binding proteins are involved in transpeptidation reactions. As Figure 3 illustrates, the interaction of a β-lactam antibiotic with Gram-positive bacteria is the result of the antibiotic diffusing through the peptidoglycan strands and binding to the penicillin-binding proteins. As a result of that binding, their will be inhibition of peptidoglycan synthesis, activation of autolytic enzymes, and cell death (Tomaz, 1979). If the compound diffuses through the peptidoglycan strands but fails to bind to the penicillin-binding proteins, the cell will survive. If a Gram-positive bacterium produces β-lactamase, an enzyme that can hydrolyze the β-lactam compound, the compound will be destroyed before it can bind to the protein target.

Table 1. Factors Involved in Antibacterial Activity of β-lactam Antibiotics (Neu and Moellering, 1985).

- Concentration of antibiotic
- Diffusion of the antibiotic through the outer cell membrane
- Ability to resist attack by inactivating enzymes
- Affinity of the antibiotic for the target enzyme

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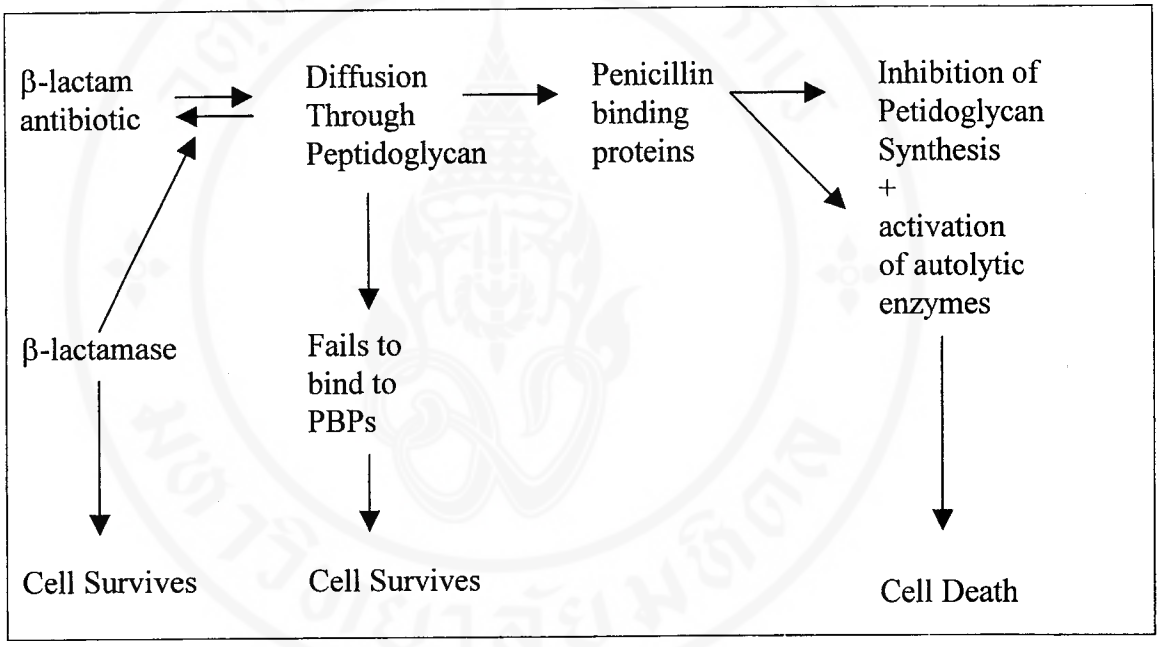

Figure 3. Interaction of a β-lactam antibiotic with a Gram-positive bacterium (Neu and Moellering, 1985).

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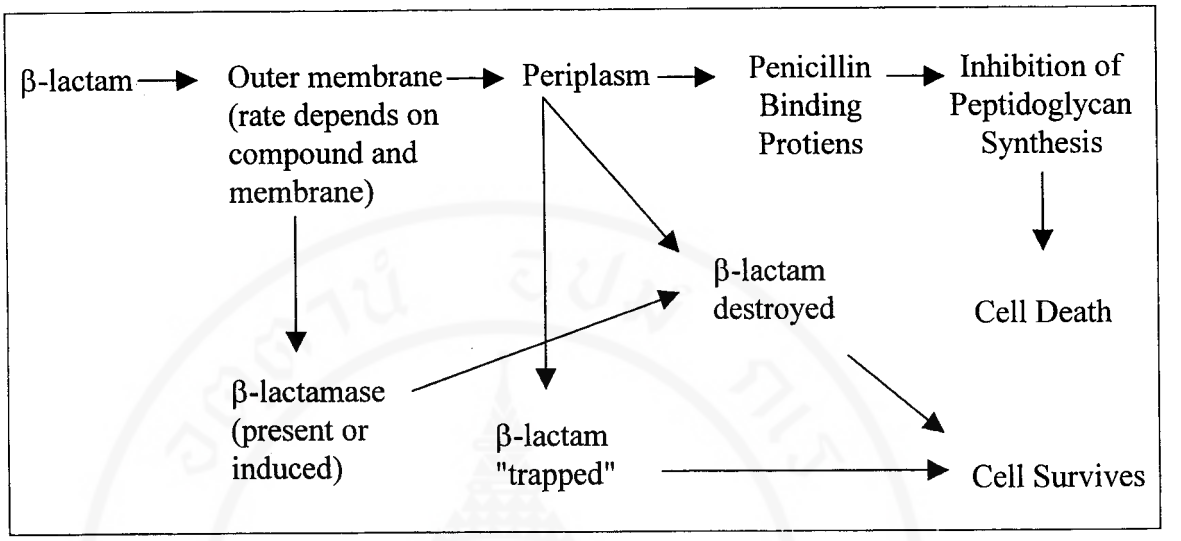

Figure 4. Interaction of a β-lactam antibiotic with a Gram-negative bacterium (Neu and Moellering, 1985).

Some Gram-negative bacteria contain β-lactamase in the periplasmic space that exists between the cell wall and the cytoplasmic membrane. Penicillin-binding proteins are present on the outer part of the cytoplasmic membrane in gram-negative bacteria. In these bacteria, there are a smaller number of peptidoglycan strands, between three and five. Peptidoglycan is exceedingly important in maintaining the integrity of gram-negative organisms. As Figure 4 illustrates, the interaction of a β-lactam antibiotic with Gram-negative bacteria is more complex. The rate at which the compound passes through the porin channels in the periplasmic space is important in terms of being destroyed by perexisting β-lactamase, or induced β-lactamase. The induced β-lactamase is the result of the induction caused by the β-lactam antibiotic in question. If the β-lactam compound readily passes through the outer membrane into

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the periplasm and reaches the penicillin-binding proteins in concentration that will inhibit peptidoglycan synthesis, the bacterial cell will die (Neu and Moellering, 1985).

2. Cephalosporins

Cephalosporin C contains a side chain derived from D-α-aminoadipic acid, which is condensed with a dihydrothiazine β-lactam ring system (7-aminocephalosporanic acid). Compounds containing 7-aminocephalosporanic acid are relatively stable in dilute acid and highly resistant to penicillinase, regardless of the nature of their side chains and their affinity for the enzyme. The cephalosporin C can be hydrolyzed by acid to 7-aminocephalosporanic acid. This compound has been subsequently modified by the addition of different side chains to create a whole family of cephalosporin antibiotics. The metabolism and the pharmacokinetics of the drugs are associated with the modification at position 3 of the dihydrothiazine ring and alteration of antibacterial activity are associated with the modification of postition 7 of the β-lactam ring (Huber *et al.*, 1972). Cephalosporins are now the most widely prescribed of all the antimicrobials (Doniwitz and Mandell, 1990). There are such large numbers of cephalosporins with similar names, varying antimicrobial coverage and uses, that the practitioner may find it challenging to keep them straight (Holdcroft, 1992). The first source of cephalosporin was the fungus *Cephalosporium acremonium*, isolated in 1948. This organism inhibited growth of a wide variety of Gram-positive and Gram-negative bacteria in vitro. In addition, broth culture filtrates of *Cephalosporium acremonium* cured staphylococcus infection and typhoid fever in

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humans (Doniwitz and Mandell, 1990; Mandell and Sande, 1990). Many clinically useful cephalosporins were developed as semisynthetic or synthetic cephalosporin C analogues (Figure 5).

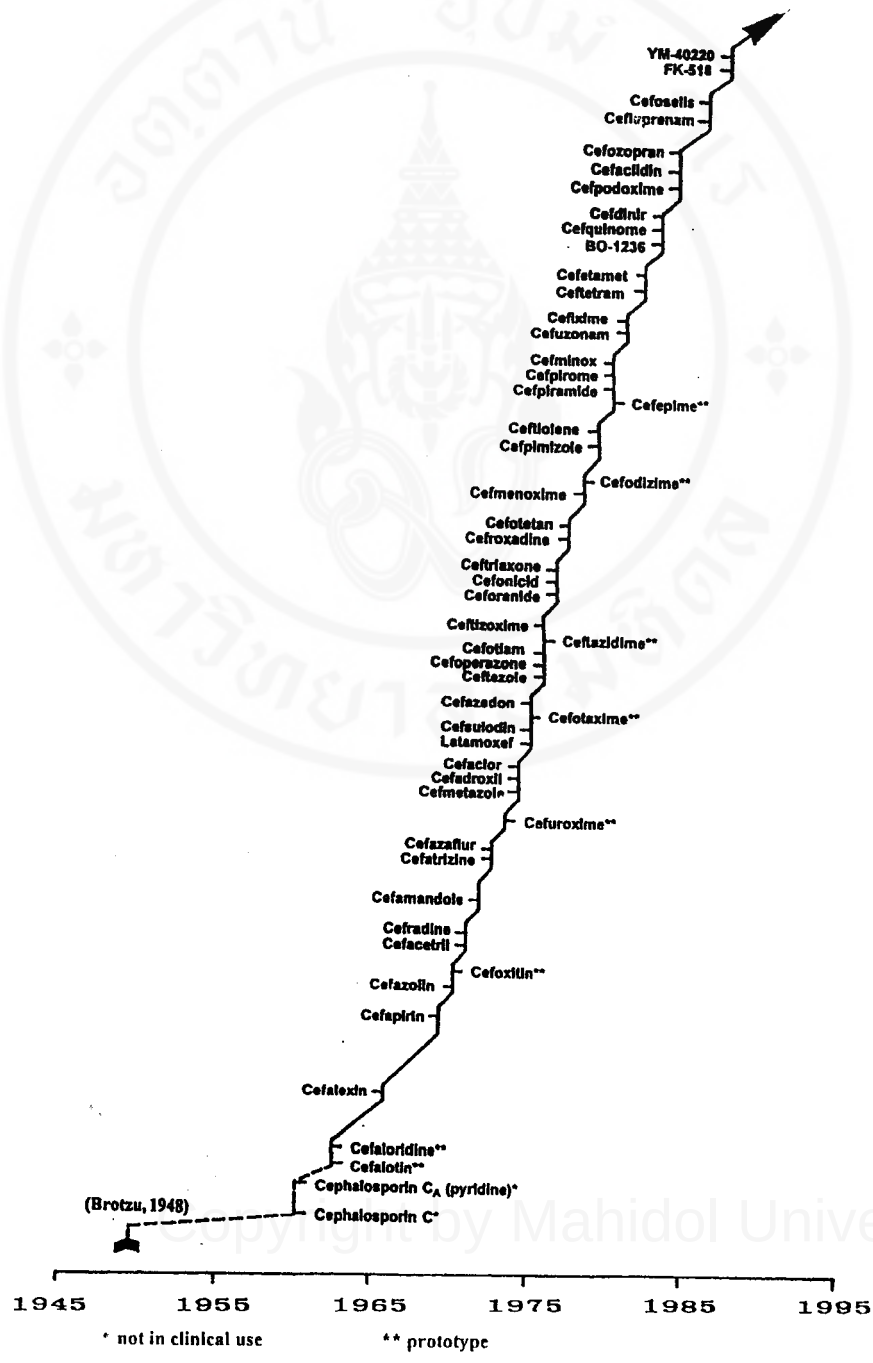

Figure 5. Development of cephem betalactams for clinical use (Periti, 1996).

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3. Cephalosporins Classification

Cephalosporin derivatives are at present numerous and different in their attributes that a rigorous chemical classification on the basis of structure-activity relationships remains an impossible dream for most cephem antibiotics (Frere *et al.*, 1988) despite several attempts at biological and chemical groupings (Periti *et al.*, 1993; Rolinson, 1986; Williams, 1987). The cephalosporins generally have been arbitrarily classified into a system of generations that has often led to the grouping of very dissimilar compounds. In fact, categorization of these antimicrobial agents into chemically similar groups was not useful because the spectrum of activity in general is not closely associated with the chemical structure even while some improvements in broad-spectrum coverage appear from generation to generation.

3.1 Classification based on the system of generations

Cephalosporins are most commonly classified in "generations" based on their antimicrobial activity. Tissue penetration by the antibiotics differs within each generation. In general, first -generation cephalosporins have good coverage against Gram-positive bacteria, including staphylococci and non-enterococcal streptococci. The antimicrobial efficacy can vary within each generation of cephalosporin.

3.1.1 First-generation cephalosporins

In addition to their activity against Gram-positive cocci, first-generation cephalosporins are effective against a few Gram-negative bacilli, including *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. First-generation cephalosporins have poor anti-pseudomonal activity. They

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are most commonly used in penicillin-allergic patients for infections due to stapphylococcus and non-enterococcal streptococci. These agents are the drugs of choice for prophylaxis during most non-gastrointestinal surgical procedures (Aharonowitz, 1992). The first useful cephalosporin compounds that were synthesized were cephalothin and cephalolidine as shown in Figure 6.

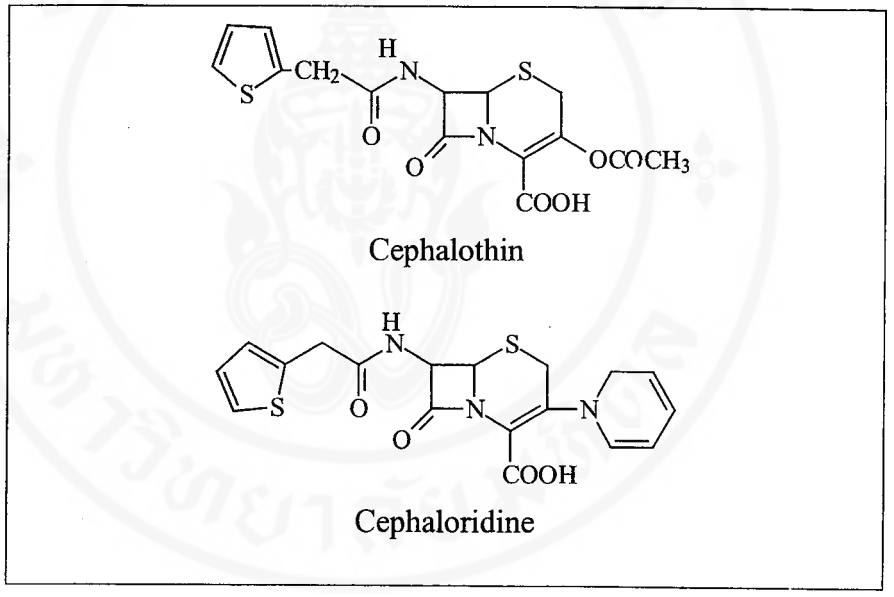

Figure 6. Chemical structures of cephalothin and cephaloridine, the first-generation cephalosporins (Neu and Moellering, 1985).

3.1.2 Second-generation cephalosporins

Second-generation cephalosporins are more active against Gram-negative bacilli such as *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, and *Proteus mirabilis* than the first-generation cephalosporins. In addition, the agents have a broader spectrum of activity against other Gram-negative

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organisms, including *Enterobacter* species, anaerobes, *Neisseria meningitidis* and *Neisseria gonorrhoeae.* Second-generation products have poor activity against *Pseudomonas* species (Holdcroft, 1992). The agents in this group are cefuroxime, cefamandole, cefotiam, and cefoxitin. The latter drug is actually a cephamycin compounds. This group of cephalosporins is shown in Figure 7.

The introduction of substituents at position 7 has several important effects on antibacterial activity. First of all, there is a reduction in activity against Gram-positive bacteria. Indeed, replacement of the hydrogen at this position with any group (methyl, methoxy, or imino) will result in loss of Gram positive activity. This is due to the poor binding of such compounds to the penicillin-binding proteins of Gram-positive species. However, substitutions at position 7 can cause a marked increase in β-lactamase stability.

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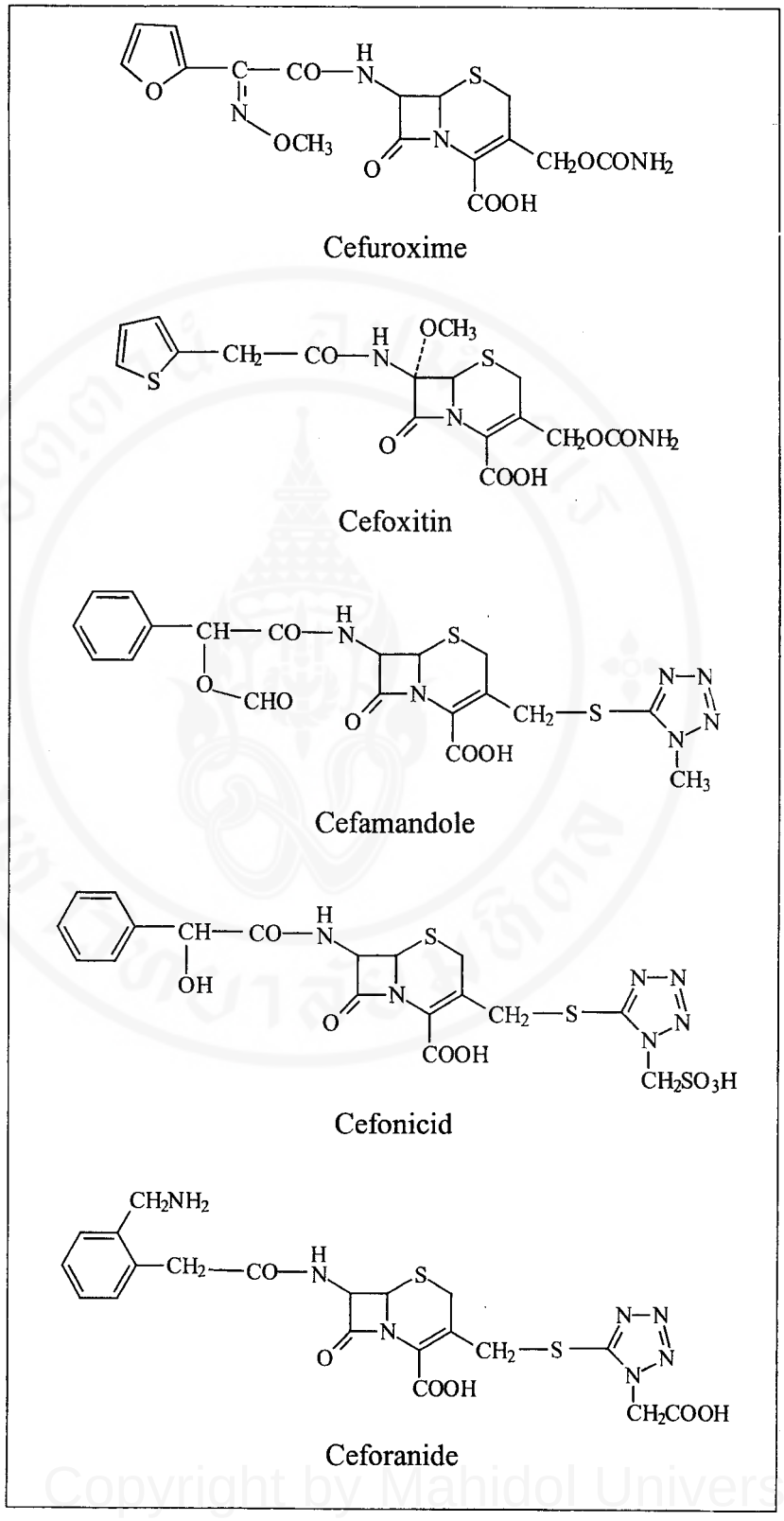

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Figure 7. Chemical structures of the second-generation cephalosporins (Neu and Moellering, 1985).

3.1.3 Third-generation cephalosporins

Third-generation cephalosporins have the broadest spectrum for infections due to Gram-negative organisms. When compared with the first- and second-generation cephalosporins, the third-generation cephalosporins have an extended spectrum of activity with generally greater activity against Gram-negative bacilli of clinical importance. The increased spectrum includes that against the Enterobacteriaceae and *Pseudomonas aeruginosa*. They are resistant to most of the β-lactamase enzymes produced by Gram-negative bacteria. However, they provide no better activity against Gram-positive than do the first-generation agents. Their activity against anaerobes is usually not superior to the second-generation compounds. Third-generation cephalosporins are further classified according to their anti-pseudomonal activity (Donowitz and Mandell, 1990). For example, Ceftriaxone (Figure 8) is the drug of choice for *Neisseria meningitidis* and *Neisseria gonorrhoeae*. The third-generation cephalosporins do not inhibit growth of *Listeria*. A majority of organisms resistant to cephalothin and cefamandole (first- and second-generation cephalosporins) and to aminoglycosides are inhibited by third-generation cephalosporins (Thornsberry, 1985).

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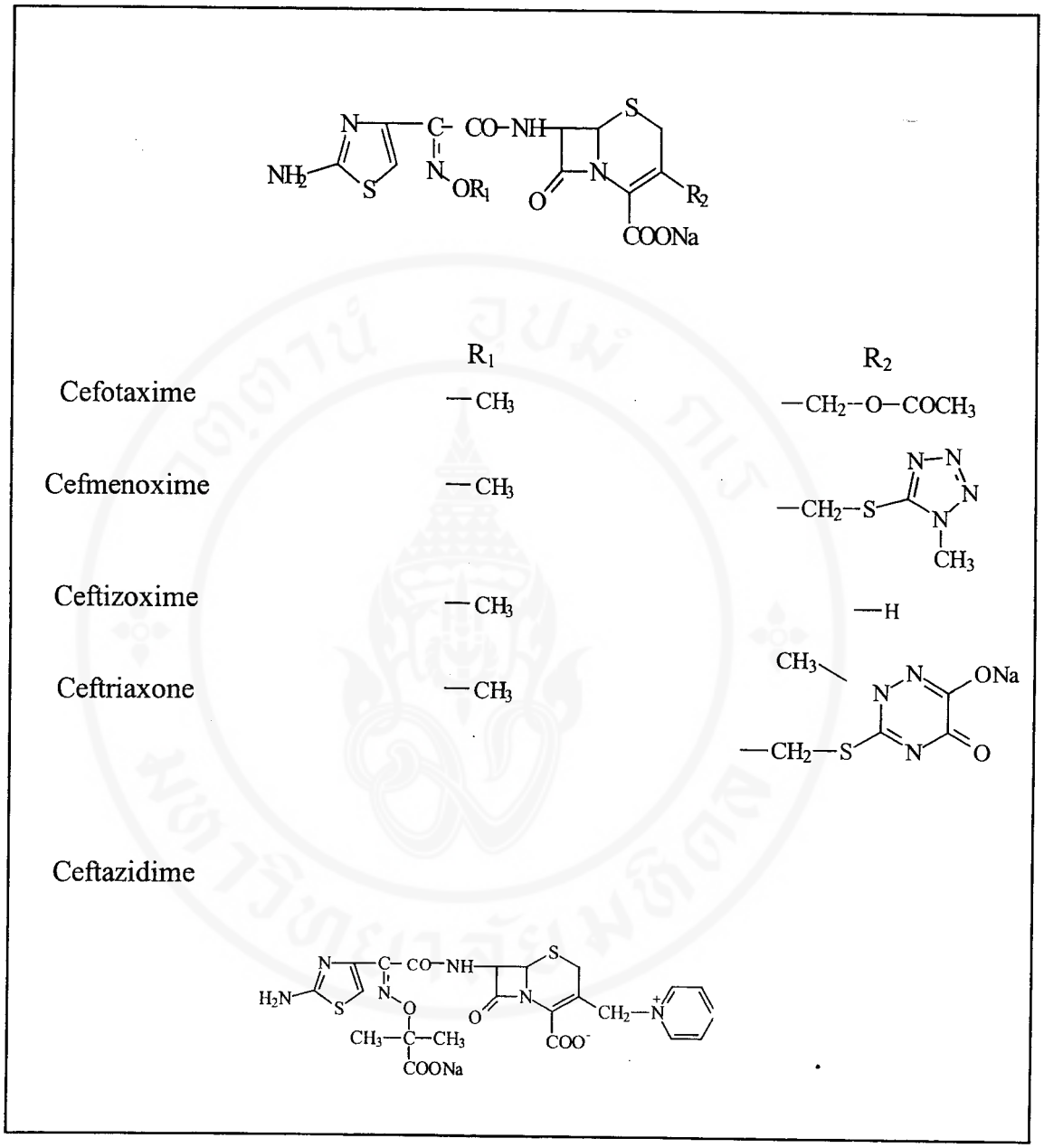

Figure 8. Chemical structures of third-generation cephalosporins (Neu and Moellering, 1985).

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3.1.4 Fourth-generation cephalosporins

Some members of the third generation were found to have excellent activity against Gram-negative bacteria but much poorer activity against Gram-

positive pathogens that do the first and second generation cephalosporins. In recent years, pharmaceutical companies have attempted to improve efficacy and spectrum of betalactams by introducing novel substitutions onto newer cephalosporin molecules, cefepime being the prototype. Cefpirome and cefepime are new quaternary ammonium cephalosporins which have been recently introduced into clinical practice (Wilson, 1998). A considerable increase in potency resulting from insertion of a quaternary ammonium group at the C-3' position has led these compounds being termed "fourth-generation" cephalosporins. Compared with the third-generation cephalosporins, fourth-generation cephalosporins possess enhanced activity against Gram-positive organisms, excellent penetration into Gram-negative bacilli, and are more stable against the activity of some β-lactamases. Cefpirome and other fourth-generation agents have activity in vitro against a broad range of Gram-positive microorganisms, including *Streptococcus pneumoniae*, viridans group streptococci, and oxacillin- (methicillin-) susceptible *Staphylococcus aureus* (MSSA) (Jones and Wilson, 1998). They also possess enhanced activity against several Gram-negative species, which are common causes of hospital-acquired infection. Stability of fourth-generation cephalosporins to β-lactamases, which inactivate many of the third-generation cephalosporins, is well-documented (Jones *et al.*, 1991). The fourth-generation cephalosporins exhibit a number of advantages over earlier generations of cephalosporins. This includes twice-daily dosing is needed, increase penetration into the spinal fluid and bronchial activity, enhanced penetration into bacteria, and greater stability to some β-lactamases. These potential benefits will likely lead to an

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increased use of these compounds such as cefpirome for the treatment of serious infections in hospitalized patients.

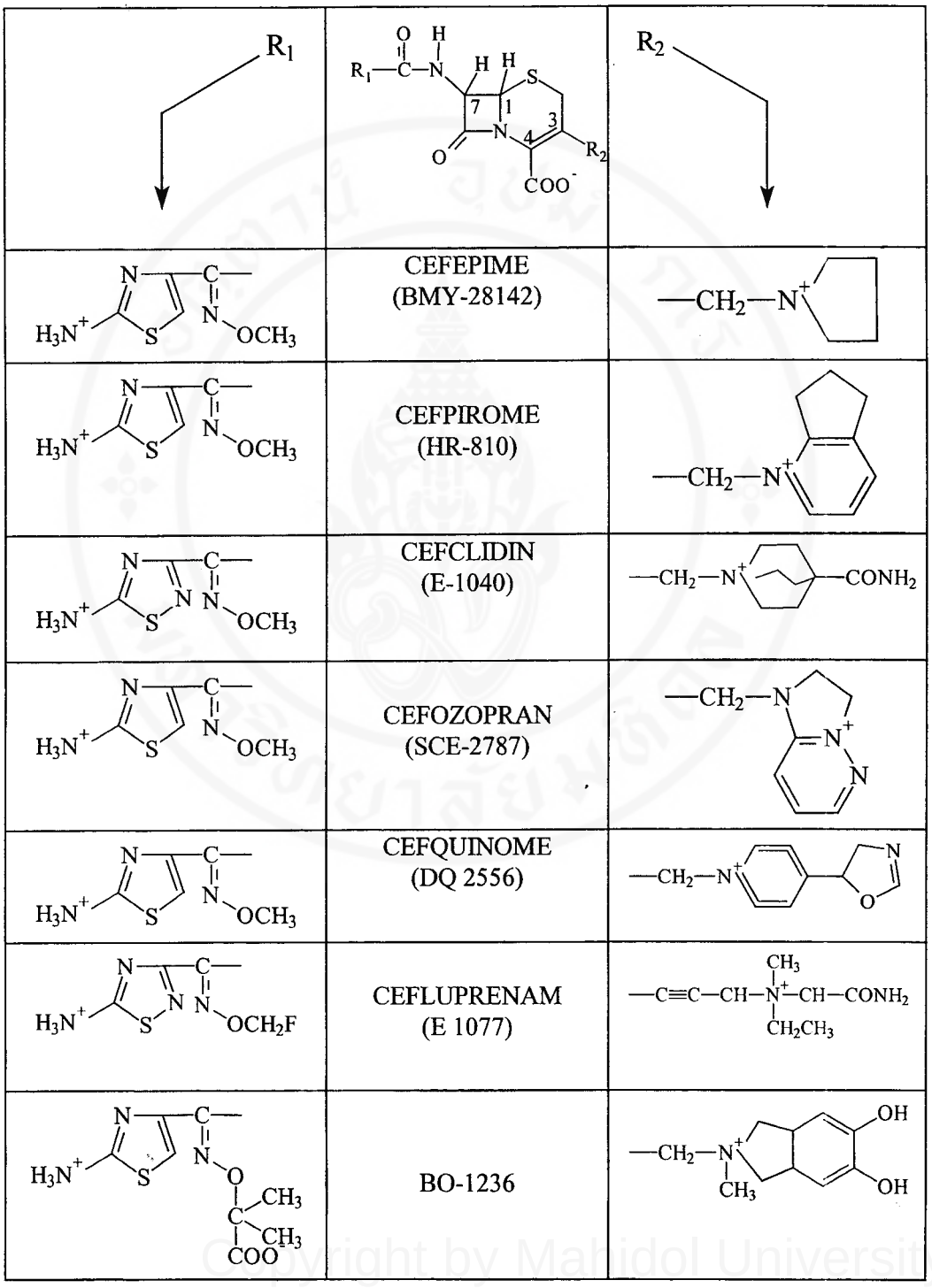

Figure 9. Chemical stuctures of new fourth generation cephalosporins (Periti, 1996).

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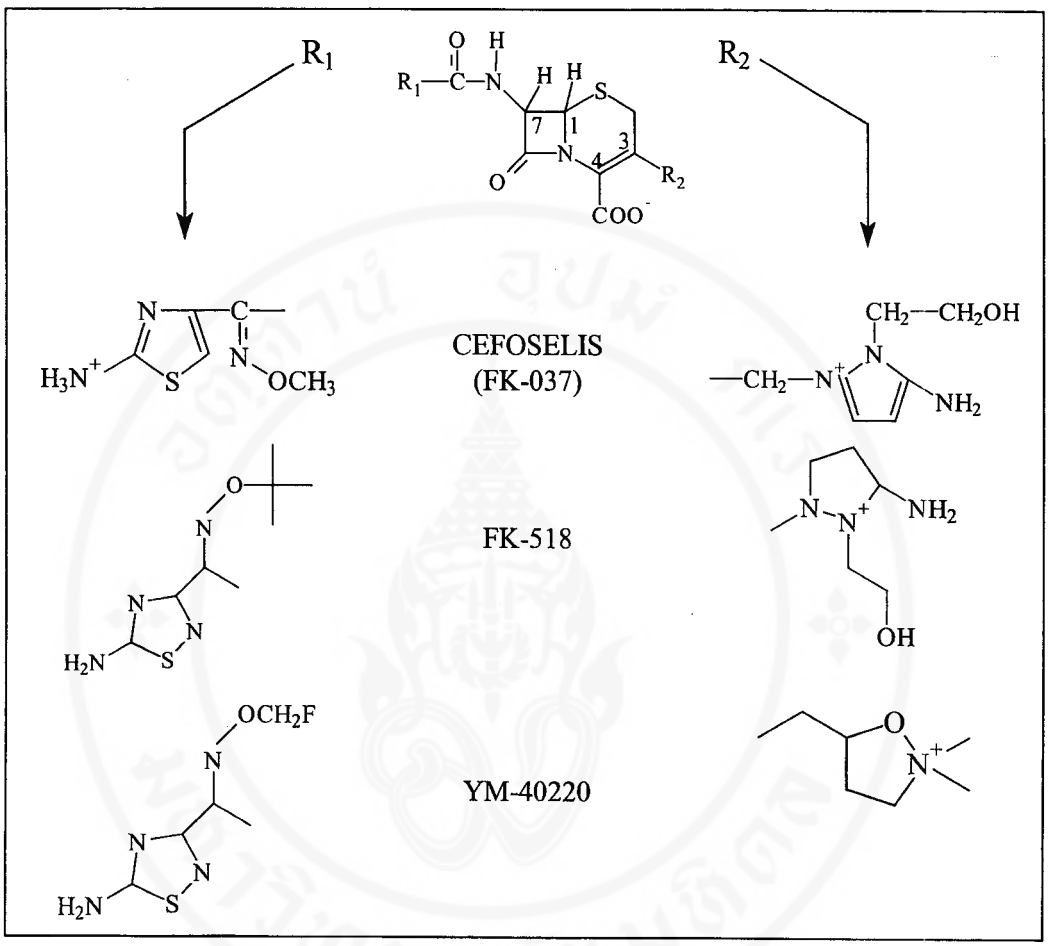

Figure 9. Chemical stuctures of new fourth generation cephalosporins (Periti, 1996) (Continued).

The general classification of cephem antimicrobial agents in clinical use is shown in Table 2 and chemical structures of the fourth generation cephem antibiotics are shown in Figure 9. All compounds in Figure 9 have a 2-aminothiazoyl (or 2-aminothiadiazoyl)-acetamido group of the side chain at position 7β with a (Z)α-oxyimino substitution and a positively-charged quaternary nitrogen present at position 3. It was found that large moiety substituent on the α-carbon of the 7β position may

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reduce betalactamase attachment (Bauernfeind *et al.*, 1991; Nikaido *et al.*, 1990). A positive charge of the side chain at position 3 was found to increase the ability of cephems to penetrate the microbial membrane (Hancock and Bellido, 1992; Benz *et al.*, 1985; Yoshimura and Nikaido, 1985; Bellido *et al.*, 1991). As a consequence, these compounds show higher activity than that of the third generation members against Gram-negative organisms in vitro, especially the Enterobateriaceae, and strong activity against *P. aeruginosa*, while maintaining or partially increasing their activity against Gram-positive organisms (Bryskier, 1995) as shown in Table 3.

Table 2. General classification of cephem antimicrobial agents in clinical use (Periti, 1996).

First	Second	Third	Fourth
Cefalotin	Cefuroxime	Cefotaxine	Cefepime
Cefaloridine	Cefoxitin	Latamoxef	Cefirome
Cefazolin	Cefamandole	Ceftizoxime	Cefclidin
Cefapirin	Cefaclor	Ceftriaxone	Cefozopran
Cefalexin	Cefonicid	Cefmenoxime	Cefquinome
Cefradine	Ceforanide	Cefodizime	Cefluprenam
Cefadroxil	Cefotetan	Ceftiolene	BO-1236
Ceftezole	Cefotiam	Cefoperazone	Cefoselis
Cefacetril	Cefmetazole	Cefpiramide	FK-518
Cefazedone	Cefatrixine	Cefpodoxime	YM-40220
Cefazaflur	Cefroxadine	Cefetamet	
Cefprozil	Cefminox	Cefixime	
	Cefuzonam	Ceftibuten	
	Ceftetram	Cefdinir	
		Loracarbef	
		Cefpimizole	
		Cefsulodin	
		Ceftazidime	

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Table 3. Relative in vitro activity of cephem antimicrobial agents (Periti, 1996).

Generation	Bacterial susceptibility	
	Gram-positive	Gram-negative
First	++++	+
Second	+++	++
Third	+	+++
Fourth	++	++++*

*More than half of the fourth generation members demonstrate good activity against *P. aeruginosa*.

3.2 O'Callaghan's classification

In 1975 O'Callaghan suggested a classification which helped to clarify the differences as well as the similarity between cephalosporins. This classification is summarized in Table 4.

Table 4. Classification of cephalosporins in 1979 (modified from O'Callaghan, 1975).

Group	Administration	β-Lactamase	Metabolism	Compounds
1	Parenteral	Sensitive	Unstable	Cephalothin
2	Parenteral	Sensitive	Stable	Cephacetril
3	Oral	Sensitive	Stable	Cephaloridine Cephazolin
4	Oral	Resistant	Stable	Cephalexin Cephradine
5	Parenteral	Resistant	Stable	Cefuroxime Cefamandole Cefoxitin

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3.3 Classification based on microbiological and pharmacological factors

Both microbiological and pharmacological factors have been taken into account in the classification of cephalosporin antibiotics as shown in Table 3. The primary subdivision was based on antimicrobial activity using four compounds, cephaloridine, cefotaxime, ceftazidime, cefoxitin, as index compounds. Subdivisions based on pharmacological properties enable all of the available cephalosporins to be classified.

Table 5. Characteristic features of cephalosporins in different groups (Abraham, 1987).

A. Primary division based upon microbiological features
I. Compound with high activity against Gram-positive cocci, in particular *S. aureus*, coagulase-negative staphylococci, β-heamolytic streptococci, pneumococci and viridans streptococci. *S. feacalis* is relatively resistant but more sensitive to cephalosporins in this group than in other. Stable to staphylococcal penicillinase but hydrolysed by Gram-negative β-lactamases.
II.Compounds with high activity against Gram-negative bacteria: Enterobacteriaceae (*E. coli*, *Proteus* spp., *Krebsiella*, *Enterobacter* and related organisms) and fastidous bacteria such as *Heamophillus influenzae*, *Neisseria gonorrhoeae*, and *N. meningitidis*. Stable to hydrolysis by some or all transferable β-lactamases. Some compounds in this group are more stable against a wide range of β-lactamases than other members of the group.
III. Compound with additional activity against *Pseudomonas aeruginosa* and related non-fermenting species such as *Acinetobacter*. Some are stable to transferable Gram-negative β-lactamases.
IV. Activity against *Bacteroides fragilis* and related anaerobic bacteria is a prominent feature. Compounds which are not hydrolysed by chromosomal or transferable β-lactamases.

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Table 5. Characteristic features of cephalosporins in different groups (Abraham, 1987) (Continued).

B. Secondary division based upon pharmacological properties.
a) Compounds, which display typical pharmacological properties, i.e. parenterally administered compounds with a serum half-life of 1 to 2 hours and excreted mainly by the kidney (75 to 95 %).
b) Compounds, which vary from the typical, pattern by being orally absorbed or having a prolonged serum half-life or by being excreted by routes other than the kidney.
c) Compounds, which are metabolically unstable following deacetylation of the ester at, position 3 by esterases in the liver or kidney of the recipient. In most cases the products have microbiologically lower activity than the parent compounds.

4. Production of Cephalosporins Intermediates

4.1 7-Aminocephalosporanic acid

Cephalosporin C, the major product of fermentation of *Cephalosporium acremonium*, is deacetylated to produce 7-aminocephalosporanic acid (Figure 10a), a key intermediate for semisynthetic cephalosporins. There was a report that this reaction was catalyzed by enzymes from species of *Achromobacter*, *Brevibacterium*, and *Flavobacterium* (Walton, 1964a). On the industrial scale this reaction is carried out chemically at high efficiency using nitrosyl chloride. A rather complicated enzymatic route from cephalosporin C via ketoadipyl and glutaryl derivatives and then hydrolysis to 7-ACA has been reported (Banyu, 1977) using D-amino acid oxidase from *Gliocladium deliquescens* and on acylase enzyme from *Pseudomonas putida*

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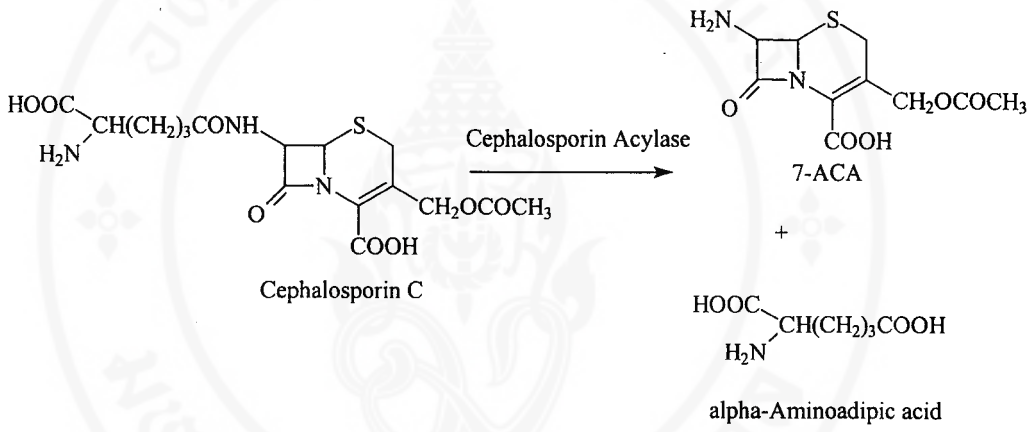
(Shibuya *et al.*, 1981). Shibuya *et al.*, (1981) isolated the acylase enzyme from *Pseudomonas putida* and showed that cephalosporin C was not the preferred substrate but rather glutaryl-7-ACA which, was the product of D-amino acid oxidase.

(a) Deacylation of Cephalosporin C



(b) Deacylation of Phenylacetyl-7-aminodesacetoxycephalosporanic acid

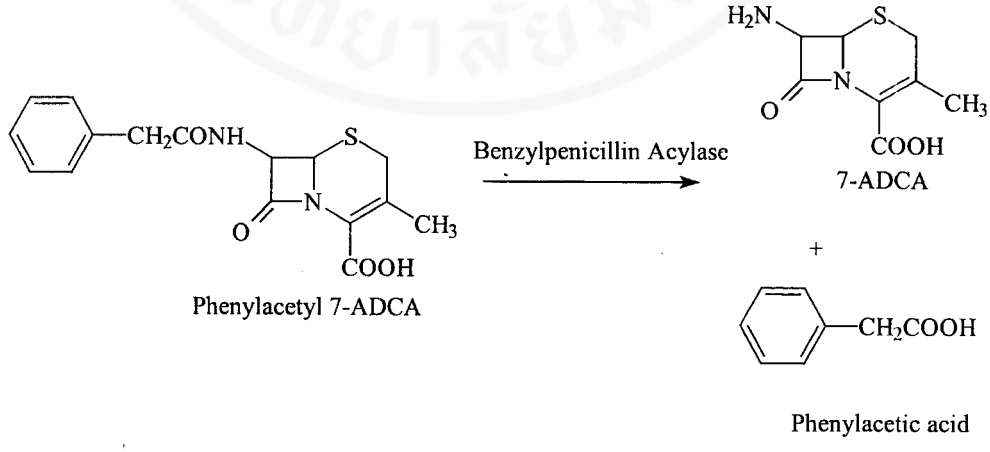


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Figure 10. Enzymatic production of cephalosporin intermediates (Vandamme, 1984).

(c) Deacetylation of 7-ACA

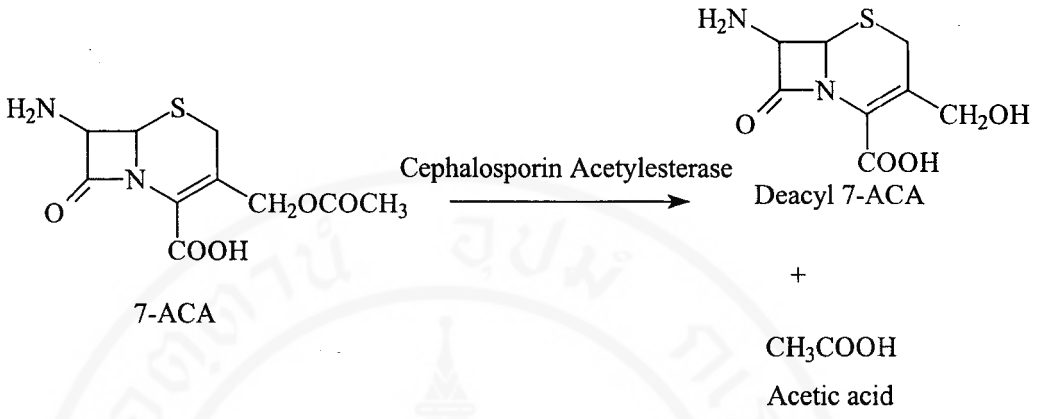

Figure 10. Enzymatic production of cephalosporin intermediates (Vandamme, 1984) (Continued).

4.2 7-Aminodesacetoxycephalosporanic acid

Cephalosporins can be produced chemically from penicillins by expanding the five-membered thiazolidine ring of penicillin to the six membered dihydrothiazine ring of cephalosporins (Cooper and Spry, 1973). Consequently, the resulting cephalosporin bears the side chain of the penicillin from which it was derived, and this side chain may be removed enzymatically to produce certain key intermediate namely, 7-amino-3-desacetoxycephalosporanic acid for making semisynthetic cephalosporins as shown in Figure 10b. The reactions for enzymatic removal of the side chain were catalyzed by either benzylpenicillin or phenoxymethylpenicillin acylases. In one process (Bayer, 1975 and 1977), immobilized benzylpenicillin acylase was used to deacylate 7-phenylacetyl-ADCA. In another one a phenoxymethylpenicillin acylase isolated from *E. aroideae* ATCC 25206 was used to deacetylate 7-phenoxyacetyl-ADCA (Fleming *et al.*, 1977).

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4.3 Deacyl-7-aminocephalosporanic acid

In order to modify the substituent at the C-3 position of cephalosporins, it is necessary to remove the acetate group. This can be achieved chemically, but yields were low owing to β-lactam degradation and lactonization between the newly formed hydroxymethyl and adjacent carboxyl group. Enzymatic hydrolysis may be preferable as shown in Figure 10c.

Acetylesterases were isolated from several sources, for examples, plants, mammalian tissues and bacteria. Particularly useful sources of the enzyme are the *Bacillus* species (Eli Lilly Co., 1976). The enzyme from *B. subtilis* NRRL-B-558 was extensively studied by Abbot and Fukuda (1975a,b) and Abbot et al. (1976). The purified enzyme was also immobilized to bentonite by adsorption.

4.4 Deacetylcephalosporin C

By the action of citrus acetylesterase, deacetylcephalosporin C is formed from cephalosporin C (Jeffery *et al.*, 1961), The deacetyl compound is one-fifth as active as cephalosporin C against *Staphylococcus aureus* and *Samonella typhi*.

5. Enzymes Act on Cephalosporin C

5.1 Cephalosporin β-lactamase

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The existence of a cephalosporinase different from penicillin β-lactamase was suspected by Newton and Abraham (1956) when they found that a crude preparation of penicillin β-lactamase from *B. cereus* destroyed cephaloporin C to a greater degree

than did the highly purified enzyme. Further work indicated that it was cephalosporinase that was present in the crude preparation and it could act as a β-lactamase (Crompton *et al.*, 1962). The occurrence of cephalosporin β-lactamase is widespread among bacteria including actinomycetes but the enzyme has not been found in yeasts and molds (Demain *et al.*, 1963). The β-lactamase activities of different microorganisms differ greatly in their ratio of activities on benzylpenicillin and on cephalosporin C. In some strains the ability to hydrolyze both penicillin and cephalosporin was the function of the same enzyme (Richmond, 1963; Hamilton-Miller *et al.*, 1965; Sabath *et al.*, 1965).

5.2 Cephalosporin acylase

None of the known penicillin acylases has ability to remove the side chain from penicillin N or cephalosporin C. This resistance to enzyme attack is attributed to the α-D-aminoadipyl side chain since 7-phenylacetamidocephalosporanic acid is hydrolyzed (Huang *et al.*, 1963; Walton, 1964b). By using special selection techniques Walton (1964b) isolated species of *Achromobacter*, *Brevibacterium*, and *Flavobacterium* possessing this enzyme.

5.3 Acetylesterase

Jeferry *et al.* (1961) found that citrus acetylesterase catalyzes the removal of the *O*-acetyl group in cephalosporin C to produce the less active deacetylcephalosporin C. This enzymatic activity was widely distributed among bacteria including actinomycetes (Demain *et al.*, 1963). Deacetylation of

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cephalosporins also occurs after administration to animals (O'Callaghan and Muggleton, 1963). The enzyme appears to be most prevalent in liver and kidney tissues.

6. Cephalosporin C Deacetylase

Cephalosporin C deacetylase (CAH), or its systematic name cephalosporin C acetylhydrolase, is an esterase that catalyzes the deacetylation of cephalosporins such as cephalosporin C and 7-aminocephalosporanic acid. Other names of this enzyme are cephalosporin acetylesterase (Abbot *et al.*, 1975a,b; Singh *et al.*, 1980), cephalosporin C deacetylase (Mitsushima *et al.*, 1995) and cephalosporin esterase (Politino *et al.*, 1997).

6.1 Sources of enzyme

Enzymes that deacetylate cephalosporins are wildespread in nature. Cephalosporin acetylesterase has been detected in variety of biological tissues as shown in Table 6. Its activity was found in many tissues or various mammalian species, notably liver and kidney (O'Callaghan and Muggleton, 1963). The esterase activity may be derived from higher plants, bacteria, yeasts and fungi. Some strains of *Bacillus subtilis* (Jeffery *et al.*, 1961; Takimoto *et al.*, 1994; Mitsushima *et al.*, 1995) have been found to have the enzyme activities. Suitable higher plant sources include the peel of citrus fruits (Jeffery *et al.*, 1961) and wheat germ (US patent no. 4,533,632). The most important sources are enzymes from microorganisms of the order Actinomycetes, e.g. in the family Mycobactericeae, Streptomycetaceae,

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Actinomycetaceae, Streptosporangiaceae, and Actinoplanaceae and of fungi imperfecti. The deacetylation of cephalosporin C can be performed with enzymes from strains of the following genera and species: mesophilic *Streptomyces*, such as *S. viridochromogenes, S. fradiae, S. griseus, S. griseoflavus, S. prasinus,* thermophilic *Actinomycetes* such as *S. violaceoruber, Thermoactinomyces vulgaris, S. thermovulgaris, Actinopycnidium* sp, *Micromonospora* sp, *Nocardia petroleophila, Streptosporangium roseum, Thermopolyspora polyspora,* and *Thermopolyspora glauca,* and others are *Mycobacterium tuberculosis var.* BCG; *Mycobacterium phlei*; *Cephalosporium* and *Aspergillus* (US patent no. 3,304,236). Microorganisms of the class Basidiomycetes which may be useful as sources of the desired esterase activity include yeast microorganisms which belong to the genera *Leucosporidium* and *Rhodosporidium*, for example strains of *Leucosporidium scottii, Rhodosporidium toruloides* (US patent no. 3,976,546) and *Rhodosporidium sphaerocarpum.* Yeast microorganisms in the Sporobolomycetaceae include organisms of the genera *Bullera, Sporidiobolus* and *Sporobolomyces*. For examples strains of *Bullera alba, Bullera tsugae, Sporodiobolus johnsonii, Sporobolomyces roseus,* and *Sporobolomyces salmonicolor* were reported. Non-yeast microorganisms of the class Basidiomycetes, which generate the desired esterase activity, may also be used. Representatives of such microorganisms are Order Polyporales e.g. strains of *Polyporus dichrous, Polyporus versicolor* and *Poria monticola.* Although the enzyme is widespread, it has not been purified to homogeneity and very little is known about its physical characteristics or kinetic behavior.

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Table 6. Sources of cephalosporin C acetylesterase.

Sources	Reference
1. Mammalian tissues: - Liver and kidney	O'Callaghan and Muggleton, 1963
2. Plants: - The peel of citrus fruits - Wheat germ	- Jeffery *et al.*, 1961. - US patent no. 4,533,632 (1985)
3. Actinomycetes: - Mesophilic *Steptomyces*, for examples; *S. viridochromogenes*, *S. fradiae*, *S, griseus*, *S griseoflavus*, *S prasinus* - Thermophilic *Steptomyces*, for examples; *S. violaceoruber*, *S. thermovulgaris*, *Thermoactinomyces vulgaricus*, *Actinopycnidium* sp., *Micromonospora* sp., *Nocardia petroleophila*, *Streptosporangium roseum*, *Thermopolyspora polyspora* and *Thermopolyspora glauca*.	- US patent no. 3,304,236 (1967)
4. Bacteria: - *Mycobacterium tuberculosis var.* BCG, *Mycobacterium phlei* - *Bacillus subtilis* - *Thermoanaerobacterium* sp.	- US patent no. 3,304,236 (1967) - Jeffery *et al.*, 1961; Takimoto *et al.*, 1994; Mitsushima *et al.*, 1995. - Lorenz and Wiegel, 1997.

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Table 6. Sources of cephalosporin C acetylesterase (Continued).

Sources	Reference
5. Fungi/Yeast: - *Cephalosporium* sp., *Aspergillus* sp. - *Luecosporidium* sp., *Rhodosporidium* sp. - *Rhodosporidium toruloides* *Sporobolomyces* sp., *Sporidiobolus* sp., *Rhodosporidium sphaerocarpum* *Polyporus versicolor*, *Schizophyllum commune*	- US patent no. 3,304,236 (1967) - US patent no. 3,976,546 (1976) - Politino *et al.*, 1997

6.2 Cephalosporin acetylesterase reaction

Cephalosporin acetylesterase is the enzyme that catalyzes cephalosporin C to deacetyl cephalosporin C. It also hydrolyzes other cephalosporins containing an *O*-acetyl group to the corresponding deacetylcephalosporins and 7-aminocephalosporinic acid to 7-amino deacetylcephalosporanic acid as shown in Figure 11 (Abraham, *et al.*, 1975 and Abbott, *et al.*, 1977). The enzyme also hydrolyzes 3-acyloxymethylceph-3em-4-carboxylic acid to 3-hydroxymethylceph-3em-4-carboxylic acid (US patent no. 3,976,546 and Takahashi, *et al.*, 1973) and converts cephaloglycin to deacetylcephaloglycin (Kukolja, 1968).

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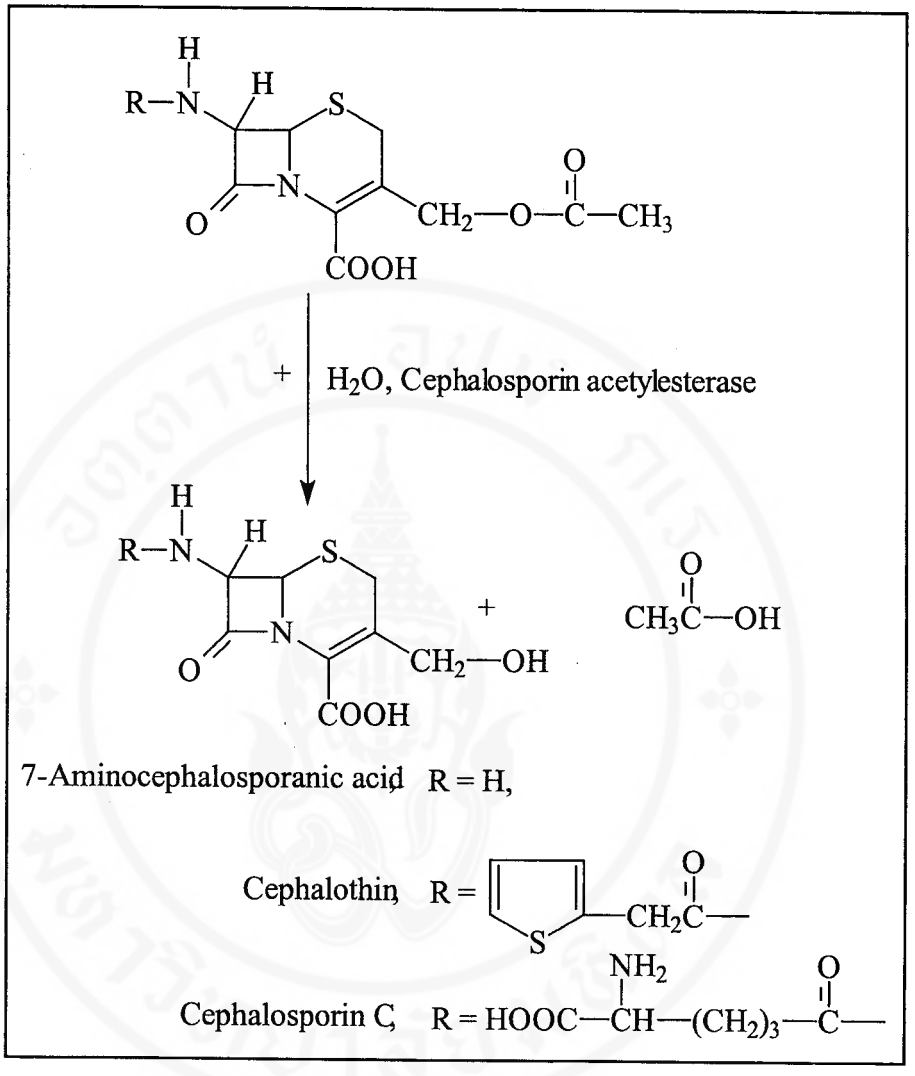

Figure 11. Cephalosporin acetylesterase reaction (Abraham, *et al.*, 1975 and Abbott, *et al.*, 1977).

6.3 Gene cloning of the cephalosporin acetylesterase genes

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Acetylesterase genes from various microorganisms have been cloned and sequenced. Cephalosporin C deacetylase enzyme from *Bacillis subtillis* SHS 0133 (FERM BP-2755) was purified by Takimoto *et al.* (1994). The native enzyme was

composed of eight identical subunits each with apparent molecular weight of 35,000 kDa. The gene encoding the cephalosporin C deacetylase (CAH) from *B. subtilis* SHS 0133 was cloned and sequenced (Mitsushima *et al.*, 1995). They found that the nucleotide sequence contained an open reading frame encoding a polypeptide consisting of 318 amino acids. The expressed CAH protein was found in an active form in the soluble fraction. The deduced amino acid sequence contained the common sequence Gly-X-Ser-X-Gly found in many esterases, lipases, and serine proteases (Brenner, 1988; Komaromy and Schotz, 1987; Read and James, 1988; Shimada *et al.*, 1994). A novel cephalosporin esterase from *Rhodosporidium toruloides* ATCC 10657 was reported by a team of Politino (Politino *et al.*, 1997). The enzyme was a glycoprotein with a molecular mass of 80 kDa after deglycosylation, a range of molecular masses were observed. They constructed a cDNA clone by 3' rapid amplification of the cDNA. The amino acid sequence of this esterase was compared with that found from the National Center for Biotechnology Information's BLAST search. They found some sequence similarities with eukaryotic lipases and esterases, particulary around the putative active site serine (G-X-S-X-G). No sequence similarities were observed with other cephalosporin esterases in the database. Lorenz and Wiegel (1997) reported an activity of cephalosporin C deacetylase after two genes form *Thermoanaerobacterium* sp JW/SL YS485 were cloned and sequenced. The deduced sequence of the *axe1*1 (encoding acetyl xylan esterase) gene product had no significant amino acid sequence similarity to any reported acetyl xylan esterase sequence but had strong similarity to cephalosporin C deacetylase from *B. subtilis*. The importance of this finding remains to be elucidated.

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6.4 Possible roles and importants of cephalosporin acetylesterase

6.4.1 Esterases

The physiological functions of many esterases are not clear. Some of these enzymes are known to be involved in metabolic pathways that provide access to carbon sources; such enzymes include the acetyl esterase and cinnamoyl esterase that are involved in degradation of hemicellulose (Dalrymple *et al.*, 1996; Ferreira *et al.*, 1993). In some plant-pathogenic bacterial and fungal strains, these cell wall-degrading esterase activities are believed to be pathogenic factors (McQueen and Schottel, 1987). Detoxification of biocides may be another their important roles. Insecticide resistance often results from amplification of genes for esterases that hydrolyze the insecticides (Blackman *et al.*, 1995). The fusidic acid resistance of *Streptomyces lividans* is due to a specific esterase which inactivates the antibiotic (von der Haar *et al.*, 1997), and a *Bacillus subtilis* esterase that hydrolyzes the phytotoxin brefeldin A has been described (Wie *et al.*, 1996).

The fact that different enantiomers interact differently in an organism and may even have hazardous effects, such as the teratogenic activity of the racemic drug thalidomide, has led to a growing demand for enantiomerically pure compounds (Schoffers *et al.*, 1996). Lipases and esterases have been used successfully in organic synthesis of optically pure substances. For instance, an esterase from *Arthrobacter globiformis* was used in the resolution of ethyl chrysanthemate derivatives (Nishizawa *et al.*, 1993; Shimizu *et al.*, 1995), which are key compounds for the synthesis of pyrethrin insecticides. A heroin specific esterase has been described, and this is

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followed by further degradation to morphinone by a morphine dehydrogenase (Rathbone *et al.*, 1997). A *Bacillus* carboxyl esterase has been used for stereospecific resolution of *R,S*-naproxen esters to *S*-naproxen (Quax and Broekhuizen, 1994), which is an important anti-inflammatory drug, and a *p*-nitrobenzyl esterase was genetically engineered in order to be used for synthesise of cephalosporin-derived antibiotics (Moore and Arnold, 1996).

6.4.2 Acetylesterases

Another role of cephalosporin acetylesterase was in protection of the bacterial producer against the β-lactams action. Although most clinically important hydrolytic enzymes are β-lactamases but β-lactam antibiotics can be degraded by a number of hydrolytic enzymes as shown in Figure 4 (Sykes, *et al.*, 1981). The major inactivation of cephalosporins by pathogenic bacteria has been considered to be due to enzymatic attack of β-lactamases; therefore little attention has been paid to the degradation of cephalosporins by other enzymes. Nishida and his coworkers (1968) reported that a cephalosporin derivative, m-bromophenyl acetamidocephalosporanic acid (m-Br-PACA) was easily degraded by a clinically isolated strain of *E. coli*, but not by a clinically isolated strain of *S. aureus*. On the contrary, α-phenylacetamidocephalosporanic acid (α-Ph-POAC) was easily degraded by the same *S. aureus*, but not by the *E. coli*. Evidences suggested that the bacteria elaborated one or more β-lactamases plus at least one other enzyme (acetylesterase) and that the activities of both enzymes differed considerably in the *E. coli* and *S. aureus*. Also it seemed reasonable to assume that α-Ph-POAC had high affinity for acetylesterase

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from *S. aureus* which reduced an antibacterial activity showed around 36%, while hydrolysis of the β-lactam ring was not detectable. For m-Br-PACA was found to have a high affinity for the β-lactamase from the *E. coli*. This demonstrated that acetylesterases in some pathogenic bacteria might play an important role in degradation of some cephalosporin derivatives (Nishida, *et al.,* 1968). It was reported that many bacteria and actinomycetes were potent producers of enzymes capable of destroying or deacetylating cephalosporin C (Demain, *et al.*, 1963).

Two novel β-lactam antibiotics, A16886A [7-(5-amino-5-carboxyvaleramido)–3-carbamoyloxymethyl-3-cephem-4-carboxylic acid] and A 16886B [7-(5-amino-5-carboxyvaleramido)-7-methoxy-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid], have been isolated from *Streptomyces clavuligerus* (Nagarajan, *et al.*, 1971). These unique natural cephalosporins have cabamate attached at the C-3′ position. An examination of *S. clavuligerus* or it cultures filtrate for the ability to produce deacetylcephalosporins was attempted since it was found that A16886A could be prepared synthetically from deacetylcephalosporin C. The possible role of cephalosporin acetylesterase in formation of A16886A and A16886B necessitated an assay method other than antibiotic activity to determine whether deacetylcephalosporin C could be formed from cephalosporin C by the culture broth of *S. clavuligerus*. The accumulation of radioactivity in deacetylcephalosporin C upon addition of cephalosporin C^{14} C-9 to the culture broth was the method for measuring the rate of deacetylation. The accuracy of this method depended upon the

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paper chromatographic separation of cephalosporin C and deacetylcephalosporin C from a sample of the substrate-broth incubation (Brannon, *et al.*, 1972).

7. Properties of Cephalosporin C Acetylesterases

Cephalosporin C acetylesterases purified from various sources were characterized and their properties are shown in Table 7.

7.1 Microbial acetylesterase

Microbial enzymes are the most important and convenient sources for productions of deacetyl derivatives of cephalosporins. A study conducted for screening microorganisms capable of deacetylating cephalosporin C revealed that many microorganisms especially bacteria, actinomycete and fungi were capable of degrading cephalosporin C to its deacetyl product (Demain, *et al.*, 1963).

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Table 7. Comparison of some properties of cephalosporin C acetylesterases from various sources (Duangsonk, 1996).

Sources	Citrus peel	*B. subtilis* WRRL-B-558	*B. subtilis* SHS 0133	*B. subtilis* ATCC 6633	*E. coli* 437	*C. acremonium* C462	*C. acremonium* no. 81	*F. oxysporum* AF-298	*A. pullulans* IFO 4466
MW (Kda)	ND	190	280	150	ND	25	ND	ND	ND
Optimum temp (°C)	30	40-50	55	ND	45	50	50	28	37
Optimum pH	6.0-6.5	7.0	8.0-8.5	8.0	7.5	7.6	8.0	7.5-8.0	Below 4.0
Temp stability (°C)	ND	Below 60	Below 60	ND	ND	ND	Below 60	ND	Below 60
pH stability	5.0-8.25	ND	7.0-10.0	ND	ND	ND	6.0-12.0	ND	4.0-6.0
pI	ND	ND	5.3	4.7	ND	4.3	ND	ND	ND
Subunit	ND	ND	8	4	ND	ND	ND	ND	ND
Km (mM) (Ceph C) (7-ACA)	4.7	0.0028 0.0028	24.0 7.9	15.0 6.5	ND	20.0	ND	ND	11.5

ND = not determined

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Table 7: (Continued)

Sources	Citrus peel	*B. subtilis* WRRL-B-558	*B. subtilis* SHS 0133	*B. subtilis* ATCC 6633	*E. coli* 437	*C. acremonium* C462	*C. acremonium* no. 81	*F. oxysporum* AF-298	*A. pullulans* IFO 4466
Ki (mM) (Acetate) (Deacetyl 7-ACA)	ND	50.0 36.0	290 171	ND	ND	ND	ND	ND	ND
References	Jansen, et al., 1947	Abbot, et al., 1975 & 1977	Takimoto, et al., 1994	Konecny, et al., 1977	Nishimura, et al., 1978	Hinnen, et al., 1976	Fujisawa, et al., 1975	Singh, et al., 1980	US patent no. 4, 517,299

ND = not determined

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7.2 Mammalian acetylesterase

It is known that antibiotics may undergo metabolic changes in the body to give rise to metabolites by formation of deacetyl compounds in case of cephalosporin antibiotics (Shimizu, *et al.,* 1970 and Wick, *et al.,* 1971). In an attempt to determine the site of deacetylation, homogenates of mouse liver, kidney, lung, skeletal muscle, spleen and brain were incubated at 37°C with cephalosporins. The results showed that liver, kidney and spleen rapidly produced the deacetyl metabolites. Liver and kidney from mouse, rat, guinea pig, rabbit, monkey and man produced the metabolites from phenylacetyl cephalosporin and N-dinitrophenyl cephalosporin C. With cephalosporin C, however the rate at which the metabolite was produced varied from species to species (O' Callaghan, *et al.,* 1963). It can be concluded that cephalosporin antibiotic can be degraded in the body of mammalians by enzymes in various tissues especially liver and kidney. Variation in rate and specificity of deacetylation between species of animals tested.

7.3 Plant acetylesterase

Enzymes hydrolyzing simple esters have been found in plant sources; for example, the enzyme that hydrolyzes esters of acetic acid occurs in oranges, lemons and grapefruit (Jansen, *et al.,* 1974). The enzyme prepared from citrus peel has been used for the preparation of 7-aminodeacetylcephalosporanic acid, deacetylcephalosporin C, deacetylcephalothin and a series of other acylamido derivatives of deacetylcephalosporanic acid (US patent no. 3,459,764 and UK patent no. 1,066,347). Good substrates include 7-aminocephalosparanic acid and a variety of

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cephalosporins. Butyryl esters are hydrolyzed much less rapidly than acetyl esters, but N-acetyl compounds are not hydrolyzed. Esterase in the crude extract of citrus peel decreased its activity after oxidation and pigment formation occurred. This loss may be minimized by addition of reducing agents or activators such as NaCl. The K_m values of 4.7 mM and 8.6 mM have been reported with cephalosporin C and cephalothin, respectively (Abraham, *et al.*, 1977).

8. Applications of Cephalosporin Acetylesterase

Deacetyl derivatives of cephalosporins produced by deacetylation of cephalosporin acetylesterase are valuable and are important intermediates for the synthesis of therapeutically useful cephalosporin compounds. The production in industrial scale was conducted by hydrolysis of cephalosporins with the esterase of microorganisms. The esterase may exist in cells or be excreted in culture broth. Therefore, the following preparations can be exemplified as processed materials to produce deacetyl derivatives.

8.1. Enzyme solutions: The solution of esterase may be employed to hydrolyze cephalosporins directly. Thus, for example, a stable solution of purified citrus acetylesterase was added directly to the solution of reaction mixtures to produce 7-α-thienylactamidocephalosporadesic acid potassium salt (US patent No.3, 459,746).

8.2. Cell-free extract: An aqueous extract of the suspension resulting from rupture of the cells may similarly be used. For example, *B. subtilis* cells were extracted with acetone to obtain the intracellular enzyme before adding to the cephalosporin reaction system (Konecny, *et al.*, 1977).

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8.3. Immobilized cells or enzymes: Whole cells or enzyme may be immobilized in or on an inert matrix prior to their addition to a hydrolysis reaction system in order to protect cells or enzyme and minimize losses during their recycling. It has been reported that *B. subtilis* cephalosporin acetylesterase was ideally suited to immobilization by containment in an ultrafiltration device with high stability (Abbott, *et al.*, 1976).

8.4. Active cells: A mixed culture may preferably be conducted by co-culturing the esterase-producing strain with a cephalosporin C producing microorganism at an apprpiate time during the cultivation of cephalosporin C-producing microorganism (US patent No.4, 517,299).

8.5. Dried cells: Harvested cells with acetylesterase may be subjected to treatment by lyphilization or vacuum drying to obtain active cells. Then, the dried cell is prepared as a cell paste before use. For example, a cell paste of *Rhizobium trifolii* was used to produce 3-hydroxymethyl-7-amino-ceph-3-em-4-oic acid (US patent No.3, 436,310).

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CHAPTER III

MATERIALS AND METHODS

I. Materials

1. Chemicals

Chemicals used in this study were analytical and biotechnological grade obtaining from Amersham (Buckinghamshire, England), Bio-Rad (Hercules, USA), BRL (Gaithersburg, USA), Promega (Madison, USA), and Sigma (St. Louis, USA).

All of them were absolute ethanol, acetic acid, acetonitrile, acetone, acrylamide, agarose, albumin, ammonium persulfate, ampicillin, bisacrylamide, boric acid, bromophenol blue, cephalosporin C, chloroform, Coomassie Brilliant Blue R-250, disodium tetraborate decahydrate (Borax), ethanol, ethidium bromide, ethylene diamine tetraacetic acid (EDTA), Fast blue BB salt, formaldehyde, glycerol, glycine, glutaraldehyde, hydrochloric acid, isopropanol, 2-mercaptoethanol, methanol, MOPS [3-(*N*-morhpolino) propanesulfonic acid], α-naphthol, α-naphthylacetate, N-N-N'-N'-tetramethyl ethylenediamine (TEMED), phenol, polyethylene glyclol (PEG), potassium acetate, potassium dihydrogen phosphate, patassium hydroxide, dipotassium hydrogen phosphate, rubidium chloride, silver chloride, silver nitrate, sodium acetate, sodium chloride, sodium citrate, sodium dodecyl sulfate, sodium hydroxide, Tris hydromethyl amino methane (Tris), Triton X-100, Tween 20.

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2. Enzymes and Miscellaneous Materials

Restriction and DNA modifying enzymes were acquired from Boehringer Mannheim (Mannheim Germany). The products for polymerase chain reaction (PCR) were supplied by Stratagene (La Jolla CA, USA) and Boehringer Mannheim. The DIG nonradioactive DNA labelling and detection kit were purchased from Boehringer Mannheim. The Prep-A-Gene DNA purification kit was purchased from Bio-Rad. The Bradford^{R} reagents for protein assay were supplied by Bio-Rad. Standard DNA markers, λ DNA cut with *Eco*RI-*Hin*dIII, was supplied by Boehringer Mannheim. Molecular weight for SDS-PAGE was obtained from Sigma. Partially purified acetylesterase (orange peel) was obtained from Sigma. Oligonucleotides were supplied by Invitrogen (Carlsbad CA, USA). The nylon membrane (charge modified, 0.2 μm pore size) for Southern hybridization was supplied by Sigma.

3. Culture Media

Microbiological media were obtained from Difco (Detroit, USA), Oxoid (Hampshire, England), Sigma and Merck (Dam stadt, Germany), and prepared according to the manufacturers's recommendation.

4. Bacterial Strains and Plasmids

Bacterial strains and plasmids are summarized in Table 8 and Table 9, respectively.

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Table 8. List of bacterial strains.

Bacterial strains	Genotypes/ Phenotypes	Purposes	Sources
Bacillus cereus BT-24	Cephalosporin C deacetylase (CAH) producer	Source of DNA for gene cloning	This study
Bacillus subtillis WRRL-B558	Wild type, CAH producer	Positive control for screening of CAH producer	NCAUR[1]
Escherichia coli JM109	(e14$^-$) *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB*$^+$ *lacI*^{q}ZΔM15]	Host cell	Promega
Escherichia coli DH-5α	F′ (ϕ80d *lacZ*ΔM15) Δ(*lac*ZYA-argF)U169 *endA1 recA1 hsdR17*(r_K-m_K+) *deoR thi-1 supE44 gyrA96 relA1* λ^-	Host cell	BRL
Staphylococcus aureus ATCC 25923	Cephalosporin C sensitive	Test organism for microbiological test	ATCC[2]

Note: [1]NCAUR = National Center for Agriculture Utilization Research, USA

[2]ATCC = American Type Culture Collection

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Table 9. List of plasmids.

Plasmids	Characteristics	Sources
pBS II SK+ (2.96 kb)	2.96 kb, *lacZ*, Ap^r	Stratagene®
pKK223-3 (4.58 kb)	4.5 kb, *tac* promoter (inducible with 1-5 mM IPTG), *rrn*B ribosomal terminator, Ap^r	Amersham Pharmacia Biotech
pSR1 (5.7 kb)	2.7 kb *Sau*3AI fragment from *B. cereus* BT-24 in pBS II SK+, Ap^r	This study
pSR2 (5.3 kb)	2.4 kb *Eco*RI-*Eco*RI fragment from pSR1 in pBS II SK+, Ap^r	This study
pSR3 (4.3 kb)	1.4 kb *Hin*dIII-*Hin*dIII fragment from pSR1 in pBS II SK+, Ap^r	This study
pSR4 (4.4 kb)	1.5 kb *Hin*dIII-*Hin*dIII fragment from pSR1 in pBS II SK+, Ap^r	This study
pSR5 (3.8 kb)	0.9 kb *Xba*I-*Xba*I fragment from pSR1 in pBS II SK+, Ap^r	This study
pSR6 (3.8 kb)	0.9 kb *Eco*RV-*Eco*RV fragment from pSR1 in pBS II SK+, Ap^r	This study
pSR7 (4.8 kb)	1.8 kb *Bam*HI/*Sau*3AI-*Eco*RV fragment from pSR1 in pBS II SK+, Ap^r	This study
pSR8 (3.3 kb)	0.4 kb *Bam*HI/*Sau*3AI -*Eco*RI fragment from pSR1 in pBS II SK+, Ap^r	This study
pSR9 (4.8 kb)	1.8 kb *Xba*I- *Bam*HI/*Sau*3AI fragment from pSR1 in pBS II SK+, Ap^r	This study
pSR11 (5.4 kb)	0.9 kb *Eco*RI-*Eco*RV PCR product of CAH gene coding region in pKK223-3, Ap^r	This study

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II. Methods

1. Bacterial Growth and Maintenance

Escherichia coli host strains, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* were usually cultivated on Luria Bertani (LB: 1.0 % tryptone, 0.5 % yeast extract, 1.0 % NaCl, pH 7.0, agar 1.5 %) agar plates whereas the recombinant *E. coli* clones were grown on LB agar supplemented with 50 μg/ml of ampicillin. They were incubated at 37°C for overnight except that *B. subtilis* was incubated at 30°C for 1-2 days. All of them were maintained at 4°C for routine use. For long term preservation, bacteria were grown in LB broth at 37°C or 30°C until mid to late log phase of growth, then 500 μl of the culture was transferred to a vial containing 500 μl of 30 % glycerol, mixed and kept at -80°C.

For selection of recombinant *E. coli* clone, LB agar were supplemented with 50 μg/ml of ampicillin. When blue-white selection was required, the plate was overlayed with 4 μl IPTG (200 mg/ml isopropyl thio-β-D-galactoside) and 40 μl X-gal (20 mg/ml in dimethylformamide of 5-bromo-4-chloro-3-indoyl-β-D-galactoside).

2. Isolation of Cephalosporin C Deacetylase Producing Bacteria from Soil Samples

2.1 Isolation of bacteria

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Fifty five soil samples were collected from various parts of Thailand. They were taken from the soil surface and kept in plastic bags that assigned number and place of collection.

One gram of soil was resuspended vigorously in 9 ml sterial distilled water. Ten fold serial dilution were prepared and the dilutions 10^{-5}-10^{-6} were heated at 80°C for 15 min. A 0.1 ml of each dilution was spread on LB agar plates and then the plates were incubated at 30°C for 1-2 days.

2.2 Screening methods for cephalosporin C deacetylase producing bacteria

To screen the esterase producing bacteria, the chemical method and microbiological method were used as the primary and secondary screening, respectively.

2.2.1 Chemical method

The chemical method was used for screening of esterase producing bacteria. The method was previously described by Higerd (1977). The colonies grown on LB agar plate were picked and spotted in grid pattern onto the same medium and incubated at 30°C for 1-2 days. Then plates were overlaid with 10 ml of 0.8 % soft agar in 50 mM potassium phosphate buffer pH 7.5 containing 5 mg α-naphthylacetate and 5 mg Fast Blue BB dye. Around 30 min after overlaying the colonies of esterase producing strains turned into dark brown colony. Appearances of all colonies were compared with that of the positive control, *B. subtilis* WRRL-B558, which was spotted onto the same plate.

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2.2.2 Microbiological method

The microbiological method was developed for screening of cephalosporin C deacetylase producing bacteria. It was modified from the biological assay method as described by Meevootisom *et al.* (1983). The positive colonies from the primary screening were grown on LB agar for 1-2 days, then, the plates were overlayered with 5 ml of soft agar (0.8% agar) containing 300 μg/ml cephalosporin C and 1% (v/v) of overnight culture *S. aureus* ATCC 25923. The plates were incubated at 37 °C for overnight. Cephalosporin deacetylase producing strains were observed for the presence of larger satellite zone of *S. aureus* ATCC 25923 around their colonies as compared to that of the positive control *B. subtilis* WRRL-B558.

3. Preparation of DNA

3.1 Bacterial genomic DNA preparation

Bacterial genomic DNA was prepared by the method of Miura, 1967. An overnight culture of *B. cereus* BT-24 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 30°C with shaking for another 4 h. Cells were pelleted by centrifugation at 8,000 rpm for 10 min at room temperature and washed once with 50 ml TE buffer (25 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0). The cell pellet was resuspended in 20 ml TE buffer containing 2 mg/ml lysozyme and incubated at 37°C for 30 min. Then 0.5 ml of 20% SDS and 1 mg/ml Proteinase K were added and mixed slowly by inversion. After incubation at 37°C for 30 min, an equal volume of phenol-chloroform solution (1:1 v/v saturated with Tris-HCl, pH 7.0) was added and mixed gently by inversion at least 50 times

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until a milky solution was formed. This was followed by centrifugation at 10,000 rpm for 5 min at 4°C 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 an equal volume of chloroform saturated with water. After centrifugation at 10,000 rpm for 5 min at 4°C, the genomic DNA was precipitated with 2 volumes of cold absolute ethanol and 1:10 volume of 3 M sodium acetate pH 5.2. The mixture was kept at -20°C for overnight, followed by centrifugation at 10,000 rpm for 10 min at 4°C. The DNA was washed once with cold 70% ethanol and centrifuged at 10,000 rpm for 5 min at 4°C. The DNA was air dried and resuspended in 1 ml TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). This DNA solution was treated with 1 μg/ml boiled Rnase A (stock solution: 10 mg/ml, kept at -20°C) at 37°C for 30 min and then extracted with phenol-chloroform repeatly until the white interface disappeared. The final extraction was done with an equal volume of chloroform. The DNA in the aqueous solution was precipitated, pelleted, washed and air dried as described above. Finally the DNA was resuspended in 0.5 ml TE buffer and kept at 4°C.

3.2 Small scale preparation of plasmid

The rapid boiling method of Holmes *et al.*, 1981 was employed for small scale preparation of plasmid. A single colony of *E. coli* was inoculated into 5 ml of LB broth containing 50 mg/ml ampicillin and incubated overnight at 37°C with vigorous shaking. Cells were packed by centrifugation at 8,000 rpm for 1 min at room temperature in a microcentrifuge tube. The supernatant was discarded and the cell

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pellet was resuspended in 350 µl of STET solution (8% sucrose, 5% w/v triton X-100, 50 mM EDTA pH 8.0 and 50 mM Tris-HCl pH 8.0). Then 50 µl of STET solution containing 10 mg/ml lysozyme was added and immediately mixed by inverting the tube 3 to 4 times. The tube was then boiled for 1 min and centrifuged at 10,000 rpm for 10 min at room temperature. The slimy gelatinous pellet was removed by picking with a sterile toothpick. An equal volume of phenol-chloroform was added to the supernatant and the tubes was vigorously shaked with vortex mixer followed by centrifugation at 10,000 rpm for 5 min at room temperature. The protein and other impurities were removed by this step. Subsequently, an aqueous phase was transferred to a new tube and equal volume of chloroform was added, mixed and recentrifuged. To precipitate DNA, 1:10 volume of 3 M sodium acetate pH 5.2 and 0.6 volume of isopropanol were added to the supernate. The solution were mixed by inversion and kept at -20°C for at least 30 min. After the tube was spun at 10,000 rpm for 5 min at room temperature, the DNA pellet was washed once with cold 70% ethanol, air dried and resuspended in 50 µl of TE buffer. To remove RNA in the sample, the DNA was incubated with 1 µl of 10 mg/ml boiled RNase A at 37°C for 30 min. This DNA was then ready for restriction endonuclease analysis.

3.3 Large scale preparation of plasmid

The modified alkaline lysis method described by Birnboim and Doly, 1979 was used to prepare large scale preparation of plasmid. Fresh *E. coli* culture was grown in 100 ml of Terrific Broth (TB: 12 g/l tryptone, 24 g/l yeast extract, 4 ml/l of glycerol, 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4) containing 50 µg/ml ampicillin at

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37°C with shaking overnight. The cells were harvested by centrifugation at 8,000 rpm for 5-10 min at room temperature. Then the pellet was completely resuspended in 8 ml of solution I (25 mM Tris-HCl pH 7.5, 10 mM EDTA, 15% sucrose, and 2 mg/ml lysozyme) and incubated on ice bath for 20 min. To lyse the cells, 16 ml of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added to the cell suspension, the mixture was mixed with gentle whirl and kept on ice bath for 10 min. The mixture was subsequently neutralized by 12 ml of cold solution III (3 M potassium acetate pH 5.2), mixed and incubated on ice bath for another 10 min. The supernatant was collected by centrifugation at 10,000 rpm for 15 min at 4°C and subsequently filtered through layers of gauze. To precipitate DNA, 0.6 volume of isopropanol was added to the filtrate. The mixture was standed at -20 °C for 30 min and then spun at 10,000 rpm for 15 min at 4°C. The DNA pellet was washed once with 70% ethanol and dissolved in 5-10 ml TE buffer. The DNA solution were incubated with 50-100 μg/ml boiled RNase A and 100-200 μg/ml of Proteinase K at 37°C for 1 h each, respectively. The protein and other impurities were removed by extraction with equal volume of phenol-chlorofor, vigorously shaked and centrifuged at 10,000 rpm for 5 min at 4°C. The DNA in the aqueous solution was removed to a new tube. The process was repeated until the white interface disappeared. The final extraction was done with an equal volume of chloroform, shaked briefly with vortex mixer and recentrifuged. The supernatant containing DNA was precipitated with 2 volumes of chilled absolute ethanol, mixed and kept at -20°C for at least 30 min. The nucleic acid was collected by centrifugation at 10,000 rpm for 10 min at 4°C, then rinsed twice with cold 70 %

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ethanol. The pellet was dried and dissolved in 0.5-1 ml TE and the DNA solution was stored at 4°C or -20 °C.

4. DNA Manipulation and Analysis

4.1 Restriction enzyme digestion

The restriction enzymes used in this study including their recognition sequences and their optimal conditions are shown in Table 10, 11.

Table 10. Restriction enzymes with their recognition sequences and optimal conditions.

Enzyme	Buffer	Optimal temperature (°C)	Recognition sequence
Bam HI	A, B, M	37	5′ G↓GATCC 3′
Eco RI	A, B, H	37	5′ G↓AATTC 3′
Eco RV	B	37	5′ GAT↓ATC 3′
*Hin*d III	A, B, M	37	5′ A↓AGCTT 3′
Pst I	H	37	5′ CTGCA↓G 3′
Pvu I	H	37	5′ CGAT↓CG 3′
Sau 3AI	A	37	5′ ↓GATC 3′
Sma I	A	25	5′ CCC↓GGG 3′
Xba I	A, H	37	5′ T↓CTAGA 3′

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Table 11. Restriction enzyme buffer composition.

Buffer components	Final concentration in mM			
	Buffer A	Buffer B	Buffer H	Buffer M
Tris-acetate	33	-	-	-
Tris-HCl	-	10	50	10
Magnesium-acetate	10	-	-	-
$MgCl_2$	-	5	10	10
Potassium-acetate	66	-	-	-
NaCl	-	100	100	50
Dithioerythritol (DTE)	-	-	1	1
Dithiothreitol (DTT)	0.5	-	-	-
2-Mercaptoethanol	-	1	-	-
PH at 37°C	7.9	8.0	7.5	7.5

All buffers store at -20°C until used.

Restriction enzyme digestion of plasmids and chromosomal DNA were performed according to the manufacturer's recommendations. In principle, the preferable reaction mixture was consisted of appropriate buffer of particular pH and salt, the restriction enzyme (not more than 10 % of total volume) and the DNA. The reaction was terminated by extracting with phenol-chloroform depended on the nature of restriction enzyme. When digestion of DNA with 2 enzymes were required, both enzymes were simultaneously in case that the buffer condition were compatible.

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However, if 2 different buffer conditions were required, one phenol-chloroform extraction and ethanol precipitation were performed after the first enzyme digestion.

4.2 Agarose gel electrophoresis

Agarose gel electrophoresis was performed by the standard method (Maniatis *et al.*, 1989). It was used for analysis and isolation of DNA fragments. The condition usually used in this study was 0.7 % agarose gel in Tris Borate EDTA buffer (TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA). Agarose concentration was occasionally varied depend on the size of DNA fragments. DNA samples were mixed in a ratio of 3:1 with gel loading buffer type III (0.25 % bromophenol blue, 30 % glycerol in water) before loading. The minicell electrophoresis sets (Mupid, Tokyo, Japan) were commonly used for routine analysis. The gel was run at a constant voltage of 50 or 100 volts at room temperature for 30-40 min or until the loading dye reached 1 cm from the bottom of the gel. The gel was then removed from the tray and stained with 0.5 μg/ml ethidium bromide solution for 10-15 min, then destained with distilled water for 10-30 min. The DNA was visualized with a long wavelength transilluminator and photographed using polariod camera (Polariod MP4, Fotodyne, New Berlin, USA) with Polariod film type 667 (Hertfordshire, England). The size of DNA fragments were estimated by standard DNA marker, λ DNA cut with *Hin*dIII and *Eco*RI, which contain 12 fragments of 21.2, 5.1, 4.9, 4.2, 3.5, 2.0, 1.9, 1.5, 1.3, 0.94, 0.83, and 0.56 kb.

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4.3 Recovery of DNA fragment

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 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 binding buffer were added onto the sliced gel and the tube was incubated at 37°C -55°C to dissolve the gel. Then 5 µl of matrix 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 at room temperature and the supernatant was discarded. Then 250 µl (50x volumes of matrix) of the binding buffer was added, mixed and standed for 5-10 min. After centrifugation at 10,000 rpm for 30 seconds at 4°C, 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 buffer). The tube was then incubated at 37°C -55°C for 5 min and centrifuged at 10,000 rpm for 1-2 min at room temperature. The aqueous DNA was collected. The elution step was repeated twice to recover most DNA as much as possible.

4.4 Dephosphorylation of DNA vector

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To remove the 5′-phosphate from both ends of the linear DNA, dephosphorelation of DNA was performed. This procedure was required in order to decrease self-recirculalization of DNA vector when it was digested with one

restriction enzyme. The plasmid DNA in the amount of approximately 1-20 pmol was completely digested with the restriction enzyme. The digested DNA was extracted once with phenol-chloroform and precipitated with ethanol for 15 min at -20°C. The precipitated DNA was dissolved in 50 µl sterile distilled water, then dephosphorylation was performed in the CIP dephosphorylation buffer (10x CIP dephosphorylation buffer: 10 mM $ZnCl_2$, 10 mM $MgCl_2$ and 100 mM Tris-HCl, pH 8.3) and 0.1 unit of calf intestinal alkaline phosphatase (CIP). The mixture was incubated at 37°C for 1 h, then enzyme was inactivated by heating at 65°C for 15 min. The mixture was extracted twice with phenol-chloroform and once with chloroform. The DNA was precipitated with ethanol and dried. It was ready to use for ligation.

4.5 Ligation of DNA

The vector and target DNA were digested with the same restriction or different restriction enzymes, which generated compatible sticky ends or blunt ends. Typically, digested vector or digested-dephosphorelated vector DNA (see method 4.4) was mixed with target DNA (see method 4.3) in a molar ratio of 1:3 and the total DNA was more than 50 ng. The mixed DNA was precipitated with 0.3 M sodium acetate at pH 5.2 and 2 volume of cold absolute ethanol at -20°C for at least 5 min to overnight. After centrifugation at 10,000 rpm for 10 min at room temperature, the DNA pellet was washed with cold 70 % ethanol and air dried. Routinely, the ligation was performed in total volume 10 µl. The dried DNA was resuspended in 8 µl TE buffer, warmed at 68°C for 10 min, and immediately chilled on ice for 1 min. Then, 1 µl of 10x ligation buffer (0.5 M Tris-HCl pH7.6, 100 mM $MgCl_2$, 100 mM dithiothreitol,

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500 μg/ml bovine serum albumin) and 1 μl T_4 DNA ligase (10 unit) were added to the mixed DNA. The ligation was allowed to occur at 16 °C for at least 3 h or overnight.

4.6 DNA amplification of cephalosporin C deacetylase (CAH) gene by PCR

PCR was used to generated gene fragment of CAH coding region.

4.6.1 Primer designs

A pair of primers was designed from the upstream sequence of the 5′ end and the downstream sequence of the 3′ end of ORF CAH gene as shown in Figure 1. To facilitate the cloning into a vector, the primers contained recognition sequences for *Eco*RI site in forward primer (EST-3) and *Eco*RV site in reverse primer (EST-2).

Forward primer: EST-3, 5′ GG CCG GAA TTC ATG TCA TTA ACA TCA G 3′, 27 mer.
(*Eco*RI)

Reverse primer: EST-2, 3′ CG TTT ACT CAT CTA TAG TTG CGC 5′, 23 mer.
(*Eco*RV)

Figure 1. Primers used for DNA amplification of cephalosporin C deacetylase gene. The restriction sites for *Eco*RI and *Eco*RV are shown in underline.

4.6.2 PCR reaction and condition of amplification

PCR reaction was performed based on the method of Saiki *et al.* (1988) using Mastercycler gradient (Hamburg, Germany).

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The reaction was done in 50 μl mixed solution containing 10 mM Tris-HCl pH 9.0, 2.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100, 200 μM of each dNTP, 10 μM

of each primers, a total concentration 20 ng of pSR1 template and 2.5 of *Pyrococcus furiosus* (*Pfu*) polymerase. The condition for PCR was shown in Table 12. After amplification, the PCR products was analysed in 0.7% agarose gel electrophoresis and the DNA fragment was purified from gel by using the Prep-A-Gene kit (see method 4.2 and 4.3).

Table 12. Condition for amplification of cephalosporin C deacetylase gene by PCR.

Step	Temperature (°C)	Time (min)	Number of cycles
1. Denaturation Anealing Extension	94 60 72	3 2 2	1
2. Denaturation Anealing Extension	94 60 72	1 1 2	33
3. Denaturation Anealing Extension	94 60 72	1 1 5	1

4.7 Southern Blot Analysis

4.7.1 Southern blot transfer

Southern blot transfer was performed by the standard method (Maniatis *et. al.*, 1989)

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The chromosomal DNA of was digested completcly with *Hin*dIII or *Eco*RI. DNA fragments were separated on 0.7% agarose gel by electroprhoresis. After taking photograph, gel was denatured by soaking twice in denaturing solution (0.5 M NaOH,

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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-HCl pH 7.4, 1.5 M NaCl) for 45 min at room temperature with constant shaking. The DNA from agarose gel was transferred into a nylon menbrane by using Blot Transfer System (Gibco, BRL). A piece of Whatman 3 MM paper was cut to form salt bridge (standing on a tray containing 20x SSC). The gel was placed on the damp 3 MM paper and air bubbles were carefully removed, then a wet nylon membrane cut exactly to the size of the gel was placed on top of the gel. All air bubbles trapped between the gel and the filter were removed with glass rod.

A stack of paper towel cut to the same size as the gel and the filter was placed on top of the membrane. An object was placed on top of the stack to weight the stack of the towel down. The objective of this arrangement was to set up a flow of liquid from the reservior through the gel and the membrane, so that the DNA fragment was transferred from the gel and deposited on the membrane by capillary force. DNA fragments were allowed to transfer for 12-24 hours. Next day, the positions of wells were marked on the membrane. The membrane was rinsed with distilled water to remove any impurities. The membrane could be subjected for hybridizaiton immediately or kept dry in desicator.

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4.7.2. Labeling DNA with digoxigenin

The DIG-DNA labeling kit was used to prepare the DNA probe. A 10 ng to 1 μg of 0.68 kb *Hin*dIII-*Eco*RV purified DNA fragment from pSR7 was labeled

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in a total volume of 20 µl of reaction mixture [10x Hexanucleotide mixture, 10x dNTP labeling mixture, 2 units/µl Klenow enzyme) at 37°C for overnight. After labeling, the reaction was stopped with 2 µl 0.2 M EDTA pH 8.0 and the DIG-labeled DNA probe was precipitated with 0.6 volume of isopropanol and 0.3 M sodium acetate, pH 5.2 at -70°C for at least 30 min. The DIG-labeled DNA was pelletted by centrifugation at 12,000 rpm at 4°C for 15 min and washed once with 40 µl 70% cold ethanol. Then, the precipitated probe was resuspended in 50 µl of TE buffer. The labeled DNA probe can be used immediately of stored at -20°C until used.

4.7.3. Hybridization with DIG-labeled DNA

Pre-hybridization :

The blotted nylon menbrane was pre-hybridized in the hybridizing tube containing 10 ml of pre-hybridization solution [5xSSC (0.75 M NaCl, 0.075 M sodium citrate), 50% v/v formamide, 0.1% w/v N-lauroylsarcosine, 0.02% w/v SDS and 5.0% w/v blocking reagent (Boehringer Mannheim)] and incubated in a Hybaid Mini hybridization oven (Hybaid Co., Ashford, England) at 42°C for at least 1 hour.

Hybridization step:

Pre-hybridization solution was removed and replaced with the same volume of new pre-hybridization solution then 5-25 ng/ml of denatured DNA probe was added. The DNA probe was denatured by boiling for 10 min and chilled on ice immediately for 3 min. Hybridization procedure was continued at 42°C for overnight.

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Washing step:

The hybridized membrane was washed twice with a washing solution I (2xSSC, 0.1% w/v SDS) for 5 min and followed by twice with a washing solution II (0.1xSSC, 0.1% SDS) at 68°C. The membrane was then detected directly or air-dried and stored at room temperature in sealed plastic bag for later detection.

Detection step:

The detection step was performed in plastic bag with small volume of solution. The membrane was rinsed with buffer I solution (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) prior to incubation in buffer II (Blocking solution: 0.5% w/v blocking reagent in buffer I) at room temperature for 30 min. Then the membrane was rewashed briefly in buffer I, and buffer II containing anti-DIG-AP Fab fragments was added. After incubation at room temperature for 30 min, the membrane was rewashed twice in buffer I for 15 min, followed by soaking in buffer III (100 mM Tris-HCl pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$) at room temperature for 2 min. Finally, colorimetric detection step was performed by addition of 45 µl of 4-Nitroblue Tetrazolium chloride (NBT) and 35 µl of 5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate) in buffer III. The membrane was kept in the dark until the desired bands appeared. The detection reaction was terminated by washing the membrane with sterile water to prevent over-development.

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4.8 DNA sequencing and analysis

Principle of DNA sequencing was based on the dideoxy-mediated chain termination method described by Sanger *et al.* (1977) using fluorescent-labeled terminator (Smith *et al.*, 1986). The DNA was sequenced by an automate ABI PRISM 377 DNA sequencer which was performed by Institute of Molecular Biology and Genetics, Mahidol University. The DNA sequence was analysed by DNASIS program and vector NTI.

5. Transformation of *Escherichia coli*

5.1 Preparation of competent cells

The competent *E. coli* cells were prepared by using the modified standard method (Hanahan, 1983). A single colony of fresh *E. coli* was grown in 3 ml LB medium for overnight at 37°C with vigorous shaking. A 1 % of this culture was inoculated into fresh LB broth and the culture was continued shaking at 37°C until the OD_{600} reached the level of 0.3-0.4. The culture was chilled on ice bath for 5 min then centrifuged at 3,000 rpm for 10 min at 4°C. Then the cell pellet was resuspended with 2:5 volume of filterd sterile Tfb I solution (30 mM potassium acetate, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, adjust pH to 5.8 with 0.2 M acetic acid) and kept on ice bath for 5 min. The cells were pelletted by centrifugation at 3,000 rpm for 5 min at 4°C and subsequently resuspended thoroughly in 1:50 volume of filtered sterile TfbII solution (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$ and 15% v/v glycerol, adjust pH to 6.5 with KOH). The suspended cells were kept on ice bath for 15 min.

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An aliquot of 200 µl was distributed into sterile ice cold microcentrifuge tube and stored at -80 °C until used.

5.2 Transformation technique

Transformation of *E. coli* was done using the standard method (Miniatis *et al.*, 1989). The frozen competent *E. coli* cells were thawed on ice bath for 15-20 min, then a 20-100 ng ligated DNA was added and swirled to mix. The tube was incubated on ice bath for another 30 min and heated at 37°C for 5 min. The tube was immediately cooled on ice bath for 1-2 min. Then 800 µl of LB broth was added to each tube and the transformed celled was further incubated at 37°C for another 1 hour. An aliquot of 100-200 µl of the transformed cells was spread on LA agar supplemented with 50 µg/ml ampicillin. The plates were incubated at 37°C for overnight.

6. Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE and NATIVE-PAGE used in this study were based on the procedure described by Laemmli (1970), and Ornstein and Davis (1964), respectively.

6.1 SDS-PAGE

6.1.1 Gel preparation

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The SDS-PAGE was carried out with 14% separating gel using Bio-Rad Miniprotein II apparatus. Preparations of separating gel and stacking gel are shown in Table 13. The separating gels were poured into the assembled glass plates

to a height of 3 cm from the top edge. Then the gel solution was overlayered with distilled water and the gel was allowed to polymerize at room temperature for 30 min. Water was poured off prior to pouring the stacking gel on top of the separating gel and the well comb was inserted between the 2 plates. The gels were allowed to polymerized at room temperature for 30 min.

Table 13. Preparations of separating gel and stacking gel (Laemmli, 1970).

Stock solution	14% Separating gel	4% Stacking gel
Distilled water	2.15 ml	3.05 ml
1.5 M Tris-HCl, pH 8.8	2.0 ml	-
0.5 M Tris-HCl, pH 6.8	-	1.25 ml
10% w/v SDS	80 µl	50 µl
Acrylamide/Bis (30 %T, 2.7 %C: 29.2 g/100 ml acrylamide, 0.8 g/100 ml N′ N′-bis-methylene-acrylamide, make to 100 ml with distilled water)	3.73 ml	0.65 ml
10% ammonium persulfate (freshly prepared)	40 µl	25 µl
TEMED	4 µl	5 µl
Total volume	8 ml	5 ml

6.1.2 Sample loading and electrophoresis

After polymerization, the comb was carefully removed and the wells were flushed several times with electrophoresis buffer (0.25 M Tris-HCl containing 0.192 M glycine, pH 8.3 and 0.1% SDS) to remove unpolymerized gel and gel debris. The sample were prepared by mixing of 40 µl of cell suspension (method 7) with 10 µ l of the 5x sample buffer (0.5 M Tris-HCl pH 6.8, 10% glycerol, 2% w/v SDS, 1% 2-

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b-mercaptoethanol, 0.025% bromophenol blue). After heating were heated in boiling water for 5 min, 20 µl of samples were loaded on the gel. The molecular weight markers prepared by the same procedure were loaded in paralleled. The two gels were electrophoresed in parallel at 150 volts for about 1 hour or until the dye reached the bottom of the gel. The molecular weight 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).

6.2 NATIVE-PAGE

6.2.1 Gel preparation

Native-PAGE was carried out with a 14 % separating gel and in 4 % stacking gel 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. After the separating gel (0.05 % w/v ammonium persulfate and 0.025 % v/v TEMED) were poured and saturated with DW, the gel was allowed to polymerized at room temperature for 30 min. A stacking gel (4 % acrylamide in 0.5 M Tris-HCl pH 6.8, 0.025 % v/v TEMED, 0.05 % w/v ammonium persulfate) was prepared 30 min before loading the protein. 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 buffer (0.06 M Tris-HCl pH 6.8, 10 % glycerol, 0.025 % bromophenol blue). The well were flushed with the electrophoresis buffer (0.025 M Tris-HCl containing 0.192 M glycine, pH8.3), before 20 µl of about 20-25 µg proteins per ml of samples with dye was loaded. The

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preparation of both gels was same as method 6.1.1 (Table 13) except that of 10 % w/v SDS was replaced by water.

6.2.2 Sample loading and electrophoresis

Sample buffer and electrophoresis buffer were prepared according to method 6.1.2 except that SDS and 2-b-mercaptoethanol were omitted. The samples were prepared by mixing and appropriate volume of protein sample (method 7) of about 20-25 μg with 5x sample buffer and the total volume was adjusted to 20 μl with 50 mM phosphate buffer. The samples were loaded directly in the gel without boiling.

6.3 Coomassie blue staining

For analysis of protein bands, the gel was stained with Coomassie Brilliant Blue R-250 staning solution [0.1% Coomassie Brilliant Blue R-250 in fixative solution (40% MeOH, 10% acetic acid)] for 1-2 h. Then the gel was destained by soaking in destaining solution (40% MeOH, 10% acetic acid) for 1-3 h. The stained gel was air dried in double layers of cellophane.

6.4 Esterase activity staining

For detection of esterase activity, the gel obtained by SDS-PAGE was renatured according to Rosenberg *et al.* (1975). After electrophoresis the gel was immediately soaked in 40 mM Tris-HCl, pH 6.0 containing 0.5% Triton X-100 at 4°C for overnight. Then, the gel was washed with 0.1 M phosphate buffer pH 7.5 and

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shaked at room temperature for 15 min. The detection of esterase activity was followed by the method of Higerd *et al.* (1973). The renatured gel obtained by SDS-PAGE or by NATIVE-PAGE was soaked in freshly prepared esterase staining solution (1 ml of 20 mg/ml α-naphthylacetate in acetone, 150 mg Fast Blue BB dye, 99 ml 0.1 M phosphate buffer pH 7.5) and shaked constantly at room temperature for 6 h. The stained gel was washed several times with distilled water and air dried in double layers of cellophane.

7. Preparation of Cell Free Extract

Cells grown in broth were harvested by centrifugation at 8,000 rpm for 10 min at 4°C, and washed twice with 50 mM phosphate buffer pH 7.0. Then, the pellets were resuspended in 1 ml of 50 mM potassium phosphate buffer, pH 7.0, and disrupted by using an ultrasonicator (VibraCell, Danburg, USA). For cell disruption, sonication obtained in short burst for 30 seconds using a microtip probe. The tube should be immersed in ice water and allowed the sample to cool for 30 seconds between each burst of sonication. After the sonication was performed for 3 min, cell debries was removed by centrifugation at 8,000 rpm for 10 minutes at 4°C, and the resulting cell free extract was used for esterase activity assay toward α-naphthylacetate or cephalosporin C substrates.

8. Total Protein Measurement

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Protein concentration was determined by a procedure described by Bradford, 1976. A 1 ml of Coomassie Brilliant blue Reagent (Bradford^{R}), containing 10 %

Coomassie Brilliant blue R, 95 % ethanol and 85 % phosphoric acid was added to 20 µl of the sample. After mixing, the mixture was allowed to stand at room temperature for 5 min. The absorbance at 595 nm was read against a blank preparation with the same buffer instead of the sample. The concentration of protein was determined by comparing with standard curve of bovine serum albumin.

9. High Performance Liquid Chromatography (HPLC)

HPLC was used to assay esterolytic activity toward cephalosporin C substrate. Culture broth and cell free extracts were used in the reaction mixture. A mixture of 500 µl of 100 mM ml phosphate buffer pH 7.0 containing 10 mg/ml cephalosporin C and 500 µl of culture broth or cell free extract in microcentrifuge tube was incubated at 30°C for 1 hour. At the end of incubation, the mixtures were centrifuged at 10,000 rpm for 10 min and the 20 µl of supernatants was loaded to HPLC. HPLC analysis was used under the following conditions:

HPLC: Hewlett Packard® (HP 1100 series, Waldbronn Germamy)

Column: Waters Spherisorb® S5 ODS2 (3.0mm x 250mm Cartridge), Waters Corporation (Milford MA, USA).

Mobile phase: 2 mM K_2HPO_4- KH_2PO_4 buffer, pH 7.0.

Flow rate: 0.5 ml/min.

Injection volumn: 20 µl.

UV detection wavelength : 265 nm.

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Deacetylcephalosporin C to be used as a reference was prepared by incubating 20 µl of a commercial acetylesterase enzyme in 480 µl of 100 mM phosphate buffer, pH 7.0 with 500 µl of 10 mg/ml cephalosporin C solution as described above.

10. Enzyme Activity Assay

The esterase activity assay was based on the method of Castro *et al.* (1992). The reactions were performed by adding 100 µl of cell free extract into 200 µl of substrate (500 µg/ml α-naphthylacetate dissoved in ethanol: water in ratio of 1:2) and 100 µl 50 mM phosphate buffer pH 7.0, which prewarmed at 30°C for 5 min. After incubation 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 dark pink color developed in the reaction mixture was measured spectrophotometrically at 515 nm against a blank which contained 50 mM phosphate buffer pH 7.0 instead of the enzyme in the reaction mixture.

The enzyme activity was measured as unit per mg protein. One unit of esterase activity was defined as the amount of enzyme required releasing 1 nmol α-naphthol per ml per min. Standard curve was set up using α-naphthol at concentrations between 5-60 nmole/ml instead of the enzyme in the reaction mixture.

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CHAPTER IV

RESULTS

1. Screening for Cephalosporin C Deacetylase (CAH) Producing Bacteria from Soil Samples

This study was focused on cephalosporin C deacetylase (CAH) that catalyzes deacetylation of the ester bond at the 3´position of cephalosporin C nucleus. This enzyme has been isolated from different strains of *Bacillus subtilis* (Abbot and Fukuda, 1975; Konecny and Voser, 1977; Takimoto *et al.*, 1994). We pay attention on isolation of the new CAH producer by screening for spore forming bacteria from soil samples.

Fifty soil samples were collected from various parts of Thailand. Altogether 550 bacterial isolates were isolated by heat shock technique and screened for the ability to produce esterase. The screening was performed by overlaying techniques using chemical and microbiological methods, as the primary and secondary screenings, respectively. In the chemical method, β-naphthylacetate (β-NA) was used as a substrate and Fast Blue BB dye was used for color development. As shown in Figure 12, the colonies of esterase producing bacteria turned into dark brown after being overlayered with the chemicals. Results of the primary screening are shown in Table 14, 30 out of 550 isolates were positive. The result indicated that 30 isolates

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could produce esterase enzymes. In order to screen for esterolytic activity of the positive isolates towards cephalosporin C substrate, the microbiological method was further tested. The principle of the test is that deacetyl derivative of cephalosporin C has no antibacterial activity against *Streptococcus aureus*. As shown in Figure 13, the colonies of positive control, *B. subtilis* WRRL-B558, showed satellite growth of *S. aureus* ATCC 25923 on the top layer medium containing 300 μg/ml cephalosporin C and 1% *S. aureus* ATCC 25923. Results of the screening are shown in Table 14, 25 out of 30 positive isolates from the primary screening were positive. The result indicated that 25 isolates seem to possess ability to convert cephalosporin C to inactive form which could not inhibit growth of *S. aureus* and it was presumably deacetylcephalosporin C. The possible CAH producing isolates that gave positive results with both screening methods were further confirmed for their esterolytic activity towards cephalosporin C substrate using HPLC as described in Materials and Methods.

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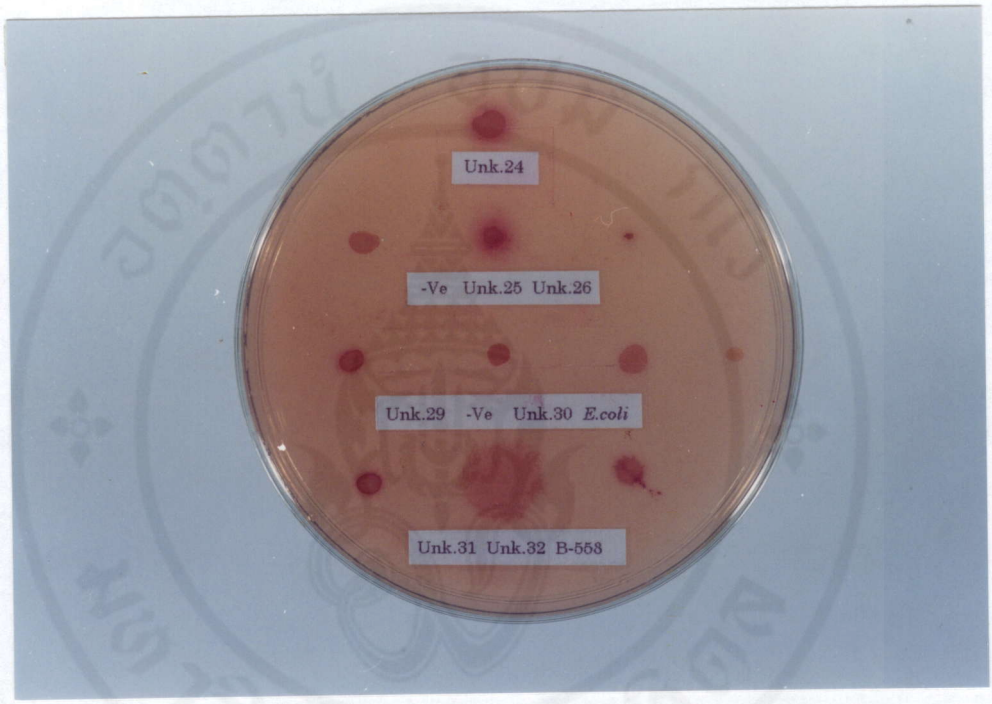

Figure 12. Screening of esterase producing bacteria by chemical mehtod.

The colonies were overlayed with 10 ml of 5 mg β-NA and 5 mg Fast Blue BB dye. The positive colonies showed dark brown colonies as compared to that of *B. subtilis* WRRL-B558.

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Figure 13. Screening of cephalosporin C deacetylase producing bacteria by microbiological method.

The colonies were overlayed with 300 μg/ml cephalosporin C and 1% (v/v) *S. aureus* ATCC 25923. The positive colonies showed satellite growth of *S. aureus*.

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Table 14. Screening for cephalosporin C deacetylase producing bacteria.

Sources (Province)	No. of samples	No. of isolates	No. of positive with		
			Chemical method	Microbiological method	HPLC
Angthong	3	40	6	6	1*
Bangkok	5	70	3	2	ND
Chaingmai	3	36	-	-	ND
Chonburi	7	65	3	3	ND
Karnchanaburi	3	30	2	1	ND
Nakorn-nayok	2	25	-	-	ND
Nakorn-prathom	6	64	6	5	ND
Phuket	1	30	-	-	ND
Prachinburi	15	120	8	6	ND
Rajchaburi	4	45	2	2	ND
Samuthprakarn	1	25	-	-	ND
Total	50	550	30	25	1

Note: ND = Non detectable when culture broth and cell free extract were tested.

* = Culture broth was negative but cell free extract was positive.

2. HPLC Analysis of Deacetylcephalosporin C Formed by Isolated Bacteria

The HPLC method was performed to detect deacetylcephalosporin C formed by 25 isolates being positive with microbiological screening method. The test was set up in 1 ml of reaction mixture containing 500 µl culture broth or cell free extract and 500 µl of 10 mg/ml cephalosporin C in 100 mM phosphate buffer pH 7.0, and

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incubated at 30°C for 1 hour. In this experiment, deacetylcephalosporin C obtained from the reaction mixture containing a commercial acetylesterase enzyme and cephalosporin C was used as a reference. A mobile phase of 2 mM KH_2PO_4-K_2HPO_4 buffer pH 7.0 at flow-rate 0.5 ml/min was used to separate cephalosporin C and deacetylcephalosporin C.

As shown in Figure 14, the reaction mixture with the commercial acetylesterase gave an increase height of the peak at retention time 3.07 min (Figure 14A) with the peak area 3.245×10^4 mA*S as compared to that of heated enzyme (Figure 14B) with the peak area 1.284×10^3 mA*S. This peak was presumed to be deacetylcephalosporin C. The typical retention time for deacetylcephalosporin C and cephalosporin C were 3.07 and 17.164-17.979 min, respectively. When the culture broth of 25 positive isolates were tested, it was found that none of them showed esterolytic activity as compared to that of the heated culture broth. However, when the cell free extract were tested, it was found that 1 out of 5 isolates, which showed large satellite growth or intense dark brown colonies, gave highest peak of deacetylacephalosporin C. As shown in Figure 15A, the cell free extract of isolate No. 24 showed an increase height of the peak at retention time 3.113 min as compared to that of the reference (Figure 14A) and heated-cell free extract (Figure 15B). The peak areas of the cell free extract and the heated-cell free extract were 1.572×10^3 mA*S and 0.869×10^3 mA*S, respectively. These data suggested that the isolate no. 24 could hydrolyse cephalosporin C to deacetylcephalosporin C. The isolate was then chosen as the CAH producer, which will be identified and used as a source of gene cloning.

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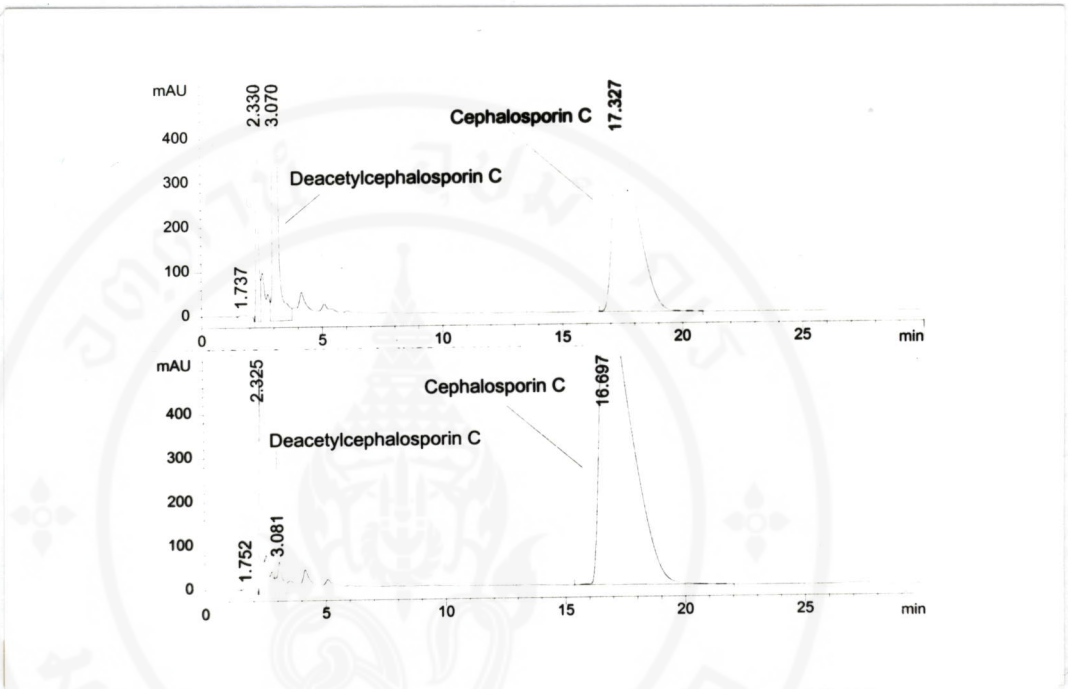

Figure 14. HPLC chromatogram of deacetylcephalosporin C obtained from the reaction mixture with a commercial acetylesterase.

Ten mg/ml cephalosporin C substrate in 100 mM phosphate buffer pH 7.0 was incubated with commercial acetylesterase (A), or heated-commercial acetylesterase (B) using 2 mM KH_2PO_4-K_2HPO_4 buffer pH 7.0 as a mobile phase. HPLC was performed under the condition as described in Materials and Methods.

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A

B

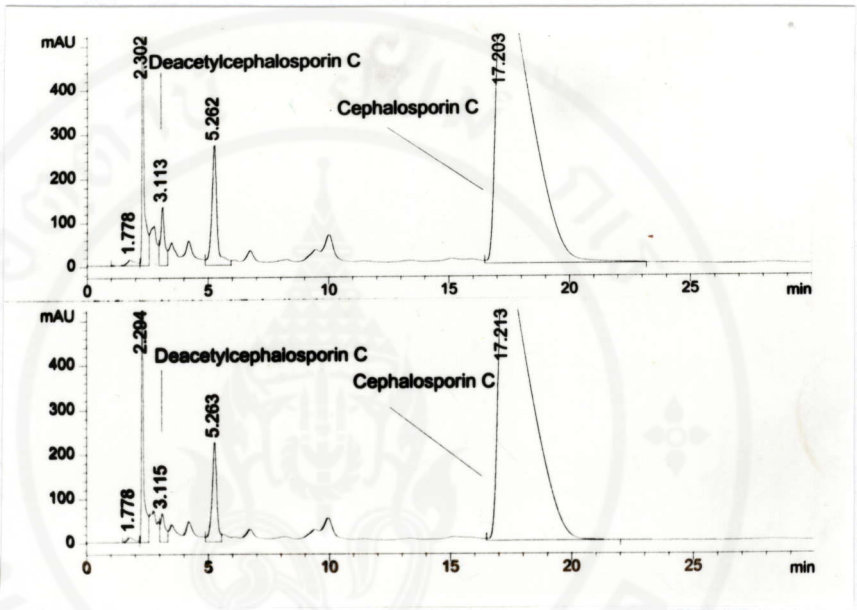

Figure 15. HPLC chromatogram of deacetylcephalosporin C obtained from the reaction mixture with cell free extract of isolated No. 24.

Ten mg/ml cephalosporin C substrate in 100 mM phosphate buffer pH 7.0 was incubated with cell free extract (A), or heated-cell free extract (B) of isolate No. 24 using 2 mM KH_2PO_4-K_2HPO_4 buffer pH 7.0 as a mobile phase. HPLC was performed under the condition as described in Materials and Methods.

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3. Identification and Characteristic of Cephalosporin C Deacetylase Producer

The isolate No. 24 was identified according to Bergey's Manual of Systematic Bacteriology Volume 2 (1986).

For identification of the bacterial isolate BT-24, microscopic examinations and biochemical tests were undertaken and the results were shown in Table 15. The isolate was gram positive rod, aerobic endospore forming of 2.5x10 μm in size. The rod tends to occur in chains. It growed well at both 30°C and 37°C. Colonies have a dull and frosted grass appearance and an undulated margin. The endospores have been observed easily under microscope after growing on LB agar for 24 hours. The API kits were used to test characteristics of this bacterium. Fermentative production of acid from various carbohydrates was tested. It was found that the bacteria could fermented ribose, glucose, fructose, N-acetylglucosamine, amygdalin, arbutin, esculin, salitin, cellobiose, maltose, sucrose, trehalose, starch, glycogen, and gluconate. As shown in Table 15. This bacterium was also shown activity for the production of arginine dihydrolase and acetoin. It can hydrolysed gelatin and gave positive result with a catalase test. The negative results were found on test for β-galactosidase production, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease production, tryptophane deaminase, indole production.

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These characteristics placed it in the genus *Bacillus* and species of *cereus*. This isolate was therefore identified as *Bacillus cereus* BT-24.

Table 15. Morphological and biochemical characteristics of *Bacillus cereus* BT-24.

Characteristics	Results
Morphologies:	
Gram staining reaction	gram positive
Shape	rod
Spore formation	yes
Biochemical testes:	
Acetoin production	positive
Arginine dihydrolase	positive
Catalase test	positive
Citrate utilization	negative
β-galactosidase production (o-nitro-phenyl-β-D-galactopyranoside)	negative
H_2S production	negative
Hydrolysis of geltin	positive
Indole production	negative
Lysine decarboxylase	negative
Ornithine decarboxylase	negative
Tryptophane deaminase	negative
Urease production	negative
Fermentative production of acid from:	
N-acetyl-glucosamine	positive
Adonital	negative
Amygdalin	positive
D-arabitol	negative
L-arabitol	negative
D-arabinose	negative
L-arabinose	negative
arbutin	positive
cellobiose	positive
dulcitol	negative
erythritol	negative
esculin	positive
fructose	positive
D-fucose	negative
L-fucose	negative

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Table 15. Morphological and biochemical characteristics of *Bacillus cereus* BT-24 (Continued).

Characteristics	Results
Fermentative production of acid from:	
galactose	negative
gentiobiose	negative
gluconate	positive
glycerol	negative
glycogen	positive
inositol	negative
inulin	negative
2-keto-gluconate	negative
5-keto-gluconate	negative
lactose	negative
D-lyxose	negative
maltose	positive
mannitol	negative
mannose	negative
melezitose	negative
melibiose	negative
α-methyl-D-glucoside	negative
α-methyl-D-mannoside	negative
β-methyl-D-xyloside	negative
raffinose	negative
rhamnose	negative
ribose	positive
salitin	positive
sorbitol	negative
sorbose	negative
sucrose	positive
starch	positive
D-tagatose	negative
trehalose	positive
D-turanose	negative
xylitol	negative
D-xylose	negative
L-xylose	negative

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4. Cloning of CAH Gene of *B. cereus* BT-24

4.1 Cloning strategy

A short gun cloning was used to clone the CAH gene from chromosomal DNA of *B. cereus* BT-24. Strategies for cloning CAH gene from *B. cereus* BT-24 was shown in Figure 16. A 30 μg of purified genomic DNA was partially digested with 0.02-0.08 units of *Sau*3AI restriction enzyme at 37°C for 15 min to obtain fragments range between 2-10 kb. The reaction was immediately extracted twice with phenol-chloroform. The purified DNA was loaded onto 0.7% agarose gel electrophoresis to examine the digestion pattern of DNA. The digested *B. cereus* DNA was then ligated to *Bam*HI dephosphorylated pBluescript II SK+ vector (pBS II SK+). The ligated products were transformed into competent *E. coli* DH-5α and transformants were selected on ampicillin-X-gal plate. The white colonies grown on ampicillin plates were selected and screened for their esterase producing ability by chemical method which overlayered with β-naphthylacetate (β-NA) and Fast Blue BB dye. Of the 5,000 transformants tested, one recombinant clone which gave positive result with the chemical method was selected. As shown in Figure 17, the recombinant clone showed dark brown colony as compared to the *E. coli* DH-5α harboring pBS II (SK+) vector. The recombinant clone was further tested with microbiological method for its ability to produce CAH by overlaying with 300 μg/ml of cephalosporin C and 1% (v/v) of an overnight culture of *S. aureus* ATCC 25923. The CAH producing clone would allow satellite growth of *S. aureus* on the top-layer medium. Since the host *E. coli* itself showed some degree satellite growth of *S. aureus*, the CAH producing clone should then allow broader satellite growth than the host. As shown in Figure

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18, the recombinant clone allowed *S. aureus* to grow around their colonies much better than that done by the host *E. coli* DH-5α harboring pBS II (SK+) vector. The results suggested that the recombinant clone harbored a plasmid, which contained a gene encoding esterase enzyme, and the enzyme could hydrolyze cephalosporin C. The recombinant clone was named pSR1.

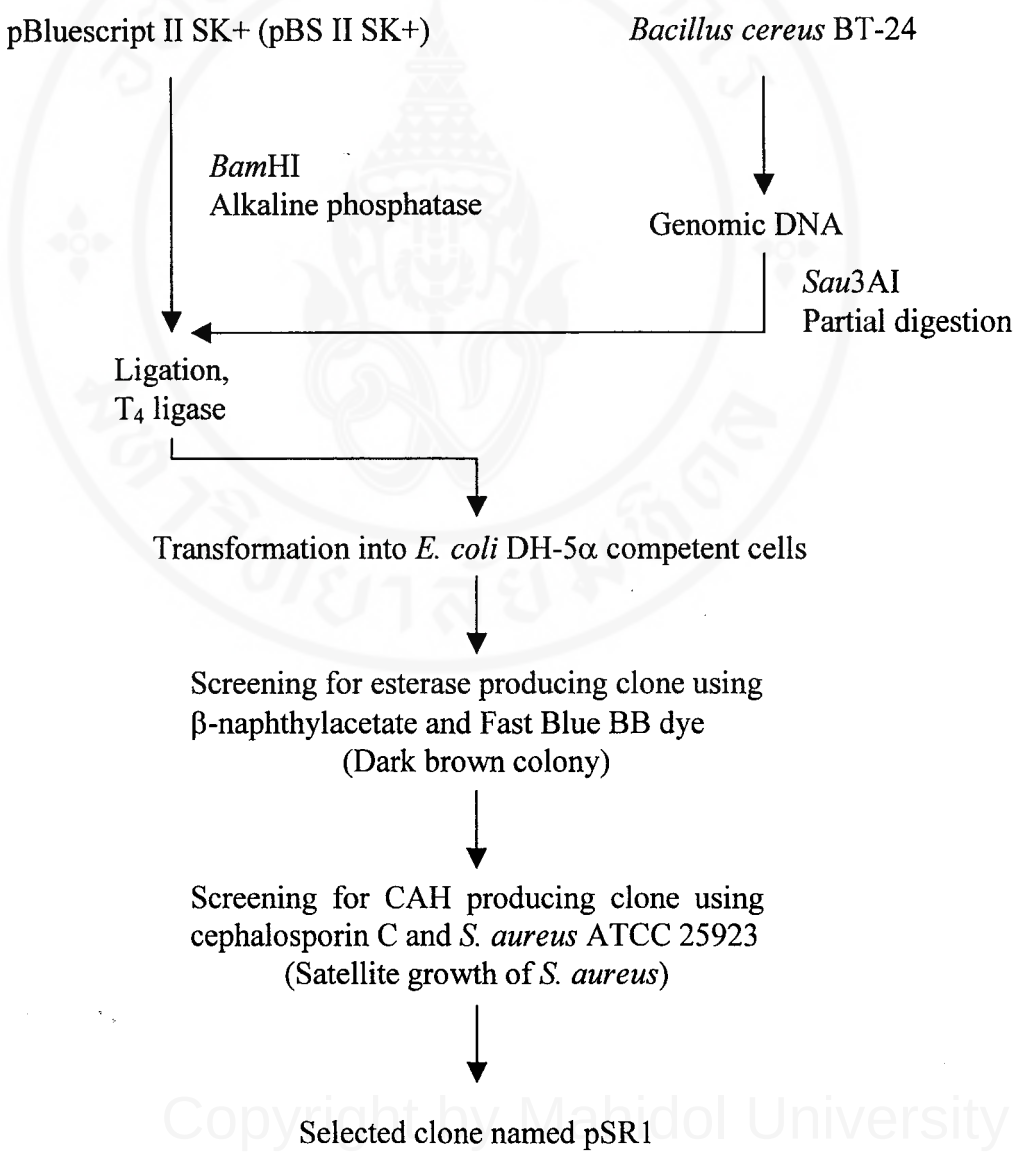

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Figure 16. Strategies for cloning of CAH gene from *B. cereus* BT-24 using pBS II (SK+) as a cloning vector.

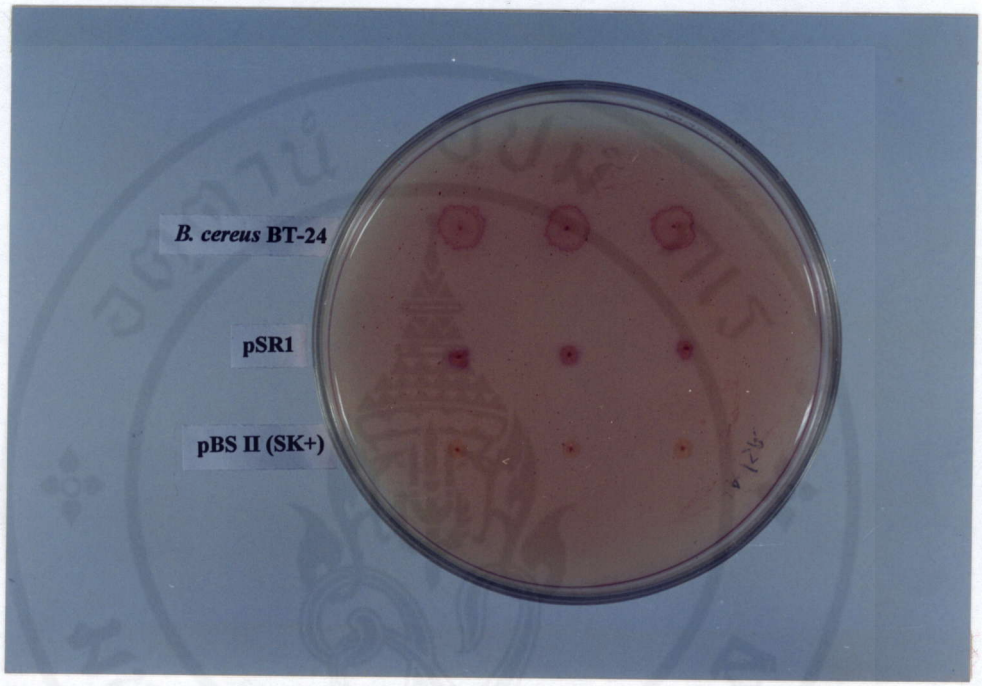

Figure 17. Detection of esterase producing clone by chemical method.

Colonies were overlayered with the β-NA and Fast Blue BB dye. The positive clone was detected as having a dark-brown colony as compared to the light–brown colony of the negative control.

B. cereus BT-24, positive control.

pSR1, recombinant clone [*E. coli* DH-5α + pSR1].

pBS II (SK+), negative control [*E. coli* DH-5α + pBS II (SK+)].

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Figure 18. Detection of cephalosporin C deacetylase producing clone by microbiological method. Colonies were overlayered with 300 μg/ml cephalosporin C and 1% (v/v) *S. aureus* ATCC 25923. The positive clone was detected as having broader satellite growth of *S. arueus* as compared to the narrow satellite growth of the negative control.

B. cereus BT-24, positive control.

pSR1, recombinant clone [*E. coli* DH-5α + pSR1].

pBS II (SK+), negative control [*E. coli* DH-5α + pBS II (SK+)].

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4.2 Esterase activity of recombinant clone pSR1

The esterase activity was assayed to detect enzyme produced by the recombinant clone pSR1. As shown in Table 16, the cell free extract of the recombinant clone pSR1 was 35.05 U/mg protein whereas that of the host was 26.14 U/mg protein. This result demonstrated that esterase specific activity of the recombinant clone pSR1 produced esterase 1.34 times higher than of host. The esterase activity of the *B. cereus* BT-24 was found to be 114.10 U/mg protein.

Table 16. Esterase activity of the cell free extract of the recombinant clone pSR1 compared with *E. coli* DH-5α host and *B. cereus* BT-24 using α-NA as a substrate.

Organisms	Esterase specific activity* (U/mg protein)
E. coli DH-5α	26.14 ± 2.38
E. coli DH-5α + pSR1	35.05 ± 1.99
B. cereus BT-24	114.10 ± 11.59

*1 unit of esterase activity was expressed as 1 nmole of α-naphthol released per min per ml at 37°C.

The values are the means of three experiments with 2 replicates each.

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4.3 HPLC analysis of deacetylcephalosporin C formed by recombinant clone pSR1

To confirm that recombinant clone pSR1 produced an esterase that could hydrolyse cephalosporin C to form deacetylcephalosporin C, the HPLC method was used to detect the product. The cell free extract of recombinant clone pSR1 and negative control [*E. coli* DH-5α + pBS II (SK+)] were used in the reaction mixture as described in result 2. As shown in Figure 19A, the cell free extract of recombinant clone pSR1 gave peak at retention time 3.113 min in HPLC chromatogram. Whereas the heated cell free extract of recombinant clone (Figure 19B) and the negative control (Figure 19C, 19D) devoided this peak.

It was suggested that the recombinant clone pSR1 could produce CAH and the enzyme was encoded by the CAH gene in the recombinant plasmid. The plasmid harboring CAH gene was named pSR1.

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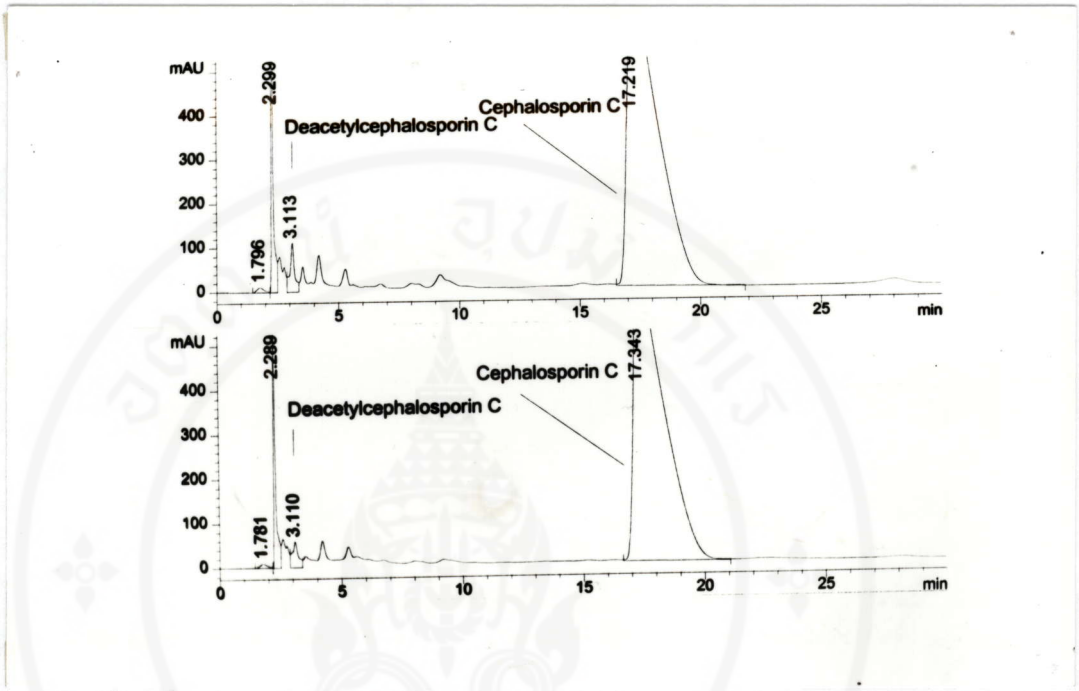

Figure 19. HPLC chromatogram of deacetylcephalosporin C obtained from the reaction mixture with cell free extract of *E. coli* harboring pSR1 and pBS II (SK+).

Ten mg/ml cephalosporin C substrate in 100 mM phosphate buffer pH 7.0 was incubated with cell free extract (A) or heated cell free extract (B) of the recombinant clone pSR1 (*E. coli* DH-5α + pSR1), cell free extract (C) or heated cell free extract (D) of *E. coli* DH-5α + pBS II (SK+), using 2 mM KH_2PO_4-K_2HPO_4 buffer pH 7.0 as mobile phase.

HPLC was performed under the condition as described in Materials and Methods.

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C

D

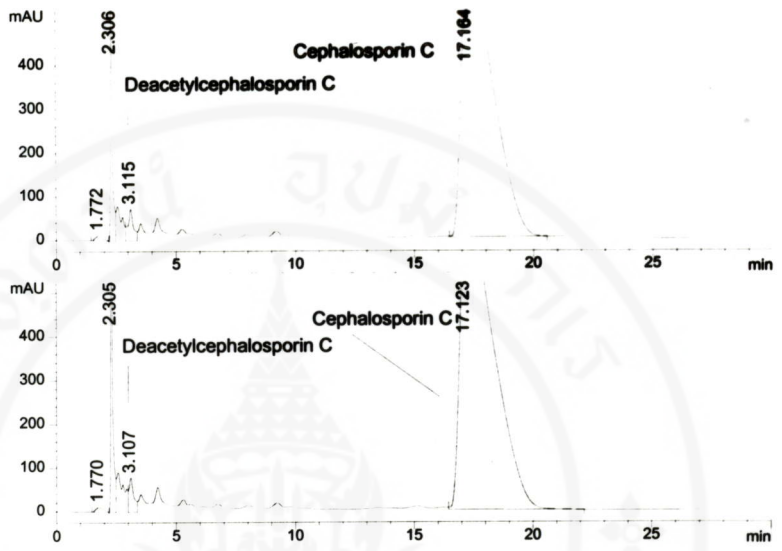

Figure 19. (Continued)

HPLC chromatogram of deacetylcephalosporin C obtained from the reaction mixture with cell free extract of *E. coli* harboring pSR1 and pBS II (SK+).

Ten mg/ml cephalosporin C substrate in 100 mM phosphate buffer pH 7.0 was incubated with cell free extract (A) or heated cell free extract (B) of the recombinant clone pSR1 (*E. coli* DH-5α + pSR1), cell free extract (C) or heated cell free extract (D) of *E. coli* DH-5α + pBS II (SK+), using 2 mM KH_2PO_4-K_2HPO_4 buffer pH 7.0 as mobile phase.

HPLC was performed under the condition as described in Materials and Methods.

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4.4 Restriction mapping of pSR1 plasmid

To analyse restriction mapping and the length of the gene fragment conferring the CAH activity, plasmid pSR1 was subjected to digestion with various restriction enzymes. The *E. coli* clone harboring pSR1 was grown in LB broth with ampicillin and plasmid was extracted by rapid boiling method. The plasmid was digested with various enzymes and the digested DNA were then loaded onto 0.7% agarose gel. The selection of enzymes was based on the restriction sites available in the polylinker of pBS II (SK+). The results of agarose gel electrophoresis were shown in Figure 20 and the sizes of DNA bands obtained from each enzyme digestion were summarized in Table 17.

It was shown that the DNA patterns were 2.2 kb and 3.5 kb from an *Eco*RI digestion; 0.85 kb and 4.9 kb from an *Eco*RV digestion; and 1.1 kb, 1.6 kb, and 3.0 kb from *Xba*I digestion. From these data, the restriction endonuclease map of cloned gene fragment in pSR1 was drawn, as shown in Figure 21. It was found that the length of cloned fragment was 2.74 kb.

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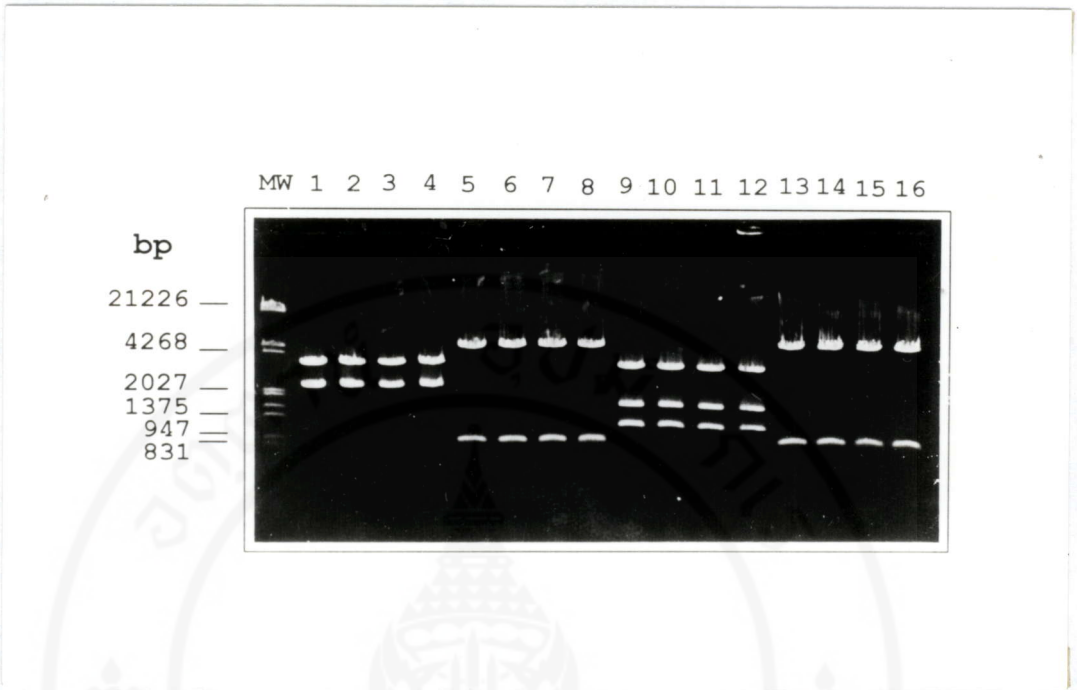

Figure 20. Restriction endonuclease analysis of pSR1 plasmid.

The digested plasmid was analysed on 0.7% agarose gel electrophoresis.

Lane MW, λ DNA cut with *Hin*dIII-*Eco*RI.

Lane 1-4, pSR1 cut with *Eco*RI. Lane 5-8, pSR1 cut with *Eco*RV.

Lane 9-12, pSR1 cut with *Hin*dIII. Lane 13-16, pSR1 cut with *Xba*I.

Table 17. The size of DNA bands of pSR1 plasmid cut with different restriction enzymes.

Restriction enzymes	Number of bands	Sizes of DNA band (Kb)
*Eco*RI	2	2.2, 3.5
*Eco*RV	2	0.85, 4.9
*Hin*dIII	3	1.1, 1.6, 3.0
*Xba*I	2	0.85, 4.9

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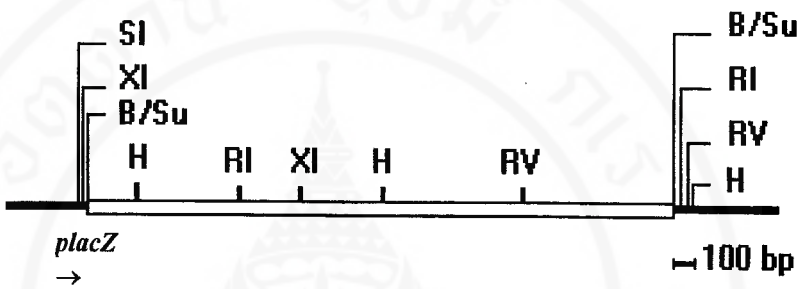

Figure 21. Restriction map of cloned gene fragment in pSR1 plasmid.

Open block represents cloned CAH gene from *B. cereus* BT-24. Thin line represents vector pBS II (SK+). The arrow indicates the direction of transcription of the *lacZ* promoter of the plasmid pBS II (SK+).

Abbriviation of restriction enzymes:

B, *Bam*HI; H, *Hin*dIII; RI, *Eco*RI;

RV, *Eco*RV; S, *Sac*I; Su, *Sau*3AI;

XI, *Xba*I.

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5. Localization of the Gene Region Conferring Cephalosporin C Deacetylase

5.1 Subcloning

To localize the region on the 2.74 kb cloned gene fragment that conferred CAH, various subclones of pSR1 plasmid were constructed. According to the restriction map of the pSR1 plasmid (Figure 21) and the size of DNA fragments obtained from digestion with various enzyme (Table 17), those DNA fragments were subjected to subcloning. The pSR1 plasmid was digested with *Eco*RI to obtain 2.2 and 3.5 kb DNA fragments. After separation in agarose gel electrophosesis, the DNA fragments were recovered from the gel. The 2.2 kb fragment was ligated to a vector pBS II (SK+) at the corresponding site. The ligated plasmid was transformed into *E. coli* DH-5α and the recombinant clones were selected by blue-white colony selection on X-gal-ampicillin plate. The resultant plasmid was designated as pSR2. Whereas the 3.5 kb fragment, which contained 2.96 kb of the vector pBS (II SK+) and 0.54 kb of the remaining clone fragment, was religated. The plasmid was designated as pSR8.

Similarly, digestion of pSR1 plasmid with *Eco*RV gave 2 fragments of 0.9 and 4.9 kb. The 0.9 kb fragment was cloned into *Eco*RV-digested pBS II (SK+) and the recombinant plasmid was named pSR6. While the 4.9 kb fragment, harboring 2.96 kb of the vector pBS II (SK+) and 1.84 kb of the remaining clone fragment, was religated. The new plasmid was name pSR7.

Digestion of pSR1 plasmid with *Hin*dIII gave 3 bands of 1.1, 1.6, and 3.0 kb, the 1.1 and 1.6 kb bands were ligated to *Hin*dIII-digested pBS II (SK+). The resultant plasmids were designated as pSR3 and pSR4, respectively.

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Digestion pSR1 plasmid with *Xba*I resulted in 2 fragments of 0.85 and 4.90 kb. The 0.85 kb was cloned into *Xba*I-digested pBS II (SK+) and the new plasmid was named pSR5. Religation of the 4.90 kb fragment, containing 2.96 kb vector and 1.94 kb of the remaining gene fragment, resulted in a new plasmid which was designated as pSR9.

Examples of construction diagrams were shown in Figure 22 and Figure 24, which illustrated the construction of plasmid pSR2 and pSR7, respectively. To confirm that the cloned DNA fragment in the pSR2 and pSR7 plasmids were derived from the pSR1 plasmid, both plasmids were subjected to restriction endonuclease analysis. The restriction analysis of pSR2 plasmid was shown in Figure 23. Digestion with *Eco*RI generated about 2.2 kb which corresponded to the size of DNA fragment from pSR1 plasmid, and 2.96 kb which corresponded to the size of pBS II (SK+) vector.

Single digestion with *Eco*RI, *Eco*RV and *Hin*dIII and double digestion with *Eco*RI-*Hin*dIII gave the expected bands as summarized in Table 18. The result suggested that the cloned fragment in pSR2 was obtained from the pSR1 plasmid. Figure 25 illustrated the restriction analysis of the pSR7 plasmid. Digestion with *Eco*RV generated only one band of about 4.9 kb which was the same size as the upper band of pSR1 plasmid digested with the same enzyme. Single digestion with *Eco*RV, *Hin*dIII and *Xba*I and double digestion with *Eco*RI-*Eco*RV generated expected bands as shown in Table 19. The result confirmed that the pSR7 plasmid was derived from pSR1 plasmid.

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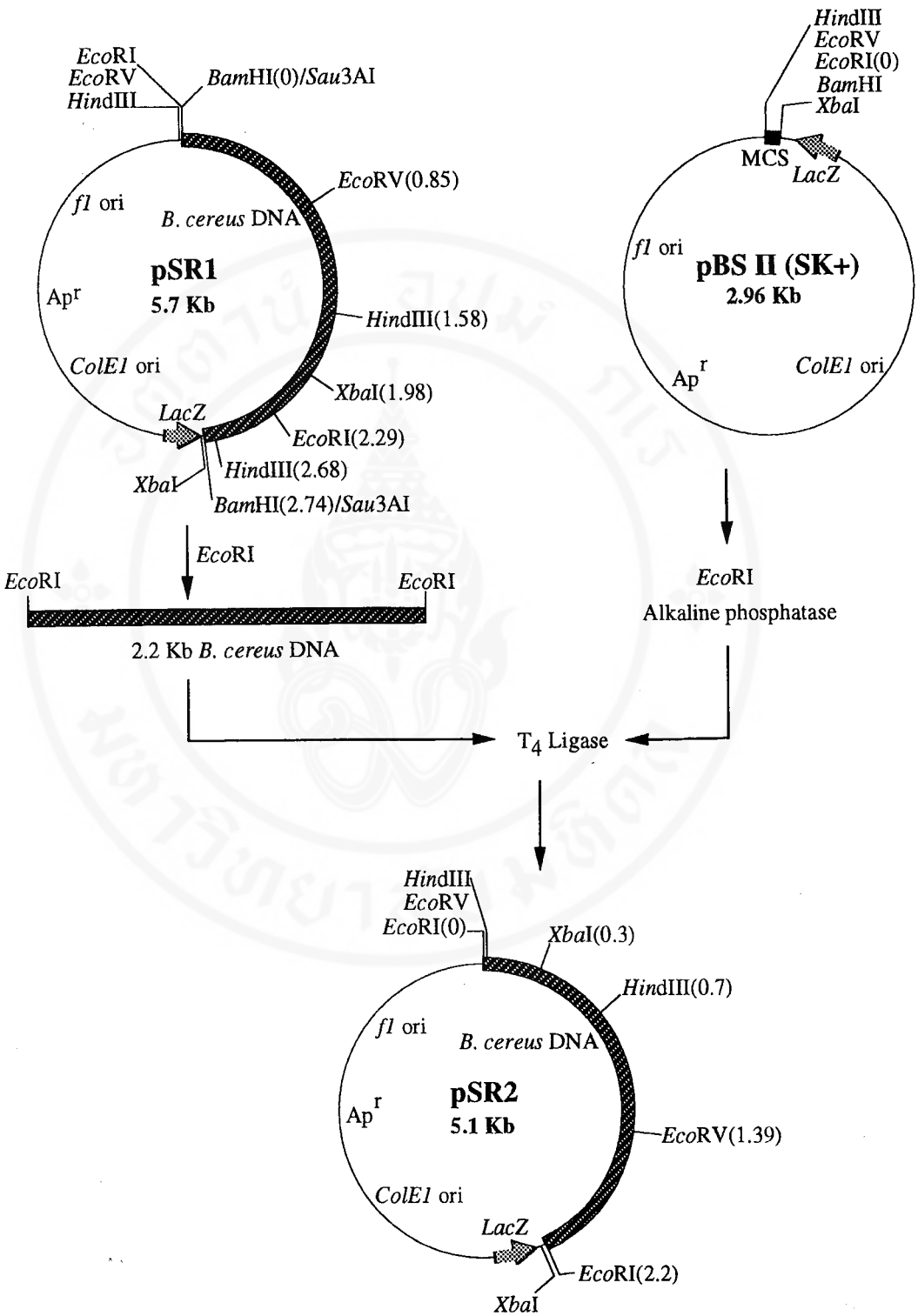

Figure 22. The construction diagram of pSR2 plasmid.

The 2.2 kb *Eco*RI-*Eco*RI fragment of pSR1 plasmid was cloned into *Eco*RI site of pBS II (SK+).

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Table 18. The expected sizes of DNA bands of pSR1 and pSR2 plasmids cut with various enzymes.

Restriction enzymes	Number of bands		Expected sizes (Kb)	
	pSR1	pSR2	pSR1	pSR2
*Eco*RI	2	2	2.2, 3.5	2.2, 2.96
*Eco*RV	-	2	-	1.5, 3.6
*Hin*dIII	-	2	-	0.7, 4.55
*Eco*RI- *Hin*dIII	-	3	-	0.7, 1.4, 3.76

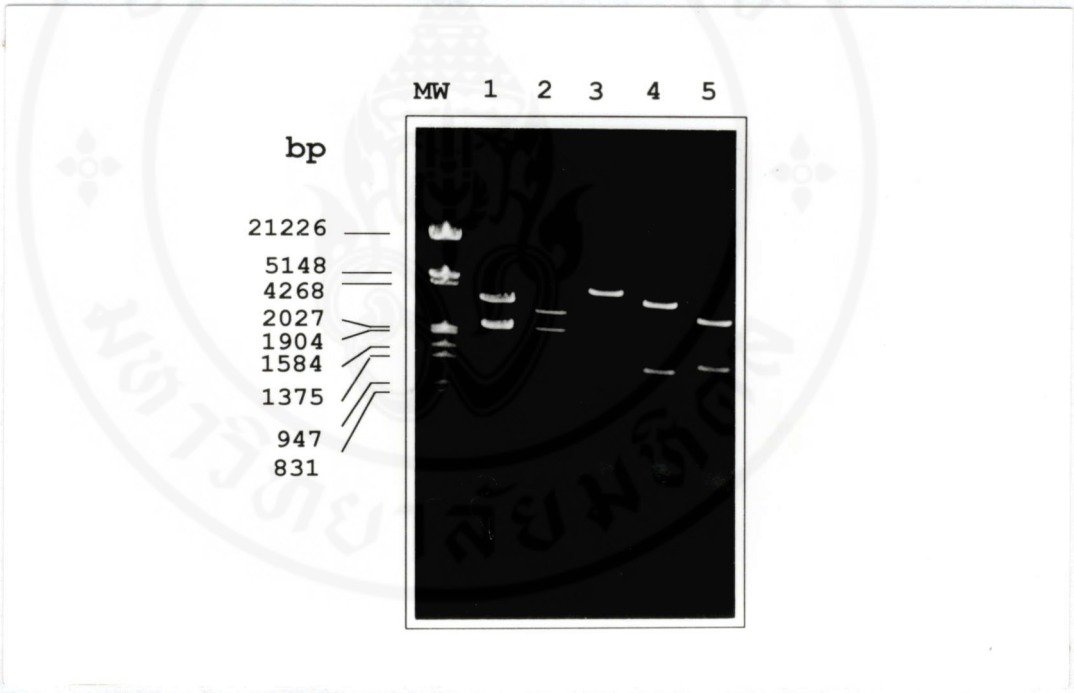

Figure 23. Restriction endonuclease analysis of pSR1 and pSR2 plasmids.

The digested plasmids were analyzed on 0.7% agarose gel electrophoresis.

Lane MW, λ DNA cut with *Hin*dIII-*Eco*RI.

Lane 1, pSR1 cut with *Eco*RI. Lane 2, pSR2 cut with *Eco*RI.

Lane 3, pSR2 cut with *Hin*dIII. Lane 4, pSR2 cut with *Eco*RV.

Lane 5, pSR2 cut with *Eco*RI- *Hin*dIII.

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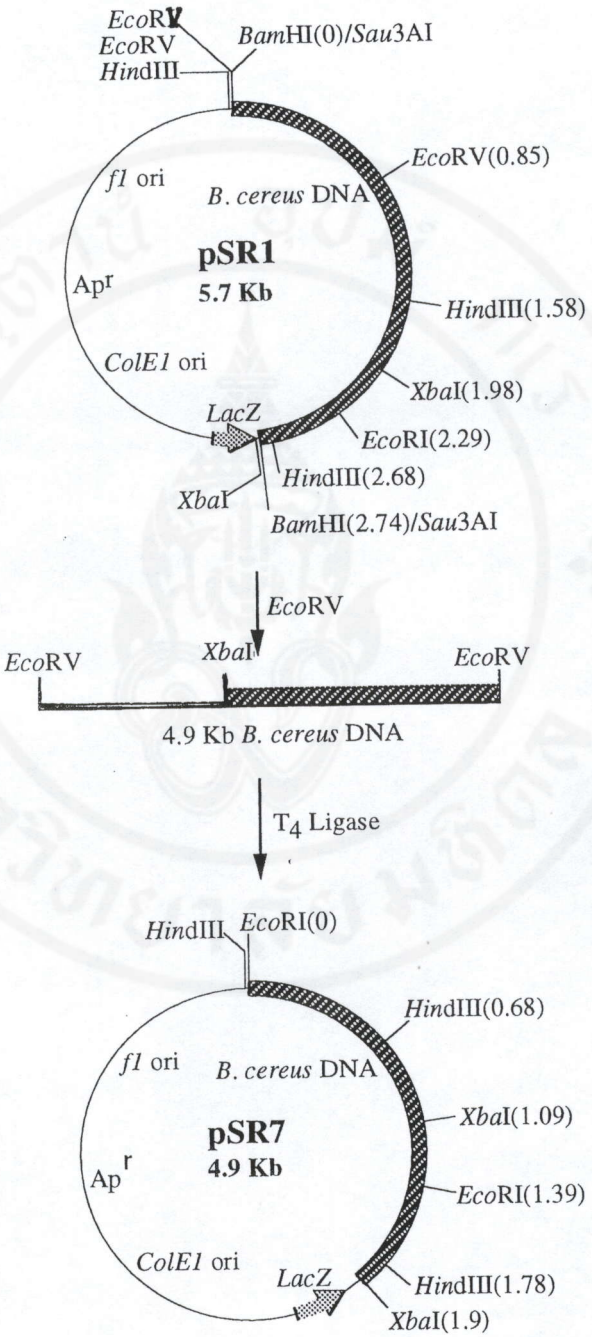

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Figure 24. The construction diagram of pSR7 plasmid.

The 4.9 kb fragment of pSR1 was religated.

Table 19. The expected sizes of DNA bands of pSR1 and pSR7 plasmids cut with various enzymes.

Restriction enzymes	Number of bands		Expected sizes (Kb)	
	PSR1	pSR7	pSR1	pSR7
*Eco*RV	2	1	0.85, 4.9	4.9
*Hin*dIII	-	3	-	0.7, 1.1, 3.1
*Xba*I	-	2	-	0.9, 4.0
*Eco*RI- *Eco*RV	-	2	-	1.4, 3.5

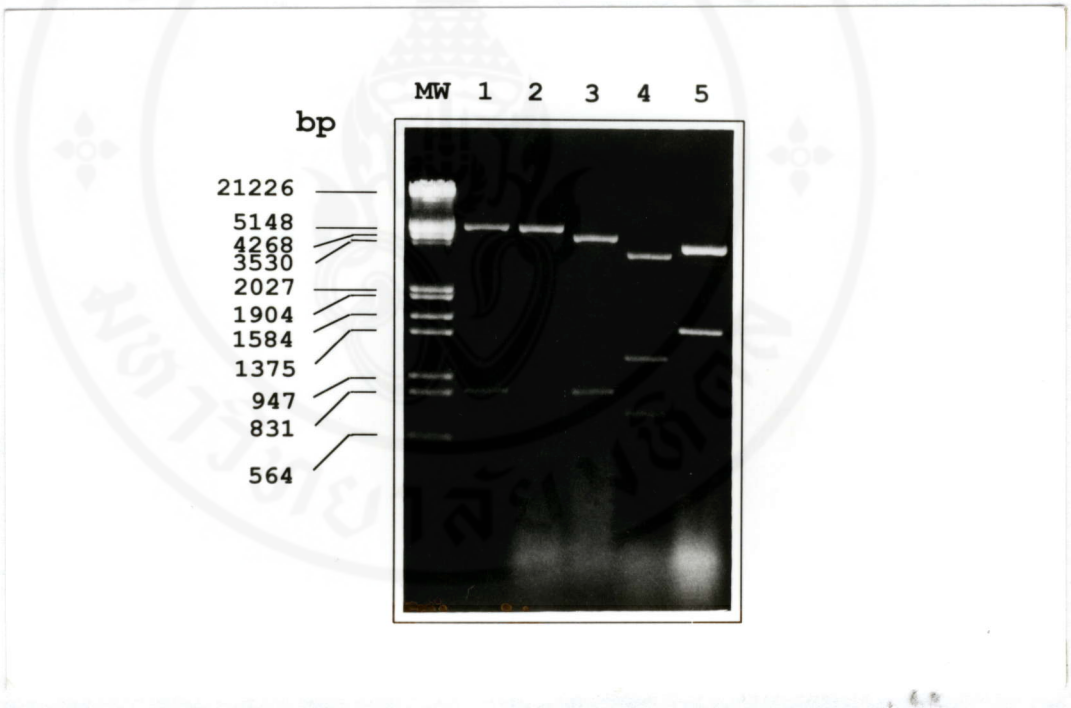

Figure 25. Restriction endonuclease analysis of pSR1 and pSR7 plasmids.

The digested plasmids were analyzed on 0.7% agarose gel electrophoresis.

Lane MW, λ DNA cut with *Hin*dIII-*Eco*RI.

Lane 1, pSR1 cut with *Eco*RV. Lane 2, pSR7 cut with *Eco*RV.

Lane 3, pSR7 cut with *Xba*I. Lane 4, pSR7 cut with *Hin*dIII.

Lane 5, pSR7 cut with *Eco*RI- *Eco*RV.

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5.2 Esterase activity of subclones

All subclones were tested for their ability to express the esterase activity by overlaying with β-NA and Fast Blue BB dye. Results of esterase activity were shown in Figure 26. The pSR2, pSR7, and pSR9 plasmids had esterase activity whereas pSR3, pSR4, pSR5, pSR6, and pSR8 plasmids devoided the activity. Altogether, the limit of the left and right side of fragment that still gave esterase activity was at *Xba*I and *Eco*RV sites, respectively. It was suggested that the CAH gene was located on *Xba*I-*Eco*RV fragment, which was about 1.4 kb.

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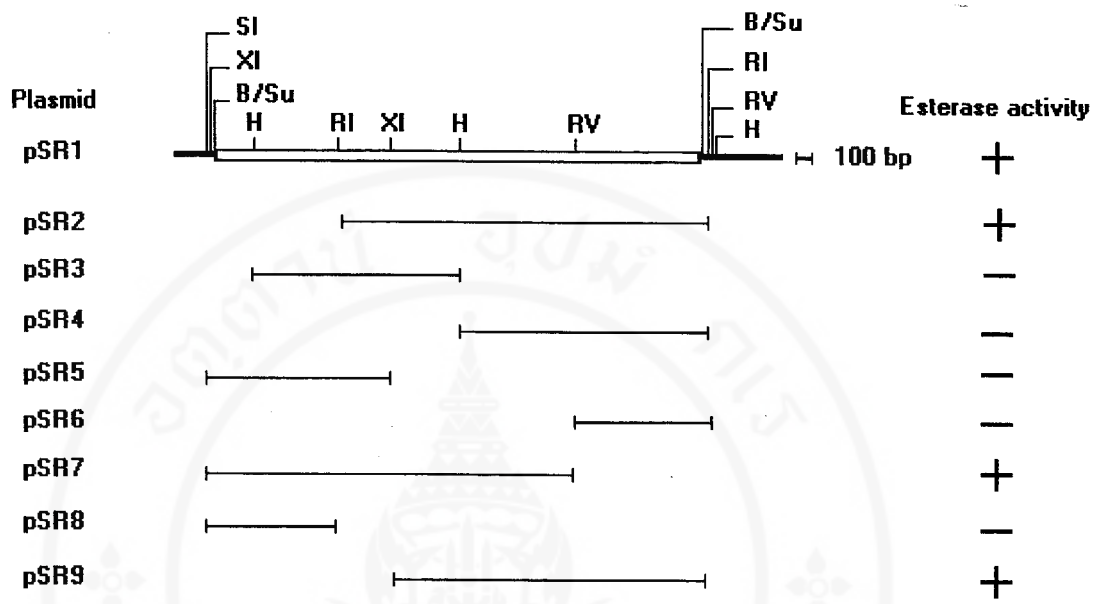

Figure 26. Localization of cephalosporin C deacetylase gene.

The bars show subcloning fragments.

All subclones were tested for esterase activity by the chemical method.

Symbol: +, colonies were dark brown (able to produce esterase);

-, colonies were not dark brown (unable to produce esterase) with β-NA and Fast Blue BB dye.

Abbreviation of restriction enzymes:

B, *Bam*HI; H, *Hin*dIII; RI, *Eco*RI;

RV, *Eco*RV; SI, *Sac*I; Su, *Sau*3AI;

X, *Xba*I.

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6. Southern Blot Analysis of *B. cereus* BT-24 Chromosomal DNA

In order to confirm that the CAH gene in pSR1 plasmid was derived from the genomic DNA of *B. cereus* BT-24, southern blot analysis was performed. The chromosomal DNA of *B. cereus* BT-24 was subjected to restriction enzyme digestion with *Eco*RI or *Hin*dIII. The digested DNA were separated on an 0.7 % agarose gel electrophoresis and blotted onto a nylon membrane. The membrane containing genomic DNA was then hybridized with the DIG labeled 0.68 kb *Hin*dIII-*Eco*RV fragment derived from pSR1 plasmid. The result of DNA hybridization was shown in Figure 27. The probe hybridized to DNA fragment of 3.5 kb in the *Eco*RI digestion (Figure 27, lane 4-5) and about 1.5 kb in the *Hin*dIII digestion (Figure 27, lane 6-7). Therefore, the CAH gene in pSR1 plasmid was derived from the genomic DNA of *B. cereus* BT-24. In addition, the analysis of hybridization pattern of the two restriction enzymes showed that the gene was a single copy in the *B. cereus* BT-24 genome. However, the probe also hybridized with pBS II (SK+) vector as shown in Figure 27 lane 1, which was vector only cut with *Eco*RI, and lane 2 and 3 which was pSR1 plasmid cut with *Eco*RI. The lower bands of 2.2 kb lane 2 and 3 were the cloned fragment whereas the upper bands of 3.5 kb was the pBS II (SK+) vector (2.96 kb) and the fragment (0.54 kb), which was out of the probe region (Figure 20 and Table 17). The cross hybridization might be resulted from nucleotides similarity between the pBS II (SK+) vector and the probe.

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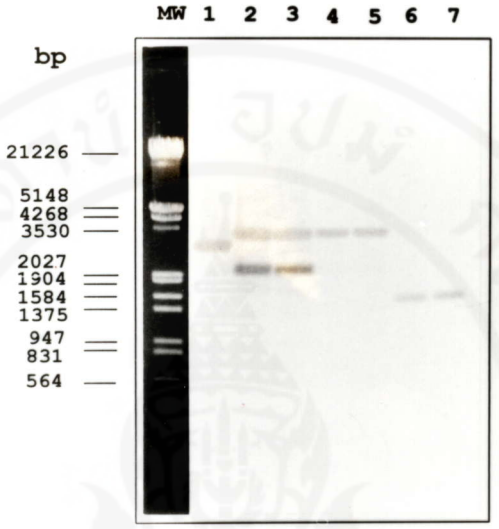

Figure 27. Southern blot analysis of *Bacillus cereus* BT-24 chromosome and the recombinant clone pSR1.

The membrane was hybridized with DIG-labeled 0.68 kb *Hin*dIII-*Eco*RV fragment derived from pSR1 plasmid as a probe.

Lane MW, λ DNA cut with *Eco*RI-*Hin*dIII.

Lane1, pBS II (SK+) cut with *Eco*RI.

Lane 2-3, pSR1 cut with *Eco*RI.

Lane 4-5, *B. cereus* DNA cut *Eco*RI.

Lane 6-7, *B. cereus* DNA cut with *Hin*dIII.

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7. Sequence Analysis of Cephalosporin C Deacetylase Gene

To analyse the DNA structure of cloned CAH, the DNA region located between *Xba*I and *Eco*RV sites in the pSR1 plasmid was sequenced. The DNA sequence was done by sequencing of various subcloness in pBS II (SK+) which obtained from the experiment localization of the gene in Figure 26. All sequencing reactions of the subclones were performed by Dye Deoxy Terminator Cycle Sequencing kit using the M-13 forward and reverse primers, and the DNA was fractionated on an automate DNA sequencer (Applied Biosystem Model 377). In order to obtain complete sequence of the gene and its both 3′ and 5′ franking region, the 1,401 bp *Eco*RI-*Eco*RV fragment was fully sequence in both orientations. The sequencing strategy was shown in Figure 28.

The DNA sequence of 1,401 bp *Eco*RI-*Eco*RV fragment of pSR1 was analyzed by using DNASIS™, BioEdit and vector NTI Programs. As shown in Figure 29, the sequence contained 1,401 bp. Analysis of the sequence data in all 6 frames showed one possible open reading frame (ORF) in the +1 frame started at nucleotide position 487 and ended at position 1,389 which contained 903 bp. Analysis of the deduced amino acid sequence of the ORF showed that the cloned CAH gene contained a sequences Gly-Asp-Ser-Ala-Gly, at amino acid 137-141, which is a conserve amino acid sequences (Gly-X-Ser-X-Gly) purposed to play an essential role in catalysis of many esterases and lipases.

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The complete nucleotide sequence upstream to the ATG start codon contained a tentative ribosome-binding site (Shine Dalgano consensus sequence), GAGG at position 474-477 and hexanucleotide sequence (the –10 region), TATAAT at position 423-428. But the –35 region, TTGACA or TTGAAAT was not be found in the sequence. Furthermore, the restriction sites for restriction endonucleases *Xba*I and *Hin*dIII as analysed by restriction mapping were found.

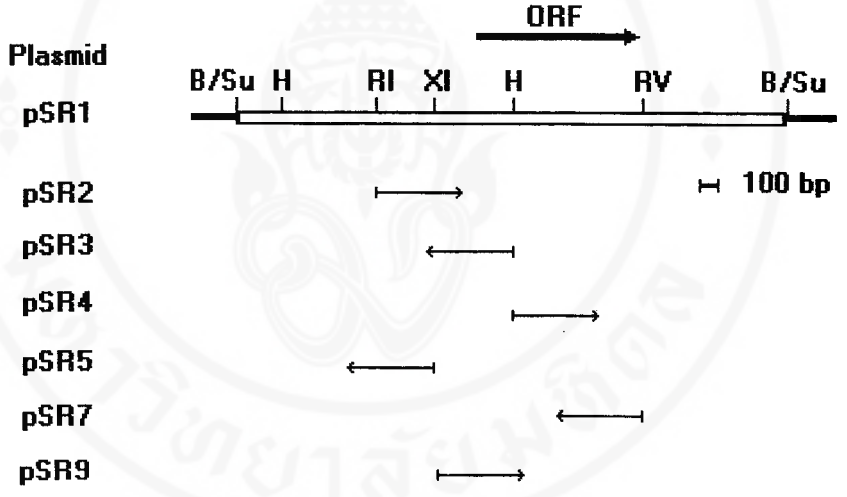

Figure 28. Sequencing strategies of the 1.4 Kb of *Eco*RI-*Eco*RV DNA fragment in pSR1 plasmid conferring CAH activity.

Arrows indicate the direction of sequencing using M13-forward (leftward arrows) and M13-reverse (rightward arrows) primers. The lengths of arrows represent the length of DNA sequence obtained from each clone. The bold arrow represents the direction and length of an open reading frame (ORF).

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```
        EcoRI
1       GAA TTC AGA ATT ACC CCA AAA CGA AAA AAT GAG CTT TTT TGT TAC   45

46      ATG TTA GTA CAA AAT TTC GTT CTG GGA TAC TAT AGA ATT TTA GCT   90

91      TAA TGG GCA TTG GGA GAG TTA CCT CTA TAC ATT TAA TTT GCC TTT   135

136     TAT GCA GGG AAA TAA GTA TTA TTT TCG AAT AGA TAG TTA GTA AAG   180

181     TTA AGT AAA TGA GGG GAA TTT AAA GAT CGG TTA AAG AAA TAT CAT   225

226     AAA AAT CAC ACA GCA ATG AAT TAT GTA GTA AAG TTA TAC AAA GAA   270
                                                          XbaI
271     GGA AAT ATG ACT GTA CTT CAA ATT TGT GAA ATT ACA AAT GTA TCT   315

316     AGA GCT TCA TTA TAT AGA AAG TTA TCG GAA GGG AAC AAA TAA TCA   360

361     TTC CGT ATT CCA TTA AAT GAA CGA ATT AAT AGA ACA AGA ATA AAT   405

406     TAT TTC TTC TAT CGA AAT ATA ATC CCA AAA TAA AAT AAT CTA TTA   450

451     AGT AAT TCT TAT TTT ACA AAA AAG AGG TGT GAA GAA ATG TCA TTA   495
                                                    Met Ser Leu    3

496     ACA TCA GTT TTA TTA AAT GTG TTA TTT GAA AAG GGG GAT GCT AAA   540
4       Thr Ser Val Leu Leu Asn Val Leu Phe Glu Lys Gly Asp Ala Lys   18

541     AGA GAT AAA GGG TTA ACT ACC CCA GAA AAT ATC GCA AGG TTT GAC   585
19      Arg Asp Lys Gly Leu Thr Thr Pro Glu Asn Ile Ala Arg Phe Asp   33

586     AAC CTT GAT TAC TAC GGG GAT AAA AGT GAA GAC CAT TTG CTT GAC   630
34      Asn Leu Asp Tyr Tyr Gly Asp Lys Ser Glu Asp His Leu Leu Asp   48

631     GTG TAT TAT CCA AAA GAT ACC GAA AAA CCT TTG CCA GCA ATT ATA   675
49      Val Tyr Tyr Pro Lys Asp Thr Glu Lys Pro Leu Pro Ala Ile Ile   63
                                                  HindIII
676     TCG ATT CAT GGT GGT GGC TGG GTG TAC GGG AAC AAG GAA GCT TAT   720
64      Ser Ile His Gly Gly Gly Trp Val Tyr Gly Asn Lys Glu Ala Tyr   78

721     CAA TTT TAC TGC ATG AAT TTA GCC CAG TAT GGG TTT ACG GTA GTA   765
79      Gln Phe Tyr Cys Met Asn Leu Ala Gln Tyr Gly Phe Thr Val Val   93
```

Figure 29. Nucleotides sequence and deduced amino acid sequence of CAH gene from *B. cereus* BT-24.

Putative hexanucleotide sequence (-10 region, TATAAT) and SD sequence (GAGG) are indicated by solid lines. The conserve amino acid sequences (Gly-X-Ser-X-Gly) are highlighted. The terminator codon is designated by an asterisk (*). The unique restriction sites are indicated by thin lines and name of enzymes.

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```
766   AAC TTC AAC TAC CGC CTT GCA CCA AAA TAT AAA TTT CCA GCG GCT   810
94    Asn Phe Asn Tyr Arg Leu Ala Pro Lys Tyr Lys Phe Pro Ala Ala   108

811   CTG GAA GAT GTA AAT TCG GTT TTT CAT TGG GTG AAG AAT AAT TCA   855
109   Leu Glu Asp Val Asn Ser Val Phe His Trp Val Lys Asn Asn Ser   123

856   ACA AAG TTT CAT ATG GAA TTA AAT AAT TTG TTT ATT GTA GGT GAT   900
124   Thr Lys Phe His Met Glu Leu Asn Asn Leu Phe Ile Val Gly Asp   138

901   TCA GCG GGG GCA CAG ATT GCA AGT CAG TAT GCA GCA ATT TTA ACT   945
139   Ser Ala Gly Ala Gln Ile Ala Ser Gln Tyr Ala Ala Ile Leu Thr   153

946   AAT TTA AAG TAT GCA AAA CTG TTT GAC TTC AAA ATG CCT GAT ATT   990
154   Asn Leu Lys Tyr Ala Lys Leu Phe Asp Phe Lys Met Pro Asp Ile   168

991   AAA ATA AAA GCA ATG GGA TTA AAC CAC GGA ATG TAT GAT CCA CTC   1035
169   Lys Ile Lys Ala Met Gly Leu Asn His Gly Met Tyr Asp Pro Leu   183

1036  GAT AGA ATA AAA AAC AAA GAA ACG AAA AAA TGG TAT AGG AAT CTT   1080
184   Asp Arg Ile Lys Asn Lys Glu Thr Lys Lys Trp Tyr Arg Asn Leu   198

1081  TTA AAT GCT TTG ATG AAG GAC TAT CTT GGA AAA GAA ATT TAT AAA   1125
199   Leu Asn Ala Leu Met Lys Asp Tyr Leu Gly Lys Glu Ile Tyr Lys   213

1126  TAT GAA AAG GAA ATG GAT TTT CAG TCA AAC AAT ACA CCA TCT TTT   1170
214   Tyr Glu Lys Glu Met Asp Phe Gln Ser Asn Asn Thr Pro Ser Phe   228

1171  CCT CCG TCC TTT GTG ACT TGC AGT GTT AAT GAT GGA TTG GTT GGG   1215
229   Pro Pro Ser Phe Val Thr Cys Ser Val Asn Asp Gly Leu Val Gly   243

1216  ATT CAA CCT GCT TTC CTT AGT AAA TTA GAA TCC GCA GGA CTT CAT   1260
244   Ile Gln Pro Ala Phe Leu Ser Lys Leu Glu Ser Ala Gly Leu His   258

1261  TAT ATT TAT AAG GAA TAT GGT CAT AAT GAC AAA GCG AGC GGT CAT   1305
259   Tyr Ile Tyr Lys Glu Tyr Gly His Asn Asp Lys Ala Ser Gly His   273

1306  GTA TTC CAC CTT GAC TTA AGA AAA GAC GAA GCA ACA ATA CTC AAC   1350
274   Val Phe His Leu Asp Leu Arg Lys Asp Glu Ala Thr Ile Leu Asn   288

1351  AAC GAA CAA ATA GAA TTT TTC CAC CAA TAC ATA TGC AAA TGA GTA   1395
289   Asn Glu Gln Ile Glu Phe Phe His Gln Tyr Ile Cys Lys  *

          EcoRV
1396  GAT ATC   1401
```

Figure 29. Nucleotides sequence and deduced amino acid sequence of CAH gene from *B. cereus* BT-24 (Continued).

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Some parameters of the complete amino acid sequences of an ORF CAH gene from *B. cereus* BT-24 analysed by ProtParam tool Program were shown in Figure 30. The deduced protein contained 301 amino acids with molecular weight of 34.68 kDa, and theoretical pI 6.8. It was found that the total number of negatively charged residues (Asp + Glu) was 36 and the total number of positively charged residues (Arg + Lys) was 35.

Hydropathy profile of the putative CAH protein determined by the method of Kyte and Doolittle (1982) with the window 13 is shown in Figure 31. It indicates that the protein is rather hydrophilic with hydrophobic N-terminal portion.

```
     1         11        21        31        41        51
     |         |         |         |         |         |
   1 MSLTSVLLNV LFEKGDAKRD KGLTTPENIA RFDNLDYYGD KSEDHLLDVY YPKDTEKPLP    60
  61 AIISIHGGGW VYGNKEAYQF YCMNLAQYGF TVVNFNYRLA PKYKFPAALN DVNSVFHWVK   120
 121 NNSTKFHMEL NNLFIVGDSA GAQIASQYAA ILTNLKYAKL FDFKMPDIKI KAMGLNHGMY   180
 181 DPLDRIKNKE TKKWYRNLLN ALMKDYLGKE IYKYEKEMDF QSNNTPSFPP SFVTCSVNDG   240
 241 LVGIQPAFLS KLESAGLHYI YKEYGHNDKA SGHVFHLDLR KDEATILNNE QIEFFHQYIC   300
 301 K
```

Number of amino acids: 301 aa
Molecular weight: 34.67 kDa
Theoretical pI: 6.80

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Figure 30. Deduced amino acid sequence, amino acid composition, molecular weight, and pI of CAH gene from *B. cereus* BT-24.

Amino acid composition:

Ala (A)	20	6.6%
Arg (R)	6	2.0%
Asn (N)	23	7.6%
Asp (D)	20	6.6%
Cys (C)	3	1.0%
Gln (Q)	8	2.7%
Glu (E)	16	5.3%
Gly (G)	18	6.0%
His (H)	10	3.3%
Ile (I)	16	5.3%
Leu (L)	30	10.0%
Lys (K)	29	9.6%
Met (M)	8	2.7%
Phe (F)	18	6.0%
Pro (P)	12	4.0%
Ser (S)	15	5.0%
Thr (T)	11	3.7%
Trp (W)	3	1.0%
Tyr (Y)	21	7.0%
Val (V)	14	4.7%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 36

Total number of positively charged residues (Arg + Lys): 35

Figure 30. Deduced amino acid sequence, amino acid composition, molecular weight, and pI of CAH gene from *B. cereus* BT-24 (Continued).

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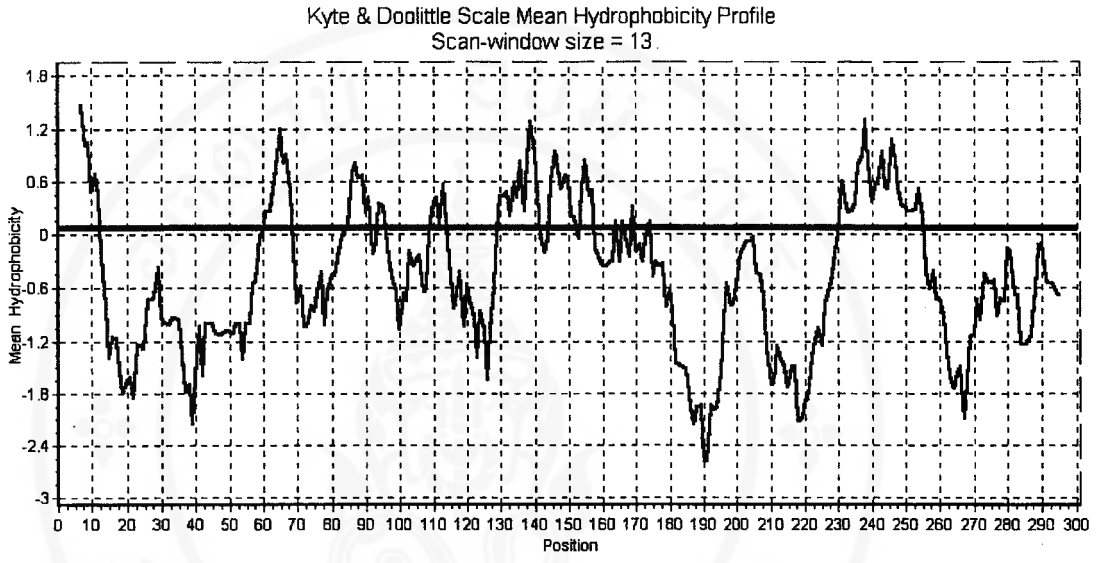

Figure 31. Hydropathy plot of the predicted CAH protein.

The plot of hydropathy value was generated according to the method of Kyte and Doolittle (1982). Positive values indicate hydrophobicity and negative values indicate hydophilicity.

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8. Comparison of Deduced Amino Acid Sequence of the Cephalosporin C Deacetylase (CAH) Gene from *B. cereus* BT-24 with that of Other Esterases

In order to compare the deduced amino acid sequence of the CAH from *B. cereus* BT-24 with that of other esterases, the CAH sequence was aligned with published sequenced in the SwissProt sequences Database. It was found that the CAH sequence was homology with several esterases and lipases at amino acid level. The three sequences having most significant alignment with *B. cereus* CAH were human arylacetamide deacetylase, triacylglecerol lipase (*Moraxella* sp, Pseudomonas group) and *E. coli* acetylesterase, as shown in Figure 32. The percent identity at amino acid level was between 21-34%. Data were analysed on March 17, 2000.

From the analysis of deduced amino acid sequence of CAH from *B. cereus* BT-24, it was found that it contained a consensus sequence Gly-X-Ser-X-Gly (Figure 32) which was found in many esterases and lipases. This finding sugested that the cloned CAH was an esterase gene.

Furthermore, amino acid sequence of CAH from *B. cereus* BT-24 was also aligned with that from *B. subtilis* SHS0133 (Mitsushima *et al.*, 1995) using Pairwise Sequence Aligment Program of BCM Search Launcher (USA). Both sequence showed 19.1% identity. The amino acid homology alignment of both sequences was illustrated in Figure 33 using Clustal W (1.7) Program.

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(A) **sp/P22760/AAAD_HUMAN ARYLACETAMIDE DEACETYLASE** (AADAC)
Length = 399, Score = 65.6 bits (157), Expect = 1e-10
Identities = 49/158 (31%), Positives = 70/158 (44%), Gaps = 14/158 (8%)

```
BT-24: 33  DNLDYYGDKSEDHLLDVYYPKDTEKPLP-AIISIHGGGWVYGNKEAYQFYCMN---LAQY 88
          +N+     K  + L+ VY PK   + L   +  IHGGGW  G+     +  ++     +
Sbjct: 77  ENVTVTETKFNNILVRVYVPKRKSEALRRGLFYIHGGGWCVGSAALSGYDLLSRWTADRL 136

BT-24: 89  GFTVVNFNYRLAPKYKFPAALEDVNSVFHWV--KNNSTKFHMELNNLFIVGDSAGAQIAS 146
             VV+ NYRLAPKY FP   EDV +   W   K    K+ +    + I GDSAG  +A+
Sbjct: 137 DAVVVSTNYRLAPKYHFPIQFEDVYNALRWFLRKKVLAKYGVNPERIGISGDSAGGNLAA 196

BT-24: 147 QYAAILTNLKYAKLFDFKMPDIKIKAMGLNHGMYDPLD 184
                  +L D     IK+K   L +    PLD
Sbjct: 197 AVT--------QQLLDDPDVKIKLKIQSLIYPALQPLD 226
```

(B) **sp/P24484//LIP *Moraxella* SP LIPASE 2** (TRIACYLGLYCEROL LIPASE)
Length = 433, Score = 62.5 bits (149), Expect = 1e-09
Identities = 30/90 (33%), Positives = 53/90 (58%), Gaps = 1/90 (1%)

```
BT-24: 61  AIISIHGGGWVYGNKEAYQFYCMNL-AQYGFTVVNFNYRLAPKYKFPAALEDVNSVFHWV 119
          A++  HGGG+  G+ + +  +C  + AQ G+ VV+ +YR+AP+Y  P AL+D  + + W+
Sbjct: 160 AMLFFHGGGFCIGDIDTHHEFCHTVCAQTGWAVVSVDYRMAPEYPAPTALKDCLAAYAWL 219

BT-24: 120 KNNSTKFHMELNNLFIVGDSAGAQIASQYA 149
             +S       + + + GDSAG  +A+  A
Sbjct: 220 AEHSQSLGASPSRIVLSGDSAGGCLAALVA 249
```

(C) **SP/P23872/AES *E. coli* ACETYL ESTERASE**
Length = 319, Score = 59.7 bits (142), Expect = 7e-09
Identities = 47/191 (24%), Positives = 88/191 (45%), Gaps = 22/191 (11%)

```
BT-24: 65  IHGGGWVYGNKEAYQFYCMNLAQYG-FTVVNFNYRLAPKYKFPAALEDVNSVFHWVKNNS 123
          +HGGG++ GN + +       LA Y   TV+  +Y L+P+ +FP A+E++ +   +    +
Sbjct: 90  LHGGGFILGNLDTHDRIMRLLASYSQCTVIGIDYTLSPEARFPQAIEEIVAACCYFHQQA 149

BT-24: 124 TKFHMELNNLFIVGDSAGAQIASQYAAILTNLKYAKLFDFKMPDIKIKAMGLNHGMYDPL 183
             + + ++ +   GDSAGA +A   A  L          D ++   K+  + L +G+Y
Sbjct: 150 EDYQINMSRIGFAGDSAGAMLALASALWLR--------DKQIDCGKVAGVLLWYGLYGLR 201

BT-24: 184 DRIKNKETKKWYRNLLNALMKDYLGKEIYKYEKEMDFQSNNTPSFPPSFVTCSVNDGLV- 242
          D +         R LL  +      +++  YE+   + SN+     P +  C  N+ L
Sbjct: 202 DSVT--------RRLLGGVWDGLTQQDLQMYEEA--YLSNDADRESPYY--CLFNNDLTR 249

BT-24: 243 GIQPAFLSKLE 253
            + P F++  E
Sbjct: 250 EVPPCFIAGAE 260
```

Figure 32. Sequence alignment of the deduced amino acid sequence of CAH from *B. cereus* BT-24 (BT-24) and sequences in SwissProt Database (A, B and C). The consensus sequence Gly-X-Ser-X-Gly is highlight and plus (+) means same group amino acid. (A) Human arylacetamide deacetylase; (B) Triacylglycerol lipase; (C) *E. coli* acetylesterase.

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CLUSTAL W (1.7) multiple sequence alignment

Sequence 1 301 aa

Sequence 2 318 aa

```
SHS0133         MQLFDLPLDQLQTYKPEK-----TAPKDFSEFWKLSLEELAKVQAEPDLQPVDYPADGVK
BT-24           MSLTSVLLNVLFEKGDAKRDKGLTTPENIARFDNLDYYGD---KSEDHLLDVYYPKDTEK
                *.* .: *: *      *     *:*:::::.* :*.       ::* .*  * ** *  *

SHS0133         VYRLTYKSFGNARITGWYAVPDKQGPHPAIVKYHGYNASYDGEIHEMVNWALHGYAAFGM
BT-24           PLPAIISIHGGGWVYGNKEAYQFYCMNLAQYGFTVVNFNYRLAPKYKFPAALEDVNSVFH
                      . .*.. : *   . :    : *   :   * .*    :  .  **..  :.

SHS0133         LVRGQQSSEDTSISLHGHALGWMTKGILDKDTYYYRGVYLDAVRALEVISSFDEVDETRI
BT-24           WVKNNS----TKFHMELNNLFIVGDSAGAQIASQYAATLTNLKYAKLFDFKMPDIKIKAM
                 *:.:.    *.: :. : *  : ..   : :  * .:  :   *  .  .: ::. . :

SHS0133         GVTGGSQGGGLTIAAAALSDIP-KAAVADYPYLSNFERAIDVALEQPYLEINSFFRRNGS
BT-24           GLNHG--------MYDPLDRIKNKETKKWYRNLLNALMKDYLGKEIYKYEKEMDFQSNNT
                *:. *          .*. *  * :   *  * *      :. *    * :  *: *.:

SHS0133         PETEVQAMKTLSYFDIMNLADRVKVPVLMSIGLIDKVTPPSTVFAAYNHLETEKELKVYR
BT-24           P-SFPPSFVTCSVNDGLVGIQPAFLSKLESAGLH-------YIYKEYGHNDKASGHVFHL
                * :   :: * *  * :   : . :. * * **        ::  *.* :. .   .:

SHS0133         YFGHEYIPAFQTEKLAFFKQHLKG
BT-24           DLRKDEATILNNEQIEFFHQYICK
                 : ::  . ::.*:: **:*::
```

Figure 33. Sequence alignment of deduced amino acid sequence of CAH from *B. cereus* BT-24 (BT-24) and sequence from *B. subtilis* SHS0133 (SHS0133). The consensus sequences that found in many esterases are shown in highlight. The identities of amino acids are indicated by asterisks. Similar amino acids are indicated by one and two dots.

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9. Overexpression of Cephalosporin C Deacetylase Gene from *B. cereus* BT-24 in *E. coli*

9.1 Subcloning

Since the expression of CAH in the pBS II (SK+) vector was low (Table 16). Attempt to increase expression of CAH was done by subcloning of the gene coding sequence of CAH into an expression vector pKK223-3 vector. The pKK223-3 vector contains *ptac* promoter, which is the extremely useful for high level expression of protein products in *E. coli* when induced with IPTG. To subclone the CAH coding region, the gene fragment was prepared by PCR amplification using pSR1 plasmid as template and EST-2 and EST-3 primers as reverse and forward primers, respectively. The EST-2 and EST-3 primers were designed to contain the recognition sequences for *Eco*RV and *Eco*RI restriction endonucleases, respectively. Construction diagram of subcloning for overexpressing was shown in Figure 34. After PCR amplification, the DNA was visualized by agarose gel electrophoresis. As shown in Figure 35, the size of PCR product was about 0.9 kb as expected from the sequence. The band was then sliced and the DNA was eluted from the agarose gel as described in Materials and Methods. The eluted DNA was double digested with the *Eco*RI and *Eco*RV and ligated to linearized plasmid pKK223-3, which was prepared by *Eco*RI and *Sma*I double digestion. The ligated DNA was transformed into *E. coli* JM109 competent cells. The transformants were selected on LB + ampicillin plate. The clone harboring CAH gene was screened by chemical method as described in Materials and Methods.

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One clone showing dark brown colony, as shown in Figure 36, was selected and named pSR11. This clone was confirmed by microbiological method, as shown

in Figure 37. It was indicated that the clone pSR11 could produced CAH enzyme. The plasmid in the selected clone which was also name pSR11 was further subjected to restriction analysis. As shown in Figure 38, digestion of pSR11 plasmid with *Eco*RI gave single band of 5.4 kb and double digestion with *Eco*RI-*Pst*I gave 2 bands of 0.9 and 4.58 kb fragments. To confirm that the 0.9 kb fragment in pSR11 plasmid was the coding region of CAH, the plasmid was digested with *Hin*dIII. As expected, two bands of 0.7 and 4.77 kb were obtained, as shown in Figure 38. To confirm that the fragment 4.5 kb in pSR11 plasmid was pKK223-3 vector, the plasmid was double digested with *Eco*RI-*Pvu*I. As expected, two bands of 1.87 and 3.6 kb were obtained, as shown in Figure 38. The expected sizes of DNA bands when the pSR11 plasmid were cut with *Eco*RI, *Hin*dIII, *Eco*RI-*Pst*I and *Eco*RI-*Pvu*I were summarized in Table 20. This result showed that pKK223-3 vector in pSR11 plasmid harbored 0.9 kb of the coding region of CAH.

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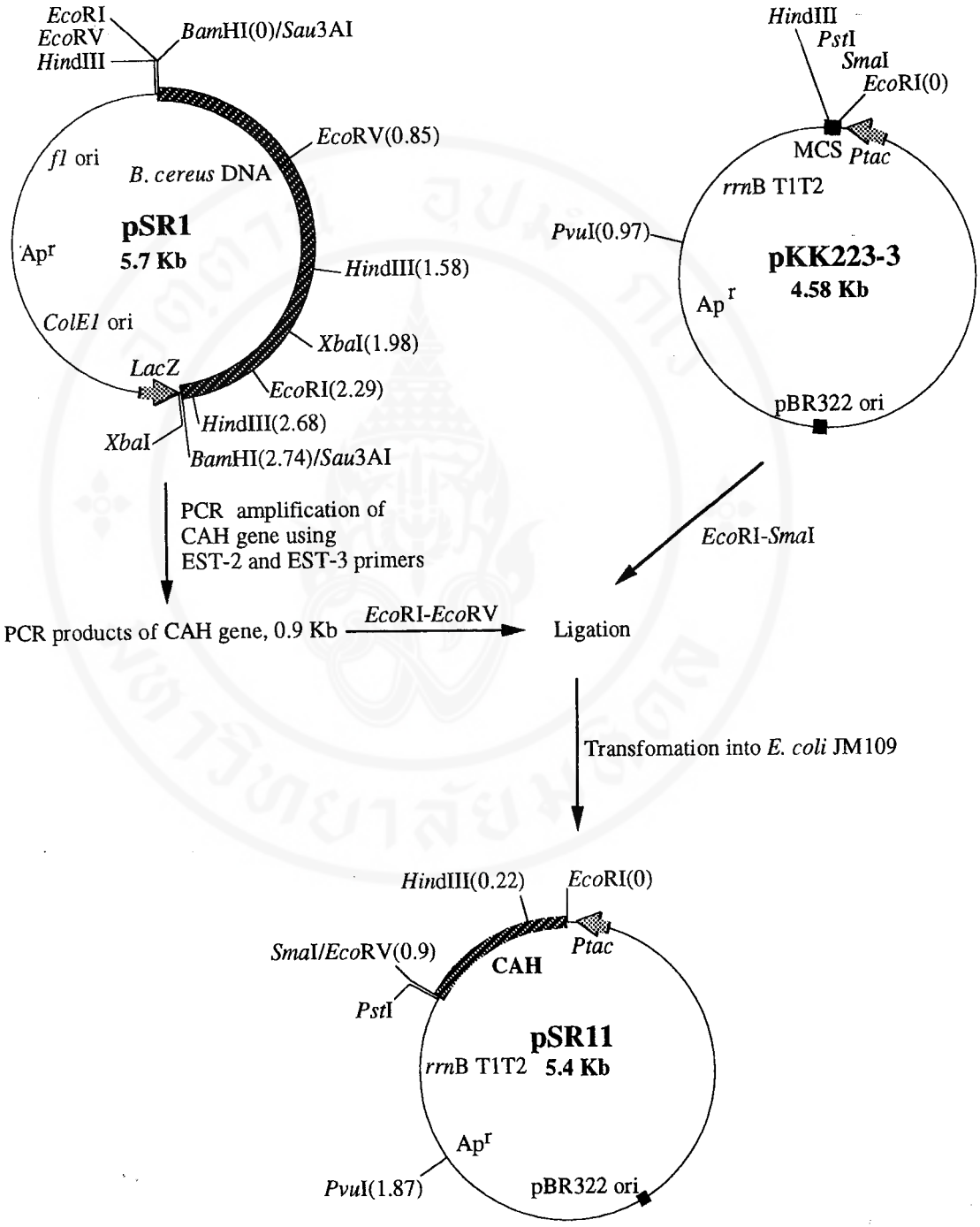

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Figure 34. Construction of pSR11 plasmid using the pKK223-3 vector.

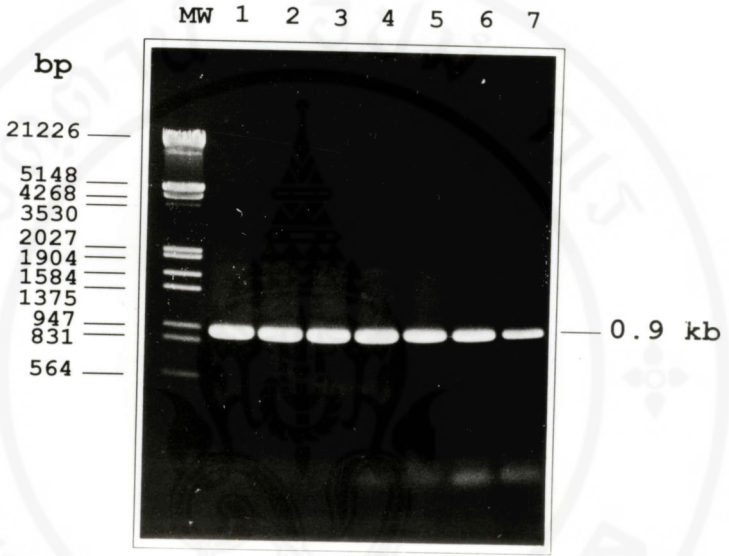

Figure 35. PCR products of CAH gene from *B. cereus* BT-24 using the pSR1 plasmid as template and the EST-2 and EST-3 as reverse and forward primers, respectively.

Lane MW, λ DNA cut with *Hin*dIII-*Eco*RI.

Lane 1-7, 0.9 kb PCR products.

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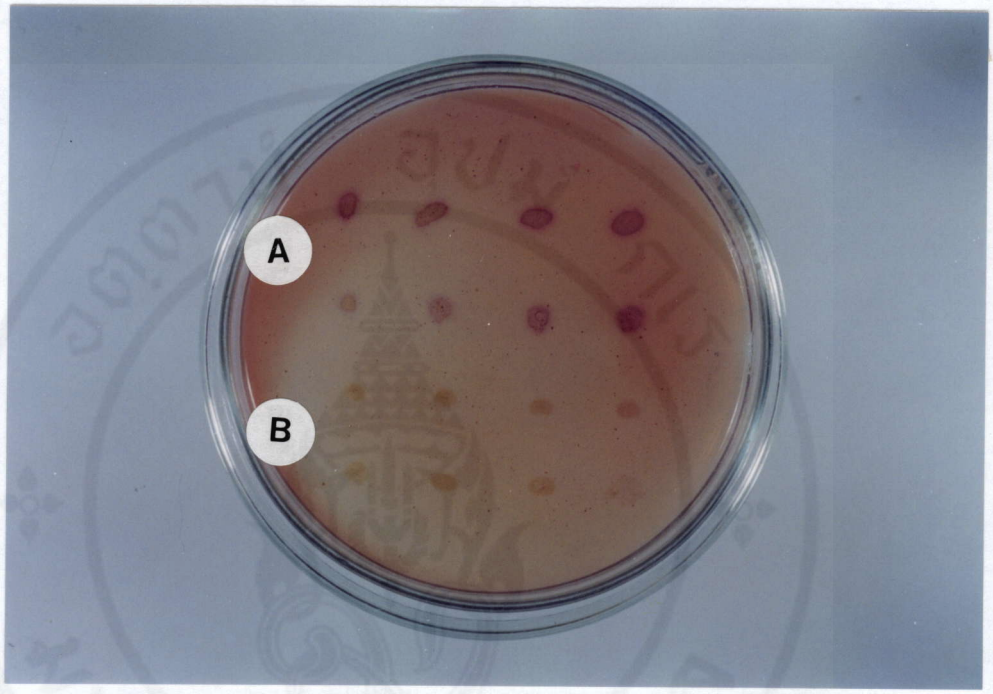

Figure 36. Confirmation of esterase activity of PCR cloned fragment in the pSR11 plasmid. The esterase producing clones were overlayed with the β-NA and Fast Blue BB dye, (the positive colony was detected as having dark-brown colony compared with the light–brown colony of negative control).

A, Clone pSR11 (*E. coli* JM109 + pSR11).

B, Negative control (*E. coli* JM109 + pKK223-3).

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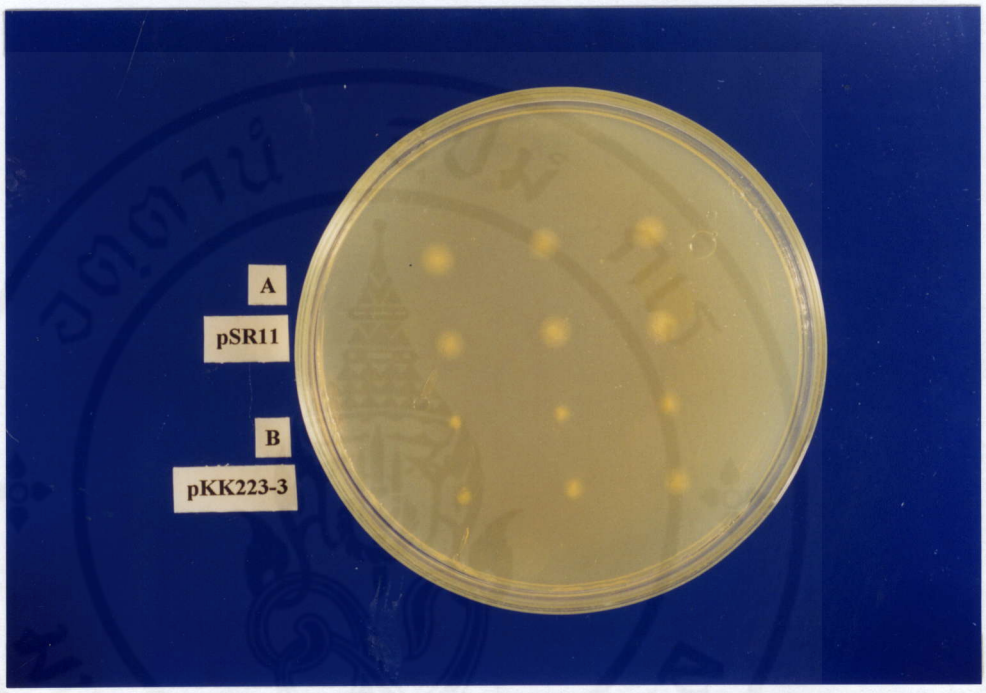

Figure 37. Confirmation of CAH activity of PCR cloned fragment in the pSR11 plasmid. The CAH clones were overlayed with 300 μg/ml cephalosporin C and 1% (v/v) *S. aureus* ATCC 25923. The positive clone was detected as having broad satellite growth of *S. aureus* as compared to the narrow satellite growth of the negative control.

A, Clone pSR11 (*E. coli* JM109 + pSR11).

B, Negative control (*E. coli* JM109 + pKK223-3).

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Table 20. The expected sizes of DNA bands of pKK223-3 vector and pSR11 plasmid cut with various enzymes.

Restriction enzymes	Number of bands		Expected sizes (Kb)	
	pKK223-3	pSR11	pKK223-3	PSR11
*Eco*RI	1	1	4.58	5.4
*Hin*dIII	-	2	-	0.7, 4.77
*Eco*RI-*Pst*I	-	2	-	0.9, 4.58
*Eco*RI-*Pvu*I	-	2	-	1.87, 3.6

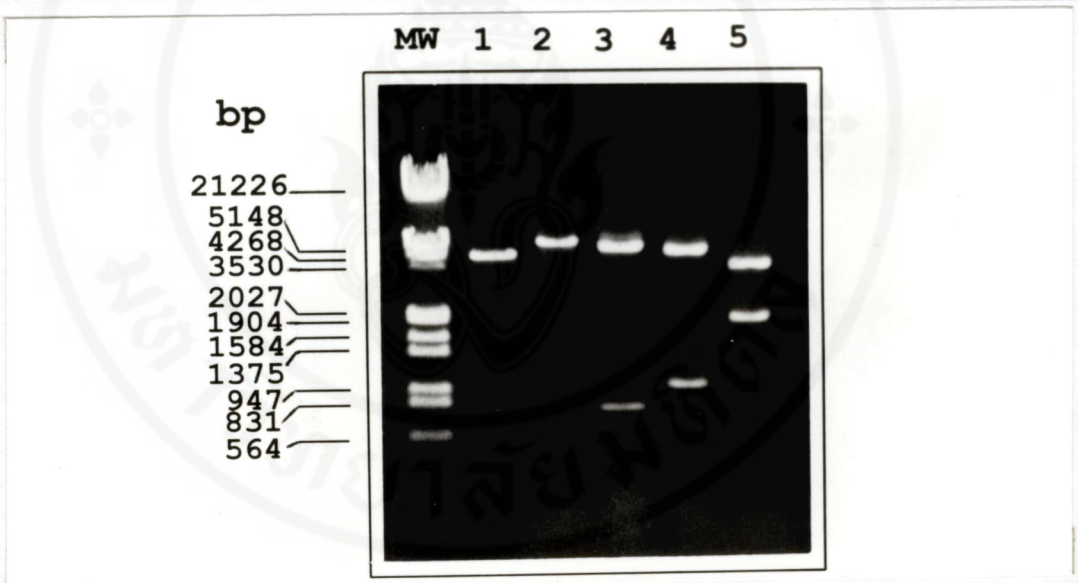

Figure 38. Restriction endonuclease analysis of pKK223-3 vector and pSR11 plasmid.

The digested plasmids were analyzed on 0.7% agarose gel electrophoresis.

Lane MW, λ DNA cut with *Hin*dIII-*Eco*RI.

Lane 1, pKK223-3 cut with *Eco*RI. Lane 2, pSR11 cut with *Eco*RI.

Lane 3, pSR11 cut with *Hin*dIII. Lane 4, pSR11 cut with *Eco*RI-*Pst*I.

Lane 5, pSR2 cut with *Eco*RI- *Pvu*I.

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9.2 Overexpression of cephalosporin C deacetylase gene in clone pSR11 under IPTG induction

Since the expression of gene under the control of *ptac* promoter can be induced for overexpression with IPTG. To overexpress the CAH gene, the clone pSR11 was grown to log phase and IPTG was then added to the culture to a final concentration of 1 mM. After incubation for 4 hr, the cells were harvested and the cell free extracts were subjected to esterolytic activity assay using α-NA as a substrate. The esterase activity of the clone pSR11 was compared with that of *E. coli* JM109 host. As shown in Table 21, the esterase specific activity of the clone pSR11 and *E. coli* JM109 host were 34.61 and 17.04 U/mg protein (with IPTG induction), respectively and were 23.92 and 17.63 U/mg protein (without IPTG induction), respectively. When the esterase activity of *E. coli* JM109 host was subtracted from that of clone pSR11, the activity of CAH was 17.57 U/mg protein with IPTG induction. Whereas, it was 6.29 U/mg protein without IPTG induction. This result showed that the expression of CAH under the control of *ptac* promoter was induced about 3 times under IPTG induction.

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Table 21. Esterase activity of cell free extract of the pSR11 clone with and without IPTG induction compared with *E. coli* JM109 host.

Organisms	Esterase specific activity* (U/mg protein)
E. coli JM109	17.63 ± 1.46
E. coli JM109 + pSR11	23.92 ± 1.84
E. coli JM109 (with IPTG)**	17.04 ± 0.00
E. coli JM109 + pSR11 (with IPTG)**	34.61 ± 0.80

*1 unit of esterase activity was expressed as 1 nmole of α-naphthol released per min per ml at 37°C.

**For IPTG induction, 4 hours culture grown in LB containing 50 μg/ml ampicillin was supplemented with 1 mM IPTG and incubated further of an additional 4 hours.

The values are the means of three experiments with 2 replicates each.

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10. Determination of Molecular Weight of Cloned CAH

To determine the molecular weight of CAH which was produced by clone pSR11, its protein extracts was subjected to SDS-PAGE. Protein extract of the *E. coli* host JM109 was also loaded on the same gel. Two gels were run in parallel at 150 V for about 1 hour. One gel was stained with Coomassie Brilliant Blue and the other was renatured immediately, and followed with the esterase activity staining using β-NA as a substrate as described in Materials and Methods. The result of SDS-PAGE was shown in Figure 39A and B. When the gel was stained with Coomassie Brilliant Blue, the intense protein bands about 34.6 kDa was observed in the cell free extract of pSR11 clone (Figure 39A, lane 2-6) as compared to that of *E. coli* host harboring pKK223-3 (Figure 39A, lane 1). When the gel was renatured and stained with esterase activity staining, the same molecular weight of an additional protein band was also observed in the protein extracted of the clone pSR11 (Figure 39B, lane 2-7) as compared to that of *E. coli* host (Figure 39B, lane 1). The results demonstrated that the protein expressed in the clone pSR11 had esterase activity and its molecular weight was about 34.6 kDa. The esterase activity staining of Native-PAGE was also performed, as shown in Figure 40. Two bands were observed in the cell free extract of the clone pSR11 with (lane 1) and without (lane 2) IPTG induction as compared to that of *E. coli* host JM109 which had only one band (lane 3). The same results were also observed when the cell free extract of the recombinant clone pSR1 (lane 4) and the *E. coli* host DH-5α (lane 5) were tested. The results confirmed that the additional band observed in esterase activity staining was expressed by the cloned gene.

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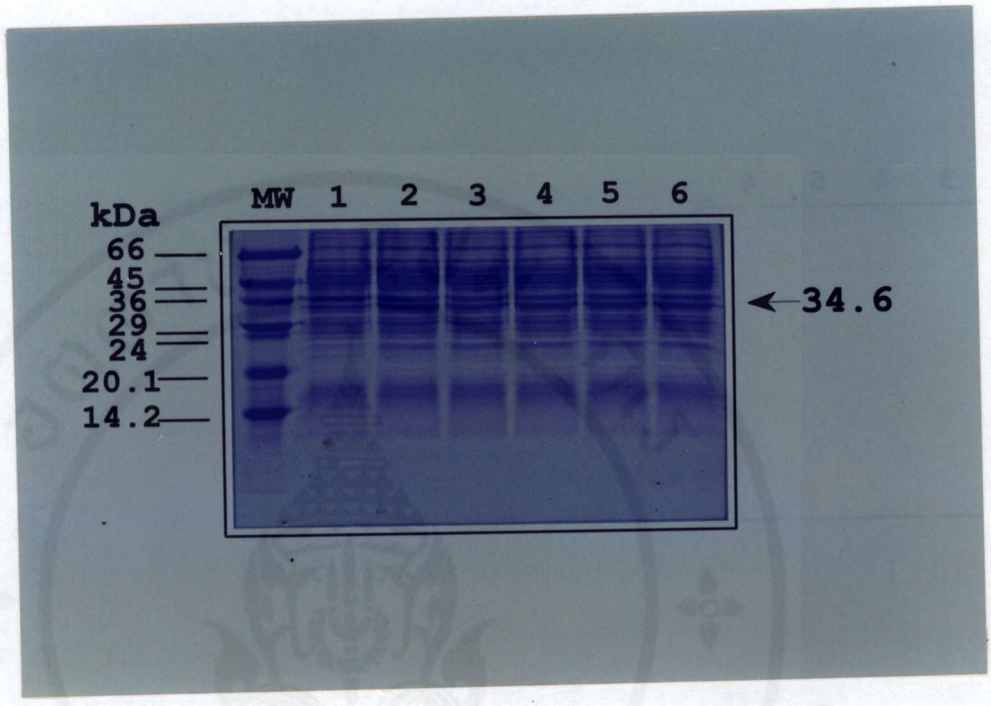
A



B

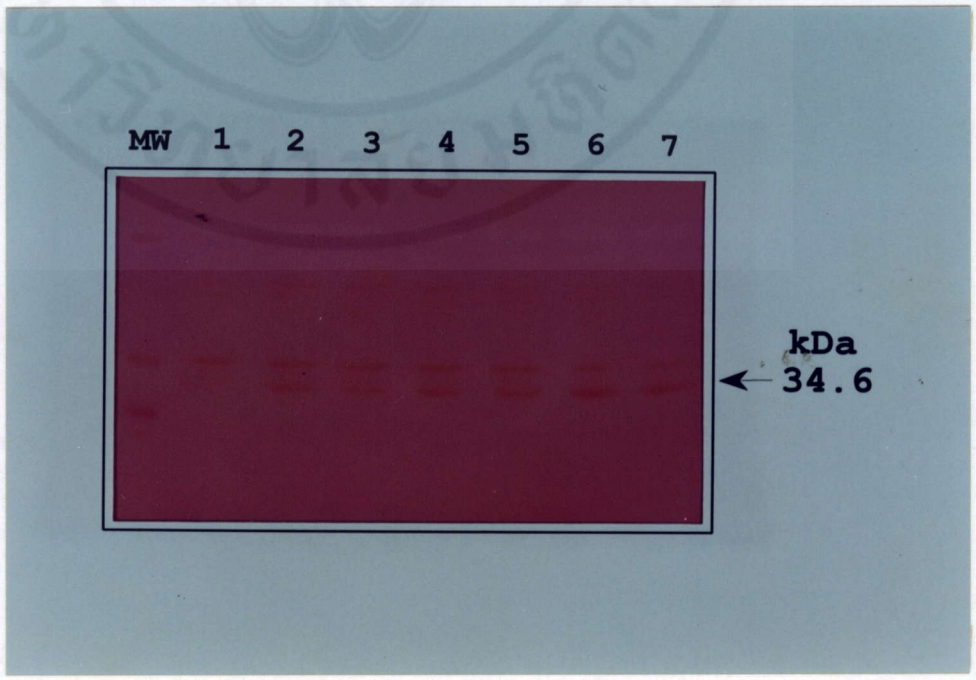


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Figure 39. SDS-PAGE of protein extract of the clone pSR11 and *E. coli* JM109.

(A). Coomassie Brilliant Blue staining;

(B). Esterase activity staining. After 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. The gel was stained with α-NA and Fast Blue BB dye in 0.1 M phosphate buffer, pH 7.0.

Lane MW, Molecular weights standards.

Lane1, pKK223-3/JM109.

Lane2-3, pSR11/JM109 uninduced with 3 mM IPTG.

Lane4-6, pSR11/JM109 induced with 3 mM IPTG.

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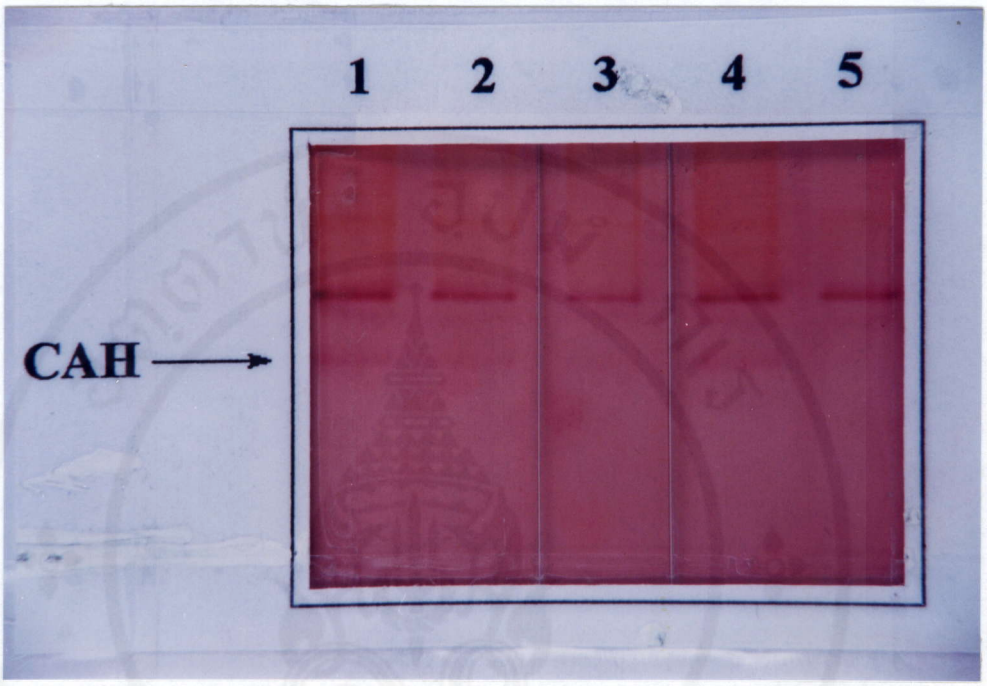

Figure 40. NATIVE-PAGE of cell free extract of the clones pSR1, pSR11 and *E. coli* JM109.

The gel was stained with esterase activity staining using β-NA and Fast Blue BB dye in 0.1 M phosphate buffer, pH 7.0.

Lane 1, *E. coli* JM109 + pSR11 (induced with 1 mM IPTG).

Lane 2, *E. coli* JM109 + pSR11.

Lane 3, *E. coli* JM109 + pKK223-3 (induced with 1 mM IPTG).

Lane 4, *E. coli* JM109 + pSR1.

Lane 5, *E. coli* JM109.

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CHAPTER V

DISCUSSION

The deacetylation of cephalosporins is of considerable interest since deacetylcephalosporins are valuable intermediates for synthesis of therapeutically cephalosporin antibiotics (O'Callaghan *et al.*, 1976 and Tossuka *et al.*, 1992). The deacetylation of cephalosporins by enzymatic reaction using cephalosporin C deacetylase (CAH) is believed to be advantageous in that it gives high yields and fewer side reactions. Several research group have reported on isolation of CAH from several sources such as citrus peel (Jeffery *et al.*, 1961), actinomycetes (Demain *et al.*, 1963), *Rhodotorula glutinis* (Sakai *et al.*, 1998), *Cephalosporium acremonium* (Fujisawa *et al.*, 1975), and different strains of *B. subtilis* (Abbott and Fukuda, 1975; Konecny and Voser, 1977; Takimoto *et al.*, 1994). CAH from each sources showed different substrate specificity and characteristics. In order to discover new CAH, attempt on isolation of the new producer was performed. This study describes the screening processes, gene cloning and characterization, and expression of CAH gene from the local isolated strain *Bacillus cereus* BT-24.

1. Screening for CAH Producing Bacteria

Previous reports showed that several CAH producers were soil organisms. The source of organism in this study was therefore soil samples collected from various

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parts of Thailand. The heat shock technique was used for isolation of spore forming bacteria which were screening as CAH producers by both the chemical and the microbiological methods using overlayered techniques. The chemical method was employed for primary screening by using β-NA as a substrate and Fast Blue BB dye as an indicator. The positive colonies were detected by observing the dark brown color compared with the positive control of CAH producer, *B. subtilis* WRRL-B558 (Abbott and Fukuda, 1975) (Figure 12). However, this method was not specific to CAH as any esterase enzymes can deacetylate β-naphthylacetate. Therefore, the microbiological method was employed in the secondary screening step. This method used cehphalosporin C as a substrate and *S. aureus* ATCC 25923 as an indicator strain. Since the deacetylcephalosporin C has no antibacterial activity against *S. aureus*, the positive colonies that could deacetylate cephalosporin C would allow *S. aureus* to grow around their colonies much better than that done by negative control *E. coli* DH-5α strain (Figure 13). From the screening process, 30 out of 550 spore forming isolates were positive with the primary screening step and 25 out of those 30 isolates were positive with the secondary screening step (Table 14).

The deacetylation of cephalosporin C to deacetylcephalosporin C by the positive isolated was further confirmed by HPLC (High Performance Liquid Chromatography). HPLC was the most suitable in terms of accuracy, sensitivity and reproducibility, thus it was chosen in this study to identify deacetylcephalosporin C product and cephalosporin C. Reverse-phase HPLC separate molecules based on their hydrophobicity. The separation occurs by differential hydrophobic interaction of

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sample components with hydrophobic functional group attatched to the metrix. The hydrophobic molecules interact strongly with the matrix and tend to be eluted out at later retention time (Snyder and Kirkland, 1979; Horvath, 1983). Since deacetylcephalosporin C was not commercially available, the reference deacetylcephalosporin C used in this study was obtained by deacetylation of cephalosporin C with a commercial acetylesterase. The deacetylcephalosporin C and cephalosporin C were eluted at retention time of 3.07 min and 17.167-17.979 min, respectively (Figure 14). By using culture broth and cell free extract as enzyme source, for deacetylation of cephalosporin C, it showed that the culture broth of all 25 positive isolates had no esterolytic activity toward cephalosporin C. However, cell free extracts of some positive isolated did and one isolate, named BT-24, showed highest activity. The isolated BT-24 gave the peak eluted at 3.113 min. (Figure 15A). Although the peak of height was low, its peak area (1.572×10^3 mA*S) clearly indicated that it was an additional peak as compared to the result obtained from heated-cell free extract (0.869×10^3 mA*S) (Figure 15B). This peak was therefore, thought to be deacetylcephalosporin C. Although the result of screening by the microbiological method demonstrated that 25 isolates allowed satellite growth of *S. aureus* around their colonies, most of them were not able to deacetylate cephalosporin C to deacetylcephalosporin C when confirmed with HPLC. It was possible that those isolates might convert cephalosporin C other inactive forms, by some enzymes other than esterase, which allowed satellite growth of *S. aureus*. β-Lactamase might be the case. The fact that most of esterases producing isolates had no esterolytic activity

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toward cephalosporin C would indicate that their esterases could not use cephalosporin C as a substrate.

From the data, it was suggested that only isolate BT-24 had esterase that could use cephalosporin C as a substrate and could convert it to deacetylcephalosporin C. Based on our interest, this enzyme was assumed to be CAH. However, study on its substrate specificity should be further performed. The bacterial isolate BT-24 was identified as *Bacillus cereus*. The result showed that this bacterial isolate was in the same genus as that reported by other researcher (Abbott and Fukuda, 1975; Takimoto *et al.*, 1994) but different in species, i.e., *Bacillus subtilis*. Therefore, a local isolate *Bacillus cereus* BT-24 was chosen to be a CAH producing organism in this study. Since the CAH of *B. cereus* BT-24 was intracellular and its amount was rather low. Several purification steps were required prior to biochemical characterization of the enzyme. In these studies, cloning and expression of CAH gene were attempted in order to obtain preliminary data for high production of enzyme for further studies.

2. Cloning and Characterization of CAH Gene of *B. cereus* BT-24

The CAH gene of *B. cereus* BT-24 was cloned by a shot gun cloning technique using vector pBS II (SK+) (Figure 16). This vector has a high copy number because it lacks the *rop* gene that involves in the control of copy number (Maniatis *et al.*, 1989), thus the large amount of libraries can be obtained. It also have a good charactertics for selection of the recombinant clones in that the expression of amino-terminal fragment of the *lacZ* β-galactosidase gene product by the plasmid displays α-

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complementation in appropriate hosts such as *E. coli* DH-5α which was used in this study. Therefore, the recombinants clones can be screened by blue-white selection. As there have been several reports demonstrated the expression of *Bacillus* gene by it own promoter in *E. coli* host, in this study the screening of the recombinant clones were based on gene function. The screening method for the recombinant clones harboring CAH gene was the same as in the screening of local isolates.

The results showed that the *Sau*3AI gene fragment of *B. cereus* BT-24 genomic DNA cloned at the *Bam*HI site of pBS II (SK+) in the selected recombinant clone, pSR1, was able to expressed and hydrolysed the ester bond of β-NA substrate when screened with the chemical method (Figure 17). The microbiological method using cephalosporin C as a substrate also indicated that the recombinant clone pSR1 had specificity toward cephalosporin C because it had ability to convert the cephalosporin C to inactive from which could not inhibit growth of *S. aureus* (Figure 18). The inactive from of cephalosporin C was presumably deacetylcephalosporin C. Result of esterase acitivity using β-NA as a substrate showed that cell free extract of recombinant clone pSR1 had esterase activity 1.34 times higher than that of *E. coli* DH-5α host (Table 16). The esterase activity detected in the *E. coli* host was probably resulted from other esterases which hydrolyse the same substrate. To confirm that the recombinant clone pSR1 could hydrolyse cephalosporin C to form deacetylcephalosporin C, the HPLC method was used to detect the product. It was demonstrated that cell free extract of the clone pSR1 increased the height of deacetylcephalosporin C peak at retention time 3.113 min as compared to that of *E.*

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coli host (Figure 19). From all of the results above suggested that the clone *Sau*3AI fragment from *B. cereus* BT-24 possessed CAH activity.

Restriction analysis of pSR1 plasmid in the selected recombinant clone showed that the length of cloned *Sau*3AI fragment was 2.7 kb and it contained several restriction sites, i.e., *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I (Figure 21). These existing restriction sites were used to create several subclones for localization of the CAH gene. By detecting esterase activity of subclones with the chemical method, the result showed that the CAH gene was located on 1.089 kb *Xba*I-*Eco*RV fragment (Figure 26). Southern hybridization between the *B. cereus* BT-24 DNA and the probe prepared from 0.68 kb *Hin*dIII-*Eco*RV of the pSR1 plasmid, confirmed that the inserted DNA was derived from the genome of locally isolated strain *B. cereus* (Figure 16). The CAH gene appeared as a single copy in the genome of *B. cereus* BT-24.

Determination of nucleotide sequencing of *Eco*RI-*Eco*RV fragment that covered *Xba*I-*Eco*RV revealed that the CAH structural gene of *B. cereus* BT-24 composed of 903 bp and coded for the protein with molecular weight 34.6 kDa (Figure 19). The size of CAH structural gene of *B. cereus* BT-24 was corresponding to that of other esterases reported so far, for instances, acetyl xylan esterase gene (AXE) from *Trichoderma reesei*, composed of 906 bp, (Clark *et al.*, 1996); AXE with CAH activity from *Thermoanaerobacteriu* sp, composed of 960 bp, (Lorenz and

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Wiegel, 1997); CAH gene from *Bacillus subtilis* SHS0133, composed of 951 bp (Mitsushima *et al.* 1995).

The primary structure of the CAH protein of *B. cereus* BT-24 was elucidated from the deduced amino acid sequence. The sequence from the position 137 to 141, Gly-Asn-Ser-Ala-Gly (Figure 29), corresponded to the common sequence Gly-X-Ser-X-Gly found in many esterase, lipases, and serine proteases. These amino acid residues matched the consensus which currently found around the active site "Ser" of cephalosporin C deacetylases (Mitsushima *et al.*, 1995; Politino *et al.*, 1997), esterases (Khalameyzer *et al.*, 1999; Brenner, 1988), lipases (Komaromy and Schotz, 1987; Shimada *et al.*, 1994; Brenner, 1988), the nonheme chloroperoxidase (Pfeifer *et al.*, 1995), acetyl xylan esterase (Lorenz and Wiegel, 1997; Clark *et al.*, 1996) as well as cutinases (Ettinger *et al.*, 1987; Soliday *et al.*, 1989; Ohnishi *et al.*, 1995). Therefore, this *B. cereus* BT-24 CAH is possible to be a serine esterase because there was serine-139 in the consensus sequence. In order to test whether it is a serine esterase, the inhibition of enzyme activity by using phenylmethylsulfonyl fluoride ($PhMeSO_2F$) should be performed.

From the nucleotide sequence shown in Figure 29, the locations of the promoter region and the ribosomal binding site can be presumed. In the upstream region of the CAH structural gene, the consensus hexanucleotide -10 region and the ribosomal binding site [Shine-Dalgarno (SD) sequence] were detected. The -10 region (5′ TATAAT 3′) was homologous to promoter of *Escherichia coli* RNA

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polymerase (Rosenberg and Court, 1979; Siebenlist *et al.*, 1980) and *B. subtilis* RNA polymerase vegetative cells (σ^{55}-RNA polymerase) (Lee *et al.*, 1980; Moran *et al.*, 1982). Another conserve sequence at -35 region was not found. This sequence should have spacing upstream of -10 region about 17-18 base pairs (Lee *et al.*, 1980). However, the possible SD sequence (GAGG) was found downstream from -10 region (Figure 18). The SD sequence of *B. cereus* CAH gene was similar to that of others genes in Gram-positive bacteria, for instances, the ribosome binding sites of α-amylase (Palva *et al.*, 1981), β-lactamase (Kroyer and Chang, 1981; Neugebauer *et al.*, 1981). The distance between SD sequence and the initiation codon of *B. cereus* BT-24 CAH gene was 9 base pairs. This distance was corresponded to the prediction by Moran *et al.* (1982) that the distance between ribosome binding sites and initiation codons ranged from 7-14 bases.

The deduced amino acid sequence of the CAH of *B. cereus* BT-24 was compared with reported sequences through the National Center for Biotechnology Information's BLAST search. The greatest similarity was found with human arylacetamide deacetylase, triacylglecerol lipase of *Moraxella* sp. and *E. coli* acetylesterase (Figure 32). This result confirmed that the clone CAH was in the group esterases and lipases. However, hydropathy plofile of the putative CAH protein demonstrated that the protein was rather hydrophilic (Figure 31). The result suggested that the CAH of *B. cereus* BT-24 was not lipases because the hydropathy profile of lipase was rather hydrophobic (Thanomsub, 1998). When using global alignment of BCM Search Launcher (USA) to compare amino acids of *B. cereus* BT-24 CAH with

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B. subtilis SHS0133 CAH (Mitsushima *et al.*, 1995), 19.1 % homology was found (Figure 33). Furthermore, 18.8 % homology was found when compared with *Thermoanaerobacterium* sp. AXE1 (acetyl xylan esterase with cephalosporin C deacetylase activity, Lorenz and Wiegel, 1997). The low homology of *B. cereus* BT-24 CAH with that of others CAH indicated that the enzyme from *B. cereus* is unique among the class of cephalosporin C deacetylase. Moreover, the cephalosporin C deacetylase, esterase and lipase genes in *B. cereus* have never been reported, which indicated that this CAH is a novel gene.

3. Expression of CAH Gene of *Bacillus cereus* BT-24

The pKK223-3 vector was chosen for studying the expression of the CAH gene from *B. cereus* BT-24. This vector contains *ptac* promoter which bears the -35 region of the *trp* promoter, and the -10 region operator and the ribosome binding site of the *lac*UV-5 promoter. The *E. coli* JM109 was used as a bacterial host for expression of the CAH gene because this strain contained a *lacI*^{q} which was a mutant of the *lacI* gene that expressed high levels of *lac* repressor. Thus, synthesis of proteins is minimized when foreign coding sequences are placed under the control of the *lac* promoter in cells carrying *lacI*^{q}. However, the expression can be derepressed at appropriate time by the addition of IPTG. The plasmid pKK223-3 had unique *Eco*RI and *Sma*I cloning sites whereas the 0.9 kb CAH gene fragment contained *Eco*RI and *Eco*RV sites at the 5′ end and the 3′ end, respectively. The *Eco*RI and *Eco*RV sites were created by PCR amplification for convenience of cloning. Since the *Eco*RV and *Sma*I created blunt end therefore the ligation of vector and the fragment

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was occurred at the corresponding *Eco*RI site, and at *Eco*RV and *Sma*I sites. The CAH gene fragment was inserted within 5 bp next to the ribosome-binding site of pKK223-3 before the ATG start codon (Figure 35). By using the chemical method as a primary screening of expressed clones, it found that the gene was expressed in pKK223-3 vector (Figure 36).

The finding that recombinant *E. coli* harboring the pSR11 plasmid showed the esterase activity about 4 times under IPTG induction (Table 21), indicated that the expression level was increased when using an expression plasmid. However, the expression level by pSR11 plasmid was still low. The fact that the gene expression is influenced by promoter used in the expression plasmid. To increase the level expression of *B. cereus* BT-24 CAH gene, it could be done by paying attention on the selection of appropriate promoter which could increase the transcription efficiency. Mitsushima *et al.* (1995) showed that the expression of *B. subtilis* SHS0133 CAH gene was increased about 10 folds when promoter was changed from *tac* promoter to *trp* promoter. In addition, the spacing between the SD sequence and the ATG initiation codon was suggested to affect strongly the expression efficiency (Gold, 1988). Takimoto *et al.* (1999) found that the spacing of 13 nucleotides showed the highest expression of *B. subtilis* SHS0133 CAH gene. Therefore, it is interesting to maximize the expression of *B. cereus* BT-24 CAH gene by using *trp* promoter and varying the space between the SD sequence and the ATG initiation codon.

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Despite that the expression of CAH gene by *ptac* in pKK223-3 vector was low, it was possible to detect the band of expressed protein from the cell free extract of the overexpressing clone pSR11 by SDS-PAGE (Figure 39A). The result showed clearly that the molecular weight of the expressed protein was 34.6 kDa which was corresponding to the calculated molecular weight from deduced amino acid. By esterase activity staining of the renatured SDS-PAGE confirmed that the expressed protein had esterase activity, because there was one additional band in the cell free extract of the clone pSR11 (Figure 39B) which was undetectable in the host cell harboring vector only. The additional band was also detectable in the Native-PAGE and the band migrated to middle part of the gel (Figure 40). This result rules out the possibility that the clone CAH was lipase. Thanomsub (1998) demonstrated that the lipase produced by the recombinant *E. coli* tend to aggregated at the upper part of Native-PAGE because of their hydrophobicity. In order to confirm the lack of lipolytic activity of the cloned CAH, enzyme assay using specific substrate for lipases should be performed.

From the finding that the cloned gene from *B. cereus* BT-24 coded for protein containing both esterase activity and deacetylation of cephalosporin C substrate to deacetylcephalosporin C, it was suggested that the cloned gene was cephalosporin C deacetylase (CAH).

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However, it is also interesting to investigate further its substrate specificity. At present, the information about optimal condition, stability and kinetics (over

various cephalosporin C substrates) of this enzyme are still unknown. In order to study all characteristics, higher production, and purification of the enzyme are also required. Such information will be useful for final conclusion about the possibility to use this novel enzyme for commercial application.

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CHAPTER VI

CONCLUSION

1. Bacterium strain BT-24 having esterolytic activity toward cephalosporin C was isolated locally from soil and was identified as *Bacillus cereus*.
2. Cephalosporin C deacetylase (CAH) gene from *Bacillus cereus* strain BT-24 was cloned and sequenced.
3. The CAH gene was located on a 1.085 Kb *Xba*I-*Eco*RV DNA fragment.
4. The nucleotide sequence of the CAH gene contained an ORF of 0.903 kb which encoded for a 301 amino acid peptide.
5. The deduced CAH protein contained sequence Gly-Asp-Ser-Ala-Gly which is a conserved sequence "Gly-X-Ser-X-Gly" found in many esterases and lipases.
6. The deduced amino acid sequence of the cloned CAH had low homology with known CAH.
7. The gene was expressed in the pKK223-3 vector using IPTG as an inducer.
8. The molecular weight of the cloned CAH as determined by SDS-PAGE and activity staining was 34.6 kDa.
9. The CAH gene found in this study is proposed to be a novel gene.

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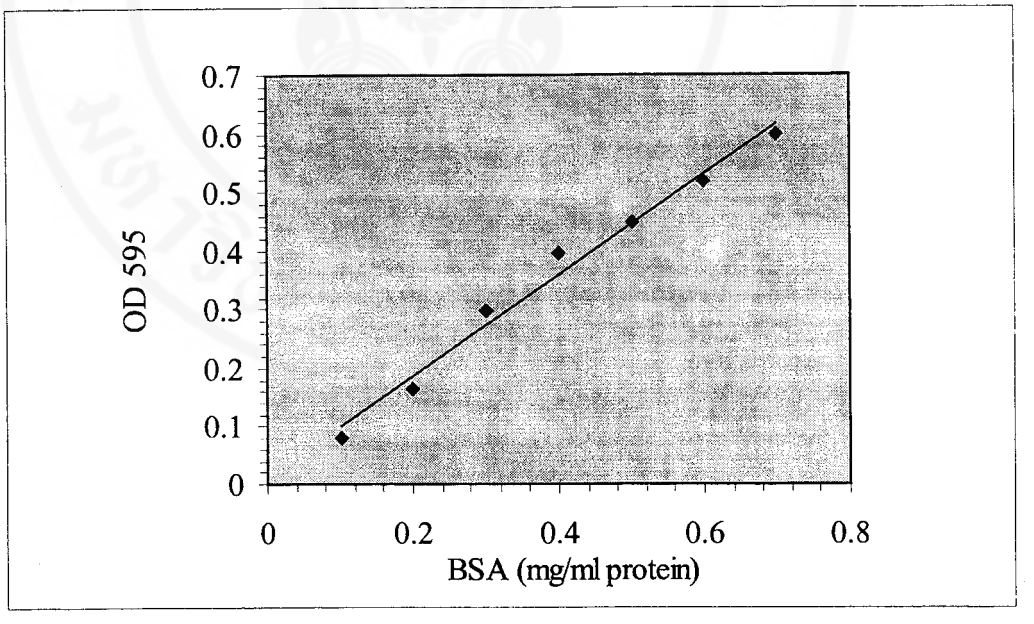
APPENDIX

Standard curves:

I. Standard curve of protein concentration determination using bovine serum albumin (BSA) measured spectrophotometrically at 595.



Concentration of protein can be calculated by a following formula:

$Y = 0.86X + 0.014$

Y = OD value at wavelength (λ) 595

X = protein concentration (mg/ml)

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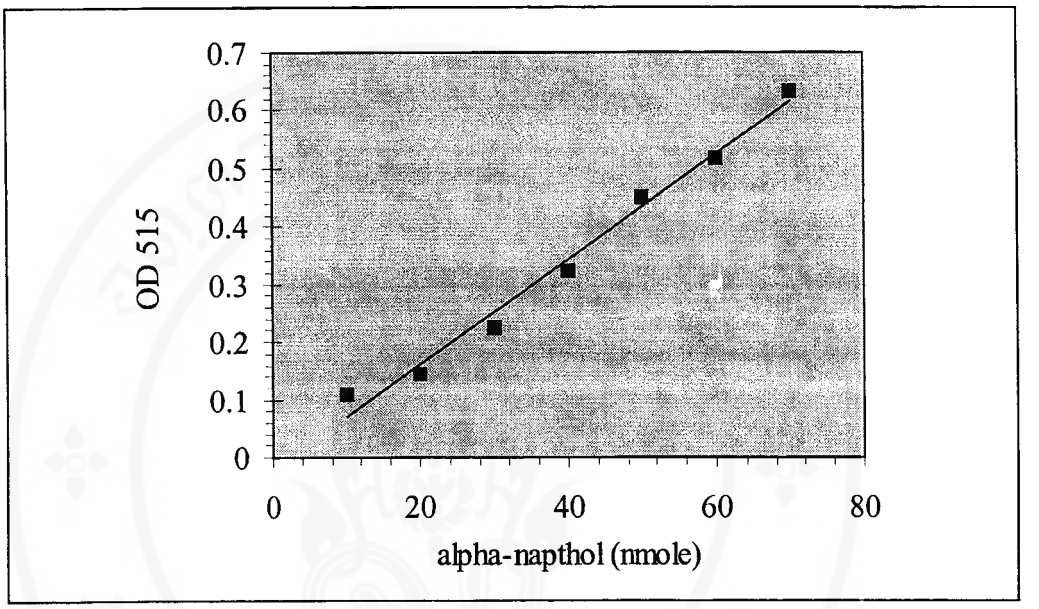
II. Standard curve of α-naphthol concentration measured spectrophotometrically at 515.



Concentration of α-naphthol can be calculated by a following formula:

$Y = 0.01X + 0.019$

Y = OD value at wavelength (λ) 515

X = α-naphthol concentration (nmole)

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